Universitat de les Illes Balears

TESIS DOCTORAL

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IMPORTANCIA DEL RATIO ERα/ERβ EN LA FUNCIÓN MITOCONDRIAL Y EL ESTRÉS OXIDATIVO EN EL CÁNCER DE MAMA. INFLUENCIA DE LA GENISTEÍNA Y LA UCP2 EN LA EFICACIA DE LOS TRATAMIENTOS ANTITUMORALES

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Daniel Gabriel Pons Miró

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Dra. Mª del Pilar Roca Salom, de la Universitat de les Illes Balears

DECLARO:

Que la tesis doctoral que lleva por título *Importancia del ratio ERα/ERβ en la función mitocondrial y el estrés oxidativo en el cáncer de mama. Influencia de la genisteína y la UCP2 en la eficacia de los tratamientos antitumorales,* presentada por Daniel Gabriel Pons Miró para la obtención del título de doctor, ha sido dirigida bajo mi supervisión y que cumple con los requisitos necesarios para optar al título de Doctor Europeo.

Y para que quede constancia de ello firmo este documento.

Firma 

Palma de Mallorca, 27 de Mayo de 2015

Dr. Jordi Oliver Oliver, de la Universitat de les Illes Balears

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Firma

Palma de Mallorca, 27 de Mayo de 2015

A mi familia y amigos

A Mar

Lo que sabemos es una gota, lo que ignoramos es el océano

Isaac Newton

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poc caparrudet :D) y por estar también a mi lado en todo momento, "No te estás portando bieeen" o "Ábremeeee" Todo esto es para nuestra historia. ¡Te quiero mazo morenito!

Un especial agradecimiento va dirigido a mi familia, ya que sin ellos todo esto no hubiera sido posible. Gracias a todos mis tíos y primos, naturales y políticos por los buenos momentos que siempre pasamos juntos. Gracias a mi familia política, que es muy importante en mi vida. *Carme i Paco, mai uns sogres han estat tan bons com voltros!, Aina cunyaaaa i es meu Cal·los! Gràcies per fer-me riure quan pareixia que era impossible, a na Duixeta, guau guau guau (tu ja m'entens)*. Me enorgullece agradecer también con palabras bien fuertes a mis padres y a mis abuelos, ellos me han educado en los valores del esfuerzo y que todo en esta vida es posible si uno quiere y se esfuerza en ello. Abuelos, gracias por mostrarme el camino y por enseñarme que lo lógico suele ser siempre la respuesta correcta, Ángel y Juani, ya sabéis que sois como unos padres para mi, y mamá y papá, sé que vuestra vida no ha sido fácil y que quizá no llegué en el momento más oportuno, pero espero que os sintáis orgullosos de mí porque yo lo estoy de vosotros. Y cuando parecía que sería hijo único y toda la herencia sería para mí llegó una personita sin la que hoy en día podría entender mi vida, *Elena, saps que sempre he confiat en tu i que ets molt especial! No canviis mai i segueix així d'autèntica!* No quiero dejar de hablar de una persona que ya no está en este mundo, pero que siempre estará en mi corazón: *Abuelita, allà on estiguis esper i sé que m'estiràs mirant i que puguis sentir-te orgullosa des teu nét que mai t'oblidarà.*

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Índice de contenidos

Acrónimos y Abreviaturas / Acronyms and Abbreviations

8PN: 8-prenilnaringenina / *8-prenylnaringenin*

ΔΨm: Potencial de membrana mitocondrial / Mitochondrial membrane potential

ADN / DNA: Ácido desoxirribonucleico /*Deoxyribonucleic Acid*

ADP: Adenosina difosfato/ *Adenosine diphosphate*

Akt: Proteína quinasa B / *Protein Kinase B*

AF-1: Función activadora 1 / *Activation Function 1*

ARN / *RNA*: Ácido ribonucleico / *Ribonucleic Acid*

ARNm / *mRNA*: ARN mensajero / *Messenger RNA*

ATM: Gen ataxia telangiectasia mutado / *Ataxia telangiectasia mutated gene*

ATP: Adenosina trifosfato / *Adenosine triphosphate*

ATPase / *ATPase:* ATP sintase / *ATP synthase*

BRCA1: Gen cáncer de mama 1 / *Breast cancer 1 gene*

BRCA2: Gen cáncer de mama 2 / *Breast cancer 2 gene*

CAT: Catalasa / *Catalase*

CDDP: Cisplatino / *Cisplatin*

COX: Citocromo c oxidasa / *Cytochrome c oxidase*

CS: Citrato Sintasa / *Citrate Synthase*

Cys: Cisteína / *Cysteine*

DCFDA: Diclorofluoresceina diacetato / *Dichlorofluorescein diacetate*

DMEM: Medio Eagle modificado por Dulbecco / *Dulbecco's modified Eagle's medium*

Drp1: Proteína relacionada con la dinamina 1 / *Dynamin-related protein* 1

E2: 17β-estradiol

ER: Receptor de estrógenos / Estrogen receptor

ERE: Elemento de respuesta a estrógenos / *Estrogen Response Element*

ERK: Quinasa regulada extracelularmente / *Extracellular signal-regulated kinase*

$FADH_2$: Flavin Adenina Dinucleótido / *Flavin Adenine Dinucleotide*

Fis1: Proteína mitocondrial de fisión 1 / *Mitochondrial fission 1 protein*

FoxO1: *Forkhead box O1*

GEN: Genisteína / *Genistein*

GPx: Glutatión Peroxidasa / *Glutathione Peroxidase*

GR: Glutatión Reductasa / *Glutathione Reductase*

Grx: Glutaredoxina / *Glutaredoxin*

GSH: Glutatión reducido / *Reduced Glutathione*

GSSG: Glutatión oxidado / *Oxidized Glutathione*

4-HNE: 4-*hydroxy-2-nonenal*

Hsp90: Proteína de choque térmico 90 / *Heat shock protein 90*

$ISDH_2$: Isocitrato deshidrogenasa / *Isocitrate dehydrogenase*

JAK2: *Janus kinase*

LC3: Proteína de cadena ligera-3 1A/1B asociada a microtúbulos / *Microtubule-associated protein 1A/1B-light chain 3*

LDH: Lactato deshidrogenasa / *Lactate dehydrogenase*

LTR: Lysotracker Red / *Lysotracker Red*

MDC: Monodansylcadaverina / *Monodansylcadaverine*

Mfn: Mitofusina / *Mitofusin*

MMP: Metaloproteinasa / *Metalloproteinase*

MRC: Cadena respiratoria mitocondrial / *Mitochondrial respiratory chain*

mtDNA: ADN mitocondrial /*Mitochondrial DNA*

MTG: Mitotracker Green / *Mitotracker Green*

mtSSB: Proteína mitocondrial de unión a cadena sencilla / *Mitochondrial single stranded DNA-binding protein*

NADH: Nicotinamida adenina dinucleótido / *Nicotinamide adenine dinucleotide*

NADPH: Nicotinamida adenina dinucleótido fosfato / *Nicotinamide adenine dinucleotide phosphate*

NAO: Naranja de acridina / *Nonyl Acridine orange*

nDNA: ADN nuclear /*Nuclear DNA*

NRF: Factor de respiración nuclear / *Nuclear respiratory factor*

OPA1: Proteína de la atrofia óptica 1 / *Optic atrophy 1 protein*

PARP: Poli ADP ribosa polimerasa / *Poly ADP ribose polymerase*

PDH: Piruvato deshigrogenasa / *Pyruvate dehydrogenase*

PGC1α: Coactivador 1α de PPARγ / *PPARγ Coactivator 1α*

PHD: Proteína con dominio prolyl hidroxilasa */ Prolyl hydroxylase domain protein*

PI3K: Fosfoinositol 3-quinasa / Phosphatidylinositol *3 kinase*

Prx: Peroxirredoxina / *Peroxiredoxin*

PTEN: Homólogo de fosfatasa y tensina/ *Phosphatase and tensin homolog*

PTX: Paclitaxel / *Paclitaxel*

ROS: Especies reactivas de oxígeno / *Reactive Oxygen Species*

SAPK: Proteína quinasa activada por estrés/ *Stress-activated protein kinase*

SERM: Modulador selectivo de los receptores de estrógenos/*Selective Estrogen Receptor Modulator*

siRNA: ARN pequeño de interferencia / *small interfering RNA*

SIRT: Sirtuina / *Sirtuin*

SOD: Superóxido dismutasa / *Superoxide dismutase*

SP-1: Proteína específica 1 / *Specificity Protein 1*

Srx: Sulfiredoxina / *Sulfiredoxin*

STAT3: Transductor de señal y activador de la transcripción 3 / *Signal transducer and activator of transcription 3*

TAM: Tamoxifeno / *Tamoxifen*

TFAM: Factor de trasncripción mitocondrial A / *Mitochondrial transcription factor A*

TMRM: Tetrametil rodamina metil éster / *Tetramethyl Rhodamine Methyl Ester*

TNFα: Factor de necrosis turmoral α / *Tumor necrosis factor alpha*

Trx: Tiorredoxina / Thioredoxin

TrxR: Tiorredoxina reductasa / Thioredoxin reductase

UCP: Proteína desacoplante / *Uncoupling protein*

XN: Xanthohumol / *Xanthohumol*

Listado de publicaciones/List of publications

Esta tesis ha originado las siguientes publicaciones / This thesis has led to the following papers:

Pons DG, Nadal-Serrano M, Torrens-Mas M, Valle A, Oliver J, Roca P. 2015. **UCP2 inhibition sensitizes breast Cancer cells to therapeutic agents by increasing oxidative stress.** Free Radic Biol Med. doi: 10.1016/j.freeradbiomed.2015.04.032. [*Epub ahead of print*].

Pons DG, Nadal-Serrano M, Blanquer-Rossello MM, Sastre-Serra J, Oliver J, Roca P. 2014. **Genistein modulates proliferation and mitochondrial functionality in breast cancer cells depending on ERalpha/ERbeta ratio.** J Cell Biochem 115:949-58.

Sastre-Serra J, Nadal-Serrano M, Pons DG, Roca P , Oliver J. 2013 **The over-expression of ERbeta modifies estradiol effects on mitochondrial dynamics in breast cancer cell line**. Int J Biochem Cell Biol 45:1509-15.

Asimismo, los siguientes artículos derivados de la tesis están en vías de publicación / Furthermore, the following papers derived from the thesis are in process of being published:

The presence of Estrogen Receptor β modulates the response of breast cancer cells to therapeutic agents.

The phytoestrogen genistein affects breast cancer cells treatment depending on the Estrogen Receptor α/Estrogen Receptor β ratio.

Anexo/Appendix

Además, durante la realización de esta tesis las colaboraciones del doctorando en otros estudios relacionados han originado las publicaciones presentadas en el anexo/*Moreover, during this thesis, the PhD student collaborations in other related studies have led to the papers presented in the appendix*:

Manuscript 6. Miro AM, Sastre-Serra J, Pons DG, Valle A, Roca P, Oliver J. 2011. **17beta-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio.** J Steroid Biochem Mol Biol 123:133-9.

Manuscript 7. Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Oliver J, Roca P. 2012. **The effects of 17beta-estradiol on mitochondrial biogenesis and function in breast cancer cell lines are dependent on the ERalpha/ERbeta ratio.** Cell Physiol Biochem 29:261-8.

Manuscript 8. Nadal-Serrano M, Sastre-Serra J, Pons DG, Miro AM, Oliver J, Roca P. 2012. **The ERalpha/ERbeta ratio determines oxidative stress in breast cancer cell lines in response to 17beta-estradiol.** J Cell Biochem 113:3178-85.

Manuscript 9. Pons DG, Sastre-Serra J, Nadal-Serrano M, Oliver A, Garcia-Bonafe M, Bover I, Roca P, Oliver J. 2012. **Initial activation status of the antioxidant response determines sensitivity to carboplatin/paclitaxel treatment of ovarian cancer.** Anticancer Res 32:4723-8.

Manuscript 10. Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Garau I, Garcia-Bonafe M, Oliver J, Roca P. 2013. **The oxidative stress in breast tumors of postmenopausal women is ERalpha/ERbeta ratio dependent.** Free Radic Biol Med 61C:11-17.

Manuscript 11. Nadal-Serrano M, Pons DG, Sastre-Serra J, Blanquer-Rossello Mdel M, Roca P, Oliver J. 2013. **Genistein modulates oxidative stress in breast cancer cell lines according to ERalpha/ERbeta ratio: effects on mitochondrial functionality, sirtuins, uncoupling protein 2 and antioxidant enzymes.** Int J Biochem Cell Biol 45:2045-51.

Manuscript 12. Roca P, Sastre-Serra J, Nadal-Serrano M, Pons DG, Blanquer-Rossello Mdel M, Oliver J. 2014. **Phytoestrogens and mitochondrial biogenesis in breast cancer. Influence of estrogen receptors ratio.** Curr Pharm Des 20:5594-618.

Esta tesis se presenta como compendio de artículos de investigación previamente publicados o aceptados. Las referencias completas de las publicaciones que constituyen el núcleo de esta tesis, así como sus indicios de calidad son:

Pons DG, Nadal-Serrano M, Torrens-Mas M, Valle A, Oliver J, Roca P. 2015. **UCP2 inhibition sensitizes breast Cancer cells to therapeutic agents by increasing oxidative stress.** Free Radic Biol Med. doi: 10.1016/j.freeradbiomed.2015.04.032. [*Epub ahead of print*]. Factor de impacto (2013): 5,710 (Q1 en las categorías de Bioquímica y Biología Molecular y de Endocrinología y Metabolismo).

Pons DG, Nadal-Serrano M, Blanquer-Rossello MM, Sastre-Serra J, Oliver J, Roca P. 2014. **Genistein modulates proliferation and mitochondrial functionality in breast cancer cells depending on ERalpha/ERbeta ratio.** J Cell Biochem 115:949-58. Factor de impacto (2013): 3,368 (Q2 en las categorías de Bioquímica y Biología Molecular y de Biología Celular).

Sastre-Serra J, Nadal-Serrano M, Pons DG, Roca P , Oliver J. 2013 **The over-expression of ERbeta modifies estradiol effects on mitochondrial dynamics in breast cancer cell line**. Int J Biochem Cell Biol 45:1509-15. Factor de impacto (2013): 4,240 (Q2 en las categorías de Bioquímica y Biología Molecular y de Biología Celular).

Resumen

El cáncer de mama es una de las enfermedades más comunes en los países desarrollados. Este cáncer suele ser hormonodependiente, es decir, se ve influenciado por la presencia de hormonas como el 17β-estradiol (E2), el estrógeno más abundante en las mujeres, que ejerce sus efectos en las células mediante su interacción con los receptores estrogénicos (ER), ERα y ERβ. El ERα presenta funciones relacionadas con la proliferación celular, mientras que el ERβ tiene efectos citostáticos y de diferenciación celular. Así pues la ratio ERα/ERβ podría influir en el desarrollo y la progresión del cáncer de mama. Además, la genisteína, un fitoestrógeno natural presente en la soja, puede unirse a ambos ERs modulando la expresión génica en las células de cáncer de mama.

La funcionalidad mitocondrial juega un papel crucial en el cáncer a través de la modulación del estrés oxidativo. Así, una menor funcionalidad mitocondrial se ha asociado a una mayor producción de especies radicales de oxígeno (ROS), lo que podría suponer un incremento en la proliferación celular. Sin embargo, un aumento excesivo en los niveles de ROS podría provocar la muerte celular si los sistemas antioxidantes de la célula no son capaces de contrarrestar estos ROS, dañando las macromoléculas. Una las proteínas que forman parte del sistema antioxidante de la célula es la proteína desacoplante 2 (UCP2), cuya función es disipar el gradiente protónico en la membrana interna mitocondrial, reduciendo así el estrés oxidativo.

Los objetivos de esta tesis fueron los siguientes: 1) estudiar cómo afecta la ratio ERα/ERβ a la función y dinámica mitocondrial después del tratamiento con concentraciones fisiológicas de E2 y genisteína, 2) investigar si la presencia del ERβ podría suponer un factor de resistencia a tratamientos antitumorales como el cisplatino, el paclitaxel y el tamoxifeno, cuyo mecanismo de acción es la generación de

estrés oxidativo en las células cancerosas para provocar la muerte celular, 3) examinar si el silenciamiento de la UCP2 podría suponer un tratamiento adyuvante en el cáncer de mama, 4) analizar la posible interferencia de la genisteína en la eficacia de los tratamientos antitumorales.

Los resultados obtenidos en esta tesis muestran como la presencia y estimulación del ERβ incrementó la funcionalidad y dinámica mitocondrial en líneas celulares de cáncer de mama tratadas con E2 y genisteína, sugiriendo que una baja ratio ERα/ERβ favorecería la resistencia de las células de cáncer de mama a tratamientos citotóxicos a través de una mejora de la funcionalidad mitocondrial, con la consiguiente reducción en la producción de ROS. Con la excepción del tamoxifeno ya que la presencia en las células del ERβ únicamente determina el tipo de respuesta a este compuesto, apoptosis o autofagia. El silenciamiento de la UCP2 produjo un aumento de la muerte celular por autofagia en las células tratadas con cisplatino y, especialmente, con tamoxifeno, debido a un aumento drástico en la producción de ROS. Finalmente, la genisteína provocó una menor respuesta a los tratamientos antitumorales en las células de cáncer de mama con una elevada ratio ERα/ERβ, desplazando al E2 de los ERs, reduciendo así el estrés oxidativo. Además, en la línea celular de cáncer de mama con una baja ratio ERα/ERβ la respuesta al tratamiento con tamoxifeno mejoró en combinación con la genisteína a través de un aumento en los niveles de apoptosis y autofagia. En conclusión, los niveles de UCP2 y la ratio ERα/ERβ, así como la concentración de E2 y genisteína que estarían interaccionando con los receptores estrogénicos, juegan un papel fundamental en la respuesta de las células de cáncer de mama a los tratamientos antitumorales, modulando la funcionalidad mitocondrial y, por tanto, la producción de ROS.

Importància de la Ràtio ERα/ERβ en la Funció Mitocondrial i l'Estrés Oxidatiu en el Càncer de Mama. Influència de la Genisteïna i l'UCP2 en l'eficàcia dels Tractaments Antitumorals

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Resum

El càncer de mama és una de les malalties més comuns del països desenvolupats. Aquest càncer sol ser hormonodependent, és a dir, es veu influenciat per la presència d'hormones com el 17β-estradiol (E2), l'estrogen més abundant a les dones, que exerceix els seus efectes a les cèl·lules mitjançant la seva interacció amb els receptors estrogènics (ER), ERα i ERβ. L'ERα presenta funcions relacionades amb la proliferació cel·lular, mentre que l'ERβ té efectes citostàtics i de diferenciació cel·lular. Així doncs la ràtio ERα/ERβ podria influir en el desenvolupament i la progressió del càncer de mama. A més, la genisteïna, un fitoestrogen natural present a la soja, pot unir-se a ambdós Ers modulant l'expressió gènica a les cèl·lules de càncer de mama.

La funcionalitat mitocondrial juga un paper crucial al càncer a través de la modulació de l'estrès oxidatiu. Així, una menor funcionalitat mitocondrial s'ha associat a una major producció d'espècies radicals d'oxigen (ROS), el que podria suposar un increment en la proliferació cel·lular. No obstant, un augment excessiu en els nivells de ROS podria provocar la mort cel·lular si els sistemes antioxidants de la cèl·lula no són capaços de contrarestar aquests ROS, danyant les macromolècules. Una de les proteïnes que formen part del sistema antioxidant de la cèl·lula és la proteïna desacoblant 2 (UCP2), la funció de la qual és dissipar el gradient protònic a la membrana interna mitocondrial, reduint l'estrès oxidatiu.

Els objectius d'aquesta tesi foren els següents: 1) estudiar com afecta la ràtio ERα/ERβ a la funció i dinàmica mitocondrial després del tractament amb concentracions fisiològiques d'E2 i genisteïna, 2) investigar si la presència de l'ERβ podria suposar un factor de resistència a tractaments antitumorals com el cisplatí, el paclitaxel i el tamoxifè, el mecanisme d'acció dels quals és la generació d'estrès oxidatiu a les cèl·lules canceroses per provocar la mort cel·lular, 3) examinar si el

silenciament de l'UCP2 podria suposar un tractament adjuvant en el càncer de mama, 4) analitzar la possible interferència de la genisteïna en l'eficàcia dels tractaments antitumorals.

Els resultats obtinguts en aquesta tesi mostren com la presència i estimulació de l'ERβ incrementà la funcionalitat i dinàmica mitocondrial en línees cel·lulars de càncer de mama tractades amb E2 i genisteïna, suggerint que una baixa ràtio ERα/ERβ afavoriria la resistència de les cèl·lules de càncer de mama als tractaments citotòxics a través d'una millora de la funcionalitat mitocondrial, amb la conseqüent reducció en la producció de ROS. Amb l'excepció del tamoxifè ja que la presència a les cèl·lules de l'ERβ únicament determina el tipus de resposta a aquesta compost, apoptosi o autofàgia. El silenciament de l'UCP2 produí una augment de la mort cel·lular per autofàgia a les cèl·lules tractades amb cisplatí i, especialment, amb tamoxifè, degut a un increment dràstic en la producció de ROS. Finalment, la genisteïna provocà una menor resposta als tractaments antitumorals a les cèl·lules de càncer de mama amb una elevada ràtio ERα/ERβ, desplaçant l'E2 dels ERs, reduint així l'estrès oxidatiu. A més, a la línea cel·lular de càncer de mama amb una baixa ràtio ERα/ERβ la resposta al tractament amb tamoxifè millorà en combinació amb la genisteïna a través d'un augment en els nivells d'apoptosi i autofàgia. En conclusió, els nivells d'UCP2 i la ràtio ERα/ERβ, així com la concentració d'E2 i genisteïna que estarien interaccionant amb els receptors estrogènics, juguen un paper fonamental en la resposta de les cèl·lules de càncer de mama als tractaments antitumorals, modulant la funcionalitat mitocondrial i, per tant, la producció de ROS.

Importance of the ERα/ERβ Ratio in the Mitochondrial Function and the Oxidative Stress in Breast Cancer. Influence of the Genistein and the UCP2 in the Efficacy of the Anticancer Treatments

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Abstract

Breast cancer is one of the most common diseases in developed countries. This type of cancer is usually hormone-dependent; it is influenced by the presence of hormones such as 17β-estradiol (E2), the most abundant estrogen in women, which exerts its effects in the cells through its interaction with the estrogen receptors (ER), ERα and ERβ. ERα has functions related to cell proliferation, while ERβ shows cytostatic and cell differentiation effects. As a result, the ERα/ERβ ratio may influence in the breast cancer development and progression. Moreover, the genistein, a natural phytoestrogen found in soybeans, is able to bind both ERs modulating the gene expression in breast cancer cells.

The mitochondrial functionality plays a crucial role in cancer through the modulation of the oxidative stress. Thus, a lower mitochondrial functionality has been associated with a greater production of radical oxygen species (ROS), which may suppose a cell proliferation increment. However, an excessive increase in ROS levels may lead to cell death if the cell antioxidant systems are unable to counteract these ROS, damaging macromolecules. One of the proteins that is part of the antioxidant system of the cell is the uncoupling protein 2 (UCP2), whose function is to dissipate the proton gradient in the inner mitochondrial membrane, so reducing oxidative stress.

The objectives of this thesis were: 1) to study how the ERα/ERβ ratio affects to the mitochondrial function and dynamics after treatment of physiological concentrations of E2 and genistein, 2) to investigate if the presence of ERβ may be a resistance factor to anticancer treatments such as cisplatin, paclitaxel and tamoxifen, whose mechanism of action is the generation of oxidative stress in cancer cells in order to provoke the cell death, 3) to review whether the UCP2 silencing could be an

adjuvant treatment against breast cancer, 4) to analyze the possible interference of the genistein in the efficacy of the anticancer treatments.

Results obtained in this thesis show that the presence and stimulation of the ERβ increased the mitochondrial functionality and dynamics in breast cancer cell lines treated with E2 and genistein, suggesting that a low ERα/ERβ ratio would favor the resistance of breast cancer cells to cytotoxic treatments through an improvement of the mitochondrial functionality, with the consequent reduction in the ROS production. With the exception of tamoxifen as the presence of the ERβ only determines the type of the response to this compound, apoptosis or autophagy. The UCP2 silencing produced an increase in the autophagic cell death in those cells treated with cisplatin and, especially, with tamoxifen, due to a drastic raise in the ROS production. Finally, the genistein triggered a lower response of breast cancer cells with a high ERα/ERβ ratio to anticancer treatments, displacing the E2 of the ERs, thereby reducing the oxidative stress. Furthermore, in that breast cancer cell line with a low ERα/ERβ ratio the response to tamoxifen treatment was enhanced in combination with the genistein through an increase in the apoptosis and autophagy levels. In conclusion, the UCP2 levels and the ERα/ERβ ratio, as well as the concentration of E2 and genistein which would be interacting with the estrogen receptors, play an important role in the response of breast cancer cells to anticancer treatments, modulating the mitochondrial functionality and, therefore, the ROS production.

1. Introducción

El cáncer es una enfermedad que se caracteriza por un crecimiento anormal de las células propias del organismo, con un cierto potencial de invasión o de propagación a otras partes del cuerpo. En una revisión del año 2000, Hanahan y Weinberg describieron seis características esenciales que toda célula cancerosa debe presentar para ser considerada como tal. Estas particularidades se van adquiriendo a lo largo de la transformación de la célula hacia un fenotipo canceroso y constituyen un marco para la comprensión del complejo proceso neoplásico. Estas seis características son el mantenimiento de señales proliferativas, la evasión de la respuesta a los supresores tumorales, la resistencia a la apoptosis, la inmortalidad replicativa, la inducción de la angiogénesis y la activación de mecanismos de invasión y metástasis[1]. Ya en 2011, los mismos autores detallaron dos características adicionales de las células cancerosas, tal y como puede observarse en la Figura 1: la modificación o reprogramación del metabolismo energético de la célula para adaptarse mejor a las características de la proliferación neoplásica y la ausencia de la respuesta inmune por parte de los macrófagos y los linfocitos T, B y citolíticos naturales (células NK, del inglés *natural killer*)[2]. Adicionalmente, son dos las particularidades de las células cancerosas que facilitan la aparición de estas dos últimas características: la inestabilidad genómica (lo que hace más probable la aparición de mutaciones en el ADN, favoreciendo el desarrollo y progresión del cáncer) y la inflamación crónica (que podría estar relacionada con la ausencia de la respuesta inmune)[2]. El concepto de cáncer es muy amplio y abarca más de 200 tipos de enfermedades. Según los datos de la Organización Mundial de la Salud (OMS), en 2012 los cánceres diagnosticados con más frecuencia fueron los de pulmón, colon y recto, próstata y mama.

1.1. Epidemiología y etiología del cáncer de mama

El cáncer de mama es el cáncer más frecuentemente diagnosticado en las mujeres, con más de 1,38 millones de nuevos casos al año estimados según datos del registro mundial de cáncer en 2008[3], produciéndose además cerca de 459.000 muertes relacionadas con este tipo de cáncer[4]. Un aspecto alarmante es que, según las

estadísticas de GLOBOCAN, 1,7 millones de mujeres fueron diagnosticadas con cáncer de mama en 2012, con 522.000 muertes relacionadas, lo que supone un incremento de la incidencia y la mortalidad del cáncer de mama en un 22% y un 13% respectivamente desde 2008[5]. Además, está previsto que la incidencia mundial del cáncer de mama femenino alcanzará los 3,2 millones de nuevos casos anuales en 2050[6]. Asimismo, el cáncer de mama masculino representa menos del 1% de todos los casos de cáncer de mama y su tratamiento y prognosis es similar al cáncer de mama en mujeres[7-9].

Figura 1. Representación esquemática de las principales características que presenta una célula tumoral para ser considerada como tal. Adaptado de Hanahan y Weinberg, 2011[2].

Al mismo tiempo, y a pesar del mayor número de casos de cáncer de mama diagnosticados en los países desarrollados, se espera que la incidencia del cáncer de mama en países en vías de desarrollo se incremente a mayor velocidad debido al aumento en la esperanza de vida acompañado de un estilo de vida más sedentario, con menos actividad física y retraso en la maternidad[10], además de cambios en los hábitos alimenticios. Estos datos reflejan la magnitud de la incidencia del cáncer de

mama, su efecto en la sociedad y la urgente necesidad de encontrar las medidas adecuadas para su prevención y tratamiento.

Los niveles circulantes de hormonas sexuales esteroideas endógenas, como los estrógenos, han sido considerados como un factor de riesgo de padecer cáncer de mama[11]. En este sentido, una menarquía temprana, un nulo o bajo número de partos, una edad tardía en el primer embarazo completo y la obesidad son factores de riesgo para el cáncer de mama[12-14]. Las hormonas sexuales podrían influir así en el riesgo de sufrir cáncer de mama promoviendo el crecimiento del tumor o incrementando la proliferación celular y, por tanto, la probabilidad de adquirir mutaciones y daño en el ADN[15]. Además, la terapia hormonal (administración de estrógenos y/o progesterona) después de la menopausia incrementa el riesgo de cáncer de mama[16]. Así pues, los estrógenos juegan un papel esencial en el desarrollo y en el progreso del cáncer de mama. La obesidad también incrementa las probabilidades de padecer cáncer de mama, ya que después de la menopausia el tejido adiposo es la principal fuente de estrógenos[17]. Además de ser la fuente principal de estrógenos debido a la aromatización de los andrógenos[17], el tejido adiposo secreta también sus propias hormonas, las adipoquinas, que podrían jugar un papel en el desarrollo del cáncer[18,19].

El cáncer de mama es sumamente heterogéneo en cuanto a su etiología y características patológicas, presentando casos de lento crecimiento con una buena prognosis, mientras que otros casos muestran un curso clínico altamente agresivo[20]. Aparte de las hormonas sexuales femeninas, existen otros factores de riesgo de padecer cáncer de mama que también podrían influir en esa heterogeneidad de la enfermedad. Algunos ejemplos son una dieta rica en carne roja y grasa animal, y pobre en frutas, verduras, calcio, vitamina D, soja y antioxidantes[21,22], el consumo de alcohol[23], una baja actividad física[24] y los factores genéticos. Entre éstos últimos cabe destacar que el historial familiar juega un papel importante en el riesgo de sufrir cáncer de mama ya que las mujeres que tienen un familiar de primer grado con cáncer de mama tienen el doble de riesgo de padecer la enfermedad[25]. Los factores genéticos más importantes son los polimorfismos genéticos (que podrían explicar por qué algunas personas son más sensibles que otras a carcinógenos ambientales como los estrógenos exógenos o el alcohol) y las mutaciones en genes claves en la regulación de

la proliferación, diferenciación y muerte celular, como por ejemplo BRCA1, BRCA2, CHEK2, PALB2, ATM, TP53, PTEN y STK1[26].

1.2. Los receptores de estrógenos

Como se ha especificado anteriormente, una exposición prolongada a los estrógenos supone un mayor riesgo de padecer cáncer de mama. En 1972 Jensen y colaboradores llevaron a cabo estudios en el útero sobre la acción del 17β-estradiol (E2), que es la forma de estrógeno más común en los mamíferos, y concluyeron que los efectos biológicos de los estrógenos se dan gracias a su interacción con el receptor de estrógenos (ER)[27], que más tarde se conocería como ERα. Además, en 1995 Gustafsson y colaboradores descubrieron un segundo tipo de receptor estrogénico, el ERβ[28]. En la glándula mamaria, por tanto, la vía de señalización de los estrógenos empieza por la unión de éstos a los receptores estrogénicos, ERα y ERβ, ejerciendo así su función correspondiente[29-38]. Normalmente el ERα se ha asociado a procesos de incremento de la proliferación de las células del epitelio mamario, mientras que el ERβ favorecería la apoptosis y la diferenciación celular[33,39,40], por lo que el balance existente entre ambos receptores estaría regulando esta actividad proliferativa[37,41-44]. Así pues, la estimulación de la proliferación celular por parte de los estrógenos vía interacción con el ERα puede ser contrarrestada por el reclutamiento del ERβ[45-47].

La distribución del ERα y el ERβ varía en función del tejido. Además, la distribución heterogénea de los correguladores de los ERs podría explicar los diferentes efectos de los estrógenos en los distintos tejidos. Se cree que en aquellos tejidos donde hay una coexistencia de ambos receptores, podrían ejercer sus actividades contrarrestándose uno al otro[33,48].

1.2.1. Estructura de los receptores de estrógenos

Ambos ERs pertenecen a la superfamilia de receptores nucleares y son factores de transcripción que se activan por ligando[49,50]. ERα (595 aa) y ERβ (530 aa) están codificados por dos genes diferentes y tienen una cierta homología (un 58% en humanos) en sus secuencias proteicas respectivas. En la Figura 2 se puede ver que ambos receptores presentan cinco dominios claramente diferenciados. El dominio A/B,

situado en la región N-terminal, es el más variable de todos y está relacionado con las interacciones proteína-proteína de la maquinaria transcripcional; este dominio contiene la función de activación de la transcripción génica (AF-1) y varios sitios de fosforilación importantes especialmente en caso de activación del receptor en ausencia de ligando. El dominio C o de unión al ADN es el más conservado, ya que tiene un 97% de homología, por lo que ERα y ERβ se unen al ADN de un modo muy similar; este dominio presenta dos dedos de zinc y un motivo corto llamado *P-box*, que confiere especificidad de unión al ADN y está, además, implicado en el proceso de dimerización[33,51,52]. Finalmente presentan un dominio D o dominio bisagra, que participa en la unión del receptor (en estado inactivo) a la chaperona HSP90; el dominio E o de unión al ligando, formado por un bolsillo de 12 hélices alfa, con un 60% de homología, y que está también envuelto en otras funciones como la dimerización, la localización nuclear o la interacción con cofactores[33]; y el dominio F situado en el extremo C-terminal, relacionado con la capacidad de transcripción de genes[33,49,50].

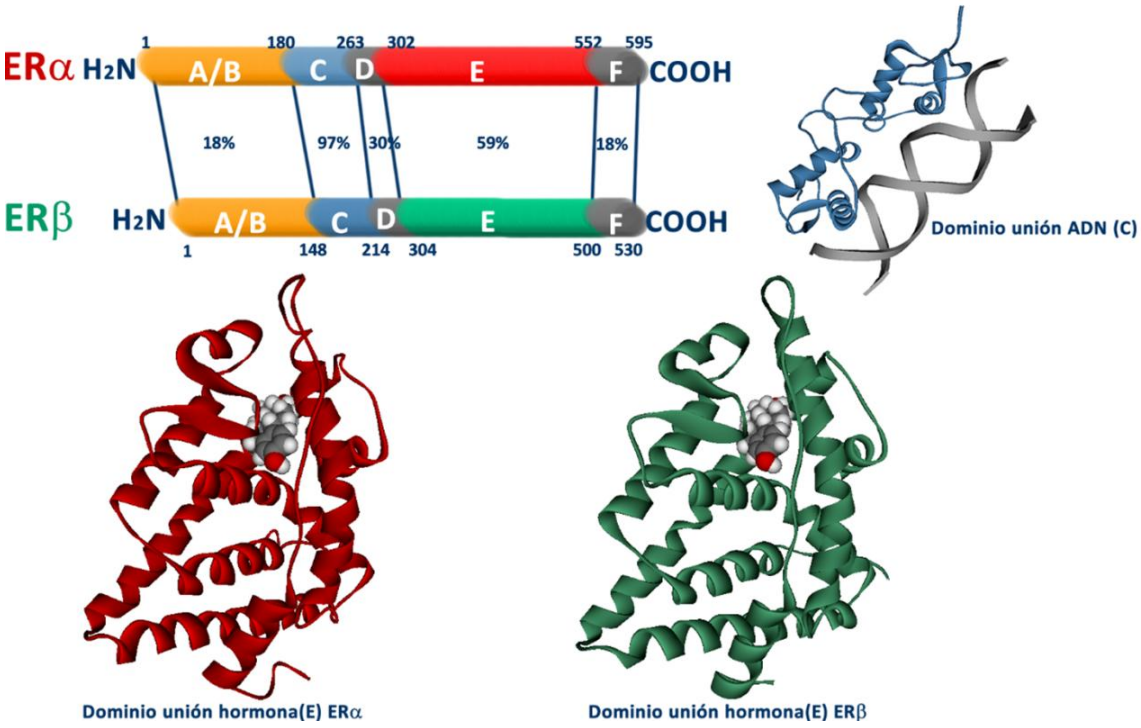


Figura 2. Representación esquemática de los cinco dominios proteicos del ERα y del ERβ. Se muestran también las estructuras tridimensionales del dominio de unión al ADN (C) y de los dominios de unión al ligando (E) de cada uno de los dos receptores estrogénicos.

1.2.2. Mecanismos de acción de los receptores de estrógenos

Los estrógenos pueden actuar a través de mecanismos y vías muy variadas en las células[52,53]. Como puede observarse en la Figura 3, la forma más habitual es la modulación de la expresión génica, típica de la superfamilia de receptores nucleares. Esta modulación puede activarse por dos mecanismos diferentes: activación directa e indirecta.

La activación directa del ER mediante su interacción con los estrógenos se puede llevar a cabo de dos formas diferentes. La primera de ellas es que la interacción E2-ER se produzca en el citoplasma de la célula y luego el complejo E2-ER sea transportado al núcleo por el citoesqueleto celular. La segunda manera sería la interacción directa del E2 con el ER en el núcleo. Una vez en el núcleo, la unión del ER al E2 favorece la disociación del receptor de la chaperona HSP90, y es cuando se produce la dimerización de los ERs (homodimerización o heterodimerización). Esta dimerización permite la unión del complejo E2-ER al elemento de respuesta a estrógeno (ERE) en el ADN, presente en los promotores de los genes diana, además de su interacción con diferentes coactivadores de la regulación génica[33,52].

Otro mecanismo de acción es el conocido como activación/inhibición indirecta. Consiste en la unión del dímero de ERs, una vez se ha producido la interacción con el ligando, con una proteína auxiliar llamada SP1, siendo esta última la que se une directamente con el elemento de unión a estrógenos (ERE) en el ADN[36,54].

Aunque habitualmente los ERs ejercen su función en las células a través de su interacción con el ADN, actuando como factores de transcripción, en muchos tejidos ejercen también acciones independientemente de su unión al ADN, es la activación no genómica de los ERs. Estos procesos, que todavía no están del todo estudiados, requieren la producción de mensajeros secundarios que tienen efectos rápidos en la célula, sin que se produzca una modulación de la transcripción génica[29].

Por último, los ERs pueden ser activados independientemente de su unión al ligando gracias a la acción de quinasas que fosforilan y activan los ERs. Esto supone que los ERs tienen mecanismos de activación no dependientes de hormonas[29].

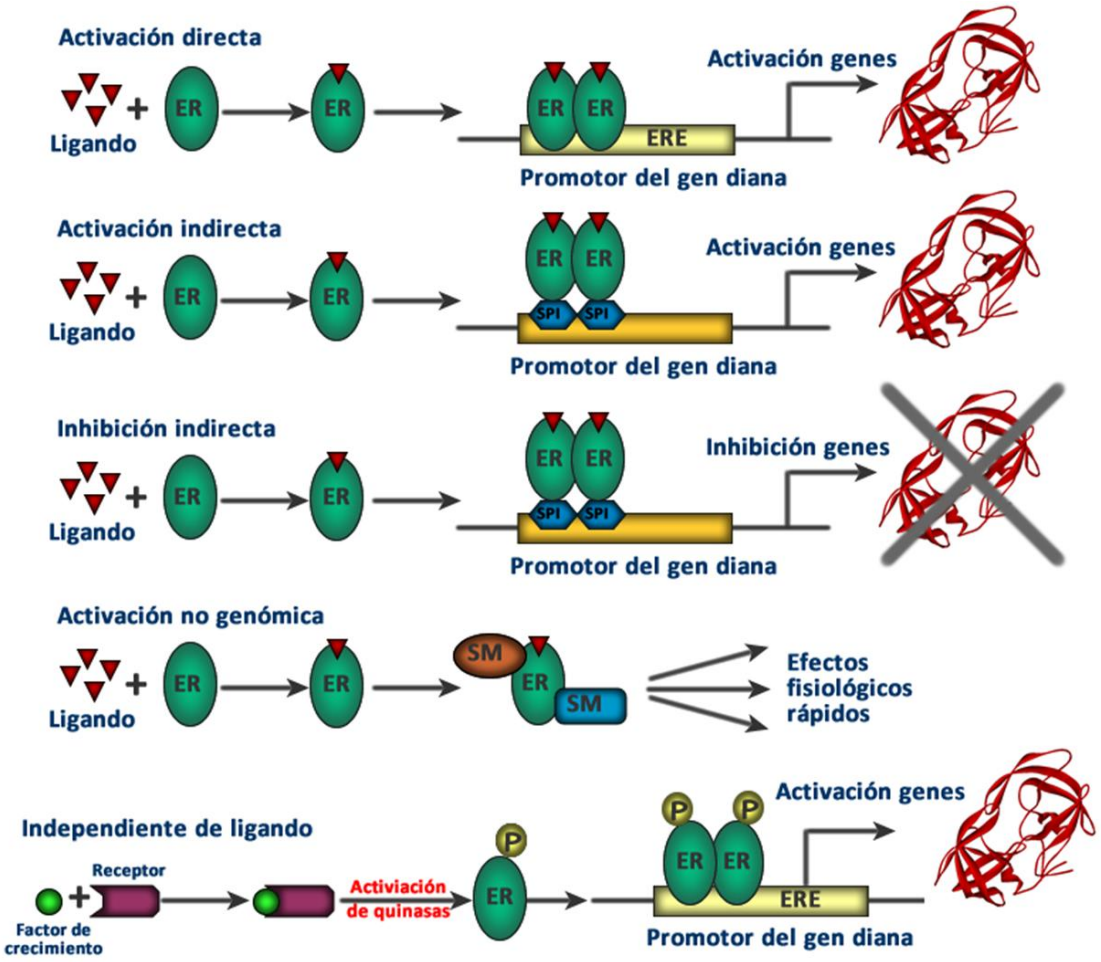


Figura 3. Mecanismos de acción de los receptores de estrógenos.

Además de los ERs por sí mismos, otras proteínas reguladoras juegan un papel importante en los mecanismos de activación de estos receptores estrogénicos. Estos cofactores actúan como coactivadores o correpresores de la actividad transcripcional de los ERs. Estas proteínas reguladoras pueden presentarse como acetilasas/deacetilasas, quinasas/fosfatasas y metilasas/demetilasas. Cabe destacar que estos correguladores pueden variar dependiendo del tejido, lo que podría explicar, al menos en parte, los diferentes efectos de los estrógenos en cada tejido[33]. No obstante, también debe tenerse en cuenta la dotación de los dos tipos de receptores estrogénicos, ERα y ERβ, que tiene cada tejido para poder evaluar y estudiar los efectos biológicos de los estrógenos y otros compuestos capaces de unirse a los ERs.

1.3. Papel de los estrógenos y los fitoestrógenos en el cáncer de mama

1.3.1. Papel de los estrógenos en la glándula mamaria y en el desarrollo del cáncer

Durante toda la vida fértil de una mujer, los estrógenos juegan un papel esencial en los cambios constantes que se producen en el desarrollo y la fisiología de la glándula mamaria. Durante la pubertad la glándula mamaria experimenta un incremento en la proliferación celular, mientras que en la etapa adulta se producen ciclos de crecimiento e involución que se corresponden con la fase menstrual de la mujer[55].

En la glándula mamaria, los receptores estrogénicos ERα y ERβ de las células ductales se contrarrestan el uno al otro en los ciclos de proliferación/involución estimulados por los estrógenos. Como se ha comentado anteriormente, la respuesta proliferativa a los estrógenos parece que depende de la ratio ERα/ERβ, donde el ERα incrementa la proliferación y el crecimiento de las células de la glándula mamaria, mientras que el papel del ERβ en la mama está relacionado con sus funciones antiproliferativas y de diferenciación[33,43]. Estudios previos realizados en nuestro grupo de investigación con líneas con diferente ratio ERα/ERβ sugieren que el tratamiento con E2 a concentraciones fisiológicas en una línea celular de cáncer de mama con una elevada ratio ERα/ERβ provoca una estimulación de la proliferación celular, mientras que en aquellas líneas con una ratio ERα/ERβ baja o con la sola presencia de ERβ, dicha estimulación con E2 no produce una mayor proliferación celular[56].

La mayoría de los tumores de mama positivos para el receptor de estrógenos (hoy en día en clínica se estudia mayoritariamente la presencia del ERα) son positivos para ambos subtipos, ERα y ERβ, aunque algunos tumores son únicamente positivos para el ERβ y podrían tener un distinto comportamiento clínico y distinta respuesta a los tratamientos. Al contrario que el ERα, algunos estudios sugieren que durante la carcinogénesis la expresión del ERβ podría verse disminuida[57-60]. Esta caída en la expresión de ERβ estaría indicando que este receptor juega un papel clave como supresor tumoral[61,62]. Desde siempre, los tumores negativos para el ERα han sido considerados resistentes a la terapia endocrina, ya que la falta del receptor los haría insensibles a la regulación estrogénica. Sin embargo, se sabe que aproximadamente el

50% de los tumores ERα-negativos expresan ERβ. Hay numerosos estudios que indican que la coexpresión de ambos ERs supone una mejor prognosis y respuesta al tratamiento, así como la presencia del ERβ en combinación con la del ERα como un marcador tumoral beneficioso[58,63-66].

Es importante remarcar que, aunque un elevado ratio ERα/ERβ es un factor importante en el desarrollo del fenotipo canceroso en el cáncer de mama[33,43,67-69], una vez generado el tumor de células malignas, una disminución de este ratio ERα/ERβ (debido a un aumento en la expresión de ERβ) puede suponer una peor prognosis y una menor respuesta al tratamiento antiestrogénico[70]. Otro factor a tener en cuenta en estos estudios es la diferente afinidad de ambos ERs por el E2, siendo 10 veces superior en el caso del ERα con respecto al ERβ[32,71], por lo que la cantidad de ERβ debe ser bastante mayor a la de ERα para que los efectos del E2 sobre el ERβ sean significativos.

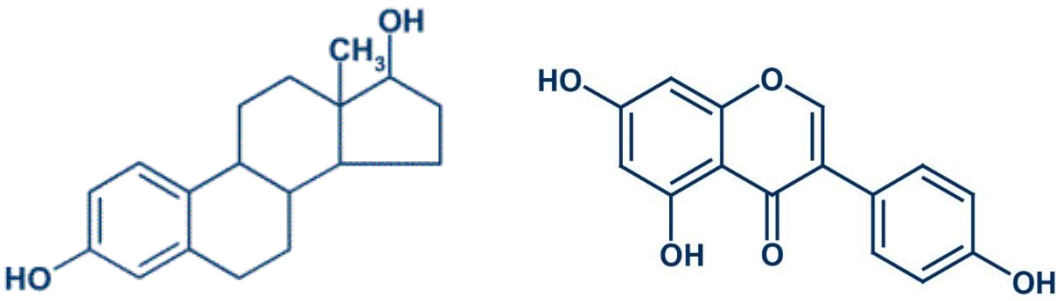
1.3.2. Fitoestrógenos y cáncer de mama. Papel de la genisteína

Los fitoestrógenos constituyen un grupo de compuestos naturales que pueden encontrarse en más de 300 plantas[72]. Estos compuestos se caracterizan por tener una estructura química muy similar a los estrógenos y por tener cierta actividad estrogénica[73]. La comunidad científica empezó a interesarse por los fitoestrógenos debido a la baja incidencia de cáncer de mama en Asia, donde la soja es un importante componente de la dieta[74]. Así, el interés por los fitoestrógenos ha ido creciendo los últimos años debido a los estudios epidemiológicos que relacionan el consumo de fitoestrógenos con un bajo riesgo de mortalidad de varios tipos de cáncer, especialmente de mama y de próstata[74-80]. Además, los productos de soja o, más recientemente, los suplementos alimenticios conteniendo los principios activos de esta planta se usan también para tratar otras enfermedades crónicas como enfermedades cardiovasculares[80] y osteoporosis[75], así como para un alivio de los síntomas climatéricos[81,82].

En general, los fitoestrógenos poseen propiedades antiinflamatorias, antioxidantes, antitrombóticas, antineoplásicas, antialérgicas y hepatoprotectoras[83], por lo que estos compuestos pueden tener efectos sobre el cáncer tanto por estas

actividades, como por sus propiedades hormonales. Otro aspecto a tener en cuenta es la concentración de fitoestrógenos en plasma y el período de exposición a éstos, ya que se cree que los efectos beneficiosos de los fitoestrógenos, entre los cuales se encontraría un posible papel quimiopreventivo del cáncer de mama, podrían estar relacionados con el consumo a lo largo de toda la vida o de etapas muy tempranas de la niñez[79,84-86]. Sin embargo, el papel de los fitoestrógenos en el cáncer de mama presenta cierta controversia debido a que las propiedades estrogénicas de estos compuestos podrían ser perjudiciales en el cáncer de mama sensible a los estrógenos[87].

El fitoestrógeno genisteína (GEN) es una isoflavona (4',5,7-trihidroxiisoflavona) que fue aislada en 1899 por primera vez por Perkin y Newbury de la planta *Genista tinctoria*[88]. Se ha visto que la GEN, debido a sus propiedades estrogénicas (como se puede observar en la Figura 4 la GEN presenta una estructura similar al E2), podría tener ciertos beneficios en enfermedades relacionadas con los estrógenos como la osteoporosis o las enfermedades cardiovasculares[89-91]. La GEN posee también otras propiedades no hormonales como la inhibición de la actividad tirosina-quinasa de algunas proteínas[92,93], efectos antioxidantes a través de la eliminación de los ROS o de la inhibición de la expresión de los genes relacionados con el estrés oxidativo[94], la inducción de la parada del ciclo celular[95], la modulación de vías de señalización intracelulares (tales como Akt/NF-κB)[96], además de la inhibición de la invasión y la metástasis de las células cancerosas[97]. Por eso, el elevado consumo de soja en Asia podría estar relacionado con la baja incidencia de cáncer de mama en este continente[98] ya que la GEN es la principal isoflavona que se encuentra en la soja.



17β-estradiol (E2) **Genisteína (GEN)**

Figura 4. Estructura molecular del 17β-estradiol (E2) y de la genisteína (GEN).

A pesar de tener otras funciones no relacionadas con sus propiedades hormonales, la GEN ejerce sus efectos principales en las células a través de su unión a los ERs[99]. En 1960, Folman y Pope fueron los primeros en establecer la afinidad relativa de unión de las isoflavonas a los ERs, las cuales se unen preferentemente al ERβ[100,101]. Kuiper y colaboradores[31,32] confirmaron que la afinidad de unión de la GEN por el ERβ es mucho mayor que por el ERα. Esta preferencia de unión al ERβ por parte de la GEN podría tener implicaciones en el riesgo de padecer cáncer de mama; así, algunos autores sugieren que cuando el ERβ es activado por ciertos ligandos podría inhibir la proliferación celular en el cáncer de mama, así como contrarrestar los efectos estimulatorios del E2 sobre el ERα, aunque todavía se desconoce el papel concreto que juega el ERβ en el cáncer de mama[43].

Sin embargo, contrastando con los efectos beneficiosos del consumo de GEN ya mencionados, algunos estudios sugieren que el consumo de GEN podría estimular el crecimiento de los tumores sensibles a estrógenos[102]. Esta controversia existente sobre los posibles efectos beneficiosos o perjudiciales de la GEN en el cáncer de mama se debe a estudios realizados en líneas celulares de cáncer de mama con un ratio ERα/ERβ elevado, donde se observó que la GEN estimulaba la proliferación de esta línea celular[103-106]. Algunos autores han revelado un posible efecto bifásico de la GEN sobre la proliferación celular: a bajas concentraciones (0,1-10 μM) la GEN mostró una estimulación de la proliferación, mientras que a altas concentraciones (a partir de 10 μM) la GEN inhibió la proliferación celular a través del bloqueo del ciclo celular[77,99,106]. Parece ser que la GEN actuaría como un inhibidor del crecimiento de las células cancerosas a altas concentraciones y, por otro lado, podría tener un efecto estimulador de la proliferación celular a bajas concentraciones[77]. En general, se cree que la actividad proliferativa de la GEN a bajas concentraciones puede ser inhibida con un compuesto antiestrogénico[107], lo que indicaría que es un mecanismo mediado por los ERs. Sin embargo, la inhibición del crecimiento a altas concentraciones de la isoflavona no se inhibe con la adición de un antiestrogénico, lo que sugiere que no es un mecanismo mediado por los ERs y que, probablemente, sea debido a concentraciones citotóxicas de la GEN[107].

1.4. La mitocondria en el cáncer de mama

Las mitocondrias son orgánulos presentes en las células eucariotas, cuya estructura está delimitada por dos membranas: la membrana externa, que establece una separación física del orgánulo con el citoplasma; y la membrana interna, formada por una serie de invaginaciones llamadas crestas mitocondriales donde está localizada la cadena respiratoria mitocondrial. Entre ambas membranas queda el denominado espacio intermembrana.

Las mitocondrias son responsables de la producción del 90% del ATP para los requerimientos energéticos de la célula y, además, son la principal fuente y diana de los ROS intracelulares. Asimismo, estos orgánulos juegan también un papel clave en la regulación de la proliferación celular y la apoptosis[53].

Como ya se ha mencionado, la principal función de las mitocondrias es la generación de ATP a través de un proceso complejo conocido como fosforilación oxidativa (*oxidative phosphorylation*, OXPHOS)[108]. Brevemente, la oxidación de las moléculas altamente reducidas contenidas en los nutrientes (carbohidratos, lípidos y proteínas) a través del metabolismo celular rinde electrones en forma de moléculas portadoras de hidrógeno reducidas como NADH o $FADH_2$. Estos cofactores reducidos donan electrones a una serie de complejos situados en la membrana interna mitocondrial, conocidos como cadena respiratoria mitocondrial. El aceptor final de electrones es una molécula de oxígeno que se reduce a una molécula de agua en el complejo IV o citocromo c oxidasa (COX). La energía conservada como un gradiente protónico entre el espacio intermembrana y la matriz mitocondrial es usada por el complejo V o F_oF_1-ATP sintasa (ATPasa) para formar ATP mediante la fosforilación del ADP gracias a la energía generada por el retorno de los protones a favor de gradiente a la matriz mitocondrial[109].

Numerosos estudios han descubierto la presencia de los ERs en la mitocondria[110-115] y, además, el genoma mitocondrial posee secuencias de ADN muy parecidas a los ERE del ADN nuclear[116], lo que hace pensar que tanto el ERα como el ERβ podrían jugar un papel esencial en la regulación de la funcionalidad mitocondrial. Chen y colaboradores demostraron en 2004 que tanto el ERα como el ERβ se unen

directamente al ADN mitocondrial a través de los EREs y que esta unión se incrementa con la presencia del E2[117]. Otros estudios han sugerido que el ERβ podría modular directamente la función mitocondrial a través de su unión directa al ADN mitocondrial y la regulación de su transcripción[115]. La presencia de los ERs tanto en el núcleo, citoplasma, mitocondrial e incluso en la membrana celular hace pensar que ambos ERs están perfectamente coordinados para modular cualquier proceso celular en el que participen[115,118-123].

1.4.1. Especies reactivas de oxígeno (ROS) y cáncer

Las mitocondrias son la fuente principal de ROS en la célula. El término especie reactiva de oxígeno (*reactive oxygen species*, ROS) hace referencia a cualquier molécula de oxígeno que haya aceptado electrones extra y sea capaz de oxidar otras moléculas[124]. Como se aprecia en la Figura 5, muchos ROS intracelulares derivan de la reducción electrónica del oxígeno (O_2), que forma el radical superóxido ($O_2^{\cdot -}$). Se estima que alrededor del 1% del O_2 consumido en la mitocondria es usado para la producción de superóxido[125,126]. Asimismo, dos moléculas de superóxido se pueden convertir en una molécula de peróxido de hidrógeno (H_2O_2) y una de agua por la acción de las superóxido dismutasas (SOD). El peróxido de hidrógeno puede también aceptar otro electrón del Fe^{2+} libre por la reacción de Fenton para formar el radical hidroxilo ($HO^{\cdot}$).

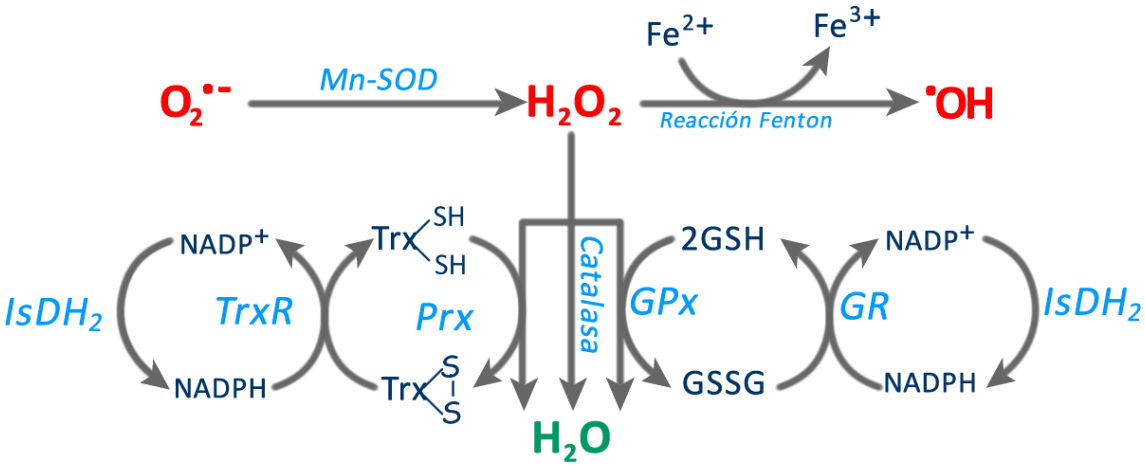


Figura 5. Mecanismos enzimáticos de detoxificación de las especies radicales de oxígeno (ROS).

Estas tres formas moleculares de ROS ($O_2^{\cdot-}$, H_2O_2 y $HO^{\cdot}$) tienen diferente reactividad y pueden tener distintos efectos en la fisiología celular[127]. Estos ROS pueden provocar tanto una mayor proliferación celular (incrementando la tumorigenicidad), como la apoptosis. Parece ser que bajos niveles de ROS podrían estimular la proliferación celular, mientras que elevados niveles podrían inducir la muerte celular[128]. En una reciente revisión, Sullivan y Chandel establecieron que mutaciones puntuales del ADN mitocondrial incrementan la tumorigenicidad a través del aumento en la producción de ROS en la mitocondria, manteniendo la capacidad de biogénesis mitocondrial; en cambio, mutaciones masivas en el ADN mitocondrial comprometen eventualmente la capacidad de biogénesis de las mitocondrias lo que haría que el gran aumento de ROS disminuyera la tumorigenicidad de las células, promoviendo la muerte celular[127].

Sin embargo, además de los efectos dañinos que tienen sobre la célula a través de la oxidación de macromoléculas, los ROS podrían también tener importancia en algunos procesos de señalización celular relacionados con la supervivencia, el crecimiento y la proliferación celular[127]. En concreto, los ROS generados en el complejo III podrían jugar un papel esencial en muchos procesos biológicos como la diferenciación celular y la inmunidad adaptativa[129]. Finalmente, los ROS pueden provocar la muerte celular, además de directamente por el estrés oxidativo generado en la célula (Figura 6), mediante la activación de las vías proapoptóticas intracelulares[130-132].

1.4.2. Función y dinámica mitocondrial en el cáncer de mama

Los ROS provocados por un mal funcionamiento de las mitocondrias podrían provocar daños irreparables ya que pueden oxidar y dañar proteínas, lípidos y ADN[133]. Por ello, la homeostasis redox de la mitocondria debe estar perfectamente regulada.

La disfunción mitocondrial se ha asociado al fenómeno del envejecimiento, a enfermedades neuronales y también al cáncer[134-136]. De hecho, reguladores clave de la muerte celular, así como otros procesos celulares que se llevan a cabo en la mitocondria están frecuentemente alterados en las células cancerosas[137], ya que las mitocondrias de las células cancerosas son distintas tanto funcionalmente como

estructuralmente de las mitocondrias de las células normales[138]. Además, las células cancerosas poseen normalmente mutaciones en el ADN nuclear y mitocondrial, lo que afecta a los componentes del complejo OXPHOS provocando una producción excesiva de ROS y daño oxidativo en las mitocondrias[138]. Es importante destacar que la falta de mecanismos de protección y reparación (como por ejemplo las histonas) en el ADN mitocondrial lo hace especialmente sensible al daño oxidativo; este ADN mitocondrial oxidado puede provocar un mayor grado de disfunción mitocondrial y, consecuentemente, una mayor producción de ROS, conduciendo a un ciclo vicioso de amplificación de la producción de ROS en la célula[139].

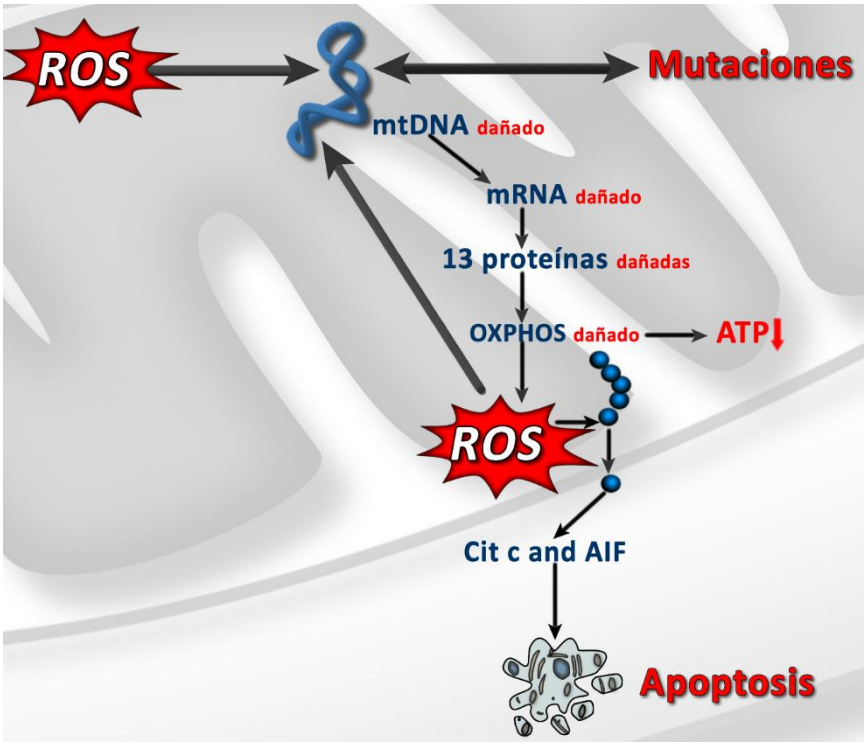


Figura 6. Esquema del daño oxidativo en una mitocondria. Los ROS pueden dañar el ADN mitocondrial, el ARN mensajero o las proteínas del OXPHOS, lo que puede llegar a provocar la apoptosis debido a la liberación del citocromo c (Cit c) o del factor inductor de la apoptosis (AIF).

En los años 20 del siglo XX, Otto Warburg ya asoció la disfunción mitocondrial con el cáncer. Así, Warburg observó que en varias muestras de células tumorales la glucólisis aeróbica estaba incrementada debido a una bajada en la capacidad respiratoria de estas células[140], describiendo lo que actualmente se conoce como

efecto Warburg. Posteriormente se han ido descubriendo más alteraciones metabólicas asociadas con la disfuncionalidad mitocondrial como un incremento en la gluconeogénesis y de la actividad glutaminolítica, una reducción en la oxidación de los ácidos grasos, así como un descenso en la oxidación del piruvato y un aumento de la producción de lactato[141-144]. Asimismo, se ha observado que las actividades de algunas enzimas relacionadas con el proceso de fosforilación oxidativa están reducidas en las células cancerosas, como por ejemplo la actividad de la ATPasa[145]. Además, la actividad de la COX también se ve reducida en las células cancerosas respecto a las normales[146,147], todo ello contribuyendo a la disfuncionalidad mitocondrial que se da en las células tumorales.

Las mitocondrias son orgánulos altamente dinámicos que continuamente se están fusionando (fusión mitocondrial) y fragmentando (fisión mitocondrial) de una forma altamente regulada[148,149], en función de las necesidades fisiológicas de la célula[150]. El proceso de fusión mitocondrial en mamíferos lo llevan a cabo principalmente tres proteínas, mitofusina 1 (Mfn1), mitofusina 2 (Mfn2) y OPA1, mientras que el de fisión lo llevan a cabo dos proteínas: Drp1 y Fis1[151]. Los cambios en la morfología y la dinámica mitocondrial juegan un papel clave en la fisiología de las células, en procesos como la generación de ROS y la inducción de la apoptosis, entre otros[150].

La célula se protege frente a la disfuncionalidad mitocondrial eliminando aquello que no funciona, desde alguna proteína mitocondrial a la mitocondria en sí[152,153]. Si la célula no es capaz de mantener un conjunto de mitocondrias funcionales, se liberan factores proapoptóticos produciéndose el fenómeno de muerte celular programada 1 o apoptosis[154].

1.5. Papel de la UCP2 en el cáncer

Las proteínas desacoplantes (*uncoupling proteins, UCPs*) son un grupo de proteínas transmembrana pertenecientes a la superfamilia de transportadores aniónicos y se localizan en la membrana interna mitocondrial (Figura 7). Su función biológica es permitir el reingreso de los protones desde el espacio intermembrana a la

matriz mitocondrial asociado a una producción de calor (proceso conocido como termogénesis), disipando el gradiente protónico, disminuyendo el potencial de membrana y, por tanto, disminuyendo la producción de ROS[155,156].

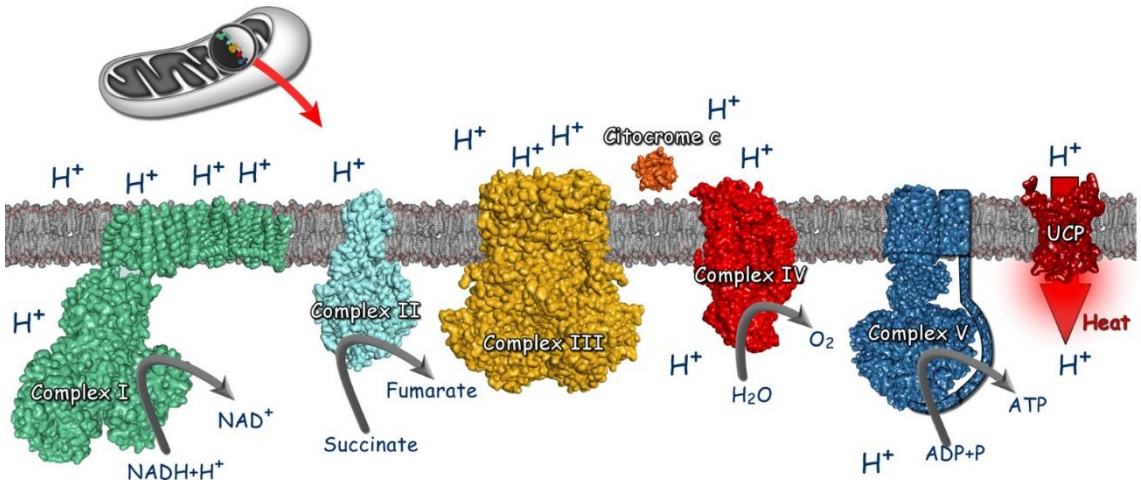


Figura 7. Representación esquemática de la cadena respiratoria mitocondrial (complejos OXPHOS) y de la proteína desacoplante (UCP), disipando el gradiente protónico con la producción de calor y disminuyendo la producción de ROS.

Así, una de las funciones claves que llevan a cabo las UCPs en las células es la disminución de la producción de ROS en la mitocondria. En condiciones de reposo, cuando el potencial de membrana es alto y el transporte electrónico está limitado por la falta de ADP y Pi, la formación de superóxido está fuertemente activada[157]. Hay una fuerte correlación positiva entre el potencial de membrana y la producción de ROS. Cuando el potencial de membrana es alto, un pequeño incremento de este potencial de membrana supone una gran producción de ROS[158], mientras que una pequeña bajada puede bajar la producción de ROS hasta el 70%[159,160]. Por lo tanto, un sutil desacoplamiento de la cadena respiratoria y, por tanto, un pequeño descenso en el potencial de membrana se ha sugerido que puede tener un efecto antioxidante natural[161]. Datos obtenidos en experimentos de inhibición de las UCPs apoyan esta teoría ya que esta inhibición produjo un aumento del potencial de membrana y una mayor producción de ROS[162,163].

El primer tipo de las proteínas desacoplantes (UCP1) fue descubierto en el tejido adiposo marrón de hámster[164]. La función principal del tejido adiposo marrón es la termogénesis y está presente en hibernadores, pequeños mamíferos y en los recién nacidos en humanos, aunque recientemente se ha demostrado también su presencia en humanos adultos[165]. Contrariamente al tejido adiposo blanco, el marrón posee una gran cantidad de mitocondrias caracterizadas por una membrana interna muy desarrollada[156]. Desde 1997 se han descubierto cuatro nuevos homólogos de las UCPs, nombrados consecutivamente del 2 al 5, identificados en roedores y humanos. Al contrario de la UCP1 cuya localización es específica del tejido adiposo marrón, el resto de homólogos tienen una expresión tisular más amplia. La UCP2 es la más importante en el cáncer, ya que la regulación de su expresión se ha relacionado con la tumorigénesis y la resistencia a la quimioterapia[166,167].

La UCP2 es el homólogo con una distribución más ubicua, ya que está presente en muchos tejidos como el tejido adiposo, músculo, corazón, riñón, tracto digestivo, cerebro, bazo y timo[168,169]. Además de una escasa función termogenética, la UCP2 desempeña otras funciones relacionadas con la obesidad y la restricción calórica, como por ejemplo la regulación del gasto energético basal[168,170-172]. Asimismo, la UCP2 regula la secreción de insulina en los islotes beta pancreáticos[173]. Esta regulación se produce debido a la función desacoplante de la cadena respiratoria mitocondrial de la UCP2 que reduce la producción de ATP, lo que provoca una disminución de la secreción de insulina estimulada por glucosa[174].

La UCP2 juega un papel fundamental en la disminución de la producción de ROS en la mitocondria. La pérdida de la UCP2 en ratones incrementó la producción de ROS y el potencial de membrana, principalmente en aquellos tejidos donde se expresa normalmente esta proteína[175]. Esto sugiere que la UCP2 podría mantener el potencial de membrana a unos niveles lo suficientemente bajos para no generar una cantidad de ROS excesiva[175]. Además, la UCP2 parece ser que es activada por los mismos ROS o por productos derivados de la peroxidación lipídica, sugiriendo que la UCP2 podría formar parte de un mecanismo de retroalimentación negativa dirigido a mitigar la producción excesiva de ROS y el daño oxidativo en las mitocondrias[176,177].

Estudios llevados a cabo con ratones *knockouts* para la UCP2 indicaron que la ausencia de esta proteína supuso una menor esperanza de vida para estos ratones, con un aumento significativo del daño oxidativo mitocondrial, sobre todo en el sistema nervioso central y en el corazón[178,179]. Otros autores estudiaron la causa de la muerte de estos ratones *knockout* y observaron un incremento en la incidencia de tumores con respecto a los ratones control[180]. Este efecto de la falta de UCP2 es más evidente bajo condiciones de estrés metabólico o de una infección[175,181,182]. Así pues, los ratones *knockout* para la UCP2 expuestos a un compuesto carcinogénico desarrollaron más tumores que los ratones control, con un aumento del estrés oxidativo y de la activación de NF-κB[183].

Estudios llevados a cabo en nuestro grupo de investigación sugieren que en el cáncer de mama, el tratamiento con E2 podría suponer una bajada de los niveles de las UCPs, sugiriendo que la exposición a estrógenos en aquellas células de mama positivas para ERα podría suponer un incremento de la producción de ROS que, a su vez, podrían actuar como señales mitogénicas aumentando el riesgo de padecer cáncer de mama[56,184].

Sin embargo, una vez establecido el cáncer, la expresión de la UCP2 promovería la quimioresistencia de las células cancerosas[185]. Así pues, la UCP2 podría tener un papel dual en el cáncer (Figura 8), actuando como un mecanismo protector en las células normales impidiendo su transformación en células malignas debido a mutaciones originadas por la producción excesiva de ROS; mientras que la sobreexpresión de UCP2 en células cancerígenas podría conferir una mayor supervivencia y resistencia a la quimioterapia gracias a la bajada de los niveles de ROS, que no serían suficientes para matar dichas células[166]. Estudios previos en nuestro grupo de investigación demostraron que los tumores de cáncer de mama se adaptan al estrés oxidativo incrementando la expresión de UCPs[186]. Es importante destacar que la agresividad del tumor está asociada a la adquisición de defensas antioxidantes que favorecen que las células cancerosas puedan escapar del ambiente de estrés oxidativo existente en el tumor[187,188], por lo que una sobreexpresión de la UCP2 podría conferir más agresividad a dichas células cancerosas. Estos estudios confirman que la sobreexpresión de UCP2 en el cáncer es el resultado de un proceso de selección a largo

plazo, en el que las células cancerosas se han adaptado a condiciones severas de estrés oxidativo.

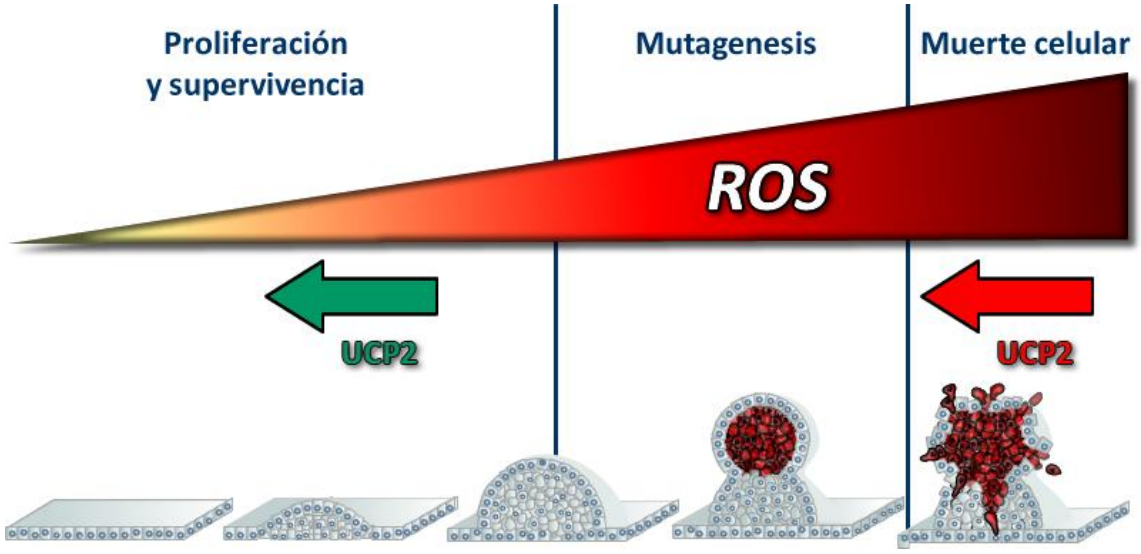


Figura 8. Niveles de ROS y efectos que producen en las células normales y cancerosas. Papel de la UCP2 en el desarrollo del cáncer y la resistencia a la muerte celular.

Recientemente, numerosos estudios han demostrado que la UCP2 está relacionada también con la quimioresistencia[189,190]. De hecho, en células de cáncer de páncreas el desacoplamiento de la cadena respiratoria mitocondrial es un mecanismo de resistencia a la quimioterapia, a través de la disminución de la producción de ROS[189]. Además, se ha descrito que la inhibición de la UCP2 produce una sensibilización de las células de leucemia a los agentes citotóxicos[190]. Todas estas evidencias llevan a pensar que la UCP2 podría ser una buena diana de actuación contra el cáncer, pudiendo incrementar los efectos de los tratamientos antitumorales, a través del aumento drástico de los ROS inhibiendo la expresión de UCP2.

1.6. La apoptosis y la autofagia en el cáncer

La apoptosis (muerte celular programada 1) y la muerte celular por autofagia (muerte celular programada 2) son dos mecanismos fisiológicos cruciales en el control del desarrollo, homeostasis y eliminación de las células cancerígenas[191,192]. Es evidente

que la eliminación de las células cancerosas después del tratamiento con quimioterapia se da, al menos en parte, por la inducción de la apoptosis[193]. Durante el proceso de apoptosis, la Poli ADP ribosa polimerasa (PARP), una proteína que está relacionada con la reparación del ADN en respuesta al estrés celular[194], es fragmentada en dos principalmente mediante la acción de la caspasa-3[195,196]. Así pues, la PARP ayuda a mantener la viabilidad celular y su rotura facilita la muerte celular y, por tanto, sirve como marcador final de la apoptosis[197].

La autofagia es un proceso altamente conservado en la evolución que permite a las células mantener la homeostasis en condiciones desfavorables, recuperando energía de los componentes subcelulares dañados o innecesarios (macromoléculas u orgánulos)[198], por lo que ha sido considerado como un proceso asociado a la supervivencia celular. Sin embargo, si el deterioro celular es demasiado severo y la autofagia se mantiene a unos elevados niveles, se produce la muerte celular por autofagia[199]. La muerte celular por autofagia es un tipo de muerte celular no apoptótica que puede ser inducida por diversos factores como por ejemplo la quimioterapia[200]. Numerosos autores sugieren que alteraciones en las vías de regulación de la autofagia podrían dar lugar al desarrollo del cáncer[201,202]. Además se ha observado que algunas células cancerosas tienen inhibida la autofagia o tienden a reducirla comparadas con las células no tumorales circundantes[201,202]. La proteína de cadena ligera-3 1A/1B asociada a microtúbulos (LC3, del inglés *Microtubule-associated protein 1A/1B-light chain 3*) sufre modificaciones post-traduccionales durante la autofagia. La rotura de la LC3 en el extremo carboxi-terminal inmediatamente después de sus síntesis produce la LC3-I localizada en el citoplasma. Durante la autofagia, LC3-I se convierte a LC3-II a través de una lipidación por un sistema de tipo ubiquitina, que permite a la LC3 asociarse con las vesículas autofágicas[203]. La presencia de LC3 en los autofagosomas y la conversión de LC3 en la forma LC3-II han sido usadas como marcadores de autofagia[204].

1.7. Tratamientos contra el cáncer

En las últimas décadas, desarrollar tratamientos eficaces para combatir el cáncer ha sido una de las prioridades de científicos de todo el mundo. La elección del tipo de tratamiento depende de distintos factores como el tipo de cáncer a tratar, las características intrínsecas del mismo, la fase en la que se encuentra o la aparición de resistencias.

Los tipos más comunes de tratamiento contra el cáncer de mama son la cirugía, la radioterapia y la terapia sistémica. La cirugía es a menudo la primera opción de tratamiento si es posible extirpar el tumor del cuerpo, aunque a veces solamente se puede eliminar parte del tumor y debe acompañarse, antes o después de la cirugía, de un tratamiento radio o quimioterapéutico, o ambos. La radioterapia utiliza la radiación producida por rayos de alta energía para destruir las células cancerosas[205,206].

La quimioterapia y la terapia hormonal son los dos principales tratamientos sistémicos. La quimioterapia se define como el uso de medicamentos dirigidos a la destrucción de las células cancerosas, entre los que se encuentran el cisplatino y el paclitaxel. Dentro de la terapia hormonal destaca el uso del tamoxifeno en el tratamiento contra el cáncer de mama[206].

1.7.1. Tamoxifeno

El tamoxifeno ha sido utilizado durante los últimos 30 años para el tratamiento y la prevención del cáncer de mama[207]. El tamoxifeno actúa principalmente a través de su unión a los ERs, modulando la expresión de genes o simplemente antagonizando los efectos del E2 sobre los ERs[207,208]. Se ha visto que es eficaz en pacientes con cáncer de mama metastásico y reduce el riesgo de recurrencia y muerte de cáncer de mama cuando se da como terapia adyuvante[209,210]. El uso del tamoxifeno está especialmente indicado en mujeres con cáncer de mama positivo para el ERα y como agente quimiopreventivo en aquellas mujeres con un alto riesgo de padecer este tipo de cáncer[206].

En los años 80 del siglo XX, el tamoxifeno se consideraba todavía simplemente un compuesto antiestrogénico no esteroideo. Sin embargo, y a pesar de sus claros efectos antiestrogénicos en el tejido mamario[211], se observó que el tamoxifeno causaba una

estimulación del crecimiento del endometrio, incrementando así el riesgo de padecer cáncer de endometrio[212,213]. Así, se hizo necesario establecer el concepto de *modulador selectivo del receptor de estrógenos* (del inglés *selective estrogen receptor modulator*, SERM), definiéndolo como un grupo de moléculas, como el tamoxifeno, que se caracterizan por su capacidad de unión a los dos tipos de ERs, ERα y ERβ, y por producir efectos agonistas o antagonistas dependiendo del tejido donde actúen[214].

La expresión de ERs es un factor importante a tener en cuenta para predecir la eficacia del tratamiento con tamoxifeno. Se ha visto que el tamoxifeno es un agonista parcial del ERα, mientras que es un antagonista puro para el ERβ[215]. Esto es, a bajas concentraciones, el tamoxifeno induce una parada del ciclo celular[216], mientras que a altas concentraciones (en el rango de micromolar) el tratamiento con tamoxifeno provoca la apoptosis de las células de cáncer de mama[217].

Además de su función como antagonista de los ERs, el tamoxifeno puede también tener otros efectos en las células cancerosas, y entre ellos destaca la disfunción mitocondrial y el aumento de la producción de ROS, llevando a las células a una situación de estrés oxidativo y, por consiguiente, a la muerte celular[218-222]. En las células de cáncer de mama esta muerte celular puede darse tanto por la inducción de la apoptosis como de la muerte celular por autofagia, incluso por una combinación de ambas[223].

1.7.2. Cisplatino

El cisplatino es uno de los tratamientos más usados y efectivos contra el cáncer. Se trata de una molécula altamente reactiva capaz de unirse al ARN, al ADN o a las proteínas formando diferentes tipos de aductos[224,225], aunque se cree que los aductos con el ADN nuclear son los que provocan, en gran medida, sus efectos citotóxicos. Las células tienen mecanismos de reparación capaces de eliminar los aductos cisplatino-ADN[226], por lo que las células con los sistemas de reparación del ADN dañados son especialmente susceptibles al tratamiento con cisplatino[227,228]. Si no son eliminados, estos aductos pueden causar un bloqueo en la replicación y/o transcripción del ADN, lo que conduce a las células a la apoptosis[229-233]. Estos efectos del cisplatino sobre el ADN nuclear explican su gran eficacia en las células con elevada capacidad de división. Sin

embargo, la citotoxicidad de este compuesto se ha detectado también en células no cancerosas, cuya capacidad de división celular está restringida[234-236], lo que sugiere que el bloqueo de la replicación del ADN no es el único mecanismo de citotoxicidad del cisplatino.

El cisplatino se puede acumular también en las mitocondrias, formando aductos con el ADN mitocondrial y las proteínas[237,238]. Como se ha comentado anteriormente, la mitocondria, aparte de ser el orgánulo responsable de la producción del 90% del ATP que necesita la célula para sobrevivir, es la principal fuente de producción de ROS. En estudios llevados a cabo en células no cancerosas, la exposición con cisplatino produjo un incremento en la producción de ROS[239-241], y el tratamiento con antioxidantes en animales de laboratorio fue capaz de revertir los efectos citotóxicos del cisplatino en diversos órganos[242-244]. Además, en las células cancerosas un incremento en los sistemas de eliminación de los ROS mitocondriales supone una mayor resistencia al cisplatino[245-248]. Todos estos estudios sugieren que la producción de ROS y el consiguiente estrés oxidativo generado en las células podría ser uno de los mecanismos de citotoxicidad del cisplatino y, además, sería un aspecto esencial en su eficacia como tratamiento anticancerígeno (Figura 9).

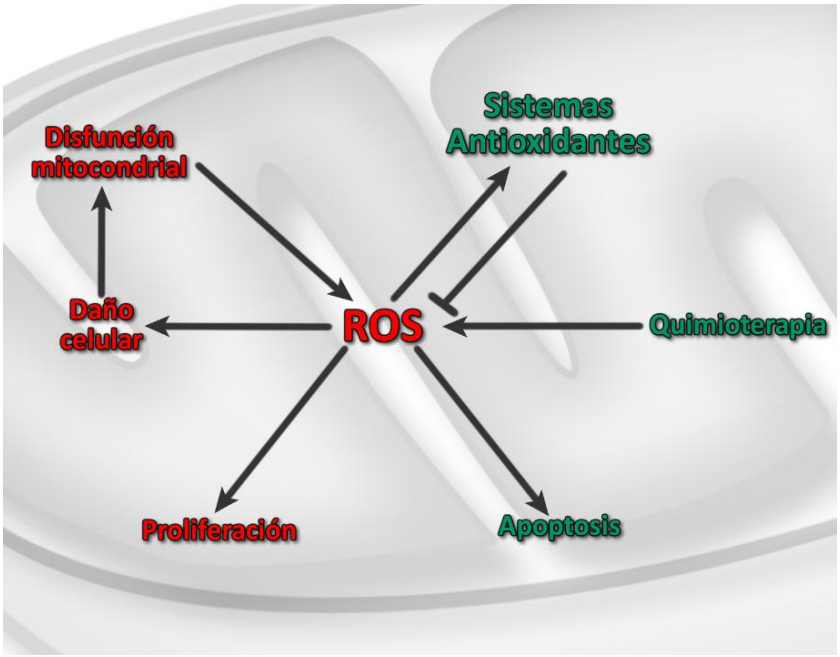


Figura 7. Representación esquemática de los efectos de la quimioterapia y el papel central que juegan los ROS.

Aunque la terapia con cisplatino no es de las más comunes en los pacientes con cáncer de mama, en una revisión de 2004, Decatris y colaboradores propusieron que una combinación del cisplatino con otros compuestos como los taxanos y la herceptina podría suponer una buena estrategia para combatir el cáncer de mama en sus distintas fases clínicas[249]. Además, en estudios recientes se ha observado que el tratamiento con cisplatino podría resultar eficaz en cáncer de mama triple negativo, caracterizado por la falta en la expresión de ER, del receptor de la progesterona y del receptor del factor de crecimiento epidérmico[250].

1.7.3. Paclitaxel

El paclitaxel es un agente citotóxico ampliamente utilizado en la terapia contra el cáncer. Su efecto principal en las células es la estabilización de los microtúbulos, impidiendo su despolimerización y, por tanto, provocando un fallo en la mitosis[251]. Asimismo, el paclitaxel también altera otras funciones celulares, donde los microtúbulos juegan un papel fundamental, como la señalización intracelular y el transporte de orgánulos[252]. Además de su principal función de estabilización de los microtúbulos, el paclitaxel puede aumentar la producción de ROS en las células cancerígenas, donde el peróxido de hidrógeno está involucrado en la muerte celular mediada por el paclitaxel *in vivo* e *in vitro*[253-255]. Sin embargo, la producción de ROS podría provenir de una disfunción en la cadena respiratoria mitocondrial ya que el peróxido de hidrógeno se forma a partir del anión superóxido (O_2^-) debido a la acción de las superóxido dismutasas, y este O_2^- es generado por la cadena respiratoria mitocondrial[256], especialmente cuando se produce una disminución de la funcionalidad de las mitocondrias. El paclitaxel es usado como tratamiento contra el cáncer de mama, aunque una de sus limitaciones es el aumento del riesgo de padecer una neuropatía periférica y reacciones de hipersensibilidad[257].

Para finalizar, cabe destacar que la selección de los tratamientos teniendo en cuenta la presencia/ausencia de ciertas proteínas importantes en el desarrollo y progresión del cáncer de mama (como la presencia del receptor de estrógenos) en cada caso ha propiciado la utilización más racional y con mayor éxito de determinadas

terapias dirigidas, lo que ha permitido que durante las dos últimas décadas la tasa de mortalidad del cáncer de mama haya disminuido aproximadamente un 30%, suponiendo una tasa de supervivencia a 5 años del 90%[258]. Por ello, en el futuro una terapia personalizada contra el cáncer de mama podría suponer la estrategia de actuación más efectiva.

2. Objetivos y planteamiento experimental / Objectives and experimental approach

El desarrollo de la presente tesis se ha enmarcado dentro de los objetivos de dos proyectos de investigación llevados a cabo en el Grupo Multidisciplinar de Oncología Traslacional (GMOT) del *Institut Universitari d'Investigació en Ciències de la Salut* (IUNICS), en la *Universitat de les Illes Balears* (UIB). El primero de estos proyectos, "Importancia de la ratio ERα/ERβ en la función mitocondrial y el estrés oxidativo en el cáncer de mama. Influencia de la leptina (PS09-01637)", tenía entre sus objetivos caracterizar la influencia de la ratio ERα/ERβ sobre la biogénesis mitocondrial y el estrés oxidativo en respuesta a dosis fisiológicas de 17β-estradiol, además de estudiar cómo la dotación de receptores estrogénicos influye sobre la respuesta a ligandos como los fitoestrógenos y el tamoxifeno, modulando la funcionalidad mitocondrial e influyendo en la proliferación celular y la apoptosis. El segundo proyecto, "Metabolismo energético de la célula tumoral: una posible diana en el tratamiento del cáncer de mama (PI12-01827)", profundiza en las diferencias existentes entre las células normales y las tumorales con especial interés en la inhibición de las UCPs como diana terapéutica y la influencia de los fitoestrógenos en los tratamientos combinados con fármacos en líneas celulares con distintas dotaciones de receptores estrogénicos prestando especial atención a la funcionalidad mitocondrial y al estrés oxidativo de las células cancerígenas.

La función y la biogénesis mitocondrial están reguladas por la unión del 17β-estradiol (E2) a los dos receptores de estrógenos (ERs), ERα y ERβ. Previos estudios llevados a cabo en nuestro laboratorio demostraron que el E2 a concentraciones fisiológicas (1 nM) afecta de manera distinta a la función mitocondrial dependiendo de la dotación de ERα y ERβ en las células de cáncer de mama[105,184]. Aunque después del tratamiento con E2 la biogénesis mitocondrial fue elevada, la funcionalidad mitocondrial no se vio afectada en las células T47D (baja ratio ERα/ERβ) ni en las MDA-MB-231 (solo pequeñas cantidades de ERβ), las cuales presentaban unos niveles bajos de estrés oxidativo y un buen funcionamiento del sistema antioxidante[56,184]. Por el contrario, el E2 provocó en las MCF-7 (alta ratio ERα/ERβ) un empeoramiento de la función mitocondrial con un aumento de los niveles de ROS[56,184]. Estas discrepancias

entre la biogénesis y la funcionalidad mitocondrial podrían ser explicadas, al menos en parte, por otros procesos de dinámica mitocondrial como la mitoptosis o mitofagia[259,260]. En condiciones normales las mitocondrias dañadas son eliminadas mediante el proceso de mitofagia[261,262], sin embargo si este proceso está inhibido se produce una acumulación de mitocondrias dañadas y poco funcionales, lo que afecta a la biogénesis y la funcionalidad mitocondrial de la célula. A partir de estos resultados, nos propusimos estudiar los efectos del E2 en la biogénesis, la dinámica y la función mitocondrial en líneas celulares de cáncer de mama con diferente dotación de ERα y ERβ. Para ello tres líneas celulares con diferente ratio ERα/ERβ, MCF-7 (alta ratio ERα/ERβ), T47D (baja ratio ERα/ERβ) y MDA-MB-231 (solo ERβ en pequeñas cantidades), además de otra línea de cáncer de mama con una sobreexpresión modulable del ERβ fueron tratadas con concentraciones fisiológicas de E2 (1 nM). La función mitocondrial se analizó mediante las actividades enzimáticas de proteínas mitocondriales como la citrato sintasa, la ATPasa y en especial la citocromo c oxidasa (COX), así como los niveles de expresión de las proteínas del complejo OXPHOS y los niveles de cardiolipina (lípido exclusivo de la membrana interna mitocondrial) mediante la naranja de acridina (NAO) y el ADN mitocondrial. La biogénesis mitocondrial se midió determinando la expresión de genes relacionados con este proceso como nrf1, nrf2, pgc1α, tfam y mtssb; y la dinámica mitocondrial se estudió midiendo los niveles de expresión génica de los cinco principales genes que participan en este proceso, mfn1, mfn2 y opa1 (fusión mitocondrial) y drp1 y fis1 (fisión mitocondrial). Los resultados y conclusiones obtenidas en este estudio se detallan en el **Manuscrito 1**.

El cáncer de mama es una de las principales causas de mortalidad en mujeres[263], y aproximadamente el 75% de casos de este tipo de cáncer son positivos para el ERα[264]. Aunque actualmente no se estudia de manera rutinaria la presencia del ERβ, estudios previos en nuestro laboratorio demostraron la importancia del ERβ en tumores de cáncer de mama, ya que su presencia hace que las células cancerígenas tengan más resistencia al estrés oxidativo[265]. Muchos tratamientos contra el cáncer de mama como el cisplatino (CDDP), el paclitaxel (PTX) y el tamoxifeno (TAM) tienen como uno de sus mecanismos de acción la disminución de la funcionalidad

mitocondrial con el consiguiente aumento en la producción de ROS y, en muchos casos, la muerte celular[247,266,267]. Teniendo en cuenta que según la ratio ERα/ERβ que tengan las células cancerígenas podrían ser más o menos resistentes al estrés oxidativo generado por los agentes citotóxicos ya mencionados, nos propusimos como objetivo encontrar la relación entre esta ratio ERα/ERβ y la respuesta a los tratamientos citotóxicos en las células de cáncer de mama con diferente dotación de ERs: MCF-7, T47D, MCF7+ERβ (línea celular obtenida a partir de las MCF-7 en la cual se ha insertado establemente el gen del ERβ) y T47D-ERβ (células T47D a las que se les ha inhibido transitoriamente el ERβ mediante un siRNA —del inglés *small interfering RNA*— específico). Parámetros como la viabilidad celular (Cristal Violeta), la producción de ROS (mediante un kit de reactivo Amplex Red®), el potencial de membrana interna mitocondrial (TMRM), la masa mitocondrial (Mitotracker Green FM®) tanto en una medida fluorimétrica cuantitativa como en una medida visual con el microscopio confocal, la apoptosis (Anexina V), la autofagia (*monodansylcadaverine*) y la funcionalidad mitocondrial (actividades y niveles proteicos de ATPasa y COX) fueron estudiados después del tratamiento con los agentes citotóxicos durante 48 horas. Los resultados y las conclusiones que generaron estos experimentos se detallan en el **Manuscrito 2**.

El estrés oxidativo juega un papel crucial en numerosas patologías, en las que se incluye el cáncer[268]. Resultados previos en nuestro laboratorio han demostrado la influencia que tienen los ROS en la inducción y la progresión del cáncer de mama[56,184,265]. Sin embargo, unos elevados niveles de ROS pueden provocar la muerte celular[188,269] y, como ya se ha comentado anteriormente, los tratamientos contra el cáncer como el CDDP y el TAM tienen como uno de sus mecanismos de acción precisamente la generación de estrés oxidativo para inducir la muerte celular en las células cancerígenas[222,247]. Numerosos estudios sugieren que el desacoplamiento mitocondrial podría ser un mecanismo de regulación del estrés oxidativo[155,156,270] y las proteínas desacoplantes (UCPs) podrían jugar un papel clave en la respuesta de la célula al estrés oxidativo disminuyendo los niveles de ROS, por lo que se cree que forman parte del sistema antioxidante de la célula[270,271]. De los cinco tipos de UCPs que se han descubierto, la UCP2 es la más ubicua, presenta una expresión significativa

en la glándula mamaria y está envuelta en la regulación del estrés oxidativo en la célula[175,184,186]. Se cree que la UCP2 podría tener un papel dual en las células, actuando como un mecanismo protector en las células no cancerosas, mientras que su sobreexpresión podría conferir quimiorresistencia a las células cancerígenas mediante la bajada de la producción de ROS[166]. Por lo tanto, con el objetivo de averiguar si la UCP2 podría ser una diana terapéutica para evitar quimiorresistencias a tratamientos que promueven el estrés oxidativo como método de inducción de la muerte celular, se analizó si la inhibición de la UCP2 con *genipin* (inhibidor de la función desacoplante de la UCP2) o su silenciamiento parcial con un siRNA específico podría incrementar el estrés oxidativo en la línea celular de cáncer de mama MCF-7, haciéndola más sensible a un tratamiento con CDDP o TAM durante 48 horas. En este estudio se midió la viabilidad celular y la clonogenicidad (Cristal Violeta), la producción de ROS (reactivo Amplex Red®), el daño oxidativo en proteínas y lípidos (grupos carbonilo y 4-HNE), el potencial de membrana (TMRM), los niveles proteicos de UCP2, la apoptosis (Anexina V y rotura de la PARP) y la autofagia (*monodansylcadaverine* y ratio LC3-II/LC3-I). Además algunos de estos parámetros (viabilidad celular, producción de ROS, apoptosis y autofagia) también se estudiaron en la línea celular de cáncer de mama T47D para evitar defectos dependientes del tipo celular. Finalmente también se representaron las curvas de supervivencia Kaplan-Meier para evaluar el impacto que tiene la expresión de UCP2 en los pacientes de cáncer de mama que están siendo tratados o no usando datos de estudios depositados en bases de datos de acceso libre. Todos los resultados y las conclusiones de este estudio se presentan en el **Manuscrito 3**.

Numerosos estudios epidemiológicos han mostrado una menor incidencia de cáncer de mama en países asiáticos, especialmente en aquellos donde el consumo de soja es habitual, como Japón[272]. El fitoestrógeno genisteína (GEN) es la principal isoflavona de la soja y se le han atribuido efectos beneficios para la salud[273]. La GEN ejerce su función a través de su interacción con los ERs, ERα y ERβ. El ERα se ha asociado a procesos proliferativos, mientras que el ERβ ha sido relacionado con efectos citostáticos y de diferenciación[274-276]. Estudios previos en nuestro grupo de investigación demostraron que el E2 provoca una mayor supervivencia celular en líneas celulares de cáncer de mama con un alta ratio ERα/ERβ, no así en aquellas con

un baja ratio ERα/ERβ[56]. Además existen estudios que sugieren que la GEN podría mejorar la funcionalidad mitocondrial[277]. Así pues el objetivo que nos planteamos fue el de investigar los efectos de concentraciones fisiológicas de E2 (1 nM) y de GEN (1 μM), y su combinación en la viabilidad y proliferación celular, el ciclo celular y la apoptosis, así como la funcionalidad y dinámica mitocondrial en líneas celulares de cáncer de mama con distinta ratio ERα/ERβ, MCF-7, T47D y MDA-MB-231. La viabilidad celular se estudió mediante la tinción con Cristal Violeta, el ciclo celular se midió con citometría de flujo con una tinción de ioduro de propidio, la proliferación celular y la apoptosis se analizaron mediante la expresión de proteínas relacionadas con dichos procesos (fosforilación de Stat-3 y rotura de la PARP, respectivamente). Para el estudio de la funcionalidad mitocondrial se examinó la actividad de la ATPasa y la COX. Finalmente se estudió también la dinámica mitocondrial mediante la expresión de los cinco principales genes relacionados con este proceso (mfn1, mfn2, opa1, drp1 y fis1). Los resultados y las principales conclusiones de este trabajo se desarrollan en el **Manuscrito 4**.

Teniendo en cuenta los resultados obtenidos en los cuatro primeros manuscritos, nos planteamos estudiar los posibles efectos que podrían tener concentraciones fisiológicas de GEN en la respuesta de las células de cáncer de mama a los tratamientos antitumorales como CDDP, PTX o TAM. Por tanto, si la presencia del ERβ promueve una mayor resistencia a los tratamientos citotóxicos mediante una mejor respuesta al estrés oxidativo y la GEN tiene una mayor afinidad por el ERβ[31,32] y disminuye la producción de ROS[278], consideramos oportuno el estudio de la eficacia de dichos tratamientos en combinación con concentraciones fisiológicas (1 μM) de GEN. Con el objetivo de conseguir unas condiciones más fisiológicas, los experimentos realizados en este trabajo se llevaron a cabo en un medio de cultivo en el que había una concentración de compuestos estrogénicos equivalente a 0,5 nM de E2, correspondiente a concentraciones plasmáticas de E2 en mujeres premenopáusicas[279]. Los análisis se desarrollaron en dos líneas celulares de cáncer de mama con diferente ratio ERα/ERβ: MCF-7 (alta ratio ERα/ERβ) y T47D (baja ratio ERα/ERβ). Se estudiaron parámetros como la viabilidad celular (Cristal Violeta), la producción de ROS (reactivo Amplex Red®), la apoptosis (Anexina V y rotura de la PARP), autofagia

(*monodansylcadaverine* y ratio LC3-II/LC3-I) y el ciclo celular por citometría de flujo después del tratamiento con GEN y/o los compuestos citotóxicos durante 48 horas. Además algunos de estos parámetros (viabilidad celular, producción de ROS, apoptosis y autofagia) se estudiaron también en la línea MCF-7 con la sobreexpresión estable del ERβ (MCF7+ERβ) con el objetivo de eliminar cualquier interferencia de algún factor intrínseco a cada una de las líneas celulares estudiadas y confirmar que los diferentes efectos que ejerce la GEN en las dos líneas celulares son debidos a la diferente ratio ERα/ERβ. Se pueden ver detallados todos los resultados y las conclusiones de este trabajo en el **Manuscrito 5**.

Las investigaciones presentadas en esta tesis doctoral fueron desarrolladas bajo la dirección de la Dra. Mª del Pilar Roca Salom y del Dr. Jordi Oliver Oliver en el Grupo Multidisciplinar de Oncología Traslacional. Durante el periodo de realización de la tesis, el doctorando recibió una beca predoctoral de personal investigador concedida por la *Conselleria d'Educació, Cultura i Universitats del Govern de les Illes Balears*, la cual está enmarcada dentro de un programa operativo cofinanciado por el Fondo Social Europeo (FSE). Además, la realización de este trabajo ha sido posible gracias a los proyectos de investigación financiados por el Fondo de Investigaciones Sanitarias del Gobierno de España (PS09-01637 concedido en el año 2009 y PI12-01827 concedido en el 2012), así como a las ayudas de la *Comunitat Autònoma de les Illes Balears* (CAIB), cofinanciadas con fondos FEDER "Una manera de hacer Europa" (31/2011 and AAEE22/2014). Asimismo, se ha contado también con la financiación del Centro de Investigaciones Biomédicas en Red de Fisiopatología de la Obesidad y la Nutrición (Ciberobn, CB06/03) del Instituto de Salud Carlos III. Finalmente, cabe destacar que el doctorando realizó una estancia de tres meses en el *Imperial College* en el *Hammersmith Hospital* de Londres, concretamente en el *Department of Surgery and Cancer, Division of Cancer* bajo la tutela del Prof. Simak Ali.

The development of this thesis has been framed within the objectives of two research projects conducted at the Multidisciplinar Group of Translational Oncology (GMOT) of University Research Institute on Health Sciences (IUNICS), at the University of Balearic Islands (UIB). The first project, called 'Importance of ERα/ERβ ratio in the mitochondrial function and the oxidative stress in breast cancer. Influence of leptin (PS09-01637)', presented several objectives such as to characterize the influence of the ERα/ERβ ratio on the mitochondrial biogenesis and oxidative stress in response to physiological doses of 17β-estradiol, in addition to studying how this ERα/ERβ ratio influences the response to ligands such as phytoestrogens and tamoxifen, modulating mitochondrial functionality and influencing cell proliferation and apoptosis. The second project, 'Energetic metabolism of cancer cell: a possible target in breast cancer treatment (PI12-01827)', explores the differences between normal and tumor cells with particular interest in inhibiting the UCPs as a therapeutic target and the influence of phytoestrogens in combination therapies with drugs in cell lines with different ERα/ERβ ratio with particular attention to the mitochondrial functionality and oxidative stress in cancer cells.

Mitochondrial function and biogenesis are regulated by the binding of the 17β-estradiol (E2) to both estrogen receptors (ERs), ERα and ERβ. Previous studies carried out in our laboratory demonstrated that physiological concentrations of E2 (1 nM) affect mitochondrial function in a different way depending on the amounts of ERα and ERβ in breast cancer cells.[105,184]. Although after E2 treatment the mitochondrial biogenesis was increased, the mitochondrial functionality was unaffected in the T47D cells (low ERα/ERβ ratio) or in the MDA-MB-231 cells (only small amounts of ERβ), which had a low level of oxidative stress and a good antioxidant system performance[56,184]. On the contrary, E2 treatment worsened the mitochondrial function in MCF-7 cells (high ERα/ERβ ratio), accompanied by an increase of ROS levels[56,184]. Such discrepancies between mitochondrial biogenesis and functionality could be explained, at least in part, by other processes of the mitochondrial dynamics such as mitoptosis or mitophagy[259,260]. In normal conditions damaged mitochondria are removed through the process called mitophagy[261,262]; however, if this process is

inhibited an accumulation of damaged and nonfunctional mitochondria occurs, affecting the mitochondrial biogenesis and functionality of the cell. From these results, we aimed to study the effects of E2 in the mitochondrial biogenesis, dynamics and function in breast cancer cells with different amounts of ERα and ERβ. To do this, three breast cancer cell lines with different ERα/ERβ ratio, MCF-7 (high ERα/ERβ ratio), T47D (low ERα/ERβ ratio) y MDA-MB-231 (only small amounts of ERβ), in addition to another breast cancer cell line with an inducible ERβ overexpression were treated with physiological concentrations of E2 (1 nM). Mitochondrial function was analyzed through the enzymatic activities of the mitochondrial proteins citrate synthase, ATPase and especially citochrome c oxidase (COX), as well as the expression levels of the OXPHOS complex proteins and the levels of cardiolipin (exclusive lipid of the inner mitochondrial membrane) by the Nonyl Acridine Orange (NAO) and the mitochondrial DNA. Mitochondrial biogenesis was measured by determining the expression of genes related to this process such as nrf1, nrf2, pgc1α, tfam and mtssb; and mitochondrial dynamics was studied measuring the expression levels of the five main genes involved in this pathway: mfn1, mfn2 and opa1 (mitochondrial fusion); drp1 and fis1 (mitochondrial fission). Results and conclusions obtained in this study are detailed in the **Manuscript 1**.

Breast cancer is a leading cause of death in women[263], and approximately 75% of cases of this cancer are positive for ERα[264]. Although currently the presence of ERβ is not studied routinely, previous studies in our laboratory demonstrated the importance of ERβ in breast cancer tumors, as its presence makes cancer cells more resistant to the oxidative stress[186]. Many breast cancer treatments such as cisplatin (CDDP), paclitaxel (PTX) and tamoxifen (TAM) are able to decrease the mitochondrial functionality leading to increased ROS production and, in many cases, cell death[247,266,267]. Considering that according to ERα/ERβ ratio cancer cells may be more or less resistant to oxidative stress generated by the aforementioned cytotoxic agents, we set the goal of finding the relationship between this ERα/ERβ ratio and the response to cytotoxic treatments of breast cancer cells with different ERα/ERβ ratio: MCF-7, T47D, MCF7+ERβ (cell line derived from MCF-7 in which is stably inserted the ERβ gene) and T47D-ERβ (T47D cells with partially silenced ERβ by a specific siRNA —

small interfering RNA—). Parameters such as cell viability (Crystal Violet), ROS production (by an Amplex Red® kit), mitochondrial membrane potential (TMRM), mitochondrial mass (Mitotracker Green FM®) both a quantitative fluorimetric measurement and a visual measurement using a confocal microscope, apoptosis (Annexin V), autophagy (monodansylcadaverine) and the mitochondrial functionality (ATPase and COX protein levels and activities) were measured after cytotoxic treatments for 48 hours. Results and conclusions generated by these experiments are detailed in the **Manuscript 2**.

The oxidative stress plays a crucial role in several pathologies, including cancer[268]. Previous results obtained in our laboratory demonstrated the influence of ROS in the breast cancer induction and progression[56,184,186]. However, high ROS levels may lead to cell death[15,16] and, as mentioned above, anticancer treatments such as CDDP and TAM are able to generate oxidative stress in order to induce cancer cells death[12,17]. Several studies have suggested that mitochondrial uncoupling could be a mechanism by which cells regulate the oxidative stress[155,156,270] and the uncoupling proteins (UCPs) may play a key role in the response of cells to oxidative stress decreasing the ROS levels, so it is believed that UCPs are part of the antioxidant system of the cell[16,18]. Of the five types of UCPs that have been discovered, the UCP2 is the most ubiquitous; it presents a significant expression in the mammary gland and it is involved in the regulation of oxidative stress in the cell[175,184,186]. UCP2 may have a dual role in cancer, acting as a protective mechanism in normal cells, while its overexpression in cancer cells may confer resistance to chemotherapy and a higher survival by the down-regulation of ROS levels[166]. Therefore, in order to determine whether the UCP2 could be a therapeutic target to prevent resistance to treatments which promote oxidative stress as a method of inducing cell death, it has been analyzed if the UCP2 inhibition with genipin (inhibitor of uncoupling function of UCP2) or its partial silencing by a specific siRNA could increase oxidative stress in the MCF-7 or T47D breast cancer cell lines, making these cells more sensitive to the treatment with CDDP or TAM for 48 hours. In this study the cell viability and the clonogenic assay (Crystal Violet), the ROS production (by an Amplex Red® kit), the oxidative damage in protein and lipids (carbonyl groups and 4-HNE), the mitochondrial membrane potential

(TMRM), the UCP2 protein levels, the apoptosis (Annexin V and PARP cleavage) and the autophagy (monodansylcadaverine and LC3-II/LC3-I ratio) were measured after cytotoxic treatments for 48 hours. Moreover, some of these parameters (cell viability, ROS production, apoptosis and autophagy) were also studied in the T47D breast cancer cell line in order to prevent cell type-dependent flaws in this study. Finally, Kaplan-Meier survival curves were performed to evaluate the impact of the UCP2 expression in breast cancer patients being treated or not using survey data stored in available free databases. All results and conclusions of this study are presented in the **Manuscript 3**.

Several epidemiological studies have showed a lower incidence of breast cancer in Asian countries, especially where soy consumption is common, as Japan[272]. The phytoestrogen genistein (GEN) is the major isoflavone found in soybeans and some beneficial health effects have been attributed to its consumption[273]. GEN exerts its function through its interaction with the ERs, ERα and ERβ. The ERα has been associated with proliferative events, while ERβ has been related to cytostatic and differentiation processes[274-276]. Previous studies carried out in our research group demonstrated that E2 provoked a greater cell survival in the breast cancer cell line with a high ERα/ERβ ratio, but not in those with a low ERα/ERβ ratio[56]. Furthermore, other studies suggest that GEN could improve mitochondrial functionality[277]. So the main objective we set was to investigate the effects of physiological doses of E2 (1 nM), GEN (1 μM) and their combination on the cell viability and proliferation, the cell cycle and the apoptosis, as well as the mitochondrial functionality and dynamics in breast cancer cell lines with different ERα/ERβ ratio: MCF-7, T47D and MDA-MB-231. Cell viability was studied by Crystal Violet staining, the cell cycle was measured by flow cytometry with propidium iodide staining, cell proliferation and apoptosis were analyzed through the expression of proteins related to these processes (Stat-3 phosphorylation and PARP cleavage, respectively). For the study of the mitochondrial functionality, ATPase and COX activities were examined. Finally, the mitochondrial dynamics was also studied through the expression of the five main genes involved in this process (mfn1, mfn2, opa1, drp1 and fis1). The results and the main conclusions of this work are developed in the **Manuscript 4**.

Considering the results obtained in the first four manuscripts, we planned to study the possible effects of physiological doses of GEN on the response of breast cancer cells to anticancer treatments such as CDDP, PTX or TAM. Therefore, if the presence of the ERβ promotes a greater resistance of breast cancer cells to cytotoxic treatments through a better response to oxidative stress and GEN has higher affinity for ERβ[31,32] and decreases ROS production[278], we consider the study of the efficacy of the mentioned treatments in combination or not with physiological concentrations of GEN (1 μM). With the aim to achieve more physiological conditions, the experiments performed in this work were carried out in a culture medium containing a concentration of estrogenic compounds equivalent to 0.5 nM E2, which correspond to plasmatic concentrations of E2 in premenopausal women[279]. The analyses were developed in two breast cancer cell lines with different ERα/ERβ ratio: MCF-7 (high ERα/ERβ ratio) and T47D (low ERα/ERβ ratio). Parameters such as cell viability (Crystal Violet), ROS production (by an Amplex Red® kit), apoptosis (Annexin V and PARP cleavage), autophagy (monodansylcadaverine and LC3-II/LC3-I ratio) and cell cycle by flow cytometry were measured after GEN and/or cytotoxic treatments for 48 hours. Moreover, some of these parameters (cell viability, ROS production, apoptosis and autophagy) were also studied in the MCF-7 cell line overexpressing the ERβ (MCF7+ERβ) in order to avoid any interference from any intrinsic factor to each of the cell lines tested and confirm that the different effects exerted by the GEN in the two cell lines studied are due to the different ERα/ERβ ratio. All the results and conclusions obtained in this work can be observed in the **Manuscript 5**.

The research presented in this thesis was developed under the direction of Prof. Pilar Roca Salom and Dr. Jordi Oliver Oliver at the Multidisciplinar Group of Translational Oncology. During the realization of the thesis, the PhD student received a PhD scholarship granted by *Conselleria d'Educació, Cultura i Universitats del Govern de les Illes Balears*, which is framed within an operational program co-financed by the *Fondo Social Europeo* (FSE). Furthermore, the achievement of this work has been possible thanks to research projects funded by *Fondo de Investigaciones Sanitarias del Gobierno de España* (PS09-01637 granted in 2009 and PI12-01827 granted in 2012), as well as to the economic assistance of the *Comunitat Autònoma de les Illes Balears*

(*CAIB*), co-financed with funds from *FEDER "Una manera de hacer Europa"* (31/2011 and AAEE22/2014). Moreover, funding from *Centro de Investigaciones Biomédicas en Red de Fisiopatología de la Obesidad y la Nutrición* (Ciberobn, CB06/03) from the *Instituto de Salud Carlos III* was also received. Finally, it is important to note that the PhD student conducted a three-month stay at the Imperial College in the Hammersmith Hospital of London, specifically in the Department of Surgery and Cancer, Division of Cancer, under the tutelage of Prof. Simak Ali.

3. Manuscripts

Manuscript 1

The over-expression of ERbeta modifies estradiol effects on mitochondrial dynamics in breast cancer cell line

Sastre-Serra J, Nadal-Serrano M, Pons DG, Roca P, Oliver J

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The over-expression of ERbeta modifies estradiol effects on mitochondrial dynamics in breast cancer cell line

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ABSTRACT

Mitochondrial biogenesis and function are under the control of 17β-estradiol, which acts through two distinct estrogen receptors (alpha or beta), and the estrogen receptors ratio can determine the final effect of 17β-estradiol on mitochondria. Our aim was to study the effects of 17β-estradiol on mitochondrial biogenesis, dynamics and function in breast cancer cell lines with different estrogen receptors ratios. Mitochondrial biogenesis was increased in MDA-MB-231 (with only estrogen receptor beta expression), T47D (normal estrogen receptors ratio) and MCF-7 (highest estrogen receptors ratio) breast cancer cell lines, in response to different mitochondrial and cellular status. In fact, mitochondria of the MDA-MB-231 and T47D cell lines maintained their functionality, although, the MCF-7 cell line did suffer an important decrease in mitochondrial function. Thus, mitochondrial biogenesis increased in MCF-7 with the aim of mitigating these defective mitochondria. In normal conditions, mitophagic processes remove defective mitochondria to refresh the mitochondrial pool. Mitochondrial dynamics were also under control by 17β-estradiol, and showed modifications in the fusion/fission processes and the modulation of mitochondrial removal. In fact, cells with only estrogen receptor beta or with a low estrogen receptors ratio, such as MDA-MB-231 and T47D, showed an increase in fusion processes. However, the MCF-7 cell line, with more estrogen receptor alpha, also showed an increase in fusion processes, even though the fission processes were diminished and led to an accumulation of unfunctional mitochondria. Finally, the importance of estrogen receptor beta in mitochondrial biogenesis, function, as well as in mitochondrial dynamics was examined. Using the T47D-estrogen receptor beta tetracycline-inducible cell line, the results confirmed that when the overexpression of estrogen receptor beta was inhibited, there was an increase in mitochondrial biogenesis, although these mitochondria were less functional, and with fewer fission events, although there was an increase in fusion processes.

1. Introduction

Mitochondria are highly dynamic organelles that undergo constant cycles of division and fusion (Rambold et al., 2011). Mitochondrial function and regulation is a very complex process that depends on mitochondrial biogenesis and mitochondrial dynamics (Rambold et al., 2011). Mitochondrial biogenesis is the combination of both proliferation (an increase in the mitochondrial population) and differentiation (an improvement of the functional capabilities of pre-existing mitochondria) processes (Justo et al., 2005), while mitochondrial dynamics is a concept that describes the morphology and distribution of mitochondria in the cell (Liesa et al., 2009).

During stress events, mitochondrial turnover can be accelerated by an autophagyc process called mitophagy, and several studies have shown that mitochondrial fission is also coordinated with mitophagy (Lemasters, 2005). Recently, it has been described that mitochondrial fission and fusion events are crucial for normal mitochondrial function (Nakada et al., 2001). In the context of mitochondrial physiology, fission is necessary for the correct redistribution of mitochondrial DNA during cell division (Ong and Hausenloy, 2010) and for transporting mitochondria to daughter cells during mitosis and meiosis (Hales, 2004), while fusion is the mechanism by which neighboring mitochondrial membranes are joined together. This latter process occurs as a means to recover the activities of damaged/depolarized membranes, thereby ensuring the proper distribution of metabolites and mitochondrial DNA (mtDNA) (Rambold et al., 2011).

Under normal conditions, mitochondrial fission and fusion occur at a balanced rate, so that a relatively constant tubular morphology is maintained (Huang et al., 2011). However, perturbation of the fission/fusion balance causes mitochondrial deformation, a

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condition that has been shown to be associated with numerous human diseases (Bossy-Wetzel et al., 2003; Chen and Chan, 2005).

In cancer, autophagy up-regulation can provide tumor cells with several survival advantages, and it has been shown that autophagy-deficient mice are more likely to develop tumors (Takamura et al., 2011). Furthermore, in response to cancer therapeutic agents, an up-regulation in autophagy may help to eliminate the surviving, yet drug-damaged cancer cells resistant to chemotherapy. In mitochondrial physiology, autophagy prevents the cancer cells from accumulating dysfunctional mitochondria (Guo et al., 2011).

The purpose of this investigation was to follow up previous studies done in breast cancer cell lines were E2 treatment provoked an increase in mitochondrial biogenesis, and depending on the prevailing estrogen receptors ratio (ERα/ERβ), dysfunctional mitochondria can be produced. The focus was to determine if mitochondrial dynamics could explain these discrepancies between mitochondrial biogenesis and function in response to E2 treatment. To address this aim, breast cancer cell lines with different ERα/ERβ ratios and a breast cancer cell line overexpressing ERβ were studied, with 17β-estradiol (E2) treatment provided at physiological concentrations. Mitochondrial function was measured by mitochondrial citrate synthase, cytochrome c oxidase and ATPase activities and OXPHOS complex expression; mitochondrial biogenesis was assayed as expression of nrf1, nrf2, pgc1α, tfam and mtssb; mtDNA and cardiolipin content (NAO); and mitofusin 1 and 2 (mfn1 and mfn2), opa1, drp1, and fis1 expression were determined for mitochondrial dynamic assay.

2. Materials and methods

2.1. Materials

Specific reagents 17β-estradiol (E2), puromycin, G418, Doxycycline (Dox) and Nonyl Acridine Orange were purchased from Sigma–Aldrich (St. Louis, MO, USA). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich (St. Louis, MO, USA), Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Cell culture and treatments

Breast cancer cell lines T47D, MDA-MB-231 and MCF-7 were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37 °C. To evaluate the effects of E2, cells were grown in phenol red-free DMEM containing 10% charcoal-stripped FBS 24 h prior to treatment. Experiments were performed when cell cultures reached confluence, which was achieved by providing fresh media supplemented with 1 nmol/l of E2 (Sigma–Aldrich) during 48 h for Western blot, mtDNA quantification and cardiolipin content assay; and 12 h for RT-PCR experiments.

The T47D-ERβ tetracycline-inducible cell line was kindly provided by Dr. J-A Gustafsson and Dr. A. Ström (CNRCS, University of Houston). T47D-ERβ cells were cultured in a 1:1 mixture of Ham's nutrient mixture F12 and DMEM (DMEM:F12) supplemented with 10% FBS and at the same temperature and humidity conditions and in the presence of 1000 ng/ml tetracycline (doxycycline) to fully inhibit ERβ expression (Strom et al., 2004). Before the experiment, cells were reselected with 0.5 μg puromycin/ml and G418 500 μg/ml was used as selection marker to prevent loss of ERβ and EGFP expression and the concurrent change in phenotype. At the same time, the cell medium for the other cell lines was changed by adding phenol red-free DMEM:F12 supplemented with 10% charcoal-stripped FBS 24 h before E2 treatment. E2 was added to the medium at final concentration of 1 nmol/l at the beginning of the treatment during 48 h for Western blot and activities determinations; and 4 h for RT-PCR experiments.

2.3. Analysis of cardiolipin content and mtDNA quantification

Cardiolipin content was assayed using Nonyl Acridine Orange (NAO) fluorescence and quantified using a microplate fluorescence reader FLx800 (Bio-Tek Winooski, Vermont, USA) (Sastre-Serra et al., 2012).

Quantification of mtDNA after DNA isolation was performed using TriPure ® Roche (Barcelona, Spain), with the primers for NADH dehydrogenase subunit 4 (mtDNA) and for 18S (nDNA) by RT-PCR with the same primers and conditions that previously described (Sastre-Serra et al., 2012).

2.4. Citrate synthase, cytochrome c oxidase and ATPase activities

The cell lysates used for enzymatic activities were obtained by scraping cells in RNAase-free water. CS (citrate synthase; EC 2.3.3.1) activity was determined by monitoring the increase in absorption of the 5-thio-2-nitrobenzoate ion at 412 nm and 30 °C (Nakano et al., 2005). Complex IV or COX (cytochrome c oxidase; EC 1.9.3.1) activity was measured by a spectrophotometric method (Chrzanowska-Lightowlers et al., 1993). ATPase (ATP phosphohydrolase, Complex V, EC 3.6.1.3) activity was measured by monitoring the oxidation of NADH at 340 nm and 37 °C (Ragan et al., 1987), with an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

2.5. Real time PCR

Total RNA was isolated from cultured cells using TriPure® isolation reagent and quantified using a spectrophotometer set at 260 nm. One μg of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10 μl volume of retrotranscription reaction mixture containing: 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNAase inhibitor and 500 μM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−70 °C) until the PCR were carried out.

PCR was done for target genes: Estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), mitofusin-1 (mfn1), mitofusin-2 (mfn2), optic atrophy-1 (opa1), dynamin-related protein-1 (drp1), mitochondrial fission protein 1 (fis1), peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC1α), nuclear respiratory factors 1 and 2 (NRF1 and NRF2), mitochondrial transcription factor A (TFAM), mitochondrial single strand DNA binding protein (mtSSB) and 18S (such as housekeeping), using specific primers (see Table 1) with SYBR Green technology and in a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μL, containing 6.5 μl Lightcycler® 480 SYBR Green I Master, 0.5 μM of the sense and antisense specific primers and 2.5 μl of the cDNA template. The amplification program consisted of a preincubation step for template cDNA denaturation (5 min, 95 °C), followed by 45 cycles consisting of a denaturation step (10 s, 95 °C), an annealing step (10 s, T^a is detailed in Table 1) and an extension step (12 s, 72 °C). A negative control lacking cDNA template was run in each assay.

The resulting PCR products were resolved on a 2% agarose gel in 45 mM Tris–borate–EDTA 1 mM buffer (pH 8.0) and visualized by ethidium bromide staining. Bands were analyzed with a Chemidoc XRS densitometer (Biorad). The Ct values of the real-time PCR were analyzed, taking into account the reaction efficiency and referring these results to the total DNA amount, using the GenEx Standard Software (MultiDAnalises, Sweden).

Table 1
Primers and conditions used for RT-PCR.

Gene	Forward primer (5′–3′) Reverse primer (5′–3′)	T^a An. (°C)	Gene	Forward primer (5′–3′) Reverse primer (5′–3′)	T^a An. (°C)	Gene	Forward primer (5′–3′) Reverse primer (5′–3′)	T^a An. (°C)
			mfn1	TTggAgCggAgACTTAgCAT TTCgATCAAgTTCCggATTC	51	nrf1	CCCgTTACAgggAggTgAg TgTAgCTCCCTgCTgCATCT	60
18S	ggACACggACAggATTgACA ACCCACggAATCgAgAAAgA	61	mfn2	AgAggCATCAgTgAggTgCT gCAgAACTTTgTCCCAgAgC	56	nrf2	gCgACggAAAgAgTATgAgC gTTggCAgATCCACTggTTT	60
ERα	AATTCAgATAATCgACgCCAg gTgTTTCAACATTCTCCCTCCTC	61	opa1	ggCCAgCAAgATTAgCTACg ACAATgTCAggCACAATCCA	51	pgc1α	TCAgTCCTCACTggTggACA TgCTTCgTCgTCAAAAACAg	60
ERβ	TAgTggTCCATCgCCAgTTAT gggAgCCACACTTCACCAT	54	drp1	AAgAACCAACCACAggCAAC gTTCACggCATgACCTTTTT	51	tfam	AgATTggggTCgggTCACT CAAgACAgATgAAAACCACCTC	61
			fis1	CTTgCTgTgTCCAAgTCCAA gCTgAAggACgAATCTCAgg	53	mtssb	TgTgAAAAAggggTCTCgAA TggCCAAAgAATCATCC	60

T^a An., annealing temperature; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; mfn-1, mitofusin 1; mfn-2, mitofusin 2; opa-1, optic atrophy 1; drp-1, dynamin-related protein q; fis-1, mitochondrial fission 1 protein; nrf1 and nrf2, nuclear respiratory factor 1 and 2; pgc1α, peroxisome proliferator-activated receptor-gamma coactivator-1alpha; tfam, mitochondrial transcription factor A; mtssb, mitochondrial single strand DNA binding protein.

2.6. Western blotting

Cell lysates were obtained by scraping cells in lysis buffer (20 mM Tris–HCl, 1.5 mM $MgCl_2$, 140 mmol/L NaCl, 0.5% Nonidet P-40, 10% Glycerol, 1 mmol/L EGTA, 1 mmol/L $NaVO_3$, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin; pH 7.4). Protein content was measured with a BCA protein assay kit (Pierce, Bonn, Germany).

For Western blot analysis, 40 μg of protein from cell lysate was fractionated by SDS-PAGE (12% polyacrylamide gel) and electro-transferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in TBS-T (20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20). Antisera against PGC1α (Millipore, MA, USA), Mitoprofile® Total OXPHOS Human WB Antibody Cocktail (Mitoscience, OR, USA), ERα, ERβ and Tubulin (Santa Cruz Biotechnologies, CA, USA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Biorad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-rad) and analyzed with Quantity One Software (Bio-rad).

2.7. Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc., Chicago, IL) was used for all statistical analyses. Data are presented as means ± standard error of the mean (SEM). Statistical differences between groups were analyzed by Student's *t*-test. Statistical significance was set at $P < 0.05$.

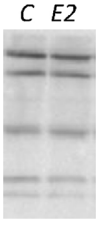
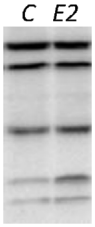
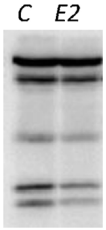
3. Results

The E2 treatments affected both mitochondrial biogenesis and dynamics in breast cancer cell lines with different levels of estrogen receptor subtypes. After a 24 h E2 treatment of the MCF-7 cell line (which has the highest ERα/ERβ ratio), all OXPHOS complexes of mitochondrial respiratory chain (except ATPase (Complex V)) showed a statistically significant decrease (Table 2), and fell off up to 70% for complex IV. There were no changes in MDA-MB-231 and T47D cell lines (Table 2), nor in lines with only the ERβ subtype or in lines with similar ERα and ERβ levels. However, it is of interest to note that the trend of an increase in complex IV due to E2 treatment was only present in the T47D cell line (86%).

The E2 modified mtDNA copy number showed a statistically significant increase in MCF-7 cell line (37%), yet there was a decrease in the MDA-MB-231 and T47D cell lines (13% and 10%, respectively), after 24 h of E2 treatment, as shown in Table 3. Furthermore, NAO fluorescence only decreased in the MDA-MB-231 cell line (17% respects to control), while E2 had no effect on the cardiolipin content of MCF-7 and T47D cell lines (Table 3). Thus, the mtDNA/NAO ratio was modified, and showed an increase in MCF-7 cell line, while no changes were noted in the MDA-MB-231 cell line, and a decrease was observed in the T47D cell line.

In an attempt to view if mitochondrial dynamics were also affected, we checked the effect of E2 on mRNA expression of the

Table 2
OXPHOS complex protein levels in MDA-MB-231, T47D and MCF-7 breast cancer cell lines.

	MDA-MB-231			T47D			MCF-7		
	Control	E2	C E2	Control	E2	C E2	Control	E2	C E2
Complex V (A.u.)	100 ± 15	89.1 ± 19.1		100 ± 16	89.3 ± 11.1		100 ± 32	69.2 ± 27.2	
Complex III (A.u.)	100 ± 26	86.5 ± 25.7		100 ± 14	80.1 ± 5.5		100 ± 12	60.9 ± 7.2*	
Complex II (A.u.)	100 ± 26	75.3 ± 12.9		100 ± 20	69.5 ± 9.0		100 ± 12	70.1 ± 2.7*	
Complex IV (A.u.)	100 ± 28	57.6 ± 15.1		100 ± 24	186 ± 66		100 ± 27	32.0 ± 6.3*	
Complex I (A.u.)	100 ± 19	64.9 ± 14.2		100 ± 24	91.6 ± 21.5		100 ± 23	35.3 ± 4.6*	

Complex V: ATP synthase subunit alpha – "CV-alpha" ~53 kD; Complex III subunit Core 2 – "CIII-core2" ~47 kD; Complex II-FeS subunit 30 kDa – "CII-30" ~30 kD; Complex IV subunit II – "CIV-II" ~24 kD; Complex I subunit NDUFB8 – "CI-20" ~20 kD. A.u.: Arbitrary units. Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) cells were set at 100, and treated cells values were represented as percentage of control values.

* Significant difference between E2-treated and vehicle-treated cells (Student's test; $P < 0.05$). Representative bands of OXPHOS complex in each cell line were showed together protein levels values.

Table 3
mtDNA and NAO quantification in MDA-MB-231, T47D and MCF-7 breast cancer cell lines.

	MDA-MB-231		T47D		MCF-7	
	Control	E2	Control	E2	Control	E2
mtDNA (A.u.)	1.00 ± 0.03	0.87 ± 0.02*	1.00 ± 0.04	0.90 ± 0.05	1.00 ± 0.07	1.37 ± 0.11*
NAO (%)	1.00 ± 0.02	0.83 ± 0.02*	1.00 ± 0.02	1.00 ± 0.03	1.00 ± 0.02	1.00 ± 0.02
mtDNA/NAO ratio	1.00	1.05	1.00	0.90	1.00	1.37

mtDNA, mitochondrial DNA; NAO, 10-N-nonyl-acridine orange.
Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) cells were set at 1.00 and treated cells values were represented as percentage of control values.
* Significant difference between E2-treated and vehicle-treated cells (Student's test; $P < 0.05$).

genes involved in mitochondrial fusion and fission processes. As shown in Fig. 1, the changes of the E2 treatment on fusion related genes (mfn1, mfn2 and opa1) and fission related genes (drp1 and fis1) can be seen; although these changes differed among cell lines. Gene expression can be classified into 2 groups: fusion genes with a statistically significant increase in all three lines (except opa1 gene in the MDA-MB-231 cell line), and the fission genes group which present different patterns, especially for fis1 gene expression. The drp1 gene expression showed an increase in all the cell lines to a different degree. However, the MCF-7 cell line had a significant decrease in fis1 gene after 12 h of E2 treatment (40%), although T47D and MDA-MB-231 cell lines had no changes in expression of this gene.

To confirm the relevance of ERβ in mitochondrial processes such as biogenesis, dynamics and function, we performed an experiment in T47D-ERβ tetracycline-inducible cell line. T47D cells were stably transfected with the ERβ expression plasmid that was under tetracycline-responsive promoter regulation. The mRNA levels of ERβ and ERα were confirmed using RT-PCR (Table 5). As shown in Table 4, the T47D-ERβ transfected cells with no ERβ overexpression (through repression by doxycycline) showed lower COX activity (55%) and higher ATPase activity (62%) than T47D-ERβ overexpressed cells. Moreover, OXPHOS protein levels decreased when ERβ overexpression was inhibited (Fig. 2).

Figs. 3 and 4 show the differences in the mRNA expression of mitochondrial biogenesis and dynamics related genes between

Table 4
Citrate synthase, cytochrome c oxidase and ATPase enzymatic activities in T47D-ERβ tetracycline-inducible cell line.

	T47D-ERβ	
	−Dox	+Dox
CS activity (mUI/mg protein)	152 ± 19	179 ± 7
COX activity (A.u.)	100 ± 6	56.5 ± 16.1*
ATPase activity (mUI/μg protein)	172 ± 23	279 ± 31*
ATPase/COX ratio (A.u.)	1.00	2.87

CS, cytrate synthase; COX, cytochrome c oxidase; A.U., arbitrary units.
Data represent the means ± SEM ($n = 6$). In COX activity values of T47D-ERbeta without doxycycline cells were set at 100.
* Significant difference between Dox-treated and untreated cells (Student's test; $P < 0.05$, $n = 6$).

Table 5
mRNA levels of estrogen receptor alpha and beta in T47D-ERβ tetracycline-inducible cell line.

	T47D-ERbeta	
	−Dox	+Dox
ERα (A.u.)	1.00 ± 0.03	1.06 ± 0.07
ERβ (A.u.)	1.00 ± 0.07	0.07 ± 0.03*

ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; A.U., arbitrary units.
Data represent the means ± SEM ($n = 6$). Values of T47D-ERbeta without doxycycline cells were set at 1.00.
*Significant difference between Dox-treated and untreated cells (Student's test; $P < 0.05$, $n = 6$).

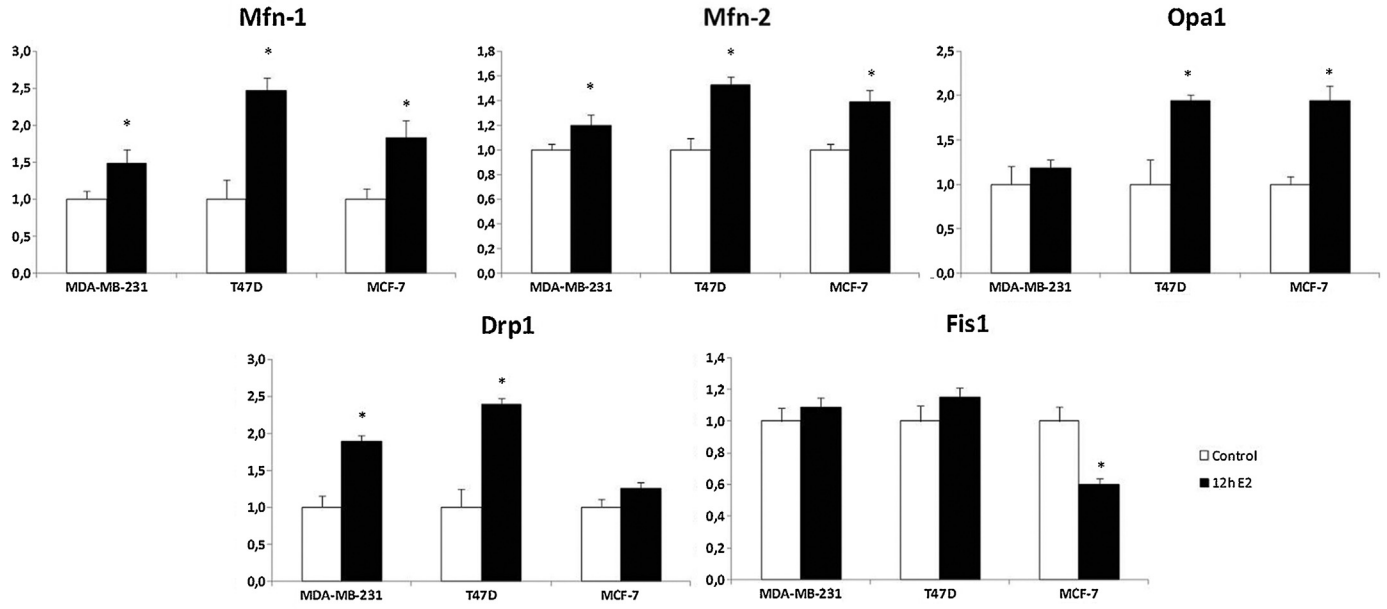


Fig. 1. Effects of E2 on mitochondrial dynamics mRNA expression in breast cancer cells with different ERα/ERβ ratio. 1 nM E2-treated cells for 12 h were shown in dark bars while white bars represent vehicle-treated cells. Data represent the means ± SEM ($n = 6$). Values of vehicle-treated cells were set at 1.00 and treated cells values were represented as percentage of control values. *Significant difference between E2-treated and vehicle-treated cells (Student's test; $P < 0.05$, $n = 6$).

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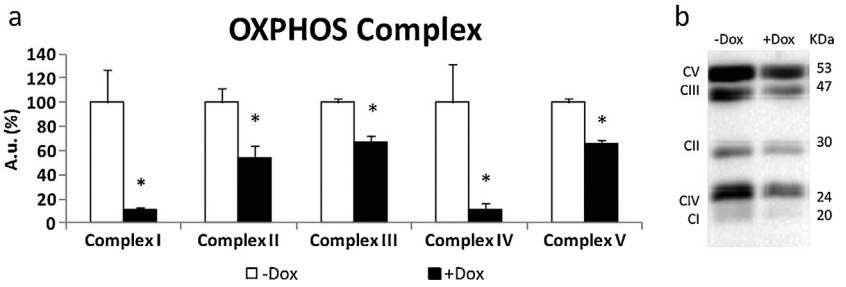

Fig. 2. ERβ maintains protein levels of OXPHOS complexes in T47D-ERβ tetracycline-inducible cell line. (a) T47D-ERβ cell line was treated (black bars) or not (white bars) with doxycycline previously to treatment with 1 nM E2 for 48 h. Data represent the means ± SEM (n = 6). Values of doxycycline untreated cells were set at 100 and doxycycline-treated cells values were represented as percentage of control values. *Significant difference between doxycycline-treated and untreated cells (Student's test; $P < 0.05$, $n = 6$). (b) Representative bands of Western blot are shown.

T47D-ERβ transfected cells with ERβ overexpression for both the inhibited and non-inhibited groups. Mitochondrial biogenesis was activated with only basal ERβ, as shown in Fig. 3, with significant increases in the pgc1α and tfam genes. A similar tendency occurred with fusion-related genes, as shown in Fig. 4, where there was an increment in mfn1, mfn2 and opa1. However, it can also be seen in Fig. 4 that a statistically significant decrease in fis1 fission-related gene occurred in the absence of ERβ overexpression.

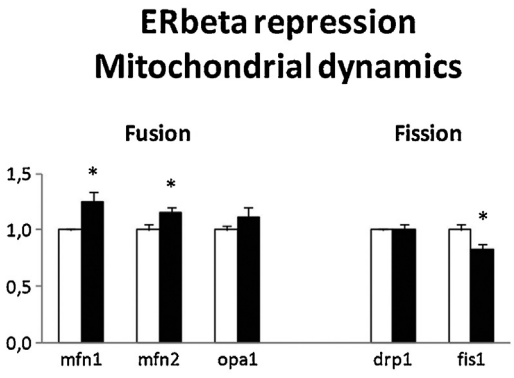

Fig. 3. Effects of ERβ repression on mitochondrial biogenesis. T47D-ERβ cell line was treated (black bars) or not (white bars) with doxycycline previously to treatment with 1 nM E2 for 4 h. Data represent the means ± SEM ($n = 6$). Values of doxycycline untreated cells were set at 1.00 and doxycycline-treated cells values were represented as percentage of control values. *Significant difference between doxycycline-treated and untreated cells (Student's test; $P < 0.05$, $n = 6$).

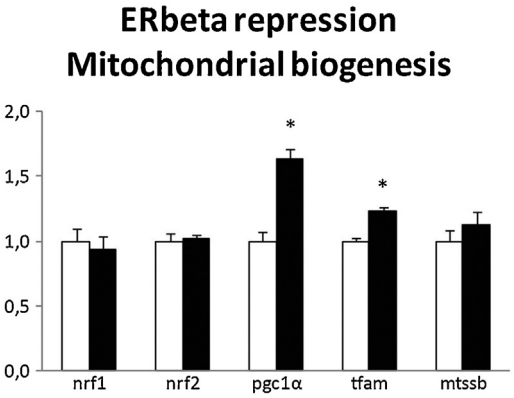

Fig. 4. Effects of ERβ repression on mitochondrial dynamics. T47D-ERβ cell line was treated (black bars) or not (white bars) with doxycycline previously to treatment with 1 nM E2 for 4 h. Data represent the means ± SEM ($n = 6$). Values of doxycycline untreated cells were set at 1.00 and doxycycline-treated cells values were represented as percentage of control values. *Significant difference between doxycycline-treated and untreated cells (Student's test; $P < 0.05$, $n = 6$).

Finally, although PGC1α did not show changes in protein levels, an increase in TFAM and a decrease in Fis1 protein levels in T47D-ERβ were observed when ERβ overexpression was inhibited, and this can be seen in Fig. 5.

4. Discussion

E2 affects mitochondrial biogenesis, dynamics and function depending on ERα/ERβ ratio in breast cancer cell lines in response to E2 treatment. The T47D, MDA-MB-231 and MCF-7 cell lines showed an increase in mitochondrial biogenesis after E2 treatment, as shown by the mRNA levels of pgc1α and tfam. However, mitochondrial dynamics exhibited different expression patterns according to the prevalent ERα/ERβ ratios, showing an increase in fusion-related genes (mfn1, mfn2 and opa1) in three cell lines, while a decrease of fis1 fission-related gene was only noted in the MCF-7 cell line. These different mitochondrial dynamics modulations modified the cell mitochondrial pool, with more functional mitochondria present in the T47D and MDA-MB-231 cell lines; while damaged, non-functional mitochondria were accumulated in MCF-7 cell line, the latter which can be observed in the results for mtDNA/NAO and mitochondrial activity. Moreover, in the T47D-ERβ tetracycline-inducible cell line, mitochondrial biogenesis and fusion were increased, although there was a decline in mitochondrial fission (fis1) expression by doxycycline inhibited

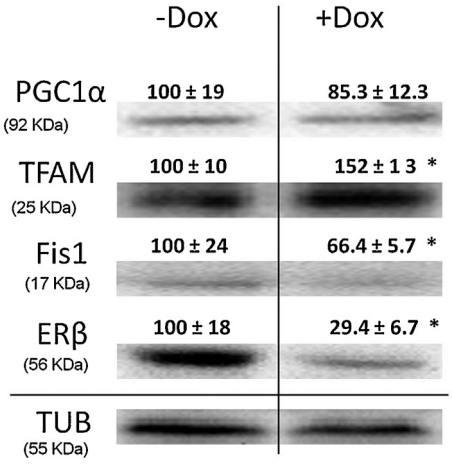

Fig. 5. Western blot representative bands of T47D-ERβ tetracycline-inducible cell line are shown. T47D-ERβ cell line was treated or not with doxycycline previously to treatment with 1 nM E2 for 48 h. Data represent the means ± SEM ($n = 6$). Values of T47D-ERbeta without doxycycline cells were set at 100 and doxycycline-treated cells values were represented as percentage of control values. *Significant difference between doxycycline-treated and untreated cells (Student's test; $P < 0.05$, $n = 6$).

overexpression of ERβ, and/or the mitochondria lacked functionality, as can be appreciated in the mitochondrial enzyme activity levels.

E2 affects mitochondrial biogenesis in T47D, MDA-MB-231 and MCF-7 cell lines, as shown in these results and in relation to previously reported results (Miro et al., 2011; Sastre-Serra et al., 2012, 2010). Mitochondrial biogenesis was elevated, although this fact was not reflected by the mitochondrial function of the three cell lines. The T47D and MDA-MB-231 cell lines had fully functional mitochondria with a lower level of oxidative stress, as well as a properly functioning antioxidant system, which has also been shown in previous results (Sastre-Serra et al., 2010; Nadal-Serrano et al., 2012). In contrast, the MCF-7 cell line had defective mitochondria, and produced a high amount of ROS (Sastre-Serra et al., 2010; Nadal-Serrano et al., 2012). This discrepancy between mitochondrial biogenesis and function could be explained, at least in part, by other processes known as mitoptosis/mitophagy (Grandemange et al., 2009; Lee et al., 2012). Under normal conditions, mitophagy removes damaged mitochondria (Mijaljica et al., 2007; Zhao et al., 2012), however, studies have also shown an accumulation of damaged mitochondria with the onset of mitophagy inhibition, which has been shown to affect mitochondrial biogenesis and function (Zhang et al., 2007). Fusion-related genes increased in the three cell lines, and this was in contrast to the fission related genes which had different patterns depending on the amount of ERβ. Yet, T47D and MDA-MB-231 cell lines maintained control levels of fission gene expression, as shown by fis1 expression. Mitophagy contributes to proper cell and mitochondrial function, and in these cell lines its increment also stimulated the replacement of defective mitochondria and the maintenance of cell bioenergetic status. However, for the MCF-7 cell line, E2 treatment reduced fission processes with a consequent accumulation of damaged mitochondria. In these cells, mitochondrial biogenesis was also stimulated in an attempt to re-establish bioenergetic status, despite negative effects of E2 on the mitochondria. This produces a doubly detrimental situation, as in addition to the detrimental effect of the accumulation of defective mitochondria, new defective mitochondria are produced. Consequently, these results were in agreement with mtDNA and NAO data, which showed that the MDA-MB-231 cell line had a decrease in mtDNA and NAO, suggesting an elimination of mitochondria after E2 treatment. Additionally, the possibility of defective mitochondrial removal could also have occurred in the T47D cell line, as a decrease in mtDNA was noted after 48 h of E2 treatment, as has also been observed in previous results (Sastre-Serra et al., 2012). However, the MCF-7 cell line presented an increase in mtDNA copy number after E2 treatment, reflecting mitochondrial accumulation, and was perhaps to a decrease in fission processes.

Furthermore, experiments executed in the T47D-ERβ tetracycline-inducible cell line confirmed the importance of ERβ in mitochondrial biogenesis and dynamics, as well as the role of the E2 regulated mitochondrial function, the latter which was demonstrated by the differences between cell lines with or without ERβ overexpression. When the overexpression of ERβ in T47D-ERβ cell line was inhibited, mitochondrial biogenesis and dynamics suffered changes according to the ERα/ERβ ratio, which reinforces the present results. Mitochondrial biogenesis was increased when ERβ was not overexpressed, and as a result showed more pgc1α and tfam mRNA levels as well as Tfam protein levels. However, these changes in mitochondrial biogenesis were not the only responses, for mitochondrial function also improved when ERβ was present and this is supported by the absence of this response in ERβ overexpression inhibited cells. Furthermore, the functionality of these mitochondria was compromised when ERβ overexpression was inhibited, which would give to this receptor a participating in mitochondrial maintenance. Moreover, when ERβ overexpression is inhibited, mitochondrial dynamics suffered an increase in fusion-related genes and a decrease in fis1 fission-related gene and protein levels, and behaved like an MCF-7 cell line in response to E2.

In conclusion, our results reinforce the protective role of ERβ in cancer mitochondria, giving special emphasis on mitochondrial dynamics as a new estrogen-modulated process. Moreover, our results could also explain why mitochondrial biogenesis and functionality in breast cancer cell lines with different ERα/ERβ ratios did not go hand in hand in their effects. Summing up, this paper showed the importance of determining the ERβ status in breast cancer tumors to provide new clues for treatment that would involve control of mitochondrial function, biogenesis and dynamics in breast cancer.

Acknowledgments

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References

Bossy-Wetzel E, Barsoum MJ, Godzik A, Schwarzenbacher R, Lipton SA. Mitochondrial fission in apoptosis, neurodegeneration and aging. Current Opinion in Cell Biology 2003;15:706–16.

Chen H, Chan DC. Emerging functions of mammalian mitochondrial fusion and fission. Human Molecular Genetics 2005;14(Spec No. 2):R283–9.

Chrzanowska-Lightowlers ZM, Turnbull DM, Lightowlers RN. A microtiter plate assay for cytochrome c oxidase in permeabilized whole cells. Analytical Biochemistry 1993;214:45–9.

Grandemange S, Herzig S, Martinou JC. Mitochondrial dynamics and cancer. Seminars in Cancer Biology 2009;19:50–6.

Guo JY, Chen HY, Mathew R, Fan J, Strohecker AM, Karsli-Uzunbas G, et al. Activated Ras requires autophagy to maintain oxidative metabolism and tumorigenesis. Genes and Development 2011;25:460–70.

Hales KG. The machinery of mitochondrial fusion, division, and distribution, and emerging connections to apoptosis. Mitochondrion 2004;4:285–308.

Huang P, Galloway CA, Yoon Y. Control of mitochondrial morphology through differential interactions of mitochondrial fusion and fission proteins. PLoS One 2011;6:e20655.

Justo R, Boada J, Frontera M, Oliver J, Bermudez J, Gianotti M. Gender dimorphism in rat liver mitochondrial oxidative metabolism and biogenesis. American Journal of Physiology Cell Physiology 2005;289:C372–8.

Lee J, Giordano S, Zhang J. Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling. Biochemical Journal 2012;441:523–40.

Lemasters JJ. Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation Research 2005;8:3–5.

Liesa M, Palacin M, Zorzano A. Mitochondrial dynamics in mammalian health and disease. Physiological Reviews 2009;89:799–845.

Mijaljica D, Prescott M, Devenish RJ. Different fates of mitochondria: alternative ways for degradation? Autophagy 2007;3:4–9.

Miro AM, Sastre-Serra J, Pons DG, Valle A, Roca P, Oliver J. 17beta-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio. Journal of Steroid Biochemistry and Molecular Biology 2011;123:133–9.

Nadal-Serrano M, Sastre-Serra J, Pons DG, Miró AM, Oliver J, Roca P. The ERalpha/ERbeta ratio determines oxidative stress in breast cancer cell lines in response to 17beta-estradiol. Journal of Cellular Biochemistry 2012;113(10):3178–85, http://dx.doi.org/10.1002/jcb.24192.

Nakada K, Inoue K, Hayashi J. Interaction theory of mammalian mitochondria. Biochemical and Biophysical Research Communications 2001;288:743–6.

Nakano K, Tarashima M, Tachikawa E, Noda N, Nakayama T, Sasaki K, et al. Platelet mitochondrial evaluation during cytochrome c and dichloroacetate treatments of MELAS. Mitochondrion 2005;5:426–33.

Ong SB, Hausenloy DJ. Mitochondrial morphology and cardiovascular disease. Cardiovascular Research 2010;88:16–29.

Ragan C, Wilson M, Darley-Usmar V, Lowe P. Subfractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. Oxford: IRL Press; 1987.

Rambold AS, Kostelecky B, Lippincott-Schwartz J. Together we are stronger: fusion protects mitochondria from autophagosomal degradation. Autophagy 2011;7:1568–9.

Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Oliver J, Roca P. The Effects of 17beta-estradiol on mitochondrial biogenesis and function in breast cancer cell lines are dependent on the ERalpha/ERbeta ratio. Cellular Physiology and Biochemistry 2012;29:261–8.

Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J, Roca P. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. Free Radical Biology and Medicine 2010;48:506–12.

Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proceedings of the National Academy of Sciences of the United States of America 2004;101:1566–71.

Takamura A, Komatsu M, Hara T, Sakamoto A, Kishi C, Waguri S, et al. Autophagy-deficient mice develop multiple liver tumors. Genes and Development 2011;25:795–800.

Zhang Y, Qi H, Taylor R, Xu W, Liu LF, Jin S. The role of autophagy in mitochondria maintenance: characterization of mitochondrial functions in autophagy-deficient *S. cerevisiae* strains. Autophagy 2007;3:337–46.

Zhao J, Zhang J, Yu M, Xie Y, Huang Y, Wolff DW, et al. Mitochondrial dynamics regulates migration and invasion of breast cancer cells. Oncogene 2012., http://dx.doi.org/10.1038/onc.2012.494 [Epub ahead of print].

Manuscript 2

The presence of Estrogen Receptor β modulates the response of breast cancer cells to therapeutic agents

Manuscript

ABSTRACT

Breast cancer is a leading cause of death for women. The estrogen receptors (ERs) ratio is important in the maintenance of mitochondrial redox status, and higher levels of ERβ increases mitochondrial functionality, decreasing ROS production. Our aim was to determine the interaction between the ERα/ERβ ratio and the response to cytotoxic treatments such as cisplatin (CDDP), paclitaxel (PTX) and tamoxifen (TAM). Cell viability, apoptosis, autophagy, ROS production, mitochondrial membrane potential, mitochondrial mass and mitochondrial functionality were analyzed in MCF-7 (high ERα/ERβ ratio) and T47D (low ERα/ERβ ratio) breast cancer cell lines. Cell viability decreased more in MCF-7 when treated with CDDP and PTX. Apoptosis was less activated after cytotoxic treatments in T47D than in MCF-7 cells. Nevertheless, autophagy was increased more in CDDP-treated MCF-7, but less in TAM-treated cells than in T47D. CDDP treatment produced a raise in mitochondrial mass in MCF-7, as well as the citochrome c oxidase (COX) and ATP synthase protein levels, however significantly reduced COX activity. In CDDP-treated cells, the overexpression of ERβ in MCF-7 caused a reduction in apoptosis, autophagy and ROS production, leading to higher cell survival; and the silencing of ERβ in T47D cells promoted the opposite effects. In TAM-treated cells, ERβ-overexpression led to less cell viability by an increment in autophagy; and the partial knockdown of ERβ in T47D triggered an increase in ROS production and apoptosis, leading to cell death. In conclusion, ERβ expression plays an important role in the response of cancer cells to cytotoxic agents, especially for cisplatin treatment.

Keywords: *Oxidative stress; ERα/ERβ ratio; ROS; breast cancer; resistance.*

INTRODUCTION

Breast cancer is a leading cause of death in women worldwide[1]. Approximately 75% of breast cancers are estrogen receptor (ER) positive[2]. There are two forms of ERs, ERα and ERβ, with distinct functions associated with: ERα is more related to cell proliferation and ERβ is linked to cytostatic and differentiation processes[3-5].

Many breast cancer treatments, such as cisplatin (CDDP), paclitaxel (PTX) or tamoxifen (TAM) have an effect on the mitochondria, diminishing the mitochondrial functionality which results in a raise in reactive oxygen species (ROS) production and, in most cases, cell death[6-8]. These cytotoxic effects on mitochondrial functionality and ROS production could be a key point in the development of resistance to these treatments, as cancer cells could develop resistance mechanisms by decreasing ROS production in order to be able to survive under oxidative stress conditions[9].

ROS can damage cellular macromolecules which can affect cell structure and viability. ROS are generated principally in mitochondria and they may play a key role in oxidative stress in cancer cells[10]. It is believed that oxidative stress is one of the main mechanisms by which cells become cancerous, especially in breast cancer, as the accumulation of ROS in cells may have a key role in cancer development and progression[10,11]. Nonetheless, for survival, cancer cells must also acquire adaptive strategies in order to counteract the deleterious effects of ROS exposure[10,12].

Previous results obtained in our laboratory demonstrated the importance of the ERα/ERβ ratio in the maintenance of mitochondrial redox homeostasis[13-16]. Thus, exposure to cells with a high ERα/ERβ ratio to 17β-estradiol (E2) or genistein (GEN), a phytoestrogen capable to bind both ERs, provoked an increase in ROS production and a loss of mitochondrial functionality. However, in those cells with a low ERα/ERβ ratio, E2 or GEN exposure triggered a better mitochondrial functionality and, therefore, a lower ROS production[13-16]. Although currently clinical diagnostics only include the analysis of the presence of ERα in breast cancer cells, we have previously reported the importance of ERβ in breast cancer tumors, whose presence leads to a greater resistance to oxidative stress[17].

With the aim of determining the potential relationship between the ERα/ERβ ratio of breast cancer cells and the response to cytotoxic treatments, we analyzed the effects of CDDP, PTX and TAM in two breast cancer cell lines: MCF-7 (high ERα/ERβ ratio) and T47D (low ERα/ERβ ratio). Parameters such as cell viability, ROS production, mitochondrial membrane potential, mitochondrial mass, apoptosis, autophagy and mitochondrial functionality (COX and ATPase protein levels and function) were studied after the cytotoxic treatments. Moreover, to avoid the differential effects between cell lines, the most important parameters (cell viability, ROS production, apoptosis and autophagy) were analyzed in a T47D with an ERβ knockdown and in a stable ERβ overexpressing MCF-7 cell line.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's medium (DMEM) high glucose was purchased from GIBCO (Paisley, UK). Cisplatin (*cis-Diammineplatinum(II) dichloride or CDDP), paclitaxel (from Taxus brevifolia or PTX) and tamoxifen (trans-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine or TAM)* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers were purchased from TIB MOLBIOL (Berlin, Germany) and from Metabion (Martinsried, Germany). Routine chemicals used were obtained from Sigma-Aldrich, Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture and cytotoxic treatments

The MCF-7 and T47D human breast cancer cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and routinely cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. For cytotoxic treatments, cells were seeded in 6-well or 96-well plates and treated the next day with 10 μM CDDP, 10 nM PTX or 10 μM TAM for 48 hours.

siRNA cell transfection

For transfection, cells were cultured in 96-well plates so that the next day cells were 60% confluent. Cells were cultured overnight and then transfected for 6 hours with a specific small interfering (si)RNA targeting ERβ mRNA purchased in Santa Cruz Biotechnology (ERβ siRNA (h): sc-35325). Lipofectamine 2000 reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) was used to perform cell transfection according to the manufacturer's protocol. After transfection the complexes were removed and cells were provided with normal growth medium.

ERβ stable transfection

ERβ cDNA clone was purchased from Origene (Rockville, MD, USA). First of all, ERβ cDNA clone was amplified in *Escherichia coli* DH5αF' Competent Cells (Life

Technologies, Paisley, UK) and isolated with MaxiPrep isolation kit (Life Technologies, Paisley, UK). After cDNA quantification using a BioSpec-nano spectrophotometer set at 260 nm (Shimadzu Biotech, Kyoto, Japan), aliquots of 1 µg/ml of cDNA were kept in -20^{o}C for cell transfections. Briefly, MCF-7 cells were seeded in 6-well plates and the next day were transfected with an ERβ cDNA clone following the manufacturer's instructions. Lipofectamine 2000 was used to perform cell transfection according to the manufacturer's protocol. After transfection the complexes were removed and cells were provided with normal growth medium. Two days after cDNA transfection, growth medium was replaced for DMEM (+10% FBS and +1% antibiotics) with 600 µg/ml of G418 (or neomycin) in order to select those cells that have incorporated the ERβ cDNA clone in their genome. After two weeks with 600 µg/ml of G418 in the growth medium, colonies were harvested with cloning discs (Sigma-Aldrich, St. Louis, MO, USA) and plated in 24-well plates (one well per colony). Ten days later, cells were ready to trypsinize and they were plated in 6-well plates. Then, cells were subcultured in order to get cells to perform the experiments. It is important to note that the MCF-7+ERβ cells obtained with this protocol must be grown routinely at a concentration of 200 µg/ml of G418 in the growth medium, except when cultured for relevant experiments.

Cell viability assay

MCF-7, MCF-7+ERβ, T47D or T47D-ERβ cells were seeded in 96-well plates. The day after, cytotoxic treatments were applied for 48h in normal growth medium. Crystal Violet was the method used to determine cell viability as described in Pons, et al.[14].

Fluorimetric determination of H_2O_2 production (ROS production)

ROS production was measured fluorimetrically by using an Amplex® Red Hydrogen Peroxide/Peroxidase assay kit (Molecular Probes). MCF-7, MCF-7+ERβ, T47D or T47D-ERβ cells were seeded in 96-well plates. The day after, cytotoxic treatments were applied for 48h. The measurement day, cells were exposed to 50 µM of Amplex Red reagent and 0.1 U/ml of horseradish peroxidase, in Krebs-Ringer buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4). Fluorescence was measured with an FLx800 microplate

fluorescence reader (Bio-Tek Winooski, Vermont, USA), set at excitation and emission wavelengths of 571 and 585 nm, to detect the maximum slope of increment in the fluorescence within 1 hour of exposure to kit reagents. Thus, the measurement obtained is the H_2O_2 produced (related to ROS production) by the cells for one hour. Values were normalized per number of viable cells determined by crystal violet assay.

Mitochondrial membrane potential, autophagy and mitochondrial mass measurement

Mitochondrial membrane potential (ΔΨm), autophagy and mitochondrial mass were measured fluorimetrically by using Tetramethylrhodamine methyl ester (TMRM), Monodansylcadaverine (MDC) and Mitotracker Green FM (MTG, Life Technologies, Paisley, UK) respectively. MCF-7, MCF-7+ERβ, T47D or T47D-ERβ cells (the latter just in the case of MDC) were seeded in 96-well plates and, the next day, cells were exposed for 48h to cytotoxic treatments. Fluorescence measurement was performed in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 552 and 576 nm (ΔΨm by TMRM, 100 nM, 15 minutes of incubation), 340 and 535 nm (autophagy by MDC, 50 µM, 15 minutes of incubation) or 490 and 516 nm (mitochondrial mass by MTG, 100 nM, 40 minutes of incubation). Values were normalized per number of viable cells determined by crystal violet assay.

Apoptosis assay

Apoptosis was measured fluorimetrically by using Annexin V method, as described previously[18]. Briefly, MCF-7, MCF-7+ERβ, T47D or T47D-ERβ cells were seeded in 96-well plates and treated with cytotoxic treatments. At the end of the treatment, cells were fixed with 2% paraformaldehyde (BD Biosciences, NJ, USA) in PBS at room temperature for 30 min and washed twice with PBS. Cells were then stained with AnnexinV/AlexaFluor350 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) in annexin binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) for 10 min at room temperature in the dark. To finish, cells were washed once with annexin binding buffer. Florescence was measured in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 346 and 442 nm, with cells kept in 100 µl of annexin binding

buffer. Values were normalized per number of viable cells determined by crystal violet assay.

Laser scanning confocal microscopy

After vehicle or CDDP treatment for 48h, cells were loaded as described previously by Rodriguez-Enriquez, et al.[19] with modifications. MCF-7 cells were loaded with Mitotracker Green FM (MTG, 0.5 μM) for 60 min (mitochondria staining) and 5 μg/ml Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) for 5 min (nucleus staining) at 37^{o}C in culture medium and the fluorescence of MTG and Hoechst was monitored with a Leica Confocal (Wetzlar, Germany) microscope using 63× oil 14 N.A. objective lens. Excitation of probes and fluorescence emission were measured also according to Rodriguez-Enriquez, et al.[19].

ATP synthase and cytochrome c oxidase activities

MCF-7 and T47D breast cancer cells were seeded in 6-well plates and, the next day, were treated with vehicle, CDDP, PTX or TAM for 48 hours. Then, cells were harvested and the enzymatic activities were performed as described in Pons, et al.[14].

Real-time quantitative PCR

MCF-7+ERβ and T47D were seeded in 6-well plates and, after transfection with ERβ siRNA in the case of T47D (T47D-ERβ), the day after total RNA was isolated from cultured cells by using TriPure® isolation reagent (Roche, Barcelona, Spain) following the manufacturer's protocol. and then quantified using a BioSpec-nano spectrophotometer set at 260 nm(Shimadzu Biotech, Kyoto, Japan). Real-time PCR was performed as described by Nadal-Serrano, et al.[13].

The primers used were forward 5'-ggACACggACAggATTgACA-3' and reverse 5'-ACCCACggAATCgAgAAAgA-3' for the 18S ribosomal RNA gene (61^{o}C annealing temperature), and forward 5'-TAgTggTCCATCgCCAgTTAT-3' and reverse 5'-gggAgCCACACTTCACCAT-3' for the ERβ gene (64^{o}C annealing temperature).

The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

Western blot analysis

MCF-7 and T47D cells were seeded in 6-well plates. The next day, cells were exposed to cytotoxic treatments for 48h. Cell protein extracts were obtained scraping cells with 200 µl of RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 1 mM EDTA, 1mM NaF, 1 mM Na_3VO_4, 10 µM leupeptin,10 µM pepstatin and 1 mM PMSF). The lysate was sonicated three times at 40% amplitude for 7 seconds. Then samples were centrifuged at 14000×*g* for 10 min at 4°C and protein content (supernatant) was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). A 20 µg protein aliquot from the cell lysate was separated on a 10% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. Membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20) for 1h. Antisera against OXPHOS complexes (Mitosciences, OR, USA) and GAPDH (Santa Cruz Biotechnology, CA, USA) were used as primary antibodies. Secondary antibodies were from Sigma-Aldrich (St. Louis, MO, USA). Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

Statistical analysis

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are expressed as mean values ± standard error of the mean (SEM). Statistical significances between vehicle-treated cells and cisplatin-, paclitaxel- or tamoxifen-treated cells were assessed by Student's t-test. The effects of changes produced by cytotoxic agents in MCF-7 and T47D cell lines and the ERβ overexpression or inhibition and cytotoxics treatments were analyzed using two-way analysis of variance (ANOVA), and when results reach significant combinatory effects Student's t-test was performed in order to

find out the significance between the experimental groups. Statistical significance was set at $P<0.05$.

RESULTS

High ERα/ERβ ratio increased breast cancer cells sensitivity to cisplatin and paclitaxel but not to tamoxifen

Cell viability assay (Figure 1A) revealed that MCF-7 cells were more affected by cytotoxic treatments than T47D cells, especially in CDDP-treated cells (-44% in MCF-7 and -30% in T47D). PTX treatment also produced a -44% and a -35% of cell viability in MCF-7 and T47D cells, respectively. However, this difference was more moderate in the case of TAM-treated cells (-38% in MCF-7 and -36% in T47D).

Apoptosis is less activated after cytotoxic treatments in T47D cells (low ERα/ERβ ratio) than in MCF-7 cells (high ERα/ERβ ratio)

As it can be observed in Figure 1B, the MCF-7 breast cancer cell line showed a significant increase in apoptosis after the three cytotoxic treatments, especially in PTX- (+281%) and CDDP-treated cells (+236%). However, in T47D this increment was slighter in the three treatments (between +38% and +55%). It is remarkable that in the TAM-treated cells had such a slight difference of the effects from the three cytotoxic treatments between the two cell lines (+84% in MCF-7 and +53% in T47D).

Autophagy is activated differently depending on the ratio of receptors and cytotoxic treatment

Similarly to apoptosis, Figure 1C shows that autophagy was increased more in the CDDP-treated MCF-7 cells (+78%) than in the T47D cells (+36%). Curiously, in the case of PTX- and specially in TAM-treated cells the opposite situation occurred as the cells with a higher autophagy compared to control were the T47D cells (+65% in PTX-treated cells and +234% in TAM-treated cells, +48% and +140% in MCF-7 cells, respectively).

ROS production is extremely more increased in CDDP- and PTX-treated MCF-7 cells than in T47D, but not in TAM-treated cells

Figure 2A displays the ROS production in MCF-7 and T47D breast cancer cell lines after cytotoxic treatments. After CDDP treatment, MCF-7 cells boosted H_2O_2 production by 175% regarding control, while T47D cells increased H_2O_2 production only by 38%. PTX treatment also increased ROS production and was cell line dependent, increasing a 70% ROS production in MCF-7 cells with only a 25% of increase in the T47D cell line. However, in TAM-treated cells no differences in H_2O_2 production were found between cell lines (+53% in MCF-7 and +47% in T47D cells).

Mitochondrial membrane potential (ΔΨm) is increased after cytotoxic treatments in both cell lines

Figure 2B shows that an increase in ΔΨm agreed with the results obtained in ROS production. Thus, in CDDP-treated cells, the high ROS production corresponded with the greatest ΔΨm measured in MCF-7 (+70% to +27% of T47D), and PTX treatment also increased ΔΨm with lesser changes between cell lines (+64% in MCF-7 and +36% in T47D), while TAM-treated cells values were the most similar between both cell lines (+52% in MCF-7 and +37% in T47D cells).

Mitochondrial mass is increased in CDDP-treated MCF-7 cells, but not in T47D cells

As can be observed in Figure 2C, mitochondrial mass, measured with MTG, increased with the three cytotoxic treatments in the MCF-7 cell line, especially in the case of CDDP-treated cells (+66%). PTX and TAM treatments also produced an increase in MTG fluorescence, but with milder changes (+23% and +25% respectively). In T47D cells, there was only a slight increment in PTX- (+11%) and TAM-treated (+21%) cells. Again, the most similar results were observed in TAM-treated cells of both cell lines (+25% in MCF-7 cells and +21% in T47D cells). With the aim to further studying this increase in mitochondrial mass of CDDP-treated MCF-7 cells, laser scanning confocal microscopy pictures (Figure 2D) were taken after MTG and Hoechst 33342 incubation, 48 hours after treatment with vehicle and CDDP. A greater mitochondrial mass can be visually observed in those MCF7 cells treated with CDDP.

Mitochondrial functionality was affected in CDDP-treated MCF-7 cells, but not in T47D cells

COX activity (Figure 3A) was reduced after CDDP treatment in MCF-7 cells (-24%), while TAM-treatment produced an increase in ATPase activity (Figure 3B) in T47D cells (+36%). Contrastingly, COX protein levels (Figure 3C) were increased in both CDDP-treated MCF-7 (+112%) and T47D cells (+50%), although with the TAM treatment, these protein levels increase only occurred in the T47D cell line (+33%). An increase in ATPase protein levels (Figure 3D) was observed in CDDP- (+95%) and TAM-treated (+50%) MCF-7 cells and no significant changes were observed in T47D treated with both cytotoxic agents.

The presence of ERβ modulated the cell viability in CDDP-treated cells, but not in TAM-treated cells

ERβ was stable overexpressed in MCF-7 cells with an increase of 734% in ERβ mRNA expression (data not shown). Furthermore, ERβ was partially silencing in T47D cells with a decrease of 46% in ERβ mRNA expression (data not shown).

As it can be observed in Figure 4A, the overexpression of ERβ in MCF-7 cells (MCF-7+ERβ) produced a greater resistance to CDDP (+5% of cells) compared to treatment in normal MCF-7 cells, but not for TAM-treated cells. In fact, the overexpression of ERβ rendered less cell viability after TAM treatment (-5.5%).

Furthermore, Figure 4B shows that the ERβ knockdown in T47D resulted in a reduced cell viability in both cytotoxic treatments, CDDP (-4.5%) and TAM (-5.5%).

The presence of ERβ inhibited apoptosis in CDDP- and TAM-treated cells, although increased autophagy in TAM-treated MCF-7+ERβ cells

The apoptosis assay (Figures 4C and 4D) showed that the presence of ERβ diminished Annexin V fluorescence in MCF-7+ERβ cells and its absence increased it in T47D-ERβ cells after both cytotoxic treatments. In MCF-7 cells, the decrease in apoptosis was enhanced after CDDP and TAM treatments by 32% and 54%, respectively, regarding the MCF-7 normal cells. In T47D with ERβ silencing, the

increment of apoptosis was especially significant in TAM-treated cells (+25% regarding TAM-treated T47D control cells).

However, autophagic vacuole formation (Figures 4E and 4F) increased significantly by 34% in TAM-treated MCF-7+ERβ respect to TAM-treated MCF-7 normal cells. Moreover, MCF7+ERβ cells underwent a decrease in this parameter in CDDP-treated cells (-21%) compared to CDDP-treated MCF-7 normal cells. In the case of T47D, the inhibition of ERβ resulted in a rise of 12% in CDDP-treated cells in comparison to T47D control cells, with no significant changes in TAM-treated cells.

ROS production is decreased in CDDP-treated cells in the presence of ERβ and it is increased after CDDP and, especially, TAM treatment in those T47D lacking ERβ

Figure 4G displays that, in MCF-7+ERβ, just the presence of ERβ caused a decrease in ROS production (-10%), when compared with MCF-7 normal cells. Interestingly, MCF-7+ERβ cells treated with CDDP showed a very significant decrease in ROS production (-124%), with no significant changes in TAM-treated cells respect to normal MCF-7 cells with the same treatment.

However, in the T47D cell line with ERβ siRNA (Figure 4H), the absence of ERβ produced an increase in ROS production with both treatments, especially in TAM-treated T47D-ERβ cells (+65%, and +13% in CDDP-treated T47D-ERβ cells).

FIGURE CAPTIONS

Figure 1. Cell viability, apoptosis and autophagy in MCF-7 and T47D cells after cytotoxic treatment for 48 hours

MCF-7 and T47D cells were treated with 10 μM cisplatin, 10 nM paclitaxel or 10 μM tamoxifen for 48h. A) Cell viability was analyzed spectrophotometrically by Crystal Violet assay. B) Apoptosis fraction was measured fluorimetrically using Annexin V method. C) Autophagic vacuoles formation was measured fluorimetrically using monodansylcadaverine reagent. Values are expressed as means ± SEM (n≥6), and normalized as percentage of the vehicle-treated value for each cell line. * Significant difference between Vehicle-treated cells and cisplatin-, paclitaxel- or tamoxifen-treated cells (Student's *t*-test; P<0.05, n≥6).

Figure 2. Effects of cytotoxic treatments on ROS production, mitochondrial membrane potential and mitochondrial mass

MCF-7 and T47D cells were treated with 10 μM cisplatin, 10 nM paclitaxel or 10 μM tamoxifen for 48h. A) ROS production was measured fluorimetrically using Amplex red reagent. B) Mitochondrial membrane potential (ΔΨm) was measured fluorimetrically using TMRM. C) Mitochondrial mass was measured fluorimetrically using Mitotracker Green FM. D) Changes in mitochondrial mass detected with Mitotracker Green FM and Hoechst 33342 fluorescence after cisplatin treatment were monitored with a Leica Confocal Microscope using 63x oil 147 N.A. objective lens. Scale bar 25 μm. Values are expressed as means ± SEM (n≥6), and normalized as percentage of the vehicle-treated value for each cell line. * Significant difference between Vehicle-treated cells and cisplatin-, paclitaxel- or tamoxifen-treated cells (Student's *t*-test; P<0.05, n≥6).

Figure 3. Effects of cisplatin and tamoxifen treatments on mitochondrial functionality

MCF-7 and T47D cells were treated with 10 µM cisplatin or 10 µM tamoxifen for 48h. After that, cells were harvested with distilled water to perform enzymatic activities or scrapped with RIPA buffer to analyze the COX and ATPase protein levels. A) COX activity was measured spectrophotometrically following the absorbance increment at 450 nm as shown in the Material and Methods section. B) ATPase activity was measured spectrophotometrically following the absorbance decrease at 340 nm as shown in the Material and Methods section. C) COX protein levels measured by western blot. D) ATPase protein levels measured by western blot. GAPDH was used as house-keeping protein. Values are expressed as means ± SEM (n=6), and normalized as percentage of the vehicle-treated value for each cell line. * Significant difference between Vehicle-treated cells and cisplatin-, paclitaxel- or tamoxifen-treated cells (Student's *t*-test; $P<0.05$, n=6).

Figure 4. Effects of cisplatin and tamoxifen on MCF-7 and T47D with modified ERα/ERβ ratio

MCF-7, MCF-7 overexpressing ERβ (MCF7+ERβ), T47D and T47D with partially silenced ERβ (T47D-ERβ) cell lines were treated with 10 µM cisplatin or 10 µM tamoxifen for 48h. For T47D with partially silenced ERβ, cells were treated one day before the beginning of cytotoxic treatment with a specific ERβ siRNA, as shown in the Material and Methods section. A) Cell viability of MCF-7 and MCF7+ERβ cells was measured spectrophotometrically by Crystal Violet assay. B) Cell viability of T47D and T47D-ERβ cells was measured spectrophotometrically by Crystal Violet assay. C) Apoptosis fraction of MCF-7 and MCF7+ERβ cells was measured fluorimetrically using Annexin V method. D) Apoptosis fraction of T47D and T47D-ERβ cells was measured fluorimetrically using Annexin V method. E) Autophagic vacuoles formation of MCF-7 and MCF7+ERβ cells was measured fluorimetrically using monodansylcadaverine reagent. F) Autophagic vacuoles formation of T47D and T47D-ERβ cells was measured fluorimetrically using monodansylcadaverine reagent. G) ROS production of MCF-7 and MCF7+ERβ cells was measured fluorimetrically using Amplex red reagent. H) ROS

production of T47D and T47D-ERβ cells was measured fluorimetrically using Amplex red reagent. Values are expressed as means ± SEM (n≥6), and normalized as percentage of the vehicle-treated value for each cell line (Cell Viability assay) or normalized as percentage of the vehicle-treated value of the *wild-type* (MCF-7 or T47D) of each cell line (Apoptosis, Autophagy and ROS production assays). ANOVA analysis was carried out where β means ERβ overexpression or silencing effect, C means cisplatin effect, T means tamoxifen effect and Cxβ or Txβ means combinatory effect of cytotoxic treatments and ERβ overexpression or silencing. As a result of combinatory effect, Student's t-test ($P<0.05$, n≥6) was carried out: ^{o} significant difference between *wild-type* cells and ERβ modified cells; * significant difference between Vehicle and Cytotoxic treated cells; $*^{o}$ significant difference between *wild-type* and ERβ modified in Cytotoxic treated cells.

FIGURES

Figure 1

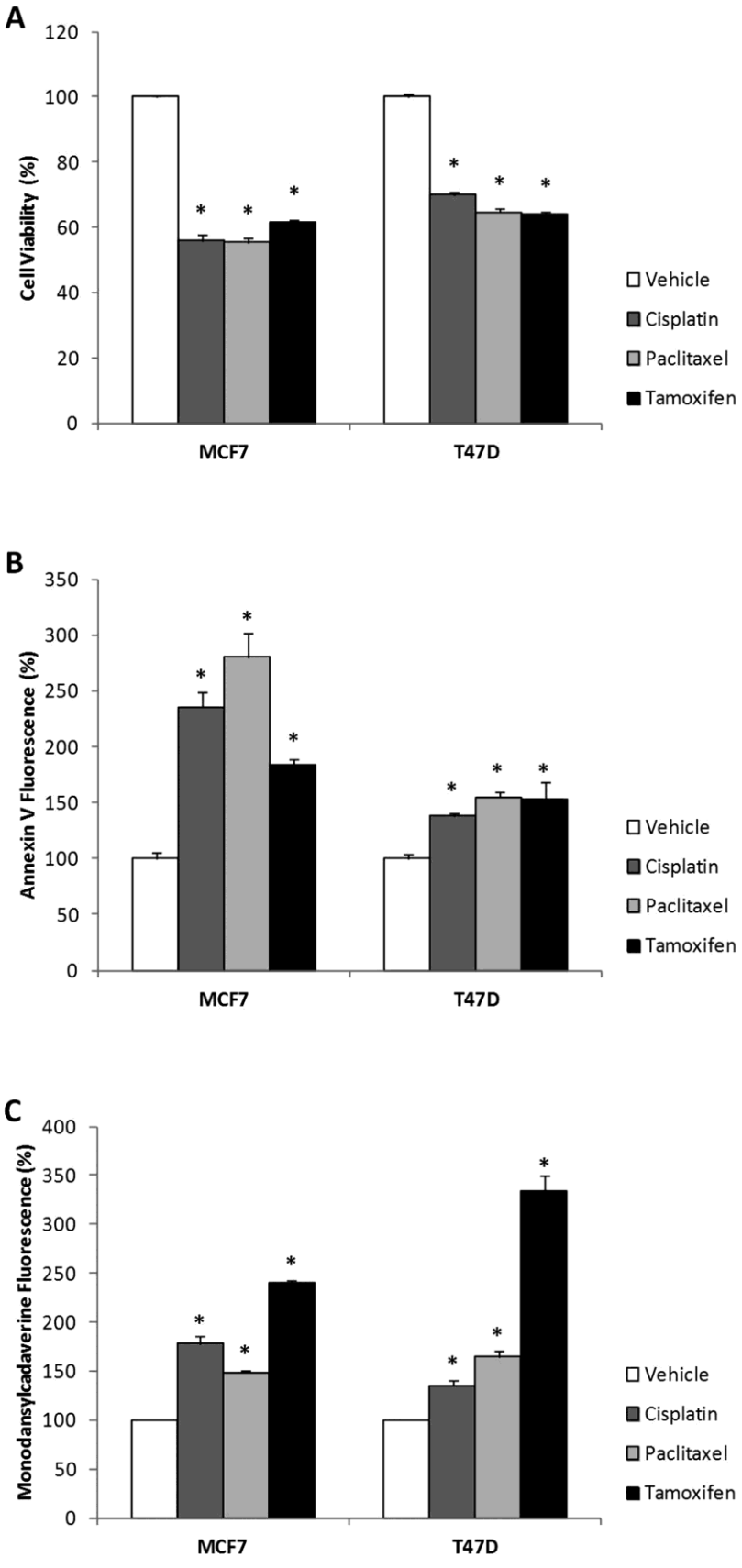


Figure 2A and 2B

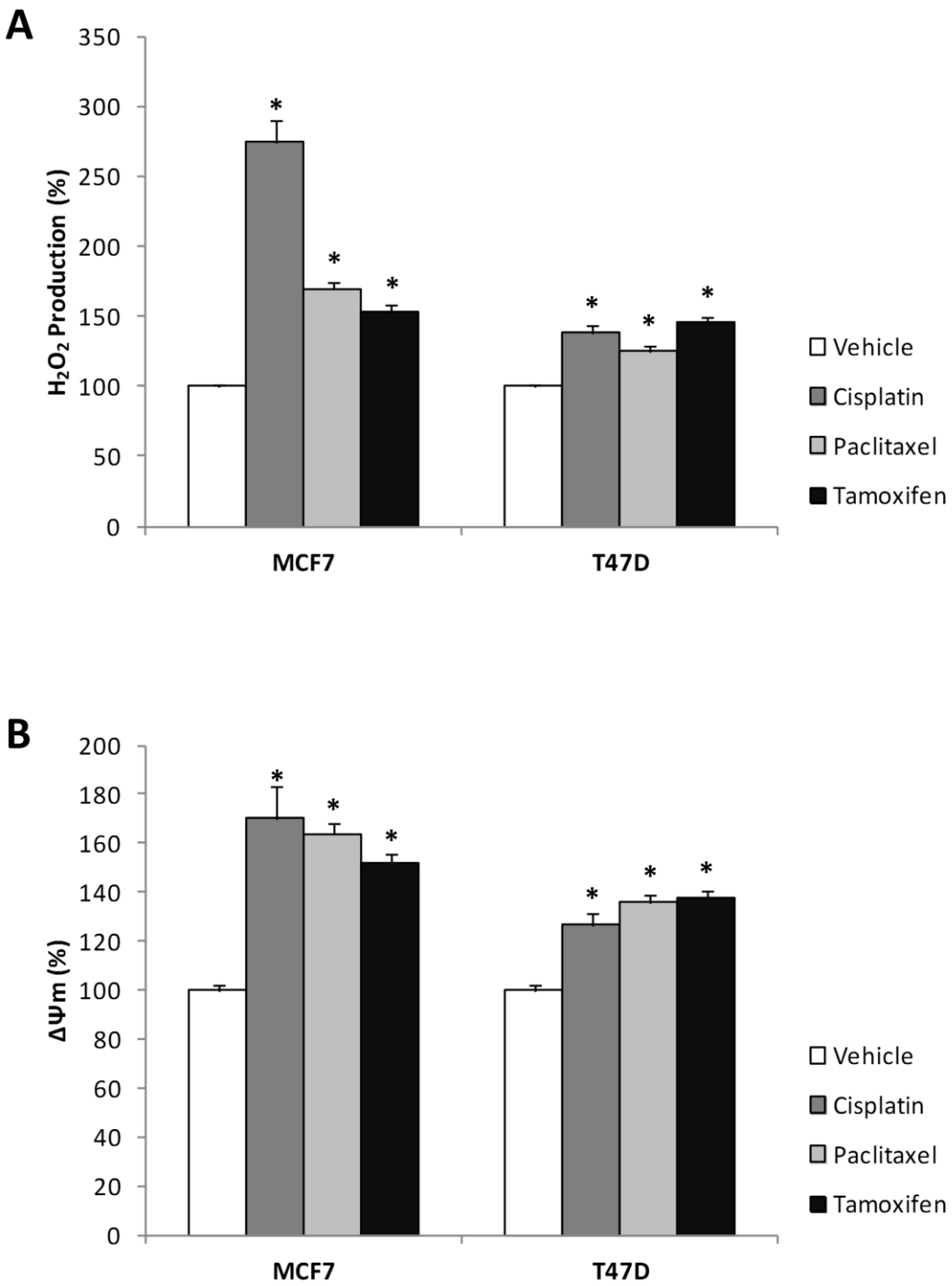
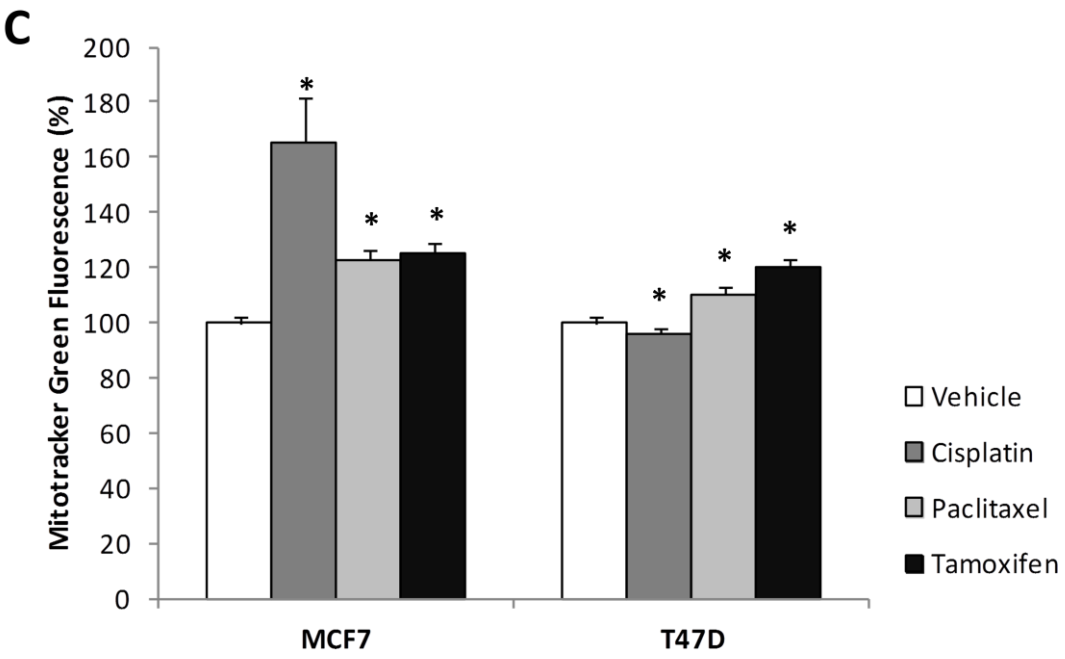


Figure 2C and 2D



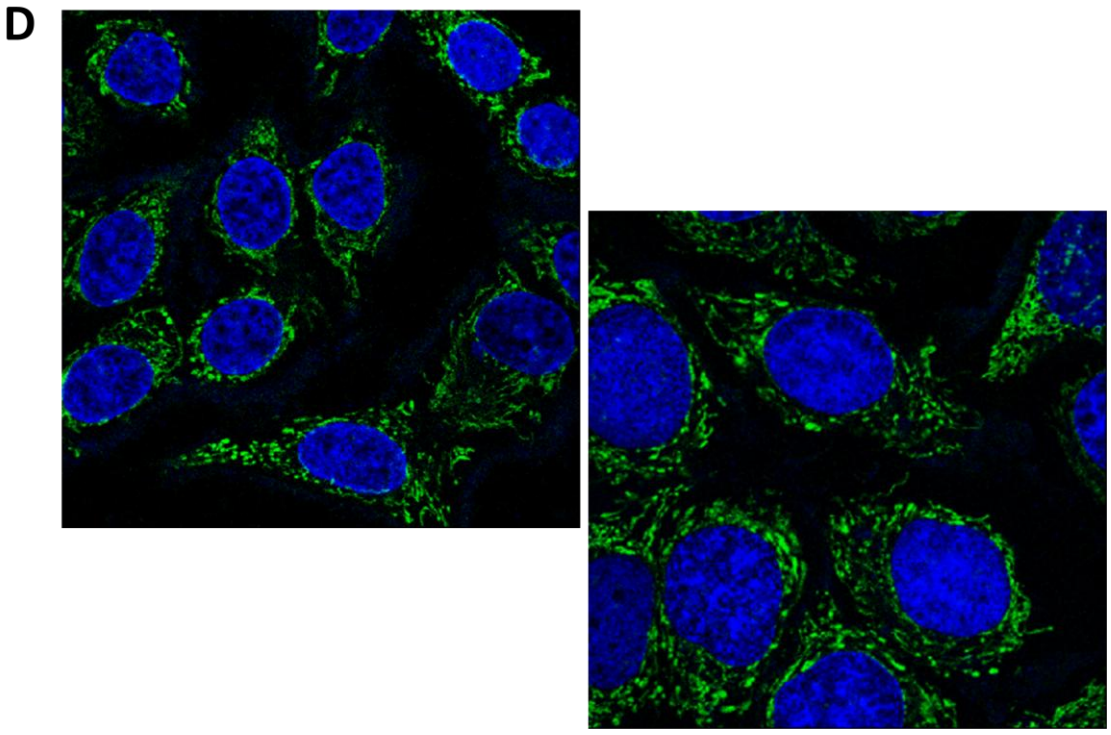


Figure 3A and 3B

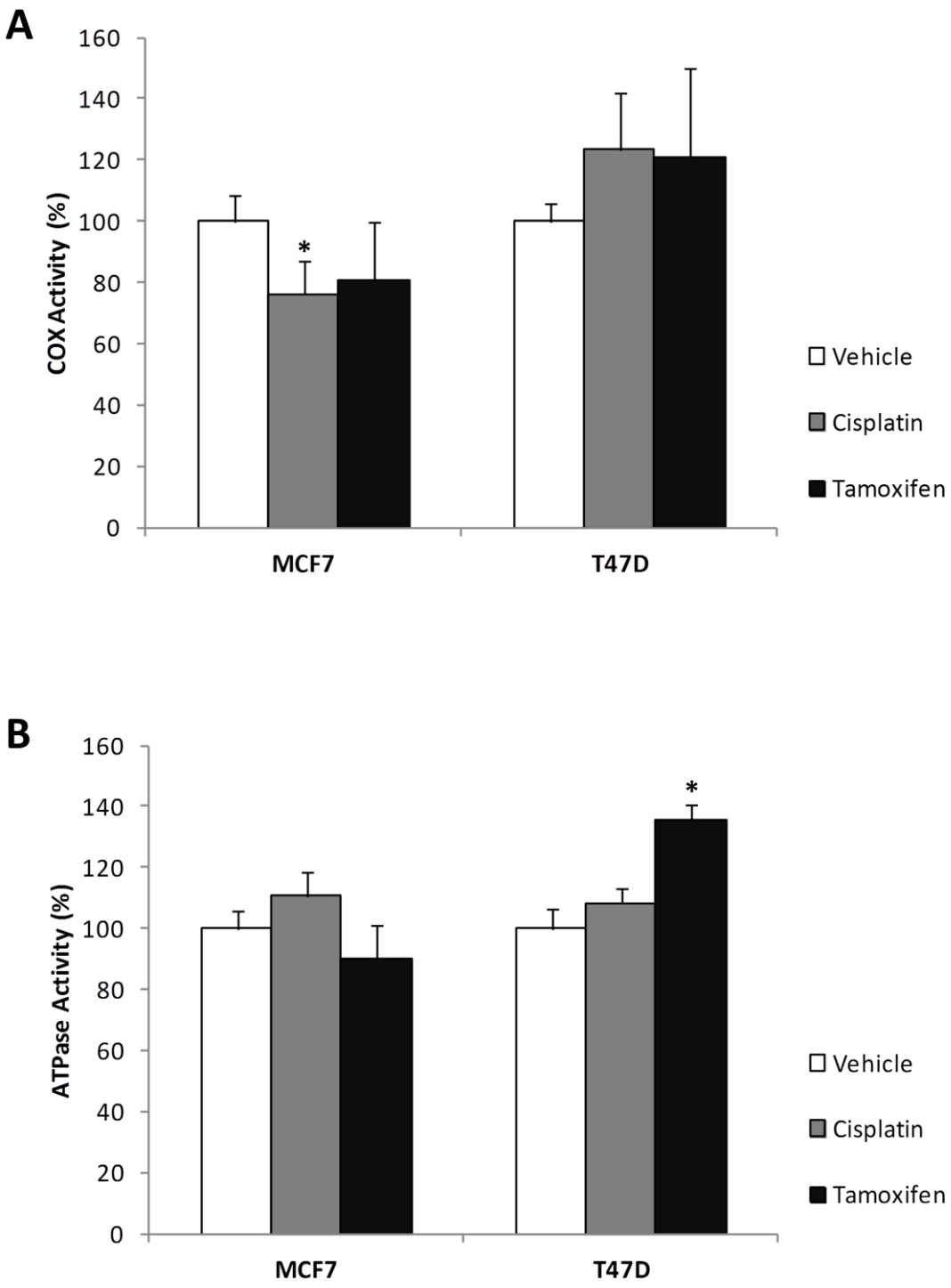


Figure 3C

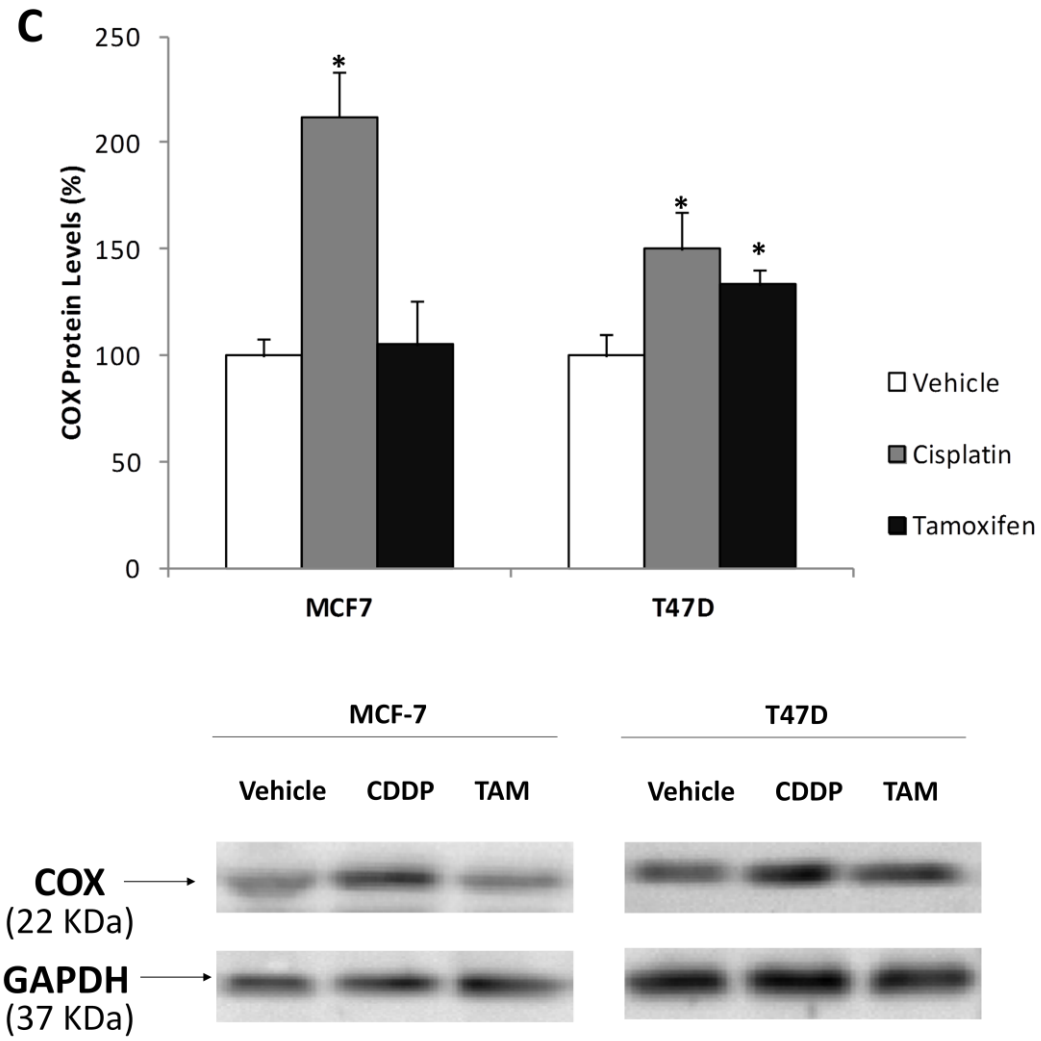

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Figure 3D

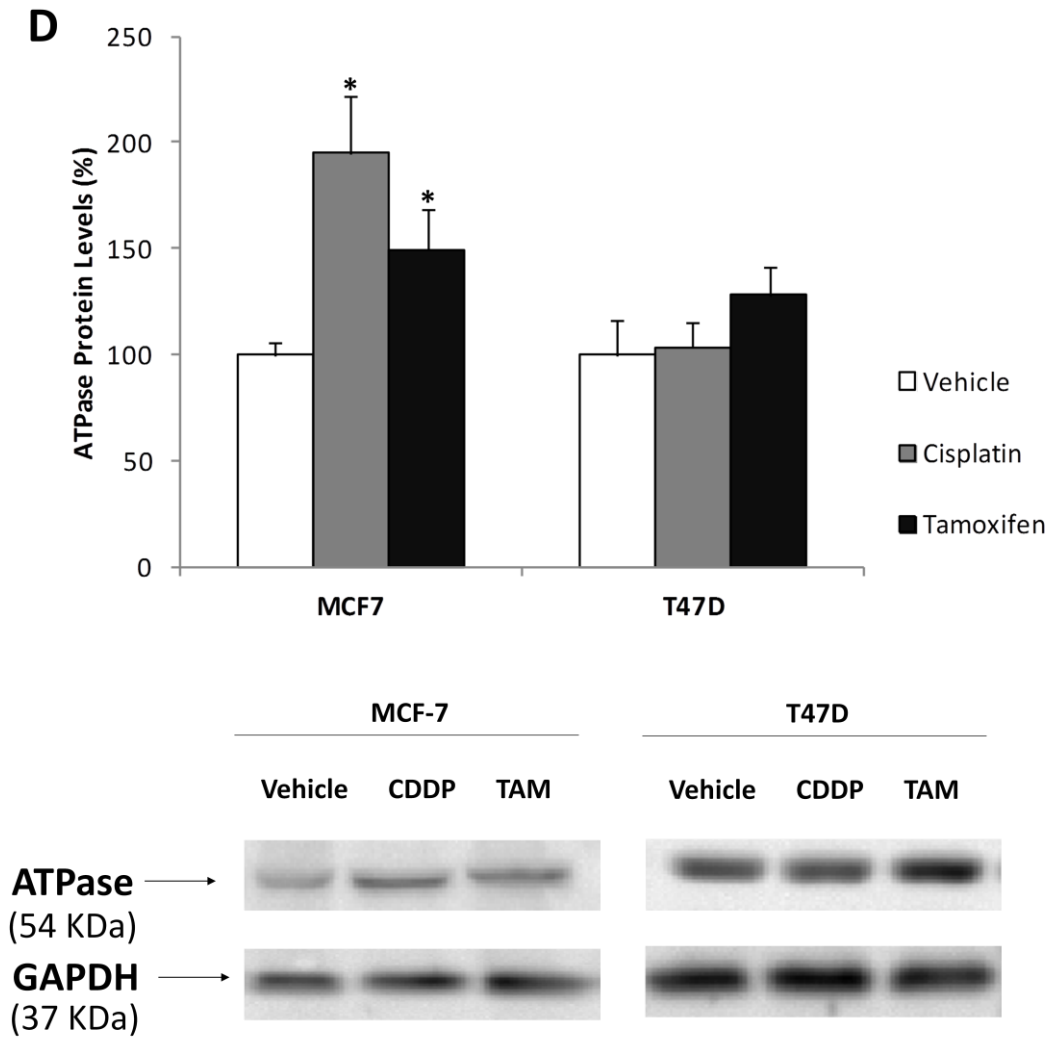
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Figure 4A and 4B

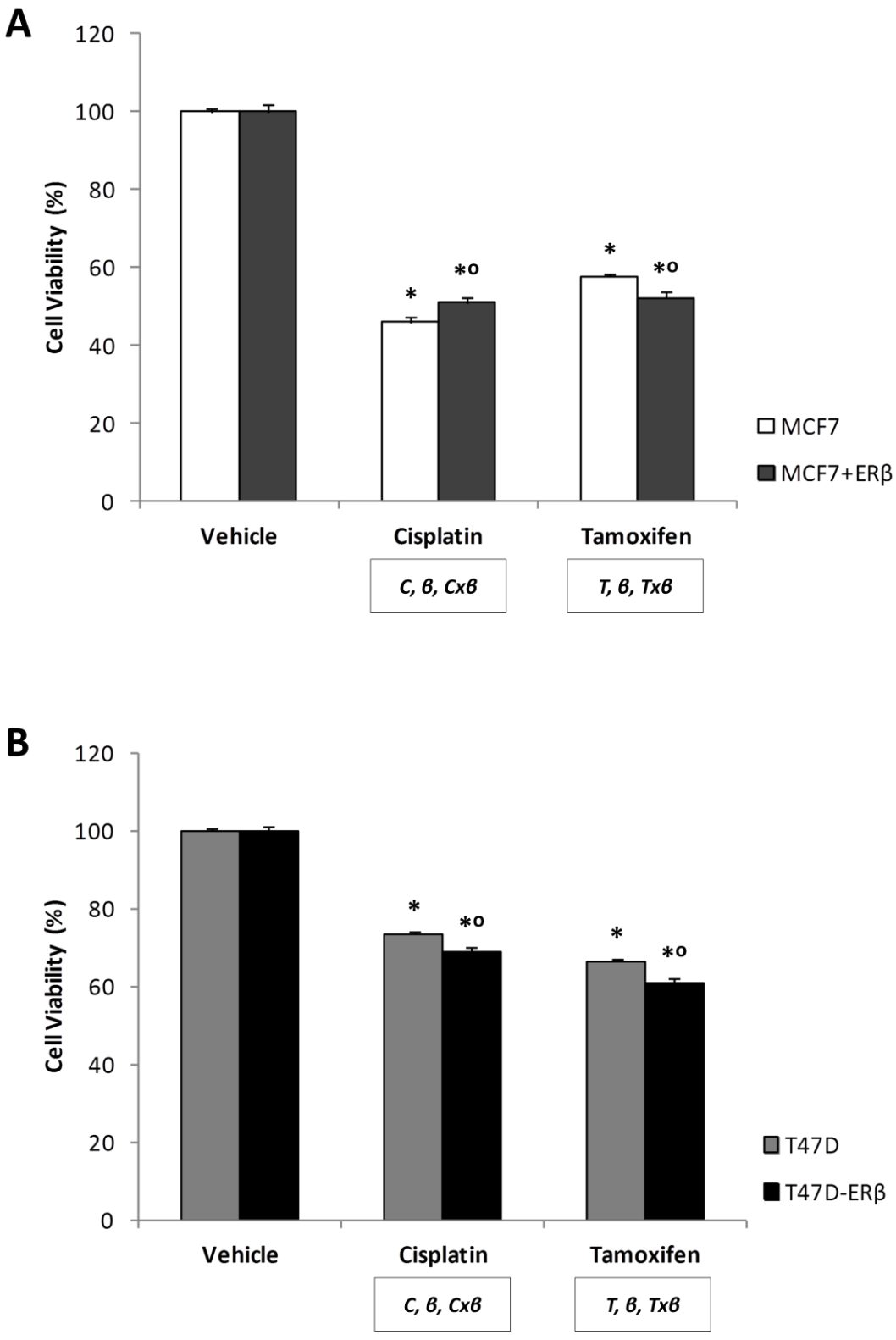
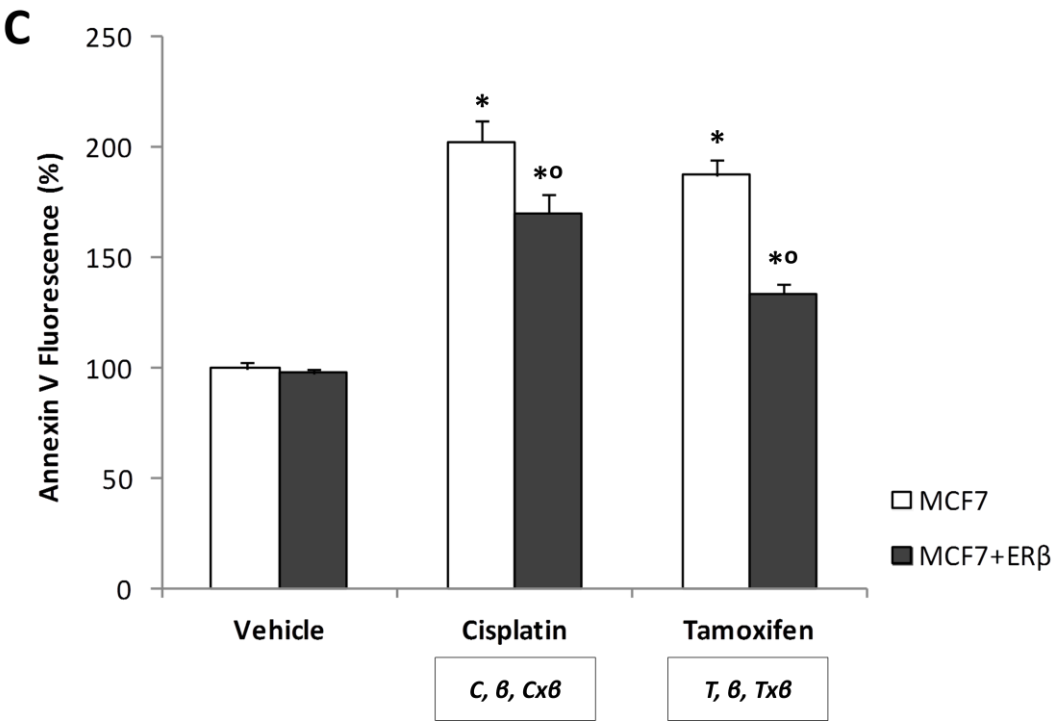


Figure 4C and 4D



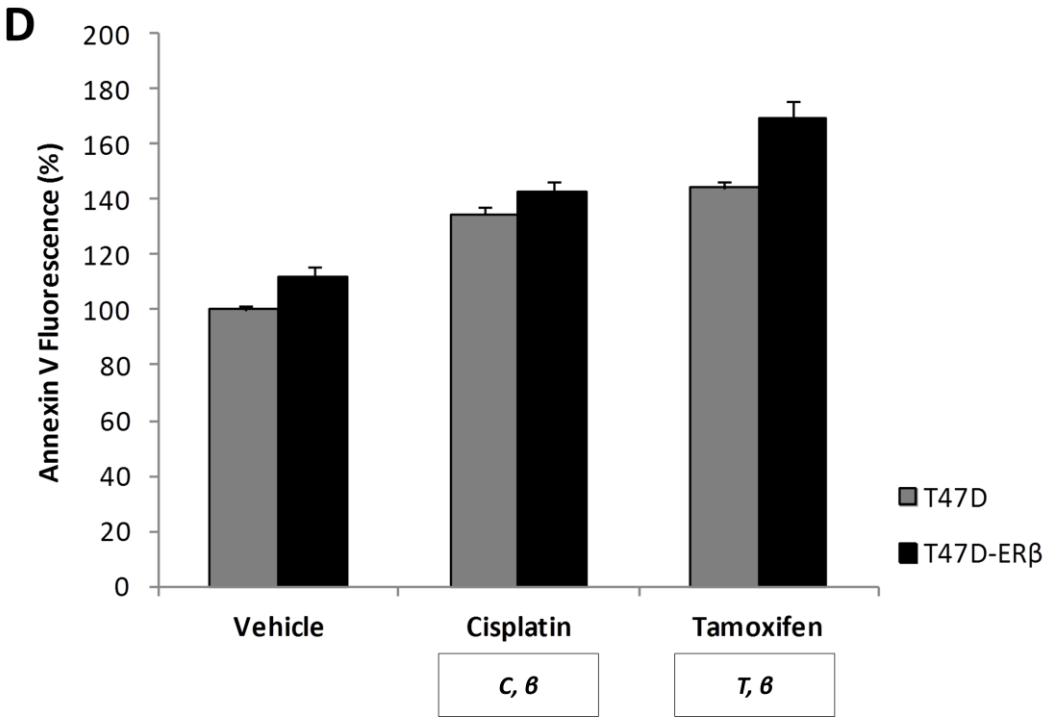
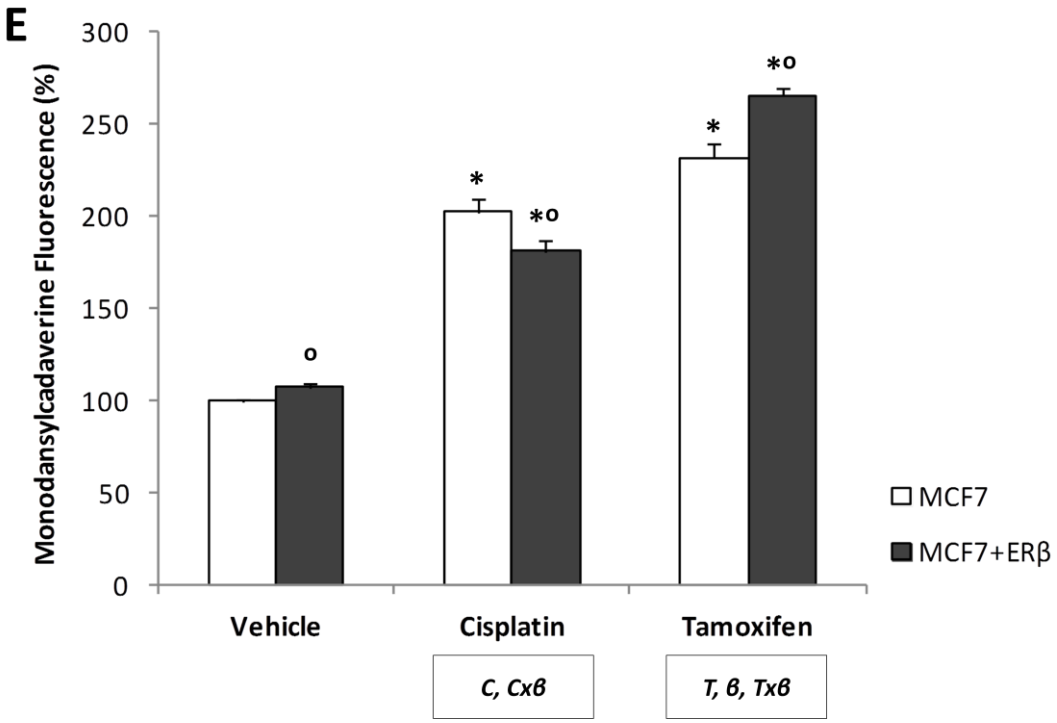


Figure 4E and 4F



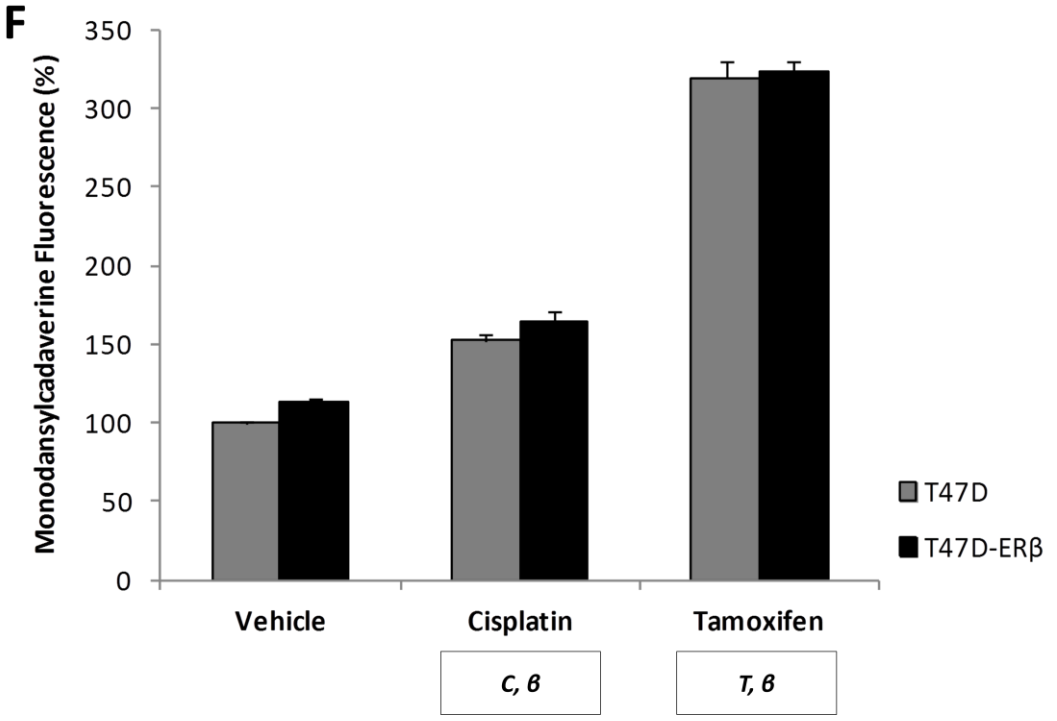
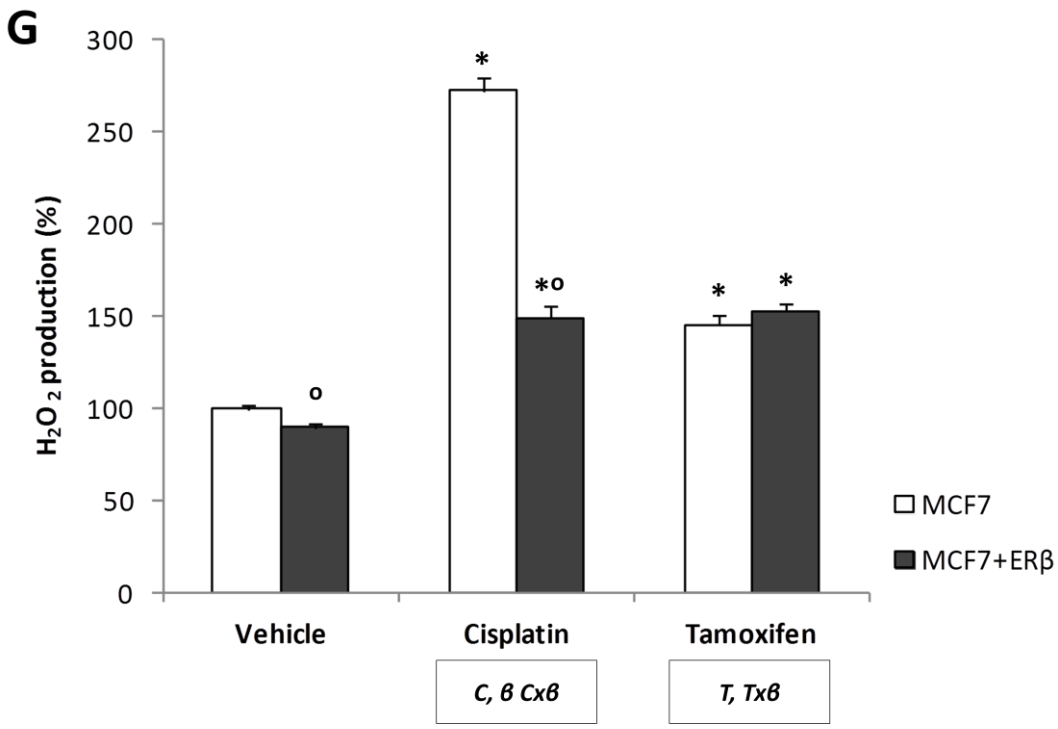
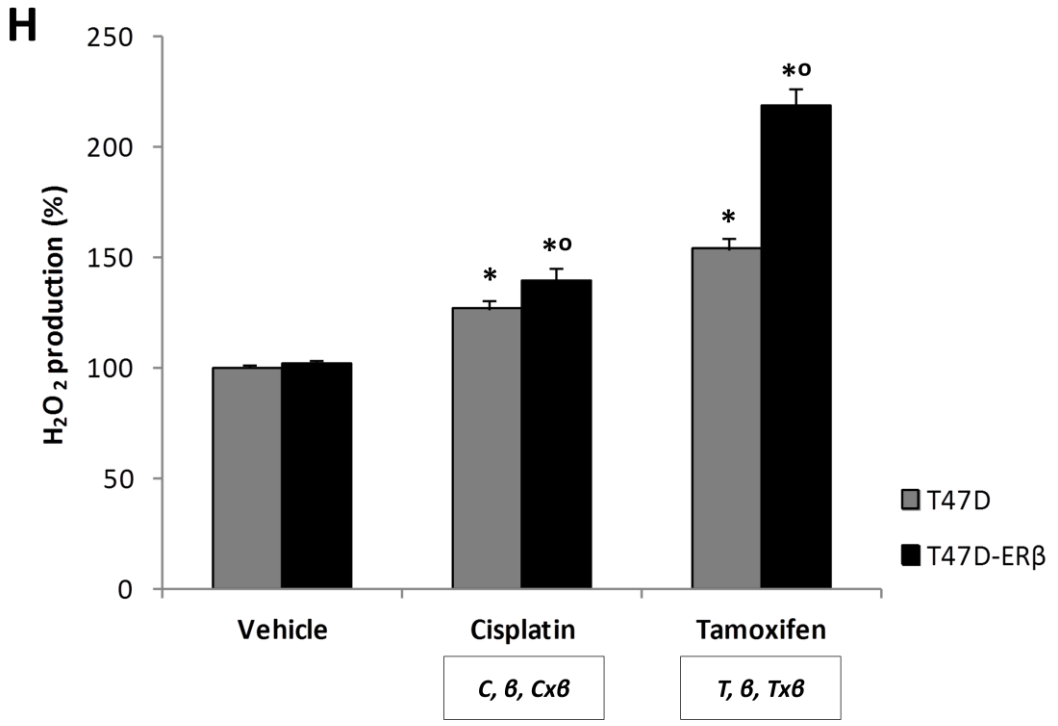


Figure 4G and 4H





DISCUSSION

In the current work, we studied the influence of the ERα/ERβ ratio in the efficacy of several anticancer treatments such as cisplatin (CDDP), paclitaxel (PTX) or tamoxifen (TAM). Thus, in T47D breast cancer cell line (low ERα/ERβ ratio) the same dose of CDDP and PTX exerted a lesser effects than in MCF-7 breast cancer cell line (high ERα/ERβ ratio), while TAM-treatment had a similar effect in both cell lines. These results were confirmed when the ERα/ERβ ratio was modified in both cell lines, with the overexpression of ERβ in the MCF-7 cells and its silencing in T47D ones. Therefore, it should be noted that the action of CDDP in those cell lines with a high ERα/ERβ ratio resulted in a substantial increase in both apoptosis and ROS production and in a drop in mitochondrial functionality, despite the increment in mitochondrial mass. Moreover, all these CDDP effects were partially reverted with the ERβ overexpression. However, TAM treatment had a similar response in both cell lines, especially through the raise of autophagic cell death; for the T47D cell line, ERβ inhibition also caused an increase in TAM treatment efficacy since there was an increase of ROS production and apoptosis.

Tamoxifen is one of the most widely used treatments against ER-responsive breast cancer[20], and for this reason cells used in this study are ERα positive[14]. Although TAM's main effect in breast cancer cells is due to its ability to bind both ERs[21,22], TAM cytotoxicity is also related to ROS production[23]. Both cell lines responded similarly to TAM treatment, and while MCF-7 (high ERα/ERβ ratio) seems to respond increasing both autophagy and apoptosis pathways, T47D (low ERα/ERβ ratio) responsiveness is more related to an increase in autophagic cell death. ROS production was increased in both cell lines in a similar manner, and a similar response was observed for the ΔΨm, as a slight increase in ΔΨm greatly stimulates ROS production[24]. Mitochondrial mass was slightly increased in both cell lines, although no significant changes were found in the COX activity, suggesting that mitochondria are affected in the same way in both cell lines. These similar results may be due to the ability of TAM to inhibit both ERs, especially ERβ because it is a pure antagonist of this receptor[21], and consequently the resulting effect on ROS production and on mitochondrial functionality would be the

same, probably due to the inhibition of the protector role of ERβ against ROS production[13,14,16].

Cisplatin is one of the most widely used chemotherapeutic anticancer agents in many cancer types[25,26]. Although the cytotoxic effects of CDDP are primarily mediated by the generation of nuclear DNA adducts[27], it also targets the mitochondria, which stimulates the ROS production that contributes to its cytotoxic effects[7]. In this case, CDDP treatment caused greater effects in MCF-7 than in T47D cells. For the same dose of CDDP, the T47D cell line (low ERα/ERβ ratio) showed a higher cell viability than the MCF-7 (high ERα/ERβ ratio) cells, accompanied by a reduced activation of apoptosis and autophagy, in addition to a very significant lesser ROS production and a lower ΔΨm, as mentioned above a slight drop in ΔΨm leads to a great decrease in ROS production[24], corroborating the protector role of ERβ and/or the proliferative effect of ERα[28-30]. Moreover, mitochondrial mass was increased only in MCF-7 cells, and the mitochondrial functionality was reduced in MCF-7 but not in T47D cells, suggesting that the greater ROS production in MCF-7 cells could be due to the mitochondrial dysfunction, since Rasbach et al. reported that a better mitochondrial functionality is related to a lower ROS production[31]. Furthermore, the accumulation of damaged mitochondria may play a key role in the increase of mitochondrial dysfunction and, consequently, ROS production, as the mitochondrial mass and the protein levels of complexes IV and V (COX and ATPase) in CDDP-treated MCF-7 cells were increased, despite the loss of mitochondrial functionality through the drop in COX activity. These results are in agreement with the fact that in normal conditions dysfunctional mitochondria are removed[32], however, in some cancers this process is inhibited and the damaged mitochondria that have accumulated in the cells would create mitochondrial dysfunction and ROS production[33]. We have demonstrated, in previous studies carried out in our laboratory, that the importance of the ERα/ERβ ratio in the regulation of the mitochondrial redox homeostasis in breast and prostate cancer[13-17]. The presence of ERβ in breast cancer cells makes them produce less ROS and provide a better mitochondrial functionality[13,14].

Paclitaxel acts against cancer cells through the stabilization of microtubules inhibiting their disassembly[34], ROS production is also involved in PTX cytotoxicity[35].

Cells after PTX treatment showed an intermediate behavior between CDDP- and TAM-treated cells, since T47D cells were slightly more resistant to treatment than MCF-7 ones. The main difference between both cell lines was in the degree of apoptosis. PTX was the treatment that provoked cell apoptosis the most in MCF-7 cells, while in T47D apoptosis was only enhanced much as other treatments. This change in apoptosis could be at least partially due to the increase in ΔΨm and, as a result, in ROS production, whose increase was significantly higher in MCF-7 than in T47D cells. These results would agree with Alexandre et al. who demonstrated that PTX treatment increases ROS production and enhanced apoptosis in lung carcinoma[35]. Again, these differences observed in both cell lines could be due to the presence of ERβ, reducing ROS production[13,36].

These observed differences between the two breast cancer cell lines could be due to their different ERα/ERβ ratio, but for the purpose of confirming this hypothesis we have modified the ERβ expression for the two cell lines, overexpressing it in MCF-7 and silencing it in T47D cells. Thus the ERα/ERβ ratio was modified and we ensured that the different effects observed after cytotoxic treatments would be due, at least in part, to the higher or lower presence of ERβ expression.

Our results that showed a better response to TAM in MCF-7 overexpressing ERβ (MCF-7+ERβ), through increasing the autophagic cell death agree with a recent study carried out by Razandi et al., who demonstrated that the presence of ERβ in the mitochondria could enhance TAM response in TAM-resistant breast cancer cells[8]. Moreover in 2009 Hartman et al. demonstrated a potential relationship between ERβ and TAM response in breast cancer patients[37]. Surprisingly, the inhibition of ERβ in T47D cells also resulted in a greater efficacy of the TAM treatment, increasing ROS production and apoptosis. These results would agree with other studies carried out in our laboratory suggesting that the presence of ERβ is involved in the reduction of ROS production[36,38,39], and thus inhibiting ERβ would lead to oxidative stress and cell death through apoptosis, since other authors have reported that an increase of ROS could trigger apoptosis[40].

Finally, the results obtained in CDDP-treated cells were very clear. The overexpression of ERβ in MCF-7+ERβ cells makes them more resistant to CDDP

treatment, confirming the results obtained in the two breast cancer cell lines mentioned above where the cell line with the lowest ERα/ERβ ratio was the more resistant one to this treatment. The presence of ERβ produced greater cell survival and was accompanied by less apoptosis and autophagy, suggesting that both programmed cell death types (apoptosis and autophagic cell death) were inhibited, at least in part, by the presence of ERβ. Moreover, the increase in ERβ expression in MCF-7 cells resulted in a very significant reduction in ROS production, suggesting a relationship between the presence of ERβ, the decrease in ROS production and the decrease, especially, in apoptosis. Furthermore, we also partially silenced the ERβ in T47D in order to confirm the influence of this receptor in the greater resistance to CDDP of this breast cancer cell line. Results obtained here indicate that the silencing of ERβ triggered a better response to CDDP in T47D cells, showing an increment in apoptosis and autophagy, as well as in ROS production. These results agree with other authors who revealed that the down-regulation of ERβ sensitized lung cancer cells to various apoptosis-inducing agents such as cisplatin or taxol[41]. Additionally, in 2013 Pereira et al. showed that the induction of ROS in colon and breast cancer cells sensitized them to CDDP-induced apoptosis[42].

To sum up, the results obtained in this study suggest the importance of the ERα/ERβ ratio and, specially, the expression of ERβ in the sensitivity to cytotoxic treatments in breast cancer cell lines. The main conclusions of this research are: 1) the presence of ERβ could mean a poorer prognosis factor in those breast cancer patients who are treated with paclitaxel and, especially, cisplatin due, at least partially, to the reduction of ROS which implies a reduction of cancer cells apoptosis and autophagic cell death; 2) in the case of the breast cancer patients treated with tamoxifen, the amount of ERβ determines the response of breast cancer cells to this treatment, because its lower presence increases the apoptosis through ROS generation and its greater presence increase autophagic cell death; and 3) further studies in other cancers are necessary to evaluate the influence of ERβ expression in cancer resistance.

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REFERENCES

1 Siegel, R., Naishadham, D. & Jemal, A. *CA: a cancer journal for clinicians* **63**, 11-30 (2013).

2 Milani, A., Geuna, E., Mittica, G. & Valabrega, G. *World journal of clinical oncology* **5**, 990-1001 (2014).

3 Chang, E. C., Frasor, J., Komm, B. & Katzenellenbogen, B. S. *Endocrinology* **147**, 4831-4842 (2006).

4 Rajah, T. T., Du, N., Drews, N. & Cohn, R. *Pharmacology* **84**, 68-73 (2009).

5 Sotoca, A. M., Ratman, D., van der Saag, P., Strom, A., Gustafsson, J. A. *et al. The Journal of steroid biochemistry and molecular biology* **112**, 171-178 (2008).

6 Andre, N., Carre, M., Brasseur, G., Pourroy, B., Kovacic, H. *et al. FEBS Lett* **532**, 256-260 (2002).

7 Marullo, R., Werner, E., Degtyareva, N., Moore, B., Altavilla, G. *et al. PloS one* **8**, e81162 (2013).

8 Razandi, M., Pedram, A., Jordan, V. C., Fuqua, S. & Levin, E. R. *Oncogene* **32**, 3274-3285 (2013).

9 Chen, J., Adikari, M., Pallai, R., Parekh, H. K. & Simpkins, H. *Cancer chemotherapy and pharmacology* **61**, 979-987 (2008).

10 Pelicano, H., Carney, D. & Huang, P. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **7**, 97-110 (2004).

11 Behrend, L., Henderson, G. & Zwacka, R. M. *Biochemical Society transactions* **31**, 1441-1444 (2003).

12 Ozben, T. *Journal of pharmaceutical sciences* **96**, 2181-2196 (2007).

13 Nadal-Serrano, M., Pons, D. G., Sastre-Serra, J., Blanquer-Rossello Mdel, M., Roca, P. *et al. The international journal of biochemistry & cell biology* **45**, 2045-2051 (2013).

14 Pons, D. G., Nadal-Serrano, M., Blanquer-Rossello, M. M., Sastre-Serra, J., Oliver, J. *et al. Journal of cellular biochemistry* **115**, 949-958 (2014).

15 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Roca, P. & Oliver, J. *The international journal of biochemistry & cell biology* **45**, 1509-1515 (2013).

16 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Valle, A., Oliver, J. *et al. Cell Physiol Biochem* **29**, 261-268 (2012).

17 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Valle, A., Garau, I. *et al. Free radical biology & medicine* **61**, 11-17 (2013).

18 Dando, I., Fiorini, C., Pozza, E. D., Padroni, C., Costanzo, C. *et al. Biochimica et biophysica acta* **1833**, 672-679 (2013).

19 Rodriguez-Enriquez, S., Kai, Y., Maldonado, E., Currin, R. T. & Lemasters, J. J. *Autophagy* **5**, 1099-1106 (2009).

20 Gnant, M., Harbeck, N. & Thomssen, C. *Breast care* **6**, 136-141 (2011).

21 Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J. *et al. Mol Pharmacol* **54**, 105-112 (1998).

22 Jensen, E. V. & Jordan, V. C. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9**, 1980-1989 (2003).

23 Lee, Y. H., Kang, B. S. & Bae, Y. S. *Life sciences* **97**, 116-122 (2014).

24 Korshunov, S. S., Skulachev, V. P. & Starkov, A. A. *FEBS letters* **416**, 15-18 (1997).

25 Byrski, T., Huzarski, T., Dent, R., Gronwald, J., Zuziak, D. *et al. Breast cancer research and treatment* **115**, 359-363 (2009).

26 Kelland, L. *Nature reviews. Cancer* **7**, 573-584 (2007).

27 Jordan, P. & Carmo-Fonseca, M. *Cellular and molecular life sciences : CMLS* **57**, 1229-1235 (2000).

28 Majumder, P. K., Mishra, N. C., Sun, X., Bharti, A., Kharbanda, S. *et al. Cell growth & differentiation : the molecular biology journal of the American Association for Cancer Research* **12**, 465-470 (2001).

29 Tamir, S., Izrael, S. & Vaya, J. *J Steroid Biochem Mol Biol* **81**, 327-332 (2002).

30 Urata, Y., Ihara, Y., Murata, H., Goto, S., Koji, T. *et al. The Journal of biological chemistry* **281**, 13092-13102 (2006).

31 Rasbach, K. A. & Schnellmann, R. G. *The Journal of pharmacology and experimental therapeutics* **325**, 536-543 (2008).

32 Mijaljica, D., Prescott, M. & Devenish, R. J. *Autophagy* **3**, 4-9 (2007).

33 Zhang, Y., Qi, H., Taylor, R., Xu, W., Liu, L. F. *et al. Autophagy* **3**, 337-346 (2007).

34 Luo, Y., Li, D., Ran, J., Yan, B., Chen, J. *et al. Protein & cell* **5**, 469-479 (2014).

35 Alexandre, J., Batteux, F., Nicco, C., Chereau, C., Laurent, A. *et al. International journal of cancer. Journal international du cancer* **119**, 41-48 (2006).

36 Nadal-Serrano, M., Sastre-Serra, J., Pons, D. G., Miro, A. M., Oliver, J. *et al. Journal of cellular biochemistry* **113**, 3178-3185 (2012).

37 Hartman, J., Strom, A. & Gustafsson, J. A. *Steroids* **74**, 635-641 (2009).

38 Miro, A. M., Sastre-Serra, J., Pons, D. G., Valle, A., Roca, P. *et al. The Journal of steroid biochemistry and molecular biology* **123**, 133-139 (2011).

39 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Roca, P. & Oliver, J. *The international journal of biochemistry & cell biology* **44**, 1901-1905 (2012).

40 Simon, H. U., Haj-Yehia, A. & Levi-Schaffer, F. *Apoptosis : an international journal on programmed cell death* **5**, 415-418 (2000).

41 Zhang, G., Yanamala, N., Lathrop, K. L., Zhang, L., Klein-Seetharaman, J. *et al. Mol Endocrinol* **24**, 1737-1747 (2010).

42 Pereira, L., Igea, A., Canovas, B., Dolado, I. & Nebreda, A. R. *EMBO molecular medicine* **5**, 1759-1774 (2013).

Manuscript 3

UCP2 inhibition sensitizes breast cancer cells to therapeutic agents by increasing oxidative stress.

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ABSTRACT

Modulation of oxidative stress in cancer cells plays an important role in the study of the resistance to anticancer therapies. Uncoupling protein 2 (UCP2) may play a dual role in cancer, acting as a protective mechanism in normal cells, while its over-expression in cancer cells could confer resistance to chemotherapy and a higher survival through down-regulation of ROS production. Thus, our aim was to check whether the inhibition of UCP2 expression and function increase oxidative stress and could render breast cancer cells more sensitive to cisplatin (CDDP) or tamoxifen (TAM). For this purpose, we studied clonogenicity, mitochondrial membrane potential (ΔΨm), cell viability, ROS production, apoptosis and autophagy in MCF-7 and T47D (only the last four determinations) breast cancer cells treated with CDDP or TAM, in combination or without a UCP2 knockdown (siRNA or genipin). Furthermore, survival curves were performed in order to check the impact of UCP2 expression in breast cancer patients. UCP2 inhibition and cytotoxic treatments produced a decrease in cell viability and clonogenicity, in addition to an increase in ΔΨm, ROS production, apoptosis and autophagy. It is important to note that CDDP decreased UCP2 protein levels, so that the greatest effects produced by the UCP2 inhibition in combination with a cytotoxic treatment, regarding to treatment alone, were observed in TAM+UCP2siRNA-treated cells. Moreover, this UCP2 inhibition caused autophagic cell death, since apoptosis parameters barely increased after UCP2 knockdown. Finally, survival curves revealed that higher UCP2 expression corresponded with a poorer prognosis. In conclusion, UCP2 could be a therapeutic target in breast cancer, especially in those patients treated with tamoxifen.

Keywords: *Oxidative stress, UCP2, ROS, cancer, cisplatin, tamoxifen, autophagic cell death, resistance.*

INTRODUCTION

Increased oxidative stress plays a crucial role in a variety of pathologic conditions including cancer[1]. Cancer cells often exhibit high levels of intracellular reactive oxygen species (ROS) with complex and controversial biological effects. Previous studies in our lab have demonstrated the influence of ROS in breast cancer induction and progression[2-4]. ROS induce DNA damage, contributing to DNA instability and mutation, which in turn promotes cancer cell growth and survival[5]. However, excessive ROS levels can lead to cell growth arrest, senescence and cell death[5,6]. Therefore, the effective regulation of endogenous and treatment-induced oxidative stress is an important factor to consider in both tumor development and the responses to anticancer therapies[7,8] such as cisplatin (CDDP) or tamoxifen (TAM) treatments.

Several studies have suggested that mitochondrial uncoupling is a major mechanism in the regulation of oxidative stress[9-11]. Uncoupling proteins (UCPs) are a family of inner mitochondrial membrane proteins whose function is to allow the reentry of protons into the matrix, dissipating the proton gradient and, therefore, decreasing mitochondrial membrane potential and ROS production[9,11]. It has been shown that UCPs are up-regulated in response to oxidative stress, acting as a feed-back mechanism to control ROS levels, suggesting that UCPs act as part of the antioxidant systems in the cell[10,12].

Specifically, the role of uncoupling protein-2 (UCP2) in cancer has recently been recognized and has attracted more attention. Derdak *et al.* found that UCP2-null mice developed more colon tumors than the wild-type controls with increased oxidative stress[13]. On the other hand, the same authors found that that UCP2 promotes chemoresistance in colon cancer cells[14]. Consequently, UCP2 may have a dual role in cancer, acting as a protective mechanism in normal cells, while its over-expression in cancer cells may confer resistance to chemotherapy and a higher survival by the down-regulation of ROS levels[15].

This dual regulation of UCP2 expression has been also reported in breast cancer. Previous results in our group demonstrated that repression of UCP2 by estrogens may play a key role in estrogen-induced breast carcinogenesis[2,4]. On the other hand, we

found that breast tumors adapted to oxidative stress showed an increase in uncoupling proteins[3]. In addition, we also observed that ovarian cancer patients were more resistant to carboplatin/paclitaxel treatment when they had lower levels of antioxidant systems, including UCP2 and UCP5, prior to be treated, suggesting that these patients could increase their UCPs levels to counteract the increase of ROS levels produced by these treatments[2]. It is worthy to note that tumor aggressiveness is associated with the ability to acquire higher antioxidant defense[6,7]. These studies, in addition to the association between UCP2 and tumor grade[16,17], suggest that UCP2 over-expression in cancer progression could be a result of a long-term selecting procedure.

Recently, several studies have demonstrated that UCP2 is involved not only in cancer cell transformation, but also in chemoresistance[18-20]. In fact, in pancreatic cancer cells, mitochondrial uncoupling by UCP2 is a mechanism of resistance to the chemotherapeutic drug gemcitabine, working through the negative regulation of mitochondrial ROS production[18]. Moreover, UCP2 inhibition sensitized multidrug resistance acute promyelocytic leukemia cell lines to cytotoxic agents[20]. This evidence suggests that UCP2 targeting may be a novel therapeutic strategy for cancer in combination with drugs that promote oxidative stress.

To approach this aim, we analyzed whether inhibition of UCP2 by siRNA or the specific inhibitor genipin[20] increase oxidative stress and could render breast cancer cells more sensitive to cytotoxic agents. Specifically, we treated the cells with CDDP, one of the most widely used chemotherapeutic anticancer agents in many cancer types[21,22], and TAM, which has been widely used for more than 30 years in breast cancer treatment and prevention[23]. Although the cytotoxic effects of CDDP are primarily mediated by the generation of nuclear DNA adducts[24] and TAM is an antagonist of the estrogen receptor (ER)[25]; CDDP targets the mitochondria, which stimulates the ROS production that contributes to its genotoxic effects[26], while ROS production is also involved in TAM cytotoxicity[8]. Therefore, the development of anti-cancer therapies based on UCP2 inhibition associated with traditional chemotherapeutic treatments could improve treatment efficacy, resulting in a higher oxidative stress and cancer cell death.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's medium (DMEM) high glucose was from GIBCO (Paisley, UK). Genipin (methyl-2-hydroxy-9-hydroxymethyl-3-oxabicyclonona-4,8-diene-5-carboxylate), cisplatin (*cis-diamminedichloroplatinum II or CDDP)* and tamoxifen *(trans-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine or TAM)* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Routine chemicals used were obtained from Sigma-Aldrich, Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture

The MCF-7 and T47D human breast cancer cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and routinely cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. Cells were seeded in plates such that they were confluent when doing the measurements.

Cell transfection and treatments

For transfection, cells were cultured in 6-well or in 96-well culture plates overnight and then, at 60% confluence, transfected for 6 hours with a specific small interfering (si)RNA targeting UCP2 mRNA purchased in Santa Cruz Biotechnology (UCP2 siRNA (h): sc-42682). Lipofectamine 2000 reagent (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) was used to perform cell transfection according to the manufacturer's protocol. After transfection the complexes were removed and cells were provided with normal growth medium. The day after, cells were exposed to vehicle (0.1% DMSO), 10 μM CDDP or 10 μM TAM for 48h.

For UCP2 inhibition, cells were exposed to 0-250 μM genipin for 24h prior the determination of the parameters.

Cell viability assay

MCF-7 or T47D cells were seeded in 96-well plates and incubated overnight. The following day, cells were transfected with UCP2 siRNA or treated with genipin (0-200 µM), and cell density was measured over time (0-72 h) by crystal violet assay. Cytotoxic treatments were applied one day after transfection with UCP2 siRNA, when cells were exposed to the vehicle, CDDP and TAM for 48h, and cell viability was determined. Briefly, cells were stained with 0.5% (p/v) crystal violet in 30% (v/v) acetic acid for 10 min. After washing, the dye was solubilized in 100 µl of methanol and absorbance was measured spectrophotometrically (A595nm) using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) to determine cell viability.

Fluorimetric determination of H_2O_2 production (ROS production)

ROS production was measured fluorimetrically by using an Amplex® Red Hydrogen Peroxide/Peroxidase assay kit (Molecular Probes). MCF-7 or T47D cells were seeded in 96-well plates, and, the day after, incubated with UCP2 siRNA or genipin. For cytotoxic treatments, the day after UCP2 inhibition by siRNA, cells were exposed for 48h to the vehicle, CDDP and TAM. To chemically inhibit UCP2, cells were treated with different concentrations of genipin (0-250 µM) for 24h. Briefly, cells were exposed to 50 µM of Amplex Red reagent and 0.1 U/ml of horseradish peroxidase, in Krebs-Ringer buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4. Fluorescence was measured with an FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA), set at excitation and emission wavelengths of 571 and 585 nm, to detect the maximum slope of increment in the fluorescence within 1 hour of exposure to kit reagents. Thus, the measurement obtained is the H_2O_2 produced (related to ROS production) by the cells for one hour. Values were normalized per number of viable cells determined by crystal violet assay.

Mitochondrial membrane potential and autophagy

Mitochondrial membrane potential (ΔΨm) and autophagy were measured fluorimetrically by using Tetramethylrhodamine methyl ester (TMRM) and

Monodansylcadaverine (MDC), respectively. MCF-7 or T47D cells (the latter only in MDC determination) were seeded in 96-well plates and, the next day, incubated with UCP2 siRNA. The day after UCP2 inhibition by siRNA, cells were exposed for 48h to the vehicle, CDDP and TAM. Fluorescence measurement was performed in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 552 and 576 nm (ΔΨm by TMRM, 100 nM, 15 minutes of incubation), and 340 and 535 nm (autophagy by MDC, 50 µM, 15 minutes of incubation). Values were normalized per number of viable cells determined by crystal violet assay.

Apoptosis assay

Apoptosis was measured fluorimetrically by using Annexin V method, as described by Dando et al.[27]. Briefly, MCF-7 or T47D cells were seeded in 96-well plates and treated as mentioned in the previous section. At the end of the treatment, cells were fixed with 2% paraformaldehyde (BD Biosciences, NJ, USA) in PBS at room temperature for 30 min and washed twice with PBS. Cells were then stained with AnnexinV/AlexaFluor350 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) in annexin binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) for 10 min at room temperature in the dark. To finish, cells were washed once with annexin binding buffer. Florescence was measured in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 346 and 442 nm, with cells kept in 100 µl of annexin binding buffer. Values were normalized per number of viable cells determined by crystal violet assay.

Determination of cell survival using colony formation

For clonogenic assay, MCF-7 breast cancer cells were plated in 6-well plates and transfected the day after with UCP2 siRNA for 6h. Afterwards, the complexes were removed and cells were provided with normal growth medium. The following day, cells were exposed to the vehicle, CDDP or TAM for 48h. After removal of the medium containing cytotoxic agents, cells were trypsinized and plated at low density (5 x 10^3 cells per 60-mm plate). Cells were cultured for 14 days, with a change of the culture

medium three times a week, and colonies were stained with crystal violet and counted for each condition at the end of the culture period.

Real-time quantitative PCR

After transfection with UCP2 siRNA, total RNA was isolated from MCF-7 and T47D cultured cells by using TriPure® isolation reagent (Roche, Barcelona, Spain) following the manufacturer's protocol and then quantified using a spectrophotometer set at 260 nm. 1 µg of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10 µl volume of retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 µM random hexamers, 10 U RNase inhibitor, and 500 µM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−80°C) until the PCR reactions were carried out.

PCR was performed in triplicate samples by SYBR Green technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). The primers used were forward 5'-ggACACggACAggATTgACA-3' and reverse 5'-ACCCACggAATCgAgAAAgA-3' for the 18S ribosomal RNA gene, and forward 5'-ggTggTCggAgATACCAAAg-3' and reverse 5'- CTCgggCAATggTCTTgTAg-3' for the UCP2 gene. Total reaction volume was 10 µL, containing 7.5 µL Lightcycler® 480 SYBR Green I Master (containing 0.5 µM of the sense and antisense specific primers) and 2.5 µl of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95 °C), followed by 45 cycles consisting of a denaturation step (10 s, 95 °C), an annealing step (10s, 61 °C for 18S and 60 °C for UCP2), and an extension step (12s, 72 °C min). A negative control lacking cDNA template was run in each assay.

The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

Western blot analysis

MCF-7 cells were seeded in 6-well plates and transfected with UCP2 siRNA the following day for 6h. Complexes were then removed and cells were provided with normal growth medium, grown overnight and were treated the next day with vehicle, CDDP or TAM. Cell protein extracts were obtained scraping cells with 200 µl of RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 1 mM EDTA, 1mM NaF, 1 mM Na_3VO_4, 10 µM leupeptin and 10 µM pepstatin; finally, 1 mM PMSF was added just before harvesting the cells with the scraper). The lysate was sonicated three times at a 40% amplitude for 7 seconds. Then samples were centrifuged at 14000×*g* for 10 min at 4°C and protein content (supernatant) was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). A 20 µg protein aliquot from the cell lysate was separated on a 10% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. Membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20) for 1h. Antisera against UCP2 and Tubulin (Santa Cruz Biotechnology, CA, USA), and PARP and LC3A/B (Cell Signaling Technology Inc, Danvers, MA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

Measurement of 4-HNE adducts and carbonyl contents

MCF-7 cells were transfected with UCP2 siRNA and total protein extracted as previously described for *Western blot analysis.* For the analysis of 4-hydroxy-2-nonenal (4-HNE) and carbonyl groups, 40 µg and 10 µg of protein from cell lysate, respectively, were separated on a 10% SDS–PAGE gel and electrotransferred onto nitrocellulose membrane. Protein carbonyls were detected using the immunological method OxySelect™ Protein Carbonyl Immunoblot kit (Cell Biolabs, San Diego, CA, USA). For derivatization of carbonyl groups, the membrane was incubated with 2, 4-dinitrophenylhydrazine (DNPH) for 5 min, and then processed according to the manufacturer's instructions. Unspecific binding sites on the membranes were blocked

in 5% non-fat milk in TBS-T (Tris-buffered saline-with 0.05% Tween-20). Antiserum against 4-HNE (Alpha Diagnostic International, San Antonio, TX, USA) and DNP were used as primary antibodies. Bands were visualized using the Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and results were analyzed with Quantity One software (Bio-Rad Laboratories).

Kaplan-Meier survival curves

Kaplan-Meier plots were made using an online (www.kmplot.com) Kaplan-Meier plotter dataset[28], selecting the KM plotter for breast cancer. The analysis was performed taking into account the relapse-free survival (RFS) or the overall survival (OS) of patients. The patients were split by median (with the best cutoff)[29], and the follow up threshold was 10 years, censoring patients surviving over this threshold. The JetSet best probe set was used in order to select the optimal probe set for UCP2 gene[30]. The ER status was fixed in ER positive, as we know that our MCF-7 breast cancer cell line is ERα positive[31].

Statistical analysis

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are expressed as mean values ± standard error of the mean (SEM). Statistical significances between control and UCP2 inhibition were assessed by Student's t-test. The effects of UCP2 inhibition and cisplatin or tamoxifen treatment were analyzed using two-way analysis of variance (ANOVA), and when results reach significant combinatory effects, Student's t-test was performed in order to find out the significance between the experimental groups. Statistical significance was set at $P<0.05$. Kaplan-Meier statistical analyses were made using an online (www.kmplot.com) Kaplan-Meier plotter dataset [28].

RESULTS

UCP2 levels after siRNA transfection

UCP2 inhibition by siRNA was determined by measuring the mRNA and protein levels 24h after siRNA transfection, as shown in Table 1, in order to ensure that UCP2 was at least partially inhibited. Indeed, UCP2 siRNA produced a 72% decrease in UCP2 mRNA levels and a 21% decrease in UCP2 protein levels in MCF-7 cells.

UCP2 inhibition reduces cell viability, and increases ROS production and macromolecules oxidative damage

To evaluate the effect of UCP2 on cell growth, a cell viability assay was performed using siRNA or the specific UCP2 inhibitor genipin. Figure 1A shows that UCP2 knockdown significantly decreased cell growth in a time-dependent manner (0-72h). UCP2 siRNA reduced MCF-7 proliferation by 10% at 24h, and 15% at both 48h and 72h. Furthermore, blocking of UCP2 activity with increasing doses of genipin (0-200 μM) reduced cell proliferation in a dose-dependent manner over the period 0-72h, reaching absolute inhibition at 200 μM (Figure 1B).

The ROS production was measured in UCP2 siRNA or genipin treated cells. As shown in Figure 2, UCP2 inhibition by both UCP2 silencing and genipin enhanced ROS production. Specifically, UCP2 siRNA increased ROS levels by 65% (Figure 2A), while genipin treatment for 24h induced ROS production with maximum levels being reached (298%) at 250 μM (Figure 2B).

In accordance with the great increase in ROS production triggered by UCP2 inhibition, it was decided to test whether UCP2 silencing and cytotoxic agents could increase oxidative damage to proteins and lipids. Table 2 shows that UCP2 silencing by siRNA for 24h increased levels of carbonyl groups and 4-HNE adducts in proteins (end markers of oxidative damage in proteins and lipids, respectively) by 13 and 19%, respectively.

Cisplatin and tamoxifen decreases cell viability and colony formation ability of MCF-7 breast cancer cells

To investigate whether UCP2 inhibition could be a therapeutic target against breast cancer, its inhibition was tested in combination with CDDP and TAM in a cell viability assay. As shown in figure 3A, UCP2 knockdown improved the effectiveness of both cytotoxic agents. CDDP and TAM treatments decreased cell growth by 54% and 42% respectively, while UCP2 silencing reduced it by 32%. Interestingly, UCP2 silencing enhanced the antiproliferative effect of the cytotoxic treatments, especially in TAM+UCP2siRNA-treated cells (18% more than TAM-only treatment). Therefore, MCF-7 breast cancer cells were more sensitive to cytotoxic agent/UCP2 silent combination.

To evaluate the long-term effect of CDDP on the MCF7 breast cancer cell line, a clonogenic assay was performed (Figure 3B). UCP2 knockdown cells resulted in a significant reduction in their capacity to form viable colonies (19%), while CDDP and TAM treatments for 48h decreased it by 75% and 23% respectively. However, only the combination of UCP2 inhibition and TAM exposure reduced significantly (regarding to the treatment alone) the clonogenic survival (-27%).

Cytotoxic agents and UCP2 knockdown increase ROS production and mitochondrial membrane potential (ΔΨm)

Furthermore, the role of UCP2 knockdown was analyzed in the ROS production mediated by the cytotoxic agents. Figure 4A shows that cells presented higher ROS production after CDDP (122%) and TAM (66%) treatments, as well as a 97% increase with the UCP2 knockdown. Moreover, these increments in ROS production were enhanced when cells were treated with UCP2 siRNA in combination with cytotoxic agents, and, once again, the combination with the greatest increase regarding to cytotoxic alone was TAM+UCP2siRNA (+57%).

As UCP2 plays a role dissipating the proton gradient in the inner mitochondrial membrane, an analysis of ΔΨm was performed to determine whether it could be affected by UCP2 inhibition or the cytotoxic agents, and the results are shown in Figure 4B. As expected, the UCP2 knockdown produced an increase of 65% in ΔΨm, as well as

did either of the cytotoxic agents acting alone (CDDP 64% and TAM 73%). The combination resulted in a rise of this parameter in the cytotoxic agents studied, CDDP (+55%) and TAM (+58%).

Furthermore, in order to determine how CDDP and TAM treatments can affect UCP2 protein levels, Western blotting was performed. As expected, Figure 4C and 4D show that UCP2 silencing by siRNA produced 22% less UCP2 protein than the untreated cells. Remarkably, results also revealed that CDDP decreased UCP2 protein levels by 22%, while TAM treatment produced a slight increase in UCP2 protein levels in reference to control vehicle-treated cells, but did not reach any significant change.

Apoptosis is activated by cytotoxic agents, but not increased in combination with UCP2 inhibition

To assess the effect of UCP2 knockdown and cytotoxic agents in programmed cell death of cancer cells, an apoptosis assay was performed. Figure 5A shows that CDDP and TAM increased apoptosis of cancer cells by 69% and 45% respectively, and UCP2 inhibition resulted in only a slight increment of this parameter (16%). However, combination of the UCP2 knockdown with both cytotoxic agents did not result in any significant difference from the cytotoxic treatments alone. Additionally, the cleaved PARP/PARP ratio (apoptosis marker) was studied in CDDP and TAM treated cells in combination or without UCP2 inhibition. As it can be observed in Figure 5C, UCP2 knockdown did not cause any significant change in cleaved PARP ratio; however the cytotoxic agents did increase PARP cleavage by 97% (CDDP) and 247% (TAM). Interestingly, treatment of cancer cells with a combination of UCP2 siRNA and TAM reduced significantly the PARP cleavage with respect to TAM-treated cells (-152%), while CDDP-treated cells in combination with UCP2 siRNA did not present any significant change with respect to CDDP treatment alone.

Activation of autophagic cell death is greater with cytotoxic agents in combination with UCP2 knockdown

As can be observed in Figure 5B, the inhibition of UCP2 triggered a 79% increase in the formation of autophagic vacuoles. Moreover, cytotoxic treatments exhibited a

rise in autophagic vacuoles formation (by 224% CDDP and 226% TAM), and only TAM-treated cells showed an increase (+170%) when combined with UCP2 knockdown, since CDDP in combination with UCP2 inhibition had no any significant increase in comparison to the cytotoxic agent alone. Moreover, Figure 5D shows that UCP2 knockdown increases the ratio LC3-II/LC3-I (autophagic marker) by 62%, as well as CDDP and TAM (by 124% and 178% respectively). Interestingly, combination of UCP2 siRNA with CDDP did not increase this LC3 ratio with respect to CDDP alone, although there was a rise in UCP2 siRNA and TAM combination with respect to cytotoxic treatment alone (+182%).

UCP2 knockdown affects cell viability, ROS production, apoptosis and autophagy in a similar manner in T47D cells after cytotoxic treatments

For the purpose of confirming the results obtained in MCF-7 cells, we have done some critical experiments in another breast cancer cell, T47D. In T47D the UCP2 specific siRNA reduced UCP2 mRNA by 48% (data not shown).

Cell viability assay in T47D (Figure 6A) revealed that UCP2 knockdown, although in a more subtle way than in MCF-7 cells, caused cell death (by 7%) and increased the effectiveness of the TAM treatment (-5% extra regarding TAM treatment alone).

Furthermore, figure 6B shows that in T47D cells the UCP2 knockdown increased the ROS production, even though milder (+11%) than in MCF-7 cells as well as the CDDP and TAM treatments (+46% and +29% respectively), and only the TAM treatment increased significantly the ROS production after UCP2 silencing (+28%).

Moreover, in figure 6C it can be observed that T47D apoptosis was increased with both cytotoxic treatments (+36% in CDDP-treated cells and +44% in TAM-treated cells) and the UCP2 knockdown resulted in a very slight raise in apoptosis (+10%); however, there were no significant changes in Annexin V fluorescence in the combination of cytotoxic and UCP2 siRNA regarding to the cytotoxic treatment alone.

Finally, figure 6D shows that UCP2 inhibition increased autophagy in T47D by 5%. In the same way, cytotoxic treatments caused an increase in autophagic vacuoles

formation by 74% (CDDP) and 317% (TAM). Interestingly, only the TAM treatment in combination with UCP2 knockdown enhanced the autophagic vacuoles formation (+49%) in comparison with TAM treatment alone.

High levels of UCP2 expression represent poorer prognosis in treated breast cancer patients

Figure 7 shows the Kaplan-Meier plots displaying the relapse-free survival (RFS) and the overall survival (OS) of patients expressing high or low levels of UCP2. As can be seen in the figure, a poor prognosis is significantly associated with high levels of UCP2 expression when all patients were taken into account (OS: P=0.0048; RFS: P=0.0073). Nevertheless, systematically untreated patients did not present any significant differences between high and low UCP2 expression patients. Finally, it is important to note a significant difference (P=0.0039) in RFS in TAM-treated patients, showing a poorer prognosis for those patients who had higher levels of UCP2 expression.

FIGURE CAPTIONS

Figure 1. *Effect of UCP2 inhibition on cell growth*

A) MCF-7 cells were incubated with the UCP2 siRNA transfection mix 6h at 37°C in a CO_2 incubator. Normal growth medium was replaced, and cells were further grown for 24h (t=0h). Cell viability was analyzed by Crystal Violet assay as described in Materials and Methods. B) MCF-7 cells were seeded in p96-well plates, incubated overnight, and provided with fresh medium containing genipin (0-200 μM) for 0-72h. Values are expressed as means ± SEM (n=6) and normalized as percentage of the control value. Every condition has significant difference regarding to Control- or Vehicle-treated cells at the time of 0h (Student's *t*-test; $P<0.05$, n=6).

Figure 2. *UCP2 inhibition increased ROS production*

A) MCF-7 cells were incubated with the siRNA transfection mix 6h at 37°C in a CO_2 incubator. Normal growth medium was replaced, and cells were further grown for 24h. ROS production was measured fluorimetrically using Amplex red reagent. B) MCF-7 cells were treated with different concentrations of genipin (0-250 μM) for 24h. ROS production was measured fluorimetrically using Amplex red reagent. Values are expressed as means ± SEM (n=6) and normalized as percentage of the control value. * Significant difference between Control- or Vehicle-treated cells and UCP2siRNA- or Genipin-treated cells, respectively (Student's *t*-test; $P<0.05$, n=6).

Figure 3. *MCF-7 cell viability and clonogenic ability after UCP2 siRNA and/or cisplatin or tamoxifen treatment*

A) MCF-7 cells were incubated with the UCP2 siRNA transfection. Next, cells were treated with 10 μM cisplatin or 10 μM tamoxifen for 48h. Cell proliferation was analyzed by Crystal Violet assay. B) Clonogenic assay of MCF-7 cells were performed after the incubation of cells with the UCP2 siRNA transfection. Then, cells were treated

with a medium containing 10 μM CDDP or 10 μM tamoxifen for 48h; the cells were then trypsinized and plated at low density (5 x 10^5 cells per 60-mm plate); after 2 weeks, formed colonies were stained with crystal violet and clones were counted for each condition. Values are expressed as means ± SEM (n=6), and normalized as percentage of the control value. ANOVA analysis was carried out where S means UCP2 siRNA effect, C means cisplatin effect, T means tamoxifen effect and CxS or TxS means combinatory effect of cytotoxic treatments and UCP2 siRNA. As a result of combinatory effect, Student's t-test ($P<0.05$, n=6) was carried out: ^{o} significant difference between Control and UCP2 siRNA treated cells; * significant difference between Vehicle and Cytotoxic treated cells; *^{o} significant difference between Control and UCP2 siRNA in Cytotoxic treated cells.

Figure 4. *Cytotoxic treatments increased ROS production, mitochondrial membrane potential and cisplatin treatment decreased UCP2 protein levels*

MCF-7 cells were incubated with the UCP2 siRNA transfection mix; next, cells were treated with 10 μM cisplatin or 10 μM tamoxifen for 48h. A) ROS production was measured fluorimetrically using Amplex red reagent. B) Mitochondrial membrane potential (ΔΨm) was measured fluorimetrically using TMRM. C) UCP2 protein expression was measured in MCF-7 cells treated with UCP2 siRNA and/or cisplatin and tamoxifen by Western blot analysis. D) UCP2 Western blot representative bands (Tubulin was used as a housekeeping protein). Values are expressed as means ± SEM (n=6), and normalized as percentage of the control value. ANOVA analysis was carried out where S means UCP2 siRNA effect, C means cisplatin effect, T means tamoxifen effect and CxS or TxS means combinatory effect of cytotoxic treatments and UCP2 siRNA. As a result of combinatory effect, Student's t-test ($P<0.05$, n=6) was carried out: ^{o} significant difference between Control and UCP2 siRNA treated cells; * significant difference between Vehicle and Cytotoxic treated cells; *^{o} significant difference between Control and UCP2 siRNA in Cytotoxic treated cells.

Figure 5. *UCP2 inhibition increased autophagic cell death instead of apoptotic cell death in MCF-7 cell line*

MCF-7 cells were incubated with the UCP2 siRNA transfection mix; next, cells were treated with 10 µM cisplatin or 10 µM tamoxifen for 48h. A) Apoptosis fraction was measured fluorimetrically using Annexin V method. B) Autophagic vacuoles formation was measured fluorimetrically using monodansylcadaverine reagent. C) Cleaved PARP/PARP protein expression was measured in MCF-7 cells treated with UCP2 siRNA and/or cisplatin and tamoxifen by Western blot analysis. PARP (116 KDa) and Cleaved-PARP (89 KDa) Western blot representative bands (Tubulin was used as a housekeeping protein). D) LC3-I/LC3-II UCP2 protein expression was measured in MCF-7 cells treated with UCP2 siRNA and/or cisplatin and tamoxifen by Western blot analysis. LC3-I (16 KDa) and LC3-II (14 KDa) Western blot representative bands (Tubulin was used as a housekeeping protein). Values are expressed as means ± SEM (n=6), and normalized as percentage of the control value. ANOVA analysis was carried out where S means UCP2 siRNA effect, C means cisplatin effect, T means tamoxifen effect and CxS or TxS means combinatory effect of cytotoxic treatments and UCP2 siRNA. As a result of combinatory effect, Student's t-test ($P<0.05$, n=6) was carried out: ^{o} significant difference between Control and UCP2 siRNA treated cells; * significant difference between Vehicle and Cytotoxic treated cells; $*^{o}$ significant difference between Control and UCP2 siRNA in Cytotoxic treated cells.

Figure 6. *Cell viability, ROS production, apoptosis and autophagy in T47D breast cancer cell line after UCP2 knockdown and cytotoxic treatments*

T47D cells were incubated with the UCP2 siRNA transfection mix. The next day, cells were treated with 10 µM cisplatin or 10 µM tamoxifen for 48h. A) Cell proliferation was analyzed by Crystal Violet assay. B) ROS production was measured fluorimetrically using Amplex red reagent. C) Apoptosis fraction was measured fluorimetrically using Annexin V method. D) Autophagic vacuoles formation was measured fluorimetrically using monodansylcadaverine reagent. Values are expressed as means ± SEM (n=6), and normalized as percentage of the control value. ANOVA analysis was carried out where

S means UCP2 siRNA effect, C means cisplatin effect, T means tamoxifen effect and CxS or TxS means combinatory effect of cytotoxic treatments and UCP2 siRNA. As a result of combinatory effect, Student's t-test ($P<0.05$, $n=6$) was carried out: ^{o} significant difference between Control and UCP2 siRNA treated cells; * significant difference between Vehicle and Cytotoxic treated cells; $*^{o}$ significant difference between Control and UCP2 siRNA in Cytotoxic treated cells.

Figure 7. *Kaplan-Meier survival curves revealed that high UCP2 expression corresponds with a poorer prognosis*

Kaplan-Meier plots were made using an online (www.kmplot.com) Kaplan-Meier plotter dataset. The analysis was performed taking into account: A) the relapse-free survival (RFS); or B) the overall survival (OS) of patients; depending on their UCP2 expression levels.

TABLES

Table 1. UCP2 mRNA and protein levels as the result of silencing by UCP2 specific siRNA in MCF-7 breast cancer cell line.

	Control	UCP2 siRNA
UCP2 mRNA levels (% AU)	100 ± 16	27.8 ± 0.4*
UCP2 protein levels (% AU)	100 ± 6	79.3 ± 9.7*

MCF-7 cells were transfected with UCP2 siRNA for 6h. Next, complexes were removed and cells were provided with normal growth medium for 24h, and then harvested for PCR and western blot analysis. Data represent the means ± SEM (n = 6). Values of Control were set at 100. AU: arbitrary units. * Significant difference between UCP2 siRNA-treated and LF-treated cells (Student's *t*-test; P≤0.05).

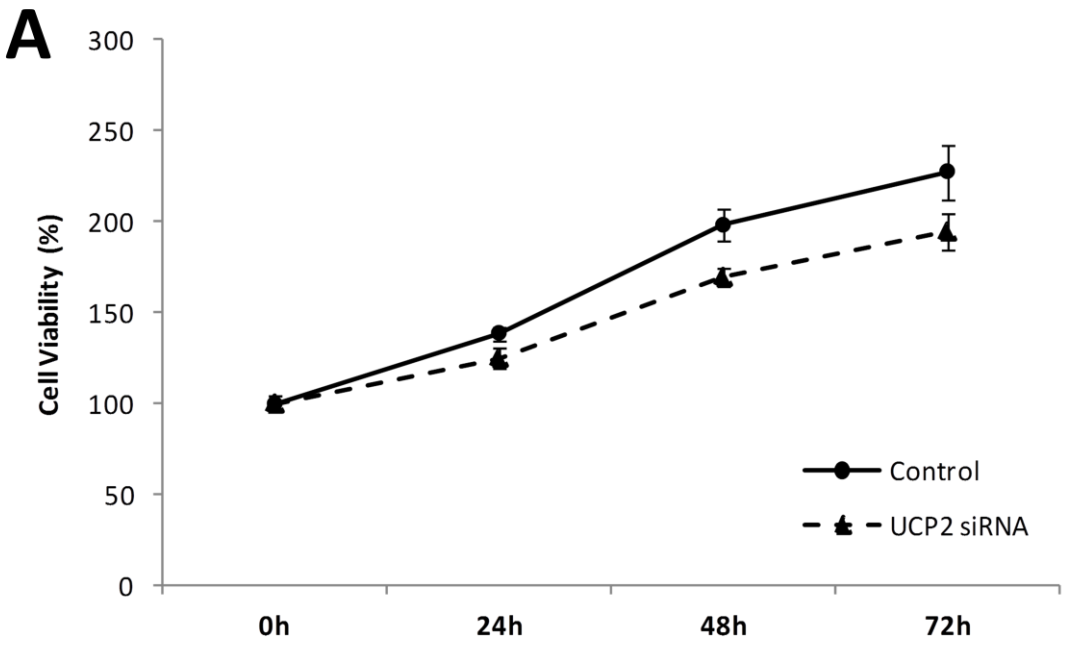
Table 2. Increase in carbonyl content and 4-HNE adduct formation after UCP2 silencing.

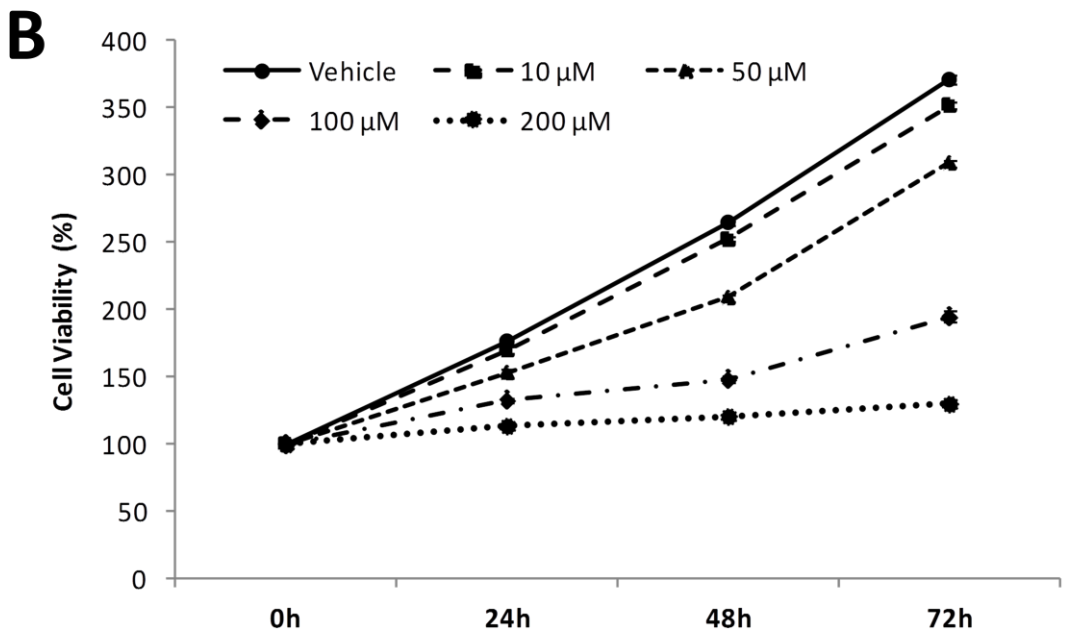
	Control	UCP2 siRNA
Protein carbonyls (% AU)	100 ± 2	113 ± 7*
4-HNE adducts (% AU)	100 ± 6	119 ± 7*

MCF-7 cells were transfected with UCP2 siRNA for 6h. Next, complexes were removed and cells were provided with normal growth medium for 24h. Whole-cell extracts were used for protein carbonyls detection (A) and 4-HNE adducts in proteins (B) by Western blot analysis. Values are expressed as means ± SEM (n=6) and normalized as percentage of the control value. * Significant difference between UCP2 siRNA-treated and Control cells (Student's *t*-test; P≤0.05).

FIGURES

Figure 1





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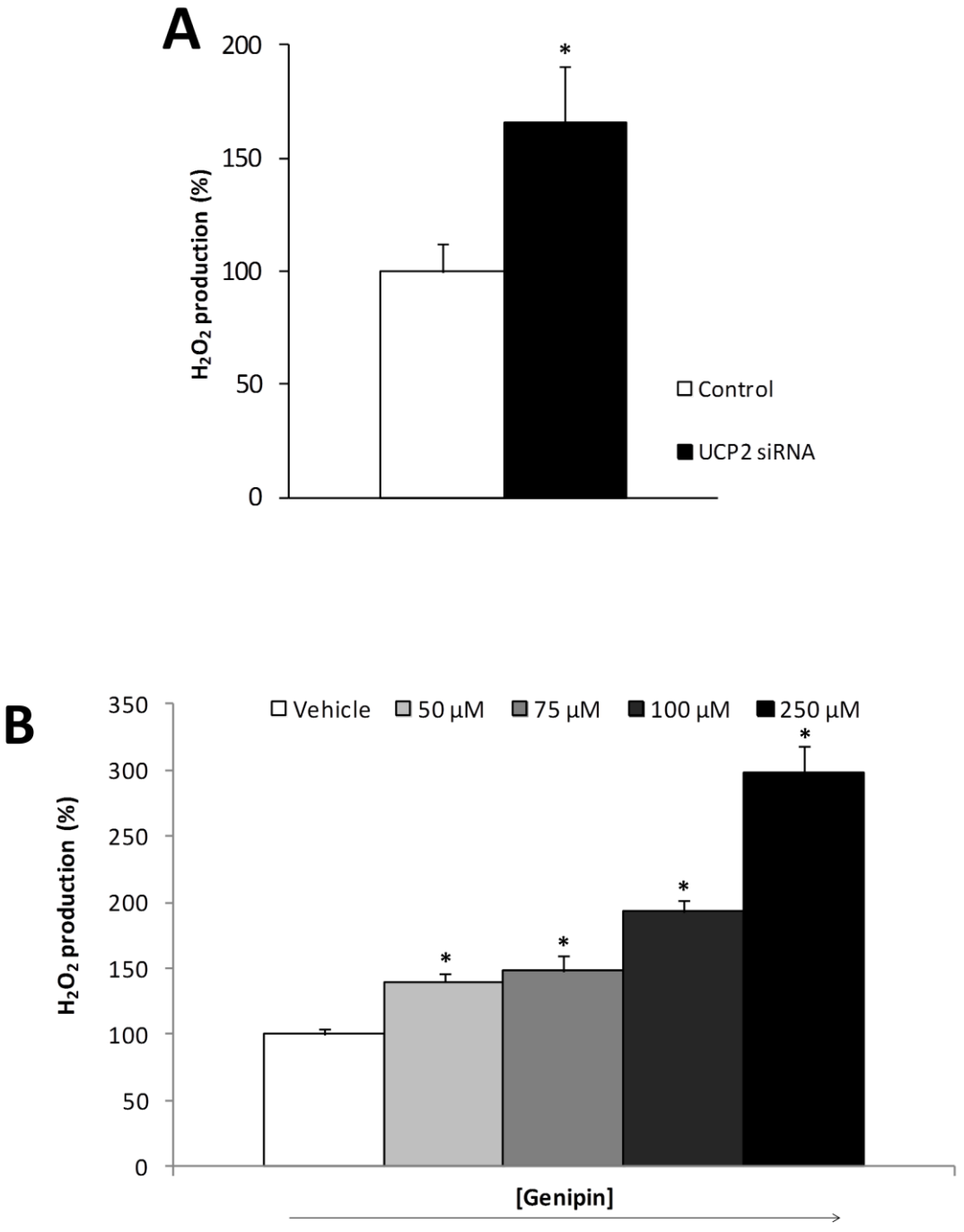
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200
150
100
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0
H_2O_2 production (%)
*
Control
UCP2 siRNA
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300
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H_2O_2 production (%)
Vehicle
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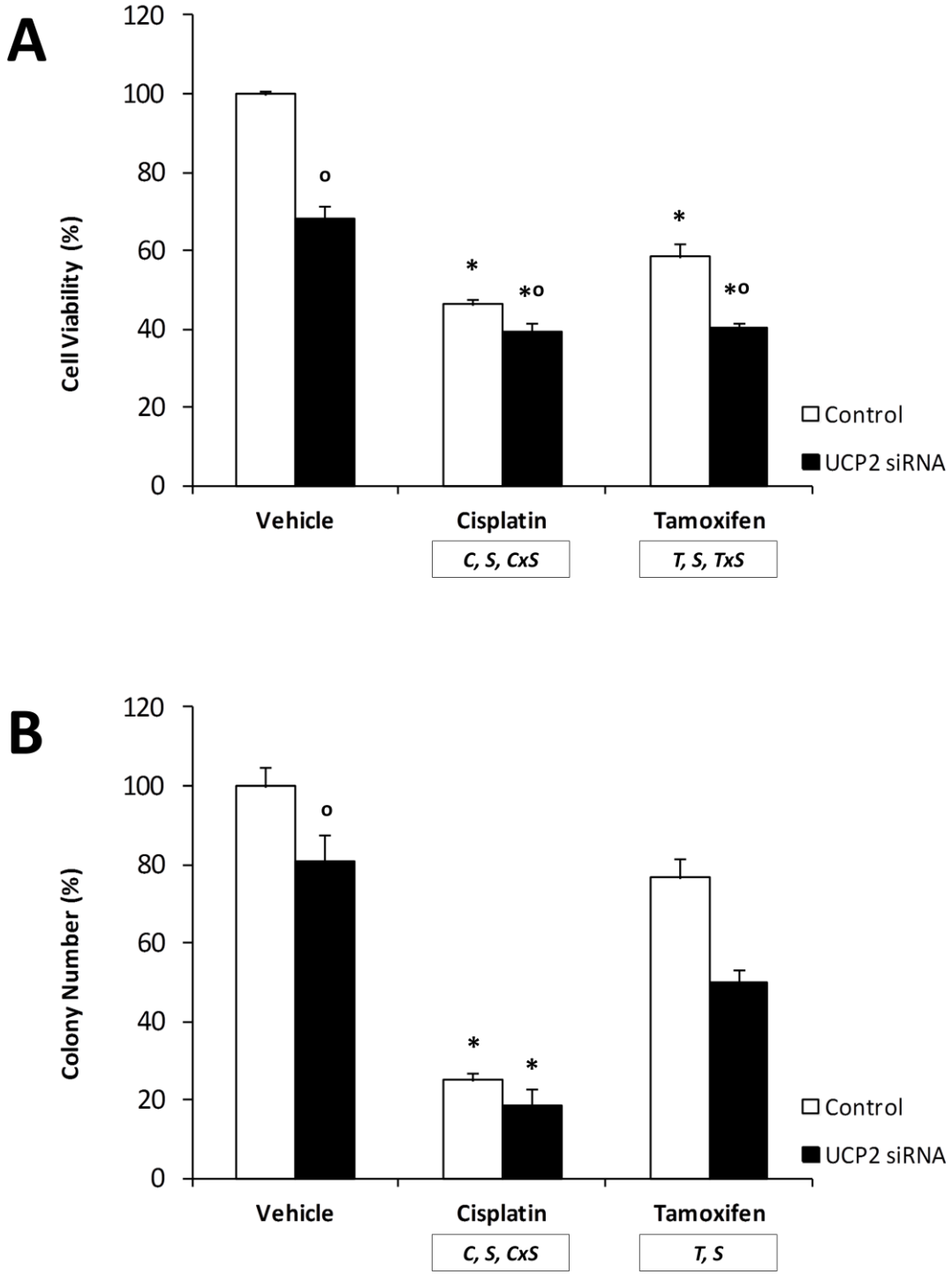
75 µM
100 µM
250 µM
*
[Genipin]

Figure 3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Cell Viability (%)
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40
20
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Control
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Vehicle
Cisplatin
Tamoxifen
C, S, CxS
T, S, TxS
B
Colony Number (%)
120
100
80
60
40
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Control
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Vehicle
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Tamoxifen
C, S, CxS
T, S

Figure 4A and 4B

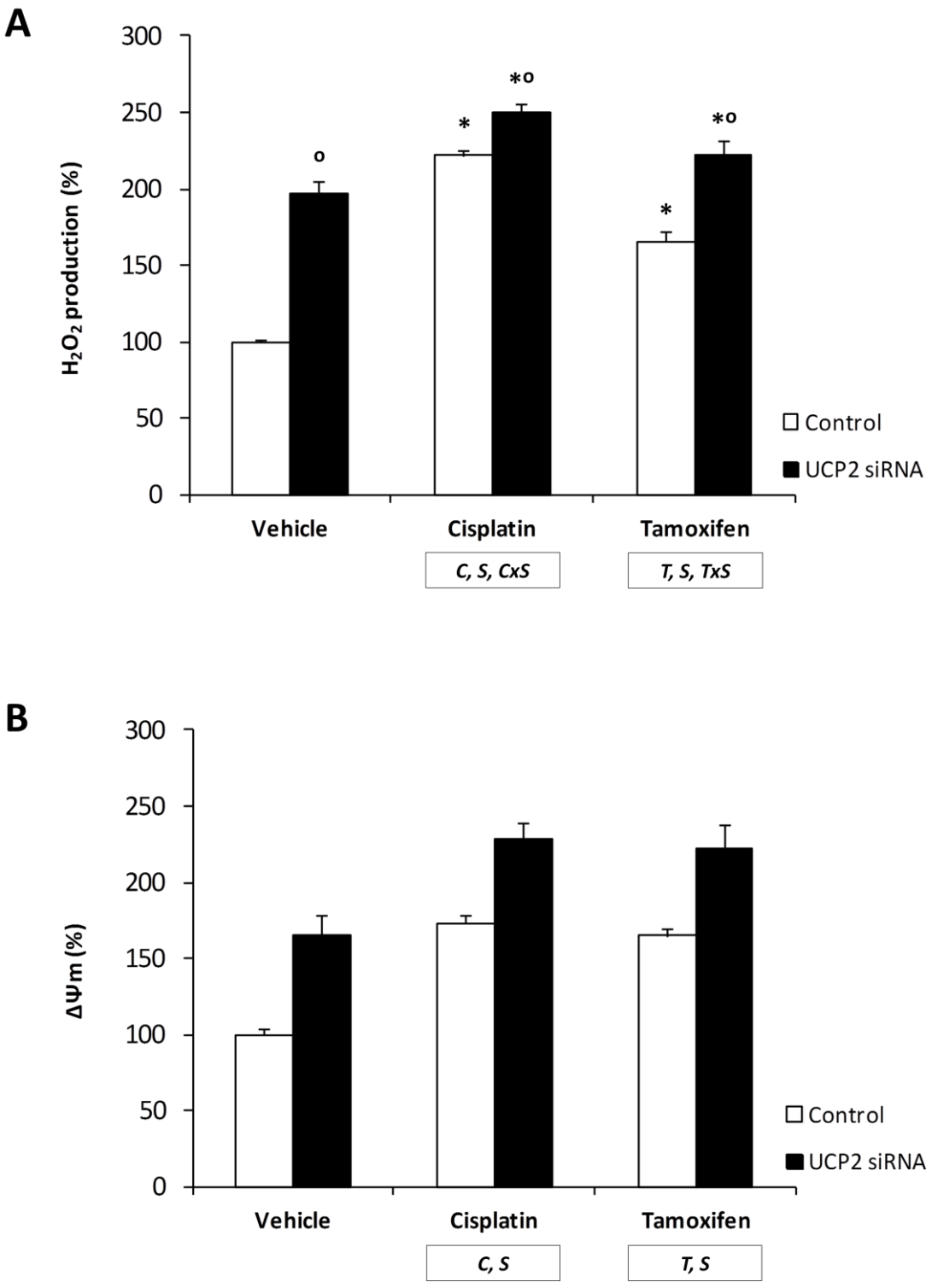
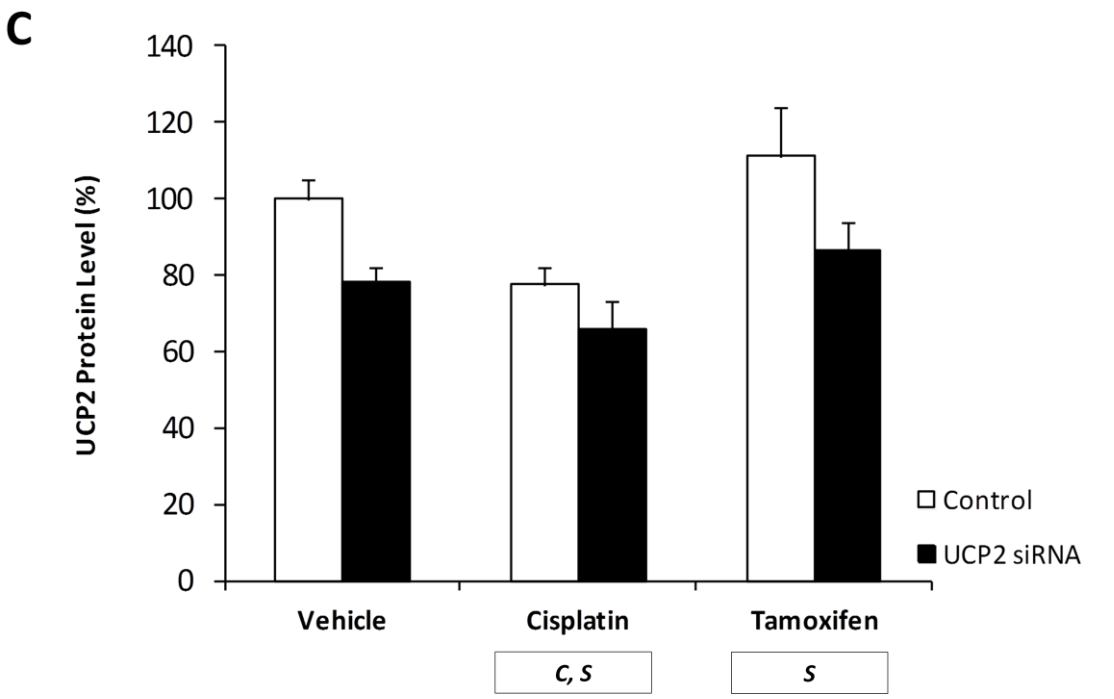


Figure 4C and 4D



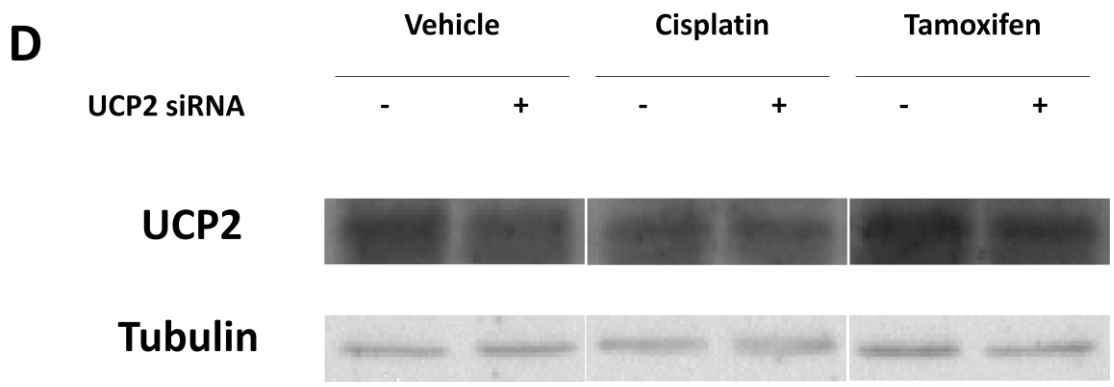


Figure 5A and 5B

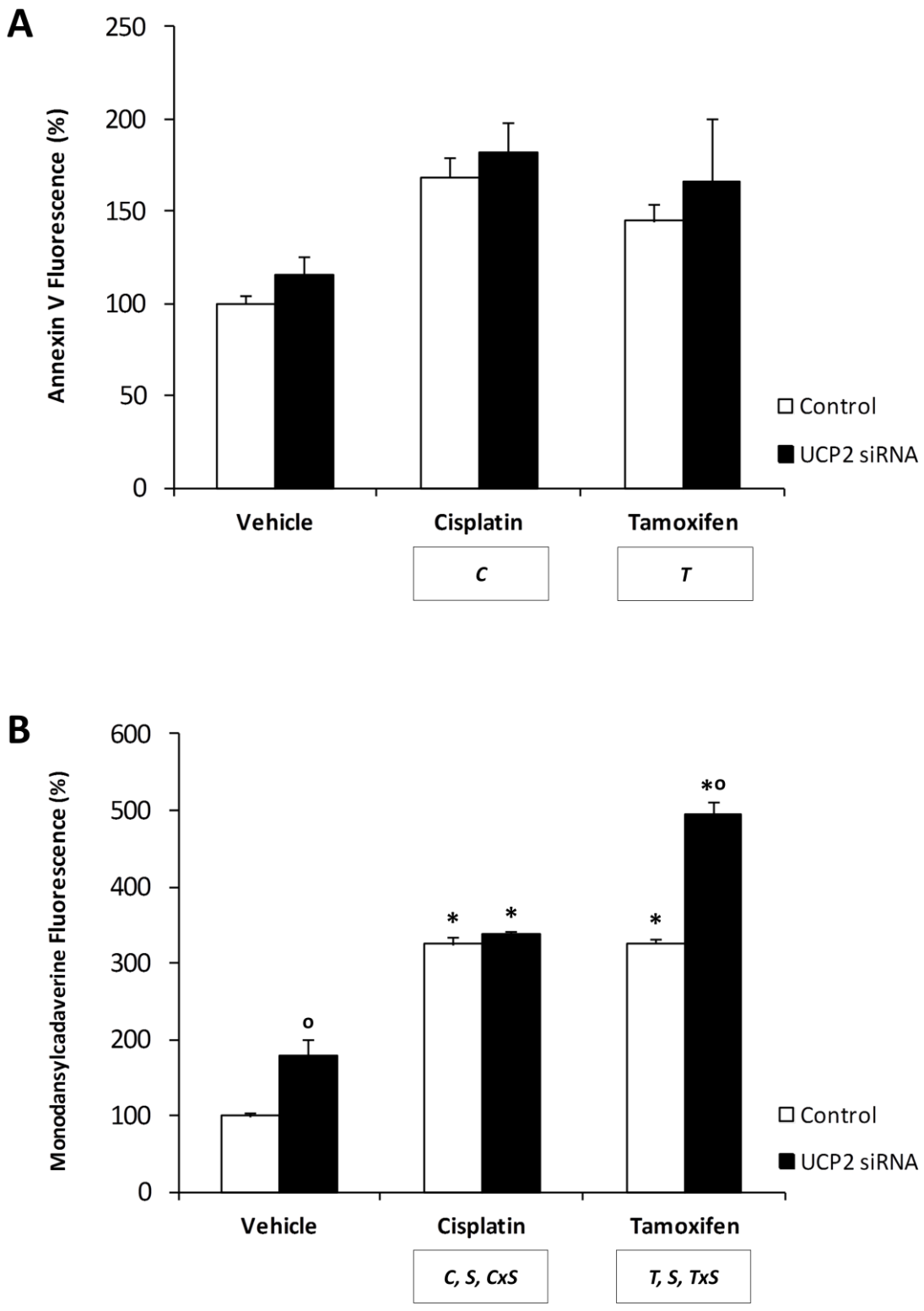
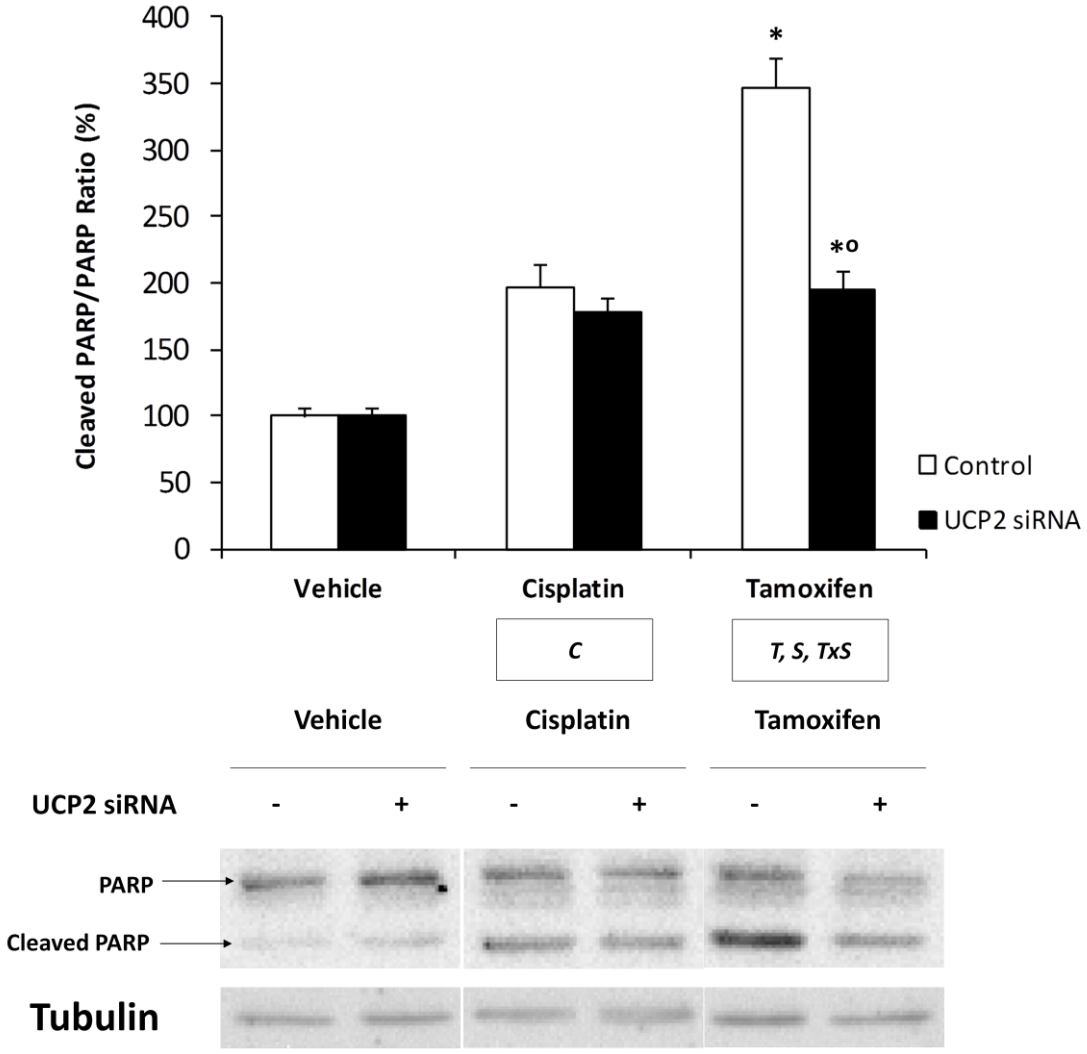


Figure 5C


Cleaved PARP/PARP Ratio (%)
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350
300
250
200
150
100
50
0
*
*o
Control
UCP2 siRNA
Vehicle
Cisplatin
Tamoxifen
C
T, S, TxS
Vehicle
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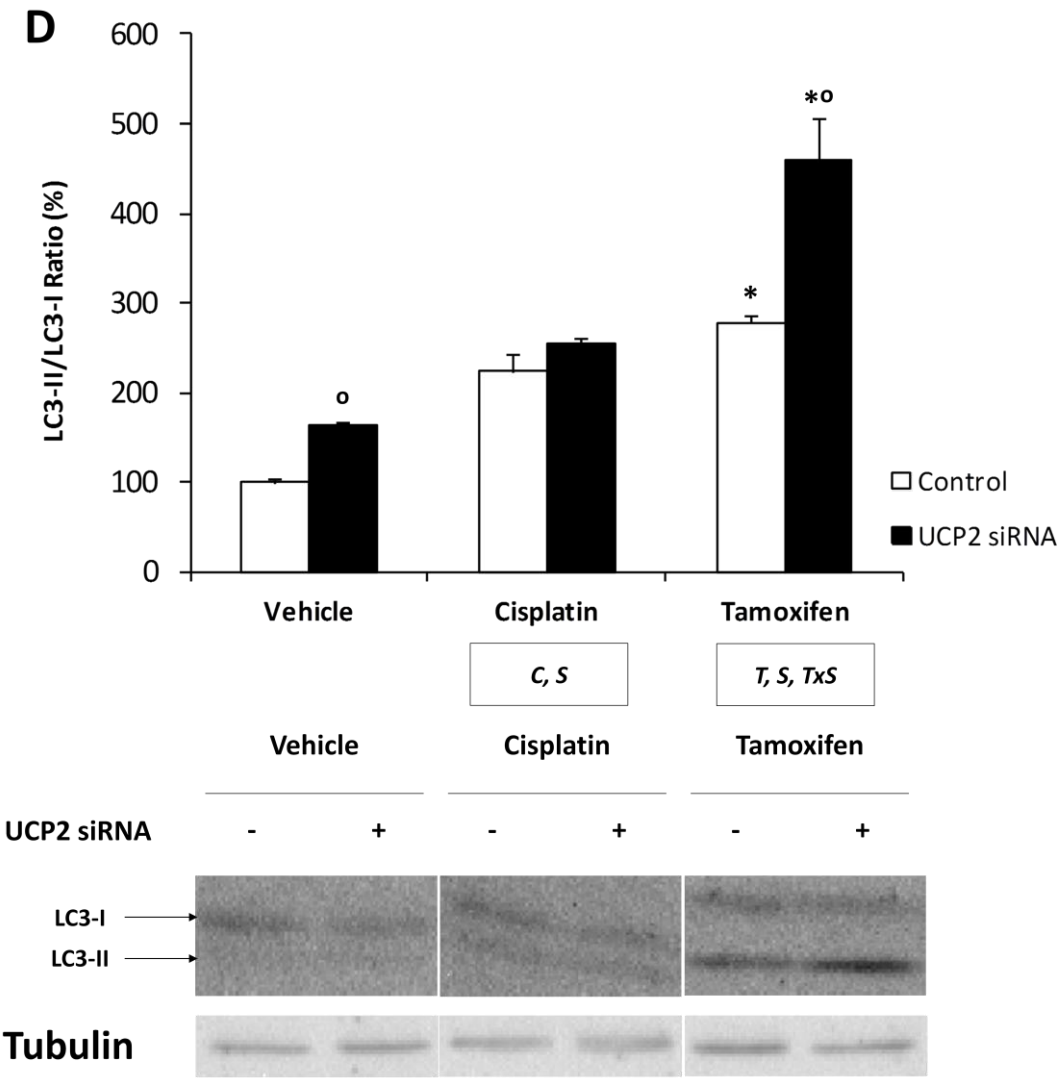
- + - + - +
PARP
Cleaved PARP
Tubulin

Figure 5D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LC3-II/LC3-I Ratio (%)
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500
400
300
200
100
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o
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Control
UCP2 siRNA
Vehicle
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Tamoxifen
C, S
T, S, TxS
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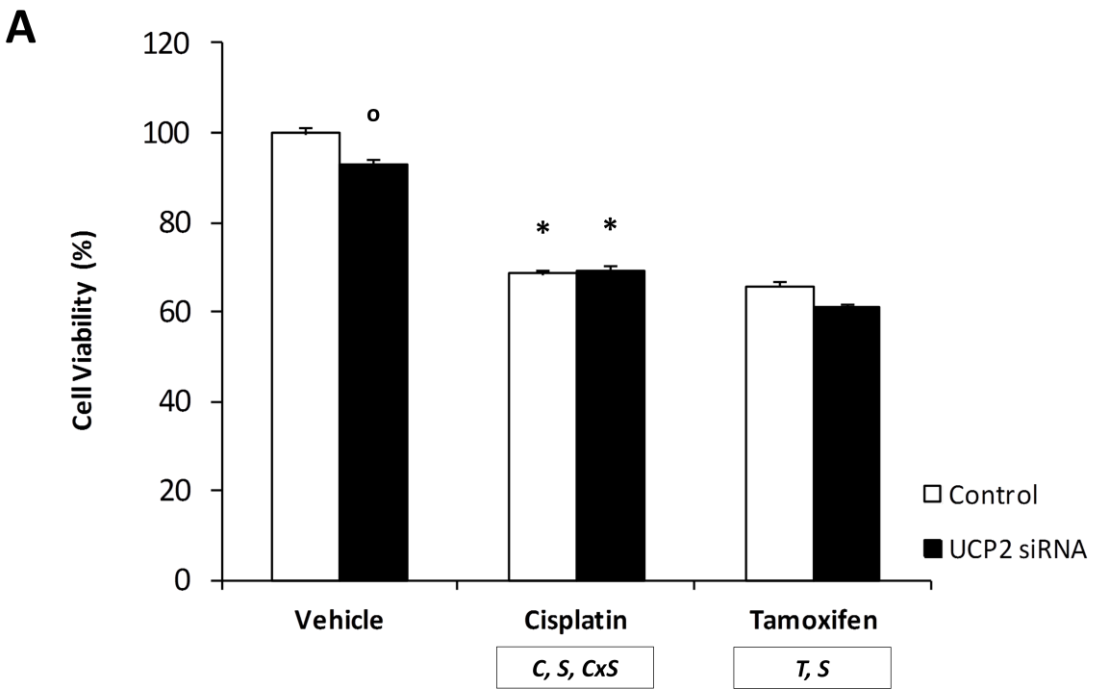
- + - + - +
LC3-I
LC3-II
Tubulin

Figure 6A and 6B



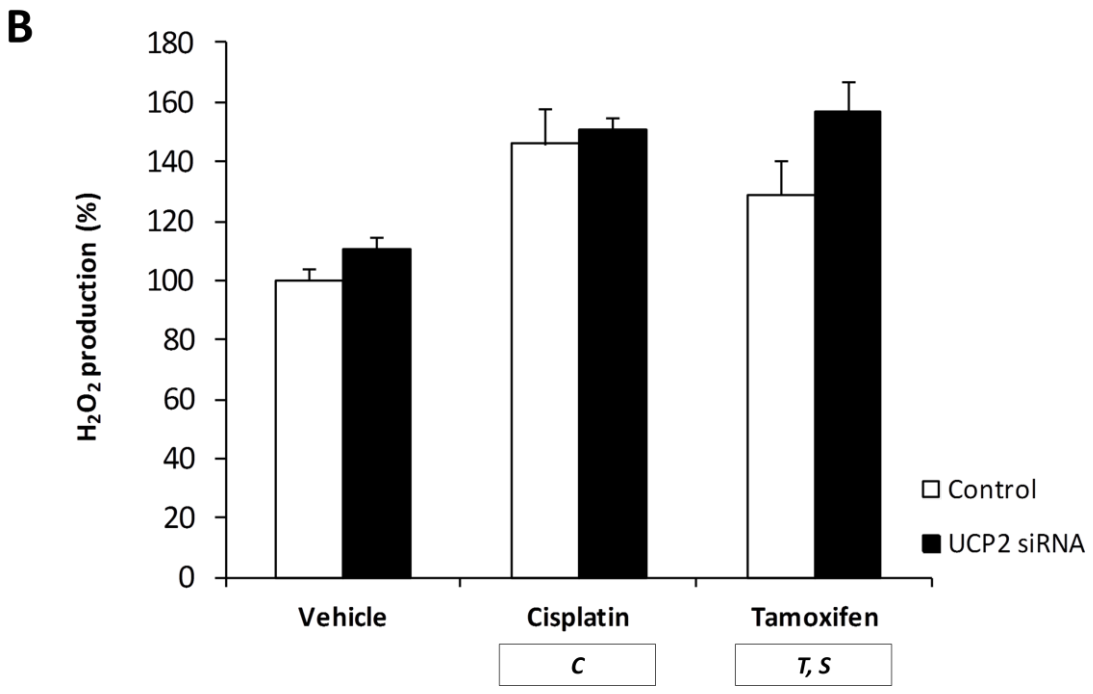
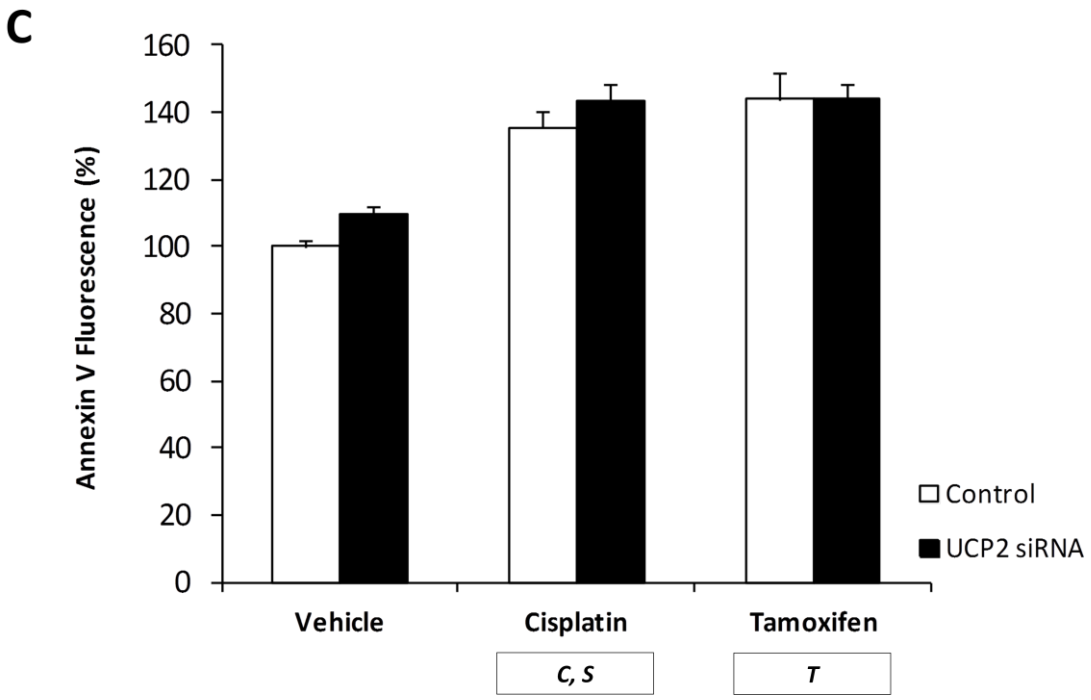


Figure 6C and 6D



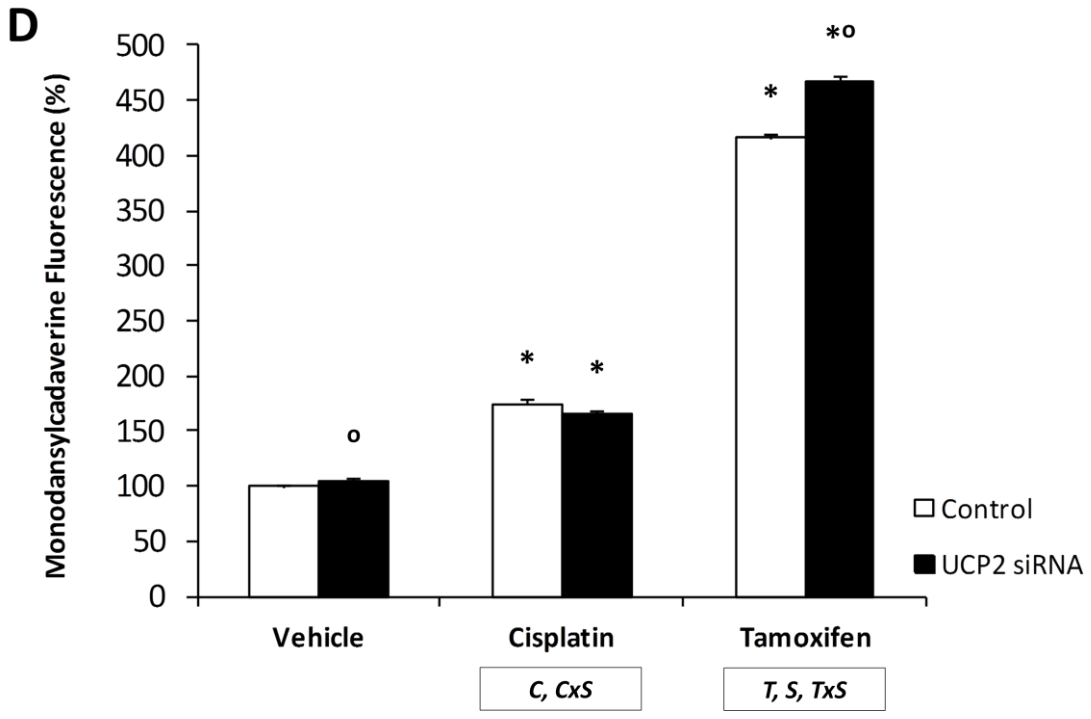
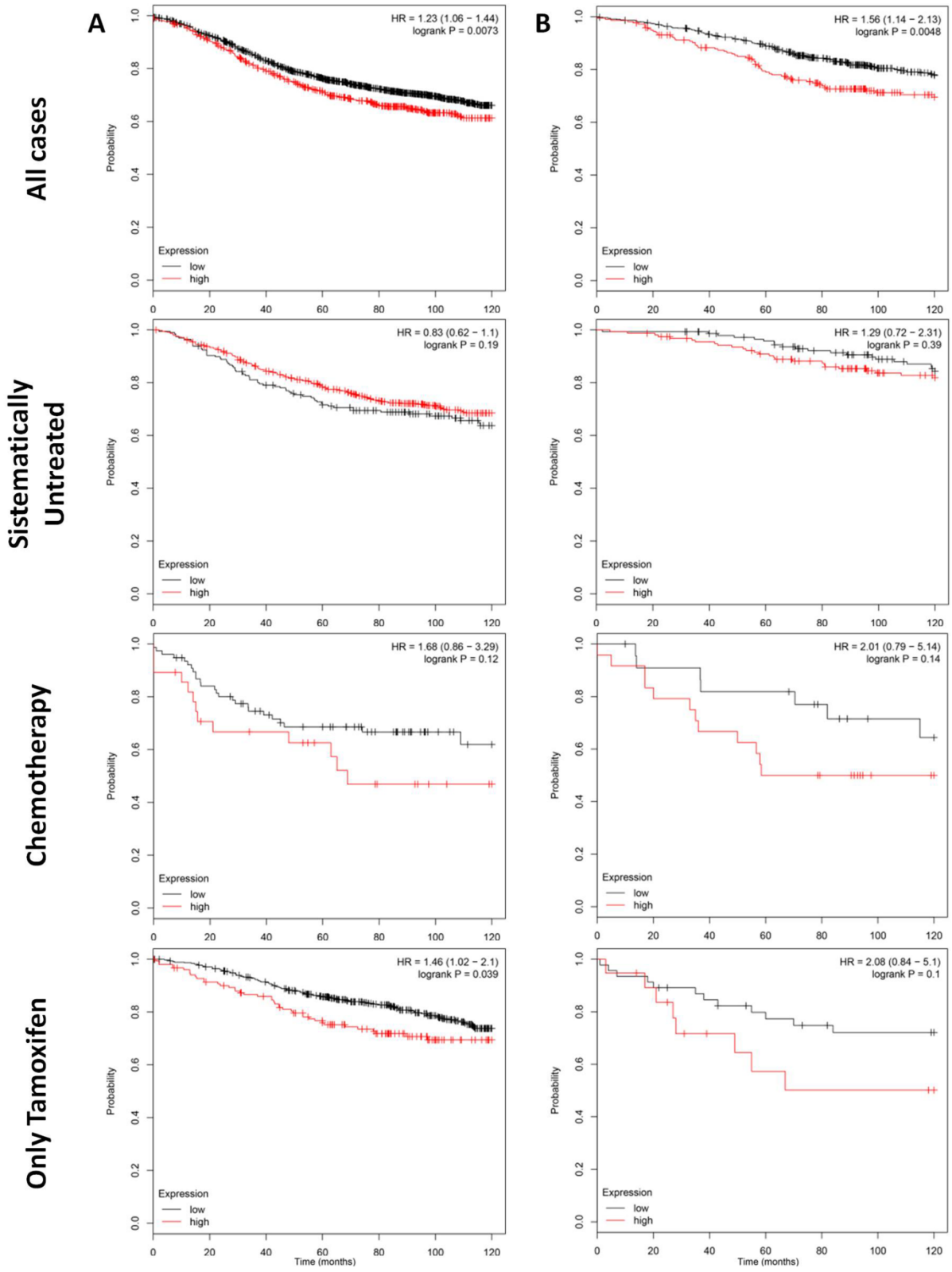


Figure 7


A
B
All cases
Sistematically Untreated
Chemotherapy
Only Tamoxifen
HR = 1.23 (1.06 − 1.44)
logrank P = 0.0073
HR = 1.56 (1.14 − 2.13)
logrank P = 0.0048
HR = 0.83 (0.62 − 1.1)
logrank P = 0.19
HR = 1.29 (0.72 − 2.31)
logrank P = 0.39
HR = 1.68 (0.86 − 3.29)
logrank P = 0.12
HR = 2.01 (0.79 − 5.14)
logrank P = 0.14
HR = 1.46 (1.02 − 2.1)
logrank P = 0.039
HR = 2.08 (0.84 − 5.1)
logrank P = 0.1
Probability
Expression
low
high
Time (months)

DISCUSSION

In the current study, we inhibited UCP2 expression and function in two breast cancer cell lines and analyzed whether this specific inhibition increases oxidative stress and affect the effectiveness of ROS-based chemotherapeutic agents, such as cisplatin and tamoxifen. Definitely, UCP2 silencing provoked oxidative stress and compromised cancer cells viability. We show here that cisplatin (CDDP) decreased UCP2 protein levels while tamoxifen (TAM) did not do so. Both cytotoxic treatments generated oxidative stress in cancer cells and decreased cell viability, with greater changes resulting from combination with the UCP2 knockdown. Moreover, although CDDP and TAM promotes both apoptosis and autophagy, UCP2 inhibition increased mainly the autophagic cell death, alone or in combination with cytotoxic agents, especially TAM. Finally, we show here the importance of UCP2 expression levels for the prognosis of ER-positive breast cancer patients, showing that higher levels of UCP2 expression correspond to a poorer prognosis.

The role of ROS in cancer remains controversial, since the induction of severe cellular damage can lead to cell death, however, mild oxidative stress promotes cell survival. Mitochondria are linked to a complex adaptive response to protect cancer cells against ROS generation[32,33], and UCP2 may contribute to this process[9,10,12]. The ability to control ROS through proton leak is considered to be the first line of defense against oxidative stress and is associated with increased cell survival[34]. We previously demonstrated that MCF7 breast cancer cells have high ROS levels and altered redox status which may contribute to cell growth[2,4]. Moreover, the MCF7 and T47D cells express the UCP2 protein, which may be modulating oxidative stress[2,4,35], and therefore UCP2 inhibition plays an important role in modulating oxidative stress and cell viability. Inhibition of UCP2 expression and function compromised cell viability in a time and dose-dependent way, and reduced the capacity of cancer cells to form viable clones. These results are in agreement with others showing that UCP2 inhibition can be considered a valuable antiproliferative target in several cancers[16,18,36,37]. Moreover, in a recent paper, it has been reported that genipin is able to decrease cell proliferation and clonogenic survival in UCP2 over-expressing breast cancer cells[16].

The loss of cell viability induced by UCP2 inhibition may be due to higher ROS levels, since the increased ROS production observed with siRNA and genipin treatments seems to affect cancer cell viability, as it is dose-dependent in genipin-treated cells. However, the strong enhanced ROS production at high concentrations of genipin could not only be due to its effects on UCP2 but also to its protein crosslinking abilities[38]. For this reason, we decided to inhibit UCP2 just by using UCP2 specific siRNA in the experiments that followed.

These high levels of ROS produced by UCP2 knockdown triggered a rise of carbonyl groups and 4-HNE adducts, which supports that this UCP2 inhibition results in oxidative damage in cancer cells. These deleterious effects on the proteins and lipids of cancer cells may explain in part the antiproliferative effect of UCP2 silencing, as well as the role that mitochondrial uncoupling plays in the regulation of oxidative stress. In this regard, many studies have found that ectopic UCP2 improves control of oxidative stress in various cancer cells and promotes cell survival, leading to chemoresistance[14,36].

ROS production is one of the cytotoxic effects of CDDP and TAM treatments[8,26], so we suspected that inhibiting UCP2 could enhance treatment efficacy increasing ROS production leading to cancer cells death. UCP2 knockdown has greater impact when used in combination with TAM, because CDDP treatment reduced UCP2 protein levels, while TAM did not do so. Previously, it has been demonstrated that CDDP reduces UCP2 levels in colon cancer cells[37]. Furthermore, since MCF-7 cells are ERα-positive[31] and TAM is an antagonist of ERα[25], the addition of this cytotoxic agent could increase UCP2 levels because we have previously demonstrated that 17β-estradiol decreases the levels of UCP2 in MCF-7 cells[4].

UCP2 inhibition, as well as the cytotoxic treatments, produced an increase in mitochondrial membrane potential (ΔΨm), which was accompanied by an increment in the ROS production (regarding to cytotoxic treatment alone), principally in TAM+siRNAUCP2-treated cells, in accordance with previous studies that have shown CDDP and TAM increase ROS levels in cancer cells[37,39]. It is important to note that a little increase in ΔΨm give large stimulation of ROS production[40], suggesting that a

small drop in UCP2 levels could mean a rise in the ΔΨm and, consequently, in ROS production. Likewise, this greater increase in ROS production could increase the drop in cell viability and the decrease in the capacity of cancer cells to form viable clones. This would be in agreement with Dalla Pozza et al., suggesting that the chemical inhibition of UCP2 has a synergistic antiproliferative effect with the cytotoxic treatment in pancreatic cancer cells[18]. Altogether, these findings suggest that UCP2 targeting may decrease cell growth, and enhance antiproliferative effects of cytotoxic agents, such as CDDP and TAM, modulating the oxidative stress in breast cancer cells. We can conclude that MCF-7 breast cancer cells are more sensitive to cytotoxic treatments in combination with UCP2 silencing due, at least in part, to an increase in ROS production.

Autophagy is a conserved evolutionary process that can enable cells to maintain homeostasis in unfavorable environmental conditions[41]. This process allows the cell to recover energy from damaged or unnecessary subcellular components (macromolecules or organelles)[41], therefore it has been considered as a process associated with cell survival. However, if the damage is too severe and a high level of autophagy persists, autophagic cell death or programmed cell death-2 will occur, which is a different phenomenon than apoptosis or programmed cell death-1[42]. Therefore, our results indicate that inhibition of UCP2 causes autophagic cell death without a significant increase of apoptosis. CDDP and TAM treatments, nevertheless, promote both apoptosis and autophagy[43-46]. Although both treatments triggered an increase in both apoptosis and autophagy, their combination with UCP2 knockdown only increased the autophagic cell death, particularly in TAM-treated cells (probably due to UCP2 inhibition caused by CDDP treatment), suggesting that inhibition of UCP2 could be a future therapeutic target, especially in those patients with endocrine therapy with TAM. These results coincide with Dando et al., who recently demonstrated that UCP2 inhibition triggers autophagic cell death through ROS generation in pancreatic adenocarcinoma cells[27].

Results obtained in this study could be useful in clinical applications because the survival curves performed with data from over 1800 breast cancer patients revealed the importance of UCP2 levels in prognosis of breast cancer patients. Our results

revealed that, when all cases are taken into account, high UCP2 expression was a poor prognosis indicator. The UCP2 dual role in cancer, which may have a protective mechanism in normal cells preventing malignant transformation although its over-expression in cancer cells may confer resistance to chemotherapy and a higher survival by down-regulation of ROS levels[15], could be explained by the Kaplan-Meier survival curves since untreated patients did not present any significant change in both disease-free patients or relapse-free survival (RFS), and overall survival (OS) parameters. In fact, in the RFS analysis of these patients, the higher UCP2 expression levels proved to be a better prognosis factor (non-significant data). Interestingly, TAM-treated patients had a poorer prognosis when they had greater UCP2 expression, which is not so obvious in chemotherapy-treated patients, probably due to the aforementioned effects of some cytotoxics such as CDDP over UCP2 protein.

In conclusion, we show here that the inhibition of the mitochondrial uncoupling protein 2 (UCP2) may cause the autophagic cell death in cancer cells through the ROS generation. Therefore, UCP2 could be a therapeutic target against breast cancer combined with cytotoxic agents which generate ROS, especially in combination with tamoxifen.

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REFERENCES

1 Waris, G. & Ahsan, H. Journal of carcinogenesis 5, 14 (2006).

2 Nadal-Serrano, M., Sastre-Serra, J., Pons, D. G., Miro, A. M., Oliver, J. et al. Journal of cellular biochemistry 113, 3178-3185 (2012).

3 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Valle, A., Garau, I. et al. Free radical biology & medicine 61C, 11-17 (2013).

4 Sastre-Serra, J., Valle, A., Company, M. M., Garau, I., Oliver, J. et al. Free radical biology & medicine 48, 506-512 (2010).

5 Hussain, S. P., Hofseth, L. J. & Harris, C. C. Nature reviews. Cancer 3, 276-285 (2003).

6 Sainz, R. M., Lombo, F. & Mayo, J. C. Cancers 4, 442-474 (2012).

7 Nogueira, V. & Hay, N. Clinical cancer research : an official journal of the American Association for Cancer Research 19, 4309-4314 (2013).

8 Lee, Y. H., Kang, B. S. & Bae, Y. S. Life sciences 97, 116-122 (2014).

9 Baffy, G., Derdak, Z. & Robson, S. C. British journal of cancer 105, 469-474 (2011).

10 Echtay, K. S. Free radical biology & medicine 43, 1351-1371 (2007).

11 Valle, A., Oliver, J. & Roca, P. Cancers 2, 567-591 (2010).

12 Baffy, G. Mitochondrion 10, 243-252 (2010).

13 Derdak, Z., Fulop, P., Sabo, E., Tavares, R., Berthiaume, E. P. et al. Carcinogenesis 27, 956-961 (2006).

14 Derdak, Z., Mark, N. M., Beldi, G., Robson, S. C., Wands, J. R. et al. Cancer research 68, 2813-2819 (2008).

15 Robbins, D. & Zhao, Y. International journal of molecular sciences 12, 5285-5293 (2011).

16 Ayyasamy, V., Owens, K. M., Desouki, M. M., Liang, P., Bakin, A. et al. PloS one 6, e24792 (2011).

17 Sayeed, A., Meng, Z., Luciani, G., Chen, L. C., Bennington, J. L. et al. Cell death & disease 1, e53 (2010).

18 Dalla Pozza, E., Fiorini, C., Dando, I., Menegazzi, M., Sgarbossa, A. et al. Biochimica et biophysica acta 1823, 1856-1863 (2012).

19 Ho, P. W., Liu, H. F., Ho, J. W., Zhang, W. Y., Chu, A. C. et al. Neurotoxicity research 17, 332-343 (2010).

20 Mailloux, R. J., Adjeitey, C. N. & Harper, M. E. PloS one 5, e13289 (2010).

21 Byrski, T., Huzarski, T., Dent, R., Gronwald, J., Zuziak, D. et al. Breast cancer research and treatment 115, 359-363 (2009).

22 Kelland, L. Nature reviews. Cancer 7, 573-584 (2007).

23 Rivera-Guevara, C. & Camacho, J. Recent patents on anti-cancer drug discovery 6, 237-245 (2011).

24 Jordan, P. & Carmo-Fonseca, M. Cellular and molecular life sciences : CMLS 57, 1229-1235 (2000).

25 Jensen, E. V. & Jordan, V. C. Clinical cancer research : an official journal of the American Association for Cancer Research 9, 1980-1989 (2003).

26 Marullo, R., Werner, E., Degtyareva, N., Moore, B., Altavilla, G. et al. PloS one 8, e81162 (2013).

27 Dando, I., Fiorini, C., Pozza, E. D., Padroni, C., Costanzo, C. et al. Biochimica et biophysica acta 1833, 672-679 (2013).

28 Gyorffy, B., Lanczky, A., Eklund, A. C., Denkert, C., Budczies, J. et al. Breast cancer research and treatment 123, 725-731 (2010).

29 Gyorffy, B., Surowiak, P., Budczies, J. & Lanczky, A. PloS one 8, e82241 (2013).

30 Li, Q., Birkbak, N. J., Gyorffy, B., Szallasi, Z. & Eklund, A. C. BMC bioinformatics 12, 474 (2011).

31 Pons, D. G., Nadal-Serrano, M., Blanquer-Rossello, M. M., Sastre-Serra, J., Oliver, J. et al. Journal of cellular biochemistry 115, 949-958 (2014).

32 Martindale, J. L. & Holbrook, N. J. J Cell Physiol 192, 1-15 (2002).

33 Trachootham, D., Alexandre, J. & Huang, P. Nature reviews. Drug discovery 8, 579-591 (2009).

34 Brand, M. D. & Esteves, T. C. Cell metabolism 2, 85-93 (2005).

35 Nadal-Serrano, M., Pons, D. G., Sastre-Serra, J., Blanquer-Rossello Mdel, M., Roca, P. et al. The international journal of biochemistry & cell biology 45, 2045-2051 (2013).

36 Collins, P., Jones, C., Choudhury, S., Damelin, L. & Hodgson, H. Liver international : official journal of the International Association for the Study of the Liver 25, 880-887 (2005).

37 Santandreu, F. M., Roca, P. & Oliver, J. Free radical biology & medicine 49, 658-666 (2010).

38 Sung, H. W., Chang, W. H., Ma, C. Y. & Lee, M. H. Journal of biomedical materials research. Part A 64, 427-438 (2003).

39 Hwang, J. J., Kim, H. N., Kim, J., Cho, D. H., Kim, M. J. et al. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 23, 997-1013 (2010).

40 Korshunov, S. S., Skulachev, V. P. & Starkov, A. A. FEBS letters 416, 15-18 (1997).

41 Cook, K. L., Shajahan, A. N. & Clarke, R. Expert review of anticancer therapy 11, 1283-1294 (2011).

42 Akar, U., Chaves-Reyez, A., Barria, M., Tari, A., Sanguino, A. et al. Autophagy 4, 669-679 (2008).

43 Bursch, W., Ellinger, A., Kienzl, H., Torok, L., Pandey, S. et al. Carcinogenesis 17, 1595-1607 (1996).

44 Jakubowicz-Gil, J., Paduch, R., Ulz, Z., Badziul, D., Glowniak, K. et al. Folia histochemica et cytobiologica / Polish Academy of Sciences, Polish Histochemical and Cytochemical Society 50, 381-391 (2012).

45 Mandlekar, S. & Kong, A. N. Apoptosis : an international journal on programmed cell death 6, 469-477 (2001).

46 Seki, K., Yoshikawa, H., Shiiki, K., Hamada, Y., Akamatsu, N. et al. Cancer chemotherapy and pharmacology 45, 199-206 (2000).

Manuscript 4

Genistein modulates proliferation and mitochondrial functionality in breast cancer cells depending on ERalpha/ERbeta ratio

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Genistein Modulates Proliferation and Mitochondrial Functionality in Breast Cancer Cells Depending on ERalpha/ERbeta Ratio

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ABSTRACT

Breast cancer is the most common malignancy in women of developed countries. The aim of this study was to investigate whether genistein, a soy phytoestrogen, and 17β-estradiol (E2) could have effects on the cell cycle and mitochondrial function and dynamics. Three human breast cancer cell lines with different estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) ratio were used: MCF-7 (high ERα/ERβ ratio), T47D (low ERα/ERβ ratio) and MDA-MB-231 (ER-negative). Cell proliferation, cell cycle, mitochondrial functionality, and mitochondrial dynamics parameters were analyzed. E2 and genistein treatment induced cell proliferation and apoptosis inhibition in MCF-7, but not in T47D and MDA-MB-231. Moreover, genistein treatment produced an up-regulation of ERβ and a rise in cytochrome *c* oxidase activity in T47D cells, decreasing the ATP synthase/cytochrome *c* oxidase ratio. Finally, genistein treatment produced a drop in mitochondrial dynamics only in MCF-7 cells. In summary, the beneficial effects of genistein consumption depend on the ERα/ERβ ratio in breast cells. Therefore, genistein treatment produces cell cycle arrest and an improvement of mitochondrial functionality in T47D cells with a low ERα/ERβ ratio, but not in MCF-7 (high ERα/ERβ ratio) and MDA-MB-231 (ER-negative) ones. J. Cell. Biochem. 115: 949–958, 2014.

KEY WORDS: GENISTEIN; MITOCHONDRIA; 17β-ESTRADIOL; ESTROGEN RECEPTORS; CELL CYCLE; APOPTOSIS; CELL PROLIFERATION

Breast cancer is the most commonly diagnosed malignancy in women of developed countries [Siegel et al., 2011]. Epidemiological studies have showed a disparity incidence of breast cancer between Eastern and Western countries, where on average 1 in every 8 women will suffer breast cancer compared to 1 in every 30 women in Japan [Bouker and Hilakivi-Clarke, 2000].

Estrogens, especially 17β-estradiol (E2), are risk factors for the development of breast cancer, and produce tumorigenesis in epithelial breast cells [Yager and Liehr, 1996]. Through diet, humans are exposed to many different phytoestrogens [Patisaul and Jefferson, 2010], and especially those originating from soy products or legumes were initially identified as cancer potential causative factors [Messina et al., 1994]. However, phytoestrogens consumption has also been linked to cancer prevention, most notably prostate and breast cancer [Adlercreutz, 2002; Orlando et al., 2011]. Moreover, high phytoestrogen consumption leads to a lower risk of cardiovascular disease [Klaunig and Kamendulis, 2004] and osteoporosis [Mantovani et al., 2008]; as well as it has been shown to relieve climacteric symptoms [Manju and Nalini, 2007].

The phytoestrogen genistein is the major isoflavonoid found in soybeans, and some beneficial effects have been attributed to its consumption, including anti-carcinogenic effects [Zhou et al., 1998]. Previous studies showed that people in the Eastern countries have higher blood levels of genistein, 10 times than people from Western

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countries [Morton et al., 2002]. Genistein has a very similar chemical structure to E2 and it binds and activates both estrogen receptors (ER), ERα and ERβ. Different functions have been associated with the two ER; while ERα mediates proliferative effects of estrogens, ERβ is more related to cytostatic effects [Chang et al., 2006; Sotoca et al., 2008; Rajah et al., 2009].

Previous studies in our laboratory demonstrate that E2 increases cell proliferation in MCF-7 breast cancer cells with a high ERα/ERβ ratio, but not in the T47D cell line, with a lower ERα/ERβ ratio than MCF-7, nor in the MDA-MB-231 breast cancer cell line, ER-negative [Sastre-Serra et al., 2010; Nadal-Serrano et al., 2012]. Moreover, similar results were obtained in prostate cancer cell lines [Miro et al., 2011]. Genistein has been postulated to be an inhibitor of cell proliferation and inductor of apoptosis in breast cancer cell lines [Davis et al., 2008] and as a cytostatic agent in colon cells [Schleipen et al., 2011].

Recently, Adams et al. [2012] observed that genistein treatment could restore mitochondrial functionality, while some studies have shown that E2 treatment produced mitochondrial dysfunction and an increase in radical oxygen species (ROS) production [Sastre-Serra et al., 2012b], and a greater production of ROS has been related with a higher cell proliferation [Sastre-Serra et al., 2010]. Mitochondria are organelles which are involved in constant cycles of division and fusion, processes related to mitochondrial dynamics [Rambold et al., 2011]. This process of mitochondrial dynamics has been related to mitochondrial function and cell apoptosis [Grandemange et al., 2009; Youle and van der Bliek, 2012].

The aim of this study was to investigate the effects of physiological concentrations of 17β-estradiol and phytoestrogen genistein on cell proliferation, cell cycle and apoptosis, and mitochondrial functionality in breast cancer cell lines with different ERα/ERβ ratios. To tackle this aim we performed a cell proliferation and cell cycle analysis and we studied the proliferation- and apoptosis-related proteins, the ATP synthase and cytochrome *c* oxidase activities and mitochondrial dynamics-related genes mRNA expression in MCF-7 (high ERα/ERβ ratio), T47D (low ERα/ERβ ratio), and MDA-MB-231 (ER-negative) breast cancer cell lines.

MATERIALS AND METHODS

MATERIALS

17β-Estradiol (E2), genistein, dimethyl sulfoxide (DMSO), and Propidium Iodide were purchased from Sigma–Aldrich (St. Louis, MO). Mitotracker Green (MTG) and Lysotracker Red (LTR) were purchased from Life Technologies Ltd (Paisley, UK). Primers were purchased from TIB MOLBIOL (Berlin, Germany) and from Metabion (Martinsried, Germany). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich, Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA).

CELL CULTURE AND TREATMENTS

Breast cancer cell lines MCF-7, T47D, and MDA-MB-231 were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37°C. To evaluate the effects of E2 and genistein, cells were grown in phenol red-free DMEM containing 10% charcoal-stripped FBS 24 h prior to treatment. Experiments were performed when cell cultures reached confluence by providing fresh media supplemented with 1 nmol/L E2, 1 μmol/L genistein, or 0.001% DMSO as vehicle for 48 h. For the cell proliferation assay with different genistein concentrations, cells were cultured in the same way as described above and treated with genistein concentrations from 10 nM to 10 μM. For cell cycle analysis in subconfluence, experiments were performed when cell cultures reached 75% of confluence.

CELL LINES ESTROGEN RECEPTORS CHARACTERIZATION

Figure 1 shows estrogen receptor (α and β) level analysis which was carried out by Western blot in normal conditions of each breast cancer cell line. MCF-7 was considered as high ERα/ERβ ratio cell line, T47D as a low ERα/ERβ ratio cell line and MDA-MB-231 as ERα-negative, in spite of the lowest levels of ERβ, so that we considered this cell line to be an ER-negative breast cancer cell line.

CELL CYCLE ANALYSIS BY FLOW CYTOMETRY

After harvesting the cells with trypsin–EDTA, cells were fixed with methanol 100% and DNA staining was carried out with an RNAase and propidium iodide mix. After 30 min of room temperature incubation, samples were analyzed using an Epics XL flow cytometer (Beckman Coulter, Miami, FL). The red fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. Ten thousand events were acquired and analyzed with Expo32 ADC analysis software (Beckman Coulter).

CELL PROLIFERATION ASSAY

Cells were plated at 10,000 cells per well in 96-well plates in DMEM, supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37°C, and shifted to phenol red-free DMEM with 10% charcoal-FBS and 1% antibiotics (penicillin and streptomycin) 24 h before treatment with the vehicle, either E2 or

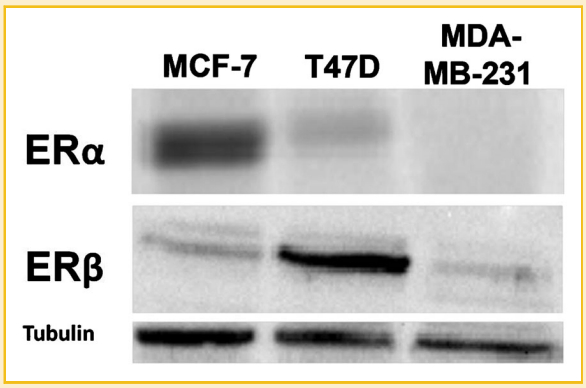


Fig. 1. Cell lines estrogen receptors characterization by Western blot analysis. Measurements were made with cells in basal conditions. ERα, estrogen receptor alpha; ERβ, estrogen receptor beta and Tubulin as a housekeeping protein.

genistein, or both treatments. The number of cells was determined by the crystal violet method [Nagamine et al., 2009] with modifications. Briefly, after 48 h of treatment, 20 μl of a Violet Crystal solution (0.5% of Violet Crystal in 30% acetic acid) was added and plates were incubated for 10 min at room temperature. The medium was removed and washed twice with distilled water. Finally, water was removed and 100 μl of methanol were added to wells and plate was shaken for 1 min. Absorbance was measured at 570 nm using a microplate reader (Power Wave XS, Bio-Tek).

WESTERN BLOT ANALYSIS

For Western blot analysis, 40 μg of cell lysate protein was fractioned by SDS–PAGE (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. ERα (1:200) and ERβ (1:200) primary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Stat3 (1:1,000), Phospho-Stat3 (1:1,000), and PARP/Cleaved PARP (1:1,000) primary antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Protein bands were visualized by the Immun-Star Western C kit reagent (Bio-Rad Laboratories) Western blotting detection system. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One software (Bio-Rad Laboratories).

ATP SYNTHASE AND CYTOCHROME *C* OXIDASE ACTIVITIES

After 48 h of vehicle, E2, genistein, or both E2 and genistein treatments, cells were harvested by scraping the culture into PBS buffer and then centrifuged at 5,000 rpm for 5 min at 4°C to remove cell debris. Cell pellet were resuspended in RNAase free water. Lysates were kept on ice and protein content was determined with the bicinchoninic acid protein assay kit (Pierce, Bonn, Germany), with the enzyme assays run immediately after.

Cytochrome *c* oxidase (Complex IV, EC 1.9.3.1) activity was measured using a spectrophotometric method [Chrzanowska-Lightowlers et al., 1993]. Briefly, cell lysate was incubated in 0.1 M $NaPO_4H_2$, pH 7.0, in the presence of 2 μg/ml catalase and 5 mM substrate DAB (3,3′-diaminebenzidine-tetrachloride) and then 100 μM of reduced cytochrome *c* was added to start the reaction, with the absorbance variation followed for 20 min at 450 nm.

ATP synthase (ATP phosphohydrolase, Complex V, EC 3.6.1.3) activity was measured by following the oxidation of NADH at 340 nm and 37°C [Ragan et al., 1987]. The extinction coefficient used was 6.22 mM^{-1} cm^{-1}.

REAL-TIME PCR

After 4 h of genistein or vehicle (0.001% DMSO), total RNA was isolated from cultured cells using TriPure® Isolation Reagent (Roche) and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U of MuLV reverse transcriptase in a 10 μl volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNAase inhibitor, and 500 μM each dNTP. Each resulting cDNA was diluted 1/10.

PCR was done for six target genes: mitofusin-1 (mfn1), mitofusin-2 (mfn2), optic atrophy 1 (opa1), dynamin-related protein (drp1), fission 1 (fis1), and 18S, using SYBR green technology on a LightCycler rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μl, containing 1 μl Lightcycler-FastStart DNA Master SYBR Green I, 0.5 μM sense and antisense specific primers, 2 mM $MgCl_2$, and 3 μl of the cDNA template. The amplification program consisted of a preincubation step for denaturation (10 min 95°C) followed by 40 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, temperature depends on each pair of specific primers), and an extension step (12 s, 72°C for all). A negative control without cDNA template was run in each assay.

The specific primers used were forward 5′-ttcgatcaagttccggattc-3′ and reverse 5′-ttggagcggagacttagcat-3′ for mfn1 with 51°C as annealing step, forward 5′-gcagaactttgtcccagagc-3′ and reverse 5′-agaggcatcagtgaggtgct-3′ for mfn2 with 56°C as annealing step, forward 5′-acaatgtcaggcacaatcca-3′ and reverse 5′-ggccagcaagattagctacg-3′ for opa1 with 51°C as annealing step, forward 5′-gttcacggcatgacctttt-3′ and reverse 5′-aagaaccaaccacaggcaac-3′ for drp1 with 51°C as annealing step, forward 5′-gctgaaggacgaatctcagg-3′ and reverse 5′-cttgctgtgtccaagtccaa-3′ for fis1 with 53°C as annealing step, and forward 5′-ggacacggacaggattgaca-3′ and reverse 5′-acccacggaatcgagaaaga-3′ for 18S with 61°C as annealing step.

The C_t values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (MultiDAnalises, Sweden).

STATISTICAL ANALYSIS

All statistical analyses were performed with the Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc, Chicago, IL). Data are presented as means ± standard error of the mean (SEM). Statistical differences between control, E2-treated, genistein-treated, and E2 + genistein-treated cells were analyzed by univariate ANOVA and the Student's *t*-test. Statistical significance was set at $P < 0.05$.

RESULTS

EFFECTS OF E2 AND GENISTEIN TREATMENT ON CELL PROLIFERATION

In Table I can be observed that after 48 h of treatment with E2, genistein, and their combination the cell proliferation was significantly increased in the order of 20% in MCF-7 breast cancer cell line. However, in T47D breast cancer cell line there were no differences between control and treated cells. In MDA-MB-231 there is a small increase (5% maximum) in cell proliferation in genistein-treated cells.

Figure 2 shows the effect of different concentrations of genistein (10 nM–10 μM) on the proliferation of cell lines studied in the absence (solid line) or presence (dashed line) of E2 at 1 nM concentration. Genistein-treated MCF-7 cells were always above the control line for the vehicle-treated ones (the solid line representing 100% of control) and around the E2-treated cells (the dotted line representing E2-

TABLE I. Effects of 17β-Estradiol and Genistein on Cell Proliferation in MCF-7, T47D, and MDA-MB-231 Cells Measured by Violet Crystal Assay

	C	E2	GEN	E2 + GEN	ANOVA
MCF-7	100 ± 1.79	118 ± 0.50*	118 ± 2.01°	121 ± 2.29°	E, G, E × G
T47D	100 ± 2.69	96.7 ± 1.35	101 ± 1.18	104 ± 2.11	NS
MDA-MB-231	100 ± 0.51	102 ± 1.07	105 ± 0.62	105 ± 1.67	G

C, vehicle-treated cells; E2, 17β-estradiol-treated cells; GEN, genistein-treated cells.
All measurements were made after 48 h of 1 nM 17β-estradiol, 1 μM genistein, combination of 1 nM 17β-estradiol, and 1 μM genistein or vehicle (0.1% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%. ANOVA analysis was carried out where E means E2 effect, G means genistein effect and E × G means combinatory effect of E2 and genistein and NS means no significant differences. As a result, in MCF-7 a Student's *t*-test ($P < 0.05$, n = 8) was carried out:
*Differences between vehicle- and E2-treated cells.
°Differences between vehicle- and GEN-treated cells.

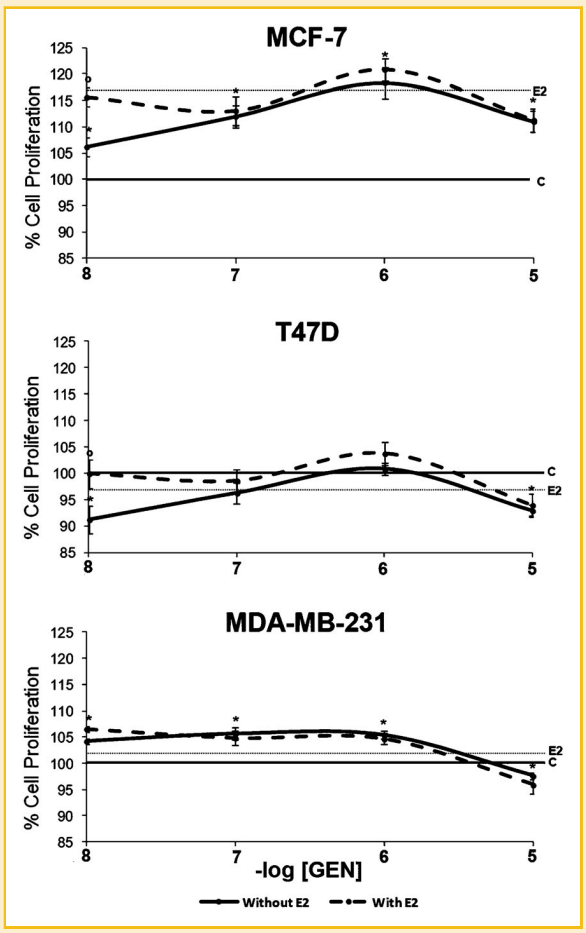


Fig. 2. Effects of different concentrations of genistein in combination or not with 17β-estradiol on cell proliferation in MCF-7, T47D, and MDA-MB-231 cells measured by Crystal Violet assay. All measurements were made after 48 h of 0.01, 0.1, 1, and 10 μM genistein or vehicle (0.001% DMSO) treatment in the absence or presence of 17β-estradiol at 1 nM. The value of vehicle-treated cells was set at 100% as an abscissa perpendicular solid line and the value of 17β-estradiol-treated cells is represented as an abscissa perpendicular dotted line. Values in ordinate represent −log of genistein concentration (mol/L). C, Vehicle-treated; E2, 17β-estradiol; GEN, Genistein. *Significant difference between vehicle- and genistein-treated cells and ° significant difference between genistein- and genistein + E2-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

treated cells) indicating a cell proliferation induction by genistein treatment. In T47D cells genistein treatment produced no differences or a small drop in cell proliferation, while MDA-MB-231 cells presented a biphasic effect: low concentrations of genistein (10 nM–1 μM) produced a small increase in cell proliferation although higher concentrations (10 μM) produced a slight drop in cell proliferation. There are no differences between E2 and genistein treatments but in MCF-7 and T47D cells the lowest genistein concentration (10 nM) without E2 treatment shows lower proliferation than the E2-treated one, probably due to the presence of ERα in these breast cancer cell lines. GEN have higher affinity for ERβ than for ERα [Kuiper et al., 1997], but at low concentrations of this phytoestrogen the presence of 1 nM of E2 could act through ERα enhancing cell proliferation in those cells that possess ERα. The physiological concentrations of genistein, 1 μM [Morton et al., 2002], mask the effects of E2 because genistein is binding to both estrogen receptors.

EFFECTS OF E2 AND GENISTEIN TREATMENT ON CELL CYCLE

The effect of E2 and genistein treatments on cell cycle of breast cancer cell lines in confluence and subconfluence with different ERα/ERβ ratio is represented in Figure 3. Cell cycle analysis determined that both treatments, E2 and genistein, produced an increase in proliferating cells (S + G2/M) and a decrease in apoptotic and cytostatic cells (G0/G1) in the MCF-7 breast cancer cell line. Furthermore, the genistein effect is in the manner but more attenuated than E2. These effects were more pronounced with subconfluence.

However, in T47D breast cancer cell line, E2 and genistein treatments produced a decrease in proliferating cells, and, moreover, genistein treatment produced an increase in cytostatic cells. In the subconfluence there was no significant difference between treated and non-treated cells.

Finally, in MDA-MB-231 breast cancer cells there was no significant difference between control and treated cells in the confluence situation. However, in the subconfluence cells treated with E2 showed a decrease in apoptosis and cytostatic cells, and a significant rise in proliferating cells.

EFFECTS OF GENISTEIN TREATMENT ON ESTROGEN RECEPTORS LEVELS

Genistein treatment produced a strongly increase (more than 11 times) of ERβ protein levels in T47D breast cancer cell line.

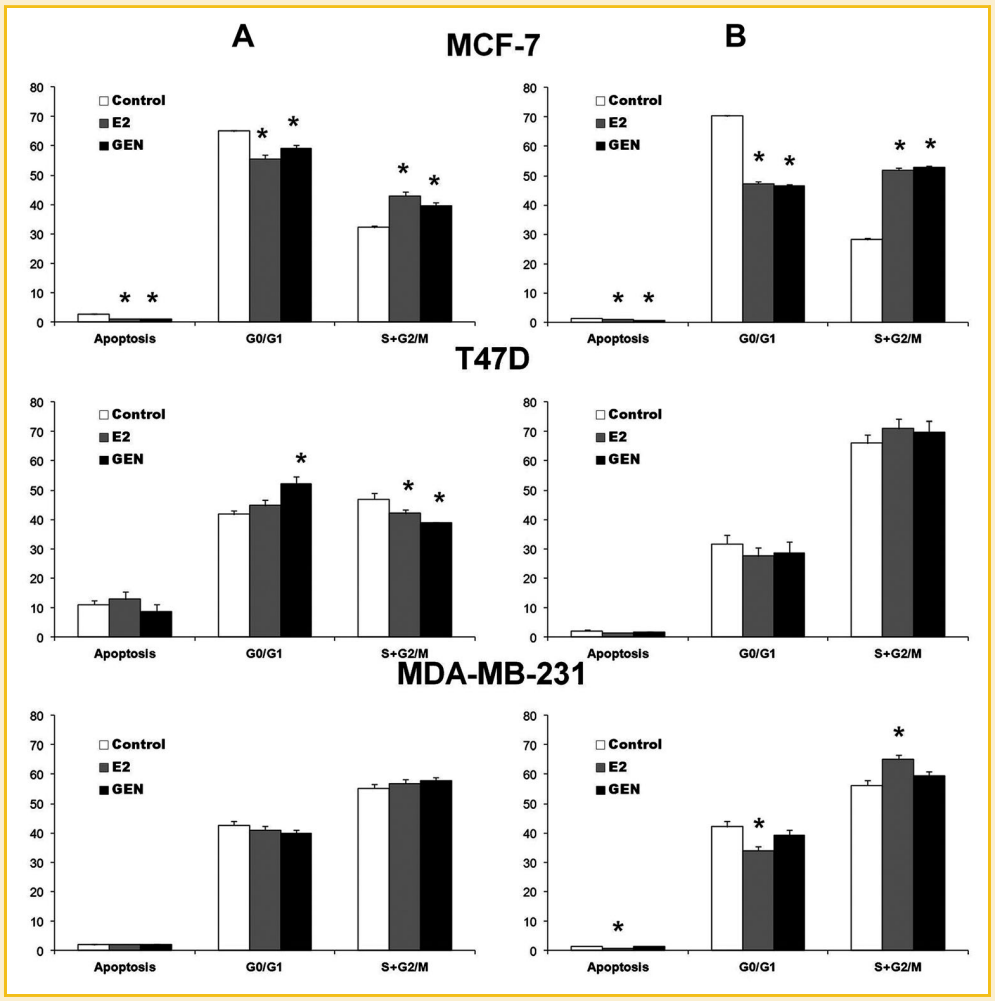


Fig. 3. Effects of E2 and genistein on cell cycle in MCF-7, T47D, and MDA-MB-231 cells measured by propidium iodide staining in flow cytometer. All measurements were made after 48 h of 1 μM genistein (dark bar), 1 nM E2 (gray bar), or vehicle (white bar) (0.001% DMSO) treatment. A-column represents the cell cycle analysis in confluence conditions and B-column represents the cell cycle analysis in subconfluence conditions. Bars represent means ± SEM of percentage of a total of 10,000 cells present in different cell cycle phases. *Significant difference between genistein- and E2-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

Nevertheless, genistein treatment produced no changes in ERβ protein levels in both MCF-7 and MDA-MB-231 cells. ERα protein levels showed no variation with genistein treatment in MCF-7 and T47D breast cancer cell lines, considering that MDA-MB-231 does not have this receptor. These results can be observed in Table II.

STAT3 PHOSPHORYLATION AND PARP CLEAVAGE ANALYSIS

The results of P-STAT3/STAT3 and Cleaved PARP/PARP protein levels are represented in Table III.

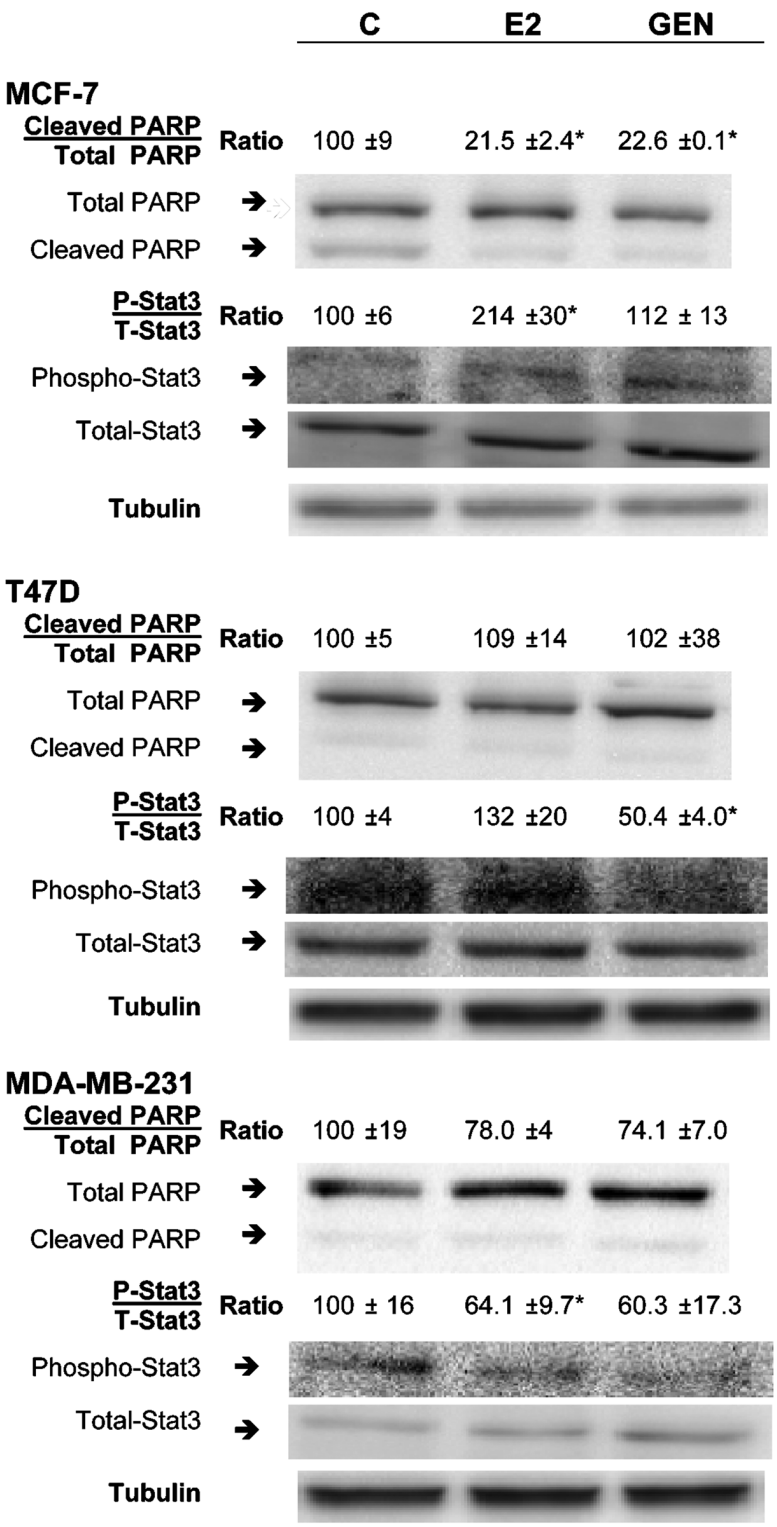
The MCF-7 breast cancer cell line showed no significant differences with E2 and genistein treatment in P-STAT3/STAT3 ratio. In the

TABLE II. Estrogen Receptors Protein Levels Analysis in MCF-7, T47D, and MDA-MB-231 Cells by Western Blot

	T47D		MCF-7		MDA-MB-231	
	C	GEN	C	GEN	C	GEN
ERα	100 ± 17.5	86.3 ± 14.5	100 ± 11.4	91.1 ± 6.84	UND	UND
ERβ	100 ± 9.18	93.7 ± 14.7	100 ± 30.9	1,100 ± 353*	100 ± 15.0	120 ± 23.0

C, vehicle-treated cells; GEN, genistein-treated cells; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; UND, undetected.
All measurements were made after 48 h of 1 μM genistein or vehicle (0.001% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%.
*Significant difference between genistein-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

TABLE III. Cleaved PARP/PARP and P-STAT3/STAT3 Ratios Analysis Measured by Western Blot in MCF-7, T47D, and MDA-MB-231 Cells



C, vehicle-treated cells; E2, 17β-estradiol-treated cells; GEN, genistein-treated cells; PARP, poly-ADP ribose polymerase; P-STAT3, phosphorylated signal transducer and activator of transcription 3, T-STAT3, total signal transducer and activator of transcription 3.

All measurements were made after 48 h of 1 μM genistein, 1 nM 17β-estradiol, or vehicle (0.001% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%. Tubulin was used as a housekeeping protein.

*Significant difference between GEN- and E2-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

T47D breast cancer cell line the genistein treatment caused a significant decrease in P-STAT3/STAT3 ratio, while the E2 treatment had no effects. In the MDA-MB-231 breast cancer cell line, both treatments produced significant lower levels of P-STAT3/STAT3 ratio, although the genistein treatment produced a greater decrease than the E2 one.

Likewise, both treatments caused a significant decrease in Cleavage PARP/PARP ratio in MCF-7 breast cancer cell line. However, in T47D and MDA-MB-231 breast cancer cell lines, the E2 and genistein treatment did not make a significant difference in Cleaved PARP/PARP ratio compared to vehicle treated cells.

MITOCHONDRIAL FUNCTIONALITY

Mitochondrial functionality was measured by ATP synthase and cytochrome *c* oxidase enzymatic activities and the resulting ratio of these activities, represented in Table IV, is considered to be the mitochondrial functionality parameter.

In the MCF-7 breast cancer cell line, treatment with E2 triggered a decrease of the cytochrome *c* oxidase activity with no change in ATP synthase activity, resulting in an increase of the ATP synthase/cytochrome *c* oxidase ratio. However, genistein treatment, as well as E2 + genistein treatment, produced no changes in enzyme activities and the ATP synthase/cytochrome *c* oxidase ratio had no alterations caused by this phytoestrogen.

In the T47D breast cancer cell line, treatment with E2 caused no differences between treated and control cells for either enzyme activity. Nevertheless, genistein treatment and, to a lesser extent, combination of E2 and genistein treatment resulted in an increase in cytochrome *c* oxidase activity and a subsequent decrease in ATP synthase/cytochrome *c* oxidase ratio in both treatments.

In MDA-MB-231 breast cancer cell line, E2, genistein, and combination of E2 and genistein treatments produced no changes in the ATP synthase/cytochrome *c* oxidase activity ratio.

MITOCHONDRIAL DYNAMICS IS AFFECTED BY GENISTEIN TREATMENT IN MCF-7 CELLS

As shown in Table V, mitochondrial dynamics is affected just in MCF-7 cells after genistein treatment, resulting in a drop in Opa1 (mitochondrial fusion) and Fis1 (mitochondrial fission) mRNA expression. However, in T47D and MDA-MB-231 cells genistein treatment did not produce any alteration in mitochondrial fusion and fission.

DISCUSSION

In this study we demonstrated the importance of ERα/ERβ ratio on the effects of 17β-estradiol and genistein at physiological concentrations in breast cancer cell lines. While in MCF-7 cells (high ERα/ERβ ratio) E2 and genistein treatment produced proliferation effects and decreased mitochondrial functionality; in T47D cells (low ERα/ERβ ratio) E2 and especially genistein, exerted antiproliferative and proapoptotic effects, and a better mitochondrial functionality.

The present study shows a relationship between ERα/ERβ ratio and genistein effects, suggesting that a high ERα/ERβ ratio is related to cell proliferation induction and apoptosis inhibition, while a low ERα/ERβ ratio is linked to cell cycle arrest and apoptosis induction. According to our results, previous studies in our laboratory have shown that E2 has proliferative effects in MCF-7 cells and no effects in T47D and MDA-MB-231 ones [Sastre-Serra et al., 2010, 2012b; Nadal-Serrano et al., 2012]. Sotoca et al. [2008] observed that a breast cancer cell line with only ERα, showed an induction of cell proliferation after E2 (1 nM) and genistein (1 μM) treatments, and over-expression of ERβ reversed this situation producing no changes in cell proliferation. In fact, the presence of ERβ in the MCF-7 breast cancer cell line could induce cell proliferation inhibition [Paruthiyil et al., 2004] and studies in vivo have observed that βERKO mice prostate cells have lower apoptosis and differentiation and higher cell

TABLE IV. ATP Synthase and Cytochrome Oxidase Enzymatic Activities in MCF-7, T47D, and MDA-MB-231 Cells, Measured by Spectrophotometric Method

	C	E_2	GEN	E2 + GEN	ANOVA
MCF-7					
ATPase	100 ± 5.17	95.6 ± 8.82	97.2 ± 3.25	92.3 ± 3.14	NS
COX	100 ± 8.75	51.3 ± 8.50*	106 ± 8.06	100 ± 6.60	E, G, E × G
ATPase/COX ratio	1.00	1.86	0.92	0.92	
T47D					
ATPase	100 ± 1.99	111 ± 8.98	100 ± 4.41	102 ± 3.52	NS
COX	100 ± 5.85	107 ± 11.8	130 ± 9.70	119 ± 8.95	G
ATPase/COX ratio	1.00	1.04	0.77	0.86	
MDA-MB-231					
ATPase	100 ± 1.95	108 ± 3.54	96.9 ± 2.13	102 ± 11.1	NS
COX	100 ± 4.03	87.0 ± 10.4	102 ± 16.8	106 ± 5.91	NS
ATPase/COX ratio	1.00	1.24	0.95	0.96	

C, vehicle-treated cells; E2, 17β-estradiol-treated cells; GEN, genistein-treated cells; ATPase, ATP synthase and COX, cytochrome *c* oxidase.
All measurements were made after 48 h of 1 nM 17β-estradiol, 1 μM genistein, combination of 1 nM 17β-estradiol and 1 μM genistein or vehicle (0.01% DMSO) treatment. Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%. ANOVA analysis was carried out where E means E2 effect, G means GEN effect and E × G means combinatory effect of E2 and GEN and NS means no significant differences. As a result, in MCF-7 a Student's *t*-test ($P < 0.05$ n = 6) was carried out:
*Differences between vehicle- and E2-treated cells.

TABLE V. Fusion- and Fission-Related Genes mRNA Expression in Breast Cancer Cell Lines After 4 h of 1 μM Genistein or Vehicle (0.001% DMSO) Treatment Analyzed by Real-Time PCR

	MCF-7		T47D		MDA-MB-231	
	C	GEN	C	GEN	C	GEN
mfn1	1.00 ± 0.05	0.98 ± 0.12	1.00 ± 0.09	1.07 ± 0.06	1.00 ± 0.08	1.00 ± 0.13
mfn2	1.00 ± 0.10	0.84 ± 0.07	1.00 ± 0.07	0.99 ± 0.06	1.00 ± 0.05	0.83 ± 0.10
opa1	1.00 ± 0.10	0.71 ± 0.07*	1.00 ± 0.11	1.06 ± 0.05	1.00 ± 0.05	1.01 ± 0.16
drp1	1.00 ± 0.07	0.85 ± 0.07	1.00 ± 0.09	0.96 ± 0.03	1.00 ± 0.08	1.19 ± 0.11
fis1	1.00 ± 0.05	0.77 ± 0.02*	1.00 ± 0.06	0.98 ± 0.04	1.00 ± 0.07	1.11 ± 0.06

C, vehicle-treated cells; GEN, genistein-treated cells; mfn1, mitofusin 1; mfn2, mitofusin 2; opa1, optic atrophy 1; drp1, dynamin-related protein q; fis1, mitochondrial fission 1 protein.
Data are represented as the mean ± SEM with the value of vehicle-treated cells set at 100%.
*Significant difference between genistein-treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

proliferation [Imamov et al., 2004]. Some authors have described that genistein treatment has proapoptotic effects in the colon and could prevent colorectal cancer, as colon cells are close to absorption sites for genistein-bearing foods [Schleipen et al., 2011]. Our results indicate that the presence of ERβ and, therefore, a low ERα/ERβ ratio in the T47D breast cancer cell line, either produced no effects or inhibited cell proliferation after E2, genistein or their combination treatment. Cell cycle analysis revealed a cell cycle arrest in G0/G1 phase in the T47D cell line after genistein treatment only in confluence conditions. Similar results were described by Yu et al. [2008], who observed an inhibition of cell proliferation after genistein treatment through a G0/G1 cell cycle phase arrest in rat aortic smooth muscle cell. In breast cancer, another phytoestrogen, formononetin, inhibits cell growth through G0/G1 cell cycle arrest [Chen et al., 2011]. The increase in P-STAT3/STAT3 ratio, cell proliferation indicator [Catlett-Falcone et al., 1999], after E2 treatment and the reduction in cleaved PARP/PARP ratio, apoptosis indicator [Hoffman et al., 2012], after both treatments in MCF-7 cells agree with our cell cycle analysis results and with previous similar studies involving regulation of PARP cleavage and STAT3 phosphorylation in an ER-dependent manner [Catlett-Falcone et al., 1999; Bjornstrom and Sjoberg, 2005; Hoffman et al., 2012]. Here we observed a biphasic effect of genistein treatment on MDA-MB-231 cell proliferation depending on genistein concentrations, with slight changes. Regarding to cell cycle analysis, MDA-MB-231 showed an induction of cell cycle progression after E2 treatment in subconfluence. This effect (among others) could be due to an ERx membrane element able to induce ERs independent gene transcription leading to the modulation of significant cellular functions such as apoptosis [Kampa et al., 2012]. A down-regulation of ERα and ERβ after E2 treatment has been reported [Nadal-Serrano et al., 2012]. In contrast, genistein treatment induced an over-expression of ERβ in T47D cells, effect described previously with a soy extract treatment by other authors [Gallo et al., 2005]. Therefore, all effects produced by genistein treatment in T47D could be magnified due to the over-expression of ERβ when cells are treated with this phytoestrogen, taking into account the fact that genistein possesses higher affinity for ERβ than for ERα [Kuiper et al., 1997; Muthyala et al., 2004] is considered.

In this study, we observed a loss of mitochondrial function (drop in cytochrome *c* oxidase activity) in high ERα/ERβ ratio cells after E2 treatment, and no effects in low ERα/ERβ ratio cells. In contrast, genistein treatment produced no changes in mitochondrial functionality in MCF-7 breast cancer cell line, as well as the combination of both treatments; while in T47D breast cancer cell line genistein treatment produced a better mitochondrial functionality due to a significant increase in cytochrome *c* oxidase activity. Some studies have shown that E2 treatment produces a mitochondrial dysfunction [Sastre-Serra et al., 2012b], and a greater production of free oxygen radicals related to a higher cell proliferation [Sastre-Serra et al., 2010]. These different effects of genistein compared to the E2 treatment could be due to the higher affinity of genistein for ERβ than for ERα [Kuiper et al., 1997; Muthyala et al., 2004]. Results obtained have also been observed in neurons which can restore the mitochondrial functionality after genistein treatment [Adams et al., 2012]. These results related to the improvement of mitochondrial functionality after genistein treatment in T47D cells may be linked to the inhibition of cell proliferation due to the reduction of free oxygen radicals [Sastre-Serra et al., 2010]. In the same way, previous studies in our lab showed an increased mitochondrial fission and a decreased mitochondrial fusion gene expression in MCF-7 cells after E2 treatment associated with a lower expression of mitochondrial respiratory chain proteins [Sastre-Serra et al., 2012a, 2013]. In the present study we have observed a drop in mitochondrial fission gene expression in MCF-7 cells after genistein treatment which has been associated with lower apoptosis [Lee et al., 2004; Parra et al., 2008]. Lee et al. [2004] found that a drop in Opa1 seemed to be correlated with an increase in apoptosis, but they postulated that Opa1 could act counteracting the proapoptotic effect of Fis1 [Lee et al., 2004], therefore if Fis1 is down-regulated the action of Opa1 could not be necessary for avoiding this proapoptotic effect of Fis1.

In summary, this study demonstrates that genistein treatment could inhibit cancer cell proliferation and induce apoptosis and cell cycle arrest depending on the ERα/ERβ ratio. While high ERα/ERβ ratio breast cancer cells genistein-treatment, as well as E2- and E2 + genistein-treatment, increase cell proliferation; genistein treatment in low ERα/ERβ ratio breast cancer cells show cytostatic effects and a better mitochondrial functionality. However, further studies are necessary to investigate genistein as a possible anticancer therapy that would be dependent on patient ERα/ERβ ratios in malignant

cells, so it would be appropriate the study of the endowment of these two estrogen receptors subtypes in breast cancer patients' cells.

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REFERENCES

Adams SM, Aksenova MV, Aksenov MY, Mactutus CF, Booze RM. 2012. Soy isoflavones genistein and daidzein exert anti-apoptotic actions via a selective ER-mediated mechanism in neurons following HIV-1 Tat(1-86) exposure. PLoS ONE 7:e37540.

Adlercreutz H. 2002. Phyto-oestrogens and cancer. Lancet Oncol 3:364–373.

Bjornstrom L, Sjoberg M. 2005. Mechanisms of estrogen receptor signaling: Convergence of genomic and nongenomic actions on target genes. Mol Endocrinol 19:833–842.

Bouker KB, Hilakivi-Clarke L. 2000. Genistein: Does it prevent or promote breast cancer? Environ Health Perspect 108:701–708.

Catlett-Falcone R, Landowski TH, Oshiro MM, Turkson J, Levitzki A, Savino R, Ciliberto G, Moscinski L, Fernandez-Luna JL, Nunez G, Dalton WS, Jove R. 1999. Constitutive activation of Stat3 signaling confers resistance to apoptosis in human U266 myeloma cells. Immunity 10:105–115.

Chang EC, Frasor J, Komm B, Katzenellenbogen BS. 2006. Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology 147:4831–4842.

Chen J, Zeng J, Xin M, Huang W, Chen X. 2011. Formononetin induces cell cycle arrest of human breast cancer cells via IGF1/PI3K/Akt pathways in vitro and in vivo. Horm Metab Res 43:681–686.

Chrzanowska-Lightowlers ZM, Turnbull DM, Lightowlers RN. 1993. A microtiter plate assay for cytochrome *c* oxidase in permeabilized whole cells. Anal Biochem 214:45–49.

Davis DD, Diaz-Cruz ES, Landini S, Kim YW, Brueggemeier RW. 2008. Evaluation of synthetic isoflavones on cell proliferation, estrogen receptor binding affinity, and apoptosis in human breast cancer cells. J Steroid Biochem Mol Biol 108:23–31.

Gallo D, Zannoni GF, Apollonio P, Martinelli E, Ferlini C, Passetti G, Riva A, Morazzoni P, Bombardelli E, Scambia G. 2005. Characterization of the pharmacologic profile of a standardized soy extract in the ovariectomized rat model of menopause: Effects on bone, uterus, and lipid profile. Menopause 12:589–600.

Grandemange S, Herzig S, Martinou JC. 2009. Mitochondrial dynamics and cancer. Semin Cancer Biol 19:50–56.

Hoffman KL, Lerner SP, Smith CL. 2013. Raloxifene inhibits growth of RT4 urothelial carcinoma cells via estrogen receptor-dependent induction of apoptosis and inhibition of proliferation. Horm Cancer 4:24–35.

Imamov O, Morani A, Shim GJ, Omoto Y, Thulin-Andersson C, Warner M, Gustafsson JA. 2004. Estrogen receptor beta regulates epithelial cellular differentiation in the mouse ventral prostate. Proc Natl Acad Sci USA 101: 9375–9380.

Kampa M, Notas G, Pelekanou V, Troullinaki M, Andrianaki M, Azariadis K, Kampouri E, Lavrentaki K, Castanas E. 2012. Early membrane initiated transcriptional effects of estrogens in breast cancer cells: First pharmacological evidence for a novel membrane estrogen receptor element (ERx). Steroids 77:959–967.

Klaunig JE, Kamendulis LM. 2004. The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol 44:239–267.

Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138:863–870.

Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ. 2004. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell 15:5001–5011.

Manju V, Nalini N. 2007. Protective role of luteolin in 1, 2-dimethylhydrazine induced experimental colon carcinogenesis. Cell Biochem Funct 25:189–194.

Mantovani A, Allavena P, Sica A, Balkwill F. 2008. Cancer-related inflammation. Nature 454:436–444.

Messina MJ, Persky V, Setchell KD, Barnes S. 1994. Soy intake and cancer risk: A review of the in vitro and in vivo data. Nutr Cancer 21:113–131.

Miro AM, Sastre-Serra J, Pons DG, Valle A, Roca P, Oliver J. 2011. 17beta-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio. J Steroid Biochem Mol Biol 123:133–139.

Morton MS, Arisaka O, Miyake N, Morgan LD, Evans BA. 2002. Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. J Nutr 132:3168–3171.

Muthyala RS, Ju YH, Sheng S, Williams LD, Doerge DR, Katzenellenbogen BS, Helferich WG, Katzenellenbogen JA. 2004. Equol, a natural estrogenic metabolite from soy isoflavones: Convenient preparation and resolution of R- and S-equols and their differing binding and biological activity through estrogen receptors alpha and beta. Bioorg Med Chem 12:1559–1567.

Nadal-Serrano M, Sastre-Serra J, Pons DG, Miro AM, Oliver J, Roca P. 2012. The ERalpha/ERbeta ratio determines oxidative stress in breast cancer cell lines in response to 17Beta-estradiol. J Cell Biochem 113:3178–3185.

Nagamine MK, da Silva TC, Matsuzaki P, Pinello KC, Cogliati B, Pizzo CR, Akisue G, Haraguchi M, Górniak SL, Sinhorini IL, Rao KVK, Barbuto JAM, Dagli MLZ. 2009. Cytotoxic effects of butanolic extract from *Pfaffia paniculata* (Brazilian Ginseng) on cultured human breast cancer cell line MCF-7. Exp Toxicol Pathol 61:75–82.

Orlando L, Schiavone P, Cinieri S. 2011. Genistein: The future of prevention and treatment of breast cancer? Cancer Biol Ther 11:918–920.

Parra V, Eisner V, Chiong M, Criollo A, Moraga F, Garcia A, Hartel S, Jaimovich E, Zorzano A, Hidalgo C, Lavandero S. 2008. Changes in mitochondrial dynamics during ceramide-induced cardiomyocyte early apoptosis. Cardiovasc Res 77:387–397.

Paruthiyil S, Parmar H, Kerekatte V, Cunha GR, Firestone GL, Leitman DC. 2004. Estrogen receptor beta inhibits human breast cancer cell proliferation and tumor formation by causing a G2 cell cycle arrest. Cancer Res 64:423–428.

Patisaul HB, Jefferson W. 2010. The pros and cons of phytoestrogens. Front Neuroendocrinol 31:400–419.

Ragan C, Wilson M, Darley-Usmar V, Lowe P. 1987. Subfractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. Oxford: IRL Press.

Rajah TT, Du N, Drews N, Cohn R. 2009. Genistein in the presence of 17beta-estradiol inhibits proliferation of ERbeta breast cancer cells. Pharmacology 84:68–73.

Rambold AS, Kostelecky B, Lippincott-Schwartz J. 2011. Together we are stronger: Fusion protects mitochondria from autophagosomal degradation. Autophagy 7:1568–1569.

Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J, Roca P. 2010. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. Free Radic Biol Med 48:506–512.

Sastre-Serra J, Nadal-Serrano M, Pons DG, Roca P, Oliver J. 2012a. Mitochondrial dynamics is affected by 17beta-estradiol in the MCF-7 breast cancer cell line. Effects on fusion and fission related genes. Int J Biochem Cell Biol 44:1901–1905.

Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Oliver J, Roca P. 2012b. The effects of 17beta-estradiol on mitochondrial biogenesis and function in breast cancer cell lines are dependent on the ERalpha/ERbeta ratio. Cell Physiol Biochem 29:261–268.

Sastre-Serra J, Nadal-Serrano M, Pons DG, Roca P, Oliver J. 2013. The over-expression of ERbeta modifies estradiol effects on mitochondrial dynamics in breast cancer cell line. Int J Biochem Cell Biol 45:1509–1515.

Schleipen B, Hertrampf T, Fritzemeier KH, Kluxen FM, Lorenz A, Molzberger A, Velders M, Diel P. 2011. ERbeta-specific agonists and genistein inhibit proliferation and induce apoptosis in the large and small intestine. Carcinogenesis 32:1675–1683.

Siegel R, Ward E, Brawley O, Jemal A. 2011. Cancer statistics 2011: The impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 61:212–236.

Sotoca AM, Ratman D, van der Saag P, Strom A, Gustafsson JA, Vervoort J, Rietjens IM, Murk AJ. 2008. Phytoestrogen-mediated inhibition of proliferation of the human T47D breast cancer cells depends on the ERalpha/ERbeta ratio. J Steroid Biochem Mol Biol 112:171–178.

Yager JD, Liehr JG. 1996. Molecular mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol 36:203–232.

Youle RJ, van der Bliek AM. 2012. Mitochondrial fission, fusion, and stress. Science 337:1062–1065.

Yu JY, Lee JJ, Lim Y, Kim TJ, Jin YR, Sheen YY, Yun YP. 2008. Genistein inhibits rat aortic smooth muscle cell proliferation through the induction of p27kip1. J Pharmacol Sci 107:90–98.

Zhou JR, Mukherjee P, Gugger ET, Tanaka T, Blackburn GL, Clinton SK. 1998. Inhibition of murine bladder tumorigenesis by soy isoflavones via alterations in the cell cycle, apoptosis, and angiogenesis. Cancer Res 58:5231–5238.

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The phytoestrogen genistein affects breast cancer cells treatment depending on the Estrogen Receptor α/Estrogen Receptor β ratio

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ABSTRACT

Genistein (GEN) is a phytoestrogen found in soybeans. GEN exerts its functions through its interaction with the estrogen receptors (ER), ERα and ERβ, and we previously reported that the ERα/ERβ ratio is an important factor to consider in GEN-treated breast cancer cells. The aim of this study was to investigate the effects of GEN treatment in breast cancer cells with different ERα/ERβ ratio: MCF-7 (high ERα/ERβ ratio), T47D (low ERα/ERβ ratio) and MCF-7 overexpressing ERβ (MCF7+ERβ) treated with cisplatin (CDDP), paclitaxel (PTX) or tamoxifen (TAM). Cell viability, ROS production, autophagy, apoptosis and cell cycle were analyzed. GEN treatment provoked an increase in cell viability in MCF-7 cells in combination with any cytotoxic agent, decreasing ROS production (CDDP+GEN and TAM+GEN) and autophagy (TAM+GEN) or apoptosis (CDDP+GEN and TAM+GEN). Moreover GEN treatment enhanced the cell cycle S phase entry in CDDP+GEN- and TAM+GEN-treated MCF-7 cells and, in the case of CDDP+GEN, decreased the proportion of cells in the G2/M phase and increased it in the subG_0/G_1 phase. Otherwise, in the T47D and MCF7+ERβ cells the combination of GEN with cytotoxic treatments did not cause significant changes in these parameters, even TAM+GEN-treated T47D cells showed less cell viability due to an increment in the autophagic cell death. In conclusion, GEN consumption may be counterproductive in those patients receiving anticancer treatment with a high ERα/ERβ ratio diagnosed breast cancer and it could be harmless or even beneficial in those patients with a lower ERα/ERβ ratio breast cancer cells.

Keywords: *Genistein, breast cancer, cisplatin, paclitaxel, tamoxifen, ROS production, apoptosis, autophagy, cell cycle, ERα/ERβ ratio*

INTRODUCTION

Phytoestrogens are a large group of natural compounds which have been found in more than 300 plants[1]. These compounds are characterized by having a close similarity in chemical structure to estrogens and harboring estrogenic activity[2]. It is believed that phytoestrogens have a lot of health-beneficial properties against several diseases such as osteoporosis, immunity and inflammation, reproduction and fertility, cardiovascular disease and cancer[3-6].

Genistein (GEN) is the major isoflavone, which is a type of phytoestrogen, found in soybeans. The lower incidence of breast cancer in Asia has been associated with higher consumption of GEN, as soybean intake is higher in Asia and blood levels of GEN in people from Eastern countries are 10 times higher than people from Western countries[7-9].

GEN exerts its functions through its interaction with estrogen receptors (ER), ERα and ERβ, and GEN has stronger affinity for ERβ than for ERα[10,11]. The ERα is associated with cell growth and proliferation, while ERβ activation is more related to cytostatic and differentiation processes through inhibition of mammary cancer cell growth as well as counteracting the proliferative effects of ERα activation[12-15].

However, the GEN consumption presents some controversy, especially once breast cancer is diagnosed. Previous studies in our research group have demonstrated that the ERα/ERβ ratio is an important factor in order to determine the benefits of the GEN consumption. Thus, GEN treatment promoted cell proliferation as well as worsened mitochondrial functionality leading to an increase in ROS production in MCF-7 breast cancer cells, with a high ERα/ERβ ratio[16,17]. On the other hand, GEN treatment produced a better mitochondrial functionality, less ROS production and cell cycle arrest in T47D breast cancer cells, with a low ERα/ERβ ratio[16,17]. Finally, GEN treatment hardly affected MDA-MB-231 cells, which is considered negative for ERs, although it has a very low presence of ERβ[17]. It is important to note that in these experiments GEN treatment was applied in the absence of 17β-estradiol (E2), the main estrogen in women. Unlike GEN, E2 has higher affinity for ERα than for ERβ[10,11], which may be important in the response to GEN treatment.

Some anticancer treatments have among its mechanisms of action the generation of radical oxygen species (ROS), producing oxidative stress in cancer cells leading to cell death. Cisplatin (CDDP), paclitaxel (PTX) and tamoxifen (TAM), whose main cytotoxic effects on cancer cells are the generation of DNA adducts[18], microtubule stabilization blocking their disassembly[19] and estrogen receptor antagonism[20] respectively, can affect mitochondria, diminishing the mitochondrial functionality, which results in a rise in ROS production and, in most cases, cell death[21-23], contributing to the action of these cytotoxic compounds on cancer cells.

The main objective of the current study was to investigate the effects of GEN treatment in breast cancer cells with different ERα/ERβ ratio, MCF-7 (high ERα/ERβ ratio) and T47D (low ERα/ERβ ratio) treated with cisplatin, paclitaxel and tamoxifen. For this purpose we analyzed parameters such as cell viability, ROS production, apoptosis, autophagy and cell cycle in order to find out whether the consumption of soy-related products may be beneficial or detrimental in patients being treated for breast cancer. Moreover, some of the experiments (cell viability, ROS production, apoptosis and autophagy) were performed in MCF-7 cells overexpressing ERβ (MCF7+ERβ) to confirm the results obtained in the two breast cancer cells with different ERα/ERβ ratio.

MATERIALS AND METHODS

Reagents

Dulbecco's Modified Eagle's medium (DMEM) high glucose was purchased from GIBCO (Paisley, UK). Genistein (*4',5,7-Trihydroxyisoflavone or GEN*), cisplatin (*cis-Diammineplatinum(II) dichloride or CDDP), paclitaxel (from Taxus brevifolia or PTX) and tamoxifen (trans-2-[4-(1,2-Diphenyl-1-butenyl)phenoxy]-N,N-dimethylethylamine or TAM)* were purchased from Sigma-Aldrich (St. Louis, MO, USA). Primers were purchased from TIB MOLBIOL (Berlin, Germany) and from Metabion (Martinsried, Germany). Routine chemicals used were obtained from Sigma-Aldrich, Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

Quantification of 17β-estradiol of fetal bovine serum

The concentration of 17β-estradiol of the serum was quantified with an Estradiol ELISA kit purchased from DRG Diagnostic (Marburg, Germany), following the manusfacturer's instructions.

Cell culture and treatments

The MCF-7 and T47D human breast cancer cell line were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and routinely cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) in a humidified atmosphere of 5% CO_2 at 37°C. For cytotoxic treatments, cells were seeded in 6-well or 96-well plates and treated the next day with 1 µM GEN, the cytotoxic treatments 10 µM CDDP, 10 nM PTX or 10 µM TAM, and the combination of GEN with each cytotoxic agent for 48 hours.

Estrogen receptor β stable transfection

ERβ cDNA clone was purchased from Origene (Rockville, MD, USA). First of all, ERβ cDNA clone was amplified in *Escherichia coli* DH5αF' Competent Cells (Life Technologies, Paisley, UK) and isolated with MaxiPrep isolation kit (Life Technologies, Paisley, UK). Afterwards cDNA quantification using a BioSpec-nano spectrophotometer set at 260 nm (Shimadzu Biotech, Kyoto, Japan), aliquots of 1 µg/ml of cDNA were kept

in -20^{o}C for cell transfections. Briefly, MCF-7 cells were seeded in 6-well plates and the next day were transfected with an ERβ cDNA clone following the manufacturer's instructions. Lipofectamine 2000 was used to perform cell transfection according to the manufacturer's protocol. After transfection the complexes were removed and cells were provided with normal growth medium. Two days after cDNA transfection, growth medium was replaced for DMEM (+10% FBS and +1% antibiotics) with 600 µg/ml of G418 (or neomycin) in order to select those cells that have incorporated the ERβ cDNA clone in their genome. After two weeks with 600 µg/ml of G418 in the growth medium, colonies were harvested with cloning discs (Sigma-Aldrich, St. Louis, MO, USA) and plated in 24-well plates (one well per colony). Ten days later, cells were ready to trypsinize and they were plated in 6-well plates. Then, cells were subcultured in order to get cells to perform the experiments. It is important to note that the MCF-7+ERβ cells obtained with this protocol must be grown routinely at a concentration of 200 µg/ml of G418 in the growth medium, except when cultured for relevant experiments.

Cell viability assay

MCF-7, T47D or MCF-7+ERβ cells were seeded in 96-well plates. Genistein and cytotoxic treatments were applied the day after for 48h. Crystal Violet was the method used to determine cell viability at the time of 0h (at the moment of applying the treatments), 24h and 48h. Briefly, cells were stained with 0.5% (p/v) crystal violet in 30% (v/v) acetic acid for 10 min. After washing with distilled water, the dye was solubilized in 100 µl of methanol and absorbance *was measured photometrically (*A595nm) using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) to determine cell viability.

Cell cycle analysis by flow cytometry

MCF-7, T47D or MCF-7+ERβ cells were seeded in 6-well plates. Genistein and cytotoxic treatments were applied the day after for 48h. After harvesting the cells with trypsin-EDTA, cells were fixed incubating cells for 1 hour at -20^{o}C with methanol 100% and DNA staining was carried out with an RNAase and propidium iodide mix. After 30 minutes of room temperature incubation in the dark, samples were analyzed using an

Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). The red fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. Ten thousand events were acquired and analyzed with Expo32 ADC analysis software (Beckman Coulter, Miami, FL, USA).

Fluorimetric determination of H2O2 production (ROS production)

ROS production was measured fluorimetrically by using an Amplex® Red Hydrogen Peroxide/Peroxidase assay kit (Molecular Probes). MCF-7, T47D or MCF-7+ERβ cells were seeded in 96-well plates. The day after, genistein and cytotoxic treatments were applied for 48h. The measurement day, cells were exposed to 50 µM of Amplex Red reagent and 0.1 U/ml of horseradish peroxidase, in Krebs-Ringer buffer (145 mM NaCl, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, 5.7 mM sodium phosphate, pH 7.4). Fluorescence was measured with an FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA), set at excitation and emission wavelengths of 571 and 585 nm, to detect the maximum slope of increment in the fluorescence within 1 hour of exposure to kit reagents. Thus, the measurement obtained is the H_2O_2 produced (related to ROS production) by the cells for one hour. Values were normalized per number of viable cells determined by crystal violet assay.

Autophagic vacuoles determination

Autophagic vacuoles were measured fluorimetrically by using Monodansylcadaverine (MDC) respectively. MCF-7, T47D or MCF-7+ERβ cells were seeded in 96-well plates and, the next day, cells were exposed for 48h to genistein and cytotoxic treatments. Fluorescence measurement was performed in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 340 and 535 nm after an incubation of 15 minutes with 50 µM MDC at 37^{o}C. Values were normalized per number of viable cells determined by crystal violet assay.

Apoptosis assay

Apoptosis was measured fluorimetrically by using Annexin V method, as described by Dando et al.[24]. Briefly, MCF-7, T47D or MCF-7+ERβ cells were seeded in 96-well plates and treated with genistein and cytotoxic treatments for 48h. At the end of the treatment, cells were fixed with 2% paraformaldehyde (BD Biosciences, NJ, USA) in PBS at room temperature for 30 min and washed twice with PBS. Cells were then stained with AnnexinV/AlexaFluor350 (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) in annexin binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) for 10 min at room temperature in the dark. To finish, cells were washed once with annexin binding buffer. Florescence was measured in a FLx800 microplate fluorescence reader (Bio-Tek Winooski, Vermont, USA) set at excitation and emission wavelengths of 346 and 442 nm, with cells kept in 100 µl of annexin binding buffer. Values were normalized per number of viable cells determined by crystal violet.

Real-time quantitative PCR

MCF-7 and MCF-7+ERβ were seeded in 6-well plates and the day after total RNA was isolated from cultured cells by using TriPure® isolation reagent (Roche, Barcelona, Spain) following the manufacturer's protocol and then quantified using a BioSpec-nano spectrophotometer set at 260 nm (Shimadzu Biotech, Kyoto, Japan). 1 µg of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10 µl volume of retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 µM random hexamers, 10 U RNase inhibitor, and 500 µM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−80°C) until the PCR reactions were carried out.

PCR was performed in triplicate samples by SYBR Green technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). The primers used were forward 5'-ggACACggACAggATTgACA-3' and reverse 5'-ACCCACggAATCgAgAAAgA-3' for the 18S ribosomal RNA gene, and forward 5'-TAgTggTCCATCgCCAgTTAT-3' and reverse 5'-gggAgCCACACTTCACCAT-3' for the ERβ gene. Total reaction volume was 10 µL, containing 7.5 µL Lightcycler® 480 SYBR Green I Master (containing 0.5 µM of the sense and antisense specific primers) and 2.5 µl of

the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95 °C), followed by 45 cycles consisting of a denaturation step (10 s, 95 °C), an annealing step (10s, 61 °C for 18S and 64 °C for ERβ), and an extension step (12s, 72 °C min). A negative control lacking cDNA template was run in each assay.

The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

Western blot analysis

MCF-7 and T47D cells were seeded in 6-well plates. The next day, cells were exposed to genistein and cytotoxic treatments for 48h. With the aim of checking the ERβ overexpression in MCF7+ERβ cells, these cells were seeded as well in 6-well plates and harvested 24h after seeding. Cell protein extracts were obtained scraping cells with 200 µl of RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X-100, 1 mM EDTA, 1mM NaF, 1 mM Na_3VO_4, 10 µM leupeptin and 10 µM pepstatin; finally, 1 mM PMSF was added just before harvesting the cells with the scraper). The lysate was sonicated three times at 40% amplitude for 7 seconds. Then samples were centrifuged at 14000×*g* for 10 min at 4°C and protein content (supernatant) was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). A 20 µg protein aliquot from the cell lysate was separated on a 12% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. Membranes were incubated in a blocking solution of 5% non-fat powdered milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20) for 1h. Antisera against PARP and LC3A/B (Cell Signaling Technology Inc, Danvers, MA) and GAPDH (Santa Cruz Biotechnology, CA, USA) as the house-keeping were used as primary antibodies. Anti-rabbit secondary antibody was from Sigma-Aldrich (St. Louis, MO, USA). Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and results were analyzed with Quantity One Software (Bio-Rad).

Statistical analysis

The Statistical Program for the Social Sciences software for Windows (SPSS, version 21.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are expressed as mean values ± standard error of the mean (SEM). Statistical significances between control and the overexpression of ERβ in ERβ mRNA levels were assessed by Student's t-test. In the survival curves, Student's t-*test was performed in order to find out the significance between vehicle- and genistein-treated cells, as well as the significance between cytotoxic- and cytotoxic+genistein-treated MCF-7 and T47D cells. The effects of changes produced by cytotoxic agents and their combination or not with genistein in MCF-7, T47D and MCF7+ERβ cell lines were analyzed using two-way analysis of variance (ANOVA), and when results reach significant combinatory effects, Student's t-test was performed in order to find out the significance between the experimental groups. Statistical significance was set at $P<0.05$.

RESULTS

Estrogenic compounds in culture medium

The Estradiol ELISA kit revealed that the fetal bovine serum contains 1nM of 17β-estradiol (data not shown), which means that in the culture medium there is 0.1 nM of E2 (because of the dilution of FBS in the DMEM at 10%). It is important to note the estrogenic capacity of phenol red, and the concentration of phenol red in the DMEM (0.04 mM) is equivalent to 0.4 nM of E2[25]. Therefore, the concentrations of estrogenic compounds are equivalent to 0.5 nM of E2.

ERβ stable overexpression in MCF-7 cells

In order to ensure the levels of ERβ expression, RT-PCR for this gene was carried out in the MCF-7 clone overexpressing ERβ (MCF7+ERβ) selected to perform the experiments. Results indicated that ERβ mRNA expression levels were increased by 734% compared to MCF-7 *wild-type* cells (data not shown).

Genistein decreased breast cancer cells sensitivity to cytotoxic treatments, especially in those with a high ERα/ERβ ratio

Figure 1 shows that GEN treatment increased cancer cells viability alone or in combination with any cytotoxic treatment in MCF-7 cells in a time-dependent manner, but in T47D cells GEN treatment even reduced cell viability in TAM+GEN-treated cells at 24 and 48 hours. In Figure 2, it can be observed that GEN treatment (1 μM) produced an increase in cell viability (+9.6% in MCF-7 and +4.9% in T47D) in control cells. Moreover, GEN treatment significantly raised cell viability in CDDP- (+5.1%), PTX- (+5.3%) and TAM-treated (+5.6%) MCF-7 cells, but not in T47D. Interestingly, the TAM+GEN treated cells in the T47D cell line showed a significant decrease in cell viability in comparison with TAM-treated cells (-3.4%).

Genistein treatment decreased ROS production in combination with cytotoxic agents

As shown in Figure 3 GEN treatment caused a reduction in ROS production in a similar way in the three cell lines (-11% in MCF-7 and -9% in T47D). In MCF-7 cells, the combination of GEN with CDDP and TAM treatment caused a significant reduction in

ROS production (-28% in CDDP+GEN-treated cells and -29% in TAM+GEN-treated cells) compared to cytotoxic treatments alone. In T47D, GEN treatment also produced a decrease in ROS production in combination with CDDP- and TAM-treated cells, but it was milder than in MCF-7 cells (-8% and -9% in CDDP+GEN- and TAM+GEN-treated cells respectively). The combination of GEN and PTX treatment reached no significant difference in both cell lines.

The combination of genistein treatment with cytotoxic affected autophagy especially in TAM-treated breast cancer cells

Figure 4 shows that GEN treatment produced a reduction in autophagy in MCF-7 control cells (-4%), which is not observed in T47D cells. Furthermore, autophagic vacuoles formation was reduced especially after TAM+GEN treatment in MCF-7 cells (-28%), which was accompanied by a decrease in the LC3-II/LC3-I ratio (-96%). Interestingly, in the T47D cell line the combination of GEN and TAM triggered an increase in autophagic vacuoles formation in comparison with TAM-treated T47D cells (+21%).

Apoptosis is decreased in CDDP+GEN- and TAM+GEN-treated MCF-7 cells and PARP cleavage is increased in TAM+GEN T47D cells with respect to cytotoxic treatment alone

For the analysis of apoptosis (Figure 5), GEN combination with CDDP and TAM provoked a drop in Annexin V Fluorescence (-25% and -20% respectively) regarding to cytotoxic treatment alone in MCF-7 cells, in addition to a reduction in the cleavage of the PARP protein in CDDP+GEN-treated cells (-112%). However, the combination of GEN and the three cytotoxic treatments studied did not reach any significant difference in Annexin V Fluorescence compared to cytotoxic treatments alone in T47D cells, although in TAM+GEN-treated cells there was an increase in the PARP cleavage (+84%).

Genistein stimulated the entry into the cell cycle S-phase in MCF-7 cells when combined with cisplatin and tamoxifen treatments

Cell cycle analysis (Table 1) revealed an increase in cells in S-phase after GEN treatment in vehicle- (+0.9%), CDDP- (+0.9%) and TAM-treated cells (+0.9%). Moreover, despite CDDP provoked an S-phase arrest, the combination of CDDP with GEN increased the proportion of cells in the G2/M phase (+3.2%) and decreased the percentage of cells in subG0 phase (-0.38%).

The overexpression of ERβ moderated changes in response to combination of genistein with cisplatin and tamoxifen in MCF-7 cells

In MCF7+ERβ cells, as shown in Figure 6 combination of GEN with CDDP did not reach any significant difference in cell viability regarding to CDDP-treated cells, but in TAM-treated cells GEN treatment caused a slight increase in cell viability compared to TAM-treated cells (+4%). Moreover, the decrease in ROS production after combination of GEN with cytotoxic agents was milder (-15% in CDDP+GEN-treated cells and -26% in TAM+GEN-treated cells) than in MCF-7 cells. Finally, the study of autophagy and apoptosis revealed that there were no significant changes in the combined treatment with GEN regarding to cytotoxic agent alone.

FIGURE CAPTIONS

Figure 1. Survival curves in MCF-7 and T47D cells after genistein and cytotoxic treatments for 0, 24 and 48 hours

MCF-7 and T47D cells were treated with 10 µM cisplatin, 10 nM paclitaxel or 10 µM tamoxifen in combination or not with 1 µM genistein for 0h, 24h and 48h. Cell viability was analyzed with Cristal Violet method. A) Cisplatin-treated MCF-7 cells. B) Cisplatin-treated T47D cells C) Paclitaxel-treated MCF-7 cells. D) Paclitaxel-treated T47D cells. E) Tamoxifen-treated MCF-7 cells. F) Tamoxifen-treated T47D cells. Values are expressed as means ± SEM (n≥6), and normalized as percentage of the 0 hours value for each cell line and treatment. * Significant difference between Vehicle-treated cells and Genistein-treated cells. # Significant difference between cytotoxic-treated cells and cytotoxic+genistein-treated cells (Student's *t*-test; $P<0.05$, n≥6).

Figure 2. Effects of genistein and cytotoxic treatments on cell viability

MCF-7 and T47D cells were treated with 10 µM cisplatin, 10 nM paclitaxel or 10 µM tamoxifen in combination or not with 1 µM genistein for 48h. Cell viability was analyzed with Cristal Violet method. A) MCF-7 breast cancer cells. B) T47D breast cancer cells. Values are expressed as means ± SEM (n=6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and TxG means combinatory effect of tamoxifen treatment and genistein. As a result of combinatory effect, Student's t-test ($P<0.05$, n≥6) was carried out: ^{o} significant difference between control vehicle- and genistein-treated cells; * significant difference between vehicle- and cytotoxic-treated cells; $*^{o}$ significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein.

Figure 3. Effects of genistein and cytotoxic treatments on ROS production

MCF-7 and T47D cells were treated with 10 µM cisplatin, 10 nM paclitaxel or 10 µM tamoxifen in combination or not with 1 µM genistein for 48h. ROS production was analyzed with Amplex® Red Hydrogen Peroxide/Peroxidase assay kit as shown in the Materials and Methods section. A) MCF-7 breast cancer cells. B) T47D breast cancer cells. Values are expressed as means ± SEM (n=6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and CxG, PxG or TxG means combinatory effect of cytotoxic treatments and genistein. As a result of combinatory effect, Student's t-test ($P<0.05$, $n\geq6$) was carried out: ^{o} significant difference between control vehicle- and genistein-treated cells; * significant difference between vehicle- and cytotoxic-treated cells; $*^{o}$ significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein.

Figure 4. Effects of genistein and cytotoxic treatments on autophagy

MCF-7 and T47D cells were treated with 10 µM cisplatin, 10 nM paclitaxel or 10 µM tamoxifen in combination or not with 1 µM genistein for 48h. Autophagic vacuoles formation was analyzed with the fluorescence of monodansylcadaverine and the lipidation of LC3-I to LC3-II as mentioned in the Materials and Methods section. A) Autophagic vacuoles formation in MCF-7 cells. B) Autophagic vacuoles formation in T47D cells. C) LC3-II/LC3-I Ratio measured by western blot in MCF-7 cells. D) LC3-II/LC3-I Ratio measured by western blot in T47D cells. Values are expressed as means ± SEM (n=6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and TxG means combinatory effect of tamoxifen treatment and genistein. As a result of combinatory effect, Student's t-test ($P<0.05$, $n\geq6$) was carried out: ^{o} significant difference between control vehicle- and genistein-treated cells; * significant difference between vehicle- and cytotoxic-treated

cells; *^{o} significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein; NS no significant difference in the ANOVA analysis.

Figure 5. Effects of genistein and cytotoxic treatments on apoptosis

MCF-7 and T47D cells were treated with 10 µM cisplatin, 10 nM paclitaxel or 10 µM tamoxifen in combination or not with 1 µM genistein for 48h. Apoptosis was analyzed with the fluorescence of Annexin V and the cleavage of PARP as mentioned in the Materials and Methods section. A) Apoptosis in MCF-7 cells. B) Apoptosis in T47D cells. C) Cleaved PARP/PARP Ratio measured by western blot in MCF-7 cells. D) Cleaved PARP/PARP Ratio measured by western blot in T47D cells. Values are expressed as means ± SEM (n=6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect, P means paclitaxel effect, T means tamoxifen effect and CxG, PxG or TxG means combinatory effect of cytotoxic treatments and genistein. As a result of combinatory effect, Student's t-test ($P<0.05$, $n\geq6$) was carried out: * significant difference between vehicle- and cytotoxic-treated cells; *^{o} significant difference between cytotoxic-treated cells and cytotoxic-treated cells in combination with genistein.

Figure 6. Effects of genistein and cytotoxic treatments on cell viability, ROS production, autophagy and apoptosis in MCF-7 overexpressing ERβ (MCF7+ERβ)

MCF7+ERβ cells were treated with 10 µM cisplatin or 10 µM tamoxifen in combination or not with 1 µM genistein for 48h. A) Cell proliferation was analyzed by Crystal Violet assay. B) ROS production was measured fluorimetrically using Amplex red reagent. C) Autophagic vacuoles formation was measured fluorimetrically using monodansylcadaverine reagent. D) Apoptosis fraction was measured fluorimetrically using Annexin V method. Values are expressed as means ± SEM (n=6), and normalized as percentage of the control-vehicle value. ANOVA analysis was carried out where G means genistein effect, C means cisplatin effect and T means tamoxifen effect.

TABLES

Table 1. Cell cycle analysis measured by flow cytometry.

	Control			Genistein			ANOVA	
Cell cycle phase	**Vehicle**	**Cisplatin**	**Tamoxifen**	**Vehicle**	**Cisplatin**	**Tamoxifen**	**ANOVA Cisplatin**	**ANOVA Tamoxifen**
Sub G_0/G_1 (%)	0.90 ± 0.07	1.74 ± 0.17	0.98 ± 0.08	0.78 ± 0.14	1.36 ± 0.10	0.95 ± 0.06	***C***	***NS***
G_0/G_1 (%)	49.4 ± 0.5	43.1 ± 0.7*	54.1 ± 1.2	53.1 ± 0.9^{o}	39.4 ± 0.8*^{o}	56.0 ± 1.6	***C, CxG***	***T, G***
S (%)	10.1 ± 0.2	20.0 ± 0.2	4.29 ± 0.21	11.0 ± 0.4	20.9 ± 0.2	5.20 ± 0.33	***C, G***	***T, G***
G_2/M (%)	39.6 ± 0.3	35.2 ± 0.7*	40.6 ± 1.2	35.1 ± 0.7^{o}	38.4 ± 0.6*^{o}	37.9 ± 1.3	***CxG***	***G***

The values in the table represents the percentage of cells in each cell cycle phase after cytotoxic treatments (10 μM) in combination (Genistein) or not (Control) with genistein treatment (1 μM) for 48 hours in MCF-7 breast cancer cells. ANOVA analysis was carried out where C means cisplatin effect, T means tamoxifen effect, G means genistein effect and CxG means combinatory effect of cisplatin and genistein treatments. As a result of combinatory effect, Student's t-test ($P<0.05$, $n=6$) was carried out: ^{o} significant difference between control vehicle- and genistein-treated cells; * significant difference between Vehicle and Cisplatin-treated cells; *^{o} significant difference between Cisplatin-treated cells and Cisplatin-treated cells in combination with genistein; NS no significant difference in the ANOVA analysis.

FIGURES

Figure 1

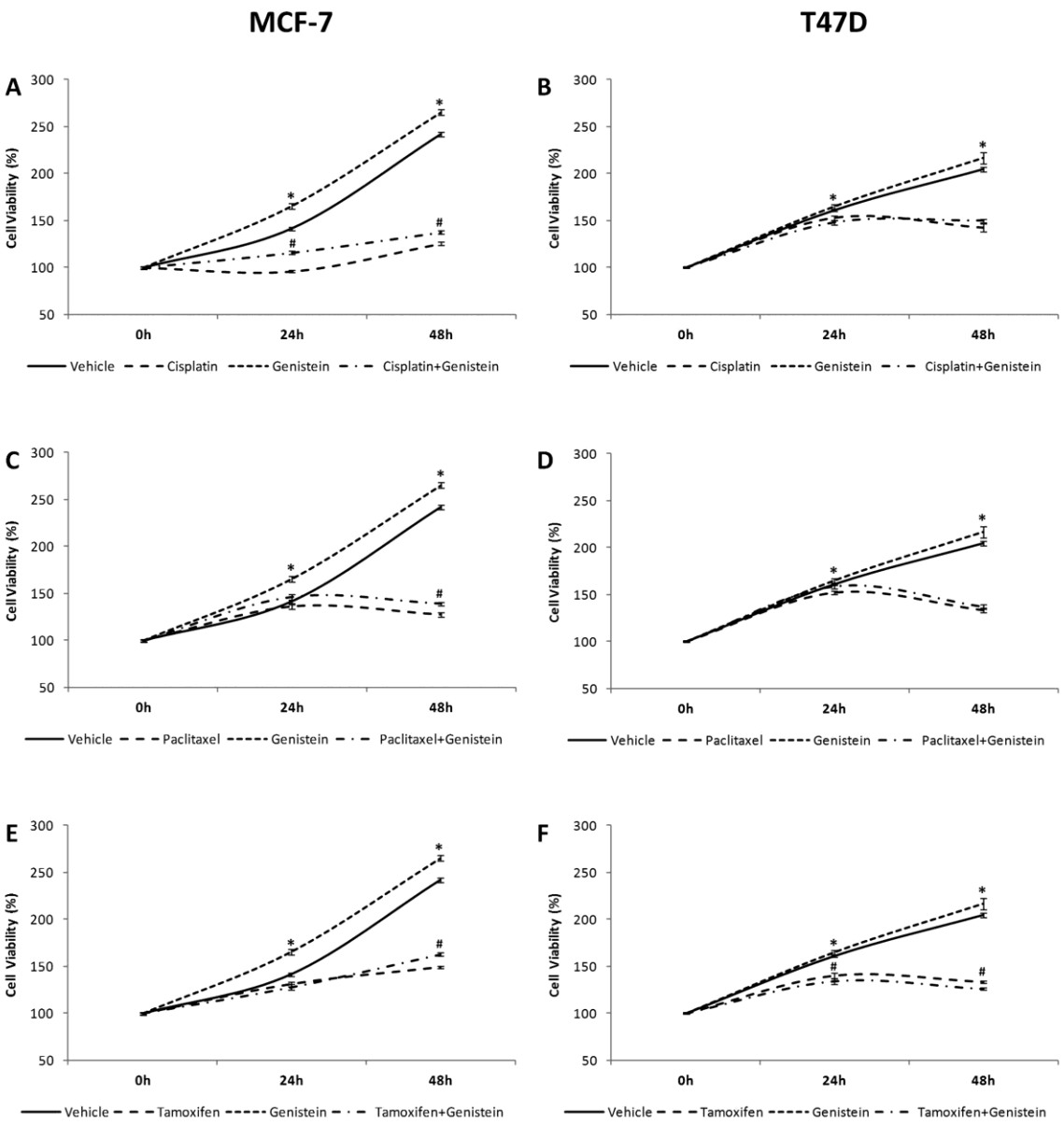


Figure 2

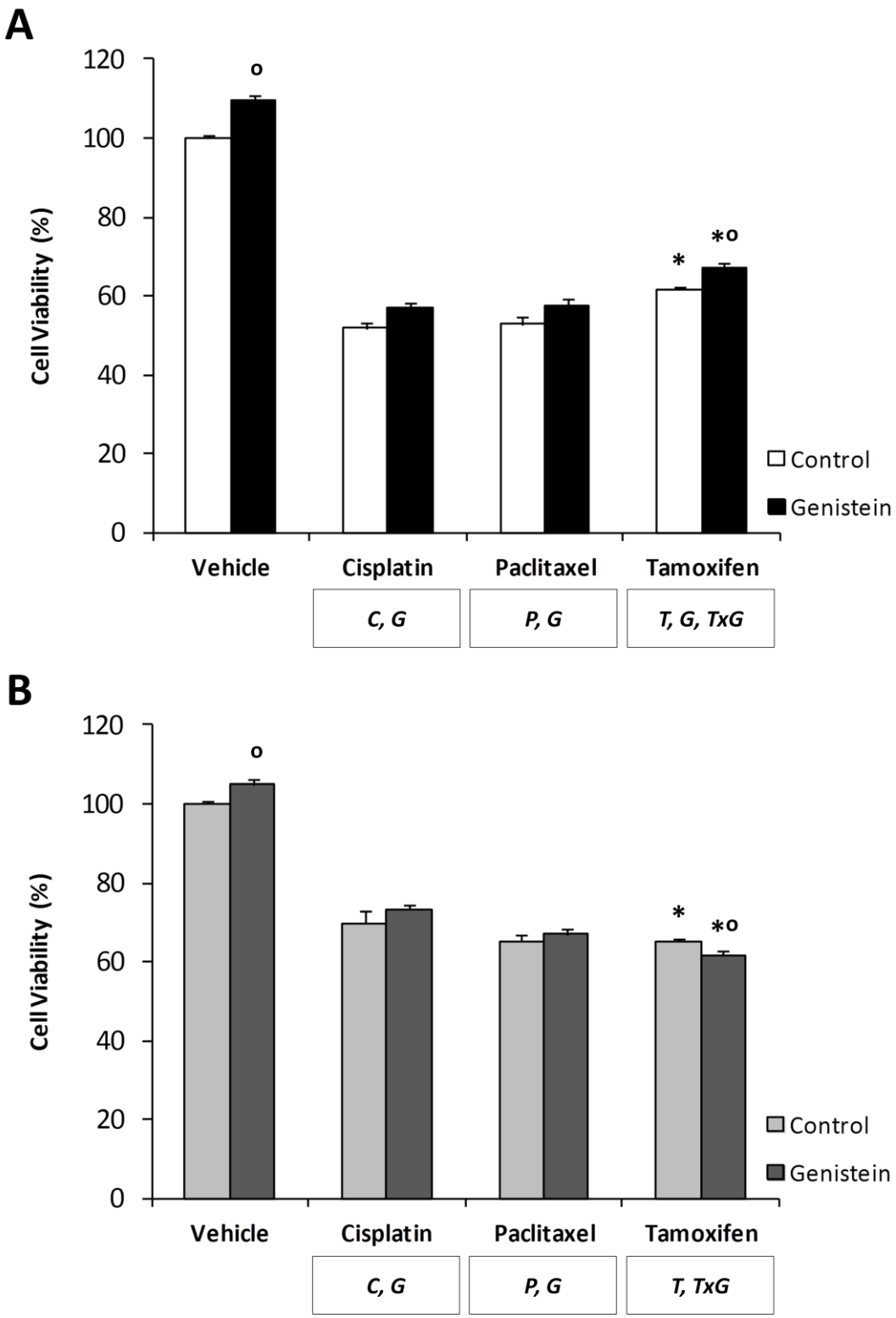

A
120
100
80
60
40
20
0
Cell Viability (%)
o
*
*o
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
C, G
P, G
T, G, TxG
B
120
100
80
60
40
20
0
Cell Viability (%)
o
*
*o
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
C, G
P, G
T, TxG

Figure 3

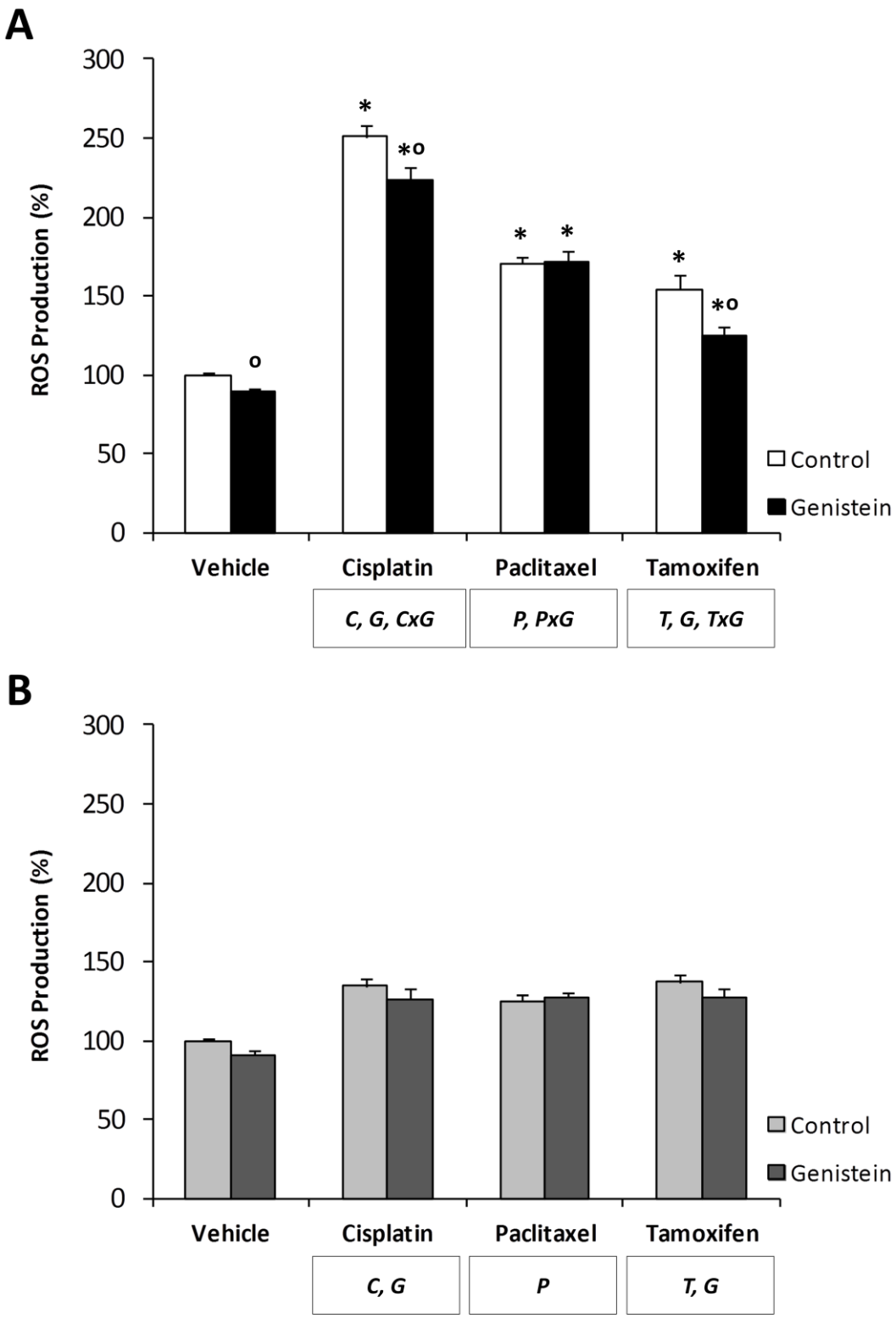
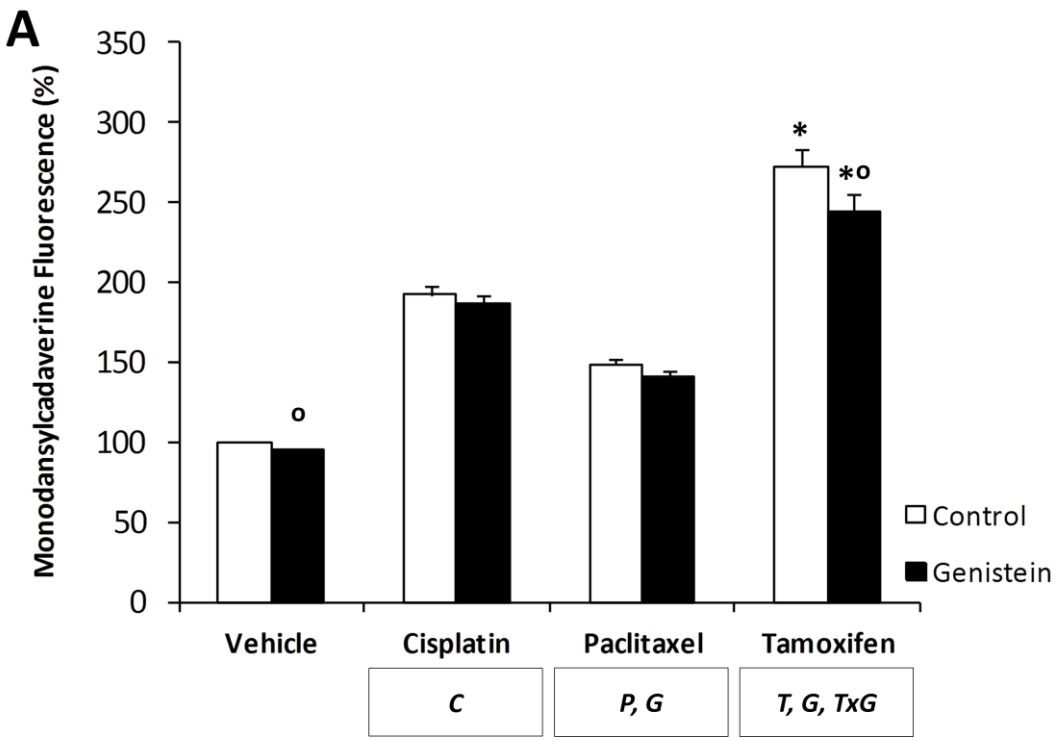

A
300
250
200
150
100
50
0
ROS Production (%)
*
*o
*
*
*
*o
o
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
C, G, CxG
P, PxG
T, G, TxG
B
300
250
200
150
100
50
0
ROS Production (%)
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
C, G
P
T, G

Figure 4A and 4B



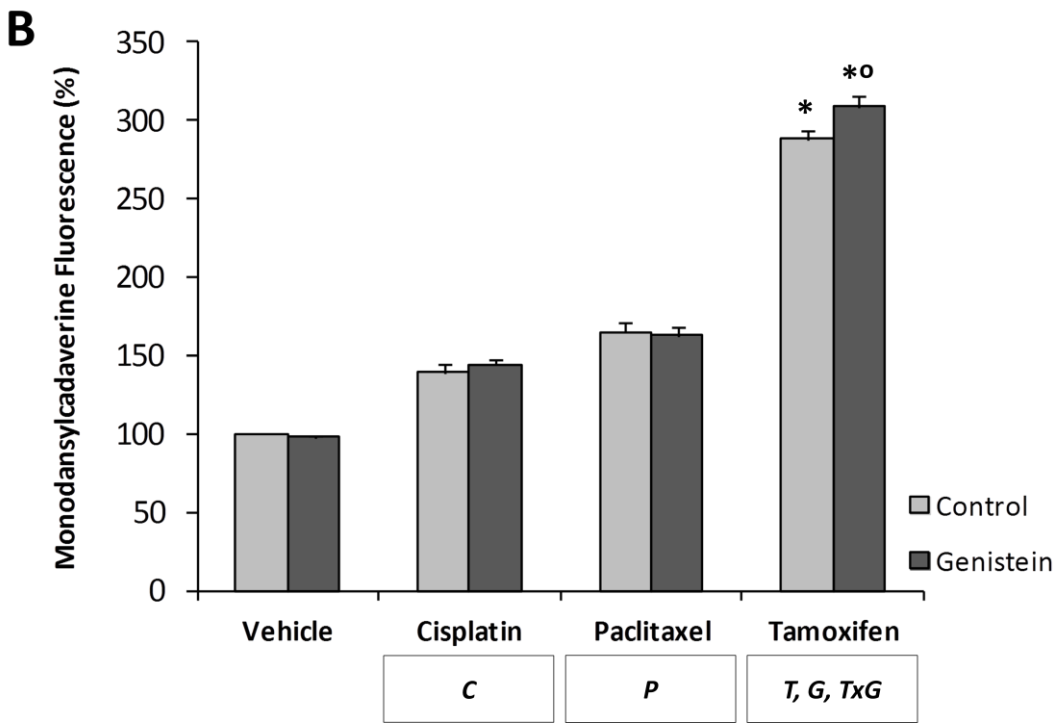


Figure 4C

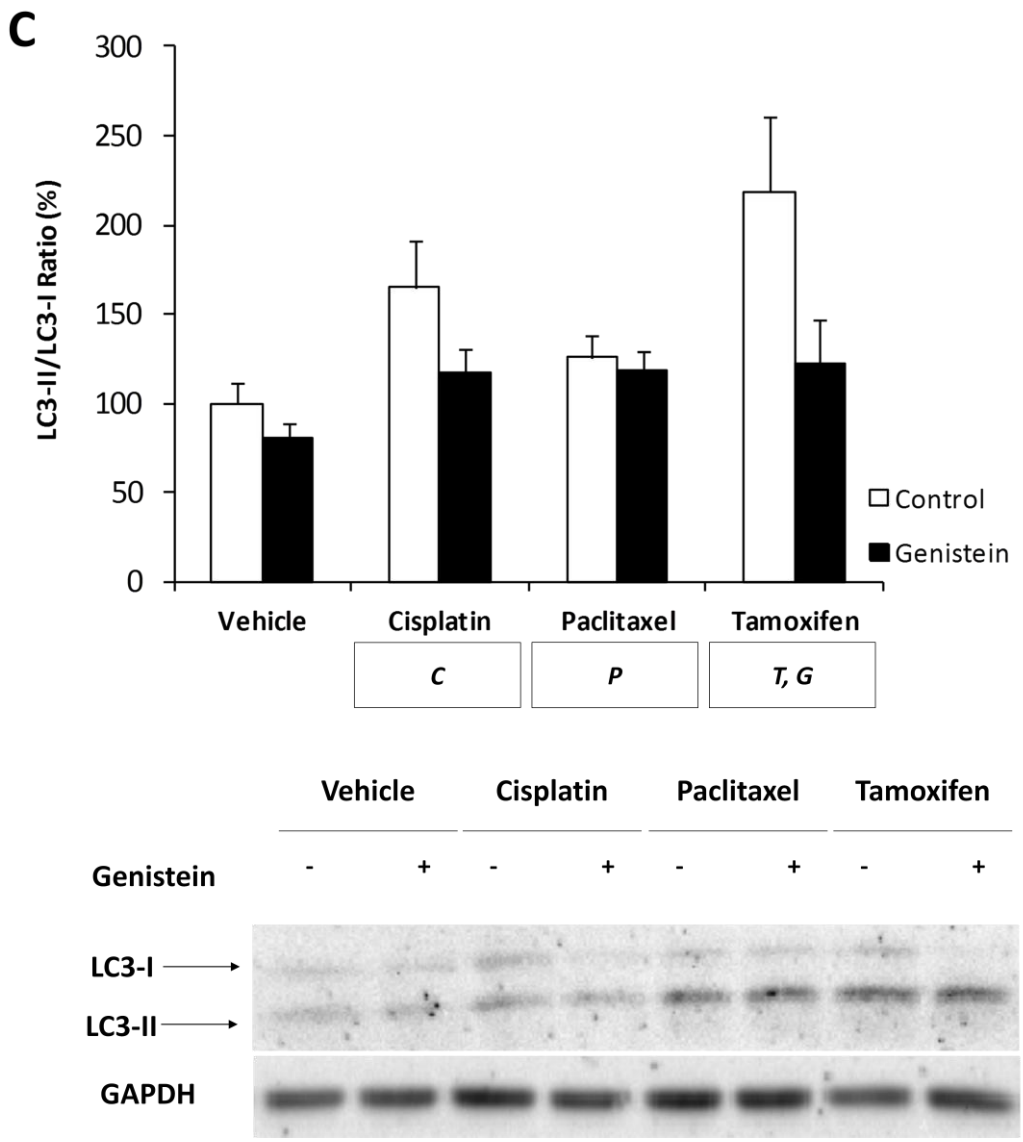

C
LC3-II/LC3-I Ratio (%)
300
250
200
150
100
50
0
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
C
P
T, G
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
Genistein
- + - + - + - +
LC3-I
LC3-II
GAPDH

Figure 4D

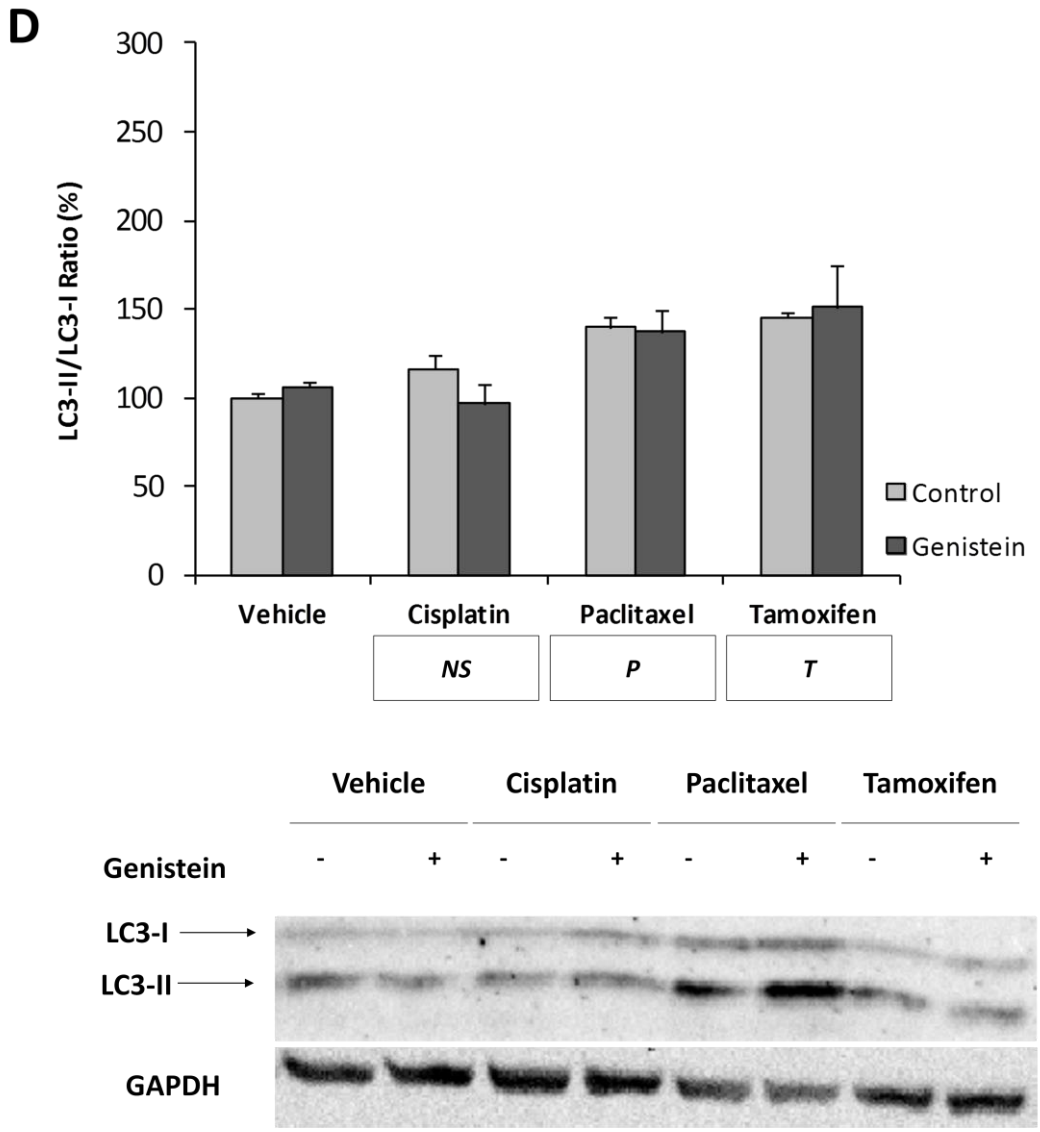
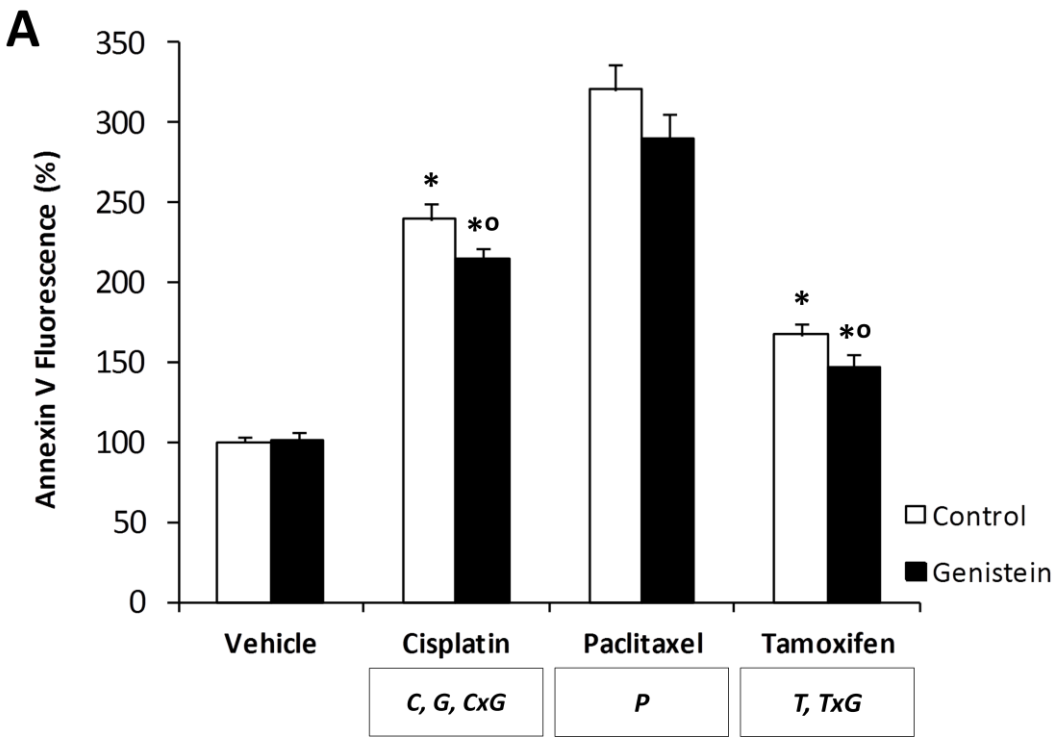

D
LC3-II/LC3-I Ratio (%)
300
250
200
150
100
50
0
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
NS
P
T
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
Genistein
- + - + - + - +
LC3-I
LC3-II
GAPDH

Figure 5A and 5B



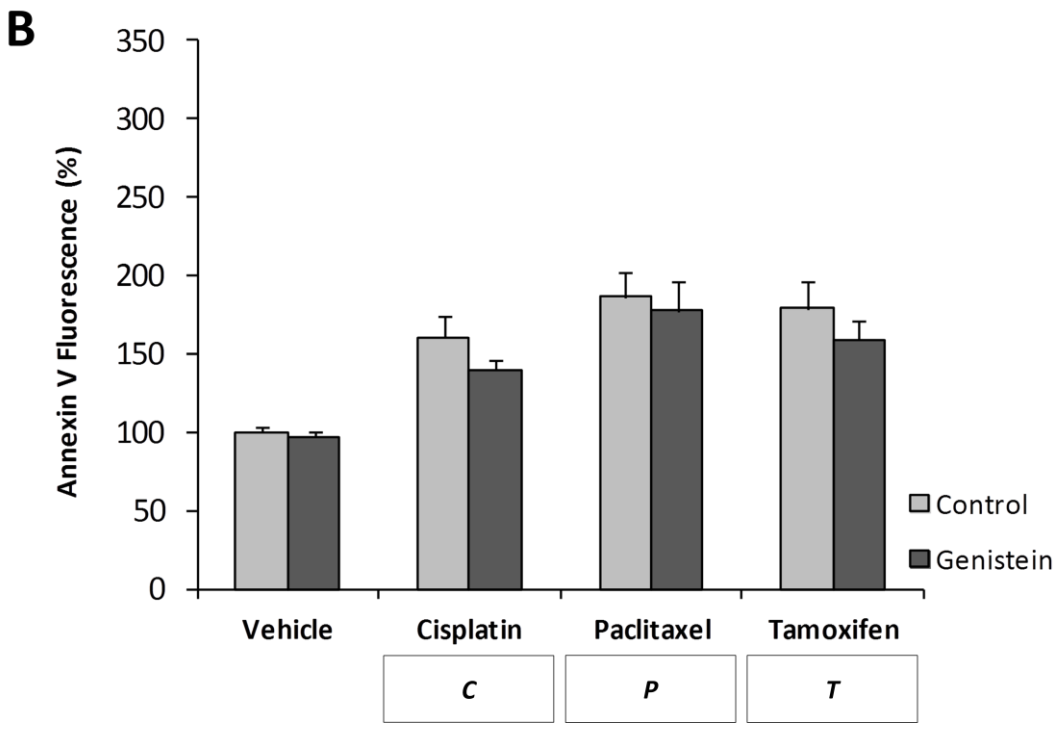


Figure 5C

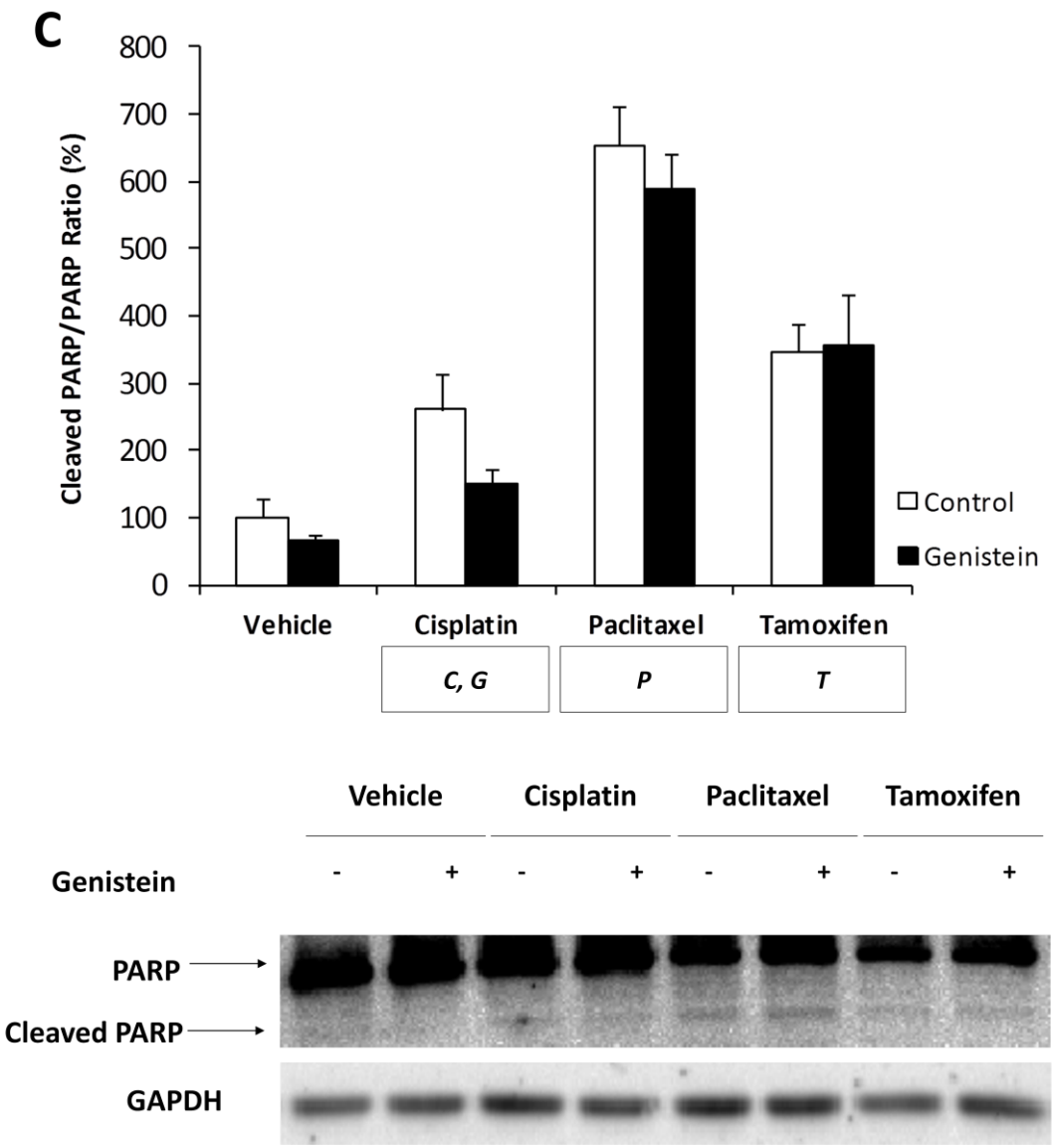

C
Cleaved PARP/PARP Ratio (%)
800
700
600
500
400
300
200
100
0
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
C, G
P
T
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
Genistein
- + - + - + - +
PARP
Cleaved PARP
GAPDH

Figure 5D

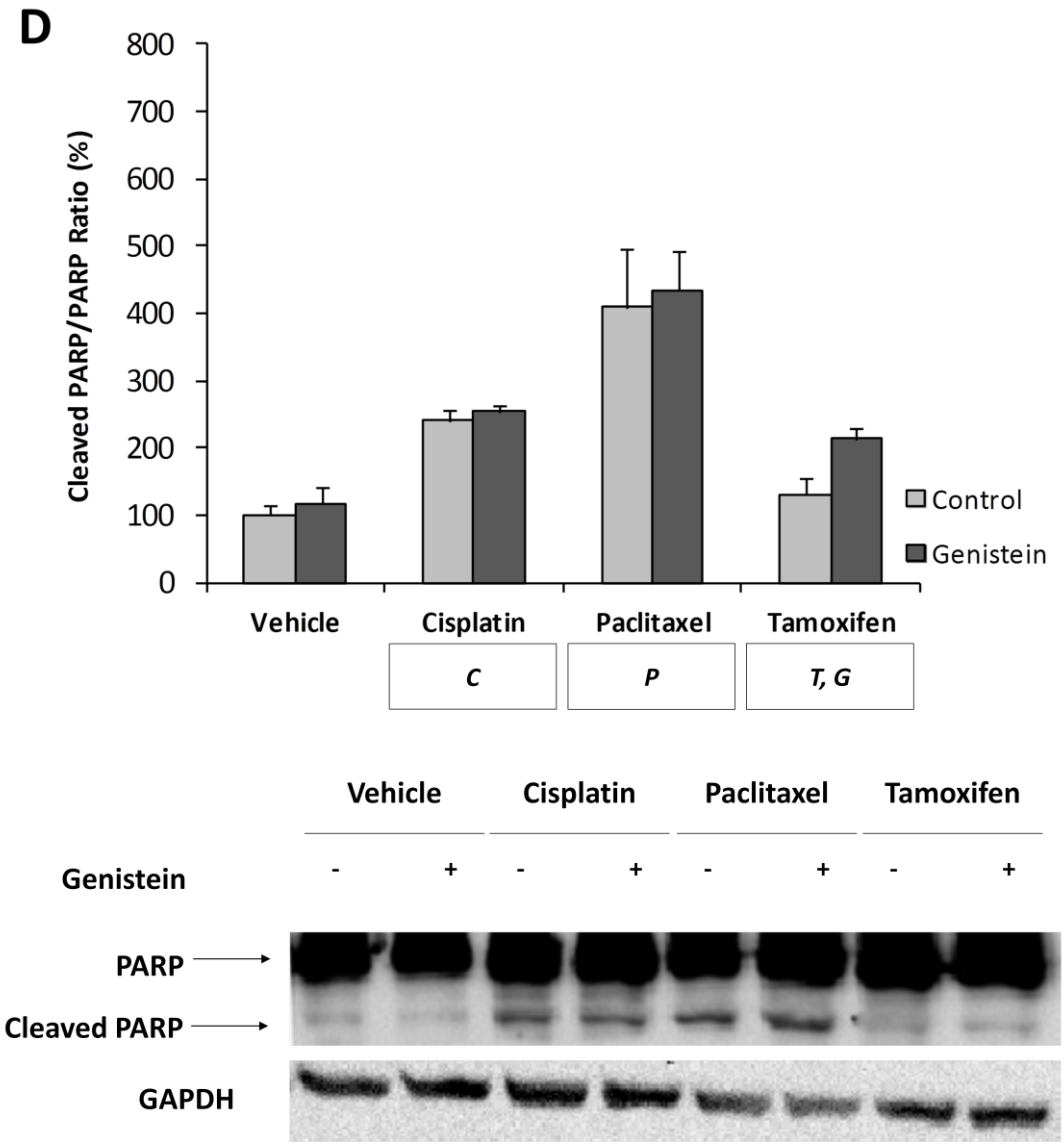

D
Cleaved PARP/PARP Ratio (%)
800
700
600
500
400
300
200
100
0
Control
Genistein
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
C
P
T, G
Vehicle
Cisplatin
Paclitaxel
Tamoxifen
Genistein
- + - + - + - +
PARP
Cleaved PARP
GAPDH

Figure 6A and 6B

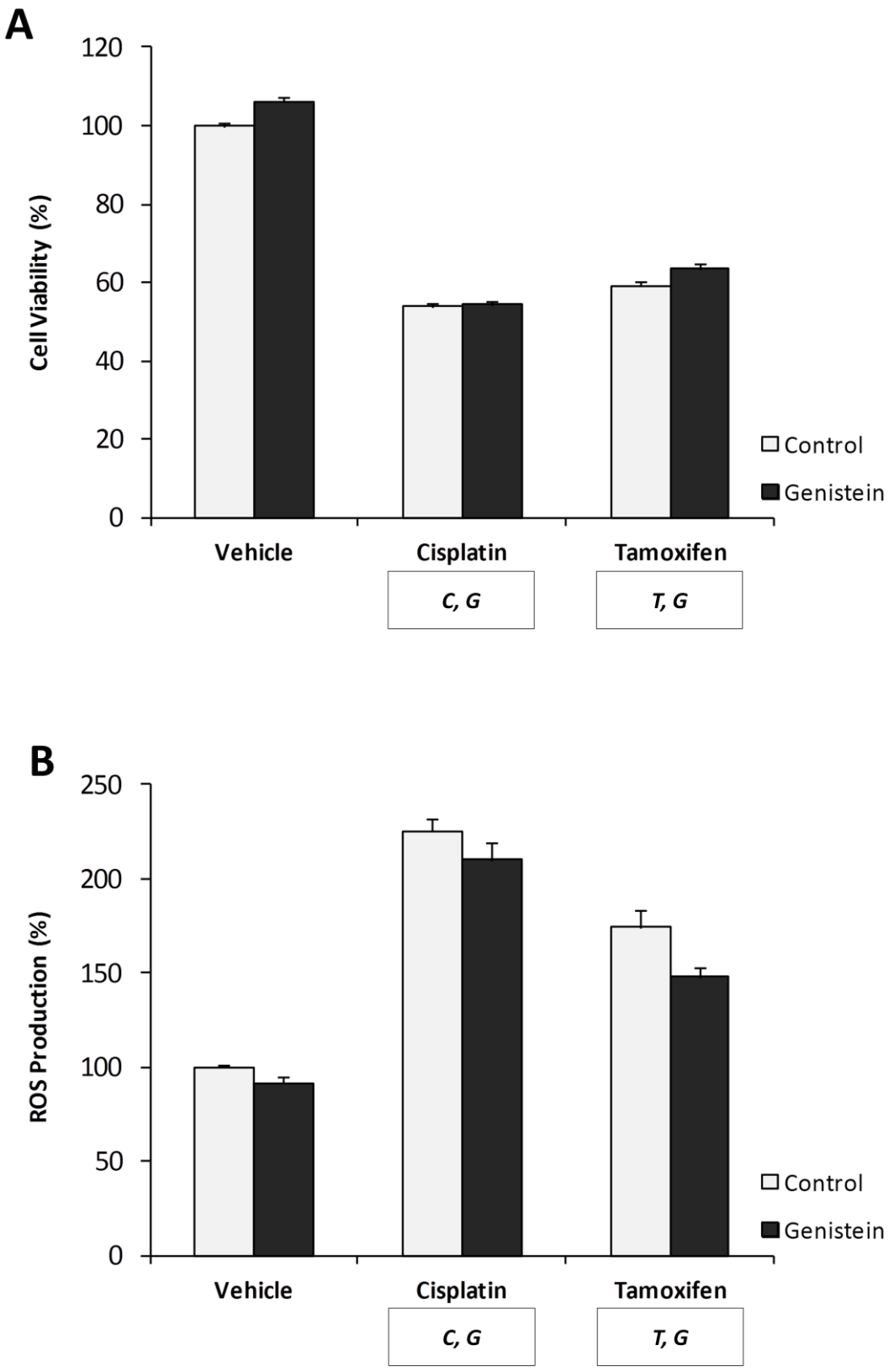
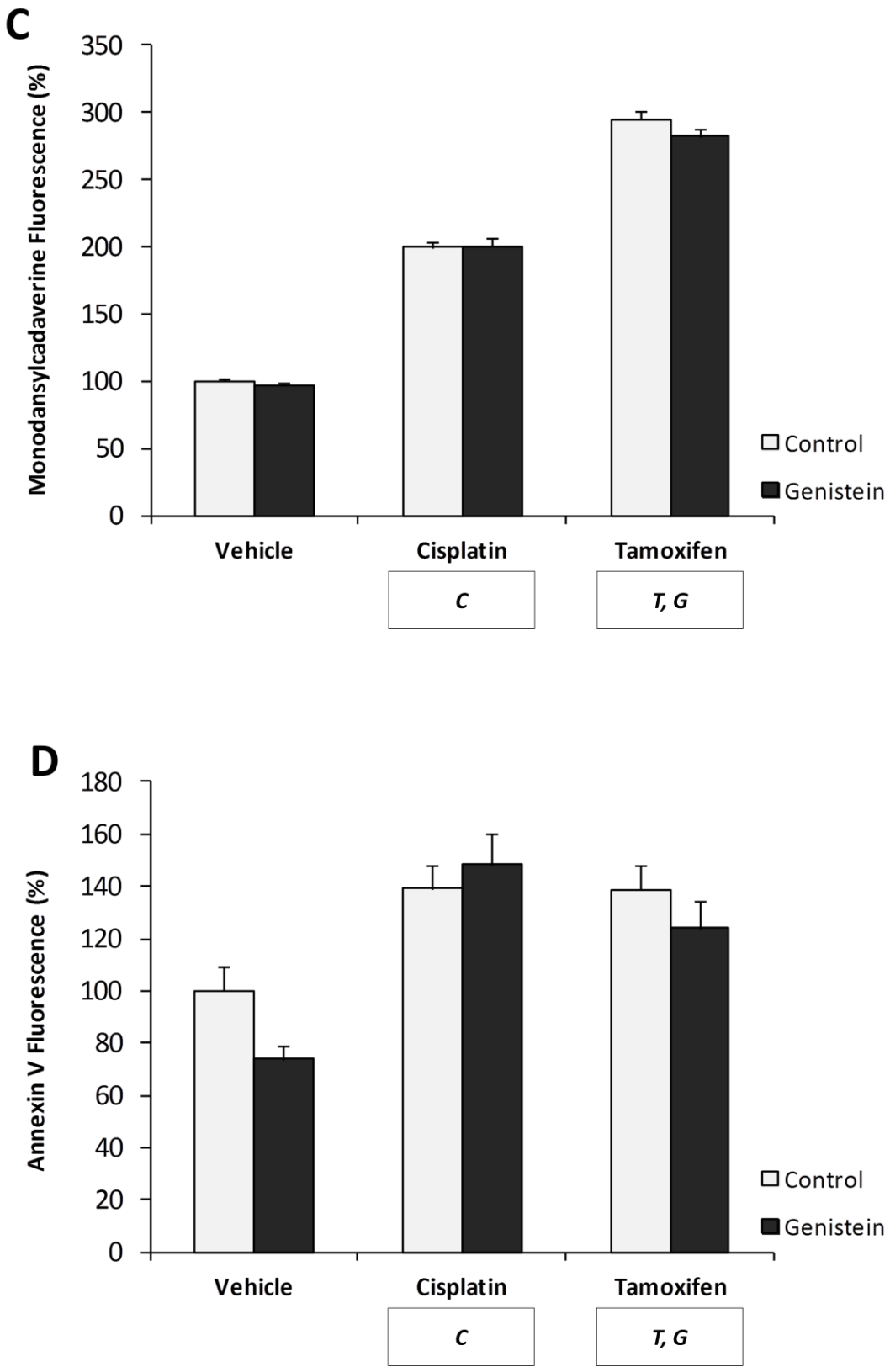


Figure 6C and 6D



DISCUSSION

We have studied the effects of the phytoestrogen genistein (GEN) in the efficacy of the anticancer treatments cisplatin (CDDP), paclitaxel (PTX) and tamoxifen (TAM) in breast cancer cell lines with different estrogen receptors (ERα and ERβ) ratio. Thus, the analysis of cell viability revealed that the combination of GEN with any cytotoxic treatment increased the cell survival in MCF-7 cells (high ERα/ERβ ratio) regarding to cytotoxic-treated cells. This increment in cell viability was accompanied by a decrease in ROS production and in the PARP cleavage (an indicator of apoptosis) in CDDP+GEN-treated cells and in autophagy in TAM+GEN-treated cells. Moreover, cell cycle analysis showed a greater entrance in the S phase with GEN treatment and, in the case of CDDP, a lesser proportion of cells in sub-G0/G1 (apoptotic cells) and, furthermore, a higher number of cells in the G2/M phase, explaining the greater cell viability in the CDDP+GEN-treated cells. Nevertheless, in T47D cells (low ERα/ERβ ratio) and in MCF-7 cells overexpressing ERβ (MCF7+ERβ), the increase in cell viability after GEN treatment was milder than in MCF-7 cells and, in some cases such as TAM+GEN-treated T47D cells, there was a decrease in cell viability after GEN treatment through a raise in the autophagic cell death.

We previously reported that GEN treatment, in a phenol red-free cell culture medium supplemented with 10% charcoal-stripped FBS, affects cancer cells in a different way depending on the ERα/ERβ ratio[16,17]. In the current study, cells were cultured in medium containing 10% FBS and phenol red, with an equivalent E2 concentration of 0.5 nM, which corresponds to a physiological concentration of E2[26]. Thus, GEN treatment produced a greater cell survival in MCF-7 cells, probably due to its estrogenic activity through its binding to ERα[27], but also displacing the E2 bound to the receptor and interacting with the ERβ also present in this cell line[17]. This interaction with the ERβ provoked a lower ROS production[16] decreasing the apoptosis and, in the case of CDDP+GEN-treated cells, decreasing the cleaved PARP/PARP ratio, a final indicator of apoptosis[28], in comparison with cytotoxic alone-treated MCF-7 cells. These results suggest that GEN could reduce ROS production and therefore apoptosis in high ERα/ERβ ratio breast cancer cells cultured with physiological concentrations of E2. Other authors have demonstrated the relationship between ROS production and

the induction of cancer cells apoptosis[29,30]. Moreover, GEN treatment in control cells as well as in combination with cytotoxic agents raised the entrance in S phase of cell cycle, as described in 2004 by Chen and Wong, who demonstrated that GEN treatment at 1 μM stimulated the growth of MCF-7 cells and significantly increased cells in the S phase[31]. Cell cycle analysis also revealed that, despite CDDP causes cell cycle arrest in the S phase[32], the CDDP+GEN-treated cells showed an increase in G2/M phase and a drop in subG_0/G_1 phase (corresponding to apoptotic cells[33]).

On the other hand, the combination of GEN with PTX and especially with TAM produced a decrease in autophagic cell death, leading to more viable breast cancer cells than cytotoxic alone-treated cells. Precisely, TAM+GEN-treated MCF-7 cells showed a decrease in the LC3 II/LC3 I ratio, an indicator of autophagy[34], confirming the reduction of autophagy after GEN treatment in TAM-treated cells, as other authors have reported previously in pancreas cancer cells showing that GEN treatment produced a decrease in the LC3 II/LC3 I ratio[35]. The study of apoptosis revealed that there was a decrease in apoptosis after GEN treatment in CDDP and TAM combined treatments and, in the case of CDDP+GEN-treated cells it was confirmed by a decrease in the PARP cleavage. These results do confirm the effects of the combination of physiological concentrations of GEN with cytotoxic agents such as CDDP and TAM, reducing their efficacy. Other authors have demonstrated that GEN reduces CDDP-induced apoptosis in kidney through the regulation of p53 induction[36]. Other authors have published that dietary GEN negated/overwhelmed the inhibitory effect of TAM on MCF-7 tumor growth in mice, lowered E_2 level in plasma and increased expression of E2-responsive genes[37].

In those cells with a low ERα/ERβ ratio such as T47D cells and the MCF-7 cells overexpressing ERβ (MCF7+ERβ), GEN treatment did not produce any significant increment in cell viability when incubated in combination with cytotoxic agents (with the exception of a small rise in the TAM+GEN-treated MCF7+ERβ cells). This is probably due to the E2 present in the medium stimulating the ERβ in these cells; therefore the protective effects of the ERβ against ROS production[38] are already occurring, so that the addition of GEN would have no significant effect. Curiously, TAM+GEN-treated MCF7+ERβ cells were the only ones (of those cells with a low ERα/ERβ ratio) that

combination of GEN with the cytotoxic agents caused an increase in cell viability, maybe due to the decrease in ROS production, as a drop in this parameter could lead to a greater cell survival[39].

Interestingly, TAM+GEN-treated T47D cells had lower cell viability than TAM-treated T47D cells through an increase in the autophagic vacuoles formation. Autophagy is a conserved evolutionary process that may enable cells to maintain homeostasis in unfavorable environmental conditions[40]. This process allows the cell to recover energy from damaged or unnecessary subcellular components (macromolecules or organelles)[40]; therefore it has been considered as a process associated with cell survival. However, if the damage is too severe and a high level of autophagy persists, autophagic cell death or programmed cell death-2 will occur, which is a different phenomenon than apoptosis or programmed cell death-1[41]. So T47D cells treated with GEN+TAM showed an increase in the autophagic cell death, leading to a poorer cell survival than TAM-treated T47D cells. Moreover, PARP cleavage is increased in TAM+GEN-treated T47D cells suggesting an increase in apoptosis as well. The raise in autophagic vacuoles formation and the cleavage of the PARP are contributing to the lesser cell survival than TAM-treated T47D cells. These results confirm the importance of the presence of ERβ when cancer cells are treated with ERβ agonists, validating the results recently obtained by Ruddy et al. who demonstrated that apoptosis and autophagy are increased in those breast cancer cells treated with a combination of TAM and an ERβ agonist[42].

In conclusion, these results indicate that in women diagnosed with breast cancer whose cancer cells have a high ERα/ERβ ratio, the genistein consumption (soy products or dietary supplements) may have a counterproductive effect in the anticancer treatment mainly due to the decrease in the ROS production (in the case of cisplatin and tamoxifen treatments, since ROS production is one of the mechanism of action of these cytotoxic agents). The consequence of that is a reduction in the apoptosis (in cisplatin-treated cells) or the autophagic cell death (in tamoxifen-treated cells), therefore increasing cancer cells viability. However, in those cells with a low ERα/ERβ ratio genistein consumption seems to have a lesser impact in the efficacy of anticancer therapies, and even increases that effectiveness in the tamoxifen-treated cells in

combination with the genistein, raising the autophagic cell death. Further studies are necessary to better understand the role of the genistein in the efficacy of the anticancer treatments, as well as the role of the ERα/ERβ ratio in those patients consuming high amounts of genistein in that efficacy.

ACKNOWLEDGMENTS

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REFERENCES

1 Farnsworth, N. R., Bingel, A. S., Cordell, G. A., Crane, F. A. & Fong, H. S. J Pharm Sci 64, 717-754 (1975).

2 Kurzer, M. S. & Xu, X. Annu Rev Nutr 17, 353-381 (1997).

3 Dixon, R. A. Annu Rev Plant Biol 55, 225-261 (2004).

4 Mense, S. M., Hei, T. K., Ganju, R. K. & Bhat, H. K. Environ Health Perspect 116, 426-433 (2008).

5 Messina, M. J. & Loprinzi, C. L. J Nutr 131, 3095S-3108S (2001).

6 Orlando, L., Schiavone, P. & Cinieri, S. Cancer Biol Ther 11, 918-920 (2011).

7 Morton, M. S., Arisaka, O., Miyake, N., Morgan, L. D. & Evans, B. A. J Nutr 132, 3168-3171 (2002).

8 Zhou, J. R., Mukherjee, P., Gugger, E. T., Tanaka, T., Blackburn, G. L. et al. Cancer research 58, 5231-5238 (1998).

9 Adlercreutz, H. The Lancet. Oncology 3, 364-373 (2002).

10 Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J. et al. Endocrinology 138, 863-870 (1997).

11 Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H. et al. Endocrinology 139, 4252-4263 (1998).

12 Chang, E. C., Frasor, J., Komm, B. & Katzenellenbogen, B. S. Endocrinology 147, 4831-4842 (2006).

13 Rajah, T. T., Du, N., Drews, N. & Cohn, R. Pharmacology 84, 68-73 (2009).

14 Sotoca, A. M., Ratman, D., van der Saag, P., Strom, A., Gustafsson, J. A. et al. The Journal of steroid biochemistry and molecular biology 112, 171-178 (2008).

15 Strom, A., Hartman, J., Foster, J. S., Kietz, S., Wimalasena, J. et al. Proceedings of the National Academy of Sciences of the United States of America 101, 1566-1571 (2004).

16 Nadal-Serrano, M., Pons, D. G., Sastre-Serra, J., Blanquer-Rossello Mdel, M., Roca, P. et al. The international journal of biochemistry & cell biology 45, 2045-2051 (2013).

17 Pons, D. G., Nadal-Serrano, M., Blanquer-Rossello, M. M., Sastre-Serra, J., Oliver, J. et al. Journal of cellular biochemistry 115, 949-958 (2014).

18 Jordan, P. & Carmo-Fonseca, M. Cellular and molecular life sciences : CMLS 57, 1229-1235 (2000).

19 Luo, Y., Li, D., Ran, J., Yan, B., Chen, J. et al. Protein & cell 5, 469-479 (2014).

20 Jensen, E. V. & Jordan, V. C. Clinical cancer research : an official journal of the American Association for Cancer Research 9, 1980-1989 (2003).

21 Andre, N., Carre, M., Brasseur, G., Pourroy, B., Kovacic, H. et al. FEBS Lett 532, 256-260 (2002).

22 Marullo, R., Werner, E., Degtyareva, N., Moore, B., Altavilla, G. et al. PloS one 8, e81162 (2013).

23 Razandi, M., Pedram, A., Jordan, V. C., Fuqua, S. & Levin, E. R. Oncogene 32, 3274-3285 (2013).

24 Dando, I., Fiorini, C., Pozza, E. D., Padroni, C., Costanzo, C. et al. Biochimica et biophysica acta 1833, 672-679 (2013).

25 Berthois, Y., Katzenellenbogen, J. A. & Katzenellenbogen, B. S. Proc Natl Acad Sci U S A 83, 2496-2500 (1986).

26 Rajah, T. T., Peine, K. J., Du, N., Serret, C. A. & Drews, N. R. Anticancer research 32, 1181-1191 (2012).

27 Hsieh, C. Y., Santell, R. C., Haslam, S. Z. & Helferich, W. G. Cancer research 58, 3833-3838 (1998).

28 Hoffman, K. L., Lerner, S. P. & Smith, C. L. Horm Cancer (2012).

29 Gotoh, Y. & Cooper, J. A. J Biol Chem 273, 17477-17482 (1998).

30 Shen, H. M. & Liu, Z. G. Free radical biology & medicine 40, 928-939 (2006).

31 Chen, W. F. & Wong, M. S. The Journal of clinical endocrinology and metabolism 89, 2351-2359 (2004).

32 Wagner, J. M. & Karnitz, L. M. Molecular Pharmacology 76, 208-214 (2009).

33 Chuang, J. Y., Tsai, Y. Y., Chen, S. C., Hsieh, T. J. & Chung, J. G. In vivo 19, 683-688 (2005).

34 Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y. et al. Journal of cell science 117, 2805-2812 (2004).

35 Suzuki, R., Kang, Y. a., Li, X., Roife, D., Zhang, R. et al. Anticancer research 34, 4685-4692 (2014).

36 Sung, M. J., Kim, D. H., Jung, Y. J., Kang, K. P., Lee, A. S. et al. Kidney Int 74, 1538-1547 (2008).

37 Ju, Y. H., Doerge, D. R., Allred, K. F., Allred, C. D. & Helferich, W. G. Cancer research 62, 2474-2477 (2002).

38 Nadal-Serrano, M., Sastre-Serra, J., Pons, D. G., Miro, A. M., Oliver, J. et al. Journal of cellular biochemistry 113, 3178-3185 (2012).

39 Trachootham, D., Lu, W., Ogasawara, M. A., Valle, N. R.-D. & Huang, P. Antioxidants & Redox Signaling 10, 1343-1374 (2008).

40 Cook, K. L., Shajahan, A. N. & Clarke, R. Expert review of anticancer therapy 11, 1283-1294 (2011).

41 Akar, U., Chaves-Reyez, A., Barria, M., Tari, A., Sanguino, A. et al. Autophagy 4, 669-679 (2008).

42 Ruddy, S. C., Lau, R., Cabrita, M. A., McGregor, C., McKay, B. C. et al. Molecular cancer therapeutics 13, 1882-1893 (2014).

4. Discusión / Discussion

En la presente tesis se ha profundizado en la importancia de la ratio ERα/ERβ en la funcionalidad y dinámica mitocondrial, y el estrés oxidativo en el cáncer de mama. Adicionalmente, se ha estudiado cómo esta ratio ERα/ERβ afecta a la respuesta de las células cancerígenas a los tratamientos antitumorales como el cisplatino, el paclitaxel o el tamoxifeno, así como el silenciamiento de la UCP2. Finalmente, se han analizado los efectos de la genisteína, un fitoestrógeno presente en la soja, sobre la funcionalidad mitocondrial y la respuesta a los tratamientos contra el cáncer de mama en función de la ratio ERα/ERβ.

La presencia del ERβ en líneas celulares de cáncer de mama provocó, después del tratamiento con concentraciones fisiológicas de 17β-estradiol (E2), una mejora en la funcionalidad mitocondrial con un aumento significativo de la dinámica mitocondrial que ayuda a mantener un conjunto de mitocondrias más funcionales en la célula. Así, en células con una alta ratio ERα/ERβ el tratamiento con E2 provocó una bajada en los niveles de las proteínas del complejo OXPHOS y un aumento de la cantidad de mitocondrias, aunque éstas tenían una menor cantidad de crestas mitocondriales, por lo que serían menos funcionales[280]. Además, la fisión mitocondrial se vio reducida sugiriendo una acumulación de mitocondrias poco funcionales en las células por una probable disminución de la mitofagia, un proceso por el cual las mitocondrias dañadas y poco funcionales son eliminadas[151,281,282]. Todos estos cambios no se observaron en aquellas células de cáncer de mama con una baja ratio ERα/ERβ, en las que el tratamiento con E2 no provocó una disminución ni de la funcionalidad ni de la fisión mitocondrial, apuntando que las mitocondrias no se vieron afectadas en estas células. Todos estos resultados obtenidos en líneas celulares de cáncer de mama con distinto ratio ERα/ERβ se confirmaron con el estudio de una línea celular de cáncer de mama con una sobreexpresión modulable del ERβ. Así, la presencia del ERβ con el tratamiento de E2 mejoró la funcionalidad mitocondrial a través del aumento en los niveles de las proteínas del complejo OXPHOS y en la actividad enzimática de la COX, disminuyendo el ratio de actividades ATPasa/COX, un indicador de funcionalidad mitocondrial[105]. Además la sobreexpresión del ERβ aumentó la fisión mitocondrial y

disminuyó la biogénesis, sugiriendo que la existencia de este receptor contribuiría a mantener un conjunto de mitocondrias más funcionales, lo que contribuiría a reducir el estrés oxidativo[151,282].

La presencia del ERβ, que contribuye a mejorar la funcionalidad mitocondrial, podría determinar la respuesta de las células de cáncer de mama a tratamientos antitumorales como el cisplatino (CDDP), el paclitaxel (PTX) y el tamoxifeno (TAM) en líneas celulares con distinta ratio ERα/ERβ. Uno de los mecanismos de acción de estos compuestos citotóxicos es el daño a las mitocondrias, aumentando el estrés oxidativo de las células cancerosas y provocando la muerte celular[222,247,253]. Así pues, se observó que los tratamientos con PTX y sobre todo con CDDP provocaron una menor viabilidad celular en aquellas líneas celulares con una mayor ratio ERα/ERβ, acompañada por una menor funcionalidad mitocondrial y un incremento sustancial de la producción de ROS. Sin embargo, en las células con una baja ratio ERα/ERβ estos cambios también se observaron pero de una forma mucho menos acusada. Estos resultados apuntan a la importancia del ERβ en la eficacia de los tratamientos antitumorales. Así, la sobreexpresión o el silenciamiento parcial del ERβ modula la respuesta al CDDP y, por tanto, la presencia del ERβ disminuiría la respuesta de las células cancerosas a este tratamiento, indicando el posible papel protector que tiene el ERβ en la mitocondria, favoreciendo la reducción en la producción de ROS[56,105,278].

La respuesta de las líneas celulares con distinta ratio ERα/ERβ al tratamiento con TAM fue bastante similar, probablemente debido a que el TAM actúa uniéndose a ambos ERs y es, además, un antagonista puro del ERβ[215]. Esto provocaría que el E2 unido al ERβ fuera desplazado por el TAM y quedaran desactivados los mecanismos de protección sobre la función mitocondrial inducidos por la unión al ERβ del E2 presente en el medio[56,105]. En este caso la presencia del ERβ únicamente determinó el tipo de respuesta de las células cancerosas al tratamiento, ya que aumenta la apoptosis o la muerte celular por autofagia. La existencia de grandes cantidades de ERβ estimularía la muerte celular por autofagia, mientras que el silenciamiento parcial del ERβ condujo a las células a un aumento de la apoptosis debido, al menos en parte, al aumento significativo en la producción de ROS, ya que la apoptosis se ha asociado a un aumento sustancial en los niveles de ROS en células cancerígenas[283].

En esta tesis se ha comprobado que la inhibición o el silenciamiento parcial de la proteína desacoplante 2 (UCP2) podría suponer una terapia adyuvante para el cáncer de mama, sobre todo en aquellas células con una alta ratio ERα/ERβ, ya que son más sensibles al estrés oxidativo generado por los tratamientos antitumorales. Así, la inhibición o el silenciamiento parcial de la UCP2 redujo la viabilidad de las células de cáncer de mama a través de un aumento de la producción de ROS, confirmando el papel que juega la UCP2 en los sistemas antioxidantes de la célula[175-177]. La combinación del tratamiento con TAM y el silenciamiento de la UCP2 provocó una mejor respuesta de las células cancerosas al tratamiento citotóxico, ya que presentaron una menor viabilidad celular debido a un aumento en la muerte celular por autofagia provocado por la subida en la producción de ROS como consecuencia del aumento en el potencial de membrana mitocondrial causado por el silenciamiento de la UCP2, evitando el reingreso de protones a la matriz mitocondrial[175]. Es importante destacar que un pequeño aumento en el potencial de membrana mitocondrial es suficiente para provocar un aumento considerable en la producción de ROS[158], por lo que una pequeña bajada de los niveles de UCP2 incrementaría sustancialmente el estrés oxidativo de la célula.

Por otra parte, la combinación del tratamiento con CDDP y el silenciamiento de la UCP2 tuvo efectos mucho menores que en el caso del tratamiento con TAM, probablemente debido a que el CDDP reduce los niveles proteicos de UCP2, algo que previamente ya observamos en células de cáncer de colon[284]. Esta disminución de los niveles de UCP2 podría ser uno de los mecanismos de acción del CDDP para inducir estrés oxidativo en las células cancerígenas.

Los resultados obtenidos en las líneas celulares concordaron con el estudio de supervivencia general y la supervivencia libre de recaída en más de 1800 pacientes de cáncer de mama. Se separaron estos pacientes según tuvieran una baja o alta expresión génica de UCP2 con el objetivo de averiguar si la presencia de la UCP2 podría ser un indicador de buena o mala prognosis en el cáncer de mama. Los análisis estadísticos indicaron que una alta expresión de UCP2 podría ser un factor de mala prognosis, sobre todo en aquellos pacientes de cáncer de mama que están siendo tratados con TAM, corroborando los resultados obtenidos en las líneas celulares.

Los efectos de la genisteína (GEN), el principal fitoestrógeno presente en la soja, en la funcionalidad y dinámica mitocondrial, así como de sus efectos sobre la eficacia de los tratamientos antitumorales fueron estudiados en líneas celulares de cáncer de mama con distinto ratio ERα/ERβ. Así pues, tanto la GEN como el E2 a concentraciones fisiológicas provocaron un incremento de la viabilidad en aquellas células con una alta ratio ERα/ERβ, ya que su unión al ERα podría estimular la proliferación celular[103,107]. Además, en estas mismas células con una alta ratio ERα/ERβ, tanto el E2 como la GEN produjeron una bajada en los niveles de apoptosis, contribuyendo a una mayor supervivencia celular. Es importante destacar que el E2 produjo una bajada en la funcionalidad mitocondrial a través de una disminución en la actividad COX[105], lo que no ocurrió con la GEN ni con la combinación de E2 y GEN, sugiriendo que la presencia de concentraciones fisiológicas de GEN en el medio de las células de cáncer de mama con una alta ratio ERα/ERβ no afectaría a la función mitocondrial, incluso con la presencia de concentraciones fisiológicas de E2. En cambio, el tratamiento con GEN sí afectó a la dinámica mitocondrial en estas células, disminuyendo la fisión mitocondrial, lo que ha sido asociado con una menor apoptosis[285,286].

En las células con una baja ratio ERα/ERβ, los tratamientos con E2 y GEN, así como su combinación no provocaron cambios en la viabilidad celular e incluso el tratamiento con GEN disminuyó la proliferación de estas células inhibiendo la fosforilación de Stat-3, un mecanismo de proliferación celular vinculado a los ERs[287,288]. Además la GEN produjo un bloqueo del ciclo celular en la fase G0/G1, algo que también observaron otros autores en células de músculo liso[289]. Es importante recalcar que en estas células con una baja ratio ERα/ERβ (T47D) el tratamiento con GEN incrementó de manera sustancial los niveles de ERβ, induciendo un sistema de retroalimentación positiva, ya que la GEN tiene una mayor afinidad por el ERβ que por el ERα[31]. Asimismo, en estas células el tratamiento con GEN provocó una mejora de la funcionalidad mitocondrial, algo que también se ha observado en neuronas después del tratamiento con GEN[277].

La GEN juega un papel clave en la respuesta a los tratamientos antitumorales en células de cáncer de mama cultivadas en presencia de concentraciones fisiológicas de E2 en función de la ratio ERα/ERβ. Se observó que en la línea celular con una alta ratio

ERα/ERβ la GEN produjo una peor respuesta de las células a tratamientos anticancerígenos como el CDDP, el PTX o el TAM. La combinación de la GEN con el CDDP o el TAM produjo una bajada significativa de la producción de ROS y, con ello, una menor muerte celular. Esto podría ser debido a un desplazamiento del E2 unido al ERα por parte de la GEN o a la unión de ésta al poco ERβ presente en estas células mejorando la funcionalidad mitocondrial y, con ello, reduciendo la producción de ROS[277,278].

No obstante, en la línea celular de cáncer de mama con una baja ratio ERα/ERβ la GEN prácticamente no influyó en los tratamientos con CDDP y PTX, probablemente debido a que el E2 presente en el medio está estimulando los efectos protectores del ERβ contra la producción de ROS[56], por lo que la adición de la GEN no tendría prácticamente ninguna consecuencia. Además, la sobreexpresión del ERβ en las MCF-7 revirtió, en gran parte, los efectos protectores de la GEN, sugiriendo que el E2 presente en el medio de cultivo estaría estimulando al ERβ y, por tanto, dichos efectos protectores ya estarían siendo inducidos en estas células.

De hecho, la GEN incluso incrementó la respuesta de estas células con una baja ratio ERα/ERβ al tratamiento con TAM debido a un aumento en la apoptosis y en la autofagia, lo que se correspondería con los resultados obtenidos recientemente por Ruddy y colaboradores que observaron que la apoptosis y la autofagia están aumentadas en aquellas células tratadas con TAM en combinación con un agonista del ERβ[290].

En su conjunto, los resultados obtenidos en esta tesis confirman la importancia de la ratio ERα/ERβ en el mantenimiento de la homeostasis mitocondrial, en concreto el papel crucial que juega el ERβ en el equilibrio entre biogénesis, funcionalidad y dinámica mitocondrial, ayudando a mantener un conjunto de mitocondrias funcionales. Además la presencia del ERβ influye sobre la acción de los tratamientos citotótoxicos disminuyendo su eficacia en el caso del cisplatino y paclitaxel mediante una disminución sustancial de la producción de ROS y un aumento significativo de la funcionalidad mitocondrial. Sin embargo, el tratamiento con el tamoxifeno deparó resultados bastante parecidos independientemente de la dotación de receptores estrogénicos, probablemente debido al carácter antagonista de este compuesto sobre

los receptores estrogénicos, apuntando a que la presencia del ERβ no tendría consecuencias en las células tratadas con tamoxifeno.

Además, la UCP2 podría tener un papel fundamental en la eficacia de los tratamientos antitumorales ya que actuaría disminuyendo la producción de ROS y, por tanto, aumentando la supervivencia celular. Así pues, la inhibición o el silenciamiento de la UCP2 podrían mejorar la efectividad de los tratamientos contra el cáncer aumentando la muerte celular por autofagia de las células tumorales.

La genisteína promovería la supervivencia celular en aquellas células con una alta ratio ERα/ERβ, disminuyendo la funcionalidad y la dinámica mitocondrial. Adicionalmente, en estas mismas células la genisteína, en presencia de concentraciones fisiológicas de 17β-estradiol, podría disminuir la eficacia de los tratamientos citotóxicos favoreciendo la supervivencia celular, disminuyendo la producción de ROS. Sin embargo, la genisteína promovería una mejor funcionalidad mitocondrial en las células de cáncer de mama con predominancia de ERβ. Además el tratamiento con genisteína, también en presencia de concentraciones fisiológicas de 17β-estradiol, prácticamente no tiene impacto alguno en los tratamientos antitumorales en estas células; e incluso la genisteína podría mejorar la eficiencia del tamoxifeno aumentando la muerte celular.

This thesis has deepened the importance of ERα/ERβ ratio in the mitochondrial functionality and dynamics, and oxidative stress in breast cancer. Additionally, it has been studied how this ERα/ERβ ratio affects the response of cancer cells to anticancer drugs such as cisplatin, paclitaxel or tamoxifen, as well as the silencing of UCP2. Finally, it has analyzed the effects of the genistein, a phytoestrogen present in soybeans, on the mitochondrial functionality and on the response to anticancer treatments depending on the ERα/ERβ ratio.

The presence of ERβ in breast cancer cell lines provoked a better mitochondrial functionality after E2 treatment at physiological concentration, accompanied by a significant increment in the mitochondrial dynamics which allows the cell to maintain a more functional pool of mitochondria. Thus, in cells with a high ERα/ERβ ratio the E2 treatment produced a decrease in the levels of OXPHOS complex proteins and an increase in the number of mitochondria, although with a lower amount of mitochondrial cristae, so they would be less functional[280]. Moreover, mitochondrial fission was reduced suggesting an accumulation of dysfunctional mitochondria in cells probably due to a diminishment of the mitophagy, a process by which damaged and dysfunctional mitochondria are removed[151,281,282]. All these changes were not observed in those breast cancer cells with a low ERα/ERβ ratio, where E2 treatment did not provoke a decrease neither in the mitochondrial functionality nor fission, pointing out that mitochondria were not affected by the E2 treatment in these cells. Results obtained in breast cancer cell lines with different ERα/ERβ ratio were confirmed by the study of a breast cancer cell line with an inducible ERβ overexpression. Thus, the presence and the stimulation of the ERβ by E2 treatment improved the mitochondrial functionality through the increase of the OXPHOS complexes protein levels and the enzymatic activity of the COX, decreasing the ratio of the activities ATPase/COX, an indicator of mitochondrial functionality[105]. Furthermore, the overexpression of the ERβ augmented the mitochondrial fission and decreased the mitochondrial biogenesis, suggesting that the existence of this receptor may contribute to maintain a more functional pool of mitochondria, with lesser oxidative stress[151,282].

The expression of ERβ, which contributes to improve the mitochondrial functionality, may determine the response of breast cancer cells to anticancer treatments such as cisplatin (CDDP), paclitaxel (PTX) and tamoxifen (TAM) in cell lines with different ERα/ERβ ratio. One of the mechanisms of action of these cytotoxic compounds is the damage to the mitochondria, increasing the oxidative stress in cancer cells causing cell death[222,247,253]. Therefore, PTX and especially CDDP treatments provoked a lesser cell viability in those cell lines with the highest ERα/ERβ ratio, accompanied by a lesser mitochondrial functionality and a substantial increase in ROS production. However, in the cells with a low ERα/ERβ ratio these changes were also observed, but in a much less pronounced way. All these results point to the importance of the ERβ in the efficacy of the anticancer treatments. In that sense, the overexpression or the partial silencing of ERβ modulate the response of cancer cells to the CDDP and, therefore, the presence of the ERβ might diminish the response of cancer cells to this treatment, indicating a possible protector role of ERβ in the mitochondrion, favoring the reduction of ROS production[56,105,278].

The response of the cell lines with different ERα/ERβ ratio to the TAM treatment was quiet similar, probably because the TAM acts binding both ERs and TAM is also a pure antagonist of the ERβ[215]. This situation may provoke that the E2 (present in the culture medium) bound to the ERβ would be displaced by the TAM and the protective mechanisms on the mitochondrial function induced by the interaction of the ERβ with the E2 would be deactivated[56,105]. In this case, the presence of the ERβ only determined the type of the response of cancer cells to the TAM treatment, since the apoptosis or the autophagic cell death is increased. The existence of great amounts of ERβ would stimulate the autophagic cell death, while the partial silencing of the ERβ led the cells to an increase of the apoptosis due, at least in part, to the significant increment in ROS production, since apoptosis has been associated with a substantial raise in the ROS levels in cancer cells[283].

In this thesis it has been shown that the inhibition or the partial silencing of the uncoupling protein 2 (UCP2) could be an adjuvant therapy for breast cancer, especially in those cells with a high ERα/ERβ ratio, since these cells are more sensitive to the oxidative stress generated by the anticancer treatments. Thus, the UCP2 inhibition or

partial silencing reduced the cell viability of breast cancer cells through an increment in ROS production, confirming the role of the UCP2 in the antioxidant systems of the cell[175-177]. The combination of the TAM treatment and the UCP2 silencing provoked a better response of cancer cells to the cytotoxic treatment, since these cells presented a lesser cell viability due to an increase in the autophagic cell death triggered by the raise in the ROS production as a consequence of the increment in the mitochondrial membrane potential caused by the UCP2 silencing, avoiding the re-entry of the protons to the mitochondrial matrix[175]. It is important to note that a small increment in the mitochondrial membrane potential is sufficient to cause a significant increase in the ROS production[158], so a small drop in the UCP2 levels would substantially increase the oxidative stress in the cell.

On the other hand, the effects of the combination of CDDP treatment and UCP2 silencing were smaller than in the case of the TAM treatment, probably because the CDDP reduces the protein levels of UCP2, previously noted in colon cancer cells[284]. This diminishment of the protein levels of UCP2 could be one of the mechanisms of action of CDDP to induce oxidative stress in cancer cells.

Results obtained in cell lines agree with the study of overall survival and relapse-free survival in more than 1800 breast cancer patients. Patients were split depending on the gene expression of UCP2 (high or low expression) in order to determine whether de presence of UCP2 could be an indicator of better or poorer prognosis in breast cancer. Statistical analysis indicated that a high UCP2 expression could be a poorer prognosis factor, especially in those breast cancer patients who are being treated with TAM, corroborating the results obtained in the cell lines.

The effects of the genistein (GEN), the major phytoestrogen present in soybeans, on the mitochondrial functionality and dynamics, as well as the effects of this compound on the efficacy of the anticancer treatments, were studied in breast cancer cell lines with different ERα/ERβ ratio. Thus, both GEN and E2 at physiological concentrations triggered an increment in the cell viability in those cells with a high ERα/ERβ ratio, since the binding of these compounds to the ERα may stimulate the cell proliferation[103,107]. Moreover, both E2 and GEN treatments provoked a drop in the apoptosis levels, contributing to a greater survival in these cells with a high ERα/ERβ

ratio. It is important to highlight that E2 produced a drop in the mitochondrial functionality through a decrease in the COX activity[105], and this did not occur neither with the GEN nor with the combination of E2 and GEN treatments, suggesting that the presence of physiological concentrations of GEN in the medium of the breast cancer cells with a high ERα/ERβ ratio would not affect the mitochondrial function even though the presence of physiological concentrations of E2. However, GEN treatment did affect the mitochondrial dynamics in these cells with a high ERα/ERβ ratio, decreasing the mitochondrial fission, what has been associated with a lower apoptosis[285,286].

In those cells with a low ERα/ERβ ratio, E2 and GEN treatments, as well as their combination did not provoke any change in cell viability and even GEN treatment decreased cell proliferation inhibiting the Stat-3 phosphorylation, a cell proliferation mechanisms linked to ERs[287,288]. Moreover, GEN also triggered a cell cycle arrest in G0/G1 phase, situation also observed by other authors in smooth muscle cells[289]. It is important to note that in those cells with a low ERα/ERβ ratio (T47D) GEN treatment increased significantly the ERβ protein levels, inducing a positive feedback, since GEN has a greater affinity for ERβ than for ERα[31]. Furthermore, in these cells GEN treatment provoked a better mitochondrial functionality, what has also been observed in neurons after GEN treatment[277].

GEN plays an important role in the response to the anticancer treatments in breast cancer cells cultured with physiological concentrations of E2 depending on the ERα/ERβ ratio. In the cell line with a high ERα/ERβ ratio, GEN produced a worse response of these cells to the cytotoxic treatments such as CDDP, PTX or TAM. The combination of the GEN with CDDP or TAM triggered a significant drop in the ROS production and, therefore, a lesser cell death. This could be due to the displacement of the E2 bound to the ERα by the GEN or could be due as well to the binding of GEN to the small amounts of ERβ present in these cells improving the mitochondrial functionality and, consequently, reducing the ROS production[277,278].

Nevertheless, in the breast cancer cell line with a low ERα/ERβ ratio GEN treatment practically did not influence the CDDP and PTX treatments, probably due to the E2 present in the culture medium stimulating the protective effects of the ERβ

against the ROS production[56], so the addition of GEN would have virtually no consequences. In addition, the overexpression of the ERβ reverted to a great extent the protective effects of the GEN, suggesting that the E2 of the culture medium would be stimulating the ERβ and, therefore, such protective effects would already be induced in these cells.

Actually, GEN treatment even increased the response of these cells with a low ERα/ERβ ratio to the TAM treatment because of an increase in the apoptosis and autophagy, accordingly to the results obtained recently by Ruddy *et al.* who observed that apoptosis and autophagy are increased in those cells treated with TAM in combination with an ERβ agonist[290].

Overall, the results obtained in this thesis confirm the importance of the ERα/ERβ ratio in the maintenance of the mitochondrial homeostasis, in particular the crucial role that ERβ plays in the balance between mitochondrial biogenesis, functionality and dynamics, contributing to maintain a functional pool of mitochondria. Moreover, the presence of ERβ influences the action of the cytotoxic agents diminishing their efficacy in the case of the cisplatin and paclitaxel treatments through a substantial decrease in the ROS production and a significant increase in the mitochondrial functionality. However, tamoxifen treatment caused very similar results independently of the estrogen receptors endowment, probably due to the antagonism of this compound over the estrogen receptors, pointing that the presence of ERβ would not have consequences in those cells treated with tamoxifen.

Furthermore, the UCP2 may have an important role in the efficacy of the anticancer treatments given that it would act decreasing the ROS production and, consequently, increasing the cell survival. Thus, the inhibition or the silencing of the UCP2 may improve the effectiveness of the anticancer treatments mainly increasing the autophagic cell death of the cancer cells.

The genistein treatment would promote the cell survival in those cells with a high ERα/ERβ ratio, diminishing the mitochondrial functionality and dynamics. Additionally, in these cells the genistein treatment, in the presence of physiological concentrations of 17β-estradiol, could decrease the efficacy of the cytotoxic treatments, favoring the cell survival, reducing the ROS production. However, the genistein treatment would

promote a better mitochondrial functionality in those breast cancer cells with predominant levels of ERβ. Moreover, the genistein treatment, again in the presence of physiological concentrations of 17β-estradiol, practically does not have any impact on the anticancer treatments in these cells; and genistein could even improve the effectiveness of the tamoxifen increasing cell death.

5. Conclusiones / Conclusions

1. La estimulación del ERβ ejerce un papel protector en las mitocondrias regulando los procesos de biogénesis y dinámica mitocondrial, mejorando la funcionalidad de las mitocondrias, lo que contribuye a que la célula tenga un conjunto de mitocondrias más funcionales disminuyendo con ello la producción de ROS.

2. La presencia del ERβ en líneas celulares de cáncer de mama reduce la respuesta de las células al tratamiento con paclitaxel y sobre todo cisplatino debido, al menos en parte, al descenso del estrés oxidativo que implica una reducción de la muerte celular por apoptosis y autofagia. Sin embargo, en las células de cáncer de mama tratadas con tamoxifeno, niveles bajos de ERβ (alta ratio ERα/ERβ) incrementan la apoptosis y elevados niveles de ERβ (baja ratio ERα/ERβ) aumentan la muerte celular por autofagia.

3. La inhibición o silenciamiento parcial de la UCP2 incrementa la eficacia de los tratamientos antitumorales como el cisplatino y especialmente el tamoxifeno a causa del incremento en la producción de ROS y a un aumento significativo de la muerte celular por autofagia. Además, los pacientes de cáncer de mama con elevados niveles de expresión de UCP2 tienen una peor prognosis, especialmente aquellos pacientes tratados con tamoxifeno.

4. El fitoestrógeno genisteína afecta a las líneas celulares de cáncer de mama de forma distinta en función de la ratio ERα/ERβ. En células con una alta ratio ERα/ERβ, el tratamiento con concentraciones fisiológicas de genisteína estimula la proliferación celular y disminuye la apoptosis, disminuyendo también la dinámica mitocondrial. En cambio, la genisteína tiene un papel citostático en las células con una baja ratio ERα/ERβ, en las que se produce un incremento de la funcionalidad mitocondrial.

5. En líneas celulares de cáncer de mama con una alta ratio ERα/ERβ, el tratamiento con genisteína reduce la respuesta de las células cancerosas a los tratamientos antitumorales como el cisplatino, el paclitaxel o el tamoxifeno, debido principalmente a la bajada en la producción de ROS. La consecuencia es una bajada de la apoptosis o de la muerte celular por autofagia con respecto al tratamiento únicamente con el citotóxico, incrementándose la viabilidad de las células cancerosas.

6. En las células con una baja ratio ERα/ERβ el tratamiento con genisteína no tiene impacto sobre la eficacia de los tratamientos antitumorales cisplatino y paclitaxel. El tratamiento con genisteína incluso aumenta la eficiencia del tamoxifeno incrementando la muerte celular por autofagia y la apoptosis.

7. La evaluación clínica rutinaria de la expresión de los niveles de ERβ y UCP2 en tumores en pacientes de cáncer de mama, así como la evaluación del consumo de fitoestrógenos, en concreto de genisteína, en dichos pacientes podría contribuir a un mejor diseño de las estrategias de tratamiento contra el cáncer de mama.

1. The stimulation of ERβ has a protective role in the mitochondria modulating the mitochondrial biogenesis and dynamics processes, improving the mitochondrial functionality, allowing the cell to have a more functional pool of mitochondria and thereby reducing the ROS production.

2. The presence of the ERβ in breast cancer cell lines reduces the response of these cells to the treatment with paclitaxel and especially cisplatin due, at least in part, to the decrease in the oxidative stress which implies a reduction in the apoptosis and autophagic cell death. However, in breast cancer cells treated with tamoxifen, low ERβ levels (high ERα/ERβ ratio) increase the apoptosis and high levels of ERβ (low ERα/ERβ ratio) raise the autophagic cell death.

3. The inhibition or the partial silencing of the UCP2 increases the efficacy of the anticancer treatments such as cisplatin and especially tamoxifen because of the increment in the ROS production and a significant raise in the autophagic cell death. Moreover, breast cancer patients with high expression levels of UCP2 have a poorer prognosis, especially those patients treated with tamoxifen.

4. The phytoestrogen genistein affects breast cancer cell lines in a different way depending on the ERα/ERβ ratio. In cells with a high ERα/ERβ ratio, the treatment with physiological concentrations of genistein stimulates the cell proliferation and decreases the apoptosis, as well as diminishes the mitochondrial dynamics. On the other hand, the genistein has a cytostatic role in those cells with a low ERα/ERβ ratio, where there is a raise in the mitochondrial functionality.

5. In breast cancer cell lines with a high ERα/ERβ ratio, the genistein treatment reduces the response of the cancer cells to anticancer treatments such as cisplatin, paclitaxel or tamoxifen, mainly due to the drop in the ROS production. The consequence is the decrease in the apoptosis or in the autophagic cell death regarding to the treatment with the cytotoxic agent alone, increasing the cancer cells viability.

6. In those cells with a low ERα/ERβ ratio the genistein treatment has no impact on the effectiveness of the anticancer drugs cisplatin and paclitaxel. The genistein treatment even increases the efficacy of the tamoxifen treatment increasing the autophagic cell death and the apoptosis.

7. Routine clinical assessment of the ERβ and UCP2 expression levels in breast cancer patients, as well as the evaluation of the phytoestrogen consumption, specifically genistein, may contribute to a better design of the breast cancer treatment strategies.

6. Bibliografía / References

1 Hanahan, D. & Weinberg, R. A. *Cell* **100**, 57-70.

2 Hanahan, D. & Weinberg, Robert A. *Cell* **144**, 646-674.

3 Druesne-Pecollo, N., Touvier, M., Barrandon, E., Chan, D. S., Norat, T. *et al. Breast cancer research and treatment* **135**, 647-654 (2012).

4 Youlden, D. R., Baade, P. D., Valery, P. C., Ward, L. J., Green, A. C. *et al. Cancer epidemiology* **36**, 476-480 (2012).

5 Tao, Z., Shi, A., Lu, C., Song, T., Zhang, Z. *et al. Cell biochemistry and biophysics* (2014).

6 Hortobagyi, G. N., de la Garza Salazar, J., Pritchard, K., Amadori, D., Haidinger, R. *et al. Clinical breast cancer* **6**, 391-401 (2005).

7 Gomez-Raposo, C., Zambrana Tevar, F., Sereno Moyano, M., Lopez Gomez, M. & Casado, E. *Cancer treatment reviews* **36**, 451-457 (2010).

8 Miao, H., Verkooijen, H. M., Chia, K.-S., Bouchardy, C., Pukkala, E. *et al. Journal of Clinical Oncology* (2011).

9 Weiss, J. R., Moysich, K. B. & Swede, H. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **14**, 20-26 (2005).

10 McCormack, V. A. & Boffetta, P. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO* **22**, 2349-2357 (2011).

11 James, R. E., Lukanova, A., Dossus, L., Becker, S., Rinaldi, S. *et al. Cancer prevention research* **4**, 1626-1635 (2011).

12 Jatoi, I. & Anderson, W. F. *Future oncology* **6**, 1781-1788 (2010).

13 Opdahl, S., Alsaker, M. D., Janszky, I., Romundstad, P. R. & Vatten, L. J. *British journal of cancer* **105**, 731-736 (2011).

14 Palmer, J. R., Boggs, D. A., Wise, L. A., Ambrosone, C. B., Adams-Campbell, L. L. *et al. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **20**, 1883-1891 (2011).

15 Ahmad, A. *Breast Cancer Metastasis and Drug Resistance: Progress and Prospects*. (Springer New York, 2012).

16 Reeves, G. K., Beral, V., Green, J., Gathani, T., Bull, D. *et al. The Lancet. Oncology* **7**, 910-918 (2006).

17 Cleary, M. P. & Grossmann, M. E. *Endocrinology* **150**, 2537-2542 (2009).

18 Newman, G. & Gonzalez-Perez, R. R. *Molecular and cellular endocrinology* **382**, 570-582 (2014).

19 Paz-Filho, G., Lim, E. L., Wong, M. L. & Licinio, J. *Frontiers in bioscience* **16**, 1634-1650 (2011).

20 Verma, R., Bowen, R. L., Slater, S. E., Mihaimeed, F. & Jones, J. L. *British medical bulletin* **103**, 129-145 (2012).

21 Dong, J. Y. & Qin, L. Q. *Breast cancer research and treatment* **125**, 315-323 (2011).

22 Pan, S. Y., Zhou, J., Gibbons, L., Morrison, H., Wen, S. W. *et al. BMC cancer* **11**, 372 (2011).

23 Chen, W. Y., Rosner, B., Hankinson, S. E., Colditz, G. A. & Willett, W. C. *Jama* **306**, 1884-1890 (2011).

24 Friedenreich, C. M., Neilson, H. K. & Lynch, B. M. *European journal of cancer* **46**, 2593-2604 (2010).

25 Newman, B., Millikan, R. C. & King, M. C. *Epidemiologic reviews* **19**, 69-79 (1997).

26 Mavaddat, N., Antoniou, A. C., Easton, D. F. & Garcia-Closas, M. *Molecular oncology* **4**, 174-191 (2010).

27 Jensen, E. V., Jacobson, H. I., Smith, S., Jungblut, P. W. & De Sombre, E. R. *Gynecologic investigation* **3**, 108-123 (1972).

28 Kuiper, G. G., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J. A. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 5925-5930 (1996).

29 Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G. *et al. Physiol Rev* **87**, 905-931 (2007).

30 Katzenellenbogen, B. S. *Biol Reprod* **54**, 287-293 (1996).

31 Kuiper, G. G., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J. *et al. Endocrinology* **138**, 863-870 (1997).

32 Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H. *et al. Endocrinology* **139**, 4252-4263 (1998).

33 Morani, A., Warner, M. & Gustafsson, J. A. *Journal of internal medicine* **264**, 128-142 (2008).

34 Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M. *et al. Proceedings of the National Academy of Sciences of the United States of America* **97**, 337-342 (2000).

35 Santen, R. J., Brodie, H., Simpson, E. R., Siiteri, P. K. & Brodie, A. *Endocr Rev* **30**, 343-375 (2009).

36 Saville, B., Wormke, M., Wang, F., Nguyen, T., Enmark, E. *et al. J Biol Chem* **275**, 5379-5387 (2000).

37 Warner, M. & Gustafsson, J. A. *Biochem Biophys Res Commun* **396**, 63-66 (2010).

38 Zhao, C., Dahlman-Wright, K. & Gustafsson, J. A. *J Biol Chem* **285**, 39575-39579 (2010).

39 Smith, C. L., Nawaz, Z. & O'Malley, B. W. *Mol Endocrinol* **11**, 657-666 (1997).

40 Webb, P., Nguyen, P. & Kushner, P. J. *J Biol Chem* **278**, 6912-6920 (2003).

41 Cheng, G., Weihua, Z., Warner, M. & Gustafsson, J. A. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 3739-3746 (2004).

42 Clarke, R. B. *Climacteric* **7**, 129-137 (2004).

43 Strom, A., Hartman, J., Foster, J. S., Kietz, S., Wimalasena, J. *et al. Proceedings of the National Academy of Sciences of the United States of America* **101**, 1566-1571 (2004).

44 Sugiyama, N., Barros, R. P., Warner, M. & Gustafsson, J. A. *Trends Endocrinol Metab* **21**, 545-552 (2010).

45 Chang, E. C., Charn, T. H., Park, S. H., Helferich, W. G., Komm, B. *et al. Mol Endocrinol* **22**, 1032-1043 (2008).

46 Jensen, E. V., Jacobson, H. I., Walf, A. A. & Frye, C. A. *Physiol Behav* **99**, 151-162 (2010).

47 Pearce, S. T. & Jordan, V. C. *Crit Rev Oncol Hematol* **50**, 3-22 (2004).

48 Drummond, A. E. & Fuller, P. J. *The Journal of endocrinology* **205**, 15-23 (2010).

49 Giguere, V., Hollenberg, S. M., Rosenfeld, M. G. & Evans, R. M. *Cell* **46**, 645-652 (1986).

50 Kumar, V., Green, S., Stack, G., Berry, M., Jin, J. R. *et al. Cell* **51**, 941-951 (1987).

51 Leung, Y. K., Lee, M. T., Lam, H. M., Tarapore, P. & Ho, S. M. *Steroids* **77**, 727-737 (2012).

52 Nilsson, S., Makela, S., Treuter, E., Tujague, M., Thomsen, J. *et al. Physiol Rev* **81**, 1535-1565 (2001).

53 Gonzalez, D. H., Bonnard, G. & Grienenberger, J. M. *Curr Genet* **24**, 248-255 (1993).

54 Kushner, P. J., Agard, D. A., Greene, G. L., Scanlan, T. S., Shiau, A. K. *et al. The Journal of steroid biochemistry and molecular biology* **74**, 311-317 (2000).

55 Russo, J., Ao, X., Grill, C. & Russo, I. H. *Breast cancer research and treatment* **53**, 217-227 (1999).

56 Nadal-Serrano, M., Sastre-Serra, J., Pons, D. G., Miro, A. M., Oliver, J. *et al. Journal of cellular biochemistry* **113**, 3178-3185 (2012).

57 Bardin, A., Boulle, N., Lazennec, G., Vignon, F. & Pujol, P. *Endocrine-related cancer* **11**, 537-551 (2004).

58 Hartman, J., Strom, A. & Gustafsson, J. A. *Steroids* **74**, 635-641 (2009).

59 Roger, P., Sahla, M. E., Makela, S., Gustafsson, J. A., Baldet, P. *et al. Cancer research* **61**, 2537-2541 (2001).

60 Skliris, G. P., Munot, K., Bell, S. M., Carder, P. J., Lane, S. *et al. The Journal of pathology* **201**, 213-220 (2003).

61 Fox, E. M., Davis, R. J. & Shupnik, M. A. *Steroids* **73**, 1039-1051 (2008).

62 Novelli, F., Milella, M., Melucci, E., Di Benedetto, A., Sperduti, I. *et al. Breast cancer research : BCR* **10**, R74 (2008).

63 Gruvberger-Saal, S. K., Bendahl, P. O., Saal, L. H., Laakso, M., Hegardt, C. *et al. Clinical cancer research : an official journal of the American Association for Cancer Research* **13**, 1987-1994 (2007).

64 Murphy, L. C. & Watson, P. H. *Endocrine-related cancer* **13**, 327-334 (2006).

65 Skliris, G. P., Leygue, E., Curtis-Snell, L., Watson, P. H. & Murphy, L. C. *British journal of cancer* **95**, 616-626 (2006).

66 Skliris, G. P., Leygue, E., Watson, P. H. & Murphy, L. C. *The Journal of steroid biochemistry and molecular biology* **109**, 1-10 (2008).

67 Adam, A. C., Bornhovd, C., Prokisch, H., Neupert, W. & Hell, K. *The EMBO journal* **25**, 174-183 (2006).

68 Garcia-Roves, P., Huss, J. M., Han, D. H., Hancock, C. R., Iglesias-Gutierrez, E. *et al. Proceedings of the National Academy of Sciences of the United States of America* **104**, 10709-10713 (2007).

69 Stossi, F., Barnett, D. H., Frasor, J., Komm, B., Lyttle, C. R. *et al. Endocrinology* **145**, 3473-3486 (2004).

70 Power, K. A. & Thompson, L. U. *Breast cancer research and treatment* **81**, 209-221 (2003).

71 Quaedackers, M. E., Van Den Brink, C. E., Wissink, S., Schreurs, R. H., Gustafsson, J. A. *et al. Endocrinology* **142**, 1156-1166 (2001).

72 Farnsworth, N. R., Bingel, A. S., Cordell, G. A., Crane, F. A. & Fong, H. S. *J Pharm Sci* **64**, 717-754 (1975).

73 Kurzer, M. S. & Xu, X. *Annu Rev Nutr* **17**, 353-381 (1997).

74 Adlercreutz, H. *The Lancet. Oncology* **3**, 364-373 (2002).

75 Cotter, A. & Cashman, K. D. *Nutr Rev* **61**, 346-351 (2003).

76 Dixon, R. A. *Annu Rev Plant Biol* **55**, 225-261 (2004).

77 Mense, S. M., Hei, T. K., Ganju, R. K. & Bhat, H. K. *Environ Health Perspect* **116**, 426-433 (2008).

78 Messina, M. J. & Loprinzi, C. L. *J Nutr* **131**, 3095S-3108S (2001).

79 Orlando, L., Schiavone, P. & Cinieri, S. *Cancer Biol Ther* **11**, 918-920 (2011).

80 van der Schouw, Y. T., de Kleijn, M. J., Peeters, P. H. & Grobbee, D. E. *Nutr Metab Cardiovasc Dis* **10**, 154-167 (2000).

81 Anderson, J. J., Anthony, M. S., Cline, J. M., Washburn, S. A. & Garner, S. C. *Public Health Nutr* **2**, 489-504 (1999).

82 Vincent, A. & Fitzpatrick, L. A. *Mayo Clin Proc* **75**, 1174-1184 (2000).

83 Middleton, E., Jr., Kandaswami, C. & Theoharides, T. C. *Pharmacol Rev* **52**, 673-751 (2000).

84 Messina, M. & Wu, A. H. *Am J Clin Nutr* **89**, 1673S-1679S (2009).

85 Shu, X. O., Jin, F., Dai, Q., Wen, W., Potter, J. D. *et al. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology* **10**, 483-488 (2001).

86 Messina, M. & Hilakivi-Clarke, L. *Nutr Cancer* **61**, 792-798 (2009).

87 Setchell, K. D., Brown, N. M., Zhao, X., Lindley, S. L., Heubi, J. E. *et al. Am J Clin Nutr* **94**, 1284-1294 (2011).

88 Perkin, A. G. & Newbury, F. G. *J Chem Soc* **75**, 830-839 (1899).

89 Adlercreutz, H. *Environ Health Perspect* **103 Suppl 7**, 103-112 (1995).

90 Marini, H., Minutoli, L., Polito, F., Bitto, A., Altavilla, D. *et al. Ann Intern Med* **146**, 839-847 (2007).

91 Si, H. & Liu, D. *Curr Med Chem* **14**, 2581-2589 (2007).

92 Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S. *et al. J Biol Chem* **262**, 5592-5595 (1987).

93 Banerjee, S., Li, Y., Wang, Z. & Sarkar, F. H. *Cancer Lett* **269**, 226-242 (2008).

94 Ruiz-Larrea, M. B., Mohan, A. R., Paganga, G., Miller, N. J., Bolwell, G. P. *et al. Free Radic Res* **26**, 63-70 (1997).

95 Ouyang, G., Yao, L., Ruan, K., Song, G., Mao, Y. *et al. Cell Biol Int* **33**, 1237-1244 (2009).

96 Peng, J. H., Zhu, J. D., Mi, M. T., Li, F. J., Cai, L. *et al. Eur J Cancer Prev* **19**, 110-119 (2010).

97 Pavese, J. M., Farmer, R. L. & Bergan, R. C. *Cancer Metastasis Rev* **29**, 465-482 (2010).

98 Adlercreutz, H. *Scand J Clin Lab Invest Suppl* **201**, 3-23 (1990).

99 Matsumura, A., Ghosh, A., Pope, G. S. & Darbre, P. D. *The Journal of steroid biochemistry and molecular biology* **94**, 431-443 (2005).

100 An, J., Tzagarakis-Foster, C., Scharschmidt, T. C., Lomri, N. & Leitman, D. C. *J Biol Chem* **276**, 17808-17814 (2001).

101 Kostelac, D., Rechkemmer, G. & Briviba, K. *J Agric Food Chem* **51**, 7632-7635 (2003).

102 Messina, M., McCaskill-Stevens, W. & Lampe, J. W. *J Natl Cancer Inst* **98**, 1275-1284 (2006).

103 Hsieh, C. Y., Santell, R. C., Haslam, S. Z. & Helferich, W. G. *Cancer research* **58**, 3833-3838 (1998).

104 Ju, Y. H., Allred, C. D., Allred, K. F., Karko, K. L., Doerge, D. R. *et al. J Nutr* **131**, 2957-2962 (2001).

105 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Valle, A., Oliver, J. *et al. Cell Physiol Biochem* **29**, 261-268 (2012).

106 Seo, H. S., DeNardo, D. G., Jacquot, Y., Laios, I., Vidal, D. S. *et al. Breast cancer research and treatment* **99**, 121-134 (2006).

107 Maggiolini, M., Bonofiglio, D., Marsico, S., Panno, M. L., Cenni, B. *et al. Mol Pharmacol* **60**, 595-602 (2001).

108 Mitchell, P. *Nature* **191**, 144-148 (1961).

109 Nicholls, D. G. *Bioenergetics : an introduction to the chemiosmotic theory*. (Academic Press, 1982).

110 Irwin, R. W., Yao, J., To, J., Hamilton, R. T., Cadenas, E. *et al. Journal of Neuroendocrinology* **24**, 236-248 (2012).

111 Milner, T. A., Ayoola, K., Drake, C. T., Herrick, S. P., Tabori, N. E. *et al. The Journal of Comparative Neurology* **491**, 81-95 (2005).

112 Mitterling, K. L., Spencer, J. L., Dziedzic, N., Shenoy, S., McCarthy, K. *et al. The Journal of comparative neurology* **518**, 2729-2743 (2010).

113 Stirone, C., Duckles, S. P., Krause, D. N. & Procaccio, V. *Molecular Pharmacology* **68**, 959-965 (2005).

114 Yager, J. D. & Chen, J. Q. *Trends in Endocrinology & Metabolism* **18**, 89-91.

115 Yang, S.-H., Liu, R., Perez, E. J., Wen, Y., Stevens, S. M. *et al. Proceedings of the National Academy of Sciences of the United States of America* **101**, 4130-4135 (2004).

116 Demonacos, C. V., Karayanni, N., Hatzoglou, E., Tsiriyiotis, C., Spandidos, D. A. *et al. Steroids* **61**, 226-232 (1996).

117 Chen, J. Q., Eshete, M., Alworth, W. L. & Yager, J. D. *Journal of cellular biochemistry* **93**, 358-373 (2004).

118 Brinton, R. D. *Trends in neurosciences* **31**, 529-537 (2008).

119 Brinton, R. D. *Trends in pharmacological sciences* **30**, 212-222 (2009).

120 McEwen, B., Akama, K., Alves, S., Brake, W. G., Bulloch, K. *et al. Proceedings of the National Academy of Sciences of the United States of America* **98**, 7093-7100 (2001).

121 Milner, T. A., Ayoola, K., Drake, C. T., Herrick, S. P., Tabori, N. E. *et al. J Comp Neurol* **491**, 81-95 (2005).

122 Milner, T. A., Lubbers, L. S., Alves, S. E. & McEwen, B. S. *Endocrinology* **149**, 3306-3312 (2008).

123 Milner, T. A., McEwen, B. S., Hayashi, S., Li, C. J., Reagan, L. P. *et al. J Comp Neurol* **429**, 355-371 (2001).

124 Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N. *et al. Annals of Internal Medicine* **107**, 526-545 (1987).

125 Handy, D. E. & Loscalzo, J. *Antioxidants & Redox Signaling* **16**, 1323-1367 (2011).

126 Quinlan, C. L., Treberg, J. R., Perevoshchikova, I. V., Orr, A. L. & Brand, M. D. *Free Radical Biology and Medicine* **53**, 1807-1817 (2012).

127 Sullivan, L. B. & Chandel, N. S. *Cancer & metabolism* **2**, 17 (2014).

128 Martindale, J. L. & Holbrook, N. J. *J Cell Physiol* **192**, 1-15 (2002).

129 Sena, Laura A. & Chandel, Navdeep S. *Molecular Cell* **48**, 158-167.

130 Gotoh, Y. & Cooper, J. A. *J Biol Chem* **273**, 17477-17482 (1998).

131 Madesh, M. & Hajnoczky, G. *The Journal of cell biology* **155**, 1003-1015 (2001).

132 Shen, H. M. & Liu, Z. G. *Free radical biology & medicine* **40**, 928-939 (2006).

133 Scherz-Shouval, R. & Elazar, Z. *Trends in biochemical sciences* **36**, 30-38 (2011).

134 Cho, D.-H., Nakamura, T. & Lipton, S. *Cell. Mol. Life Sci.* **67**, 3435-3447 (2010).

135 Koopman, W. J. H., Willems, P. H. G. M. & Smeitink, J. A. M. *New England Journal of Medicine* **366**, 1132-1141 (2012).

136 Nunnari, J. & Suomalainen, A. *Cell* **148**, 1145-1159.

137 Gogvadze, V., Orrenius, S. & Zhivotovsky, B. *Trends in Cell Biology* **18**, 165-173.

138 Modica-Napolitano, J. S. & Singh, K. K. *Mitochondrion* **4**, 755-762 (2004).

139 Fariss, M. W., Chan, C. B., Patel, M., Van Houten, B. & Orrenius, S. *Mol Interv* **5**, 94-111 (2005).

140 Warburg, O., Wind, F. & Negelein, E. *The Journal of general physiology* **8**, 519-530 (1927).

141 Fischer, C. P., Bode, B. P. & Souba, W. W. *Annals of surgery* **227**, 627-634; discussion 634-626 (1998).

142 Lundholm, K., Edstrom, S., Karlberg, I., Ekman, L. & Schersten, T. *Cancer* **50**, 1142-1150 (1982).

143 Mazurek, S., Boschek, C. B. & Eigenbrodt, E. *Journal of bioenergetics and biomembranes* **29**, 315-330 (1997).

144 Ockner, R. K., Kaikaus, R. M. & Bass, N. M. *Hepatology* **18**, 669-676 (1993).

145 Pedersen, P. L. & Morris, H. P. *J Biol Chem* **249**, 3327-3334 (1974).

146 Sun, A. S. & Cederbaum, A. I. *Cancer research* **40**, 4677-4681 (1980).

147 Sun, A. S., Sepkowitz, K. & Geller, S. A. *Laboratory investigation; a journal of technical methods and pathology* **44**, 13-17 (1981).

148 Palmer, C. S., Osellame, L. D., Stojanovski, D. & Ryan, M. T. *Cell Signal* **23**, 1534-1545 (2011).

149 Westermann, B. *Nature reviews. Molecular cell biology* **11**, 872-884 (2010).

150 Campello, S. & Scorrano, L. *Mitochondrial shape changes: orchestrating cell pathophysiology*. Vol. 11 (2010).

151 Youle, R. J. & van der Bliek, A. M. *Science* **337**, 1062-1065 (2012).

152 Anand, R., Langer, T. & Baker, M. J. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1833**, 195-204 (2013).

153 Baker, M. J., Tatsuta, T. & Langer, T. *Cold Spring Harbor perspectives in biology* **3** (2011).

154 Wasilewski, M. & Scorrano, L. *Trends in Endocrinology & Metabolism* **20**, 287-294.

155 Baffy, G., Derdak, Z. & Robson, S. C. *British journal of cancer* **105**, 469-474 (2011).

156 Valle, A., Oliver, J. & Roca, P. *Cancers* **2**, 567-591 (2010).

157 Echtay, K. S. *Free Radical Biology and Medicine* **43**, 1351-1371 (2007).

158 Korshunov, S. S., Skulachev, V. P. & Starkov, A. A. *FEBS Letters* **416**, 15-18.

159 Hansford, R., Hogue, B. & Mildaziene, V. *Journal of bioenergetics and biomembranes* **29**, 89-95 (1997).

160 Votyakova, T. V. & Reynolds, I. J. *Journal of Neurochemistry* **79**, 266-277 (2001).

161 Skulachev, V. P. *Bioscience reports* **17**, 347-366 (1997).

162 Nègre-Salvayre, A., Hirtz, C., Carrera, G., Cazenave, R., Troly, M. *et al. The FASEB Journal* **11**, 809-815 (1997).

163 Talbot, D. A., Lambert, A. J. & Brand, M. D. *FEBS Letters* **556**, 111-115.

164 Lin, C. S. & Klingenberg, M. *Biochemistry* **21**, 2950-2956 (1982).

165 Nedergaard, J., Bengtsson, T. & Cannon, B. *Unexpected evidence for active brown adipose tissue in adult humans*. Vol. 293 (2007).

166 Robbins, D. & Zhao, Y. *International journal of molecular sciences* **12**, 5285-5293 (2011).

167 Sayeed, A., Meng, Z., Luciani, G., Chen, L. C., Bennington, J. L. *et al. Cell death & disease* **1**, e53 (2010).

168 Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O. *et al. Nature genetics* **15**, 269-272 (1997).

169 Gimeno, R. E., Dembski, M., Weng, X., Deng, N., Shyjan, A. W. *et al. Diabetes* **46**, 900-906 (1997).

170 Stuart, J. A., Cadenas, S., Jekabsons, M. B., Roussel, D. & Brand, M. D. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* **1504**, 144-158 (2001).

171 Walder, K., Norman, R. A., Hanson, R. L., Schrauwen, P., Neverova, M. *et al. Human Molecular Genetics* **7**, 1431-1435 (1998).

172 Yamada, M., Hashida, T., Shibusawa, N., Iwasaki, T., Murakami, M. *et al. FEBS Letters* **432**, 65-69.

173 Zhou, Y.-T., Shimabukuro, M., Koyama, K., Lee, Y., Wang, M.-Y. *et al. Proceedings of the National Academy of Sciences* **94**, 6386-6390 (1997).

174 Chan, C. B., MacDonald, P. E., Saleh, M. C., Johns, D. C., Marbàn, E. *et al. Diabetes* **48**, 1482-1486 (1999).

175 Arsenijevic, D., Onuma, H., Pecqueur, C., Raimbault, S., Manning, B. S. *et al. Nature genetics* **26**, 435-439 (2000).

176 Echtay, K. S., Murphy, M. P., Smith, R. A. J., Talbot, D. A. & Brand, M. D. *Journal of Biological Chemistry* **277**, 47129-47135 (2002).

177 Krauss, S., Zhang, C.-Y., Scorrano, L., Dalgaard, L. T., St-Pierre, J. *et al. Journal of Clinical Investigation* **112**, 1831-1842 (2003).

178 Andrews, Z. B. & Horvath, T. L. *Uncoupling protein-2 regulates lifespan in mice*. Vol. 296 (2009).

179 Lebovitz, R. M., Zhang, H., Vogel, H., Cartwright, J., Dionne, L. *et al. Proceedings of the National Academy of Sciences* **93**, 9782-9787 (1996).

180 McDonald, R. B., Walker, K. M., Warman, D. B., Griffey, S. M., Warden, C. H. *et al. Experimental Gerontology* **43**, 1061-1068 (2008).

181 Horimoto, M., Fülöp, P., Derdák, Z., Wands, J. R. & Baffy, G. *Hepatology* **39**, 386-392 (2004).

182 Zhang, C.-Y., Baffy, G., Perret, P., Krauss, S., Peroni, O. *et al. Cell* **105**, 745-755.

183 Derdák, Z., Fülöp, P., Sabo, E., Tavares, R., Berthiaume, E. P. *et al. Carcinogenesis* **27**, 956-961 (2006).

184 Sastre-Serra, J., Valle, A., Company, M. M., Garau, I., Oliver, J. *et al. Free Radical Biology and Medicine* **48**, 506-512 (2010).

185 Derdak, Z., Mark, N. M., Beldi, G., Robson, S. C., Wands, J. R. *et al. Cancer research* **68**, 2813-2819 (2008).

186 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Valle, A., Garau, I. *et al. Free radical biology & medicine* **61C**, 11-17 (2013).

187 Nogueira, V. & Hay, N. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 4309-4314 (2013).

188 Sainz, R. M., Lombo, F. & Mayo, J. C. *Cancers* **4**, 442-474 (2012).

189 Dalla Pozza, E., Fiorini, C., Dando, I., Menegazzi, M., Sgarbossa, A. *et al. Biochimica et biophysica acta* **1823**, 1856-1863 (2012).

190 Mailloux, R. J., Adjeitey, C. N. & Harper, M. E. *PloS one* **5**, e13289 (2010).

191 Hockenbery, D., Nunez, G., Milliman, C., Schreiber, R. D. & Korsmeyer, S. J. *Nature* **348**, 334-336 (1990).

192 Wang, C. W. & Klionsky, D. J. *Molecular medicine (Cambridge, Mass.)* **9**, 65-76 (2003).

193 Kerr, J. F., Winterford, C. M. & Harmon, B. V. *Cancer* **73**, 2013-2026 (1994).

194 Satoh, M. S. & Lindahl, T. *Nature* **356**, 356-358 (1992).

195 Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K. *et al. Nature* **376**, 37-43 (1995).

196 Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z. *et al. Cell* **81**, 801-809 (1995).

197 Oliver, F. J., de la Rubia, G., Rolli, V., Ruiz-Ruiz, M. C., de Murcia, G. *et al. J Biol Chem* **273**, 33533-33539 (1998).

198 Akar, U., Chaves-Reyez, A., Barria, M., Tari, A., Sanguino, A. *et al. Autophagy* **4**, 669-679 (2008).

199 Cook, K. L., Shajahan, A. N. & Clarke, R. *Expert review of anticancer therapy* **11**, 1283-1294 (2011).

200 Kondo, Y., Kanzawa, T., Sawaya, R. & Kondo, S. *Nature reviews. Cancer* **5**, 726-734 (2005).

201 Edinger, A. L. & Thompson, C. B. *Cancer cell* **4**, 422-424 (2003).

202 Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B. *et al. Nature* **402**, 672-676 (1999).

203 Wu, J., Dang, Y., Su, W., Liu, C., Ma, H. *et al. Biochem Biophys Res Commun* **339**, 437-442 (2006).

204 Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y. *et al. Journal of cell science* **117**, 2805-2812 (2004).

205 Iwata, H. *Japanese journal of clinical oncology* **42**, 14-20 (2012).

206 Kotwall, C. A. *Canadian family physician Medecin de famille canadien* **45**, 1917-1924 (1999).

207 Rivera-Guevara, C. & Camacho, J. *Recent patents on anti-cancer drug discovery* **6**, 237-245 (2011).

208 Jensen, E. V. & Jordan, V. C. *Clinical cancer research : an official journal of the American Association for Cancer Research* **9**, 1980-1989 (2003).

209 Love, R. R. *Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **7**, 803-815 (1989).

210 Mazur, W. *Baillieres Clin Endocrinol Metab* **12**, 729-742 (1998).

211 Perry, R. R., Kang, Y. & Greaves, B. *Annals of surgical oncology* **2**, 238-245 (1995).

212 Barakat, R. R. *Clinical obstetrics and gynecology* **39**, 629-640 (1996).

213 Jordan, V. C. *British journal of pharmacology* **147 Suppl 1**, S269-276 (2006).

214 Barakat, R. R. *Cancer treatment and research* **94**, 195-207 (1998).

215 Barkhem, T., Carlsson, B., Nilsson, Y., Enmark, E., Gustafsson, J. *et al. Mol Pharmacol* **54**, 105-112 (1998).

216 McDonnell, D. P. *Maturitas* **48 Suppl 1**, S7-12 (2004).

217 Otto, A. M., Paddenberg, R., Schubert, S. & Mannherz, H. G. *Journal of cancer research and clinical oncology* **122**, 603-612 (1996).

218 Cardoso, C. M., Custodio, J. B., Almeida, L. M. & Moreno, A. J. *Toxicology and applied pharmacology* **176**, 145-152 (2001).

219 Cardoso, C. M., Moreno, A. J., Almeida, L. M. & Custodio, J. B. *Toxicology in vitro : an international journal published in association with BIBRA* **17**, 663-670 (2003).

220 Dietze, E. C., Caldwell, L. E., Grupin, S. L., Mancini, M. & Seewaldt, V. L. *J Biol Chem* **276**, 5384-5394 (2001).

221 Zheng, A., Kallio, A. & Harkonen, P. *Endocrinology* **148**, 2764-2777 (2007).

222 Lee, Y. H., Kang, B. S. & Bae, Y. S. *Life sciences* **97**, 116-122 (2014).

223 Bursch, W., Ellinger, A., Kienzl, H., Torok, L., Pandey, S. *et al. Carcinogenesis* **17**, 1595-1607 (1996).

224 Fichtinger-Schepman, A. M., van der Veer, J. L., den Hartog, J. H., Lohman, P. H. & Reedijk, J. *Biochemistry* **24**, 707-713 (1985).

225 Pascoe, J. M. & Roberts, J. J. *Biochemical pharmacology* **23**, 1345-1357 (1974).

226 Damia, G., Imperatori, L., Stefanini, M. & D'Incalci, M. *International journal of cancer. Journal international du cancer* **66**, 779-783 (1996).

227 Beljanski, V., Marzilli, L. G. & Doetsch, P. W. *Molecular Pharmacology* **65**, 1496-1506 (2004).

228 Lee, K. B., Parker, R. J., Bohr, V., Cornelison, T. & Reed, E. *Carcinogenesis* **14**, 2177-2180 (1993).

229 Alazard, R., Germanier, M. & Johnson, N. P. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **93**, 327-337 (1982).

230 Ciccarelli, R. B., Solomon, M. J., Varshavsky, A. & Lippard, S. J. *Biochemistry* **24**, 7533-7540 (1985).

231 Mello, J. A., Lippard, S. J. & Essigmann, J. M. *Biochemistry* **34**, 14783-14791 (1995).

232 Ormerod, M. G., Orr, R. M. & Peacock, J. H. *British journal of cancer* **69**, 93-100 (1994).

233 Pinto, A. L. & Lippard, S. J. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 4616-4619 (1985).

234 De Koning, P., Neijt, J. P., Jennekens, F. G. I. & Gispen, W. H. *Toxicology and applied pharmacology* **89**, 81-87 (1987).

235 Stadnicki, S. W., Fleischman, R. W., Schaeppi, U. & Merriam, P. *Cancer chemotherapy reports. Part 1* **59**, 467-480 (1975).

236 Ward, J. M. & Fauvie, K. A. *Toxicology and applied pharmacology* **38**, 535-547 (1976).

237 Olivero, O. A., Chang, P. K., Lopez-Larraza, D. M., Cristina Semino-Mora, M. & Poirier, M. C. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **391**, 79-86 (1997).

238 Yang, Z., Schumaker, L. M., Egorin, M. J., Zuhowski, E. G., Guo, Z. *et al. Clinical Cancer Research* **12**, 5817-5825 (2006).

239 Dehne, N., Lautermann, J., Petrat, F., Rauen, U. & de Groot, H. *Toxicology and applied pharmacology* **174**, 27-34 (2001).

240 Jiang, Y., Guo, C., Vasko, M. R. & Kelley, M. R. *Cancer research* **68**, 6425-6434 (2008).

241 Santos, N. A. G., Catão, C. S., Martins, N. M., Curti, C., Bianchi, M. L. P. *et al. Arch Toxicol* **81**, 495-504 (2007).

242 Campbell, K. C., Rybak, L. P., Meech, R. P. & Hughes, L. *Hearing research* **102**, 90-98 (1996).

243 El-Beshbishy, H. A., Bahashwan, S. A., Aly, H. A. A. & Fakher, H. A. *European Journal of Pharmacology* **668**, 278-284 (2011).

244 Santos, N. A. G., Bezerra, C. S. C., Martins, N. M., Curti, C., Bianchi, M. L. P. *et al. Cancer chemotherapy and pharmacology* **61**, 145-155 (2008).

245 Brown, D. G., Chin-Sinex, H., Nie, B., Mendonca, M. & Wang, M. *Cancer chemotherapy and pharmacology* **63**, 723-730 (2009).

246 Hour, T.-C., Lai, Y.-L., Kuan, C.-I., Chou, C.-K., Wang, J.-M. *et al. Biochemical pharmacology* **80**, 325-334 (2010).

247 Marullo, R., Werner, E., Degtyareva, N., Moore, B., Altavilla, G. *et al. PloS one* **8**, e81162 (2013).

248 Xiong, P., Li, Y.-x., Tang, Y.-t. & Chen, H.-g. *Protein J* **30**, 499-508 (2011).

249 Decatris, M. P., Sundar, S. & O'Byrne, K. J. *Cancer treatment reviews* **30**, 53-81 (2004).

250 Silver, D. P., Richardson, A. L., Eklund, A. C., Wang, Z. C., Szallasi, Z. *et al. Journal of clinical oncology : official journal of the American Society of Clinical Oncology* **28**, 1145-1153 (2010).

251 Alexandre, J., Hu, Y., Lu, W., Pelicano, H. & Huang, P. *Cancer research* **67**, 3512-3517 (2007).

252 Honore, S., Pasquier, E. & Braguer, D. *Cellular and Molecular Life Sciences CMLS* **62**, 3039-3056 (2005).

253 Alexandre, J., Batteux, F., Nicco, C., Chéreau, C., Laurent, A. *et al. International Journal of Cancer* **119**, 41-48 (2006).

254 Alexandre, J., Nicco, C., Chéreau, C., Laurent, A., Weill, B. *et al. Journal of the National Cancer Institute* **98**, 236-244 (2006).

255 Ramanathan, B., Jan, K.-Y., Chen, C.-H., Hour, T.-C., Yu, H.-J. *et al. Cancer research* **65**, 8455-8460 (2005).

256 Pelicano, H., Feng, L., Zhou, Y., Carew, J. S., Hileman, E. O. *et al. Journal of Biological Chemistry* **278**, 37832-37839 (2003).

257 Binder, S. *Clinical journal of oncology nursing* **17 Suppl**, 9-14 (2013).

258 Siegel, R., Ward, E., Brawley, O. & Jemal, A. *CA: a cancer journal for clinicians* **61**, 212-236 (2011).

259 Grandemange, S., Herzig, S. & Martinou, J. C. *Seminars in cancer biology* **19**, 50-56 (2009).

260 Lee, J., Giordano, S. & Zhang, J. *The Biochemical journal* **441**, 523-540 (2012).

261 Mijaljica, D., Prescott, M. & Devenish, R. J. *Autophagy* **3**, 4-9 (2007).

262 Zhao, J., Zhang, J., Yu, M., Xie, Y., Huang, Y. *et al. Oncogene* **32**, 4814-4824 (2013).

263 Siegel, R., Naishadham, D. & Jemal, A. *CA: a cancer journal for clinicians* **63**, 11-30 (2013).

264 Milani, A., Geuna, E., Mittica, G. & Valabrega, G. *World journal of clinical oncology* **5**, 990-1001 (2014).

265 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Valle, A., Garau, I. *et al. Free radical biology & medicine* **61**, 11-17 (2013).

266 Andre, N., Carre, M., Brasseur, G., Pourroy, B., Kovacic, H. *et al. FEBS Lett* **532**, 256-260 (2002).

267 Razandi, M., Pedram, A., Jordan, V. C., Fuqua, S. & Levin, E. R. *Oncogene* **32**, 3274-3285 (2013).

268 Waris, G. & Ahsan, H. *Journal of carcinogenesis* **5**, 14 (2006).

269 Hussain, S. P., Hofseth, L. J. & Harris, C. C. *Nature reviews. Cancer* **3**, 276-285 (2003).

270 Echtay, K. S. *Free radical biology & medicine* **43**, 1351-1371 (2007).

271 Baffy, G. *Mitochondrion* **10**, 243-252 (2010).

272 Bouker, K. B. & Hilakivi-Clarke, L. *Environ Health Perspect* **108**, 701-708 (2000).

273 Zhou, J. R., Mukherjee, P., Gugger, E. T., Tanaka, T., Blackburn, G. L. *et al. Cancer research* **58**, 5231-5238 (1998).

274 Chang, E. C., Frasor, J., Komm, B. & Katzenellenbogen, B. S. *Endocrinology* **147**, 4831-4842 (2006).

275 Rajah, T. T., Du, N., Drews, N. & Cohn, R. *Pharmacology* **84**, 68-73 (2009).

276 Sotoca, A. M., Ratman, D., van der Saag, P., Strom, A., Gustafsson, J. A. *et al. The Journal of steroid biochemistry and molecular biology* **112**, 171-178 (2008).

277 Adams, S. M., Aksenova, M. V., Aksenov, M. Y., Mactutus, C. F. & Booze, R. M. *PloS one* **7**, e37540 (2012).

278 Nadal-Serrano, M., Pons, D. G., Sastre-Serra, J., Blanquer-Rossello Mdel, M., Roca, P. *et al. The international journal of biochemistry & cell biology* **45**, 2045-2051 (2013).

279 Folkerd, E. J., Lønning, P. E. & Dowsett, M. *Journal of Clinical Oncology* **32**, 1396-1400 (2014).

280 Sastre-Serra, J., Nadal-Serrano, M., Pons, D. G., Roca, P. & Oliver, J. *The international journal of biochemistry & cell biology* **44**, 1901-1905 (2012).

281 Lemasters, J. J. *Rejuvenation research* **8**, 3-5 (2005).

282 Twig, G., Elorza, A., Molina, A. J. A., Mohamed, H., Wikstrom, J. D. *et al. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy*. Vol. 27 (2008).

283 Simon, H. U., Haj-Yehia, A. & Levi-Schaffer, F. *Apoptosis : an international journal on programmed cell death* **5**, 415-418 (2000).

284 Santandreu, F. M., Roca, P. & Oliver, J. *Free radical biology & medicine* **49**, 658-666 (2010).

285 Lee, Y. J., Jeong, S. Y., Karbowski, M., Smith, C. L. & Youle, R. J. *Molecular biology of the cell* **15**, 5001-5011 (2004).

286 Parra, V., Eisner, V., Chiong, M., Criollo, A., Moraga, F. *et al. Cardiovascular research* **77**, 387-397 (2008).

287 Bjornstrom, L. & Sjoberg, M. *Mol Endocrinol* **19**, 833-842 (2005).

288 Catlett-Falcone, R., Landowski, T. H., Oshiro, M. M., Turkson, J., Levitzki, A. *et al. Immunity* **10**, 105-115 (1999).

289 Yu, J. Y., Lee, J. J., Lim, Y., Kim, T. J., Jin, Y. R. *et al. Journal of pharmacological sciences* **107**, 90-98 (2008).

290 Ruddy, S. C., Lau, R., Cabrita, M. A., McGregor, C., McKay, B. C. *et al. Molecular cancer therapeutics* **13**, 1882-1893 (2014).

7. Anexo / Appendix

Manuscript 6

17β-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio

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17β-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio

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ABSTRACT

Estrogen action is mediated by the two receptor isoforms: estrogen receptor alpha and beta. Both receptors are expressed in human prostate tissue and have different action profiles. ERalpha is positively correlated with the malignancy of prostate cancer, while ERbeta may protect against abnormal prostate cell growth. 17β-Estradiol (E2), at least in part, induces cancerous transformations by causing deleterious mutations through the formation of reactive oxygen species (ROS).

The aim was to study the effect of E2 on oxidative stress and the expression of uncoupling proteins (UCPs) and antioxidant enzymes in several prostate cancer cell lines with different ERalpha/ERbeta ratios.

The cell prostate lines with a lower ERalpha/ERbeta ratio had lower oxidative stress, which could be partially explained by the increased expression of antioxidant enzymes and UCPs. Moreover, the action of E2 on the expression of antioxidant enzymes and UCPs was dual and dependent on the ERalpha/ERbeta ratio. Treatments with 0.1 nM E2 in cell lines with high ERalpha/ERbeta ratio produced a decrease in antioxidant enzymes and UCPs levels, with an increase in ROS production. These effects disappeared when the treatment was done in the presence of an ERalpha antagonist (MPP). In the cell lines with greatest levels of ERbeta and the lowest ERalpha/ERbeta ratio, E2 treatment caused the up-regulation of antioxidant enzymes and UCPs with a look-up decrease in ROS production. These effects were reversed when the cells were treated with E2 in the presence of an ERbeta antagonist (R,R-THC).

On the whole, our results suggest a dual E2 effect; increasing or decreasing oxidative stress in part by modulation of UCPs and antioxidant enzymes according to the abundance ERbeta and ERalpha/ERbeta ratio in prostate cancer cell lines.

1. Introduction

Prostate cancer is the most common cancer in men and the second leading cause of cancer death [1]. In industrialized nations, prostate cancer incidence is on the increase, although the number of deaths has declined [2,3]. Epidemiological and experimental studies suggest that estrogens may have cancerogenic and chemopreventative effects on the prostatic epithelium [4,5]. Estrogen action is mediated by specific nuclear receptors that regulate transcription of the target genes by binding to their DNA response elements. Estrogen action is mediated by two receptors, ERalpha and ERbeta, which are often antagonistic to one another [6]. Estrogen via ERalpha stimulates proliferation in the breast, uterus, and developing prostate, while estrogen via ERbeta inhibits proliferation and promotes differentiation in the prostate, mammary gland, colon, lung, and bone marrow stem cells [6].

Both ERalpha and ERbeta receptors are expressed in human prostate tissue [7,8]. The function of ERalpha, the classical receptor, has been thoroughly studied and several groups have reported greater ERalpha expression in cancer specimens than in benign hyperplasia and normal prostate tissue. These data are consistent with other reports in which abundance of ERalpha is positively correlated with the malignancy of prostate cancer [9,10]. Nevertheless, recent reports have shown a frequent loss of ERbeta expression in prostate cancer samples relative to normal prostatic tissue [11,12]. In the primary prostate tumor sites, ERbeta is strongly expressed in low grade prostate carcinoma and is markedly diminished in higher grade tumors [11–13]. These data suggest that ERbeta may protect against abnormal prostate cell growth [11,14]. In fact, even the use of ERbeta agonists has been suggested as a therapy to prevent grade progression in prostate tumors [6].

In the prostate, estrogens have been described to induce cancerous transformation by their genotoxic metabolites, and in part,

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these metabolites may directly induce genomic damage or via the formation of reactive oxygen species (ROS) [15]. During the onset of cancer, ROS may cause DNA damage and mutagenesis, and they also have a role as secondary messengers, stimulating proliferation while inhibiting apoptosis, which confers a growth advantage to established cancer cells [16,17]. In breast cancer, it has been suggested that 17β-estradiol (E2) induced oxidative stress that resulted from a modulation of antioxidant enzyme status through an estrogen receptor (ER)-dependent mechanism [15,18]. Recently, in our laboratory, we have found that E2, through an ER-dependent mechanism, may increase mitochondrial ROS production by repressing the uncoupling proteins (UCPs) [19]. UCPs are a family of inner mitochondrial membrane proteins whose function is to allow the re-entry of protons into the mitochondrial matrix by dissipating the proton gradient by subsequently decreasing membrane potential and ROS production. In these last years, it has described the importance of these proteins in both cancer development and progression [20].

It is of interest to investigate whether E2 influenced UCPs expression and consequently oxidative stress in prostate cancer cells with a different ERalpha/ERbeta ratios. To tackle this aim, the effect of E2 on oxidative stress, antioxidant enzymes and uncoupling protein expression was analysed in several prostate cancer cell lines with different endowment levels of ERalpha and ERbeta which were concretely: VCaP cancer cell lines with a high ERalpha/ERbeta ratio, DU145 cell lines that only express ERbeta, and PC3 cell lines with an average of ERalpha/ERbeta ratio.

2. Materials and methods

2.1. Materials and reagents

17β-Estradiol (E2), genistein and testosterone were purchased from Sigma–Aldrich (St. Louis, MO, USA). 1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidiniylethoxy) phenol]-1H-pyrazole dihydrochloride (MPP) and (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol (R,R-THC) were purchased from Tocris (Ellisville, MO, USA). Antisera against uncoupling protein 2 (UCP2) and uncoupling protein 5 (UCP5) were purchased from Alpha Diagnostic International (San Antonio, TX, USA); catalase and glutathione peroxidase (GPx) from Calbiochem (San Diego, CA, USA); ERalpha, ERbeta and actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and antisera against 4-hydroxy-2-nonenal (HNE) were purchased from Alpha Diagnostic International (San Antonio, TX, USA). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich, Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Cell culture and treatments

Prostate cancer cell lines VCaP, PC3 and DU145 were purchased from the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37 °C. To evaluate the effects of E2, testosterone, genistein, MPP and R,R-THC cells were grown in phenol red-free DMEM containing 10% charcoal-stripped FBS 24 h before treatment. Experiments were performed when cell cultures reached confluence by providing fresh medium supplemented with 1 nmol/L testosterone or 1 μM genistein or 0.1 nmol/L E2 with or without MPP (1 μM) and R,R-THC (1 μM) for 48 h. Cell lysates were obtained by scraping cells in lysis buffer (20 mM Tris–HCl, 1.5 mM $MgCl_2$, 140 mmol/L NaCl, 0.5%, Nonidet P-40, 10% glycerol, 1 mmol/L EGTA, 1 mmol/L $NaVO_3$, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml pepstatin; pH 7.4). Protein content was measured with a BCA protein assay kit (Pierce, Bonn, Germany).

2.3. Measurement of carbonyl content

Carbonyl groups were quantified using the Oxyblot protein oxidation detection kit (Chemicon, Chandlers Ford, UK). Derivatization by 2,4-dinitrophenylhydrazine (DNPH) was carried out for 15 min on 5 μg of total cell lysate protein following the manufacturer's instructions. Proteins were transferred onto nitrocellulose membrane by means of a slot-blot system (Bio-Rad). After incubation with anti-DNP antibody, bands were visualized using Immun-Star Western C Kit reagent (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analysed with Quantity One software (Bio-Rad). To ensure specificity, the oxidized proteins provided by the kit were included as a positive control. Treatment of sample with a control solution served as a negative control for DNPH derivatization.

2.4. Measurement of 4-hydroxy-2-nonenal

For 4-hydroxy-2-nonenal (HNE) analysis, 5 μg of protein from cell lysate was transferred onto nitrocellulose membrane by means of a slot-blot system (Bio-Rad). Membrane was incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. Antisera against 4-HNE were used as primary antibody. Bands were visualized using the Immun-Star Western C Kit reagent (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analysed with Quantity One software (Bio-Rad).

2.5. Western blotting

For Western blot analysis, 40 μg of protein from cell lysate was fractioned by SDS-PAGE (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. Antisera against UCP2 (32 kDa, Primary Ab 1:500), UCP5 (36 kDa, Primary Ab 1:500), Catalase (60 kDa, Primary Ab 1:1000), glutathione peroxidase (22.5 kDa, Primary Ab 1:500), ERalpha (66 kDa, Primary Ab 1:1000), ERbeta (54 kDa, Primary Ab 1:200) and actin (45 kDa, Primary Ab 1:1000) (such as housekeeping) were used as primary antibodies. A negative control without primary antibody template was run in each assay. Protein bands were visualized by the Immun-Star Western C kit reagent (Bio-Rad) Western blotting detection system. The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analysed with Quantity One software (Bio-Rad).

2.6. Real-time RT-PCR analysis

Total RNA was isolated from cultured cells using TriPure isolation reagent and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10-μl volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNase inhibitor, and 500 μM each dNTP. Each cDNA was diluted to 1/10 and aliquots were frozen (−70 °C) until the PCRs were carried out.

Real-time PCR was done for seven target genes, UCP1, UCP2, UCP3, UCP4, UCP5, catalase and GPx; and one housekeeping gene: 18S ribosomal RNA (18S).

PCR was carried out using SYBR green technology, purchased by Sigma–Aldrich, on a LightCycler rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μl, containing 5 μl SYBR Green JumpStart *Taq* ReadyMix (containing 20 mM Tris–HCl, pH 8.3, 100 mM KCl, 0.4 mM each dATP, dCTP,

dGTP, TTP, glass passivator, stabilizers, 0.05 U/μl *Taq* DNA Polymerase, JumpStart *Taq* antibody, and SYBR Green I dye), 0.5 μM of the sense and antisense primers, 2 mM $MgCl_2$, and 3 μl of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (2 min, 94 °C), followed by 40 cycles consisting of a denaturation step (15 s, 94 °C), and annealing, extension and read fluorescence step (1 min, 60 °C for UCP2, UCP5 and catalase; 61 °C for UCP1, UCP3, UCP4 and 18S; and 66 °C for GPx). A negative control without cDNA template was run in each assay.

The primers used were forward 5′-CTTGGTGTCGGCTCTTATCG-3′ and reverse 5′-CCGTTGGTCCTTCGTTAGTG-3′ for UCP1, forward 5′-GGTGGTCGGAGATACCAAAG-3′ and reverse 5′-CTCGGGC-AATGGTCTTGTAG-3′ for UCP2, forward 5′-GGGATTCTGGTCTT-CACTGC-3′ and reverse 5′-TCCAACCTTCCATTTTGTCC-3′ for UCP3, forward 5′-GCGACAAGGAGTGCGTTATC-3′ and reverse 5′-ATCCAGGGGAAAGGTTGCTA-3′ for UCP4, and forward 5′-CAAGCCGTTGGTCTCCTAAG-3′ and reverse 5′-CGTTTTCAAT-GTCACCCATC-3′ for UCP5, forward 5′-CATCGCCACATGAATGGATA-3′ and reverse 5′-CCAACTGGGATGAGAGGGTA-3′ for CAT, forward 5′-GCGGCGGCCCAGTCGGTGTA-3′and reverse 5′-GAGCTTGGGGT-CGGTCATAA-3′ for GPx, and forward 5′-GGACACGGACAGGATTGA-CA-3′ and reverse 5′-ACCCACGGAATCGAGAAAGA-3′ for 18S.

The Ct values of the real-time PCR were analysed, taking into account the efficiency of the reaction and referring the results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

2.7. Cell proliferation assay

Cells were plated at 5000 cells per well in 96-well plates in phenol red-free DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37 °C and shifted to phenol red-free DMEM 10% charcoal-FBS 1% antibiotics (penicillin and streptomycin) 24 h before treatment with E2 or testosterone during 48 h. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml) was added and plates were incubated for 1 h at 37 °C. The medium was removed and formazan crystals were dissolved by adding 100 μl dimethyl sulfoxide (DMSO). Absorbance of the converted dye was measured at 570 nm with background subtraction at 620 nm using a microplate reader (Power Wave XS, BIO-TEK).

2.8. Measurement of ROS production

Cells were plated at 5000 cells per well in 96-well plates in phenol red-free DMEM supplemented with 10% FBS and 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37 °C and shifted to phenol red-free DMEM 10% charcoal-FBS 1% antibiotics (penicillin and streptomycin) 24 h before treatment with E2, genistein or E2 with MPP or R,R-THC during 48 h. After treatment, 10 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) was added and plates were incubated for 90 min at 37 °C. Assay was performed at 37 °C in a 96-well microplate fluorimeter FLx800 (BIO-TEK instruments, Winooski, VT, USA). Hydrogen peroxide production was assayed by measuring the increase in fluorescence (485 nm excitation, 530 nm emission).

To correct the effect of treatment in the proliferation 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml) was added and plates were incubated for 1 h at 37 °C. The medium was removed and formazan crystals were dissolved by adding 100 μl dimethyl sulfoxide (DMSO). Absorbance of the converted dye was measured at 570 nm with background subtraction at 620 nm using a microplate reader (Power Wave XS, BIO-TEK).

Table 1
Effect of E2 on VCaP, PC3 and DU145 cell proliferation.

	VCaP	PC3	DU145
Control	100 ± 1.7	100 ± 3.1	100 ± 8.3
E2	112 ± 3.1*	96 ± 1.9	100 ± 2.2
T	113 ± 3.8*		
E2 + T	117 ± 2.5*		

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) cells were set at 100. E2, 17β-estradiol 0,1 nM; T, testosterone 1 nM.
* Significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$).

2.9. Measurement of ROS levels by flow cytometry

Cells were stained with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) for 15 min, washed with PBS containing 10 mM glucose, and analysed immediately using an Epics XL flow cytometer (Beckman–Coulter, Miami, FL, USA). The green fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. 10,000 events were acquired and analysis was performed with Expo32 ADC analysis software (Beckman Coulter, Miami, FL).

2.10. Analysis of mitochondrial membrane potential (MMP) by flow cytometry

Cells were stained with 250 nM tetramethylrhodamine methyl (TMRM) ester for 15 min, washed with PBS containing 10 mM glucose, and analysed by flow cytometry as described above. TMRM is a lipophilic cationic dye that accumulates within mitochondria according to the MMP. The red fluorescence was measured using the FL-2 setting (log mode) after the cell debris was electronically gated out. In each analysis, 10,000 events were recorded and analysed with Expo32 ADC analysis software (Beckman Coulter, Miami, FL).

2.11. Statistical analysis

All data were expressed as means ± SEM from at least three independent experiments performed in duplicate. Statistical analysis was carried out using the Statistical Program package (SPSS 18.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups were analysed with unpaired Student's *t*-test. A level of $P < 0.05$ was accepted as significant.

3. Results

3.1. Effect of E2 on VCaP PC3 and DU145 cell proliferation

E2 treatment (0.1 M) increased the proliferation of VCaP and had no effect on the proliferation of other prostate cancer cell lines studied (Table 1). Due to the presence of androgen receptor in VCaP cells, the effect of testosterone (1 nM) alone and in combination with E2 also had been evaluated in this cell line with no difference between treatments.

3.2. VCaP, PC3 and DU145 prostate cell lines characterization

The protein levels of estrogen receptor isoforms ERalpha and ERbeta were determined to characterize the prostate cancer cell lines used in this study: VCaP, PC3 and DU145 (Table 2 and Fig. 1). No differences between VCaP and PC3 cancer cell lines were found in ERalpha. In DU145 the levels of ERalpha were undetected (see Fig. 1). ERbeta protein levels were statistically different between all cell lines, with VCaP cells presented the lower levels, PC3 the higher levels (206% considering VCaP as 100%) and DU145 intermediate

Table 2
VCaP, PC3 and DU145 prostate cell lines characterization.

	VCaP	PC3	DU145
ERα	100 ± 9	110 ± 19	UD
ERβ	100 ± 14	206 ± 18*	151 ± 22*,§
Ratio ERα/ERβ	1.00	0.48	0
ROS levels	100 ± 3	88 ± 2*	87 ± 5*
TMRM fluorescence	100 ± 2	124 ± 3*	180 ± 10*,§
Carbonil content	100 ± 7	63 ± 9*	75 ± 13*
4-HNE	100 ± 9	72 ± 14*	65 ± 16*

Data represent the means ± SEM ($n = 6$). Values of VCaP cell line were set at 100. ERα, estrogen receptor alpha; ERβ, estrogen receptor beta, ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester; 4-HNE, 4-hydroxy-2-nonenal; UD, undetected.

* Significant difference between VCaP cell line and PC3 or DU145 cell lines (Student's *t*-test; $P < 0.05$, $n = 6$).

§ Significant difference between PC3 cell line and DU145 cell line (Student's *t*-test; $P < 0.05$, $n = 6$).

levels (151%). Considering both receptors, ERalpha/ERbeta ratio has also been calculated and VCaP presented the higher range, PC3 the mid-range and DU145 the lower value of this ratio, which was 0, as a result of undetected levels of ERalpha.

Table 2 shows the indicators of mitochondrial function and oxidative stress balance in prostate cancer cell lines: VCaP, PC3 and DU145. Specifically, we measured ROS levels by DCFDA (as an oxidative stress indicator), TMRM fluorescence (as an indicator of mitochondrial membrane potential), and carbonyl and 4-HNE content (as an indicator of protein and lipids oxidative damage, respectively). The highest mitochondrial membrane potential was found in DU145 cells followed by those of PC3, with the VCaP cells presenting the lowest value.

To complete the oxidative stress balance characterization in cancer prostate cell lines, we studied the expression of antioxidant enzymes and UCPs by real time PCR and the protein levels by Western blot. The crossing points (Cp) of catalase, GPx and UCP1–UCP5 of VCaP, PC3 and DU145 cell lines in culture basal conditions are summarized in Table 3, as well as 18S (such as housekeeping). Catalase and GPx were expressed in significant levels in all cell lines studied ($Cp < 21$), only UCP2 and UCP5 presented a Cp high enough to be considered as a significant gene expression ($Cp < 24$) in VCaP, PC3 and DU145 cells, while the other UCPs (UCP1, UCP3 and UCP4) presented, in most cases, had a Cp higher than 25, and therefore the expression of these genes may be considered as basal. Only UCP3 in the DU145 cell line presents a Cp value of 22.3.

Table 4 shows protein levels of antioxidant enzymes (GPx and catalase) and main uncoupling proteins (UCP2 and UCP5), in the VCaP, PC3 and DU145 cancer prostate cell lines. Despite of no differences in GPx between the cell lines analysed, the protein catalase levels presented the highest values in PC3 lines and the lowest values of the DU145 cell line, both levels were statistically significant. UCP2 shows no statistically significant differences between lines, although UCP5 protein levels were lower in VCaP with respect to PC3 and DU145.

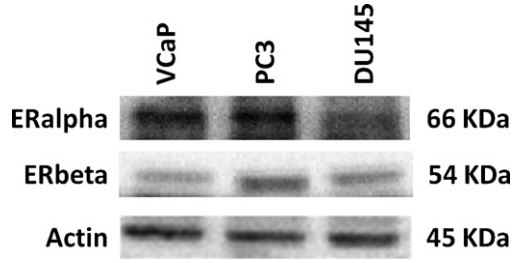

Fig. 1. Representative bands of Western blot are shown. ERalpha, estrogen receptor alpha; ERbeta, estrogen receptor beta.

Table 3
Antioxidant enzymes and UCPs expression in VCaP, PC3 and DU145 cell lines.

	VCaP	PC3	DU145
UCP1	28.3 ± 0.7	28.7 ± 0.2	27.6 ± 0.5
UCP2	20.6 ± 0.1	23.4 ± 0.1	21.7 ± 0.4
UCP3	25.1 ± 0.4	26.2 ± 0.2	22.3 ± 0.4
UCP4	26.5 ± 0.2	26.4 ± 0.3	26.2 ± 0.5
UCP5	21.3 ± 0.1	21.1 ± 0.1	23.3 ± 0.4
Catalase	19.1 ± 0.2	18.6 ± 0.2	21.0 ± 0.4
GPx	17.4 ± 0.2	18.1 ± 0.2	18.7 ± 0.3
18S	8.2 ± 0.1	8.3 ± 0.1	9.4 ± 0.3

Data represent the means of Ct values ± SEM ($n = 6$). UCP, uncoupling protein; GPx, glutathione peroxidase.

Table 4
Protein levels of UCPs and antioxidant enzymes in VCap, PC3 and DU145 cancer prostate cell lines.

	VCaP	PC3	DU145
UCP2	100 ± 10	81 ± 8	92 ± 9
UCP5	100 ± 8	148 ± 11*	175 ± 45*
GPx	100 ± 12	90 ± 13	90 ± 2
CAT	100 ± 11	343 ± 31*	79 ± 4*,§

Data represent the means ± SEM ($n = 6$). Values of VCaP cells were set at 100. UCP, uncoupling protein; GPx, glutathione peroxidase.

* Significant difference between VCaP cell line and PC3 or DU145 cell lines (Student's *t*-test; $P < 0.05$, $n = 6$).

§ Significant difference between PC3 cell line and DU145 cell line (Student's *t*-test; $P < 0.05$, $n = 6$).

3.3. Effect of E2 on antioxidant enzymes and UCPs mRNA levels in VCap PC3 and DU145

The effect of a 48 h E2-treatment on antioxidant enzymes catalase and GPx, and UCP2 and UCP5 mRNA levels was evaluated in order to establish the influence of this hormone on the antioxidant capacity of prostate VCaP, PC3 and DU145 cancer cell lines (Table 5). In Table 5, the fold change for hormone treatment is shown to better elucidate whether E2 treatment has a positive or negative effect on the mRNA expression of these genes. In VCaP cells, E2 treatment had no effect on GPx, but there was a decrease in catalase, UCP2 and UCP5 mRNA levels. Given that an androgen receptor is expressed in the VCaP cell line, the effect of testosterone (1 nM) has also been evaluated and this treatment had no effect on antioxidant enzymes and the UCPs analysed. E2 increased expression of antioxidant enzymes and UCPs, in PC3 cells, was only statisti-

Table 5
Fold-change of UCPs and antioxidant enzymes ARNm in prostate cancer cell lines after hormonal treatment.

		Control	E2	T
VCaP	UCP2	0.00 ± 0.09	−0.44 ± 0.09*	0.05 ± 0.08
	UCP5	0.00 ± 0.08	−0,15 ± 0.02*	0.12 ± 0.08
	Catalase	0.00 ± 0.08	−0.29 ± 0.12*	0.05 ± 0.1
	GPx	0.00 ± 0.09	−0.08 ± 0.15	0.08 ± 0.03
PC3	UCP2	0.00 ± 0.10	0,17 ± 0.18	
	UCP5	0.00 ± 0.09	0.41 ± 0.06*	
	Catalase	0.00 ± 0.12	0.41 ± 0.21	
	GPx	0.00 ± 0.13	0.42 ± 0.06*	
DU145	UCP2	0.00 ± 0.11	0,15 ± 0.12	
	UCP5	0.00 ± 0.18	0.23 ± 0.17	
	Catalase	0.00 ± 0.12	0.20 ± 0.15	
	GPx	0.00 ± 0.06	0.22 ± 0.09*	

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) cells were set at 0. E2, 17β-estradiol 0,1 nM; T, testosterone 1 nM; UCP, uncoupling protein; GPx, Glutathione peroxidase.

* Significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$).

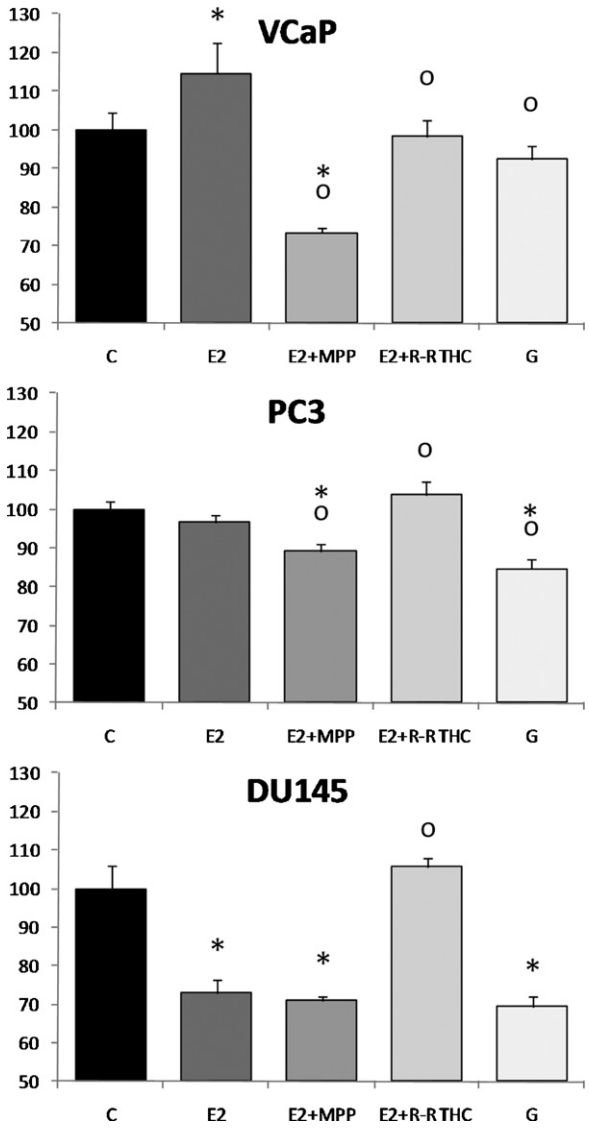

Fig. 2. Effects of 17β-estradiol, genistein and antagonists from estrogen receptors on ROS production. E2, 17β-estradiol 1 nM; MPP1 μM, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidiniylethoxy)phenol]-1H-pyrazole dihydrochloride; R,R-THC 1 μM, (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol; G, genistein. *Significant difference between treated and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$). ○ Significant difference between antagonists or genistein-treated cells versus E2-treated. cells (Student's *t*-test; $P < 0.05$, $n = 6$).

cally significant for GPx and UCP5 and showed a high tendency in catalase levels ($p = 0.083$). In DU145 cells, the E2 treatment only increases the expression of GPx and had no effect on the catalase, UCP2 and UCP5 mRNA levels.

3.4. Effect of ERalpha and ERbeta antagonists on E2 stimulation of ROS production in VCaP, PC3 and DU145

ROS production of vehicle-treated cells was used as a reference (100%). The effect of estrogen with and without estrogen receptor antagonists (MPP and R,R-THC), and genistein was also determined in all prostate cancer cell lines studied (Fig. 2). Treatment effects were more evident in the VCaP cell line, which showed a higher ERalpha/ERbeta ratio with the statistically significant increase in the ROS production of E2-treated cells (+15% with respect to vehicle-treated cells), while experiencing a decrease in cell ROS production with the ERalpha antagonist (MPP −41% with respect to E2-treated cells) or the genistein (−7% with respect to vehicle-treated cells) treatment. In PC3 cells with a lower ERalpha/ERbeta ratio, ERalpha antagonist and genistein also statistically decreased ROS production, although this inhibition was lower than in VCaP (−7% MPP with respect to E2-treated cells). Moreover, the ERbeta antagonist increased ROS production (R,R-THC, 8% with respect to vehicle-treated cells). ROS production in DU145 cells, which only expresses ERbeta, was affected by E2 (−27% with respect to vehicle-treated cells) and was not affected by ERalpha antagonist or genistein (with respect to vehicle-treated cells), while the ERbeta antagonist statistically significant increased oxidative stress (+33% with respect to E2-treated cells).

3.5. Effect of E2 with and without ER antagonists on antioxidant enzyme and UCPs protein levels in VCaP and PC3

At this point of the experiment it was of interest to check if the E2 effects in VCaP cell line were caused by ERalpha and in PC3 cell line by ERbeta. For this reason we used MPP (antagonist of ERalpha) in VCaP cell lines, and R,R-THC (antagonist of ERbeta) in PC3 cell line. Fig. 3 shows effect of E2 with and without MPP in the VCaP cell line and the effect of E2 with and without R,R-THC in PC3 cell line on protein levels of antioxidant enzymes, GPx and catalase, and UCP2 and UCP5. E2 treatment in VCaP line decreased the expression of GPx, catalase and UCP5, although it was only statistically significant in GPx and UCP5. These effects were prevented by ERalpha antagonist addition in all the cases. Moreover, in PC3 line E2 treatment increased statistically expression of GPx, catalase and UCP2, effects that were reversed by the ERbeta antagonist reversed.

4. Discussion

Cell lines with a lower ERalpha/ERbeta ratio and higher levels of ERbeta have less oxidative stress than cell lines with a predominance of ERalpha. This state may be explained, at least in part, by the greater levels of antioxidant systems as well as UCP5 protein levels. UCPs are a family of inner mitochondrial membrane proteins whose function is to allow the re-entry of protons into the mitochondrial matrix, dissipate the proton gradient and, subsequently, decrease membrane potential and ROS production [20]. Taking into account the genotoxic effect of ROS, it can be speculated that one of the predictable consequences of UCPs function may be in cancer prevention [21,22] and may offer a clinical benefits for cancer treatment studies [22].

Additionally, the action of E2 on antioxidant enzymes and UCPs expression was dual and dependent on the ERalpha/ERbeta ratio and ERbeta amounts. Thus, treatment with 0.1 nM E2 (physiological concentrations on obese men plasma [23]) in cell lines with a high ERalpha/ERbeta ratio (VCaP) produced a reduction of antioxidant enzymes and UCPs levels, and an increase in ROS production. These effects disappeared when treatment was done in the presence of an ERalpha antagonist. In the cell lines which had high levels of ERbeta and a lower ERalpha/ERbeta ratio (PC3), E2 treatment caused an increase in antioxidant enzymes and UCPs, with a concomitant decrease in ROS production. These effects were reversed when cells were treated with E2 in the presence of an ERbeta antagonist.

These results would agree with those of several authors who have shown in different tissues and cell lines that UCPs and antioxidant enzymes are under the control of sex hormones [24–28]. Moreover, similar results have been described in breast cancer cell lines. In MCF-7 cell lines with a higher expression of ERalpha E2 down-regulates UCPs and antioxidant enzymes, increases oxidative stress, while in MDA-MB-231, which only expresses ERbeta, estrogen cannot down-regulate these proteins and does not increase oxidative stress [19].

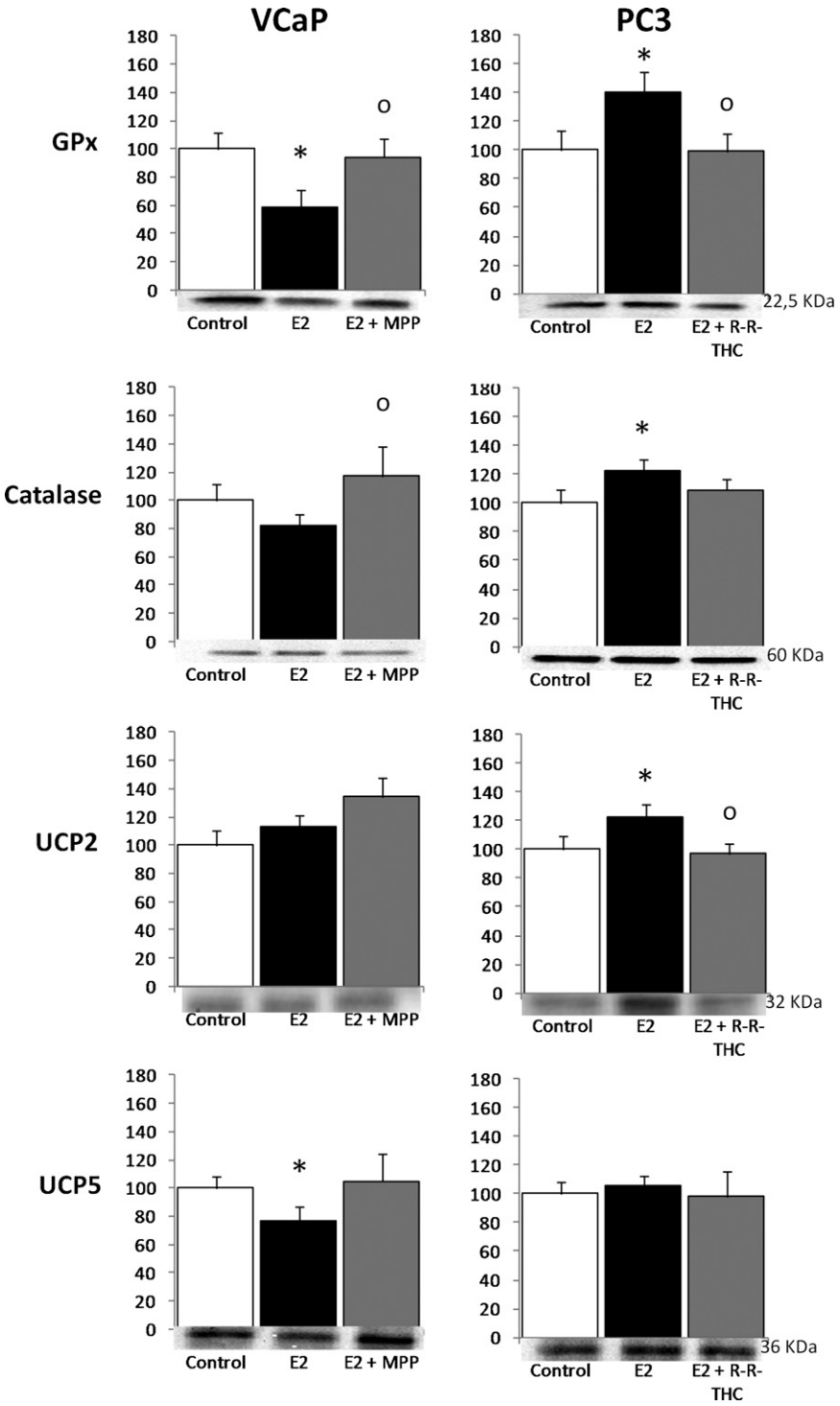

Fig. 3. Effect of 17β-estradiol on GPx, catalase and UCPs protein levels. E2, 17β-estradiol 0.1 nM; MPP 1 μM, 1,3-bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidiniylethoxy)phenol]-1H-pyrazole dihydrochloride; R,R-THC 1 μM, (R,R)-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol. GPx, glutathione peroxidase; UCP, uncoupling protein. Representative bands of Western blot are shown. *Significant difference between E2-treated cells and vehicle-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$). ○Significant difference between antagonists-treated cells and E2-treated cells (Student's *t*-test; $P < 0.05$, $n = 6$).

The results of this paper would be consistent with the knowledge that in different cancers the effects that E2 exerts through ERalpha bring about greater oxidative stress and proliferation, whereas the effects through ERbeta have a protective nature when faced with oxidative stress. Thus, in tissues where ERalpha is abundant, E2 is considered a cancer risk, whereas in those tissues in which the predominant isoform receptor is ERbeta, protective effects of E2 when facing oxidative stress have been described [19,29–31]. Moreover, these results agree with the fact that abundance of ERalpha is positively correlated with the malignant prostate cancer and that ERbeta is strongly expressed in low grade prostate carcinoma, while it is markedly diminished in high grade tumors [6]. Several authors have been suggested that ERbeta may protect against abnormal prostate cell growth. Even the use of ERbeta agonists has been suggested as a therapy to prevent grade progression in prostate tumors [6].

Likewise, the antioxidant effects caused by genistein agree with the beneficial effects of phytoestrogens in cancer, as phytoestrogens have reported a 70-fold higher binding affinity for ERbeta than for ERalpha [32], and its difference in affinity for estrogen receptors could explain the protective effects of phytoestrogen.

Another aspect to consider is that some studies have demonstrated that PC3 cancer cells are more aggressive than other prostate cancer cells. These authors believe in the possibility that the aggressive nature of PC3 cells is caused by the lower sensitivity to ROS [33], which could explain why these cells have a greater number of antioxidant systems in response to the higher ERbeta levels.

5. Conclusion

On the whole, our results suggest a dual E2 effect; that an increase or decrease in oxidative stress is regulated by modulating the UCPs and antioxidant enzymes according to the abundance of ERbeta and the ERalpha/ERbeta ratio. Thus, it would be interesting to study these two estrogen receptor isoforms for prostate cancer diagnosis in order to establish possible future treatments. Likewise, the continuation of the study of the role of UCPs in the development of prostate cancer, which could provide valuable information for diagnosis and treatment, since the expression of these proteins has been shown to decrease prostate cancer risk, yet at the same time can have non-desirable effects in neoplastic development, as they confer to these cancer cells a higher resistance to oxidative stress, facilitating their malignancy and chemoresistance.

Acknowledgements

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References

[1] A. Jemal, R. Siegel, E. Ward, T. Murray, J. Xu, M.J. Thun, Cancer statistics, CA Cancer J. Clin. 57 (1) (2007) 43–66.
[2] P.D. Baade, M.D. Coory, J.F. Aitken, International trends in prostate-cancer mortality: the decrease is continuing and spreading, Cancer Causes Control 15 (3) (2004) 237–241.
[3] D.M. Parkin, F. Bray, J. Ferlay, P. Pisani, Global cancer statistics 2002, CA Cancer J. Clin. 55 (2) (2005) 74–108.
[4] W.Y. Chang, G.S. Prins, Estrogen receptor-beta: implications for the prostate gland, Prostate 40 (2) (1999) 115–124.
[5] M.S. Steiner, S. Raghow, B.L. Neubauer, Selective estrogen receptor modulators for the chemoprevention of prostate cancer, Urology 57 (4 Suppl. 1) (2001) 68–72.
[6] M. Warner, J.A. Gustafsson, The role of estrogen receptor beta (ERbeta) in malignant diseases—a new potential target for antiproliferative drugs in prevention and treatment of cancer, Biochem. Biophys. Res. Commun. 396 (1) (2010) 63–66.
[7] G.G. Kuiper, B. Carlsson, K. Grandien, E. Enmark, J. Haggblad, S. Nilsson, J.A. Gustafsson, Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta, Endocrinology 138 (3) (1997) 863–870.
[8] S. Mosselman, J. Polman, R. Dijkema, ER beta: identification and characterization of a novel human estrogen receptor, FEBS Lett. 392 (1) (1996) 49–53.
[9] H. Bonkhoff, T. Fixemer, I. Hunsicker, K. Remberger, Estrogen receptor expression in prostate cancer and premalignant prostatic lesions, Am. J. Pathol. 155 (2) (1999) 641–647.
[10] B.G. Mobbs, I.E. Johnson, Y. Liu, Quantitation of cytosolic and nuclear estrogen and progesterone receptor in benign, untreated, and treated malignant human prostatic tissue by radioligand binding and enzyme-immunoassays, Prostate 16 (3) (1990) 235–244.
[11] L.G Horvath, S.M. Henshall, C.S. Lee, D.R. Head, D.I. Quinn, S. Makela, W. Delprado, D. Golovsky, P.C. Brenner, G. O'Neill, R. Kooner, P.D. Stricker, J.J. Grygiel, J.A. Gustafsson, R.L. Sutherland, Frequent loss of estrogen receptor-beta expression in prostate cancer, Cancer Res. 61 (14) (2001) 5331–5335.
[12] A. Latil, I. Bieche, D. Vidaud, R. Lidereau, P. Berthon, O. Cussenot, M. Vidaud, Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays, Cancer Res. 61 (5) (2001) 1919–1926.
[13] T Tsurusaki, D. Aoki, H. Kanetake, S. Inoue, M. Muramatsu, Y. Hishikawa, T. Koji, Zone-dependent expression of estrogen receptors alpha and beta in human benign prostatic hyperplasia, J. Clin. Endocrinol. Metab. 88 (3) (2003) 1333–1340.
[14] K.M. Lau, M. LaSpina, J. Long, S.M. Ho, Expression of estrogen receptor (ER)-alpha and ER-beta in normal and malignant prostatic epithelial cells: regulation by methylation and involvement in growth regulation, Cancer Res. 60 (12) (2000) 3175–3182.
[15] M.C.S. Yeh, J. Ni, Y. Yin, E. Chang, M. Zang, X. Wen, Functions of estrogen receptor in prostate and prostate cancer, in: Basic Mechanisms and Therapeutic Approaches, W.S. Publishing, New Jersey, 2005, pp. 293–313.
[16] B. Halliwell, Oxidative stress and cancer: have we moved forward? Biochem. J. 401 (1) (2007) 1–11.
[17] J.L. Martindale, N.J. Holbrook, Cellular response to oxidative stress: signaling for suicide and survival, J. Cell Physiol. 192 (1) (2002) 1–15.
[18] G.G. Kuiper, J.G. Lemmen, B. Carlsson, J.C. Corton, S.H. Safe, P.T. van der Saag, B. van der Burg, J.A. Gustafsson, Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta, Endocrinology 139 (10) (1998) 4252–4263.
[19] J. Sastre-Serra, A. Valle, M.M. Company, I. Garau, J. Oliver, P. Roca, Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer, Free Radic Biol Med 48 (4) (2007) 506–512.
[20] A. Valle, J. Oliver, P. Roca, Role of uncoupling proteins in cancer, Cancers 2 (2) (2010) 567–591.
[21] F.M. Santandreu, A.S. Valle, P. Fernandez de Mattos, P. Roca, J. Oliver, Hydrogen peroxide regulates the mitochondrial content of uncoupling protein 5 in colon cancer cells, Cell Physiol. Biochem. 24 (5–6) (2009) 379–390.
[22] F.M. Santandreu, P. Roca, J. Oliver, Uncoupling protein-2 knockdown mediates the cytotoxic effects of cisplatin, Free Radic Biol Med 49 (4) (2006) 658–666.
[23] H.K. Kley, T. Deselaers, H. Peerenboom, H.L. Kruskemper, Enhanced conversion of androstenedione to estrogens in obese males, J. Clin. Endocrinol. Metab. 51 (5) (1980) 1128–1132.
[24] R. Guevara, F.M. Santandreu, A. Valle, M. Gianotti, J. Oliver, P. Roca, Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress, Free Radic. Biol. Med. 46 (2) (2009) 169–175.
[25] A.M. Rodriguez, M. Monjo, P. Roca, A. Palou, Opposite actions of testosterone and progesterone on UCP1 mRNA expression in cultured brown adipocytes, Cell Mol. Life Sci. 59 (10) (2002) 1714–1723.
[26] S. Rodriguez-Cuenca, E. Pujol, R. Justo, M. Frontera, J. Oliver, M. Gianotti, P. Roca, Sex-dependent thermogenesis, differences in mitochondrial morphology and function, and adrenergic response in brown adipose tissue, J. Biol. Chem. 277 (45) (2002) 42958–42963.
[27] C Borras, J. Gambini, M.C. Gomez-Cabrera, J. Sastre, F.V. Pallardo, G.E. Mann, J. Vina, 17beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade, Aging Cell 4 (3) (2005) 113–118.
[28] J. Vina, J. Sastre, F.V. Pallardo, J. Gambini, C. Borras, Modulation of longevity-associated genes by estrogens or phytoestrogens, Biol. Chem. 389 (3) (2008) 273–277.
[29] J. Hartman, A. Strom, J.A. Gustafsson, Estrogen receptor beta in breast cancer—diagnostic and therapeutic implications, Steroids 74 (8) (2009) 635–641.
[30] S. Tamir, S. Izrael, J. Vaya, The effect of oxidative stress on ERalpha and ERbeta expression, J. Steroid Biochem. Mol. Biol. 81 (4–5) (2002) 327–332.
[31] Y. Urata, Y. Ihara, H. Murata, S. Goto, T. Koji, J. Yodoi, S. Inoue, T. Kondo, 17Beta-estradiol protects against oxidative stress-induced cell death through the glutathione/glutaredoxin-dependent redox regulation of Akt in myocardiac H9c2 cells, J. Biol. Chem. 281 (19) (2006) 13092–13102.
[32] A.M. Sotoca, H. van den Berg, J. Vervoort, P. van der Saag, A. Strom, J.A. Gustafsson, I. Rietjens, A.J. Murk, Influence of cellular ERalpha/ERbeta ratio on the ERalpha-agonist induced proliferation of human T47D breast cancer cells, Toxicol. Sci. 105 (2) (2008) 303–311.
[33] L. Chaiswing, J.M. Bourdeau-Heller, W. Zhong, T.D. Oberley, Characterization of redox state of two human prostate carcinoma cell lines with different degrees of aggressiveness, Free Radic. Biol. Med. 43 (2) (2007) 202–215.

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The Effects of 17β-estradiol on Mitochondrial Biogenesis and Function in Breast Cancer Cell Lines are Dependent on the ERα/ERβ Ratio

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Key Words

Estrogen receptor alpha and beta ratio • Breast cancer • 17β-estradiol (E2) • Mitochondrial biogenesis

Abstract

Background/Aims: 17β-estradiol (E2) is a risk factor for the development of breast cancer, and cause tumorigenesis in epithelial breast cells. Moreover, E2 has distinct effects on different tissues that are attributed to the presence of two estrogen receptor isoforms, ERα and ERβ. Methods: The effect of E2 on mitochondrial biogenesis and function was investigated in two breast cancer cell lines with different estrogen receptor ratios, MCF-7 (high ERα/ERβ ratio) and T47D (low ERα/ERβ ratio) cell lines treated with physiological concentrations of E2 (1 nM). Results: Mitochondria of the MCF-7 cell line showed an increase in proliferation but a decrease in functionality, while the T47D cell line, with low ERα/ERβ ratio, maintained functionality with fewer mitochondria. Conclusion: Our results suggest that ERs endowment and its subtypes relation have an effect on treatment response and could contribute new ideas about mitochondria and ERs in breast cancer, as well as new indicators to the disease progression.

Introduction

Estrogens are hormones that have different roles in a number of physiological processes and pathologic conditions such as cancer [1, 2]. It is well known that estrogens, and particularly 17β-estradiol (E2), are risk factors for the development of breast cancer, and cause tumorigenesis in epithelial breast cells [3]. In the last decades, the focus on estrogen effects has been on the action of these hormones by their binding to estrogen receptors [4, 5]. There are two estrogen receptors: the classical receptor called estrogen receptor alpha (ERα) and the second receptor estrogen receptor beta (ERβ) [6]. ERβ may have different biological effects than ERα and display different intracellular and tissue distribution patterns [7-9], whilst ERα mediates the proliferative actions of E2 and these effects can be opposed by ERβ [10]. In the last few years, researchers have studied the pathways of these receptors with the aim to understand

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the importance of these signals in breast cancer.

Moreover, it is known that in mtDNA there are estrogen response elements (ERE) as well as estrogen receptors that can localize in mitochondria in response to several signals [11-13]. On the other hand, mitochondrial biogenesis and reactive oxygen species (ROS) production are under estrogen influence [14, 15]. Similarly, it is demonstrated that mitochondria can modulate the expression of nuclear cell cycle genes and human breast tumor growth [16-18]. For this reason, some authors have given estrogens a new role in the breast carcinogenesis process through the modulation of mitochondrial function and ROS production [14, 19-21].

Additionally, although it is well known that mitochondrial function is altered in cancer cells [15], the supposed theories on the effects of estrogen on mitochondria are controversial, as several studies have shown an increased mitochondrial biogenesis in response to E2 treatment while others have found a mitochondrial dysfunction under the same conditions [12, 14, 22, 23].

The purpose of this article is to investigate whether the influence of E2 on mitochondrial biogenesis and function is dependent on the estrogen receptor ratio. To tackle this aim, we used two breast cancer cell lines: the MCF-7 cell line with a high ERα/ERβ ratio; and T47D cell line with a low ERα/ERβ ratio.

Materials and Methods

Materials

17β-estradiol (E2) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mitotracker Green (MTG) was purchased from Molecular Probes (Eugene, OR, USA). Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma-Aldrich (St Louis, MO, USA), Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA, USA).

Cell culture and treatments

Breast cancer cell lines MCF-7 and T47D bear a great resemblance to one another as human breast adenocarcinoma cell lines with a great difference in ERα/ERβ with MCF-7 possessing the highest ratio. Cell lines were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin) in 5% CO_2 in air at 37°C. To evaluate the effects of 17β-estradiol, cells were grown in phenol red-free DMEM containing 10% charcoal-stripped FBS 24 hours prior to treatment. Experiments were performed when cell cultures reached confluence by providing fresh media supplemented with 1 *n*mol/l E2 (Sigma-Aldrich).

Analysis of Cardiolipin content

Cardiolipin content was assayed using Nonyl Acridine Orange (NAO) fluorescence and quantified using a microplate fluorescence reader FLx800 (BIO-TEK Winooski, Vermont, USA) set at 485/20 nm to excitation and 528/20 to emission. The fluorescence was corrected by Hoescht 33342 (Sigma-Aldrich) fluorescence with the same fluorescence reader set at 360/40 nm to excitation and 460/40 to emission.

Citrate synthase, cytochrome c oxidase and ATPase activities

The cell lysates used for enzymatic activities were obtained by scrapping cells in RNAase-free water. CS (citrate synthase; EC 2.3.3.1 [previously 4.1.3.7]) activity was determined by following the increase in absorption of the 5-thio-2-nitrobenzoate ion at 412 nm and 30°C as previously reported [24]. Complex IV or COX (cytochrome c oxidase; EC 1.9.3.1) activity was measured using a spectrophotometric method [25]. Briefly, cell lysate was incubated in 0.1 M $NaPO_4H_2$, pH 7.0, in the presence of 2 μg/mL catalase and 5 mM substrate DAB (3, 3' diaminebenzidine-tetrachloride). After 30 s, 100 μM reduced cytochrome c was added to start the reaction, and the absorbance variation was recorded for 15 min at 450 nm. ATPase (ATP phosphohydrolase, Complex V, EC 3.6.1.3) activity was measured by monitoring the oxidation of NADH at 340 nm and 37°C [26], with an extinction coefficient was 6.22 mM^{-1} cm^{-1}.

Real time PCR

Total RNA was isolated from cultured cells using TriPure® isolation reagent and quantified using a spectrophotometer set at 260 nm. One μg of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10 μl volume of retrotranscription reaction mixture containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNAase inhibitor and 500 μM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (-70°C) until the PCR reactions were carried out.

PCR was done for seven target genes: Presenilin 2 (pS2), peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC1α), nuclear respiratory factors 1 and 2 (NRF1 and NRF2), mitochondrial transcription factor A (TFAM), mitochondrial single strand DNA binding protein (mtSSB) and 18S, using specific primers (see Table 1) SYBR Green technology on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μL, containing 6,5 μl Lightcycler® 480 SYBR Green I Master, 0.5 μM of the sense and antisense specific primers and 2,5 μl of the cDNA template. The amplification program consisted of a preincubation step for template cDNA denaturation (5 min, 95°C), followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, 60°C for PGC1α, NRF1, NRF2 and mtSSB; and 61°C for 18S, pS2 and TFAM) and an extension step (12s, 72°C). A negative control lacking cDNA template was run in each assay.

Gene	Forwar Primer (5'-3') Reverse Primer (5'-3')	Tª An. (°C)	Gene	Forwar Primer (5'-3') Reverse Primer (5'-3')	Tª An. (°C)
			NRF1	CCCgTTACAgggAggTgAg TgTAgCTCCCTgCTgCATCT	60
18S	ggACACggACAggATTgACA ACCCACggAATCgAgAAAgA	61			
pS2	TTgTggTTTTCCTggTgTCA gCAgATCCCTgCAgAAgTgT	61	NRF2	gCgACggAAAgAgTATgAgC gTTggCAgATCCACTggTTT	60
PGC1α	TCAgTCCTCACTggTggACA TgCTTCgTCgTCAAAAACAg	60	TFAM	AgATTggggTCgggTCACT CAAgACAgATgAAAACCACCTC	61
			mtSSB	TgTgAAAAAggggTCTCgAA TggCCAAAgAATCATCC	60

Table 1. Table and conditions used for RT-PCR. Tº An.: Annealing temperature; pS2: presenilin 2; PGC1α: peroxisome proliferator-activated receptor-gamma coactivator-1alpha; NRF1 and NRF2: nuclear respiratory factor 1 and 2; TFAM: mitochondrial transcription factor A; mtSSB: mitochondrial single strand DNA binding protein.

The resulting PCR products were resolved on a 2% agarose gel in 45 mM Tris-borate- EDTA 1 mM buffer (pH 8.0) and visualized by ethidium bromide staining. Bands were analysed with a Chemidoc XRS densitometer (Biorad).The Ct values of the real-time PCR were analysed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (MultiDAnalises, Sweden).

mtDNA quantification

DNA was isolated from cultured cells using TriPure® isolation reagent following the manufacture protocol and used to quantify mtDNA. 5 ng of the total DNA (quantified using a spectrophotometer set at 260 nm) was amplified using specific primers for 18S and the NADH dehydrogenase subunit 4, with SYBR Green technology on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland).

Total reaction volume was 10 µL, containing 6,5 µl Lightcycler® 480 SYBR Green I Master, 0.5 µM of the sense and antisense specific primers and 2,5 µl of the cDNA template. The amplification program consisted of a preincubation step for denaturation (5 min, 95°C) followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, 6°C for 18S and mtDNA), and an extension step (12 s, 72°C). A negative control without cDNA template was run in each assay. The primers used were forward 5'-ggA CAC ggA CAg gAT TgA CA-3' and reverse 5'-ACC CAC ggA ATC gAg AAA gA-3' for 18S and forward 5'-CgT gAC TCC TAC CCC TCA CA-3' and reverse 5'-ATC ggg TgA TgA TAg CCA Ag- 3' for the NADH dehydrogenase subunit 4 (mtDNA).

The resulting PCR products were analysed with the same method before described in section 2.5.

Western blotting

Proteins were isolated from cultured cells using TriPure® isolation reagent following the manufacture protocol. For western blot analysis 50 µg of protein from cell lysate was fractioned by SDS-PAGE (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in TBS-T (20 mM Tris HCl, 0.13 mM NaCl, and 0.1% Tween 20). Antisera against COXIV (Mitoscience, OR, USA), ATPasa, ERα, ERβ TFAM and Tubulin (Santa Cruz Biotechnologies, CA, USA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Biorad) western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Biorad) and analysed with Quantity One Software (Biorad).

Visualization of mitochondria

Mitochondria were visualized with the mitochondria-specific dye MitoTracker Green as previously described by Rodriguez-Enriquez et al. [27]. The confocal images were adquired on a Leica TCS-SP2 confocal microscope.

Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are presented as means ± standard error of the mean (SEM). Statistical differences between control and E2-treated cells were analysed by Student's t-test. Statistical significance was set at $P < 0.05$.

Results

E2 had an effect over estrogen receptor alpha and beta (ERα and ERβ), as shown in Table 2, with a statistically significant decreases in both MCF-7 and T47D cell lines. We used two breast cancer cell lines with different estrogen receptor levels and ratios (Table 2). It can be observed in Table 3 that E2 had a proliferative effect in MCF-7 cell line but not in T47D cell line, with all

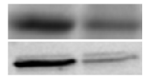
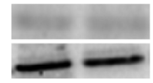
	MCF-7			T47D		
	Control	17β-Estradiol	MCF7 Control E2	Control	17β-Estradiol	T47D Control E2
ERα (AU)	100 ± 11	40.1 ± 9.3*		13.7 ± 1.8	10.1 ± 1.6	
ERβ (AU)	100 ± 21	35.5 ± 8.2*		64.4 ± 8.4	50.3 ± 8.3	
ERα/ERβ	1.00	1.13		0.21	0.20	

Table 2. Estrogen receptor alpha and beta protein levels in MCF-7 and T47D breast cancer cell lines after 48 hours E2-treatment. ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; A.U.: Arbitrary units. Data represent the means ± SEM (n=6). Values of control (vehicle-treated) cells of MCF-7 were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

	MCF-7		T47D	
	Control	E2	Control	E2
MTT assay (%)	100 ± 4	111 ± 3*	100 ± 7	96.7 ± 5.0
mtDNA/18S	100 ± 6	114 ± 4*	100 ± 3	75.0 ± 4.0*
NAO (%)	100 ± 1	104 ± 1*	100 ± 2	100 ± 2
NAO/mtDNA Ratio	1.00	0.91	1.00	1.33

Table 3. Cellular proliferation and mitochondrial proliferation and differentiation in MCF-7 and T47D breast cancer cell lines. mtDNA: mitochondrial DNA; NAO: 10-N-nonyl-acridine orange. A.U.: Arbitrary units. Data represent the means ± SEM (n=6). Values of control (vehicle-treated) cells were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

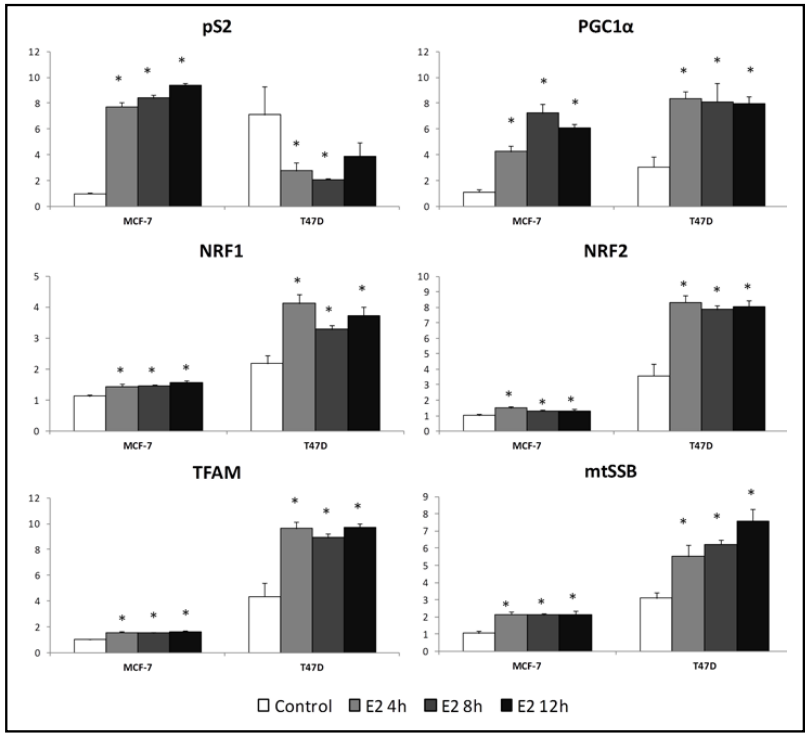


Fig. 1. Mitochondrial biogenesis mRNA expression in MCF-7 and T47D breast cancer cell lines after E2-treatment for 48h. mRNA levels were detected by RT-PCR using SYBR Green technology. pS2: presenilin 2; PGC1α: peroxisome proliferator-activated receptor-gamma coactivator-1alpha; NRF1 and NRF2: nuclear respiratory factor 1 and 2; TFAM: mitochondrial transcription factor A; mtSSB: mitochondrial single strand DNA binding protein. E2: 17β-estradiol. Bars represent the means ± SEM (n=6) with the values of control (vehicle-treated) cells of MCF-7 were set at 1. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

results obtained by the MTT assay. The MCF-7 cell line showed significant increases in mtDNA copy number (14 %) and cardiolipin content (4 %). However, T47D cell line showed a statistically significant decrease

	MCF-7			T47D		
	Control	E2	MCF7 Control E2	Control	E2	T47D Control E2
TFAM A.u. (%)	100 ± 9	65.1 ± 6*		100 ± 16	113 ± 13	
COXIV A.u. (%)	100 ± 22	39.9 ± 8.2*		100 ± 45	92.9 ± 29.6	
ATPase A.u. (%)	100 ± 35	100 ± 43		100 ± 4	87.5 ± 2.5*	
ATPase/COX Ratio	1.00	2.51		1.00	0.94	

Table 4. TFAM, COXIV and ATPase protein levels in MCF-7 and T47D breast cancer cell lines. TFAM: mitochondrial transcription factor A; COXIV: citochrome c oxidase subunit IV. A.U.: Arbitrary units. Data represent the means ± SEM (n=6). Values of control (vehicle-treated) cells were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

	MCF-7		T47D	
	Control	E2	Control	E2
CS A.U. (%)	100 ± 8	72.4 ± 10*	100 ± 6	127 ± 18
COX A.U. (%)	100 ± 9	56.7 ± 8*	100 ± 7	103 ± 12*
ATPase A.U. (%)	100 ± 4.0	83.8 ± 4.8*	100 ± 5	100 ± 6
ATPase/COX Ratio	1.00	1.48	1.00	0.97

Table 5. Cytrate Synthase, Cytochrome c oxidase and ATPase enzymatic assay in MCF-7 and T47D breast cancer cell lines. CS: Cytrate synthase; COX: citochrome c oxidase. A.U.: Arbitrary units. Data represent the means ± SEM (n=6). Values of control (vehicle-treated) cells were set at 100. *Significant difference between E2-treated and non-treated cells (Student's test; P<0.05, n=6).

in mtDNA copy number (25 %) but not in cardiolipin content. For this reason, the NAO/mtDNA ratio was higher in T47D cell line than in MCF-7 cell line (0,91 and 1,33 respect to control as 1,00) after treatment with E2.

In an attempt to localize these differences in mitochondrial biogenesis, we checked the effect of E2 on mRNA expression of different genes involved in this pathway. As shown in Fig. 1, PGC1α, NRF1, NRF2, TFAM and mtSSB had a time-dependent increase with the E2 treatment. It is worth noting that initial mRNA levels and the amount of the increase observed differed, depending on the cell line. Moreover, these effects were also monitored by the TFAM, COX and ATPase protein levels. Table 4 shows the decrease in TFAM and COX protein levels in MCF-7 cell line (35 % and 60 %, respectively), while in T47D cell line there were no changes after 48 hours of E2-treatment. Furthermore, ATPase protein levels decreased in the T47D cell line (22 %). All of these changes are statistically significant. Consequently, the ATPase/COX ratio showed an increase in MCF-7 cell line and a decrease in T47D cell line.

At this point of the experiment, it was considered of interest to investigate the activities of the mitochondrial-related enzymes. As shown in Table 5, citrate synthase activity showed a statistically significant decrease in the

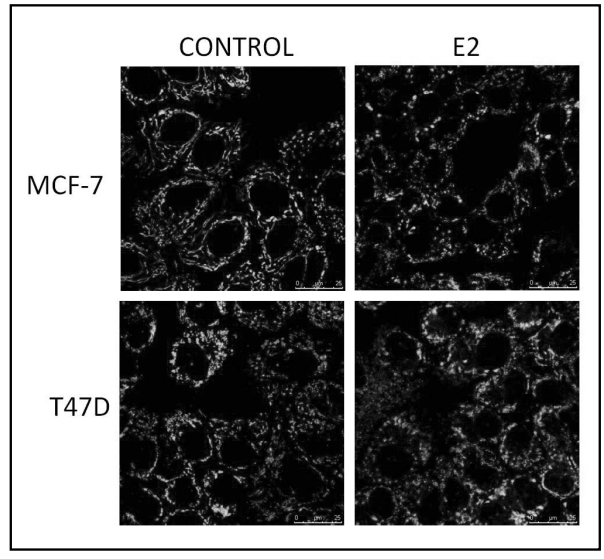


Fig. 2. Changes in mitochondrial morphology in response to E2. Breast cancer cells were cultured normally and labeled with Mitotracker Green nad imaged, as described in Material and Methods.

MCF-7 cell line (28 %) while the T47D cell line showed an increase (27 %). Furthermore, COX and ATPase activities were lower in MCF-7 E2-treated cells than in treated controls. Moreover, as shown in Fig. 2, morphology of mitochondria were different in E2-treated cell line, while T47D cell line not showed differences in mitochondrial morphology after treatment.

Discussion

E2 increased mitochondrial biogenesis in MCF-7 and T47D breast cancer cell lines. However, there is a clear difference in the response in the mitochondrial biogenesis of these cell lines when faced this E2 stimulation. MCF-7 cell line (high ERα/ERβ ratio) has mitochondria with lower mitochondrial function than T47D cell line (with low ERα/ERβ ratio) although this latter line had more functional mitochondria in response to the E2 treatment. These results would be in agreement with the different physiological functions of estrogens displayed once bound to their corresponding receptors [8, 21, 28].

In the MCF-7 cell line, which has highest reported ERα/ERβ ratio [29, 30] E2 increased mtDNA copy number, although mitochondrial function was diminished since Citrate Synthase, cytochrome c oxidase and ATPase activities suffered a statistically significant decreases. Moreover, cytochrome c oxidase protein levels were also lower after E2 treatment. However, for the T47D cell line, with lower ERα levels than the MCF-7 cell line, E2, despite a decrease in mtDNA copy number per cell, maintained mitochondrial function, as enzymatic activities did not decrease per cell with E2. This is noteworthy as given that even citrate synthase has a tendency to increase, the enzymatic activities indicate functional mitochondria.

These estrogen receptor isoform dependent differences in mitochondria fit with the dual effect of E2 found in different tissues [31]. Thus, E2 has a beneficial role in some tissues such as: heart, brain, muscle, brown adipose tissue, and liver [32-34], where this hormone increases mitochondrial function. This effect agrees with studies that shown more functional mitochondria in female rats and these findings have been associated with a lower oxidative stress [35, 36]. However, E2 has a role in tissue malignancy in: ovary, mammary gland and uterus; and has been related to lifetime exposure to estradiol and other cancer risk factors [8]. These effects could be attributed to the difference in the predominant estrogen receptor isoform in the tissues [9].

Expression of mitochondrial biogenesis genes was increased in MCF-7 cell line, but to a lesser degree than in T47D cell line. Amongst all genes studied, it should be underscored that TFAM, had a higher expression in the T47D cell line than in the MCF-7 in response to E2 treatment. Moreover, there was a decrease in TFAM protein levels after a 48h E2 treatment respect to its control in the MCF-7 cell line, while in the T47D cell line, there was an increase in this protein. High TFAM levels are known to stimulate differentiation, while low levels of TFAM stimulate mitochondrial proliferation [37-40]. Thus, the resultant TFAM levels would be in agreement with mitochondrial functionality observed in these breast cancer cell lines. Another important mitochondria biogenesis factor is NRF-2, which also increased with E2, although differed in its magnitude of expression according to cell line, showing a higher increase in the T47D cell line than in the MCF-7cell line. NRF-2 has been related to mitochondrial maturation, owing to the fact that it regulates traffic and assembly related-genes of the mitochondrial proteins [41]. Thus, the effect of E2 on mitochondrial biogenesis genes indicate different responses in the genes involved in mitochondrial biogenesis, as the MCF-7 cell line showed a response to proliferation while the T47D showed a differentiation response.

Lately, mechanisms has been proposed to modulate mitochondrial number and the deleterious effects from signals like ROS or UV light known as mitoptosis [42]. Mitoptosis in normal conditions removes dysfunctional mitochondria [43]. There are studies that show that in some cancers, mitoptosis is inhibited, and for this reason, the damaged mitochondria accumulated in the cells would create mitochondrial dysfunction, affecting mitochondrial biogenesis [44]. Mitoptosis could be explained by the increase in MCF-7 mtDNA copy number caused by an accumulation of dysfunctional mitochondria, or as well by the decrease observed in the T47D cell line, when removing the damaged mitochondria accumulated in the pretreatment with Charcoal, where the beneficial effects of E2 in this cell line there are not present.

In fact, if we observed mitochondrial morphology, we appreciate a several change in morphology of E2-treated MCF-7 mitochondria according to all the results.

In summing up, the results suggest that E2 acts over function, proliferation and mitochondrial differentiation, according to the estrogen receptor ratio (ERα/ERβ), with lesser mitochondrial activity present in the cell line with a predominance of ERα, while in contrast, the cell line with a lower ERα/ERβ ratio had more functional mitochondria.

Thus, our results reinforce the idea that depending on the estrogen receptor endowment and its subtype relation, breast tumors could have different response to treatment and prognosis, with ERβ clinical analyses could provide an indicator to the disease progression and provide new insight of the role of mitochondria and estrogen receptors in breast cancer.

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References

1 Clemons M, Goss P: Estrogen and the risk of breast cancer. N Engl J Med 2001;344:276-285.

2 Harrington WR, Sheng S, Barnett DH, Petz LN, Katzenellenbogen JA, Katzenellenbogen BS: Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Mol Cell Endocrinol 2003;206:13-22.

3 Yager JD, Liehr JG: Molecular mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol 1996;36:203-232.

4 Pearce ST, Jordan VC: The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol 2004;50:3-22.

5 Pettersson K, Delaunay F, Gustafsson JA: Estrogen receptor beta acts as a dominant regulator of estrogen signaling. Oncogene 2000;19:4970-4978.

6 Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA: Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci U S A 1996;93:5925-5930.

7 Heldring N, Pike A, Andersson S, Matthews J, Cheng G, Hartman J, Tujague M, Strom A, Treuter E, Warner M, Gustafsson JA: Estrogen receptors: How do they signal and what are their targets. Physiol Rev 2007;87:905-931.

8 Palmieri C, Cheng GJ, Saji S, Zelada-Hedman M, Warri A, Weihua Z, Van Noorden S, Wahlstrom T, Coombes RC, Warner M, Gustafsson JA: Estrogen receptor beta in breast cancer. Endocr Relat Cancer 2002;9:1-13.

9 Drummond AE, Fuller PJ: The importance of erbeta signalling in the ovary. J Endocrinol 2010;205:15-23.

10 Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA: Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line t47d. Proc Natl Acad Sci USA 2004;101:1566-1571.

11 Pedram A, Razandi M, Wallace DC, Levin ER: Functional estrogen receptors in the mitochondria of breast cancer cells. Mol Biol Cell 2006;17:2125-2137.

12 Chen JQ, Eshete M, Alworth WL, Yager JD: Binding of mcf-7 cell mitochondrial proteins and recombinant human estrogen receptors alpha and beta to human mitochondrial DNA estrogen response elements. J Cell Biochem 2004;93:358-373.

13 Yang SH, Liu R, Perez EJ, Wen Y, Stevens SM, Jr., Valencia T, Brun-Zinkernagel AM, Prokai L, Will Y, Dykens J, Koulen P, Simpkins JW: Mitochondrial localization of estrogen receptor beta. Proc Natl Acad Sci USA 2004;101:4130-4135.

14 Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J, Roca P: Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. Free Radic Biol Med 2010;48:506-512.

15 Chen JQ, Cammarata PR, Baines CP, Yager JD: Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. Biochim Biophys Acta 2009;1793:1540-1570.

16 Felty Q, Xiong WC, Sun D, Sarkar S, Singh KP, Parkash J, Roy D: Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. Biochemistry 2005;44:6900-6909.

17 Felty Q, Roy D: Estrogen, mitochondria, and growth of cancer and non-cancer cells. J Carcinog 2005;4:1.

18 Parkash J, Felty Q, Roy D: Estrogen exerts a spatial and temporal influence on reactive oxygen species generation that precedes calcium uptake in high-capacity mitochondria: Implications for rapid nongenomic signaling of cell growth. Biochemistry 2006;45:2872-2881.

19 Addison R, McCormick DB: Biogenesis of flavoprotein and cytochrome components in hepatic mitochondria from riboflavin-deficient rats. Biochem Biophys Res Commun 1978;81:133-138.

20 Sogl B, Gellissen G, Wiesner RJ: Biogenesis of giant mitochondria during insect flight muscle development in the locust, locusta migratoria (l.). Transcription, translation and copy number of mitochondrial DNA. Eur J Biochem 2000;267:11-17.

21 Miro AM, Sastre-Serra J, Pons DG, Valle A, Roca P, Oliver J: 17beta-estradiol regulates oxidative stress in prostate cancer cell lines according to eralpha/erbeta ratio. J Steroid Biochem Mol Biol 2011;123:133-139.

22 Ivanova MM, Luken KH, Zimmer AS, Lenzo FL, Smith RJ, Arteel MW, Kollenberg TJ, Mattingly KA, Klinge CM: Tamoxifen increases nuclear respiratory factor 1 transcription by activating estrogen receptor {beta} and ap-1 recruitment to adjacent promoter binding sites. FASEB J 2011;25:1402-1416.

23 Mattingly KA, Ivanova MM, Riggs KA, Wickramasinghe NS, Barch MJ, Klinge CM: Estradiol stimulates transcription of nuclear respiratory factor-1 and increases mitochondrial biogenesis. Mol Endocrinol 2008;22:609-622.

24 Nakano K, Tarashima M, Tachikawa E, Noda N, Nakayama T, Sasaki K, Mizoguchi E, Matsuzaki M, Osawa M: Platelet mitochondrial evaluation during cytochrome c and dichloroacetate treatments of melas. Mitochondrion 2005;5:426-433.

25 Chrzanowska-Lightowlers ZM, Turnbull DM, Lightowlers RN: A microtiter plate assay for cytochrome c oxidase in permeabilized whole cells. Anal Biochem 1993;214:45-49.

26 Ragan C, Wilson M, Darley-Usmar V, Lowe P: Subfractionation of mitochondria and isolation of the proteins of oxidative phosphorylation. IRL Press, OXFORD 1987

27 Rodriguez-Enriquez S, Kai Y, Maldonado E, Currin RT, Lemasters JJ: Roles of mitophagy and the mitochondrial permeability transition in remodeling of cultured rat hepatocytes. Autophagy 2009;5:1099-1106.

28 Chen JQ, Yager JD, Russo J: Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. Biochim Biophys Acta 2005;1746:1-17.

29 Buterin T, Koch C, Naegeli H: Convergent transcriptional profiles induced by endogenous estrogen and distinct xenoestrogens in breast cancer cells. Carcinogenesis 2006;27:1567-1578.

30 Sartippour MR, Pietras R, Marquez-Garban DC, Chen HW, Heber D, Henning SM, Sartippour G, Zhang L, Lu M, Weinberg O, Rao JY, Brooks MN: The combination of green tea and tamoxifen is effective against breast cancer. Carcinogenesis 2006;27:2424-2433.

31 Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA: Mechanisms of estrogen action. Physiol Rev 2001;81:1535-1565.

32 Moreira PI, Custodio JB, Nunes E, Oliveira PJ, Moreno A, Seica R, Oliveira CR, Santos MS: Mitochondria from distinct tissues are differently affected by 17beta-estradiol and tamoxifen. J Steroid Biochem Mol Biol 2011;123:8-16.

33 Rodriguez-Cuenca S, Monjo M, Gianotti M, Proenza AM, Roca P: Expression of mitochondrial biogenesis-signaling factors in brown adipocytes is influenced specifically by 17beta-estradiol, testosterone, and progesterone. Am J Physiol Endocrinol Metab 2007;292:E340-346.

34 Nadal-Casellas A, Amengual-Cladera E, Proenza AM, Llado I, Gianotti M: Long-term high-fat-diet feeding impairs mitochondrial biogenesis in liver of male and female rats. Cell Physiol Biochem 2010;26:291-302.

35 Justo R, Boada J, Frontera M, Oliver J, Bermudez J, Gianotti M: Gender dimorphism in rat liver mitochondrial oxidative metabolism and biogenesis. Am J Physiol Cell Physiol 2005;289:C372-378.

36 Colom B, Alcolea MP, Valle A, Oliver J, Roca P, Garcia-Palmer FJ: Skeletal muscle of female rats exhibit higher mitochondrial mass and oxidative-phosphorylative capacities compared to males. Cell Physiol Biochem 2007;19:205-212.

37 Topper JN, Clayton DA: Identification of transcriptional regulatory elements in human mitochondrial DNA by linker substitution analysis. Mol Cell Biol 1989;9:1200-1211.

38 Dairaghi DJ, Shadel GS, Clayton DA: Human mitochondrial transcription factor a and promoter spacing integrity are required for transcription initiation. Biochim Biophys Acta 1995;1271:127-134.

39 Falkenberg M, Gaspari M, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM: Mitochondrial transcription factors b1 and b2 activate transcription of human mtdna. Nat Genet 2002;31:289-294.

40 Fernandez-Silva P, Enriquez JA, Montoya J: Replication and transcription of mammalian mitochondrial DNA. Exp Physiol 2003;88:41-56.

41 Hood DA, Irrcher I, Ljubicic V, Joseph AM: Coordination of metabolic plasticity in skeletal muscle. J Exp Biol 2006;209:2265-2275.

42 Grandemange S, Herzig S, Martinou JC: Mitochondrial dynamics and cancer. Semin Cancer Biol 2009;19:50-56.

43 Mijaljica D, Prescott M, Devenish RJ: Different fates of mitochondria: Alternative ways for degradation? Autophagy 2007;3:4-9.

44 Zhang Y, Qi H, Taylor R, Xu W, Liu LF, Jin S: The role of autophagy in mitochondria maintenance: Characterization of mitochondrial functions in autophagy-deficient s. Cerevisiae strains. Autophagy 2007;3:337-346.

Manuscript 8

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The ERalpha/ERbeta Ratio Determines Oxidative Stress in Breast Cancer Cell Lines in Response to 17Beta-Estradiol

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ABSTRACT

The effects of 17beta-estradiol (E2) are mediated through activation of estrogen receptors (ER): ERalpha and ERbeta. It is known that ERalpha/ERbeta ratio is higher in breast tumors than in normal tissue. Since antioxidant enzymes and uncoupling proteins (UCPs) are reactive oxygen species (ROS) production and mitochondrial biogenesis regulators, our aim was to study the E2-effect on oxidative stress, antioxidant enzyme expression, and UCPs in breast cancer cell lines with different ERalpha/ERbeta ratios. The lower ERalpha/ERbeta ratio T47D cell line showed low ROS production and high UCP5 levels. However, the higher ERalpha/ERbeta ratio MCF-7 cell line showed an up-regulation of antioxidant enzymes and UCPs, yet exhibited high oxidative stress. As a result, a decrease in antioxidant enzyme activities and UCP2 protein levels, coupled with an increase in oxidative damage was found. On the whole, these results show different E2-effects on oxidative stress regulation, modulating UCPs, and antioxidant enzymes, which were ERalpha/ERbeta ratio dependent in breast cancer cell lines. J. Cell. Biochem. 113: 3178–3185, 2012.

KEY WORDS: ESTROGEN RECEPTORS; BREAST CANCER; ERalpha/ERbeta RATIO; OXIDATIVE STRESS; REACTIVE OXYGEN SPECIES; ANTIOXIDANT ENZYMES

Breast cancer is the most commonly diagnosed malignancy in women of developed countries [Siegel et al., 2011]. Estrogens, and particularly 17beta-estradiol (E2), play an important role in physiology of the reproductive process, such as the development of the mammary gland in menarche [Russo et al., 1999]. However, it is known that E2 is also implicated in pathological processes such as in breast carcinogenesis. Lifetime exposure to estradiol is associated with an increased risk of breast cancer development, and contributes to tumor progression [Clemons and Goss, 2001; Nilsson et al., 2001]. Classically, it has been described that estrogens induce cancerous transformations because they cause growth on the epithelial cells of the mammary gland, and this proliferation increases susceptibility to the acquirement of mutations during DNA replication [Chen et al., 2009]. Moreover, there are a number of reports that point out a main role of oxidative stress in E2-induced breast cancer pathogenesis, establishing a relation between estrogen-induced breast cancer and oxidative stress [Roy et al., 2007; Okoh et al., 2011]. E2 action is mediated by its binding to two estrogen receptor (ER) subtypes, ERalpha and ERbeta [Jensen and DeSombre, 1973; Kuiper et al., 1996]. In breast cancer, ERalpha plays an important proliferative role, increasing tumor growth and oxidative stress [Mense et al., 2008]. However, the role of ERbeta is not yet clear, because this receptor could mediate effects opposed to those of ERalpha. ERbeta appears to reduce cell proliferation induced by ERalpha and promote differentiation in the mammary gland [Strom et al., 2004; Warner and Gustafsson, 2010]. In fact, E2 also affects non-reproductive tissues such as: skeletal muscle, cardiovascular, brain, liver, and adipose, were it has been shown to play a protective role [Valle et al., 2007, 2008; Colom et al., 2007ab; Irwin et al., 2008; Guevara et al., 2009; Song et al., 2009;

Mercedes Nadal-Serrano and Jorge Sastre-Serra contributed equally to the present work.

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Borras et al., 2010]. Moreover, other authors have described a different response to E2 in prostate cancer cell lines, which is ERalpha/ERbeta ratio dependant [Miro et al., 2011]. This different tissue-specific effect of E2 could thus be attributed to the different ERalpha and ERbeta ratios within these tissues.

Mitochondria are the main source of reactive oxygen species (ROS) in the cell [Fariss et al., 2005]. The imbalance between the oxidative damage caused by E2-induced ROS production and the ability of cells to metabolize them, coupled with the excessive accumulation of ROS that overwhelms cellular defenses, leads to oxidative stress [Ziech et al., 2011]. Control mechanisms of ROS play a crucial role in tumor development. The systems that can protect against oxidative damages are antioxidant systems and uncoupling proteins (UCPs) [Fariss et al., 2005; Echtay, 2007]. Mitochondrial biogenesis and ROS production are under the influence of estrogen, and for this reason, some authors have given estrogens a new role in the process of carcinogenesis and in the modulation of mitochondrial function [Chen et al., 2009; Sastre-Serra et al., 2010]. The damage exerted by ROS on mitochondria and mitochondrial DNA may lead to a high degree of mitochondrial dysfunction, and in turn, to a high ROS production, creating a positive feedback of ROS amplification [Fariss et al., 2005].

UCPs have been proposed to be involved in pathological conditions such as cancer [Derdak et al., 2008; Azzu et al., 2010; Sastre-Serra et al., 2010; Baffy et al., 2011]. There is abundant evidence about UCPs, such as their important role as metabolic regulators in attenuating free radical formation [Baffy, 2010; Sastre-Serra et al., 2010].

Thus, the aim of this article is to investigate the effect of E2 on the oxidative stress parameters, and the role of UCPs in this process, in breast cancer cell lines with different ERalpha/ERbeta ratios.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

17β-Estradiol (E2) was purchased from Sigma–Aldrich (St. Louis, MO). Routine chemicals used were obtained from Roche (Barcelona, Spain), Sigma–Aldrich, Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA).

CELL CULTURE AND TREATMENTS

Human breast cancer cell lines MCF-7 (ERalpha and ERbeta positive, with predominance of ERalpha), T47D (ERalpha and ERbeta positive, with predominance of ERbeta), and MDA-MB-231 (ERalpha negative and ERbeta positive) were purchased from American Type Culture Collection (ATCC; Manassas, VA) and cultured in 100 mm culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin and streptomycin) in a 5% CO_2 humidified atmosphere at 37°C. To evaluate the effects of E2, cells were grown in a phenol red-free DMEM containing 10% charcoal-stripped FBS for 24 h before treatment. Estrogen treatments were begun when cell cultures reached confluence and by the addition of 1 nmol/L E2 for 24 h for enzymatic activity determination and mRNA expression assays, and for 48 h, for Western blot determination.

CATALASE, SUPEROXIDE DISMUTASE, GLUTATHIONE PEROXIDASE, AND GLUTATHIONE REDUCTASE ACTIVITY

Cells were harvested by scraping the culture into a PBS buffer and then were centrifuged at 5,000 rpm for 5 min at 4°C to remove cell debris. Cell pellets were resuspended in RNAse-free water. Lysates were kept on ice and protein content was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). Subsequent enzyme assays were carried out immediately.

Catalase (CAT; EC 1.11.1.6) activity was measured by the method of Aebi based on the consumption of H_2O_2 at 240 nm [Aebi, 1984] in a UV-2401 PC spectrophotometer (Shimadzu) at 37°C.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by following the reduction of cytochrome c by measuring the absorbance at 550 nm on a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.) at 37°C, as described previously [Quick et al., 2000].

Glutathione peroxidase (GPx; 1.11.1.9) activity was determined by the *tert*-butyl-hydroperoxide/glutathione oxidized (GSSG) recycling method that monitored the decrease in absorbance of NADPH at 340 nm [Paglia and Valentine, 1967].

Glutathione reductase (GRd; 1.8.1.7) activity was measured by following the oxidation of NADPH at 340 nm, according to an adaptation of the Carlberg method [Carlberg and Mannervik, 1985].

MEASUREMENT OF CARBONYL CONTENT

The presence of carbonyl groups, a measure of protein oxidation, was determined by an immunological method using the OxySelect™ Protein Carbonyl Immunoblot kit (Cell Biolabs, San Diego, CA). Protein carbonyls were detected by labeling the 20 μg sample of total protein with 2,4-dinitrophenylhydrazine (DNPH) for 5 min and then read according to the manufacturer's instructions. For this assay carbonyl groups were derivitized after electrophoresis and transblotted to avoid alterations of the electrophoretic properties of the stained protein. After incubation with the DNP-antibody, bands were visualized using the Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad Laboratories) and analyzed with Quantity One software (Bio-Rad Laboratories).

MEASUREMENT OF 4-HYDROXY-2-NONENAL

For 4-hydroxy-2-nonenal (4-HNE) analysis, 40 μg of protein from cell lysate was transferred onto a nitrocellulose membrane. Antiserum against 4-HNE (HNE11-S) was used as primary antibody (Alpha Diagnostic International, San Antonio, TX). Bands were visualized by Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

MEASUREMENT OF ROS LEVELS BY FLOW CYTOMETRY

The ROS levels in the breast cancer cell lines were measured as described previously by Sastre-Serra et al. [2010], measuring fluorescence of DCF using an Epics XL flow cytometer (Beckman-Coulter, Miami, FL). The green fluorescence was measured using the FL-1 setting (log mode) after electronically gating out the cell debris.

Ten thousand events were acquired and analyzed with Expo32 ADC analysis software (Beckman Coulter).

ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIAL BY FLOW CYTOMETRY

Cell were dyed with 250 nM tetramethylrhodamine methyl ester (TMRM) for 15 min, washed by PBS containing 10 mM glucose, and analyzed by flow cytometry as described above. TMRM is a lipophilic cationic dye that accumulates within the inner membrane of functional mitochondria according to the membrane potential. The orange-red fluorescence was measured using the FL-2 setting (log mode) after the cell debris were electronically gated out. In each analysis, ten thousand events were recorded and analyzed with Expo32 ADC analysis software (Beckman Coulter; $n = 12$).

MTT ASSAY

MTT assay was performed as described before [Sastre-Serra et al., 2010]. Absorbance of the converted dye was measured at 570 nm with background subtraction at 610 nm using a PowerWave XS Microplate Spectrophotometer (BioTek Instruments, Inc.; $n = 8$).

WESTERN BLOT ANALYSIS

Cells were harvested by scraping them out with lysis buffer [Miro et al., 2011] and disrupted by sonication. Afterwards, protein content was determined with a bicinchoninic acid protein assay kit (Pierce). For Western blot analysis, 40 μg of protein from cell lysate was separated on a 12% SDS–PAGE gel and electrotransferred onto nitrocellulose membranes. After the transfer, unspecific biding sites on the membranes were blocked in 5% non-fat milk in Tris-buffered saline-Tween (TBS with 0.05% Tween-20). Antisera against ERα (D-12), ERβ (H-150), and α-tubulina (B-7), the latter used as a housekeeping protein, were from Santa Cruz Biotechnology (Santa Cruz, CA); and UCP2 (UCP21-A) and UCP5 (UCP51-A) were from Alpha Diagnostic and were used as primary antibodies. Finally, protein bands were visualized by Immun-Star© Western C© Chemiluminescent Kit (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analyzed with Quantity One Software (Bio-Rad).

REAL-TIME QUANTITATIVE PCR OF ANTIOXIDANT SYSTEMS AND UCPs

Total RNA was isolated from MCF-7, T47D and MDA-MB-23I cells, using TriPure® isolation reagent following the manufacture's recommendations and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42°C for 60 min with 25 U MuLV reverse transcriptase in a 10 μl volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNase inhibitor, and 500 μM of each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−80°C) until the PCR reactions were carried out.

PCR was done for seven target genes, Mn-SOD, CuZn-SOD, CAT, GPx, GRd, ucp2 and ucp5, with 18S ribosomal RNA (18S) used as a housekeeping gene. PCR was carried out using specific primers (see Table I) and SYBR Green Technology© on a LightCycler 480 System II Rapid Thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μl, containing 6.5 μl Lightcycler© 480 SYBR Green I Master, 0.5 μM of sense and antisense specific primers, and 2.5 μl of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95°C), followed by 45 cycles consisting of a denaturation step (10 s, 95°C), an annealing step (10 s, 60°C for UCP2, UCP5; 61°C for CAT, GPx, and 18S; 64°C for GRd, Mn-SOD, and CuZn-SOD), and an extension step (12 s, 72°C min). A negative control lacking cDNA template was run in each assay.

The resulting PCR products were resolved on a 2% agarose gel in 45 mM Tris-borate-EDTA 1 mM buffer (pH 8.0) and visualized by ethidium bromide staining. Bands were analyzed with a Chemidoc XRS densitometer (Biorad). The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

STATISTICS

The Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc, Chicago, IL) was used for all statistical analyses. Data are presented as means ± standard error of the mean (SEM). Statistical differences between control and E2-treated cells were analyzed by Student's *t*-test. Statistical significance was set at $P \leq 0.05$.

TABLE I. RT-PCR Primer Configurations and Conditions

Gene	Forward primer (5′–3′) / Reverse primer (5′–3′)	T° An. (°C)	Gene	Forward primer (5′–3′) / Reverse primer (5′–3′)	T° An. (°C)
18S	ggACACggACAggATTgACA ACCCACggAATCgAgAAAgA	61	Mn-SOD	CgTgCTCCCACACATCAATC TgAACgTCACCgAggAgAAg	64
ucp2	ggTggTCggAgATACCAAAg CTCgggCAATggTCTTgTAg	60	CuZn-SOD	TCAggAgACCATTgCATCATT CgCTTTCCTgTCTTTgTACTTTCTTC	64
ucp5	CAAgCCgTTggTCTCCTAAg CgTTTTCAATgTCACCCATC	60	GPx	gCggCggCCCAgTCggTgTA gAgCTTggggTCggTCATAA	61
CAT	CATCgCCACATgAATggATA CCAACTgggATgAgAgggTA	61	GRD	TCACgCAgTTACCAAAAggAAA CACACCCAAgTCCCCTgCATAT	64

T° An, annealing temperature; CAT, catalase; CuZn-SOD, copper and zinc superoxide dismutase; GPx, glutathione peroxidase; GRd, glutathione reductase; Mn-SOD, manganese superoxide dismutase; UCP2 and UCP5, uncoupling protein 2 and uncoupling protein 5.

TABLE II. Effects of E2 on ERalpha and ERbeta Protein Levels in MCF-7, T47D, and MDA-MB-231 Breast Cancer Cell Lines

	MCF-7		T47D		MDA-MB-231	
	Control	E2	Control	E2	Control	E2
ERα (AU)	100 ± 6	38.5 ± 3.7*	9.00 ± 0.49	7.48 ± 0.60*	UD	UD
ERβ (AU)	100 ± 15	43.7 ± 10.1*	79.2 ± 10.3	56.6 ± 1.1*	68.5 ± 6.0	63.6 ± 17.7
ERα/ERβ Ratio	1.00	0.88	0.11	0.13	–	–

ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; UD, undetected.
Data represent the means ± SEM (n = 6). Values of control MCF-7 cell line were set at 100.
*Significant difference between E2-treated and non-treated cell lines (Student's *t*-test; $P \leq 0.05$, n = 6).

RESULTS

ERalpha AND ERbeta PROTEIN EXPRESSION

Table II and Figure 1 show different ERalpha and ERbeta protein levels in the three cell lines studied. Our results showed a statistically significant decrease in ERs levels in E2-treated MCF-7 and T47D cell lines (61% and 17% for ERalpha, respectively; 56% and 28% for ERbeta, respectively). ERalpha/ERbeta ratio was calculated and, as shown in Table II, the MCF-7 cell line showed the highest value; while MDA-MB-231 had a zero value, because MDA-MB-231 is a negative-ERalpha cell line, and indicates undetected values of ERalpha.

EFFECTS OF E2 ON CELL PROLIFERATION, MITOCHONDRIAL MEMBRANE POTENTIAL, AND OXIDATIVE STRESS

In Table III it can be observed that E2 had a proliferative effect in MCF-7 cell line (11%) but not in T47D and MDA-MB-231 cells lines, with the results obtained by the MTT assay. Moreover, E2-treated MCF-7 cells showed a statistically significant increase in mitochondrial membrane potential (10%) measured by flow cytometry with TMRM fluorescence (Table III). Furthermore, the effect of 48 h treatment of 1 nmol/L E2 on oxidative stress in the cell lines studied is shown in Table III. Specifically, ROS levels were determined by oxidation of DCFDA, and a statistically significant increase (33%) was observed in E2-treated MCF-7 cells, whereas E2-treated T47D and MDA-MB-231 cells showed a significant decrease of ROS levels (35% and 11%, respectively). MCF-7 treated with E2 showed an increase of protein carbonyl content ($P = 0.059$) and 4-HNE content (indicator of lipid peroxidation), showed a significant increase (76%) in E2-treated MCF-7.

ANTIOXIDANT ENZYMES AND UCPs GENE EXPRESSION

To further examine the possible mechanisms involved in the control of oxidative stress, the main antioxidant enzyme genes and the genes encoding proteins UCP2 and UCP5 were analyzed and the results are shown in Table IV. The cell line with the highest ERalpha/ERbeta ratio, MCF-7, showed a significant increase of Mn-SOD and CuZn-SOD genes (22% and 34%, respectively), which also showed an enhancement of ucp2 and ucp5 (21% and 16%, respectively), after treatment with E2. T47D and MDA-MB-231 E2-treated cell lines did not show any important changes in gene expressions.

ANTIOXIDANT ENZYMES ACTIVITIES AND UCPs PROTEIN LEVELS

To study the relevance of antioxidant activity under E2-related oxidative stress situations, the activity of antioxidant enzymes (Table V), as well as the UCP2 and UCP5 protein levels were determined (Fig. 2). Representative bands are shown in Figure 1.

The enzymatic activity in the E2-treated MCF-7 cell lines was significantly lower for all of the antioxidant enzymes studied (CAT (37%), SOD (37%), and GRd (54%)), than in the vehicle-treated cells; and with no GPx activity. The lack of detected activity of this enzyme is not surprising as it has been previously reported that

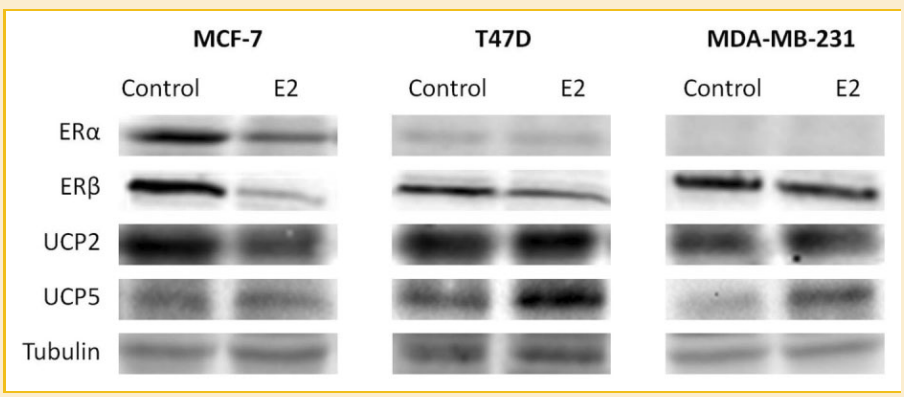

Fig. 1. Representative bands of Western blot are shown. ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; UCP2: uncoupling protein 2; UCP5: uncoupling protein 5 and Tubulina as a housekeeping protein.

TABLE III. Cell Proliferation, Mitochondrial Membrane Potential, ROS Levels, and Oxidative Damage Parameters in MCF-7, T47D, and MDA-MB-231 Cell Lines After E2 Treatment

	MCF-7		T47D		MDA-MB-231	
	Control	E2	Control	E2	Control	E2
MTT assay (AU)	100 ± 4	111 ± 3*	100 ± 6	103 ± 2	100 ± 1	103 ± 3
TMRM fluorescence (AU)	100 ± 3	110 ± 5*	100 ± 5	100 ± 8	100 ± 4	102 ± 1
ROS levels (AU)	100 ± 11	133 ± 14*	100 ± 5	65.2 ± 0.3*	100 ± 3	88.7 ± 3.9*
Carbonil content (AU)	100 ± 13	125 ± 4	100 ± 6	96.5 ± 12.8	100 ± 11	110 ± 17
4-HNE (AU)	100 ± 9	176 ± 16*	100 ± 9	123 ± 10	100 ± 9	124 ± 16

AU, arbitrary units; 4-HNE, 4-hydroxy-2-nonenal; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester.
Data represent the means ± SEM (n = 6). Values of control MCF-7, T47D, and MDA-MB-231 cell lines were set at 100.
*Significant difference between E2-treated and non-treated cell lines (Student's *t*-test; P≤0.05, n = 6).

ERalpha-positive breast cancer cell lines have very low GPx expression, and this would explain the absence of GPx activity in this line [Esworthy et al., 1995]. However, the cell lines with either lower ERalpha/ERbeta ratio or those with only ERbeta (T47D and MDA-MB-231, respectively) did not show changes in the activity of the main antioxidant enzymes, as shown in Table V.

Moreover, in relation to the UCPs, it was observed that in cells with high amounts of ERbeta or only with ERbeta (T47D and MDA-MB-231, respectively), E2 treatment significantly increased UCP5 protein levels (51% in the T47D cells and 81% in the MDA-MB-231 cells), whereas in the cell line with the highest ERalpha/ERbeta ratio (MCF-7), the treatment with E2 caused a statistically significant decrease in UCP2 protein levels (35%) (Figs. 1 and 2).

DISCUSSION

The results obtained in these breast cancer cell lines show that the E2-effect on oxidative stress depends on the amount of ERs, ERalpha and ERbeta, and more importantly the ERalpha/ERbeta ratio. Therefore, in T47D cell line, with lower ERalpha/ERbeta ratio, or in MDA-MB-231 cell line, which only presents ERbeta, E2 treatment decreased ROS production and oxidative stress. However, in MCF-7 cell line, with a higher ERalpha/ERbeta ratio, treatment with E2 produced an increase in both ROS production and oxidative stress. In agreement with this, the cell line with higher ratio showed a slight increase in mitochondrial membrane potential, that enhance mitochondrial free radical production [Brand et al., 2004; Negre-Salvayre et al., 1997; Sastre-Serra et al., 2010]. Moreover, E2 treatment increased the proliferation of the cell line with a higher ERalpha/ERbeta ratio. These effects could be explained at least in part to changes in antioxidant enzymes expression and/or activity, as well as modulation of the UCPs.

The T47D cell line, with a lower ERalpha/ERbeta ratio than the MCF-7 cell line, and the MDA-MB-231 cell line, which only expresses ERbeta, when treated with 1 nmol/L E2 (a physiological concentration) did not show changes in lipid and protein oxidative damage, although the T47D cell line showed a lower significant ROS levels after E2 treatment, and the MDA-MB-231 showed a tendency to reduce these levels. Regarding the antioxidant enzymes, it is worth noting that neither gene expression nor activity showed any change in the E2-treated T47D and MDA-MB-231 cell lines. It must be emphasized however, that after E2-treatment, UCP5 protein levels increased considerably in both cell lines. This fact may explain the lower ROS production, as previous studies that indicate an UCP decrease related to an increase in mitochondrial ROS production [Horimoto et al., 2004; Echtay, 2007; Derdak et al., 2008; Baffy et al., 2011].

Some of the effects of the E2 treatment in MCF-7 cells are in agreement with the results previously described by Sastre-Serra et al. [2010], who reported a greater oxidative damage and higher ROS levels, and moreover showed low Mn-SOD and CAT protein levels. In this present study we also found that E2 increased mitochondrial membrane potential, a fact that is strongly linked to

TABLE IV. Response of Antioxidant Enzymes and UCPs Expression in MCF-7, T47D, and MDA-MB-231 Cell Lines After E2 Treatment

	MCF-7		T47D		MDA-MB-231	
	Control	E2	Control	E2	Control	E2
Mn-SOD	1.00 ± 0.04	1.22 ± 0.05*	1.00 ± 0.18	1.06 ± 0.24	1.00 ± 0.09	1.01 ± 0.44
CuZn-SOD	1.00 ± 0.06	1.34 ± 0.03*	1.00 ± 0.14	1.01 ± 0.14	1.00 ± 0.05	1.20 ± 0.68
Catalase	1.00 ± 0.01	0.98 ± 0.00*	1.00 ± 0.02	1.00 ± 0.02	1.00 ± 0.01	1.01 ± 0.04
GPx	1.00 ± 0.19	0.83 ± 0.07	1.00 ± 0.27	0.98 ± 0.29	1.00 ± 0.07	0.96 ± 0.35
GRd	1.00 ± 0.02	0.93 ± 0.07	1.00 ± 0.20	0.92 ± 0.20	1.00 ± 0.08	0.88 ± 0.53
UPC2	1.00 ± 0.03	1.21 ± 0.08*	1.00 ± 0.08	1.17 ± 0.10	1.00 ± 0.09	0.80 ± 0.28*
UCP5	1.00 ± 0.03	1.16 ± 0.09*	1.00 ± 0.09	0.88 ± 0.16	1.00 ± 0.23	0.96 ± 0.33

CAT, catalase; CuZn-SOD, copper and zinc superoxide dismutase; GPx, glutathione peroxidase; GRd, glutathione reductase; Mn-SOD, manganese superoxide dismutase; UCP2 and UCP5, uncoupling protein 2 and uncoupling protein 5.
Data represent the means ± SEM (n = 6). Values of control (non-treated) cells of MCF-7, T47D, and MDA-MB-231 were set at 1.
*Significant difference between E2-treated and non-treated cells (Student's *t*-test; $P \leq 0.05$, n = 6).

TABLE V. Effects of E2 on Antioxidant Enzyme Activities

	MCF-7		T47D		MDA-MB-231	
	Control	E2	Control	E2	Control	E2
SOD (mUI/mg DNA)	45.5 ± 6.7	28.7 ± 5.0*	8.11 ± 0.88	6.95 ± 0.83	46.4 ± 7.4	48.9 ± 10.8
CAT (mUI/mg DNA)	64.1 ± 9.5	40.1 ± 2.9*	51.1 ± 3.6	49.7 ± 3.5	673 ± 170	539 ± 140
GPx (mUI/mg DNA)	UD	UD	UD	UD	0.04 ± 0.01	0.05 ± 0.01
GRd (mUI/mg DNA)	25.4 ± 3.3	11.5 ± 3.0*	13.1 ± 0.9	14.8 ± 2.3	9.8 ± 1.8	6.5 ± 0.8

CAT, catalase; GPx, glutathione peroxidase; GRd, glutathione reductase; SOD, superoxide dismutase; UD, undetected.
Data represent the means ± SEM (n = 6).
*Significant difference between E2-treated and non-treated cells (Student's *t*-test; $P \leq 0.05$, n = 6).

ROS production [Hansford et al., 1997; Korshunov et al., 1997; Brand et al., 2004]. Moreover this paper reports that E2 increases Mn-SOD, CuZn-SOD, and UCP2 and UCP5 mRNA levels and that some authors have also found an up-regulation of the expression of relation-stress antioxidant enzymes [Borras et al., 2005] and UCPs [Brand et al., 2004; Horimoto et al., 2004; Derdak et al., 2006] which agree with these findings, in this current experiment, the increase in antioxidant enzyme gene expression was not followed by an increase in enzymatic activity, and instead an important reduction of this activity was detected. However, this increase in antioxidant gene expression, unlike their activity, suggests that these enzymes could be target for own oxidative stress, and this has recently been described by Yamakura and Kawasaki [2010] for Mn-SOD, where an increase in oxidative stress induced post-translational modifications that caused an inactivation or down-regulation of Mn-SOD activity. Subsequently it is quite possible that this lack of activity could be due to inactivation of the enzyme by oxidative stress through translational and post-translational events.

Moreover, the oxidative stress observed in MCF-7 cell line, but not in T47D and MDA-MB-231 cell lines, is perhaps not only due to an inadequate response of the antioxidant systems but could also be due to a mitochondrial dysfunction, as showed recently by Sastre-Serra et al. [2012]. These authors report that mitochondrial function is modulated by E2, but the effect differs according to ERalpha/ERbeta ratio. In cell lines with a lower ERalpha/ERbeta ratio or that only has ERbeta, which is the case for the T47D cells and MDA-MB-231 cells, respectively, E2 treatment improved the mitochondrial function.

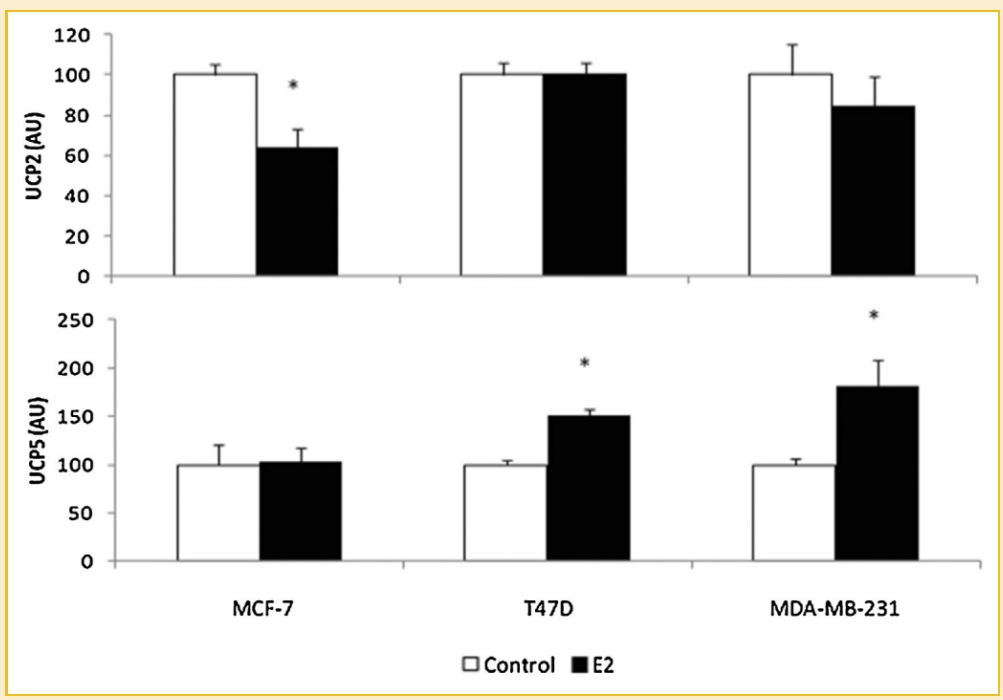

Fig. 2. UCP2 and UCP5 protein levels in response to E2-treatment in MCF-7, T47D, and MDA-MB-231 cell lines. Levels of uncoupling proteins were measured by Western blot as described in the Materials and Methods Section. AU: arbitrary units; UCP2 and UCP5: uncoupling protein 2 and uncoupling protein 5. Data represent the means ± SEM, with the value of vehicle-treated cells set at 100. *Significant difference between E2-treated and non-treated cells (Student's *t*-test; $P < 0.05$, n = 6).

On the other hand, we observed that E2-treated MCF-7 cells also showed a statistically significant increase of ucp2 and ucp5 gene expression. However, UCP2 protein levels were lower with the E2 treatment, which is in agreement with the findings of Sastre-Serra et al. [2010]. Furthermore, growing evidence indicates that UCPs decrease mitochondrial membrane potential, minimizing excessive mitochondria-derived ROS production [Negre-Salvayre et al., 1997; Derdak et al., 2008; Baffy, 2010]. Sastre-Serra et al. [2010] check the importance of UCPs in breast cancer cell lines by adding a specific inhibitor of UCP (GDP). Our results are in agreement with these findings and the idea of UCPs may play a significant role especially in ERalpha-positive breast cancer cells. From a physiological point of view, the decrease in uncoupling by E2 could be of increasing mitochondrial efficiency at ATP synthesis for cell proliferation [Santandreu et al., 2010; Sastre-Serra et al., 2010]. In fact, we observed that E2-treated MCF-7 cells showed an increase in cellular proliferation, which are associated with higher degree of malignancy. As described above, E2 increased mitochondrial membrane potential in MCF-7 cell line and increased ROS production, in concordance. Thus, some authors have suggested that oxidative stress in itself and E2 also affect the activity of the UCPs and the antioxidant enzymes, and thereby influence the electronic transport chain and free radical production [Echtay et al., 2003, 2005; Santandreu et al., 2009; Sastre-Serra et al., 2010; Yamakura and Kawasaki, 2010]. This fact is very interesting for design of new treatments because more increment of ROS production by inhibition of UCPs can lead the cell to apoptosis [Samudio et al., 2008; Santandreu et al., 2010].

Taking together, these findings reinforce the idea that E2 acts as an antioxidant or pro-oxidant, and that is tissue dependent. One role of E2 is proliferative for some tissues with high amounts ERalpha such as ovary, mammary gland, and uterus; and has been related to a lifetime exposure to estradiol [Palmieri et al., 2002]. On the other hand, E2 has a beneficial role in tissues such as heart, brain, muscle, brown adipose tissue, and liver [Colom et al., 2007ab; Valle et al., 2008; Guevara et al., 2009; Nadal-Casellas et al., 2010]. In fact, E2 had distinct effects in prostate cancer cell lines with different ERalpha/ERbeta ratios [Miro et al., 2011]. Thus it would appear that the different response to E2 could be due accounted by the different tissues ERalpha/ERbeta ratios. Within the tissues in these above-mentioned studies, more functional mitochondria were found in the non-reproductive tissues of female rats, and were also associated with a lower oxidative stress.

On the whole, our results suggest a dual E2 effect; as the E2-response differed in the breast cancer cell lines studied: MCF-7, T47D, and MDA-MB-231; and could be attributed to ERbeta presence and amount. Thus, E2 protects against oxidative stress in cell lines with either a low ERalpha/ERbeta ratio, like those of T47D cell line, or in the MDA-MB-231 cells, were only ERbeta is present. In contrast to these findings, in the MCF-7 cell line with a higher ERalpha/ERbeta ratio, there is a decrease in the antioxidant enzymes and UCPs as a result from E2 binding to ERalpha. Thus, this work indicates the importance of ERbeta in breast cancer and its implication from an oncological clinic point of view, with an interest point of view for the design of new treatments, based in UCPs inhibition, and for the understanding of cancer proliferation. Thus, it would be necessary to continue investigating the E2-effect on antioxidant enzymes and UCP levels and their activities.

REFERENCES

Aebi H. 1984. Catalase in vitro. Methods Enzymol 105:121–126.

Azzu V, Jastroch M, Divakaruni AS, Brand MD. 2010. The regulation and turnover of mitochondrial uncoupling proteins. Biochim Biophys Acta 1797:785–791.

Baffy G. 2010. Uncoupling protein-2 and cancer. Mitochondrion 10:243–252.

Baffy G, Derdak Z, Robson SC. 2011. Mitochondrial recoupling: A novel therapeutic strategy for cancer? Br J Cancer 105:469–474.

Borras C, Gambini J, Gomez-Cabrera MC, Sastre J, Pallardo FV, Mann GE, Vina J. 2005. 17Beta-oestradiol up-regulates longevity-related, antioxidant enzyme expression via the ERK1 and ERK2[MAPK]/NFkappaB cascade. Aging Cell 4:113–118.

Borras C, Gambini J, Lopez-Grueso R, Pallardo FV, Vina J. 2010. Direct antioxidant and protective effect of estradiol on isolated mitochondria. Biochim Biophys Acta 1802:205–211.

Brand MD, Affourtit C, Esteves TC, Green K, Lambert AJ, Miwa S, Pakay JL, Parker N. 2004. Mitochondrial superoxide: Production, biological effects, and activation of uncoupling proteins. Free Radic Biol Med 37:755–767.

Carlberg I, Mannervik B. 1985. Glutathione reductase. Methods Enzymol 113:484–490.

Chen JQ, Cammarata PR, Baines CP, Yager JD. 2009. Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. Biochim Biophys Acta 1793:1540–1570.

Clemons M, Goss P. 2001. Estrogen and the risk of breast cancer. N Engl J Med 344:276–285.

Colom B, Alcolea MP, Valle A, Oliver J, Roca P, Garcia-Palmer FJ. 2007a. Skeletal muscle of female rats exhibit higher mitochondrial mass and oxidative-phosphorylative capacities compared to males. Cell Physiol Biochem 19:205–212.

Colom B, Oliver J, Roca P, Garcia-Palmer FJ. 2007b. Caloric restriction and gender modulate cardiac muscle mitochondrial H_2O_2 production and oxidative damage. Cardiovasc Res 74:456–465.

Derdak Z, Fulop P, Sabo E, Tavares R, Berthiaume EP, Resnick MB, Paragh G, Wands JR, Baffy G. 2006. Enhanced colon tumor induction in uncoupling protein-2 deficient mice is associated with NF-kappaB activation and oxidative stress. Carcinogenesis 27:956–961.

Derdak Z, Mark NM, Beldi G, Robson SC, Wands JR, Baffy G. 2008. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. Cancer Res 68:2813–2819.

Echtay KS. 2007. Mitochondrial uncoupling proteins–what is their physiological role? Free Radic Biol Med 43:1351–1371.

Echtay KS, Esteves TC, Pakay JL, Jekabsons MB, Lambert AJ, Portero-Otin M, Pamplona R, Vidal-Puig AJ, Wang S, Roebuck SJ, Brand MD. 2003. A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. EMBO J 22:4103–4110.

Echtay KS, Pakay JL, Esteves TC, Brand MD. 2005. Hydroxynonenal and uncoupling proteins: A model for protection against oxidative damage. Biofactors 24:119–130.

Esworthy RS, Baker MA, Chu FF. 1995. Expression of selenium-dependent glutathione peroxidase in human breast tumor cell lines. Cancer Res 55:957–962.

Fariss MW, Chan CB, Patel M, Van Houten B, Orrenius S. 2005. Role of mitochondria in toxic oxidative stress. Mol Interv 5:94–111.

Guevara R, Santandreu FM, Valle A, Gianotti M, Oliver J, Roca P. 2009. Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress. Free Radic Biol Med 46:169–175.

Hansford RG, Hogue BA, Mildaziene V. 1997. Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. J Bioenergy Biomembr 29:89–95.

Horimoto M, Fulop P, Derdak Z, Wands JR, Baffy G. 2004. Uncoupling protein-2 deficiency promotes oxidant stress and delays liver regeneration in mice. Hepatology 39:386–392.

Irwin RW, Yao J, Hamilton RT, Cadenas E, Brinton RD, Nilsen J. 2008. Progesterone and estrogen regulate oxidative metabolism in brain mitochondria. Endocrinology 149:3167–3175.

Jensen EV, DeSombre ER. 1973. Estrogen-receptor interaction. Science 182: 126–134.

Korshunov SS, Skulachev VP, Starkov AA. 1997. High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. FEBS Lett 416:15–18.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930.

Mense SM, Remotti F, Bhan A, Singh B, El-Tamer M, Hei TK, Bhat HK. 2008. Estrogen-induced breast cancer: Alterations in breast morphology and oxidative stress as a function of estrogen exposure. Toxicol Appl Pharmacol 232:78–85.

Miro AM, Sastre-Serra J, Pons DG, Valle A, Roca P, Oliver J. 2011. 17beta-Estradiol regulates oxidative stress in prostate cancer cell lines according to ERalpha/ERbeta ratio. J Steroid Biochem Mol Biol 123:133–139.

Nadal-Casellas A, Amengual-Cladera E, Proenza AM, Llado I, Gianotti M. 2010. Long-term high-fat-diet feeding impairs mitochondrial biogenesis in liver of male and female rats. Cell Physiol Biochem 26:291–302.

Negre-Salvayre A, Hirtz C, Carrera G, Cazenave R, Troly M, Salvayre R, Penicaud L, Casteilla L. 1997. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASEB J 11:809–815.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA. 2001. Mechanisms of estrogen action. Physiol Rev 81:1535–1565.

Okoh V, Deoraj A, Roy D. 2011. Estrogen-induced reactive oxygen species-mediated signalings contribute to breast cancer. Biochim Biophys Acta 1815:115–133.

Paglia DE, Valentine WN. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J Lab Clin Med 70:158–169.

Palmieri C, Cheng GJ, Saji S, Zelada-Hedman M, Warri A, Weihua Z, Van Noorden S, Wahlstrom T, Coombes RC, Warner M, Gustafsson JA. 2002. Estrogen receptor beta in breast cancer. Endocr Relat Cancer 9:1–13.

Quick KL, Hardt JI, Dugan LL. 2000. Rapid microplate assay for superoxide scavenging efficiency. J Neurosci Methods 97:139–144.

Roy D, Cai Q, Felty Q, Narayan S. 2007. Estrogen-induced generation of reactive oxygen and nitrogen species, gene damage, and estrogen-dependent cancers. J Toxicol Environ Health B Crit Rev 10:235–257.

Russo J, Ao X, Grill C, Russo IH. 1999. Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast Cancer Res Treat 53:217–227.

Samudio I, Fiegl M, McQueen T, Clise-Dwyer K, Andreeff M. 2008. The warburg effect in leukemia-stroma cocultures is mediated by mitochondrial uncoupling associated with uncoupling protein 2 activation. Cancer Res 68:5198–5205.

Santandreu FM, Valle A, Fernandez de Mattos S, Roca P, Oliver J. 2009. Hydrogen peroxide regulates the mitochondrial content of uncoupling protein 5 in colon cancer cells. Cell Physiol Biochem 24:379–390.

Santandreu FM, Roca P, Oliver J. 2010. Uncoupling protein-2 knockdown mediates the cytotoxic effects of cisplatin. Free Radic Biol Med 49:658–666.

Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J, Roca P. 2010. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. Free Radic Biol Med 48:506–512.

Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Oliver J, Roca P. 2012. The effects of 17beta-estradiol on mitochondrial biogenesis and function in breast cancer cell lines are dependent on the ERalpha/ERbeta ratio. Cell Physiol Biochem 29:261–268.

Siegel R, Ward E, Brawley O, Jemal A. 2011. Cancer statistics, 2011: The impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin 61:212–236.

Song JY, Kim MJ, Jo HH, Hwang SJ, Chae B, Chung JE, Kwon DJ, Lew YO, Lim YT, Kim JH, Kim MR. 2009. Antioxidant effect of estrogen on bovine aortic endothelial cells. J Steroid Biochem Mol Biol 117:74–80.

Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. 2004. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci USA 101:1566–1571.

Valle A, Guevara R, Garcia-Palmer FJ, Roca P, Oliver J. 2007. Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions. Am J Physiol Cell Physiol 293:C1302–C1308.

Valle A, Guevara R, Garcia-Palmer FJ, Roca P, Oliver J. 2008. Caloric restriction retards the age-related decline in mitochondrial function of brown adipose tissue. Rejuvenation Res 11:597–604.

Warner M, Gustafsson JA. 2010. The role of estrogen receptor beta (ERbeta) in malignant diseases–a new potential target for antiproliferative drugs in prevention and treatment of cancer. Biochem Biophys Res Commun 396:63–66.

Yamakura F, Kawasaki H. 2010. Post-translational modifications of superoxide dismutase. Biochim Biophys Acta 1804:318–325.

Ziech D, Franco R, Pappa A, Panayiotidis MI. 2011. Reactive oxygen species (ROS)-induced genetic and epigenetic alterations in human carcinogenesis. Mutat Res 711:167–173.

Manuscript 9

Initial activation status of the antioxidant response determines sensitivity to carboplatin/paclitaxel treatment of ovarian cancer

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Initial Activation Status of the Antioxidant Response Determines Sensitivity to Carboplatin/ Paclitaxel Treatment of Ovarian Cancer

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Abstract. *Background/Aim: Ovarian carcinoma is the main cause of gynecological cancer related deaths. The aim of this study was to determine the activation status of the antioxidant response in samples of ovarian serous carcinoma from paraffin-embedded biopsies and compare them with the response of patients to carboplatin-paclitaxel treatment. Materials and Methods: Estrogen receptor alpha (ERα), antioxidant enzymes, and uncoupling protein (UCP) levels were analyzed by western blotting and the presence of estrogen receptor beta (ERβ) was investigated by immunohistochemistry (IHC). Results: Lower levels of ERα, antioxidant enzymes and UCPs were found in patients resistant to treatment in comparison to the carboplatin/paclitaxel-sensitive ones; IHC revealed a greater presence of ERβ in sensitive patients. Conclusion: These results indicate that patients resistant to treatment have a lower level of antioxidant response activation compared to sensitive patients, fact which may be related to the efficacy of this treatment.*

Ovarian epithelial cancer is the fifth cause of cancer-related deaths for European women (1). Moreover, ovarian carcinoma is the leading cause of gynecological cancer-related death (1).

Ovarian cancer represents approximately 3% of all of gynecological carcinomas. It is mainly a post-menopausal disease that usually appears in patients between the ages of 65-69 years. By diagnosis, 75% of patients already have advanced stage of the disease, and this is due to poor early detection techniques and the asymptomatical nature of the early stages of the disease. The overall rate of survival at five years for patients with advanced disease is 20-30% (1).

The current standard treatment for ovarian cancer involves carboplatin, followed by the administration of paclitaxel. Although more than 80% of patients respond to initial treatment, in approximately 75% of cases, there is a relapse within the first two years after treatment, which is very likely to be incurable (1).

Carboplatin [*cis*-diamine-(1,1-cyclobutanedicarboxylate) platinum (II)] is used as a cancer treatment for many types of carcinomas, such as small-cell lung cancer, ovarian cancer, head and neck carcinomas (2). The antitumor action of carboplatin is *via* DNA alkylation, which causes cancer cell death (2).

Paclitaxel is one of the best-known chemotherapeutic agents, widely used in anticancer therapy. The site of action is located at the microtubules. The primary effect on cells is abnormal stabilization of the microtubule polymerization dynamics, and this leads to mitosis failure. In addition, paclitaxel alters other cellular functions related to microtubules, such as intracellular signaling, and organelle transport and locomotion (3). It is effective against several human tumors types, including ovarian carcinomas. The efficacy of paclitaxel is limited by the acquired or intrinsic resistance of the population of malignant cells surviving the treatment (2).

Treatment of carboplatin with paclitaxel can cause oxygen free radical formation and, consequently, the appearance of oxidative stress (4-8). Reactive oxygen species (ROS) act as potent mutagens, increasing genomic instability and contributing to cancer progression (9). Estrogen receptors (ERs), alpha (ERα) and beta (ERβ), may have a modulating role in oxidative stress (9, 10). It has been described that the presence of ER in estrogen-dependent tissues results in an increase in ROS levels (10). In regards to the relationship between ERs and ovarian cancer, it has been suggested that

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there is a link between ovarian cancer and gonadal receptors (11), and this has been demonstrated by the discovery of both mRNA and proteins of ERs in normal ovarian tissue and malignant ovarian tumors (11).

The objective of this study was to determine the oxidative stress status in formalin-fixed paraffin-embedded biopsies of ovarian cancer in comparison to the sensitivity of patients for the carboplatin-paclitaxel treatment. Specifically, the levels of antioxidant enzymes, uncoupling proteins (UCPs), and oxidative damage to lipids and proteins were analyzed, in addition to assessing the ER levels.

Materials and Methods

Reagents. Routine chemicals were supplied by Roche (Barcelona, Spain), Sigma–Aldrich (St. Louis, Missouri, USA) and Panreac (Barcelona, Spain).

Study subjects and treatments. Tissue samples from twelve women with papillary serous cystadenocarcinoma, the most common form of ovarian cancer, were obtained from the Biological Specimen Bank of Son Llàtzer Hospital. These samples were added from 2003 to 2008, and were collected, stored and analyzed in accordance to permits from the Balearic Island Bioethics Committee. The individuals from this sampling group had similar age, weight, height and Body Mass Index (Table I). Any treatment or therapy was given after the completion of the tumor biopsies that were used for obtaining the data for this article. According to the standard guidelines, maximal surgical effort was attempted in all patients, resulting in complete resection in all cases. All patients received platinum-based chemotherapy [(AUC CA125)=5 for carboplatin, per cycle], and paclitaxel (175 mg/m^2 for each cycle). Disease recurrence was defined according to the GCIG CA125 criteria (12) and/or radiological confirmation of tumor progression. Chemosensitivity was defined by the common definition of platinum resistance, and patients were designated as 'sensitive' when they had experienced disease relapse 6 months or more after prior platinum-containing chemotherapy, while 'resistant' referred to those patients who had either experienced relapse in fewer than 6 months after the end of chemotherapy, or whose disease had progressed while on therapy (13).

Immunohistochemistry. Immunohistochemistry was performed with the BenchMark ULTRA (Ventana Medical Systems, Inc., Tucson, AZ, USA). Immunohistochemical staining was carried out on formalin-fixed paraffin-embedded (FFPE) with the ERβ antibody [Mouse monoclonal (988), Abcam, Cambridge, UK], dilution 1:50. Sections were evaluated independently by two observers who were unaware of clinicopathological characteristics of patients according to staining localization (nuclear or/and cytoplasmic) and intensity.

Protein extraction. Protein extracts were obtained from formalin-fixed tissues. For protein extraction, the method described by Addis *et al*. (14) was used. Briefly, two replicates of 10-μm thick microtome sections of FFPE tissue were placed in 1.5 ml Eppendorf safe-lock tubes (Eppendorf, Hamburg, Germany) and de-paraffinized by three incubations in HistoClear II (National Diagnostics, Atlanta, GA, USA) for 10 min at room temperature. After each incubation, the tissue was pelleted at 11,900 ×*g* in a microcentrifuge for 2 min. The de-paraffinized tissue pellets were then rehydrated with graded series of ethanol (100%, 96% and 70%). Then, 100 μl of extraction buffer [20 mM Tris HCl (pH 8.8), 2% sodium dodecyl sulfate (SDS), 4 mM dithiothreitol (DTT), 10 μM leupeptin and 10 μM pepstatin] was well-mixed with the samples. After that, the samples with extraction buffer were left to incubate for 5 min on ice and were then subjected to high-temperature extraction at 100˚C for 20 min and then at 80˚C for 2 h with shaking. Finally, samples were placed for 1 min at 4˚C and centrifuged at 14,000 ×g for 15 min, at 4˚C. The resulting supernatant was placed into a new tube and the protein content of each sample quantified using the BCA kit (Pierce, Bonn, Germany).

Table I. *Patients' anthropometric data*.

	Sensitive	Resistant
Age (years)	55.0±4.8	55.7±4.8
Weight (kg)	61.7±6.8	65.8±7.3
Height (cm)	157±3	157±2
BMI (kg/m^2)	24.9±2.0	26.8±2.9

Body mass index (BMI) is the body weight (kg) divided by the square of the height (m). Data are represented as the mean±SD and there are no significant differences between the two groups (p>0.05; n=6).

Western blots. For western blot analysis, 40 μg of protein from protein extracts was fractioned by SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Membranes were incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. Antisera against ERα (66 kDa; dilution 1:200) and glutathione reductase (GRd) (53 kDa; dilution 1:500) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); glutathione peroxidase (GPx) (22.5 kDa; dilution 1:200) and catalase (60 kDa; dilution 1:1000) were purchased from Calbiochem (Merck KGaA, Darmstadt, Germany); UCP2 (32 kDa; dilution 1:500), UCP5 (36 kD; dilution 1:1000) and 4-hydroxynonenal (4-HNE), (dilution 1:200) were purchased from Alpha Diagnostic International (San Antonio, Tx, USA); and Oxiselect (antisera against dinitrophenyl [αDNP]; dilution 1:2000) were purchased from Cell Biolabs (San Diego, CA, USA). All the above were used as primary antibodies. Protein bands were visualized by the Immun-Star Western C kit reagent western blotting detection system (Bio-Rad, Hercules, California, USA). The chemiluminescence signal was captured with a Chemidoc XRS densitometer and analyzed with the Quantity One software (Bio-Rad).

Statistical analysis. All data are expressed as means±SD. Statistical analysis was carried out using the statistical program SPSS 18.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical differences between experimental groups were analyzed with unpaired Student's *t*-tests. A level of p<0.05 was accepted as significant.

Results

Patients with disease resistant to treatment with carboplatin and paclitaxel had 30% lower levels of ERα in comparison with patients with non-resistant disease (Figure 1).

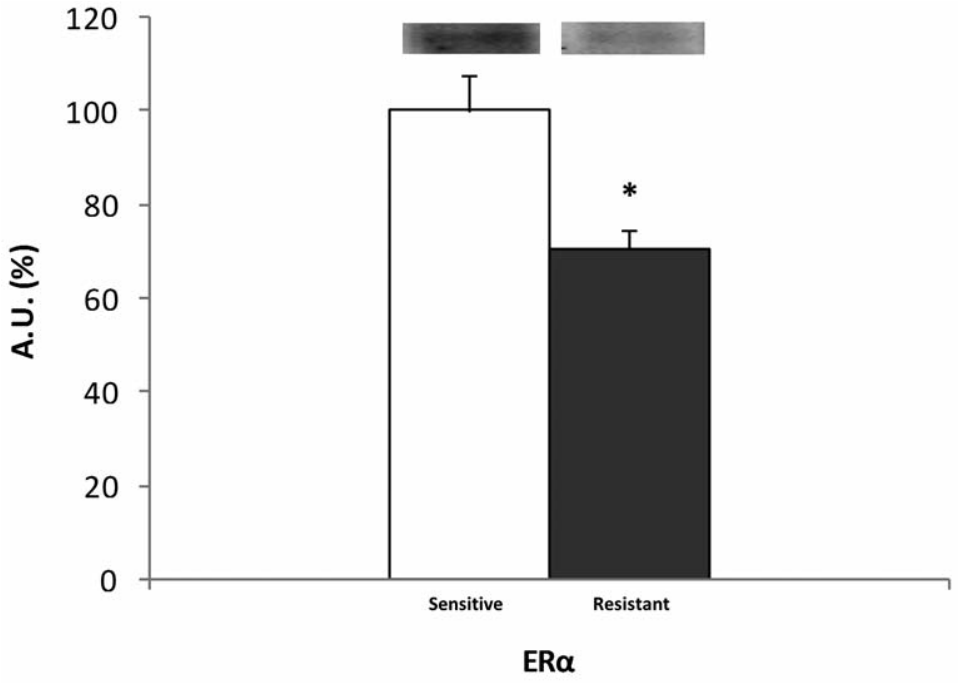

Figure 1. *Estrogen receptor alpha (ERα) protein levels in tumor samples, sensitive and resistant to treatment, as measured using western blot assays. The ERα protein levels in resistant samples were lower than those in sensitive samples (*p<0.05; mean±SD; n=6). A.U.: Arbitrary units.*

For the antioxidant enzymes, Table II shows the tumor levels of catalase and GRd, which were significantly lower in the resistant cases, while levels of GPx did not show any significant variation between the two studied groups.

Likewise, Figure 2 shows that levels of UCP2 were lower in resistant samples than in the treatment-sensitive ones, while for UCP5, the levels in the resistant samples were significantly lower than in the sensitive ones.

Oxidative damage in both protein and lipids was not significantly different between the two experimental groups (Table III), despite resistant ovarian tumors presenting lower levels of protein carbonyl adducts.

Immunohistochemical analysis of ERβ showed more staining and increased cytoplasmic staining of this receptor in patients who did respond to treatment compared to those that did not (Figure 3). From these results and those obtained by western blotting, patients who did have a response to treatment of carboplatin and paclitaxel had higher levels of both ERs.

Discussion

This study demonstrates that the initial condition of activation of the antioxidant response in ovarian cancer could be related to sensitivity to treatments such as carboplatin and paclitaxel, which act by increasing oxidative stress.

Table II. *Catalase, glutathione peroxidase (GPx) and glutathione reductase (GRd) protein levels in tumor samples, sensitive and resistant to treatment, measured using western blot assays. Next to each numerical value is the image of the corresponding western blot. Data are represented as mean±SD. There was significantly less catalase and GRd in the sensitive group than in the resistant (*p<0.05; n=6). However, for GPx levels, there is no significant difference between the groups (p>0.05; n=6).*

	Sensitive	Resistant
Catalase (A.U. %)	100±13	55.8±3.7*
GPx (A.U. %)	100±32	72.8±28.7
GRd (A.U. %)	100±15	60.1±6.9*

A.U.: Arbitrary units.

Table III. *Protein carbonyl and 4-hydroxynonenal (4-HNE) content in tumor samples, sensitive and resistant to treatment, as measured using western blot assays. Data are presented as the mean±SD, and there are no significant differences between groups (p>0.05; n=6).*

	Sensitive	Resistant
Protein carbonyl (A.U. %)	100±27	68.7±24.3
4-HNE (A.U. %)	100±22	104±3

A.U.: Arbitrary Units.

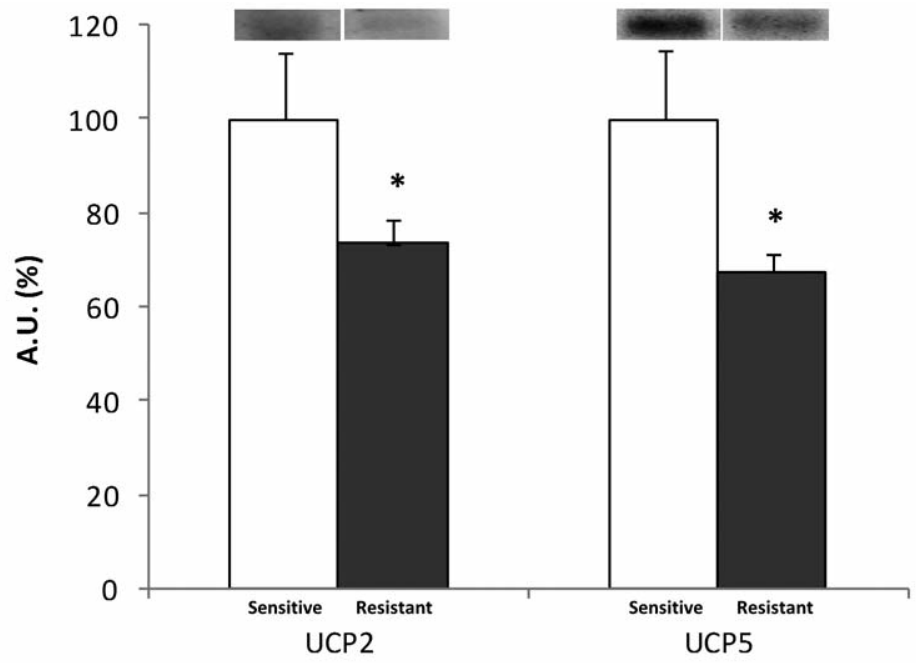

Figure 2. *Protein levels of uncoupling protein 2 (UCP2) and uncoupling protein 5 (UCP5) in tumor samples, sensitive and resistant to treatment, as measured using western blot assays. Levels of both UCPs in resistant samples were lower than those in sensitive ones (*p<0.05; mean±SD; n=6). A.U.: Arbitrary units.*

Specifically, it has been observed that patients with resistant disease have levels of oxidative damage to proteins and lipids, similar to those of sensitive patients, but with a lower activation of the antioxidant response. These treatment-resistant patients have lower levels of antioxidant enzymes and UCPs (UCP2 and UCP5). This could also be related to a lower presence of ERα and ERβ, and the oxidative stress balance in patients with resistant disease.

Both carboplatin and paclitaxel have been associated with oxidative stress induction (4-8). One of the effects produced by treatment with carboplatin is free radical formation, leading to the onset of oxidative stress (5, 7). Alexandre *et al*. (4) found that treatment with paclitaxel induced the release of ROS to the extracellular medium, increasing the apoptosis of all cells in the vicinity of treatment administration. Recently, Panis *et al*. (8) observed an increase in lipid peroxidation in patients treated with paclitaxel, which may indicate that this treatment also produces oxidative stress. Moreover, treatment with paclitaxel induced cytotoxic stress in ovarian cancer cells (6). Furthermore, the same authors have described that cell lines sensitive to paclitaxel treatment had a lower sensitivity when an antioxidant was added at the same time as the treatment (6). Accordingly, one of the mechanisms underlying treatment with carboplatin-paclitaxel is oxidative stress induction, which leads to apoptosis of cancer cells. Tamarit *et al*. showed an implication of ROS and oxidative damage in the induction of apoptosis (15). Other studies have demonstrate that the apoptotic effects of ROS have been drastically mitigated by pretreatment with intracellular ROS scavengers, indicating that intracellular ROS generation is responsible for apoptosis (16).

Regarding oxidative stress status, it has been shown that fall off in the levels of reduced glutathione (GSH) are accompanied by increased levels of ROS during apoptosis (17), which could explain why in patients sensitive to treatment levels of glutathione reductase increased, in an attempt to restore the GSH level. Despite this, it has been found in the MCF7 breast cancer cell line, which has a characteristic overexpression of catalase, that there is a rise in resistance to pro-oxidant treatments (18).

Recently, ERα and ERβ have been associated with a modulatory role of oxidative stress in other types of tumor (9, 10). Our results show that the tumor of treatment-resistant patients had lower levels of ERα and ERβ than sensitive ones. These results are similar to those obtained in previous work (19), where there was a higher presence of both receptor types in non-cancerous ovarian tissue compared to malignant ovarian tumors. Overall, our results regarding the presence of ERβ in ovarian cancer are in line with previous studies, in which this receptor had a lower presence in malignant tumors in comparison to normal tissue (20, 21).

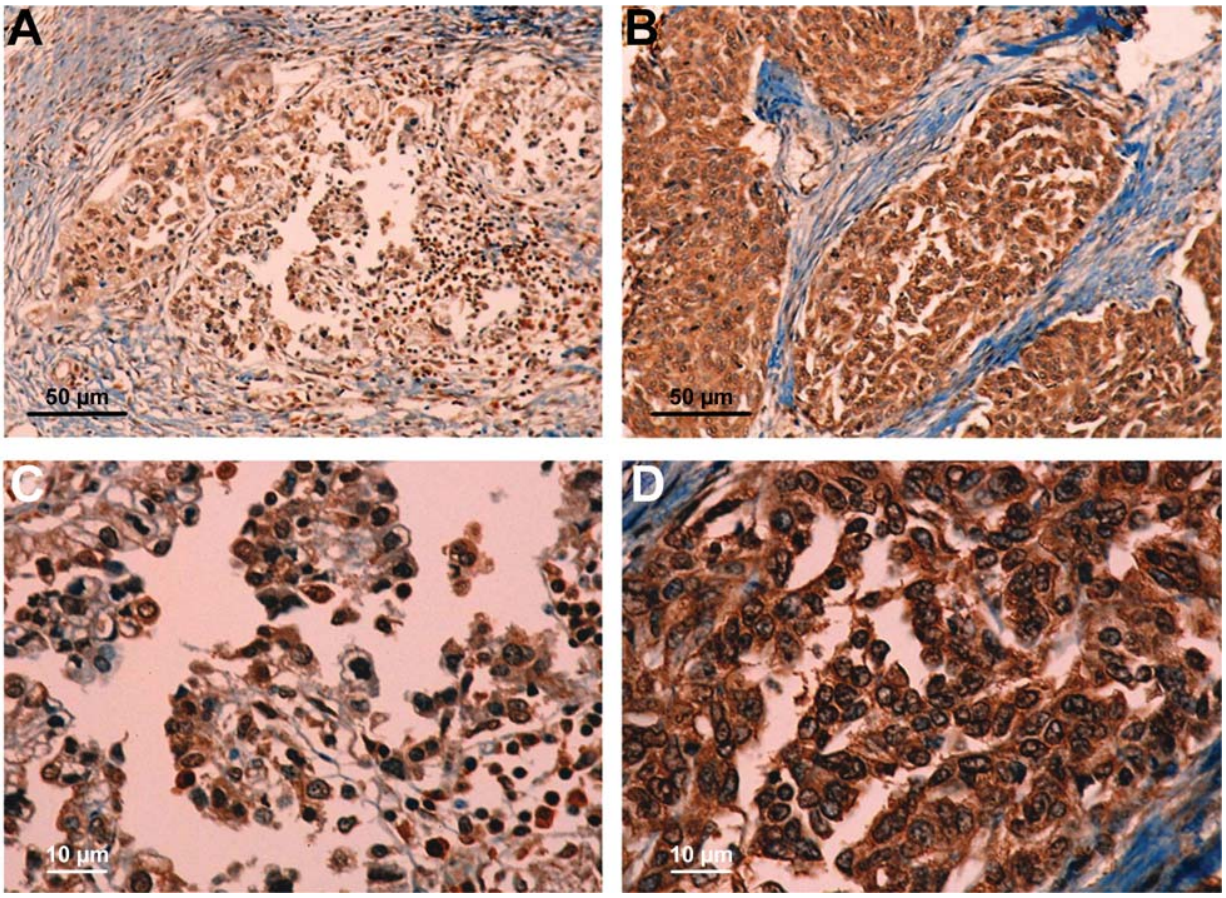

Figure 3. *Representative photographs of estrogen receptor beta immunostaining, showing expression in resistant (A and C) and sensitive ovarian cancer tissue samples (B and D).*

Bardin *et al*. (20) shows that ERβ had a different cytoplasmic localization in the two groups. Thus, sensitivity to treatment might be related to the more positive immunohistochemical staining of ERβ, localized in the cytoplasm. However, other authors have linked cytoplasmic ERβ staining with reduced patient survival (22).

There is also a controversy about the relationship between the presence of ERs and sensitivity to treatment for other tumor types. Thus, it has been described that ER^- breast cancer cell lines have a poorer response to treatment with paclitaxel than do ER^+ cell lines (23). However, other authors have found that a greater presence of ERs reduces the efficacy of paclitaxel in endometrial (24) and breast cancer (25).

In 2003, Rousset *et al*. (26) discovered the presence of UCP2 in the reproductive tract of female mice. Recently, Liu *et al*. (27) described the presence of UCP2 in human ovarian tissue. In this article, UCPs show the same pattern as do the classic antioxidant enzymes, with lower levels in patients resistant to treatment. Further reinforcing their antioxidant role, it should be noted that UCPs have been associated with a decrease of oxidative stress level in breast cancer cell lines (28).

In conclusion, the results of this study indicate that patients sensitive to treatment have higher initial levels of antioxidant enzymes and UCPs than do patients with resistant disease, despite tumor in both groups having a similar carbonyl content and lipid peroxidation. For patients with sensitive disease, treatment administration generates oxidative stress which proves to be unbearable, and tumor cells consequently respond to treatment. In contrast, in resistant cases the oxidative stress generated is not enough to remove cancer cells, because they had a lower initial antioxidant response, giving them more leeway to respond to stress caused by treatment.

This work highlights the importance of the study of oxidative stress in regard to the evolution of ovarian cancer and its relationship to standard treatment of these tumors. Further studies are required on oxidative stress, the balance

of free radicals and antioxidant defenses, and the supply of ERs in both cell lines and tumor biopsy samples.

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References

1 Cannistra SA: Cancer of the ovary. N Engl J Med *351*: 2519-2529, 2004.
2 Kelland L: The resurgence of platinum-based cancer chemotherapy. Nat Rev Cancer *7*: 573-584, 2007.
3 Honore S, Pasquier E and Braguer D: Understanding microtubule dynamics for improved cancer therapy. Cell Mol Life Sci *62*: 3039-3056, 2005.
4 Alexandre J, Hu Y, Lu W, Pelicano H and Huang P: Novel action of paclitaxel against cancer cells: Bystander effect mediated by reactive oxygen species. Cancer Res *67*: 3512-3517, 2007.
5 Cheng CF, Juan SH, Chen JJ, Chao YC, Chen HH, Lian WS, Lu CY, Chang CI, Chiu TH and Lin H: Pravastatin attenuates carboplatin-induced cardiotoxicity *via* inhibition of oxidative stress associated apoptosis. Apoptosis *13*: 883-894, 2008.
6 Goto T, Takano M, Hirata J and Tsuda H: The involvement of FOXO1 in cytotoxic stress and drug-resistance induced by paclitaxel in ovarian cancers. Br J Cancer *98*: 1068-1075, 2008.
7 Husain K, Whitworth C, Hazelrigg S and Rybak L: Carboplatin-induced oxidative injury in rat inferior colliculus. Int J Toxicol *22*: 335-342, 2003.
8 Panis C, Herrera AC, Victorino VJ, Campos FC, Freitas LF, De Rossi T, Colado Simao AN, Cecchini AL and Cecchini R: Oxidative stress and hematological profiles of advanced breast cancer patients subjected to paclitaxel or doxorubicin chemotherapy. Breast Cancer Res Treat *133*: 89-97, 2012.
9 Roca P, Oliver J, Sastre-Serra J, and Nadal-Serrano M: The Importance of ERalfa/ERbeta Ratio in Breast Cancer: Mitochondrial Function and Oxidative Stress. *In*: Breast Cancer. Carcinogenesis, Cell Growth and Signalling Pathways. Gunduz M, Gunduz E eds.. Rijeka: InTech, p. 732, 2011.
10 Roy D, Cai Q, Felty Q and Narayan S: Estrogen-induced generation of reactive oxygen and nitrogen species, gene damage, and estrogen-dependent cancers. J Toxicol Environ Health B Crit Rev *10*: 235-257, 2007.
11 Clinton GM and Hua W: Estrogen action in human ovarian cancer. Crit Rev Oncol Hematol *25*: 1-9, 1997.
12 Laurent A, Nicco C, Chereau C, Goulvestre C, Alexandre J, Alves A, Levy E, Goldwasser F, Panis Y, Soubrane O, Weill B and Batteux F: Controlling tumor growth by modulating endogenous production of reactive oxygen species. Cancer Res *65*: 948-956, 2005.
13 Fung-Kee-Fung M, Oliver T, Elit L, Oza A, Hirte HW and Bryson P: Optimal chemotherapy treatment for women with recurrent ovarian cancer. Curr Oncol *14*: 195-208, 2007.
14 Addis MF, Tanca A, Pagnozzi D, Rocca S and Uzzau S: 2-D PAGE and MS analysis of proteins from formalin-fixed, paraffin-embedded tissues. Proteomics *9*: 4329-4339, 2009.
15 Tamarit J, Cabiscol E and Ros J: Identification of the major oxidatively damaged proteins in Escherichia coli cells exposed to oxidative stress. J Biol Chem *273*: 3027-3032, 1998.
16 Matsunaga T, Kamiya T, Sumi D, Kumagai Y, Kalyanaraman B and Hara A: L-Xylulose reductase is involved in 9,10-phenanthrenequinone-induced apoptosis in human T lymphoma cells. Free Radic Biol Med *44*: 1191-1202, 2008.
17 Risch HA: Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. J Natl Cancer Inst *90*: 1774-1786, 1998.
18 Glorieux C, Dejeans N, Sid B, Beck R, Calderon PB and Verrax J: Catalase overexpression in mammary cancer cells leads to a less aggressive phenotype and an altered response to chemotherapy. Biochem Pharmacol *82*: 1384-1390, 2011.
19 Lindgren PR, Cajander S, Backstrom T, Gustafsson JA, Makela S, and Olofsson JI: Estrogen and progesterone receptors in ovarian epithelial tumors. Mol Cell Endocrinol *221*: 97-104, 2004.
20 Bardin A, Boulle N, Lazennec G, Vignon F and Pujol P: Loss of ERbeta expression as a common step in estrogen-dependent tumor progression. Endocr Relat Cancer *11*: 537-551, 2004.
21 Chan KK, Wei N, Liu SS, Xiao-Yun L, Cheung AN and Ngan HY: Estrogen receptor subtypes in ovarian cancer: a clinical correlation. Obstet Gynecol *111*: 144-151, 2008.
22 De Stefano I, Zannoni GF, Prisco MG, Fagotti A, Tortorella L, Vizzielli G, Mencaglia L, Scambia G and Gallo D: Cytoplasmic expression of estrogen receptor beta (ERbeta) predicts poor clinical outcome in advanced serous ovarian cancer. Gynecol Oncol *122*: 573-579, 2011.
23 Ciucci A, Gianferretti P, Piva R, Guyot T, Snape TJ, Roberts SM and Santoro MG: Induction of apoptosis in estrogen receptor-negative breast cancer cells by natural and synthetic cyclopentenones: role of the IkappaB kinase/nuclear factor-kappaB pathway. Mol Pharmacol *70*: 1812-1821, 2006.
24 Won YS, Lee SJ, Yeo SG and Park DC: Effects of female sex hormones on clusterin expression and paclitaxel resistance in endometrial cancer cell lines. Int J Med Sci *9*: 86-92, 2012.
25 Tokuda E, Seino Y, Arakawa A, Saito M, Kasumi F, Hayashi SI and Yamaguchi Y: Estrogen receptor-alpha directly regulates sensitivity to paclitaxel in neoadjuvant chemotherapy for breast cancer. Breast Cancer Res Treat 2011.
26 Rousset S, Alves-Guerra MC, Ouadghiri-Bencherif S, Kozak LP, Miroux B, Richard D, Bouillaud F, Ricquier D and Cassard-Doulcier AM: Uncoupling protein 2, but not uncoupling protein 1, is expressed in the female mouse reproductive tract. J Biol Chem *278*: 45843-45847, 2003.
27 Liu Y, Jiang H, He LY, Huang WJ, He XY and Xing FQ: Abnormal expression of uncoupling protein-2 correlates with CYP11A1 expression in polycystic ovary syndrome. Reprod Fertil Dev *23*: 520-526, 2011.
28 Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J and Roca P: Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. Free Radic Biol Med *48*: 506-512, 2010.

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Manuscript 10

The oxidative stress in breast tumors of postmenopausal women is ERα/ERβ ratio dependent

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Original Contribution

The oxidative stress in breast tumors of postmenopausal women is ERα/ERβ ratio dependent

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ABSTRACT

Estrogen receptor status is a diagnostic parameter in breast cancer treatment. Estrogen receptor presence is related to a better prognosis because the principal treatments attacking breast cancer tumors have their action site directed at the estrogen receptor. However, the two different subtypes of estrogen receptor, ERα and ERβ, have different functions. In this work an alternative point of view focusing on oxidative stress is shown, given that estrogen receptors regulate several proteins related to this oxidative stress, such as antioxidant enzymes, sirtuins, and uncoupling proteins. Postmenopausal human breast tumors with different ERα/ERβ ratios were analyzed to characterize the amount of oxidative stress, mitochondrial function, and proliferation-related and oxidative stress-activated signaling pathways. Results showed that tumors with a low ERα/ERβ ratio have greater oxidative damage and higher antioxidant enzyme protein levels, as well as uncoupling protein (UCP) and sirtuin 3 (SIRT3), and have high studied signaling pathway activation. Glutathione peroxidase, Complex V, Complex III, Complex II, Complex IV, AKT, SAPK, and ERα were significantly and positively correlated with ERα/ERβ ratio. However, carbonyl groups, catalase, CuZn-superoxide dismutase, UCP5, SIRT3, and ERβ were significantly and negatively correlated with ERα/ERβ ratio. From the independent variables included in the step-by-step stepwise multiple linear regression analysis, only the ERα/ERβ ratio was independently associated with carbonyl groups. Surprisingly, these low ERα/ERβ ratio tumors have poor prognosis for the patient, and these results and those of other authors suggest that these tumors are adapted to conditions of increased oxidative stress.

Breast cancer is one of the most common female malignancies and a leading cause of death among women [1]. Breast cancer occurs more frequently among postmenopausal women in comparison to premenopausal women [2], and it is known to be an estrogen-dependent malignancy. Based on this finding, the current strategy for treatment of hormone-dependent breast cancer is to block estrogen binding to the estrogen receptor (ER) by using antiestrogen agents [3] or aromatase inhibitors to diminish the conversion of testosterone into estradiol [4].

However, there is a controversy in relation to the positive/negative effects of these antiestrogen agents, which could be due to the modulatory action of the two isoforms of estrogen receptor [5,6]. In 1995, 10 years after the discovery of ERα, Gustafsson's laboratory discovered a second estrogen receptor, ERβ [7]. Much has been reported about the differences between the function and the distribution of these two receptors [8–10]. Furthermore, several studies have been published with different conclusions about the correlation of ERβ, prognostic markers, and clinical outcome [11,12].

Estrogens affect many pathways of various mechanisms involved in cancer. One of these pathways is the AKT signaling pathway, which is involved in the regulation of many cell functions, including cell proliferation, cell survival, and cell cycle progression [13,14]. Another site of regulation is the JNK/SAPK signaling pathway. This pathway becomes phosphorylated when levels of reactive oxygen species (ROS) are high and can affect several important transcription factors in breast cancer, such as c-jun, STAT3, and p53, all of which promote the growth, survival, or apoptosis of the tumor cells [15,16].

Uncoupling proteins (UCPs) are a family of proteins that can prevent ROS formation of the modulating mitochondrial transmembrane proton gradient [17,18]. In relation to these facts, the importance of these proteins in cancer development, treatment, and progression has recently been described [11,19–21].

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Another point of possible interest is the role of sirtuins (SIRTs). Sirtuins are proteins with histone deacetylase activity that have an important and well-established function in aging, but in the past few years have also been shown to play a role in cancer and in the control of oxidative stress [22,23]. Humans possess eight different types of sirtuins, but SIRT3 is of special importance because of its inner mitochondrial location, where it controls, at least in part, mitochondrial biogenesis and oxidative stress [24,25]. In fact, SIRT3 is called the guardian of mitochondria, because its multiple functions suggest that it functions as a tumor suppressor [24,26].

Taking this evidence together, this study was designed to compare various breast tumors according to their ERα/ERβ ratio under the criteria of oxidative stress, mitochondrial functionality, and signaling pathway activation status by focusing in on those factors that can provide new clues to a better understanding of this malignancy, such as uncoupling proteins and/or sirtuin 3, which are key proteins in mitochondrial dysfunction and oxidative stress status.

Materials and methods

Materials

Routine chemicals were supplied by Sigma–Aldrich (St. Louis, MO, USA), Panreac (Barcelona, Spain), and Bio-Rad Laboratories (Hercules, CA, USA).

Tissue specimens

Tissue samples were obtained from 13 women who underwent a partial or total mastectomy because of primary breast cancer during 2005–2007. All the patients presented with invasive ductal carcinoma with a postmenopausal hormonal status, with patient age ranging from 50 to 70 years. Samples of these patients were obtained from the Biological Specimen Bank of Son Llàtzer Hospital and as specified by and with the necessary permission granted from the Balearic Island Bioethics Committee. Tumor samples were collected immediately after tumor removal and were frozen in isopentane for analysis. Written informed consent was obtained from the patients before surgery.

Sample preparation

Twenty-five milligrams of frozen tissue was homogenized in STE buffer (250 nM sucrose, 5 mM Tris–HCl, and 2 mM EGTA, pH 7.4) supplemented with protease and phosphatase inhibitors: 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mmol/ml phenylmethylsulfonyl fluoride, and 1 mmol/ml $NaVO_3$. Total sample protein was quantified with a BCA kit (Pierce).

Measurement of oxidative damage

Forty micrograms of protein was transferred onto a nitrocellulose membrane using the Trans-Blot Turbo transfer system (Bio-Rad). Antiserum against 4-hydroxynonenal (4-HNE; HNE11-S), as an index of lipid peroxides, was used as primary antibody (Alpha Diagnostic International, San Antonio, TX, USA). Protein carbonyl groups, as an index of protein oxidation, were measured in tissue homogenates (20 μg of total protein was used for each sample tumor) by immunoblot detection using the OxyBlot protein oxidation detection kit (Cell Biolabs, San Diego, CA, USA) according to the manufacturer's protocol with minor modifications.

After incubation with the 4-HNE antibody or dinitrophenol antibody for analysis of oxidative damage in lipids and proteins, respectively, bands were visualized using the Immun-Star Western C chemiluminescence kit (Bio-Rad). The chemiluminescent signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

Western blotting

For Western blotting, 40 μg of total protein, prepared as described above, was fractioned by SDS–PAGE and electrotransferred onto nitrocellulose filters using the Trans-Blot Turbo transfer system (Bio-Rad). Membranes were incubated in a blocking solution of 5% nonfat powdered milk in 20 mM Tris–HCl, 0.13 mM NaCl, and 0.1% Tween 20. Antisera against UCP5 (Alpha Diagnostics); Mn-superoxide dismutase (SOD), CuZn-SOD, catalase, glutathione peroxidase (Calbiochem, San Diego, CA, USA); pAKT, AKT, pSAPK, SAPK (Cell Signaling, Danvers, MA, USA); SIRT3 (Millipore, Billerica, MA, USA); glutathione reductase (GRd), ERα, ERβ, UCP2, and tubulin (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) and MitoProfile Total OXPHOS Human WB Antibody Cocktail (Mitoscience, Eugene, OR, USA) were used as primary antibodies. Protein bands were visualized using the Immun-Star Western C kit reagent (Bio-Rad) Western blotting detection system. The chemiluminescent signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analyzed with Quantity One Software (Bio-Rad).

Statistics

All data are expressed as means ± standard error of the mean (SEM).

Statistical differences between tumors with different ERα/ERβ ratios were assessed by one-way ANOVA to analyze the ratio effect. Subsequently, the LSD test was used as a post hoc comparison. A p value of less than 0.05 was considered statistically significant. A statistical software package (SPSS 18.0 for Windows, Chicago, IL, USA) was used to perform all statistical analyses. In the search for correlations between variables, Pearson's correlation test was used. To assess the influence of tested parameters, step-by-step stepwise multiple linear regression analysis was used. The F value was set at 4.0 at each step.

Results

All the tumors were characterized by Western blotting of ERα and ERβ and then classified according to the amounts of these proteins (Table 1), creating three categories: high, medium, and low ERα/ERβ ratio (HER, MER, and LER ratios, respectively). Clinical characteristics of tumors are shown in Table 2.

Table 1
Sample distribution in groups depending on estrogen receptor ratio.

	n	ERα	ERβ	ERα/ERβ ratio
High ERα/ERβ	4	1.30 ± 0.15***	0.81 ± 0.13	1.60**,***
Medium ERα/ERβ	5	1.00 ± 0.09*	1.00 ± 0.09	1.00*,***
Low ERα/ERβ	4	0.79 ± 0.05*	1.33 ± 0.16	0.59*,**
ANOVA		R	R	R

ER, estrogen receptor. All parameters are expressed as arbitrary units; data represent the means ± SEM, with the value of tumors with medium ERα/ERβ ratio set at 1.00. The ratio effect was analyzed by one-way ANOVA ($p < 0.05$): R indicates ratio effect. Least significant difference (LSD) was applied as a post hoc analysis of ratio effect.

* Significant difference from tumors with high ERα/ERβ ratio ($p < 0.05$) determined by LSD post hoc.

** Significant difference from tumors with medium ERα/ERβ ratio ($p < 0.05$) determined by LSD post hoc.

*** Significant difference from tumors with low ERα/ERβ ratio ($p < 0.05$) determined by LSD post hoc.

As shown in Table 3, breast tumors with a LER ratio had higher oxidative damage, measured as carbonyl group content in proteins, than tumors with a HER or a MER ratio, and MER ratio tumors also had higher oxidative damage in proteins than HER ratio tumors. On the other hand, tumors with a LER ratio showed the highest levels of catalase (CAT), Mn-SOD and CuZn-SOD, and GRd protein. However, glutathione peroxidase (GPx) protein levels were lower in tumors with MER and LER ratios than in tumors with a HER ratio, as shown in Table 3.

The results of the analyses of the five mitochondrial respiratory chain complexes are shown in Fig. 1. All complexes, except Complex I (NADH:ubiquinone oxidoreductase), had a tendency to decrease when the ERα/ERβ ratio decreased. Complexes II and V (succinate dehydrogenase and ATP synthase, respectively) showed statistically significant lower levels between HER ratio tumors with respect to both MER and LER tumors.

Table 2
Clinical characteristics of tumors.

	Age (years)	Height (cm)	Weight (kg)	BMI	Menopause	PR	HER2
HER	68.5 ± 5.3	155 ± 4	66.7 ± 6.9	27.6 ± 1.8	4/4	2/4	0/4
MER	62.5 ± 3.8	154 ± 2	65.8 ± 5.2	27.6 ± 2.0	5/5	4/5	0/5
LER	68.0 ± 3.9	154 ± 3	63.0 ± 8.4	26.2 ± 2.6	4/4	0/4	0/4

High, medium, and low ERα/ERβ ratio (HER, MER, and LER, respectively). BMI, body mass index; PR, progesterone receptor.

Table 3
Oxidative damage and antioxidant enzymes in breast tumors.

	High ERα/ERβ	Medium ERα/ERβ	Low ERα/ERβ	ANOVA
4-HNE	100 ± 7	104 ± 7	101 ± 9	NS
Carbonyl groups	100 ± 11	136 ± 9	168 ± 10	*R*
GPx levels	100 ± 10	71.1 ± 7.4	75.1 ± 14.5	NS
GRd levels	100 ± 18	180 ± 28	145 ± 17	NS
CAT levels	100 ± 23	160 ± 7	266 ± 57*,**	*R*
Mn-SOD levels	100 ± 59	32.7 ± 3.7	286 ± 51*,**	*R*
CuZn-SOD levels	100 ± 32	201 ± 29	237 ± 24	*R*

4-HNE, 4-hydroxynonenal; GPx, glutathione peroxidase; GRd, glutathione reductase; CAT, catalase; Mn-SOD, manganese superoxide dismutase; CuZn-SOD, copper–zinc superoxide dismutase. All parameters are expressed as arbitrary units; data represent the means ± SEM, with the value of tumors with high ERα/ERβ ratio set at 100. High ERα/ERβ $n = 4$, medium ERα/ERβ $n = 5$, low ERα/ERβ $n = 4$. The ratio effect was analyzed by one-way ANOVA ($p < 0.05$): *R* indicates ratio effect and NS stands for nonsignificant. Least significant difference (LSD) was applied as a post hoc analysis of ratio effect.

* Significant difference from tumors with high ERα/ERβ ratio ($p < 0.05$) determined by LSD post hoc.

** Significant difference from tumors with medium ERα/ERβ ratio ($p < 0.05$) determined by LSD post hoc.

Fig. 2 shows protein levels of UCP2 and UCP5. UCP2 protein levels did not change, although UCP5 levels did increase with a lower ERα/ERβ ratio (increase in ERβ protein levels). Furthermore, as shown in Table 4, the proliferation-related and oxidative stress-activated signaling pathways AKT and JNK/SAPK, respectively, were studied. Thus, AKT and JNK/SAPK were more phosphorylated than total protein in tumors with a LER ratio. Moreover, it can be appreciated from Fig. 3 that the protein levels of SIRT3 in tumors with a LER ratio were twice as high as those with a HER ratio.

Table 5 shows the correlation matrix of variables. GPx, Complex V, Complex III, Complex II, Complex IV, AKT, SAPK, and ERα were significantly and positively correlated with ERα/ERβ ratio. However, carbonyl groups, CAT, CuZn-SOD, UCP5, SIRT3, and ERβ were significantly and negatively correlated with ERα/ERβ ratio.

Step-by-step stepwise multiple linear regression analysis was performed using carbonyl groups (as a marker of oxidative stress) as the dependent variable and 4-HNE, GPx, GRd, CAT, Mn-SOD, CuZn-SOD, Complex V, Complex III, Complex II, Complex IV, Complex I, pAKT, AKT, pSAPK, SAPK, UCP2, UCP5, SIRT3, ERα, ERβ, and ERα/ERβ ratio as independent variables. Of the many independent variables included in the analysis, the ERα/ERβ ratio was independently associated with carbonyl groups (Table 6), showing a standardized regression coefficient of 0.887.

Discussion

In breast tumors, oxidative damage has been strongly and inversely correlated with ERα/ERβ ratio. This fact may be explained, at least in part, by the decrease observed in OXPHOS protein levels, which would indicate a mitochondrial dysfunction.

During the evolution of cancer, the tumoral cells acquire mutations that modify the cell cycle and other characteristics of cells. One of the modified points is cell metabolism, and for this reason tumoral cells change their metabolism from oxidative to glycolytic. It is for this reason that tumoral cells need to adapt to the oxidative stress created by metabolism changes, a process that seems to be a clonal selection. As shown in our results, breast cancer tumors with a LER have a dysfunction in the mitochondrial OXPHOS system, which could be responsible for the high oxidative stress presented by these tumors. On the other hand, antioxidant enzymes increased in response to this oxidative damage, as well as uncoupling proteins, although these two responses were not sufficient to avoid this damage.

Moreover, breast tumors with a LER ratio had an increase in the activation of proliferation-related (AKT) and oxidative stress-activated (SAPK) signaling pathways. These results are in agreement with the findings of Cadenas et al. regarding thioredoxin reductase 1 protein, which is associated with prognosis in breast cancer [27]. Another protein of interest is SIRT3, a guardian of the mitochondria, as its multiple functions (with effects on oxidative

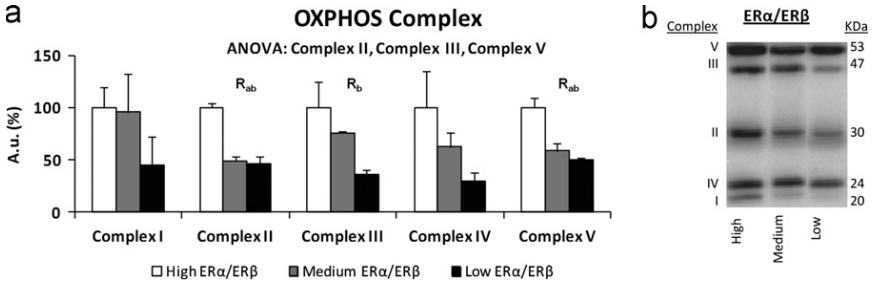


Fig. 1. Mitochondrial respiratory chain complex protein levels according to ERα/ERβ ratio. Protein levels were measured by Western blot as described under Materials and methods. Representative bands of the captured images are shown in (b). A.u., arbitrary units. Data represent the means ± SEM, with the value of tumors with high ERα/ERβ ratio set at 100. HER $n = 5$, MER $n = 3$, and LER $n = 5$. Ratio effect was analyzed by one-way ANOVA ($p < 0.05$): only variables with ratio effect are indicated. Least significant difference was applied as a post hoc analysis of ratio effect. [a] $p < 0.05$, HER versus MER. [b] $p < 0.05$, HER versus LER. [c] $p < 0.05$, MER versus LER.

stress and proliferation) suggest that this protein acts as a tumor suppressor, and the findings in this experiment that SIRT3 was increased in breast tumors with a LER ratio support this function.

All mitochondrial respiratory chain complexes were decreased in breast tumors and were concurrent with a drop in ERα/ERβ ratio, with the exception of Complex I. Complex I is the entryway for electrons into the respiratory chain, and if the other complexes are lacking, Complex I will be converted in an electron burst and cause an increase in ROS levels, which would result in oxidative damage [28,29]. In fact, oxidative damage was higher in breast tumors with a LER ratio than in those with a HER ratio.

A decrease in OXPHOS proteins indicates that mitochondria are not functioning properly and this could be one of the reasons for the higher ROS levels. ROS can contribute to an increase in cell proliferation in the beginning of tumor initiation [11,17,30]. However, a ROS excess can participate in the events that lead the cells to apoptosis and consequently in a decrease in tumor size [31].

Additionally, in tumors with high ERβ levels, we observed a strong antioxidant response, which led to the diminishment of ROS levels, even though this response was also insufficient. Catalase and superoxide dismutase (both manganese and copper–zinc isoforms) increased according to decreasing ERα/ERβ ratios; however, only glutathione peroxidase decreased. The SOD/GPx index is vital to the duration of ROS, and logically, an increase in this is harmful to the cell [21], and in this case, hydrogen peroxide can attack proteins, lipids, and DNA, causing damage to cell structure and function [32,33]. As a response to this decrease in glutathione peroxidase, glutathione reductase undergoes an important increase with the aim to reduce all the glutathione that would act as a cosubstrate for glutathione peroxidase [32,34].

ROS have various roles in the functioning of the cell and may act also as secondary messengers in the proliferative response through MAPK pathways [35], which in turn can influence key proteins in oxidative stress control. This fact agrees with our results, as the pathways studied showed an increase in tumors with a LER ratio, which also had a higher oxidative stress than tumors with low ERβ protein levels.

All of these changes in oxidative stress and mitochondrial function could be mediated by SIRT3. Recently, some authors have given sirtuins a new role in carcinogenesis and oxidative stress, in addition to its currently accepted one in the aging process [24,26]. In fact, some authors have recently demonstrated that SIRT3 knockout mice exhibited a deterioration in liver mitochondrial

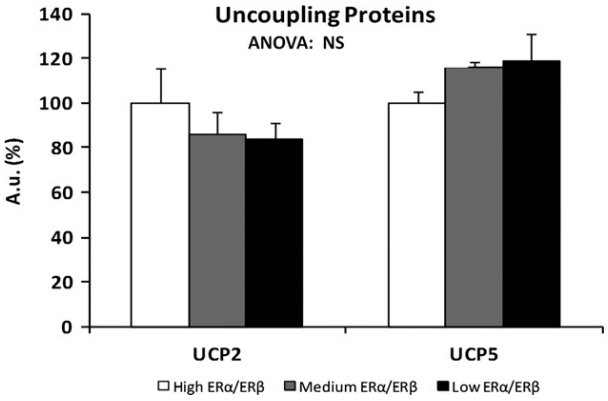


Fig. 2. Uncoupling proteins 2 and 5 in breast tumors with different ERα/ERβ ratios. Protein levels were measured by Western blot as described under Materials and methods. A.u., arbitrary units. Data represent the means ± SEM, with the value of tumors with high ERα/ERβ ratio set at 100. HER $n = 5$, MER $n = 3$, and LER $n = 5$. Ratio effect was analyzed by one-way ANOVA ($p < 0.05$): NS indicates nonsignificant effect.

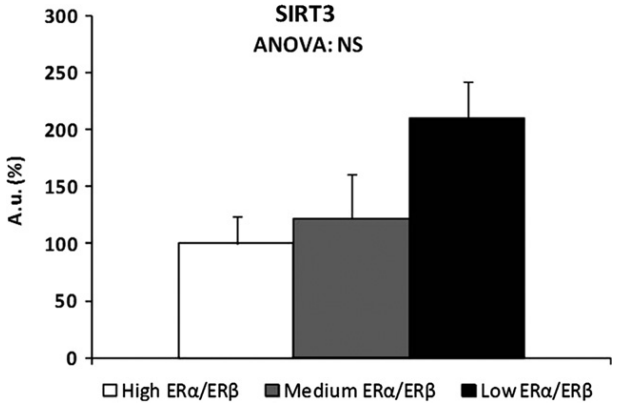


Fig. 3. SIRT3 protein levels increase when ERα/ERβ ratio diminishes. Protein levels were measured by Western blot as described under Materials and methods. A.u. arbitrary units. Data represent the means ± SEM, with the value of tumors with high ERα/ERβ ratio set at 100. HER $n = 5$, MER $n = 3$, and LER $n = 5$. Ratio effect was analyzed by one-way ANOVA ($p < 0.05$): NS indicates nonsignificant effect.

Table 4
Proliferation-related and oxidative stress-activated signaling pathways in breast tumors.

	pAKT level	AKT level	AKT ratio
High ERα/ERβ	100 ± 19	100 ± 37	1.00
Medium ERα/ERβ	183 ± 42	61.7 ± 10.1	2.97*
Low ERα/ERβ	187 ± 76	38.7 ± 9.8	4.83*,**
ANOVA	NS	NS	
	pSAPK level	**SAPK level**	**SAPK ratio**
High ERα/ERβ	100 ± 53	100 ± 26	1.00
Medium ERα/ERβ	98.1 ± 25.6	95.1 ± 10.6	1.03
Low ERα/ERβ	78.3 ± 16.4	44.0 ± 7.5	1.78*,**
ANOVA	NS	NS	

All parameters are expressed as arbitrary units; data represent the means ± SEM, with the value of tumors with high ERα/ERβ ratio set at 100 for protein levels and 1.00 for ratio. Ratio was calculated from the means. HER $n = 4$, MER $n = 5$, and LER $n = 4$. Ratio effect was analyzed by one-way ANOVA ($p < 0.05$): NS stands for nonsignificant. Least significant difference (LSD) was applied as a post hoc analysis of ratio effect.

* Significant difference from tumors with high ERα/ERβ ratio ($p < 0.05$) determined by LSD post hoc.

** Significant difference from tumors with medium ERα/ERβ ratio ($p < 0.05$) determined by LSD post hoc.

Table 5
Correlation matrix of mammary carcinoma data.

		ERα/ERβ ratio	4-HNE	Carbonyl groups	GPx	GRd	CAT	Mn-SOD	CuZn-SOD	Complex V	Complex III	Complex II	Complex IV	Complex I	pAKT	AKT	pSAPK	SAPK	UCP2	UCP5	SIRT3	ERα	ERβ
ERα/ERβ ratio	PC	1																					
	Sig.																						
4-HNE	PC	−0.158	1																				
	Sig.	0.304																					
Carbonyl groups	PC	−0.782**	0.342	1																			
	Sig.	0.001	0.138																				
GPx	PC	0.572*	−0.058	−0.402	1																		
	Sig.	0.021	0.425	0.098																			
GRd	PC	−0.365	0.529*	0.410	−0.406	1																	
	Sig.	0.122	0.038	0.093	0.095																		
CAT	PC	−0.634*	0.080	0.715**	−0.100	0.103	1																
	Sig.	0.013	0.402	0.007	0.379	0.382																	
Mn-SOD	PC	−0.335	−0.110	0.381	0.014	−0.164	0.387	1															
	Sig.	0.144	0.367	0.124	0.483	0.315	0.120																
CuZn-SOD	PC	−0.641*	−0.035	0.357	−0.410	0.127	0.536*	0.396	1														
	Sig.	0.012	0.457	0.141	0.093	0.355	0.045	0.114															
Complex V	PC	0.850**	−0.215	−0.822**	0.636*	−0.368	−0.454	−0.294	−0.634*	1													
	Sig.	0.001	0.275	0.002	0.024	0.148	0.110	0.205	0.033														
Complex III	PC	0.706*	−0.204	−0.650*	0.736**	−0.316	−0.351	−0.230	−0.347	0.462	1												
	Sig.	0.011	0.286	0.021	0.008	0.186	0.177	0.262	0.180	0.089													
Complex II	PC	0.902**	−0.211	−0.571*	0.792**	−0.477	−0.320	−0.178	−0.761**	0.814**	0.599*	1											
	Sig.	0.000	0.279	0.042	0.003	0.082	0.200	0.311	0.009	0.002	0.034												
Complex IV	PC	0.623*	−0.080	−0.479	0.747**	−0.114	−0.250	0.017	−0.566	0.469	0.869**	0.578*	1										
	Sig.	0.027	0.413	0.081	0.007	0.376	0.259	0.482	0.056	0.086	0.001	0.040											
Complex I	PC	−0.104	0.283	0.355	−0.022	−0.154	−0.173	0.109	−0.036	−0.342	−0.265	−0.201	−0.213	1									
	Sig.	0.388	0.214	0.157	0.476	0.336	0.328	0.382	0.463	0.167	0.230	0.289	0.277										
pAKT	PC	−0.312	0.399	0.526*	−0.386	0.248	−0.155	0.313	0.111	−0.572	−0.493	−0.504	−0.309	0.904**	1								
	Sig.	0.175	0.112	0.048	0.121	0.231	0.335	0.189	0.380	0.054	0.089	0.083	0.209	0.000									
AKT	PC	0.672*	0.092	−0.441	0.274	−0.226	−0.590*	−0.483	−0.465	0.621*	0.035	0.583*	−0.064	0.250	0.009	1							
	Sig.	0.012	0.394	0.087	0.207	0.252	0.036	0.079	0.088	0.037	0.464	0.050	0.435	0.258	0.490								
pSAPK	PC	−0.086	0.251	0.106	0.220	0.308	−0.298	−0.088	−0.460	−0.338	0.245	−0.350	0.447	−0.071	−0.041	−0.381	1						
	Sig.	0.390	0.204	0.371	0.235	0.165	0.174	0.393	0.066	0.169	0.248	0.161	0.098	0.423	0.453	0.124							
SAPK	PC	0.630*	−0.091	−0.626*	0.123	0.028	−0.530*	−0.587*	−0.303	0.732**	0.149	0.514	0.036	−0.047	−0.170	0.851**	−0.167	1					
	Sig.	0.011	0.383	0.015	0.344	0.465	0.038	0.022	0.169	0.008	0.340	0.064	0.460	0.448	0.309	0.000	0.292						
UCP2	PC	0.218	0.199	−0.452	0.110	−0.096	−0.143	0.147	−0.345	0.522	0.141	0.199	0.369	−0.235	−0.151	0.021	0.223	0.213	1				
	Sig.	0.237	0.257	0.070	0.360	0.384	0.328	0.325	0.136	0.061	0.349	0.291	0.147	0.257	0.329	0.475	0.232	0.242					
UCP5	PC	−0.617*	0.243	0.458	−0.724**	0.314	0.588*	0.321	0.684*	−0.594*	−0.491	−0.692*	−0.390	0.098	0.365	−0.573	−0.270	−0.384	0.144	1			
	Sig.	0.022	0.236	0.092	0.006	0.188	0.037	0.183	0.015	0.046	0.090	0.019	0.150	0.401	0.167	0.053	0.211	0.122	0.336				
SIRT3	PC	−0.526*	0.190	0.518*	−0.162	0.054	0.690**	0.410	0.137	−0.341	−0.574*	−0.266	−0.375	0.049	0.093	−0.373	0.339	−0.312	0.288	0.642*	1		
	Sig.	0.039	0.277	0.042	0.307	0.434	0.009	0.105	0.344	0.167	0.041	0.229	0.143	0.447	0.393	0.129	0.140	0.162	0.182	0.023			
ERα	PC	0.681**	0.364	−0.362	0.442	0.013	−0.518*	−0.486	−0.741**	0.592*	0.410	0.805**	0.327	−0.075	−0.275	0.771**	0.221	0.541*	0.098	−0.646*	−0.258	1	
	Sig.	0.005	0.111	0.123	0.065	0.484	0.042	0.054	0.003	0.036	0.119	0.002	0.178	0.419	0.207	0.003	0.234	0.028	0.375	0.016	0.209		
ERβ	PC	−0.755**	0.434	0.732**	−0.423	0.350	0.573*	0.255	0.351	−0.594*	−0.651*	−0.491	−0.574*	−0.104	0.069	−0.396	0.107	−0.462	−0.041	0.446	0.666**	−0.138	1
	Sig.	0.001	0.069	0.003	0.075	0.132	0.026	0.212	0.132	0.035	0.021	0.075	0.041	0.388	0.420	0.114	0.364	0.056	0.447	0.085	0.009	0.326	

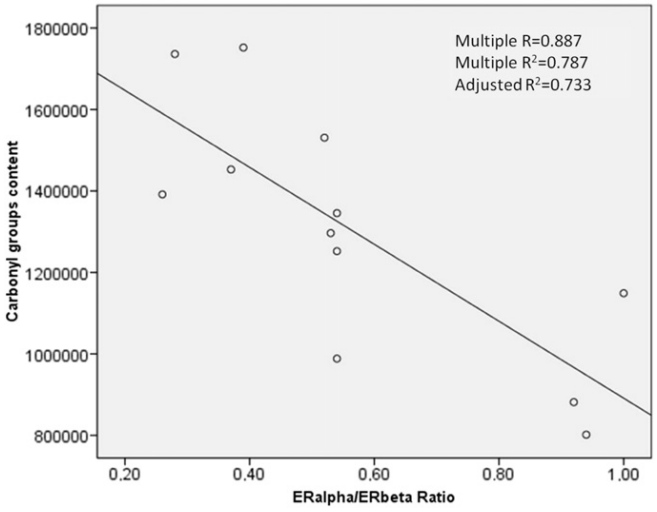
PC, Pearson correlation. Sig., significance (unilateral).

* The correlation is significant at $p < 0.05$ (unilateral).

** The correlation is significant at $p < 0.01$ (unilateral).

Table 6
Stepwise multiple linear regression analysis by step-by-step selection procedure for carbonyl groups as a dependent variable.

Independent variable	Unstandardized coefficient		Standard coefficient		
	Unstandardized regression coefficient	SE	Standardized regression coefficient	*F* value	*p* value
ERα/ERβ ratio	−1,310,352.852	341,091.197	−0.887	−3.842	0.018



DNA, lower ATP production, and an increase in mitochondrial ROS, including superoxide levels, in addition to developing ER/progesterone receptor-positive breast malignancies [24]. In this regard, loss of SIRT3 results in a series of stress- and/or aging-related phenotypes including receptor-positive breast cancer [24]. All these findings are along the same line as the results of this experiment and perhaps the most important correlation exists in SIRT3 levels versus Mn-SOD levels, as it is well known that sirtuin 3 acts not only on expression of Mn-SOD, but also on its activity [36,37].

Another point of interest related to oxidative stress is the importance of uncoupling proteins [11,17,32]. UCPs play an important role in cancer development in their control of ROS production and the sensitivity to chemotherapy of some cancers [19,30,32]. Our results show an increase in uncoupling proteins as a mechanism of response to high ROS production. For this reason, UCPs could be a therapy target to improve cancer treatment, because high inhibition with a specific inhibitor for UCPs would only increase ROS production and lead to cancer cell apoptosis [21,38].

On the whole, our results are in agreement with these two different points of view. First, the glycolytic switch (the Warburg effect) observed in malignant tissues is triggered by mitochondrial oxidative damage and/or activation of redox-sensitive transcription factors and results in an increase in cell resistance to oxidants [39–41]. Second, there are emerging lines of evidence that indicate that tumor cells must also defend themselves from oxidative damage to survive [39]. For this reason, breast tumors with a LER ratio have more oxidative damage even though these tumors cells are adapted to this status, and thus it could be postulated that UCPs and/or SIRT3 could be a new therapy target to take into account for the improvement of breast cancer treatment, because a high inhibition with a specific inhibitor for UCPs or SIRT3 would lead to an increase in ROS production and consequently cell apoptosis. In fact, taking into account previous results of our lab and results presented here, ERβ should be used as a biomarker to predict the effect of chemical agents, chiefly those that affect oxidative stress and/or estrogen signaling.

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References

[1] Shibuya, K.; Mathers, C. D.; Boschi-Pinto, C.; Lopez, A. D.; Murray, C. J. Global and regional estimates of cancer mortality and incidence by site. II. Results for the global burden of disease 2000. *BMC Cancer* **2:**37; 2002.
[2] Ray, A.; Nkhata, K. J.; Grande, J. P.; Cleary, M. P. Diet-induced obesity and mammary tumor development in relation to estrogen receptor status. *Cancer Lett.* **253:**291–300; 2007.
[3] Moreira, P. I.; Custodio, J.; Moreno, A.; Oliveira, C. R.; Santos, M. S. Tamoxifen and estradiol interact with the flavin mononucleotide site of complex I leading to mitochondrial failure. *J. Biol. Chem.* **281:**10143–10152; 2006.
[4] Sui, M.; Zhang, H.; Fan, W. The role of estrogen and estrogen receptors in chemoresistance. *Curr. Med. Chem.* **18:**4674–4683; 2011.
[5] Kumar, S.; Sacchini, V. The surgical management of ductal carcinoma in situ. *Breast J.* **16(Suppl. 1):**S49–52; 2010.
[6] Gong, Y.; Han, E. Y.; Guo, M.; Pusztai, L.; Sneige, N. Stability of estrogen receptor status in breast carcinoma: a comparison between primary and metastatic tumors with regard to disease course and intervening systemic therapy. *Cancer* **117:**705–713; 2011.
[7] Kuiper, G. G.; Enmark, E.; Pelto-Huikko, M.; Nilsson, S.; Gustafsson, J. A. Cloning of a novel receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. USA* **93:**5925–5930; 1996.
[8] Gustafsson, J. A. Estrogen receptor beta—a new dimension in estrogen mechanism of action. *J. Endocrinol* **163:**379–383; 1999.
[9] Pearce, S. T.; Jordan, V. C. The biological role of estrogen receptors alpha and beta in cancer. *Crit. Rev. Oncol. Hematol.* **50:**3–22; 2004.
[10] Jensen, E. V.; Jacobson, H. I.; Walf, A. A.; Frye, C. A. Estrogen action: a historic perspective on the implications of considering alternative approaches. *Physiol. Behav.* **99:**151–162; 2010.
[11] Roca, P.; Oliver, J.; Sastre-Serra, J.; Nadal-Serrano, M. The importance of ERα/ERβ ratio in breast cancer: mitochondrial function and oxidative stress. In: Gunduz, P., Gunduz, E., editors. *Breast Cancer—Carcinogenesis, Cell Growth and Signalling Pathways*. New York: InTech; 2011. p. 221–244.
[12] Hartman, J.; Strom, A.; Gustafsson, J. A. Estrogen receptor beta in breast cancer—diagnostic and therapeutic implications. *Steroids* **74:**635–641; 2009.
[13] Coffer, P. J.; Jin, J.; Woodgett, J. R. Protein kinase B (c-Akt): a multifunctional mediator of phosphatidylinositol 3-kinase activation. *Biochem. J.* **335:**1–13; 1998.
[14] Lee, Y. R.; Park, J.; Yu, H. N.; Kim, J. S.; Youn, H. J.; Jung, S. H. Up-regulation of PI3K/Akt signaling by 17β-estradiol through activation of estrogen receptor-α, but not estrogen receptor-β, and stimulates cell growth in breast cancer cells. *Biochem. Biophys. Res. Commun.* **336:**1221–1226; 2005.
[15] Ho, J. M.; Nguyen, M. H.; Dierov, J. K.; Badger, K. M.; Beattie, B. K.; Tartaro, P.; Haq, R.; Zanke, B. W.; Carroll, M. P.; Barber, D. L. TEL-JAK2 constitutively activates the extracellular signal-regulated kinase (ERK), stress-activated protein/Jun kinase (SAPK/JNK), and p38 signaling pathways. *Blood* **100:**1438–1448; 2002.
[16] Kumar, R.; Srinivasan, S.; Pahari, P.; Rohr, J.; Damodaran, C. Activating stress-activated protein kinase-mediated cell death and inhibiting epidermal growth factor receptor signaling: a promising therapeutic strategy for prostate cancer. *Mol. Cancer Ther* **9:**2488–2496; 2010.
[17] Sastre-Serra, J.; Valle, A.; Company, M. M.; Garau, I.; Oliver, J.; Roca, P. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. *Free Radic. Biol. Med.* **48:**506–512; 2010.
[18] Rupprecht, A.; Sokolenko, E. A.; Beck, V.; Ninnemann, O.; Jaburek, M.; Trimbuch, T.; Klishin, S. S.; Jezek, P.; Skulachev, V. P.; Pohl, E. E. Role of the transmembrane potential in the membrane proton leak. *Biophys. J.* **98:**1503–-1511; 2010.
[19] Horimoto, M.; Resnick, M. B.; Konkin, T. A.; Routhier, J.; Wands, J. R.; Baffy, G. Expression of uncoupling protein-2 in human colon cancer. *Clin. Cancer Res.* **10:**6203–6207; 2004.
[20] Derdak, Z.; Mark, N. M.; Beldi, G.; Robson, S. C.; Wands, J. R.; Baffy, G. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. *Cancer Res.* **68:**2813–2819; 2008.

[21] Santandreu, F. M.; Roca, P.; Oliver, J. Uncoupling protein-2 knockdown mediates the cytotoxic effects of cisplatin. *Free Radic. Biol. Med.* **49:**658–666; 2010.
[22] Kong, X.; Wang, R.; Xue, Y.; Liu, X.; Zhang, H.; Chen, Y.; Fang, F.; Chang, Y. Sirtuin 3, a new target of PGC-1alpha, plays an important role in the suppression of ROS and mitochondrial biogenesis. *PLoS One* **5:**e11707; 2010.
[23] Alhazzazi, T. Y.; Kamarajan, P.; Joo, N.; Huang, J. Y.; Verdin, E.; D'Silva, N. J.; Kapila, Y. L. Sirtuin-3 (SIRT3), a novel potential therapeutic target for oral cancer. *Cancer* **117:**1670–1678; 2011.
[24] Kim, H. S.; Patel, K.; Muldoon-Jacobs, K.; Bisht, K. S.; Aykin-Burns, N.; Pennington, J. D.; van der Meer, R.; Nguyen, P.; Savage, J.; Owens, K. M.; Vassilopoulos, A.; Ozden, O.; Park, S. H.; Singh, K. K.; Abdulkadir, S. A.; Spitz, D. R.; Deng, C. X.; Gius, D. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. *Cancer Cell* **17:**41–52; 2010.
[25] Cimen, H.; Han, M. J.; Yang, Y.; Tong, Q.; Koc, H.; Koc, E. C. Regulation of succinate dehydrogenase activity by SIRT3 in mammalian mitochondria. *Biochemistry* **49:**304–311; 2010.
[26] Alhazzazi, T. Y.; Kamarajan, P.; Verdin, E.; Kapila, Y. L. SIRT3 and cancer: tumor promoter or suppressor? *Biochim. Biophys. Acta* **1816:**80–88; 2011.
[27] Cadenas, C.; Franckenstein, D.; Schmidt, M.; Gehrmann, M.; Hermes, M.; Geppert, B.; Schormann, W.; Maccoux, L. J.; Schug, M.; Schumann, A.; Wilhelm, C.; Freis, E.; Ickstadt, K.; Rahnenfuhrer, J.; Baumbach, J. I.; Sickmann, A.; Hengstler, J. G. Role of thioredoxin reductase 1 and thioredoxin interacting protein in prognosis of breast cancer. *Breast Cancer Res.* **12:**R44; 2010.
[28] Chen, Q.; Vazquez, E. J.; Moghaddas, S.; Hoppel, C. L.; Lesnefsky, E. J. Production of reactive oxygen species by mitochondria: central role of complex III. *J. Biol. Chem.* **278:**36027–36031; 2003.
[29] Guo, W. J.; Ye, S. S.; Cao, N.; Huang, J.; Gao, J.; Chen, Q. Y. ROS-mediated autophagy was involved in cancer cell death induced by novel copper(II) complex. *Exp. Toxicol. Pathol.* **62:**577–582; 2010.
[30] Valle, A.; Oliver, J.; Roca, P. Role of uncoupling proteins in cancer. *Cancers* **2**; 2010.
[31] Benhar, M.; Engelberg, D.; Levitzki, A. ROS, stress-activated kinases and stress signaling in cancer. *EMBO Rep.* **3:**420–425; 2002.
[32] Santandreu, F. M.; Valle, A.; Fernandez de Mattos, S.; Roca, P.; Oliver, J. Hydrogen peroxide regulates the mitochondrial content of uncoupling protein 5 in colon cancer cells. *Cell. Physiol. Biochem.* **24:**379–390; 2009.
[33] Fukai, T.; Ushio-Fukai, M. Superoxide dismutases: role in redox signaling, vascular function, and diseases. *Antioxid. Redox Signaling* **15:**1583–1606; 2011.
[34] Woolston, C. M.; Al-Attar, A.; Storr, S. J.; Ellis, I. O.; Morgan, D. A.; Martin, S. G. Redox protein expression predicts radiotherapeutic response in early-stage invasive breast cancer patients. *Int. J. Radiat. Oncol. Biol. Phys.* **79:**1532–1540; 2011.
[35] Felty, Q.; Xiong, W. C.; Sun, D.; Sarkar, S.; Singh, K. P.; Parkash, J.; Roy, D. Estrogen-induced mitochondrial reactive oxygen species as signal-transducing messengers. *Biochemistry* **44:**6900–6909; 2005.
[36] Ozden, O.; Park, S. H.; Kim, H. S.; Jiang, H.; Coleman, M. C.; Spitz, D. R.; Gius, D. Acetylation of MnSOD directs enzymatic activity responding to cellular nutrient status or oxidative stress. *Aging (Albany NY)* **3:**102–107; 2011.
[37] Chen, Y.; Zhang, J.; Lin, Y.; Lei, Q.; Guan, K. L.; Zhao, S.; Xiong, Y. Tumour suppressor SIRT3 deacetylates and activates manganese superoxide dismutase to scavenge ROS. *EMBO Rep.* **12:**534–541; 2011.
[38] Samudio, I.; Fiegl, M.; McQueen, T.; Clise-Dwyer, K.; Andreeff, M. The Warburg effect in leukemia–stroma cocultures is mediated by mitochondrial uncoupling associated with uncoupling protein 2 activation. *Cancer Res* **68:** 5198–5205; 2008.
[39] Pani, G.; Galeotti, T.; Chiarugi, P. Metastasis: cancer cell's escape from oxidative stress. *Cancer Metastasis Rev.* **29:**351–378; 2010.
[40] Baffy, G. Uncoupling protein-2 and cancer. *Mitochondrion* **10:**243–252; 2010.
[41] Echtay, K. S.; Esteves, T. C.; Pakay, J. L.; Jekabsons, M. B.; Lambert, A. J.; Portero-Otin, M.; Pamplona, R.; Vidal-Puig, A. J.; Wang, S.; Roebuck, S. J.; Brand, M. D. A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. *EMBO J.* **22:**4103–4110; 2003.

Manuscript 11

Genistein modulates oxidative stress in breast cancer cell lines according to ERα/ERβ ratio: effects on mitochondrial functionality, sirtuins, uncoupling protein 2 and antioxidant enzymes

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Genistein modulates oxidative stress in breast cancer cell lines according to ERα/ERβ ratio: Effects on mitochondrial functionality, sirtuins, uncoupling protein 2 and antioxidant enzymes

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ABSTRACT

Genistein is a biologically active isoflavone with estrogenic activity and can be found in a variety of soy products. This natural compound displays a wide array of biological activities, but it is best known for its ability to inhibit cancer progression, especially for hormone-related ones such as breast cancer. Genistein has been shown to bind both the estrogen receptor alpha (ERα) and the estrogen receptor beta (ERβ), although it has a higher affinity for the ERβ. The ERα/ERβ ratio is a prognostic marker for breast tumors, and ERβ expression could indicate the presence of tumors more benign in state, whereas ERα indicates malignant tumors. The objective of the present study was to investigate the effects of genistein on oxidative stress and mitochondrial functionality through its interaction with the estrogen receptor in breast cancer cell lines with different ERα/ERβ ratios. The lower ERα/ERβ ratio T47D cell line showed lower oxidative stress and greater mitochondrial functionality, along with an up-regulation of uncoupling protein 2 and sirtuins. On the other hand, genistein-treated MCF-7 cell line, with the highest ERα/ERβ ratio, reported no changes for the control situation. On the whole, our results show different genistein effects depending on ERα/ERβ ratio for oxidative stress regulation, mitochondrial functionality, and modulation of UCPs, antioxidant enzymes and sirtuins in breast cancer cell lines. Effects of genistein on oxidative stress and mitochondria could be due at least in part, to a higher ERβ presence, but could also be due to up-regulation of ERβ caused by the genistein treatment.

1. Introduction

Oxidative stress leads to lipid, carbohydrate, protein and DNA damage in biological systems and affects cell structure and function. Reactive oxygen species (ROS) have a key role in oxidative stress, which are generated primarily in the mitochondria (Pelicano et al., 2004). It has been postulated that oxidative stress is one of the mechanisms triggering the carcinogenic effect, especially in breast cancer, as the accumulation of cellular damage could play a role in the development and progression of this disease (Behrend et al., 2003; Pelicano et al., 2004). To survive, cancer cells must acquire adaptive mechanisms that counteract the harmful effects of exposure to free radicals (Ozben, 2007; Pelicano et al., 2004).

Previous papers have highlighted that lifetime exposure to estrogens is associated with increased breast cancer risk, and may contribute to tumor growth (Nadal-Serrano et al., 2012; Sastre-Serra et al., 2010; Yager and Davidson, 2006). Phytoestrogens are a class of plant-derived compounds that are structurally similar to the principal mammalian estrogen, 17β-estradiol (E2), and they have a weak estrogen activity. One of the best-studied is genistein (GEN), and epidemiological studies have shown an inverse correlation between the intake of GEN and the incidence of breast cancer (Adlercreutz, 2002). In fact, in Asia, where women consume significantly higher amounts of phytoestrogens, the incidence of breast cancer is much lower than for Western women. (Adlercreutz, 2002). As mentioned, GEN structurally mimics E2 and could modulate the effects of estrogen in breast cancer cells through the estrogen receptor (ER). GEN selectively binds to ERβ with a stronger affinity than

Abbreviations: CAT, catalase; DCFDA, 2′,7′-dichlorofluorescein diacetate; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; GEN, genistein; 4-HNE, 4-hydroxy-2-nonenal; MnSOD, manganese superoxide dismutase; Δψm, mitochondrial membrane potential; NAO, Nonyl Acridine Orange; ROS, reactive oxygen species; OXPHOS, oxidative phosphorylation; SIRT, sirtuin; TMRM, tetramethylrhodamine methyl ester; UCP, uncoupling protein.

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ERα (Kuiper et al., 1997, 1998) and thereby could be a potential point of modulation for breast cancer development in cells with different ERα/ERβ ratios. Indeed, some data suggest that when ERβ is activated by certain ligands, this activated complex inhibits mammary cancer cell growth as well as the stimulatory effects of ERα (McCarty, 2006; Strom et al., 2004), and thus the preferential binding of isoflavone to ERβ may have implications in reducing breast cancer risk. Some reported possible mechanisms by which GEN may reduce the risk of breast cancer are through its antioxidant properties, achieved by free radical scavenging, or by the inhibition of the expression of stress-response related genes which induce cell cycle arrest, as well as inhibiting angiogenesis, invasion and metastasis of cancer cells (Ouyang et al., 2009; Pavese et al., 2010; Ruiz-Larrea et al., 1997).

Sirtuins (SIRTs) and uncoupling proteins (UCPs) are key protein regulators of stress responses and metabolism, and their biological functions could be involved in pathological conditions such as cancer (Derdak et al., 2008; Martinez-Pastor and Mostoslavsky, 2012). Sirtuins are a highly conserved family of NAD^+ -dependent deacetylases involved in stress response, metabolism and longevity (Milne and Denu, 2008). In humans, seven sirtuins have been identified (Michishita et al., 2005), although SIRT1 (localized mainly in the nucleus) and SIRT3 (localized mainly in the mitochondria) are of the particular interest, since they regulate both cell death and survival, and therefore a controversy has emerged in the literature about their role as a tumor promoter and/or tumor suppressor (Alhazzazi et al., 2011; Martinez-Pastor and Mostoslavsky, 2012). On the other hand, UCPs belong to the mitochondrial anion transporter superfamily and are located in the inner mitochondrial membrane, where they cause a mild uncoupling, which results in a decrease of the mitochondrial membrane potential, ($\Delta\psi m$) (Derdak et al., 2008). Consequently, UCPs decrease the mitochondrial ROS production, protecting the cell from oxidative stress (Derdak et al., 2008; Nadal-Serrano et al., 2012; Sastre-Serra et al., 2010). Moreover, the UCP2 isoform has been detected in some chemoresistant cancer cells and is one of the many adaptive mechanisms used by drug-resistant cells to maintain ROS homeostasis, and thereby reinforces the activation of signaling cascades that contribute to tumor growth, angiogenesis and metastasis (Ayyasamy et al., 2011; Baffy et al., 2011; Derdak et al., 2008).

The aim of present study, taking into account the current knowledge about free radical biology and its influence in the development and progression of breast cancer, was to investigate the effect of GEN on mitochondrial ROS production, $\Delta\psi m$ and oxidative damage, as well as the response of the antioxidant enzymes and mitochondrial functionality and its relationship to sirtuins and UCP2, in breast cancer cell lines with different ERα/ERβ ratios.

2. Materials and methods

2.1. Materials

Genistein (GEN) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Routine chemicals used were obtained from Sigma–Aldrich, Roche (Barcelona, Spain), Panreac (Barcelona, Spain) and Bio-Rad Laboratories (Hercules, CA, USA).

2.2. Cell culture and treatments

Human breast cancer cell lines T47D (ERα and ERβ positive, with predominance of ERβ) and MCF-7 (ERα and ERβ positive, with predominance of ERα) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Both cell lines are characterized by an important difference in ERα/ERβ ratio, with MCF-7 cell line possessing the highest ratio (Nadal-Serrano et al., 2012). Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) in a humidified atmosphere of 5% CO_2 at 37 °C. To evaluate the effects of GEN, cells were grown in phenol red-free DMEM containing 10% (v/v) charcoal-stripped FBS 24 h before treatment. Experiments were carried out when cell cultured reached confluence by providing fresh medium supplemented with 1 μmol/L GEN.

2.3. Measurement of ROS production by flow cytometry

ROS (reactive oxygen species) levels were measured as described previously by Sastre-Serra et al. (2010), measuring fluorescence of 2′,7′-dichlorofluorescein diacetate (DCFDA) using an Epics XL flow cytometer (Beckman-Coulter, Miami, FL, USA). The green fluorescence was measured using the FL-1 setting (log mode) after the cell debris was electronically gated out. 10 000 events were acquired and analysis was performed with Expo32 ADC analysis software (Beckman Coulter, Miami, FL, USA).

2.4. Analysis of $\Delta\psi m$ by flow cytometry

Cells were dyed with 250 nmol/L tetramethylrhodamine methyl ester (TMRM) for 15 min, washed by PBS containing 20 mmol/L glucose, and analyzed by flow cytometry (Sastre-Serra et al., 2010). The orange–red fluorescence was measured using the FL-2 setting (log mode) after the cell debris were electronically gated out. In each analysis, 10 000 events were recorded and analyzed with Expo32 ADC analysis software (Beckman Coulter, Miami, FL, USA).

2.5. Measurement of 4-HNE adducts content

For 4-hydroxy-2-nonenal (4-HNE) analysis, 40 μg of protein from cell lysate was transferred onto nitrocellulose membrane. Antiserum against 4-HNE (HNE11-S) was used as primary antibody (Alpha Diagnostic International, San Antonio, TX, USA). Bands were visualized using the by Immun-Star® Western C® Kit reagent (Bio-Rad). The chemiluminescence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analyzed with Quantity One software (Bio-Rad).

2.6. Analysis of cardiolipin content

Cardiolipin content was assayed using Nonyl Acridine Orange (NAO) fluorescent and quantified using a microplate fluorescent reader FLx800 (BIO-TEK Winooski, Vermont, USA) set at 485/20 nm to excitation and 528/20 to emission. The fluorescence was corrected by Hoescht 33342 (1 μg/well) fluorescence with the same fluorescence reader set at 360/40 nm to excitation and 460/40 to emission.

2.7. Mitochondrial DNA quantification

DNA was isolated from cultured cells using TriPure® isolation reagent (Roche, Barcelona, Spain) following the manufacturer's protocol and used to quantify mtDNA (Sastre-Serra et al., 2012b). 5 ng of the total DNA (quantified using a spectrophotometer set at 260 nm) was amplified using specific primers for 18S and the NADH dehydrogenase subunit 4, with SYBR Green technology on a LightCycler 480 System II (Roche Diagnostics, Basel, Switzerland).

The primers used were forward 5′-ggACACggACAggATTgACA-3′ and reverse 5′-ACCCACggAATCgAgAAAgA-3′ for 18S and forward 5′-CgTgACTCCTACCCCTCACA-3′ and reverse 5′-ATCggg-TgATgATAgCCAAg- 3′ for the NADH dehydrogenase subunit 4 (mtDNA).

Table 1
Table and conditions used for RT-PCR.

Gene	Forward primer (5′–3′) Reverse primer (5′–3′)	T° An. (°C)
18S	ggACACggACAggATTgACA ACCCACggAATCgAgAAAgA	61
UCP2	ggTggTCggAgATACCAAAg CTCgggCAATggTCTTgTAg	60
SIRT3	CggCTCTACACgCAgAACATC CAgAggCTCCCCAAAgAACAC	56
CAT	CATCgCCACATgAATggATA CCAACTgggATgAgAgggTA	61
Mn-SOD	CgTgCTCCCACACATCAATC TgAACgTCACCgAggAgAAg	64
CuZn-SOD	TCAggAgACCATTgCATCATT CgCTTTCCTgTCTTTgTACTTTCTTC	64
ERα	AATTCAgATAATCgACgCCAg gTgTTTCAACATTCTCCCTCCTC	61
ERβ	TAgTggTCCATCgCCAgTTAT gggAgCCACACTTCACCAT	64
NRF1	ggTCAgAgATgTgCTTgCAA AAggATTCCTgggAAggAgA	60
NRF2	gCgACggAAAgAgTATgAgC gTTggCAgATCCACTggTTT	60
PGC1α	TCATgCCgTggTAAgTACCA gTgCAAAgTTCCCTCTCTgC	60
pS2	TTgTggTTTTCCTggTgTCA gCAgATCCCTgCAgAAgTgT	61

T° An., Annealing temperature; CAT, catalase; CuZn-SOD, copper and zinc superoxide dismutase; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; Mn-SOD, manganese superoxide dismutase; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; pS2, presenilin 2; SIRT3, sirtuin 3; and UCP2, uncoupling protein 2.

2.8. Real-time quantitative PCR

Total RNA was isolated from T47D and MCF-7 cultured cells using TriPure® isolation reagent (Roche, Barcelona, Spain) following the manufacturer's protocol and quantified using a spectrophotometer set at 260 nm. One microgram of the total RNA was reverse transcribed to cDNA at 42 °C for 60 min with 25 U MuLV reverse transcriptase in a 10 μl volume of retrotranscription reaction mixture containing 10 mM Tris–HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 2.5 μM random hexamers, 10 U RNase inhibitor, and 500 μM each dNTP. Each cDNA was diluted 1/10 and aliquots were frozen (−80 °C) until the PCR reactions were carried out.

PCR was done for ten target genes: ERα, ERβ, PGC1α, NRF1, NRF2, Mn-SOD, CuZn-SOD, CAT, pS2, UCP2, SIRT3 and 18S ribosomal RNA (18S), like one housekeeping gene. PCR was carried out using specific primers (see Table 1) SYBR Green technology on a LightCycler 480 System II rapid thermal cycler (Roche Diagnostics, Basel, Switzerland). Total reaction volume was 10 μL, containing 6.5 μL Lightcycler® 480 SYBR Green I Master, 0.5 μM of the sense and antisense specific primers and 2.5 μL of the cDNA template. The amplification program consisted of a preincubation step for denaturation of the template cDNA (5 min, 95 °C), followed by 45 cycles consisting of a denaturation step (10 s, 95 °C), an annealing step (10 s, 56 °C for SIRT3; 60 °C for UCP2, PGC1α, NRF1 and NRF2; 61 °C for ERα, CAT, pS2 and 18S, and 64 °C for ERβ, Mn-SOD and CuZn-SOD), and an extension step (12 s, 72 °C min). A negative control lacking cDNA template was run in each assay.

The resulting PCR products were resolved on a 2% (w/v) agarose gel in 45 mM Tris-borate-EDTA 1 mM buffer (pH 8.0) and visualized by ethidium bromide staining. Bands were analyzed with a Chemidoc XRS densitometer (Biorad). The Ct values of the real-time PCR were analyzed, taking into account the efficiency of the reaction and referring these results to the total DNA amount, using the GenEx Standard Software (Multi-DAnalises, Sweden).

2.9. Western blot analysis

Cells were harvested by scraping in lysis buffer (20 mM Tris–HCl, 1.5 mM $MgCl_2$, 140 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin and 10 μg/mL pepstatin; pH 7.4) and disrupted by sonication. Afterwards, protein content was determined with a bicinchoninic acid protein assay kit (Pierce, Bonn, Germany). Western blot analysis of 40 μg of protein from cell lysate was done as previously described (Nadal-Serrano et al., 2012). Antisera against UCP2 and α-Tubulin, the latter used as housekeeping protein, (Santa Cruz Biotechnology, CA, USA), OXPHOS Complex (MitoSciences, OR, USA), CuZnSOD and MnSOD (Calbiochem, CA, USA), and SIRT3 (Millipore, CA, USA) were used as primary antibodies. Protein bands were visualized by Immun-Star® Western C® Kit reagent (Bio-Rad) Western blotting detection systems. The chemiluminiscence signal was captured with a Chemidoc XRS densitometer (Bio-Rad) and analyzed with Quantity One Software (Bio-Rad).

2.10. Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, version 18.0; SPSS Inc., Chicago, IL) was used for all statistical analyses. Data are presented as means ± standard error of the mean (SEM). Statistical differences between control and GEN-treated cells were analyzed by Student's *t*-test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Determination of ROS levels, Δψm and 4-HNE adducts in T47D and MCF-7 breast cancer cell lines after 48 h GEN-treatment

As shown in Table 2, the GEN-treated T47D cell line had a decrease in ROS levels (30%), whereas for the MCF-7 cell line, no changes were found for this same treatment. Moreover, the T47D cell line treated with GEN showed a significant decrease in Δψm (23%). The 4-HNE adducts content, as marker of oxidative damage, showed a tendency to decrease in T47D cell line after treatment ($p = 0.08$), while the MCF-7 GEN-treated cell line showed no change in the 4-HNE adducts.

3.2. Mitochondrial proliferation and differentiation

Table 3 reports a tendency to increase the cardiolipin content ($p = 0.07$), measured by NAO, in the T47D cell line after GEN-treatment for 48 h, whereas this cell line did not show changes in mtDNA copy number. For this reason, the NAO/mtDNA ratio was lower in T47D cell line respect to control, while the MCF-7 cell line did not show changes in these parameters.

3.3. Protein levels of mitochondrial respiratory chain complexes (OXPHOS) after treatment with 1 μmol/L GEN for 48 h

The protein levels of OXPHOS complexes were determined by Western blot method (Section 2.9), as shown in Table 4. Interestingly, in the T47D cell line complexes I and complex II had significantly higher protein level contents (32% and 19%, respectively) after GEN treatment. Moreover, it can be observed that complex IV showed a tendency to increase slightly ($p = 0.09$). However, the GEN-treated MCF-7 cell line did not present significant changes when compared to vehicle-treated cells.

Table 2
The ROS levels, Δψm and 4-HNE adducts content in T47D and MCF-7 breast cancer cell lines after 48 h GEN-treatment.

	MCF-7		T47D	
	Control	GEN	Control	GEN
ROS levels (AU)	100 ± 11	99.3 ± 11.0	100 ± 4	70.4 ± 3.7*
TMRM fluorescence (AU)	100 ± 6	90.2 ± 4.2	100 ± 7	76.9 ± 1.7*
4-HNE adducts content (AU)	100 ± 18	104 ± 22	100 ± 17	69.1 ± 8.8**

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) T47D and MCF-7 cell lines were set at 100. AU, arbitrary units; 4-HNE, 4-hydroxy-2-nonenal; ROS, reactive oxygen species; TMRM, tetramethylrhodamine methyl ester.
* Significant difference between GEN-treated and vehicle-treated cell lines (Student's *t*-test; $P < 0.05$).
** Significant difference between GEN-treated and vehicle-treated cell lines (Student's *t*-test; $P < 0.1$).

Table 3
Effects of GEN for 48 h treatment on mitochondrial proliferation and differentiation in T47D and MCF-7 cell lines.

	MCF-7		T47D	
	Control	GEN	Control	GEN
mtDNA (AU)	100 ± 9	109 ± 8	100 ± 8	86.9 ± 7.9
NAO(AU)	100 ± 3	105 ± 4	100 ± 8	121 ± 11*
mtDNA/NAO Ratio	1.00	1.04	1.00	0.72

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) T47D and MCF-7 cell lines were set at 100. AU, arbitrary units; mtDNA, mitochondrial DNA; NAO, 10-N-nonyl-acridine orange.
* Significant difference between GEN-treated and vehicle-treated cell lines (Student's *t*-test; $P < 0.1$).

3.4. Effects of GEN on mRNA expression levels of main genes involved in response to oxidative stress and mitochondrial biogenesis and function. Expression levels of ERα and ERβ

The 4 h GEN treatment for the T47D breast cancer cell line increased mRNA expression levels of UCP2, SIRT3, ERβ and NRF2, as can be observed in Table 5. However, mRNA expression levels of CuZn-SOD decreased with the treatment. In the MCF-7 breast cancer cell line, gene expression of UCP2, CAT and PGC1α diminished, while gene expression of ERβ and pS2 increased.

3.5. Effects of GEN on oxidative stress-related protein levels and mitochondrial function

Table 6 shows the changes observed in GEN-treated T47D and MCF-7 breast cancer cell lines for the main antioxidant enzymes. In the T47D cell line, CuZn-SOD and Mn-SOD protein levels showed a statistically significant increase after GEN treatment. Nevertheless, the MCF-7 GEN-treated cell line did not show significant changes. Fig. 1 presents the effect of the same treatment on target protein related mitochondrial biogenesis and function. The results

Table 5
mRNA expression in T47D and MCF-7 breast cancer cell lines after GEN-treatment for 4 h.

	MCF-7		T47D	
	Control	GEN	Control	GEN
UCP2	1.00 ± 0.22	0.55 ± 0.02*	1.00 ± 0.04	1.60 ± 0.06*
SIRT 3	1.00 ± 0.17	0.70 ± 0.04	1.00 ± 0.03	1.24 ± 0.08*
CAT	1.00 ± 0.13	0.73 ± 0.06*	1.00 ± 0.06	1.00 ± 0.06
Mn-SOD	1.00 ± 0.22	1.04 ± 0.07	1.00 ± 0.19	0.90 ± 0.14
CuZn-SOD	1.00 ± 0.15	0.85 ± 0.08	1.00 ± 0.06	0.80 ± 0.03*
ERα	1.00 ± 0.20	0.72 ± 0.09	1.00 ± 0.13	1.17 ± 0.14
ERβ	1.00 ± 0.51	1.64 ± 0.20*	1.00 ± 0.28	3.81 ± 0.93*
PGC1α	1.00 ± 0.40	0.23 ± 0.05*	1.00 ± 0.14	0.89 ± 0.09
NRF1	1.00 ± 0.33	0.41 ± 0.05	1.00 ± 0.28	1.04 ± 0.26
NRF2	1.00 ± 0.22	1.06 ± 0.08	1.00 ± 0.04	1.30 ± 0.14*
pS2	1.00 ± 0.09	1.26 ± 0.04*	1.00 ± 0.03	1.01 ± 0.05

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) T47D and MCF-7 cell lines were set at 1.00. CAT, catalase; CuZn-SOD, copper and zinc superoxide dismutase; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; Mn-SOD, manganese superoxide dismutase; NRF1, nuclear respiratory factor 1; NRF2, nuclear respiratory factor 2; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; pS2, presenilin 2; SIRT3, sirtuin 3; and UCP2, uncoupling protein 2.
* Significant difference between GEN-treated and vehicle-treated cell lines cells (Student's *t*-test; $P < 0.05$).

Table 6
Effects of GEN on antioxidant enzymes protein levels in T47D and MCF-7 cell lines.

	MCF-7		T47D	
	Control	GEN	Control	GEN
Mn-SOD (AU)	100 ± 6	103 ± 9	100 ± 8	159 ± 17*
CuZn-SOD (AU)	100 ± 7	88.8 ± 5.7	100 ± 4	127 ± 7*
CAT (AU)	100 ± 7	99.9 ± 8.6	100 ± 3	95.8 ± 1.9

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) T47D and MCF-7 cell lines were set at 100. AU, arbitrary units; CAT, catalase; CuZn-SOD, copper and zinc superoxide dismutase; Mn-SOD, manganese superoxide dismutase.
* Significant difference between GEN-treated and vehicle-treated cell lines (Student's *t*-test; $P < 0.05$).

Table 4
OXPHOS Complex protein levels in T47D and MCF -7 breast cancer cell lines after GEN treatment.

	MCF-7			T47D		
	Control	GEN		Control		GEN
Complex V (AU)	100 ± 8	111 ± 2		100 ± 9		109 ± 7
Complex III (AU)	100 ± 7	110 ± 4		100 ± 8		115 ± 9
Complex II (AU)	100 ± 4	101 ± 7		100 ± 6		119 ± 7*
Complex IV (AU)	100 ± 6	94.9 ± 11.7		100 ± 6		112 ± 6**
Complex I (AU)	100 ± 6	91.2 ± 8.5		100 ± 5		132 ± 8*

Data represent the means ± SEM ($n = 6$). Values of control (vehicle-treated) T47D and MCF-7 cell lines were set at 100. Complex V: ATP synthase subunit alpha–"CV-alpha"–53 kDa; complex III subunit core 2–"CIII-core2"–47 kDa; complex II-FeS subunit 30 kDa–"CII-30"–30 kDa; complex IV subunit II–"CIV-II"–24 kDa; complex I subunit NDUFB8–"CI-20"–20 kDa. AU: arbitrary units.
Representative bands of OXPHOS complex in each cell line are shown together protein levels values.
* Significant difference between GEN-treated and vehicle-treated cell lines (Student's *t*-test; $P < 0.05$).
** Significant difference between GEN-treated and vehicle-treated cell lines (Student's *t*-test; $P < 0.1$).

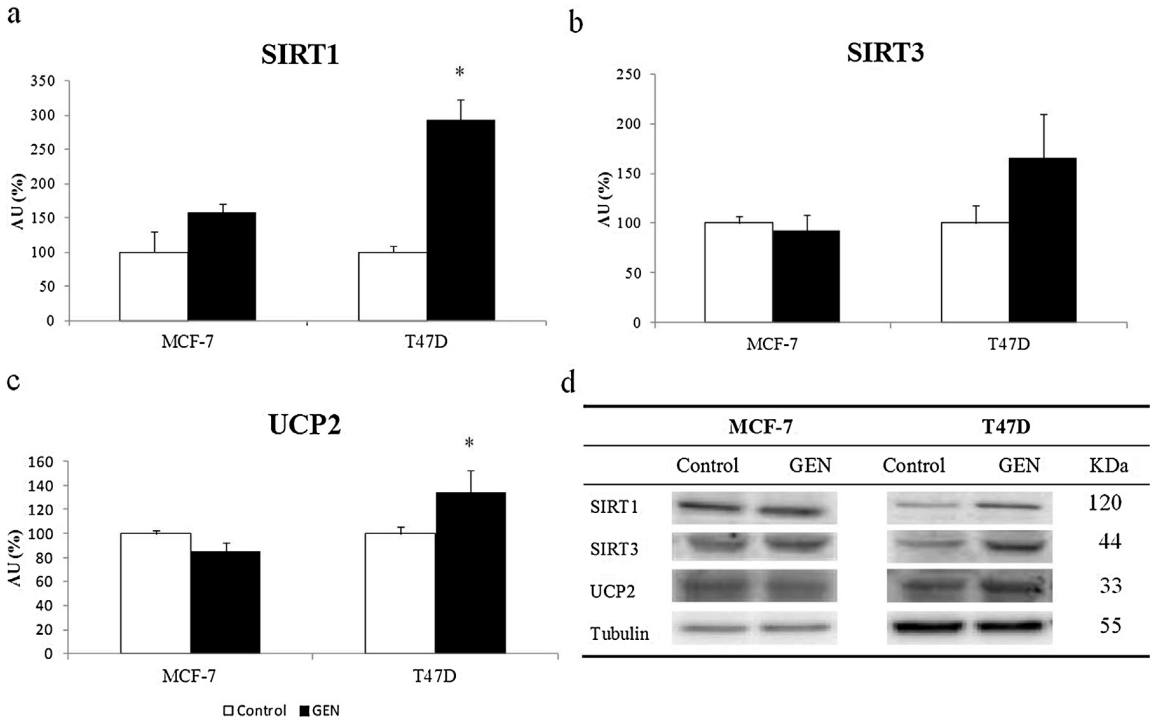

Fig. 1. SIRT1 protein levels in response to GEN-treatment in T47D and MCF-7 breast cancer cell lines (a). Effects of GEN on SIRT3 protein levels in breast cancer cell lines (b). Effects of GEN on UCP2 isoform protein levels (c). Levels of UCP2 and SIRT1 and SIRT3 after treatment were measured by Western blot as described in Section 2 (d). All measurements were made after 48 h of 1 μmol/L GEN or vehicle (DMSO) treatment. Bars represent means ± SEM ($n = 6$), with the values of vehicle-treated cell lines were set at 1.00. *Significant difference between GEN-treated and vehicle-treated cell lines (Student's t-test; $P < 0.05$, $n = 6$). C, control; GEN, GEN-treated cell line, SIRT1, sirtuin 1; SIRT3, sirtuin 3; UCP2, uncoupling protein 2.

displayed a significant up-regulation of UCP2 and SIRT1 in GEN-treated T47D cell line, while the MCF-7 line showed no effect.

4. Discussion

This present study shows that GEN treatment on T47D cell line, characterized by a low ERα/ERβ ratio (Nadal-Serrano et al., 2012), improves the antioxidant enzyme response and enhances mitochondrial biogenesis and functionality, with the consequent reduction of oxidative stress. Moreover, our results report changes in the SIRT1 and UCP2, with an increase in the protein levels after GEN treatment and these in turn diminish oxidative stress. In contrast, for the MCF-7 cell line, which has the highest ERα/ERβ ratio, GEN treatment did not cause any change in either mitochondrial functionality or antioxidant response, nor were there any changes in the sirtuin and UCP2 levels. These data contrast with the E2-effect reported in recent papers of our laboratory (Nadal-Serrano et al., 2012; Sastre-Serra et al., 2012b, 2010). Our previous studies suggest a dual E2 effect on oxidative stress and mitochondrial function according to the ERα/ERβ ratio present in breast cancer cell lines, and thus, E2 could provide protection against oxidative stress in the T47D cell line (Nadal-Serrano et al., 2012). Moreover, this cell line shows more functional mitochondria after E2 treatment (Sastre-Serra et al., 2012b), whereas, in the MCF7 cell line, with the binding of E2 to ERα, E2 increases mitochondrial-derived ROS production by repressing UCPs and antioxidant enzymes, which would result in a lower mitochondrial activity (Nadal-Serrano et al., 2012; Sastre-Serra et al., 2012b).

The GEN-treated T47D cell line, with a lower ERα/ERβ ratio, showed a decrease in oxidative damage and lower ROS levels, which is strongly linked to a decrease in $\Delta\psi m$ (Negre-Salvayre et al., 1997; Sastre-Serra et al., 2010), and which is also described in the present study (Section 3.1). This lower oxidative stress could be explained on one hand by antioxidant enzymes and on the other by enhanced mitochondrial functionality. Regarding antioxidant enzymes, the results showed (Section 3.5) that GEN treatment increased Mn-SOD and CuZn-SOD protein levels, although there were no changes in CAT protein levels. It might seem that this status could lead to a life-threatening situation for the cell, due to the accumulation of H_2O_2 in the cell; however, the GEN-treated T47D cell line did not show higher oxidative damage than the control situation. Therefore, the lower oxidative stress observed could be due to a better mitochondria functionality of the cell line, as reported also by Rasbach (Rasbach and Schnellmann, 2008). It is known that the deregulation of cellular energy metabolism has been thought to be an emerging hallmark of cancer, hence effective control of mitochondrial biogenesis and turnover is critical for the maintenance of energy production and the prevention of endogenous oxidative stress (Fogg et al., 2011; Hanahan and Weinberg, 2011). It must be emphasized that mitochondrial biogenesis is made up of two linked processes, mitochondria proliferation and differentiation. Proliferation involves an increase in mitochondrial number, with a resultant increase in mtDNA copy number, whereas differentiation affects pre-existent mitochondria, and increases both mitochondrial cristaes and protein, indicating more functional mitochondria (Sastre-Serra et al., 2012a). As shown in the results (Section 3.2), the GEN-treated T47D cell line had a slight increase in cardiolipin content, indicator of quantity of the inner mitochondrial membrane (Sastre-Serra et al., 2012a), while there were no changes in the mtDNA copy number, suggesting more differentiated mitochondria and with more functionality such as result. This result could explain that the T47D breast cancer cell line had more functional mitochondria, in part due to the increase in differentiation of the pre-existent mitochondria after treatment with GEN. Furthermore, we analyzed the mitochondrial respiratory chain complexes which

showed an overall increase in the OXPHOS complexes of T47D cell line after treatment, supporting the effect of GEN on improving mitochondrial functionality.

Nowadays, there is more evidence that points toward a central role of oxidative stress in the pathogenesis of mammary cancer (Carew and Huang, 2002). For this reason, in the present study we also examined the role that UCP2 could play in ROS production after treatment of breast cancer cells with GEN. The uncoupling protein UCP2 has been shown to be up-regulated in many human cancers due to oxidative stress, where it reduces oxidative stress and provides a growth advantage for these cancers (Derdak et al., 2008). Many authors have attributed an antioxidant effect to GEN due to its ability to scavenge free radicals or by inhibiting the expression of stress-response related genes (Ruiz-Larrea et al., 1997). In fact, our results show that GEN-treated T47D cell line presented an increase in UCP2 protein levels, which is in agreement with observed decreases in ROS levels and $\Delta\psi m$. Indeed, UCP2 allows the reentry of protons into the matrix, dissipating the proton gradient and, therefore increasing the respiration rate and decreasing the $\Delta\psi m$, and as a consequence minimizing mitochondria-derived ROS production. Altogether, these results support that mitochondria are more functional and T47D cells would be more adapted or protected against oxidative stress.

Whereas in the MCF-7 cell line, with the highest ERα/ERβ ratio (Nadal-Serrano et al., 2012; Sastre-Serra et al., 2012b), the GEN treatment had no effects on antioxidant enzymes or on mitochondrial biogenesis. The differences observed between the MCF-7 and the T47D cell lines could be explained, in part, by their distinct ERα/ERβ ratio. Our findings indicate that GEN either provides protective effects or causes no changes on oxidative stress in breast cancer cell lines, as the GEN-treated T47D cell line, with a higher predominance of ERβ, had a lower oxidative stress and greater functional mitochondria than the MCF-7 cell line, with the highest ERα/ERβ ratio. Therefore, the beneficial role of GEN could be due to the presence of ERβ, and several studies can be found in the literature show that GEN has more affinity for ERβ, than for ERα, while E2 has a greater affinity for ERα, and therefore GEN may induce antiproliferative effects (Bondesson and Gustafsson, 2010; McCarty, 2006). It should be pointed out that previous studies in our laboratory demonstrated that E2 treatment in these same cell lines have different effects (Nadal-Serrano et al., 2012; Sastre-Serra et al., 2012b, 2010). Many GEN treatment effects on the MCF-7 cells are opposite than the E2 effects previously described, since we reported a greater oxidative damage and higher ROS levels, as well as a decrease in UCPs levels in the E2-treated MCF-7 cell line. In contrast, the T47D cell line presented a lower oxidative stress and an up-regulation of the UCPs after E2 treatment, with the consequent decrease on ROS production (Nadal-Serrano et al., 2012). Moreover, results of our laboratory also reported that the T47D cell line, with a lower ERα/ERβ, shows more functional mitochondria after E2 treatment (Sastre-Serra et al., 2012b). Therefore, the effects of E2 and GEN on oxidative stress and mitochondrial function depend on the estrogen receptor ratio, which gives protective effects when the ERα/ERβ ratio is low. Furthermore, we found that GEN has beneficial effects than those of E2, and GEN could play a favorable role in breast cancer cells by attenuating ROS production and improving mitochondrial function. In addition, it must be underscored that the GEN treatment also increased ERβ levels, as shown in results, and thereby amplified GEN effects.

All the results described above would be in agreement with the findings with sirtuins, a conserved family of NAD^+ - dependent deacetylases involved in stress response, metabolism and longevity, which have been described as critical for maintaining mitochondrial integrity and function (Milne and Denu, 2008). Specifically in this study, we investigated the expression of SIRT1 and SIRT3 to explore their potential role in breast cancer cell lines. SIRT1 showed an up-regulation in the GEN-treated T47D cell line, contributing to maintain a lower oxidative stress observed, suggesting that the sirtuins could provide protection from oxidative damage. These preliminary results in cell lines agree with recent publications, which show that a loss of SIRT1 or SIRT3 might be a late event in carcinogenesis, conferring proliferative advantage to cells (Bell et al., 2011; Kim et al., 2010).

Taking together these findings, it must be emphasized that the GEN-treated T47D breast cancer cell line with a predominance of ERβ shows a better mitochondrial functionality and a lower oxidative stress situation than in the MCF-7 cell line, in which ERα is the predominant isoform of the estrogen receptor. Moreover, the present study shows GEN to have a beneficial role in the T47D cell line, greater than the E2 treatment. In addition, the effect of GEN treatment on mitochondria could be due, at least in part, to the higher presence of ERβ, and also to its up-regulation caused by this treatment.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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References

Adlercreutz H. Phytoestrogens and breast cancer. The Journal of Steroid Biochemistry and Molecular Biology 2002;83:113–8.
Alhazzazi TY, Kamarajan P, Verdin E, Kapila YL. SIRT3 and cancer: tumor promoter or suppressor. Biochimica et Biophysica Acta 2011;1816:80–8.
Ayyasamy V, Owens KM, Desouki MM, Liang P, Bakin A, Thangaraj K, et al. Cellular model of Warburg effect identifies tumor promoting function of UCP2 in breast cancer and its suppression by genipin. PloS ONE 2011;6:e24792.
Baffy G, Derdak Z, Robson SC. Mitochondrial recoupling: a novel therapeutic strategy for cancer. British Journal of Cancer 2011;105:469–74.
Behrend L, Henderson G, Zwacka RM. Reactive oxygen species in oncogenic transformation. Biochemical Society Transactions 2003;31:1441–4.
Bell EL, Emerling BM, Ricoult SJ, Guarente L. SirT3 suppresses hypoxia inducible factor 1alpha and tumor growth by inhibiting mitochondrial ROS production. Oncogene 2011;30:2986–96.
Bondesson M, Gustafsson JA. Does consuming isoflavones reduce or increase breast cancer risk? Genome Medicine 2010;2:90.
Carew JS, Huang P. Mitochondrial defects in cancer. Molecular Cancer 2002;1:9.
Derdak Z, Mark NM, Beldi G, Robson SC, Wands JR, Baffy G. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. Cancer Research 2008;68:2813–9.
Fogg VC, Lanning NJ, Mackeigan JP. Mitochondria in cancer: at the crossroads of life and death. Chinese Journal of Cancer 2011;30:526–39.
Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.
Kim HS, Patel K, Muldoon-Jacobs K, Bisht KS, Aykin-Burns N, Pennington JD, et al. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. Cancer Cell 2010;17:41–52.
Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, et al. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997;138:863–70.
Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998;139:4252–63.
Martinez-Pastor B, Mostoslavsky R. Sirtuins, metabolism, and cancer. Frontiers in Pharmacology 2012;3:22.
McCarty MF. Isoflavones made simple – genistein's agonist activity for the beta-type estrogen receptor mediates their health benefits. Medical Hypotheses 2006;66:1093–114.

Michishita E, Park JY, Burneskis JM, Barrett JC, Horikawa I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Molecular Biology of the Cell 2005;16:4623–35.
Milne JC, Denu JM. The sirtuin family: therapeutic targets to treat diseases of aging. Current Opinion in Radical Research 2008;12:11–7.
Nadal-Serrano M, Sastre-Serra J, Pons DG, Miro AM, Oliver J, Roca P. The ERalpha/ERbeta ratio determines oxidative stress in breast cancer cell lines in response to 17beta-estradiol. Journal of Cellular Biochemistry 2012;113:3178–85.
Negre-Salvayre A, Hirtz C, Carrera G, Cazenave R, Troly M, Salvayre R, et al. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASEB Journal 1997;11:809–15.
Ouyang G, Yao L, Ruan K, Song G, Mao Y, Bao S. Genistein induces G2/M cell cycle arrest and apoptosis of human ovarian cancer cells via activation of DNA damage checkpoint pathways. Cell Biology International 2009;33:1237–44.
Ozben T. Oxidative stress and apoptosis: impact on cancer therapy. Journal of Pharmaceutical Sciences 2007;96:2181–96.
Pavese JM, Farmer RL, Bergan RC. Inhibition of cancer cell invasion and metastasis by genistein. Cancer Metastasis Reviews 2010;29:465–82.
Pelicano H, Carney D, Huang P. ROS stress in cancer cells and therapeutic implications. Drug Resistance Updates 2004;7:97–110.
Rasbach KA, Schnellmann RG. Isoflavones promote mitochondrial biogenesis. The Journal of Pharmacology and Experimental Therapeutics 2008;325:536–43.
Ruiz-Larrea MB, Mohan AR, Paganga G, Miller NJ, Bolwell GP, Rice-Evans CA. Antioxidant activity of phytoestrogenic isoflavones. Free Radical Research 1997;26:63–70.
Sastre-Serra J, Nadal-Serrano M, Pons DG, Roca P, Oliver J. Mitochondrial dynamics is affected by 17beta-estradiol in the MCF-7 breast cancer cell line. Effects on fusion and fission related genes. The International Journal of Biochemistry and Cell Biology 2012a;44:1901–5.
Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Oliver J, Roca P. The effects of 17beta-estradiol on mitochondrial biogenesis and function in breast cancer cell lines are dependent on the ERalpha/ERbeta ratio. Cellular Physiology and Biochemistry 2012b;29:261–8.
Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J, Roca P. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. Free Radical Biology and Medicine 2010;48:506–12.
Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proceedings of the National Academy of Sciences of the United States of America 2004;101:1566–71.
Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. The New England Journal of Medicine 2006;354:270–82.

Manuscript 12

Phytoestrogens and mitochondrial biogenesis in breast cancer. Influence of estrogen receptors ratio

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Phytoestrogens and Mitochondrial Biogenesis in Breast Cancer. Influence of Estrogen Receptors Ratio

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Abstract: Phytoestrogens were originally identified as compounds having a close similarity in structure to estrogens and harboring weak estrogen activity. The interest in phytoestrogens as potential therapeutic agents has recently risen in the field of oncology, since population based studies have linked phytoestrogens consumption with a decreased risk of mortality due to several types of cancer. This review departs from the main focus of these articles by describing recent advances in our understanding of phytoestrogen potential action on mitochondria, specifically on mitochondrial biogenesis, dynamics and functionality, as well as mitoptosis in breast cancer. Further studies are necessary to explain the effects of individual phytoestrogens on mitochondrial biogenesis and dynamics and for designing of new therapy targets for cancer treatment, nevertheless area promising therapeutic approach.

Keywords: Phytoestrogens, Breast Cancer, Energy Metabolism, Oxidative Stress, ERalpha/ERbeta.

INTRODUCTION

Phytoestrogens constitute a large group of natural compounds which have been found in at least 300 plants [1]. These natural compounds were originally identified as having a close similarity in structure to estrogens and harboring weak estrogen activity, leading to their classification as an estrogen-like compound derived from a plant source [2]. Their perceived health beneficial properties extend beyond hormone-dependent breast and prostate cancers and osteoporosis to include cognitive function, cardiovascular disease, immunity and inflammation, reproduction and fertility [3-6].

The basis for the initial focus on breast cancer can be attributed to the historically low breast cancer incidence rates in Asia, where soy foods comprise an important dietary component [7]. For this reason, the interest in phytoestrogens as potential therapeutic agents has recently risen in the field of oncology since epidemiological studies have linked phytoestrogens consumption with a decreased risk of mortality from several types of cancer, most notably prostate and breast cancer [3-9]. Moreover, the popularity of soy products, and lately of dietary supplements, is also due to their role in prevention of other chronic health disorders such as cardiovascular disease [9] and osteoporosis [8]; as well as relieving climacteric symptoms [10,11].

Roughly speaking, the phytoestrogens show anti-inflammatory, antioxidant, anti-thrombotic, antineoplastic, anti-allergic and hepatoprotective activities [12]. These compounds may affect breast cancer through both hormonally mediated and non-hormonally related mechanisms. However, the role of phytoestrogens in breast cancer has become controversial, in contrast to the beneficial effects discussed above. Concerns have arisen that the estrogen-like properties of phytoestrogens may be harmful to patients with estrogen sensitive breast cancer[13]. Some reports suggest that phytoestrogens, especially the isoflavone genistein, may stimulate the growth of estrogen-sensitive tumors. The opposite effects could be due to the concentration of phytoestrogen intake and the period of lifelong exposure, suggesting that the protective effects may be related to lifetime or early life exposure [5,14,15].

There are several excellent reviews which document the importance of mitochondria in cancer disease [16-19] and evaluate the large and complex body of evidences which purport to demonstrate the beneficial effects of phytoestrogens for human and laboratory animals health [6,7,20-22]. This review departs from the main focus of these articles by describing recent advances in our understanding of phytoestrogen potential action on mitochondria, specifically on mitochondrial biogenesis, dynamics and functionality, as well as mitoptosis in breast cancer.

1. PHYTOESTROGENS: CLASSIFICATION AND STRUCTURE

Phytoestrogens are a diverse group of compounds found in many edible plants that have, as their common denominator, a pair of hydroxyl groups and a phenolic ring (show Fig. **1**), which is required for binding to estrogen receptors ERα and ERβ. The position of these hydroxyl groups appears to be an important factor in determining the abilities of phytoestrogen to bind the ERs and activate gene transcription; moreover, the excretion of the phenolic group plays an important role in determining the estrogenic agonist/antagonistic properties of these compounds. Phytoestrogens have been categorized according to their chemical structures as flavonoids, lignans and stilbenes; additionally, flavonoids are subdivided into isoflavonoids, flavones, flavonols, flavanones and chalcones [1]. Isoflavonoids have been distributed in isoflavones and coumestans.The major classes of phytoestrogens are concentrated in soybeans, legumes, fruits, vegetables, berries, whole grains or tea, among others [1,6].

2. PHYTOESTROGENS:SOURCE AND BIOLOGICAL FUNCTIONS

In this section, the source, the biological function and features of main individual phytoestrogens are reviewed and discussed.

2.1. Isoflavonoids - isoflavones

2.1.1. Genistein

The isoflavonegenistein (4′,5,7-trihydroxyisoflavone) was first isolated by Perkin and Newbury in 1899 from Dyer's Broom (*Geni-*

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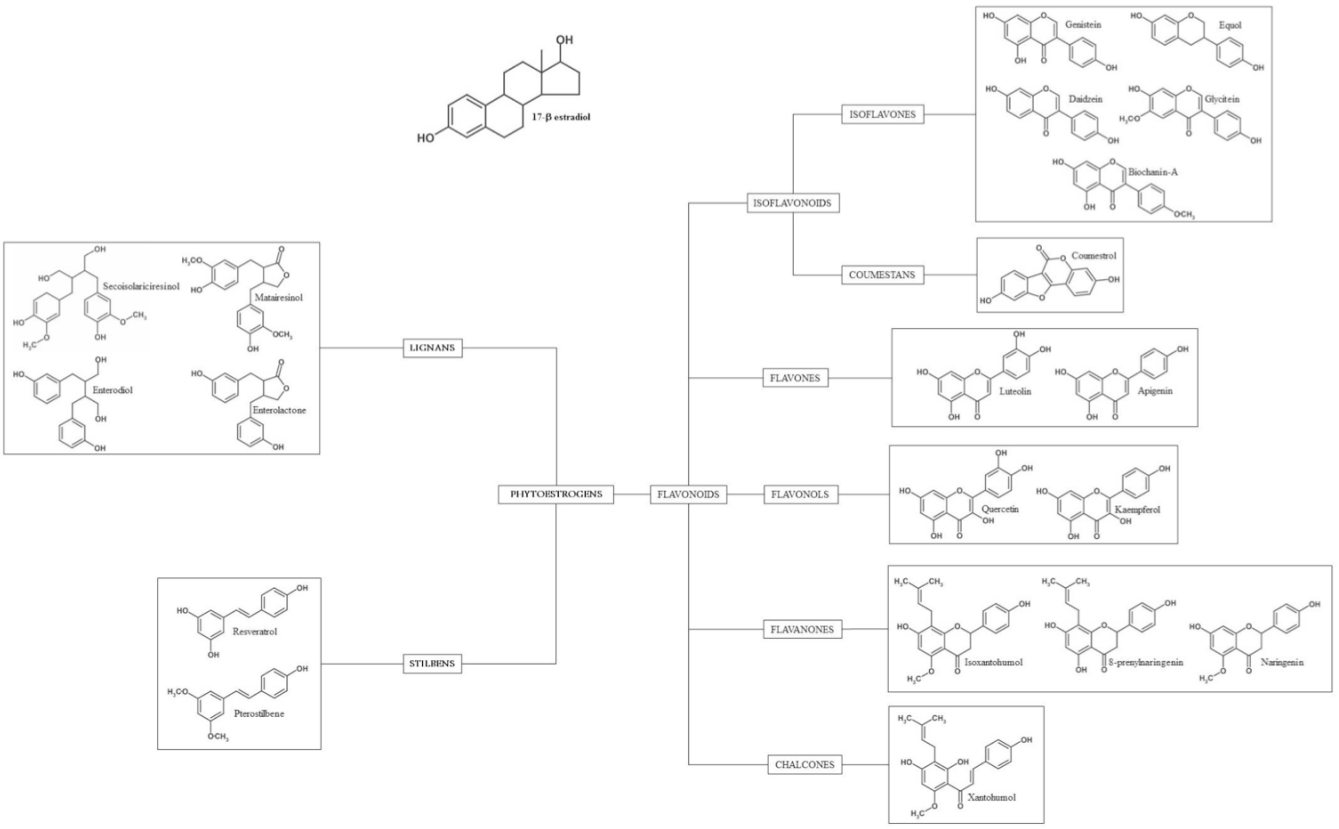

Fig. (1). Structure and classification of phytoestrogens.

statinctoria)[23]. This naturally derived compound presents a weak estrogen activity, hence diets rich in genistein could play a potential favorable role in the prevention of hormone related cancer as breast cancer [24], as well as some other estrogen-related diseases such as osteoporosis and cardiovascular diseases [22,25,26]. Genistein also possesses non-hormonal properties such as inhibition of protein-tyrosin kinase activity[24,27], antioxidant effects through scavenging free radicals or inhibiting the expression of stress-response related genes [28], induction of cell cycle arrest [29], modulation of intracellular signaling pathways (such as Akt/NF-κB family proteins)[30], inhibition of invasion and metastasis of cancer cells [31], in addition to several possible mechanisms by which soy may reduce the risk of breast cancer. However, in contrast to the possible beneficial effects, many reports suggest that genistein may stimulate the growth of estrogen-sensitive tumors [32]. It appears that on the one hand, genistein would have an inhibitory effect at high concentrations and, on the other hand, a stimulatory effect at low concentrations[3]. Moreover, other studies indicate that cancer chemoprevention of certain phytoestrogens, such as genistein, depends on lifelong exposure from childhood [15,33].

2.1.2. Daidzein

Daidzein, 7-hydroxy-3-(4-hydroxyphenyl) chromen-4-one, along with genistein, is the most prominent isoflavone found in soy products and the most intensely studied type of phytoestrogen. Daidzein can be found in nuts, fruits, soybeans and soy-based products [34]. Daidzin is the 7-O-glucoside of daidzein. Daidzein can be converted into its final metabolite S-equol in some humans, based on the presence of certain intestinal bacteria, and many research studies determined that equol has potential health benefits [35]. Daidzein has been garnered interest as a nontoxic compound capable of inducing tumor cell death in a variety of cancer types. In fact, many reports have indicated that the anticancer activity of daidzein in breast cancer is mediated through cell cycle arrest at the G1 and G2/M phases and apoptosis [36,37]. However, the specific apoptosis mechanisms are not yet well understood. Jin *et al*. suggested that daidzein could induce breast cancer cell apoptosis through the mitochondrial caspase-dependent cell death pathway [37].

2.1.3. Equol

Equol, 7-hydroxy-3-(4'-hydroxyphenyl)-chroman, was firstly isolated from an estrogenic fraction of pregnant mare's urine by Marrian and Haslewood in 1932 [38]. Equol is a non-steroidal estrogen, but in contrast to the isoflavonesgenistein, daidzein and glycitein, equol is not a phytoestrogen because it is not a natural constituent of plants. In fact, equol is exclusively a product of intestinal bacterial metabolism of isoflavonedaidzein, moreover this metabolite is not produced in all healthy adults in response to dietary challenge with soy or daidzein, only approximately 20% to 35% of the population is able to metabolize daidzein to equol, they are called "equol-producers" [39]. The ability to produce equol depends on the presence of equol-forming bacteria, however the factors promoting their colonization in the human gut are still unknown [40]. The bacterial daidzein metabolite equol has a longer half-life and exhibits biological properties that exceed those of its precursor, which may enhance the actions of soy isoflavones, supporting the theory that equol may be advantageous to be able to convert daidzein to equol. Among the potential health benefits of equol are greater affinity for estrogen receptors, although it binds preferentially to ERβ; prevention of estrogen- and androgen-mediated conditions; and superior antioxidant activity [40,41], therefore comprising a lower risk of breast cancer and cardiovascular disease, better bone health, and decreased menopausal symptoms. It is noteworthy that the main groups with a higher percentage of equol producers, such as the Japanese and vegetarians, do

have lower incidences of prostate and breast cancer, and cardiovascular disease [35,40].

2.1.4. Glycitein

Glycitein, 4',7-dihydroxy-6-methoxyisoflavone, is an O-methylated isoflavone, which has a weak estrogenic activity, comparable to that of the other soy isoflavones[42]. Glycitein is a metabolite formed by the transformation of glycitin (a β glycoside form of glycitein) by intestinal microflora, and it has been reported to be one of the best absorbed flavonoids [43]. This isoflavone is distributed among Leguminosae family, such as flowers and roots of *Puerariathunbergiana*, the bark of *Maackiaamurensis* and soybean [44]. The biological activity of this compound is less clear compared withthe other soy isoflavones, nevertheless, many studies have indicated that glycitein suppresses osteoclast generation and induces osteoclast apoptosis *in vitro* to a similar extent as genistein, and therefore suggests that glycitein may also exert bone beneficial effects *in vivo*[45]. On the other hand, there are many reports suggesting that glycitein also shows properties associated with the down-regulation of matrix metalloproteinases (MMP-3 and MM-9), which play a pivotal role in proliferation, invasion and angiogenesis [46]. Moreover, it has been described that glycitein possesses cytoprotective properties against oxidative stress and hypocholesterolemic activities, and has a neuroprotective effect against β-amyloid-induced toxicity [46-48].

2.1.5. Biochanin-A

Biochanin-A (4'-O-methylgenistein, *i.e.* a methoxy form of genistein) is an isoflavone isolated from red clover (*Trifolium pretense*) [49], but biochanin-A is also found in cabbage and alfalfa. Recently, dietary supplements containing red clover have received a great deal of attention for treating the symptoms of menopause, the maintenance/improvement of bone, and cardiovascular health [50]. Moreover, many studies have also suggested biochanin-A has chemoprotective effect on breast cancer [50,51], since this compound appears to have an inhibitory and apoptogenic effect on certain cancer cells [52]. Biochanin-A is a major phytoestrogen that shows an enzyme activity inhibition and suppresses the transcriptional control of CYP19 (aromatase) in breast cancer cells [51]. In addition, this natural compound is a potent inhibitor of protein tyrosine kinase activity, as well as serine/threonine kinase, thereby interfering with the normal cascade of signal transduction implicated in cell growth [52,53]. Finally, it has been reported that biochanin A is involved in anti-inflammatory processes and presents a useful therapeutic value against activities of cells that lead to inflammation and carcinogenesis [52].

2.2. Isoflavonoids - Coumestans

2.2.1. Coumestrol

Coumestrol (3,9-Dihydroxy-6-benzofurano[3,2-c]chromenone) belongs to the group of coumestans which is included, in turn, in the group of isoflavonoids. Coumestrol is derived from daidzein[6]. Althoughcoumestrol is found in several foods such as mung bean sprouts [54], soybeans sprout [55-57], alfalfa sprouts [54-57], supplements made from kudzu [58] as well as in many foods and Chinese medicine products[59], the amount is considerably low [54,58]. Coumestrol is an estrogen agonist that has been shown to be effective in reducing bone loss, inhibiting α-glucosidase[60], modulating production of thymic hormones [57] and decreasing both aromatase activity and estradiol concentrations in blood [61]. Coumestrol has also neuroprotective [55,60] and metabolic effects whichare independent of its estrogenicity, the latter includes an increase in lipid synthesis and glycogen catabolism[6,62]. Coumestrol induces cell apoptosis [54], exhibits a powerful antioxidant activity by free radicalscavenging, inhibits lipid peroxidation and increases protective enzyme activity [60].On the contrary, this compoundstimulates growth, but not apoptosis in breast cancer cell line MCF-7 [57]. Moreover, some studies have demonstrated that coumestrol has potential mutagenic effects in human cells [6,63].

2.3. Flavones

2.3.1. Luteolin

Luteolin [2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone] is a flavonoid which belongs to flavones group [64]. Luteolin is found abundantly in green peppers, pommel, celery, and chamomile tea [64]. The intestinal absorption of luteolin is in the aglycon form (intestinal microbacteria can hydrolyze glucosides like luteolin 7-O-β-glucoside) and that luteolin is converted to glucuronides during passing through the intestinal mucosa [65]. Deglucuronidation of luteolin has been observed during inflammation, which suggests that the plasma concentration of free luteolin could increase during inflammation [66]. Luteolin has been shown to have a wide variety of biological activities such as antioxidant, anti-inflammatory, antimicrobial and anticancer activity among others [67].

2.3.2. Apigenin

The compound 4',5,7-trihydroxyflavone (5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one), is commonly referred to apigenin[68]andbelongs to the flavones group of flavonoids. Major apigenin containing food sources include thyme, cherries, tea, olives, broccoli, celery, and legumes; with the most abundant sources being the leafy herb parsley, *Petroselinumcripspum*, and dried flowers of chamomile, *Matricariachamomilla*[69-73]. Apigenin is also commonly present in red wine and in beer [74,75]. Apigenin consumption has been related to a lower risk of several types of cancer such as breast, colon, lung, ovarian, prostate, skin, gastric and liver cancer, among others [68,76]. Moreover, apigenin has been shown to exert anti-inflammatory, antiviral and purgative effects [77].

2.4. Flavonols

2.4.1. Quercetin

Quercetin (3,30,40,5,7-pentahydroxyflavone) is a flavonoid which belongs to flavonols group, and has a relatively high bioavailability compared to the other phytochemicals [78]. Apples, onions and tomatoes are the main sources containing quercetin[79] and its bioavailability depends on the type of glycosides present in different food sources [80]. After absorption, quercetin is metabolized in the different organs andis conjugated to 3'-*O*-methylquercetin (isorhamnetin), quercetin-3-*O*-glucuronide, 3'-*O*-methylquercetin-3-*O*-glucuronide and quercetin-3'-*O*-sulfate, the major conjugates in humans [79]. Quercetin has been shown to present antioxidant and anti-inflammatory properties associated with the prevention and therapy of cardiovascular diseases and cancer [78]. Some of the beneficial effects of quercetin against cancer are senescence, apoptosis, antiangiogenic activity, antiproliferative and growth suppressive effects among others [81,82].

2.4.2. Kaempferol

Kaempferol (3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one) is a flavonoid which belongs to flavonols group [83]. Kaempferol is commonly ingested as glycoside and the main sources of this natural compound are tea, broccoli and kale [70]. The high polarity of glycosides hinders their absorption, whereas the intermediate polarity of aglycones facilitates it [83]. Kaempferol can be metabolized in the small intestine to sulfoconjugates or glucuronides by intestinal conjugation enzymes [84] andis also metabolized by the colon microflora[85]. After absorption, Kaempferol is extensively metabolized in liver to form sulfo- and glucurono-conjugates [84,86]. Kaempferol and its derivates can reach systemic circulation and tissues and then are excreted in urine [87]. Kaempferol consumption may reduce the risk of developing several types of cancer such as lung, gastric, pancreatic and ovarian cancer and cardiovascular diseases[83]. Kaempferol intake has been

also associated with a lower incidence of cerebrovascular disease [88] and some kaempferol glycosides have anti-diabetic activity [89,90].

2.5. Flavanones

2.5.1. Isoxantohumol

Isoxanthohumol is a flavanone of the group of flavonoids whichis found in the hop plant, *Humuluslupulus* (Cannabaceae), andis traditionally used to add bitterness and flavor to beer [91]. Although xanthohumol is the major prenylflavonoid in hops, its content is rather low in beer due to the thermal isomerization during the brewing process. The product of thermal isomerization is precisely isoxanthohumol[92] and, thus, isoxanthohumol is the most prevalent prenylflavonoid in beers [93-95]. For this reason, human exposure to isoxanthohumol is primarily through beer consumption [96]. Interestingly, isoxanthohumol has been shown to act as a precursor of8-prenylnaringenin, which is main estrogenic principle derived from hops [94,97,98]. Among the health effects of isoxanthohumol highlight anti-infective activity [99,100], human chorionic gonadotropin-activated steroidogenesis attenuation, anti-angiogenic effects [101] and immortalized human microvascular endothelial cell line and microcapillary tube formation inhibition [75,102,103]. In addition, chemo-preventive and anti-inflammatory properties have been described for isoxanthohumol[75]. Isoxanthohumol has anti-tumor effects by suppressing the synthesis and release of pro-inflammatory mediators providing evidences that this compound may also influence tumor-host crosstalk [104]. Moreover,isoxanthohumol induces a caspase-independent form of cell death, suggested to be autophagy. Therefore, isoxanthohumol appears to be promising candidate for further investigation in prostate and breast anticancer therapy [105-107].

2.5.2. 8-Prenylnaringenin

8–Prenylnaringenin (8-dimethylallylnaringenin) is a flavanone which belongs to the group of flavonoids. This compound has been identified as the estrogenic principle of the hop plant (*Humuluslupulus L.*) which is largely used in the brewing industry as a preservative and flavoring agent to add bitterness and aroma to beer [108,109]. During beer production, xanthohumol, the most abundand phytoestrogen in *Humuluslupulus,* can be converted to isoxanthohumol and to 8–prenylnaringenin. Otherwise, *Eubacteriumlimosum* of intestinal microbiota was identified to be capable of bioconversion process from isoxanthohumol to 8–prenylnaringenin[92,97] and *E. javanicum* was found to be an optimal fungus for 8–prenylnaringenin biosynthesis [92]. Furthermore, 8–prenylnaringenin can be synthesized in high yield from xanthohumol[107]. This compound exerts anti-aggregatory and anti-adhesive effects on human platelets [110], enhances osteoblast differentiation and mineralization [111,112], stimulates angiogenesis [101] and exerts beneficial effects against hot flushes [109,113], in fact, this compound is apotential drug in menopausal hormone therapy [107]. Moreover, 8–prenylnaringenin has antiproliferative and pro-apoptotic effects [114], possesses cancer chemopreventive activity [115], shows ability to inhibit the aromatase activity [116] and induces a caspase-independent form of cell death, suggested to be autophagy. Therefore, this flavanone also appears to be a promising candidate for further investigation in prostate anticancer therapy [105].

2.5.3. Naringenin

Naringenin [5,7-dihydroxy-2-(4-hydroxyphenyl)chroman-4-one], the aglycone form of naringin, is one of the naturally occurring flavanone, belonging to the group of flavonoids, widely distributed in tomatoes, cherries, grapefruits and predominantly present in citrus fruits [117-119]. Naringenin acts as an anticarcinogenic[118,120], anti-mutagenic [118,121], anti-inflammatory [118,119,122-124], antibacterial [119], antioxidant [118,125], aromatase inhibitor [119], carbohydrate metabolism promoter and immune system modulator [126,127]. Naringenin enhances insulin stimulated tyrosine phosphorylation and insulin tissue sensitivity [124,128,129]. Furthermore, naringenin has hepatoprotective and nephroprotective properties [118] and can prevent apoptosis induced by high glucose levels[126]. Neuroprotection offered by naringenin is attributed to its free radical-scavenging, antioxidant, anti-inflammatory properties and its ability to move across the blood–brain barrier [124,130-132]. In addition, naringenin has also been verified as an active compound responsible for the anti-invasive and anti-metastatic characteristics of dietary plants on tumors [133].

2.6. Chalcones

2.6.1. Xantohumol

Xanthohumol (3'-[3,3-dimethyl allyl]-2',4',4-trihydroxy-6'-methoxychalcone) is a flavanone and the principal prenylated flavonoid of the female inflorescences of the hop plant (*Humuluslupulus*L.), an ingredient of beer [96,134,135]. Xanthohumol is formed in lupulin glands by a specialized branch of flavonoid biosynthetic pathway that involves prenylation and O-methylation of the polyketide intermediate chalconaringenin. Although a lupulin gland-specific chalcone synthase is known, the aromatic prenyltransferase and O-methyltransferase participating in xanthohumol have not been identified [96]. Xanthohumol might be a natural compound able to prevent hyaluronan overproduction and subsequent reactions in osteoarthritis [136] and also manifests anti-angiogenic and anti-inflammatory effects [101,137]. Moreover, xanthohumolhas potential application in cancer prevention, in prevention or treatment of (post-) menopausal 'hot flashes' and osteoporosis [96]. Xanthohumol has been characterized as a 'broad-spectrum' cancer chemopreventive agent in *invitro*studies [96,134]. Among other effects, xanthohumol inhibits metastasis [138], inhibits the states of initiation, promotion and progression of carcinogenesis [75,96,139], inhibits growth and induces apoptosis in breast cancer line MCF-7[99,140,141].

2.7. Lignans

2.7.1. Secoisolariciresinol

Secoisolariciresinol [(2R,3R)-2,3-bis[(4-hydroxy-3-methoxyphenyl)methyl]butane-1,4-diol] is a precursor for mammalian lignans[7], and exists in various whole-grain cereals, seeds, nuts, legumes and vegetables [142]. Secoisolariciresinol actions are mediated byits metabolites enterolactone and enterodiol[143], anaerobically formed via colonic bacterial fermentation [144]. As well as matairesinol, secoisolariciresinol acts as anantiproliferative compound [145,146], showingcancer-preventive properties in some animal models [145].

2.7.2. Matairesinol

The compound matairesinol [(αR,βR)- α, β-bis[(4-hydroxy-3-methoxybenzyl)butyrolactone)] is another precursor for mammalian lignans[7]. Matairesinol is a plant lignan occurring in a variety of different foods, such as oilseeds, whole grains, vegetables and fruits [147,148], and is converted directly to enterolactone by the gut microflora in the proximal colon [149,150]. This phytoestrogen has been shown to have a wide variety of biological activities such as antiproliferative effects [151] or associated to lower vascular inflammation and endothelial dysfunction [146].

2.7.3. Enterodiol and Enterolactone

Enterodiol [3,3′-[2,3-Bis(hydroxymethyl)butane-1,4-diyl]diphenol] and enterolactone [trans-α,β-Bis(3-hydroxybenzyl)butyrolactone] are the mammalian lignans formed by microflora[149]. The human lignansenterodiol and enterolactone are more biologically active than their precursors,secoisolariciresinol and matairesinol, and both compounds may be defined as the real drugs in cancer prevention [152]. Enterodiol and enterolactone have beneficial health effects, including anti-tumor [152], anti-angiogenic[153], anti-inflammatory [154]and have been associated with reduced risks of ER- and PR-positive postmenopausal breast cancer [155].

2.8. Stilbens

2.8.1. Resveratrol

The phytoestrogen resveratrol (3,5,4'-trihydroxy-trans-stilbene), a polyphenolicstilbene first isolated from the root of *Veratumgrandiflorum*[156], occurs naturally in grapes, mulberries and peanuts [157,158]. Although it is known that in humans resveratrol is rapidly absorbed after its oral administration and is detected in both plasma and urine, data concerning the potential beneficial effects of the pure compound in humans are still very limited [159,160]. The cardioprotective[161], anticancer [162,163], anti-inflammatory and antioxidant [164] properties of resveratrol are quite well characterized. In some studies, resveratrol was found to reduce body weight and adiposity in obese animals, as well as blood insulin [160]. Moreover, cells exposed to resveratrol were characterized by an increase in release of reactive oxygen species (ROS), which was accompanied by up-regulated biogenesis of mitochondria and collapsed mitochondrial membrane potential [165]. There is evidence that this effect may be associated with the activation of sirtuins which belong to the family of NAD^+-dependent histone deacetylases and play an important role in the regulation of energy homeostasis, maintenance of genetic stability, and stress response [165-167].

2.8.2. Pterostilbene

Pterostilbene (trans-3,5-dimethoxy-4'-hydroxystilbene), a dimethyl ether analogue of resveratrol, is a natural antioxidant which belongs to stilbens, predominantly found in blueberries, grapes, and a tree wood [168,169]. Pterostilbene has two methoxy groups and one hydroxyl group, and this structural difference from resveratrol may contribute to the better bioavailability of pterostilbene*in vivo* when compared with resveratrol [170,171]. This phytoestrogen has been shown to have a wide variety of biological activities such as anti-cancer, anti-inflammatory, antioxidant and analgesic activity [170]. It has been described that pterostilbene possesses cytoprotective properties against oxidative stress as antioxidant [172] and activating antioxidant enzymes [173], is proapoptotic[174], inhibits wnt pathway [175], and increases autophagy [176]. Moreover, some authors indicated that pterostilbene is more potent than resveratrol as inhibitor of the proliferation of some cells [177].

3. MITOCHONDRIA

3.1. Structure and Function

Mitochondria are important organelles located in the cytoplasm of all eukaryotic cells. The structure of the mitochondrion is delimited by a double membrane. The outer membrane is wrinkled and completely surrounds the organelle, and the inner membrane has infoldings called cristae where the mitochondrial respiratory chain (MRC) machinery resides.

Mitochondria are involved in a wide array of physiological functions that are essential for cell survival and function, including apoptosis, energy production, redox control, calcium and iron homeostasis, certain metabolic and biosynthetic pathways, and signalling pathways [178]. Mitochondria are the organelles responsible of ATP supplying, being capable of generating more than 90% of its energy requirements and are also the main intracellular source and target ofROS. Moreover, mitochondria play a role in the regulation of cellular proliferation and apoptosis [179].

As mentioned above, the main role of mitochondria is the generation of ATP through a complex controlled process known as oxidative phosphorylation (OXPHOS) [180]. Concisely, oxidation of reduced nutrient molecules (carbohydrates, lipids, and proteins) through cellular metabolism yields electrons in the form of reduced hydrogen carriers such as nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$). These reduced cofactors donate electrons to a series of inner mitochondrial membrane embedded protein complexes known as the electron transport chain. The ultimate destiny of electrons is the reduction of molecular oxygen at mitochondrial complex IV. As a result of this process a molecule of water is yielded, whereas the energy, conserved as proton gradient between both sides of the inner mitochondrial membrane, is used by the F0F1-ATP synthase (or complex V) to phosphorylate ADP [181].

Conversion of metabolic fuel into ATP is not a fully efficient process. Some of the energy of the electrochemical gradient is not coupled to ATP production due to a phenomenon known as proton leak, which consists of the return of protons to the mitochondrial matrix through alternative pathways that bypass ATP synthase [182]. Although this apparently futile cycle of protons is physiologically important, accounting for 20-25% of basal metabolic rate, its function is still a subject of debate [183]. Although a part of the proton leak may be attributed to biophysical properties of the inner membrane, such as protein/lipid interfaces, the bulk of the proton conductance is linked to the action of a family of mitochondrial proteins termed uncoupling proteins (UCP) [184].

3.1.1. Mitochondrial Uncoupling Proteins

UCPs allow protons to re-enter into the mitochondrial matrix, dissipate the proton gradient and decrease the membrane potential (Fig. **2**) [183].

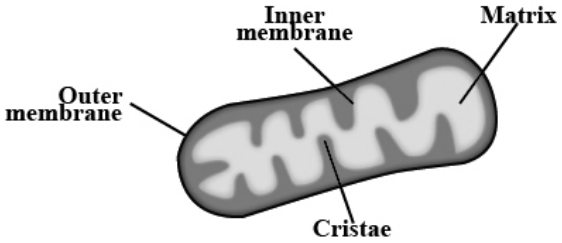

Fig. (2). Mitochondrial structure.

Tissue specificity occurs for the different UCPs and the exact methods how UCPs transfer H^+ are not resolute. There are five UCP homologs in mammals (UCP1-5):UCP1 is specifically expressed in brown adipose tissue and play a role in thermogenesis [185]; UCP2 is the most ubiquitous as is present in many tissues such as adipose tissue, muscle, heart, kidney, digestive tract, brain, spleen and thymus; UCP3 expression is principally restricted to skeletal muscle and heart [183]; and UCP4 and 5 are the most recently discovered UCP homologs and they are mainly expressed in the brain. Among others, UCPs have been proposed to be involved in the control of body weight, basal energy expenditure and fat metabolism [186]. One of the most interesting functions attributed to UCPs is their ability to decrease the formation of mitochondrial ROS [187]. UCPs have also been assigned as true antioxidant defense due to their role as carriers of fatty acid peroxide in the inner mitochondrial membrane [188,189].

Recent studies suggest that mitochondrial uncoupling is related to and facilitate the Warburg effect in cancer cells, hence promoting aerobic glycolysis even in the absence of oxidative capacity alterations, and facilitating a shift to the oxidative metabolism of non-glucose carbon source (glutamine and/or fatty acids), also mitochondrial uncoupling may contribute to chemoresistance increasing the resistance to chemotherapeutic insults [190].

3.1.2. Reactive Oxygen Species

Mitochondria are the most important source of ROS production within most mammalian cells[191-197]. The sites of ROS production along the MRC have been the subject of many studies. The two major sites of superoxide production are at complex I to the matrix side of the mitochondrial membrane and complex III to both the

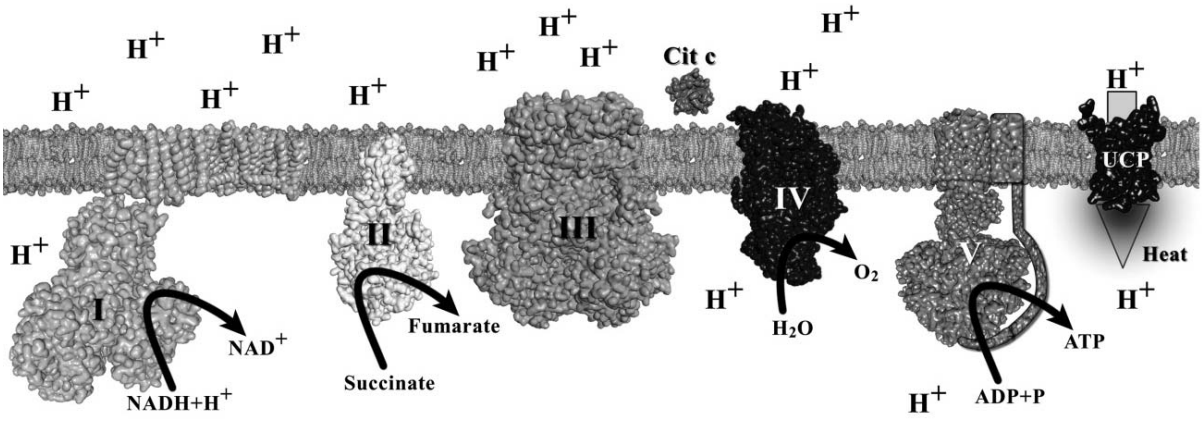

Fig. (3). Mitochondrial oxidative phosphorylation system and uncoupling protein.

matrix and the intermembrane space [198]. Complex IV (or cytochrome c oxidase) produces little or no superoxide, and catalyzes the complete reduction of oxygen to water in the aerobic respiration. Nevertheless, during the transfer of electrons along the respiratory chain, 1-5% of single electrons escape and result in a single electron reduction of molecular oxygen to form a superoxide anion ($O_2^{\bullet-}$), which is the precursor of other ROS (Fig. **3**) [183,195, 197,199,200]. $O_2^{\bullet-}$is rapidly transformed to hydrogen peroxide (H_2O_2), either catalyzed by the enzyme superoxide dismutase (SOD) or spontaneously. H_2O_2, despite the fact thatis not a free radical, can lead to the productionof the highly reactive hydroxyl radical ($^{\bullet}OH$). ROS production can be meaningfully intensifiedwith a high mitochondrial potential membrane ($\Delta\psi m$), situation linked with high NADH production, high ATP levels or functional impairment of complexes I or III of MRC[191,192,195,199,200].

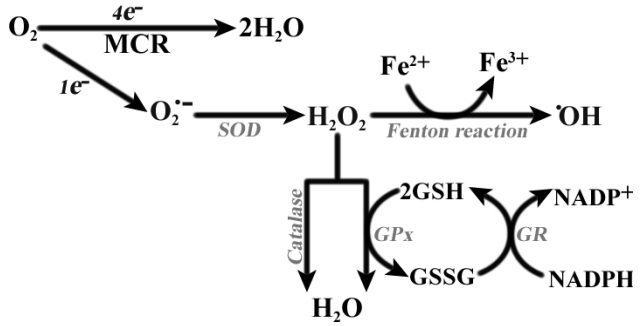

Fig. (4). ROS production and scavenging. SOD: superoxide dismutase; GPx: glutathione peroxidase; GR: glutathione reductase; GSH: reduced glutathione; GSSG: oxidized glutathione (glutathione disulfide).

ROS cause damage to cellular macromolecules such as lipids, proteins, and nucleic acids, especially within the mitochondria. Once mitochondrial enzymatic and non-enzymatic antioxidant systems are overwhelmed by these ROS, oxidative damage and cell death can occur [197]. To counteract the ROS oxidant effects and to restore redox balance, cells must have antioxidant system defences. The natural antioxidant system can be classified into two major groups: enzymes and low molecular weight antioxidants [187]. The enzymes scavenging ROS include SOD, glutathione peroxidase (GPx) and catalase. SOD converts $O_2^{\bullet-}$ into H_2O_2, which is dissipated by the action of catalase and GPx yielding H_2O. Glutathione (GSH) is regenerated from glutathione disulfide (GSSG) by the action of glutathione reductase (GR), consuming NADPH as a reducing equivalent. The low molecular weight antioxidants include ascorbate, GSH, phenolic compounds, and tocopherols [183, 192, 193,195,199-204].

In addition, one interesting issueaboutUCPs is their ability to decrease the formation of mitochondrial ROS. Mitochondria production of superoxide anion is strongly enhanced under resting (state 4) conditions when the membrane potential is high and the rate of electron transport is limited by lack of ADP and Pi [183,195,205-207]. Thus, there is a well-established strong positive correlation between membrane potential and ROS production. Consistent with such proposal, the inhibition of UCPs results in increased membrane potential and ROS production [208,209]. The hypothesis of UCPs as an antioxidant defence has been strongly supported by two facts: UCP2 and UCP3 knockouts show increased ROS production and elevated mitochondrial membrane potential in those tissues normally expressing the missing protein, and UCPs have been shown to be activated by ROS or by-products of lipid peroxidation, showing that UCPs would form part of a negative feed-back mechanism aimed to mitigate excessive ROS production and oxidative damage[183,187,195,207,210]

When cellular production of ROS overwhelms the overall antioxidant defences, these oxygen species may escape and exert their harmful effects. This situation is known as oxidative stress, and is supposed to be responsible for the accumulation of cellular damage during lifetime, thereby playing a role in the etiogenesis and progression of numerous pathologies, including cancer [183,191,193, 194,200-204,211-217].

3.2. Mitochondrial Biogenesis

Mitochondrial biogenesis is a highly coordinated process that must achieve the spatiotemporally coordinated synthesis and import of protein encoded by nuclear DNA (nDNA) and assembly of protein subunits encoded by mitochondrial DNA (mtDNA) into newly synthesized phospholipid membrane.

Even though mitochondria have their own genome, most of the proteins that reside in the mitochondria are nDNA encoded genes. MRC is one of the major structural and functional parts of mitochondria, which consists of five complexes, namely complexes I, II, III, IV and V. These complexes comprise more than 80 proteins, 30 of them are encoded by mtDNA, which is transcribed and translated within mitochondria. The remaining MRC proteins are encoded by nDNA, transcribed in nucleus, synthesized in cytosol and imported into mitochondria. The mtDNA-encoded proteins play a major, constitutive role in MRC function, whereas the nDNA-encoded MRC proteins are thought to play a regulatory role [218].

Each mammalian cell contains from several hundred to more than a thousand mitochondria, and each organelle harbours 2–10 copies of mtDNA [219]. The double-strand circular mtDNA consists of 16,500 base pairs, that encodes 13 proteins (or polypeptides), 22 transfer RNA (or tRNA) and 2 ribosomal RNA (or rRNA) necessary for the translation (Fig. **4**) [220]. A single major non-coding region, referred to as the displacement loop (D-loop), contains the primary regulatory sequences for transcription and initiation of replication [221].

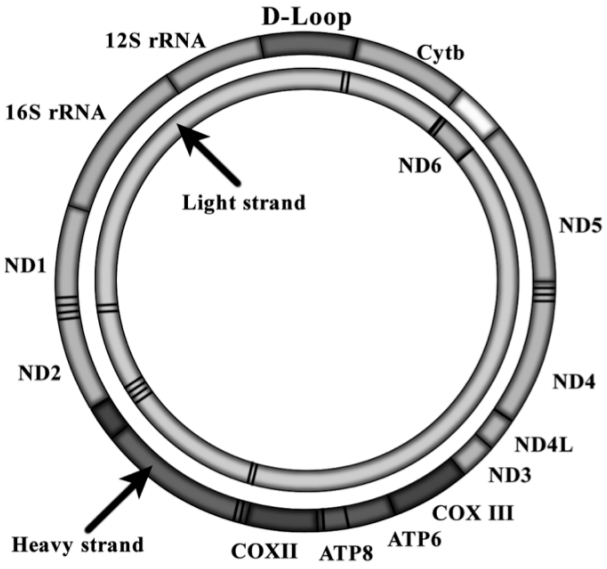

Fig. (5). The mitochondrial genome.

The proper function of MRC requires the coordinate assembly of the MRC complexes of the mtDNA encoded proteins with the nDNA-encoded proteins [221]. Replication of mtDNA, transcription and translation of mtDNA-encoded proteins require the involvements of a number of nDNA-encoded transcription factors and accessory proteins. Thus, the proper mitochondrial biogenesis depends on the spatiotemporal coordination of the nuclear synthesis and mitochondrial import of near 1000 proteins encoded by the nuclear genome [220].

Transcription and replication of mtDNA is regulated by a nuclear-encoded protein named mitochondrial transcription factor A (TFAM), which binds to a common upstream enhancer of the promoter sites of the two mtDNA strands [222]. Additionally, mitochondrial transcription factors B1 and B2 (TFB1M and TFB2M) are capable to interact with the mammalian mitochondrial RNA polymerase and TFAM [223]. Nuclear respiratory factors 1 and 2 (NRF1 and NRF2) exert an important role in the regulation of mitochondrial respiratory function, modulating nuclear transcription of the OXPHOS subunits [224]. Finally, PGC-1α (peroxisome proliferator-activated receptor-γ coactivator 1α) operates as a master transcriptional regulator for mitochondrial biogenesis in vertebrates [225].

The energy demand of a cell can change dramatically during the development, differentiation and neoplasia or due to physiologically changing circumstances. Therefore, the mitochondrial contents and MRC activities are variable and can be adjusted. Specific cell types may adapt mitochondrial function in response to cellular and environmental signals such as hormones, growth factors, changes in physiological activity and developmental signals[226].

The TFAM promoter contains response elements for NRF1 and/or NRF2. However, other mitochondrial related genes do not appear to be regulated by NRFs and need other transcription factors for their regulation. Above mentioned PGC-1α lacks DNA-binding activity but is capable to co-activate numerous transcription factors including NRFs. In fact, mitochondrial biogenesis and respiration are stimulated by PGC-1α which induces gene expression of NRF1 and NRF2 [227].

The synthesis of 30 polypeptides within mitochondria are under the control of hormones and other factors, including cortisol [228], androgens[229], glucocorticoids [230], 1,25α-dihydroxyvitamin D3 [231], thyroid hormone [232], estrogens[233] and PPARs [234]. Receptorsforglucocorticoids, thyroid hormone, estrogens and androgenshavebeenlocalized in mitochondria and specificsteroid hormone responsiveelementsforglucocorticoids, thyroid hormone and estrogen are identified in the human mtDNAregulatoryregion.

3.2.1. Mitochondrial Dynamics

Mitochondria are highly dynamic and versatile organelles, and frequently change their morphologies that undergo constant cycles of division and fusion [235]. To ensure efficiency of functionality and subsequent healthiness of the cell, mitochondria must be maintained in the finest condition [236]. Mitochondrial function and regulation is a very complex process that depends on mitochondrial biogenesis and mitochondrial dynamics [237]. Mitochondrial biogenesis is the combination of both proliferation (an increase in the mitochondrial population) and differentiation (an improvement of the functional capabilities of pre-existing mitochondria) processes [238], while mitochondrial dynamics is a concept that describes the morphology and distribution of mitochondria in the cell [239]. Degradation of dysfunctional or excess mitochondria is another mechanism to regulate the organelle, in a mechanism called mitophagy[240].

Variations in mitochondrial morphology are mainly a transition between two extreme distribution scenarios; firstly, a reticular network of fused mitochondria and secondly a fragmented arrangement, also called “network state” and “individual state”, respectively (Fig. **5**) [241]. Perturbations in this balance produce mitochondrial deformation and have been found to be associated with numerous human diseases [239]. Since mitochondria cannot be synthesized de novo, they must proliferate from pre-existing ones.

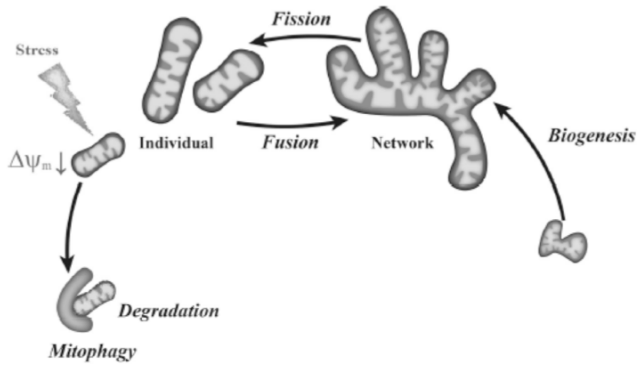

Fig. (6). The mitochondrial life cycle.

The mitochondrial fusion apparatus involves two proteins conserved in yeast and human: fuzzy onion (fzo)/Mitofusins (Mfn) and the inner membrane dynamin-like Mgm1p/Optic Atrophy 1 (Opa1) [242]. In mammals, two mitofusins, Mfn1 and Mfn2, have been described, and are capable to make homo- or heterodimers via coiled domain, allowing mitochondrial tethering and fusion [243,244]. OPA1 ispresent in the inner mitochondrial membrane, andis required for mitochondrial fusion and mtDNAand cristae maintenance [245]. Mgm1p/Opa1 is required in two isoforms, as an inner membrane bound protein and as a soluble intermembrane

space protein, and its cleavage is catalyzed by Presenilin-associated rhomboid-like (PARL) protease[246].

The key molecules of the mitochondrial fission process are conserved in yeast and mammals: Fis1p/hFis1 and Dnm1p/ Dynamin related protein 1 (Drp1). In mammals, the hFis1 protein is integrated in the Outer Mitochondrial Membrane(OMM) and serves as a receptor for Drp1 which translocates from the cytosol to the OMM where it forms a ring that drives mitochondrial fission [247]. The exact mechanism of mitochondrial fission is largely unknown.

Initially, in the fusion process, Mfn1 and Mfn2 facilitate the contact and tethering of mitochondria and the GTPase activity, which is important for the fusion efficiency. In a second step, OPA1 is responsible for the fusion of the inner membrane that must be coordinated with fusion of the inner membrane but the mechanism is still unclear [247].

As mentioned above, mitochondrial morphology is a dynamic process between "individual state" and "network state"[241]. Overall, the mitochondrial fission followed by selective fusion segregates dysfunctional mitochondria and permits their removal by autophagy [241,248,249]. Mitochondrial fusion requires and is dependent on the $\Delta\psi m$. Components of the fission and fusion machinery have been shown to be regulated at the post-translational level through phosphorylation, ubiquitination and sumoylation [235].

Mitochondria morphological changes are a key step in the cell responding to apoptotic stimuli, and initiation of the signalling cascade. Additionally, multiple components of the mitochondrial morphogenesis machinery are capable of regulating apoptosis [250]. Many death stimuli are mediated by activation of pro-apoptotic members of the Bcl-2 family such as Bax and Bak. Mitochondrial fragmentation and apoptosis can be induced by the over-expression of Fis1 [251]. Simultaneous silencing Fis1 and OPA1 can prevent the induction of apoptosis in an independently manner of mitochondrial fragmentation [250]. Other authors have reported that during apoptosis, fragmented mitochondria are sensitized to Bax insertion into the outer membrane and this can be suppressed by mitochondrial fusion [252]. The role of Bcl-2 family members as regulators of mitochondrial morphology are involved in the regulation of mitochondrial fusion through interactions with Mfn2 in healthy cells [253], but requires further investigations. A number of components of the fission and fusion machinery including OPA1, Fis1, Drp1, Mfn1 and Mfn2 have been directly implicated in the regulation of apoptosis [251].

In 1966, Hackenbrock reported that the ultrastructure of mitochondria varies according to their metabolic activity [254]. Briefly, mitochondria that displayed a dense matrix and large intercristae spaces wereassociated with sustained OXPHOS, whereas mitochondria with expanded matrix and thin cristae wereassociated with reduced oxygen consumption. In addition, the entire morphology of mitochondria appears to be modified depending on the functionality, suggesting that mitochondrial fusion and elongation could be associated with increased OXPHOS [255]. A link between mitochondrial fusion and mtDNA maintenance has been suggested and

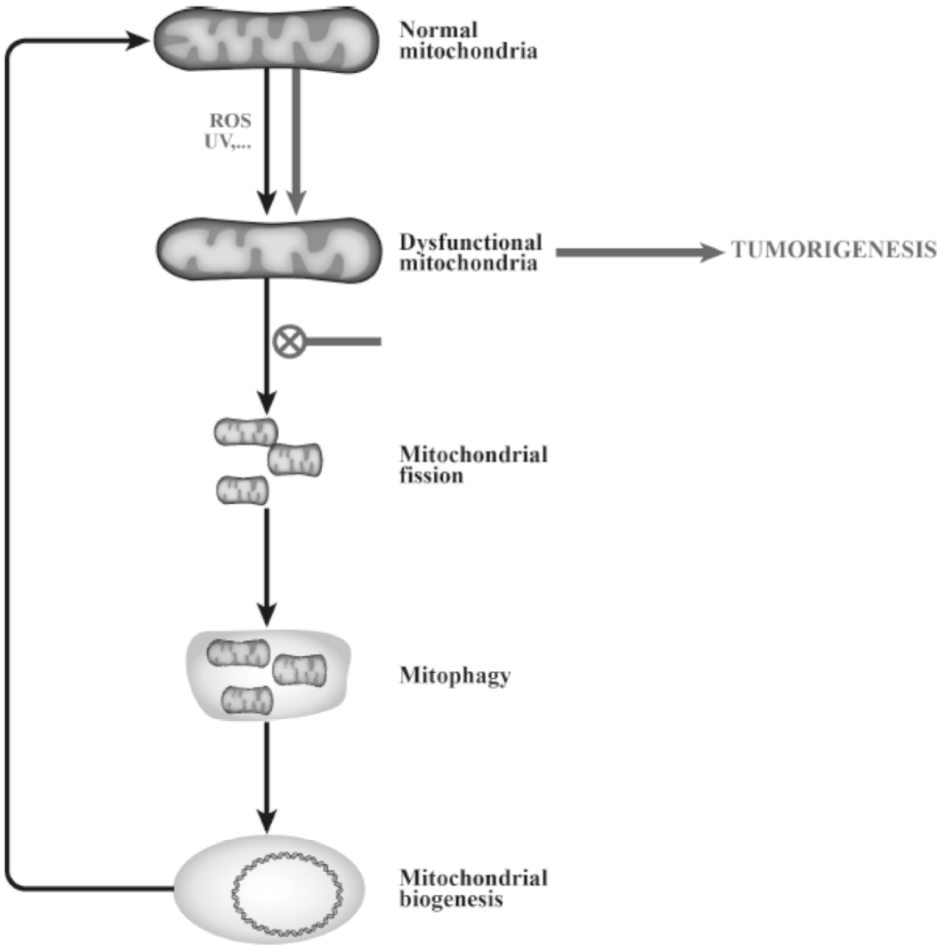

Fig. (7). Model linking a mitochondrial fission defect, accumulation of dysfunctional mitochondria and tumorigenesis (248).

provides a molecular mechanism for the dependence of respiratory activity on mitochondrial fusion [255]. mtDNA mutations caused by ROS could also lead to OXPHOS alterations and mitochondrial fission [255].

Apoptotic resistance is included into the hallmarks proposed to define a cancer cell [81] which is linked to mitochondria and to proteins involved in mitochondrial dynamics. Since mitochondrial dynamics is essential for preserving the integrity and function of the organelle, it has been postulated that mitochondrial dynamics alterations could participate in tumorigenesis by contributing to the accumulation of damaged mitochondria in cells (Fig. **6**) [255]. Warburg effect could correspond to a cell adaptation to environmental pressure and could be a way for cells to put mitochondria at rest butWarburg effect could also be the consequence of mitochondrial damages[255]. Yu and colleagues have demonstrated that reduced mtDNA copy number could be involved in breast cell transformation or progression [256]. Interestingly, a loss of mtDNA has also been involved in the resistance of anti-estrogen therapy in breast cancer [257]. Mitochondrial fusion activity is necessary for mtDNA maintenance, and an impairment of this process has been related to the loss of mtDNA observed in some cancers. Mitochondrial fission is also important for elimination of damaged mitochondria; it is possible that an impairment of this process may contribute to the accumulation of damaged mitochondria.

4. ENERGETIC METABOLISM OF TUMORAL CELL

One of the first tumor-specific metabolic alteration was discovered by the Nobel Prize winner Otto Warburg in the 1920s, the ‘‘Warburg phenomenon’’[258]. Otto Warburg found that cancer cells produce most of their ATP through glycolysis, even under aerobic conditions, and there was a correlation between glycolytic ATP production and aggressiveness of the tumor cells. Warburg assumed that such ‘aerobic glycolysis’ was a universal property of malignant cells and suggested that cancer is caused by impaired mitochondrial metabolism, which forces the cell to depend on glycolysis for energy supply [259].

Independently of whether mitochondrial respiration is low or not, cancer cells do exhibit high rates of glycolysis – aerobic or anaerobic [260]. A higher yield of ATP is observed when the cell respires via OXPHOS, whereas the rate of ATP generation becomes greater if respiration occurs through glycolysis, this enables cancer cells to gain competitive advantage [261]. In additionto generate energy, glycolysis also provides the key carbon precursors needed for the synthesis of nucleic acids, phospholipids, fatty acids, cholesterol and porphyrins [262] (see Fig. **7**).

Furthermore, the pentose phosphate shunt generates cellular reducing power in the form of NADPH necessary for lipid synthesis, thus, the switch to glycolytic metabolism in spite of oxygen availability helps the survival and proliferation of tumor cells [263,264]. In summary, actively dividing cells not only need great amounts of ATP but also macromolecules such as nucleotides, lipids and proteins, synthesis of which is facilitated by metabolic reprogramming.

4.2. Sirtuins: Metabolic Regulators and Oxidative Stress Adaptors

Sirtuins are recognized as key regulators of energy metabolism and stress adaptors (oxidative, genotoxic and metabolic stress) likely through sensing changes in levels of intracellular NAD^+, an obligated cofactor for their enzymatic activity. Sirtuins are a highly conserved family of proteins, homologs of the *Saccharomyces cerevisiae* protein Sir2 [265]. They are NAD^+-dependent protein deacetylases and/or mono-[ADP-ribosyl] transferases. In mammals seven sirtuins have been described: SIRT1, SIRT6 and SIRT7 are mainly nuclear, SIRT2 is primarily found in cytosol, and SIRT3, SIRT4 and SIRT5 are mitochondrial proteins [266].

Sirtuins are important modulators of multiple metabolic processes, including energy production, the urea cycle, fatty acid me-

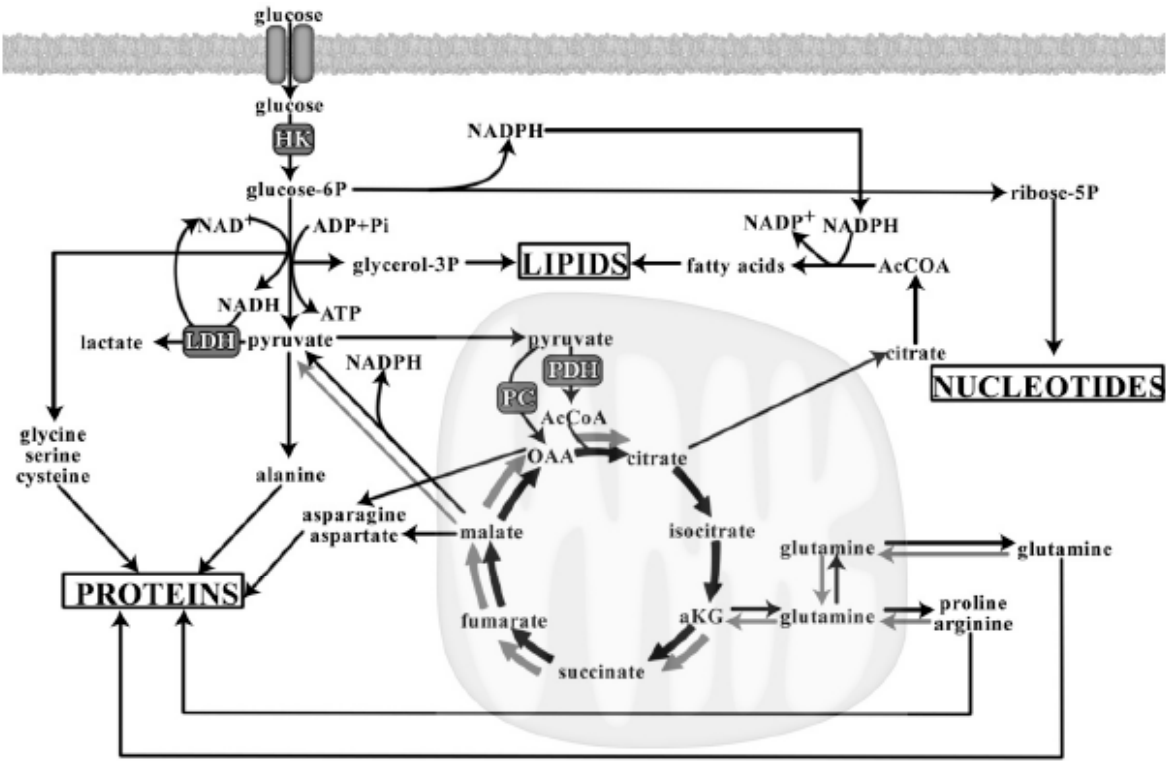

Fig. (8). Summary of how glycolytic intermediates are used for the synthesis of macromolecules in the cancer cell.

tabolism, and acetate metabolism. The mitochondria provide the hub that integrates these pathways, serving as a critical site for the production and exchange of metabolic intermediates. As the best-studied sirtuin, SIRT1 has been implicated in particular in the regulation of glucose and lipid metabolism during fasting and caloric restriction. SIRT3 and SIRT6 have a fundamental role in the regulation of glucose utilization [266].

Sirtuins are postulated ascontrolling the dynamics of mitochondrial protein acetylation, thus potentially influencing every aspect of mitochondrial function. Indeed, it has been shown that mitochondrial biogenesis itself is regulated by both SIRT1 and SIRT3, through a process involving the transcriptional coactivatorPGC1α [267]. Moreover, not only biogenesis but also mitochondrial degradation seems to be regulated by sirtuins, since mitophagy in aged kidney has been described that is dependent to SIRT1 [268].

Deletion of SIRT6 in mice causes an upregulation of glucose uptake that is preferentially used for glycolysis instead of mitochondrial respiration, a metabolic switch similar to the Warburg effect in cancer cells. SIRT6 appears to function as a corepressor of thehypoxia-inducible factor 1α (HIF-1), which is a key regulator of nutrient stress responses. SIRT6 works as a sensor of nutrient availability and as a master regulator of glucose homeostasis [269], and recently, a role as potent tumor suppressor has been revealed for SIRT6 [270]. SIRT3 is the major mitochondrial protein deacetylase and is capable to stimulate fatty acid oxidation, amino acid catabolism through urea cycle, and tricarboxylic acid (TCA) cycle, and under low nutrient conditions stimulates alternative pathways for energy metabolism [271]. It is noteworthy that SIRT1, SIRT3 and SIRT6 have also been shown to protect from genomic instability upon genotoxic and oxidative stress, protecting the genome from mutations that can drive tumorigenesis[271]. Even it has been proposed that SIRT3 might be a tumor suppressor that protects against carcinogenesis by maintaining mitochondrial integrity and efficient oxidative metabolism [272].

In humans, SIRT1 expression is higher in several types of cancer, including acute myeloid leukemia, primary colon cancer, prostate cancers, and non-melanoma skin cancers [273], reinforcing the role of SIRT1 as an oncogene. Expression of SIRT3 was found significantly reduced in human breast cancer samples [274]. These evidences are in accordance with the fact that SIRT3 works as a tumor suppressor by opposing reprogramming of cancer cell metabolism [274], in other words, repressing the Warburg effect.However, higher levels of SIRT3 have been described in node-positive breast cancer comparing with non-malignant breast tissue [275].

4.1. Tumor Microenvironment and Stabilization of HIF-1

Tumor microenvironment favors a specific metabolic profile. Oxygen levels within a tumor fluctuate both temporally and spatially, and are almost always insufficient to satisfy tumor cell growth, leading to hypoxia [276]. Cancer cells are known to adapt to the hypoxic condition via the HIF-1, which initiates a transcriptional program that provides multiple solutions to low oxygen availability by decreasing the dependence on aerobic respiration. When hypoxic stress is detected by HIF-1 protein, cell metabolism is shifted toward glycolysis by the increased expression of inhibitors of mitochondrial metabolism, glucose transporters and glycolytic enzymes such as lactate dehydrogenase A (LDH-A) [195,260,264,277-281].

HIF-1 is known to induce pyruvate dehydrogenase kinase 1 (PDK-1). The phosphorylation of the E1 subunit of pyruvate dehydrogenase (PDH) by PDK-1 inactivates PDH activity, preventing the entry of pyruvate into the mitochondria; this leads to lactate production via LDH and regenerating NAD^+ to continue glycolysis in the cytosol [278,279,281]. Thus, inhibition of PDK-1 impairs mitochondrial respiration and ROS production, thereby promoting cell proliferation[264].

The hypoxic environment of proliferating tumor tissue also facilitates ROS production. ROS levels can be increased by hypoxia when electron transport complexes are in the reduced state [282]. Compelling lines of evidence have shown that ROS are also involved in the stabilization of HIF-1 by both direct and indirect mechanisms [283,284].

Suppression of pyruvate oxidation in the mitochondria may protect cells from the hypoxia-mediated production of cytotoxic amounts of ROS [278]. However, HIF-1 stabilization may only partially inhibit OXPHOS since cancer cells need to keep TCA functional for anabolic purposes. Cancer cells have important biosynthetic requirements to sustain cell growth, which includes intense synthesis de novo of protein, DNA and lipids (Fig. **7**).

4.3. Reactive Oxygen Species: Their Role in Cancer

ROS play multiple roles during tumorigenesis, eliciting cellular responses that range from proliferation to cell death [193-195,200-204,212,213,215-217,285,286]. Among others, low levels of ROS in normal cells have been suggested to play a role in cell proliferation, induction of apoptosis, detoxification, phagocytosis and other biochemical processes [193-195,200-204,212,213,216, 217,287,288]. While higher doses of ROS induce oxidative damage in the genome of cells leading to cell apoptosis, exposure of low levels ofROS produce genomic instability as well as transduce signals for cell growth, cell transformation and cell invasion[195,203].

ROS contribute to carcinogenesis in different ways. ROS can directly produce single- or double-stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications and DNA cross-links.ROS are characterized by the fact that these compounds arerapidly-produced, short-lived, and highly diffusible, features that also perfectly fit the characteristics of a second messenger molecule. Actually, even though ROS do cause damage, low levels of ROS are well established that participate in cell signalling processes including cell proliferation, inflammation, apoptosis and phagocytosis [289]. Thus, it is well known that ROS may act as second messengers in cellular signalling transduction cascade pathways, including stress-activated protein kinases (SAPK) with both p38MAPK and c-Jun N-terminal kinase (JNK), p53 through PI3K/PKB and NF-κB pathways [183,195,197,215,217,290,291]. In this complex context, low levels of ROS activate cellular proliferation, while high levels produce apoptosis (Fig. **8**).

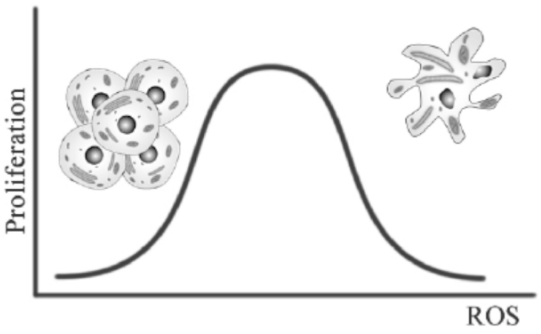

Fig. (9). Representative scheme of the effects of ROS levels on cell proliferation and apoptosis.

ROS operate as signalling molecules mainly through the oxidation of iron–sulfurcenters or the formation of cysteine oxidative adducts (glutathionylcysteine, SSG) or disulfide bonds (S–S) on their protein targets (Fig. **9**) [203,292]. Interestingly, different cysteine-based oxidative modifications may occur on the same residue of the same protein, with different biochemical and functional effects [203,293]. Reversible cysteine oxidation plays a pivotal role in redox signaling cascades, major molecular targets being protein

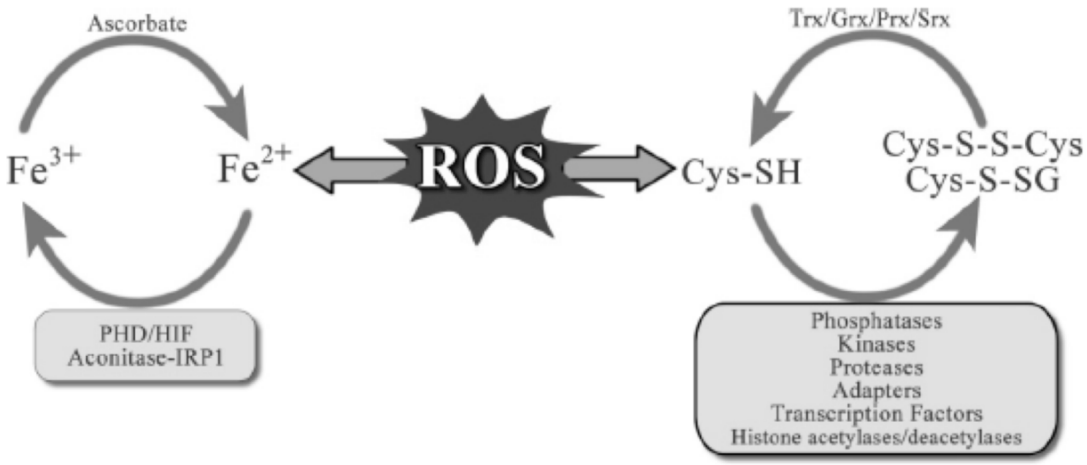

Fig. (10). Molecular targets of redox signaling.

tyrosine phosphatase or lipid phosphatases (like PTEN), proteases, signaling adaptors and transcription factors [203]. Among protein tyrosine phosphatases, protein tyrosine phosphatase 1B (PTP1B), SHP-2, PTEN and low molecular weight-PTP (LMW-PTP) are transiently inactivated by hydrogen peroxide in the context of growth factor, cytokine or integrin signaling, as a necessary step for the propagation of tyrosine phosphorylation cascades [294].

In summary, many cellular signalling pathways are sensible to ROS levels and the final cellular response depends on the final cell interpretation, which is the result of balance between apoptotic signals and proliferative and survival signals [193-195,200,201,203,204,211,212,216].

5. ESTROGEN RECEPTORS IN BREAST CANCER

In the developed countries, breast cancer is the malignancy with the highest incidence and the first leading cause of cancer deaths in women [295].Almost 1.4 million women were diagnosed with breast cancer worldwide in 2008 and approximately 459,000 deaths were recorded [296].

Hormonal environment is the major cause of breast cancer, thuselevated lifetime estrogen exposure has been shown to be a major risk factor for this malignancy [297-302]. Epidemiological studies show that early menarche, late menopause, and obesity in postmenopausal women are related to an increase in breast cancer risk[302-305].

5.1. Estrogen Signaling

Estrogensignalling pathway starts with binding the estrogens tothe estrogen receptors (ERs)[306-315]. 17β-Estradiol (E2), the principal mammalian estrogen, exerts its activity by binding to the two ERs normally present in the mammary gland, ERα and ERβ. ERα is most closely linked to the increase of mammary epithelial cell proliferation with the balance between ERα and ERβ regulating this activity [309,316-319]. Thus, estrogen stimulation of cell proliferation mediated via ERα can be counteracted by ERβ recruitment [320-322].

ERα (595 aa) and ERβ (530 aa) belong to a large superfamily of nuclear receptors that act as ligand-activated transcription factors[323,324]. Both receptors are codified by two different genes,which share only moderate homology in their protein sequences (58% in human and 55% in rat).DNA-binding domain is the most conserved with a 97% of homology, this domain has two zinc fingers and a short motif, called a P-box, which confers DNA specificity and is also implicated in dimerization [313,325,326]. Thus, ERα and ERβ bind to DNA in a similar manner. The ligand binding domain, with a 60% homology, is also involved in other functions (receptor dimerization, nuclear localization and cofactor interaction) [313]. Although, ERα and ERβ are associated with different cofactors thus they are modulated by diferential transcription genes[323,324].

The ERsalso show non-genomic actions in many tissues, this process involves the production of secondary messenger proteins with rapid effects, although this process is still not well characterized. Moreover, ERs have a ligand-independent activation mechanism, which is mediated by kinases that phosphorylate and activate ERs [310].

Other regulatory proteins have an important role in the activation mechanisms of ERs.These cofactors or coregulators act as coactivators or corepressors of the transcriptional activity of ERs. These coregulators can be in the form of acetylases/deacetylases, kinases/phosphatases and methylases/demethylases. It is noteworthy that the pool of coregulatorsmay differ according to the tissue, and this fact could explain the differential tissue effects of estrogen [313].

In additionto ER coregulator distribution, the ERα and ERβ distribution has also been reported to vary according to the tissue. In ERα and ERβ coexisting tissues, their effects appear to counteract each other. Consequently, in the immune systems, mammary glands and the uterus, ERα stimulatescell proliferation while ERβ presents proapoptotic and cell differentiation functions [313,327,328].

5.2. Breast Cancer and Estrogens

As mentioned above, estrogens represent the main risk factor for breast cancer initiation and progression, but the cellular proliferative action of this hormone could be determined by the ERα/ERβ ratio. Thus, an increased ERα/ERβ ratio comparedtonormal breast tissue has been postulated as an important factor for the development of the cancerous phenotype [313,317,329-331]. Conversely, a decrease in this ratio is indicative of a poor prognosis [332,333]. In this context, the different action of estrogens and phytoestrogens could be attributed to a variationin ERα and ERβ levels [334].

Estrogen stimulates both ERα and ERβ receptors, although the affinity for ERα is 10 times greater than for ERβ [312,335]. Additionally, ERα and ERβ expression are under estrogen regulation in cell lines such as MCF-7 and T47D,with an opposite pattern, henceestrogen treatment downregulate ERα and upregulate ERβ[332,336].

Recently, estrogen response elements have been identified in mtDNA, suggesting that the action of estrogen in the mitochondria could be related to the carcinogenic process and apoptotic pathway[179,337]. Additionally, ERα and ERβ has been localized in the mitochondria and has been related with the regulation of mitochondrial genes [179,233,337-340]. However, it is possible that the effect of ERα and ERβ in the mitochondria differsas well as in different ratio tissues.

5.2.1. Estrogens, Mitochondria and Oxidative Stress

Mitochondrial biogenesis and ROS production are modulated by estrogens. For this reason, several authors attribute to estrogens a new role in the carcinogenesis process, due to their action in modulating the mitochondrial function [211,337]. Impaired mitochondrial function could cause an increase in mitochondrial ROS levels, which could altermitochondrialcontrol ofcellular processes such as proliferation and apoptosis pathways, and this could explain in part the effect ofestrogens in cancer development [179,211].

This E2-mediated mitochondrial pathway involves the cooperation of the ERα and ERβ, both nuclear and mitochondrial,with their necessary co-activators to coordinate the regulation of bothnDNA and mtDNA-encoded genes in order tofacilitate the MRC protein assembly[337]. Estrogen and ER upregulate TFAM and possibly TFB1M and TFB2M through NRF-1 and NRF-2 [337]. Moreover, it has been found that estrogen significantly increases the amounts of mitochondrial ERα and ERβ in a time- and concentration-dependent manner and these actionsgo along with anupregulation of mtDNA-encoded genes [233].

The production of cathecolestrogens during the estrogen metabolism has been postulated as another mechanism that couldexplain the association between estrogens and cancer. Cathecolestrogens are genotoxic metabolites capable to produce DNA adducts and generate ROS [301,341,342], although this mechanism has little relevant impact at estrogen physiological concentrations.

ERα and ERβ presence and levels can be of great importance in the establishment of oxidative stress in mitochondria, and may explain the opposite effects of estrogens found in different tissues. Thus, an upregulation of the expression levels of antioxidant enzymes and UCPs has been described in liver, brain, skeletal and cardiac muscle as well as adipose tissue[233,343-348]. However, in breast and prostate cancer cell lines estrogen induce oxidative stress depending on the ERα/ERβ ratio [333,349,350]. Thus, in MCF7 breast cancer cell lines with a high ratio of ERα/ERβ estrogen induces oxidative stress either by or in combination with mitochondrial dysfunction, decrease in antioxidant enzymes and/or UCPs. On the contrary, in T47D breast cancer cell lines, which only presents ERβ, estradiol reduce oxidative stress by or in combination with improved mitochondrial function and increased expression of antioxidant enzymes and UCPs [333,349,350]. Similar results have been described in prostatecancer cell lines,where in those cell lines with low ER/ER ratio, the E2treatment producedthe up-regulation of antioxidant enzymes and UCPs with a drop in ROS production [351].

6. PHYTOESTROGENS AND BREAST CANCER

6.1. Flavonoids

There may be a link between high flavonoid-intake from Asian women and the low incidence of breast cancer in Asian countries [352].

6.1.1. Isoflavonoids - Isoflavones

As described above, lifetime exposure to E2is associated with an increased risk of breast cancer development, and contributes to tumor progression [297,304,325,349,353]. In the 1960s, Folman and Pope [354,355] were the first to establish the relative binding affinity of the isoflavones for the estrogen receptors, preferentially binding to ERβ[356,357]. Kuiper *et al.*[311,312] reported that the binding affinity of the isoflavone genistein for ERα was 4%, and for ERβ was 87%, compared to 17β-estradiol. The preferential binding of isoflavone to ERβ may have implications related to breast cancer risk; some data suggest that, when activated by certain ligands, ERβ inhibits mammary cancer cell growth as well as the stimulatory effects of ERα to E2[317]. However, the precise role of ERβ in breast cancer is unclear [317].

The relationship between isoflavones and breast cancer is very complex and remains enigmatic [3,32], since reports in support of the positive and negative effects of isoflavones maybe conflictive. These compounds, especially genistein, are involved in non-hormonal mediated mechanisms, such as inhibition of protein tyrosine kinase, inhibition of cell cycle progression, induction of apoptosis, inhibition of DNA topoisomerase, inhibition of angiogenesis and metastasis, which might reduce the carcinogenesis [24,358]. Moreover, isoflavones are well known as antioxidants. The ability to inhibit the generation of ROS results in the blockageofthe NF-κB activation [24]. However, a major mechanism of isoflavones action is thought to result from their ability to interact directly with estrogen receptors [358]. In fact, despite the proposed benefits, isoflavones and soy foods are not without controversy. Concerns have risen that estrogen-like properties of soy isoflavones may be harmful to patients with estrogen-sensitive breast cancer. This concern is based on research that showed that genistein stimulates the growth of ER-positive breast cancer cells, such as MCF-7 breast cancer cell lines, which presentthehighest ERα/ERβ ratio [350,359-361]. As shown in (Fig.**10**), experimental evidences in our laboratory suggest that 72 hours of genistein treatment in MCF7, T47D and MDA-MB-231 human breast cancer cell lines has different effects depending on their ERα/ERβ ratio. While in MCF-7 cell line, with the highest ERα/ERβ ratio, genistein treatment at 1μM presents a proliferative effect, in T47D, with thelowest ERα/ERβ ratio, the same treatment exerts a potent antiproliferative function. On the other hand, in MDA-MB-231, an ERα-negative and with low levels of ERβ cell line, genistein treatment has no significant effects on cell proliferation.

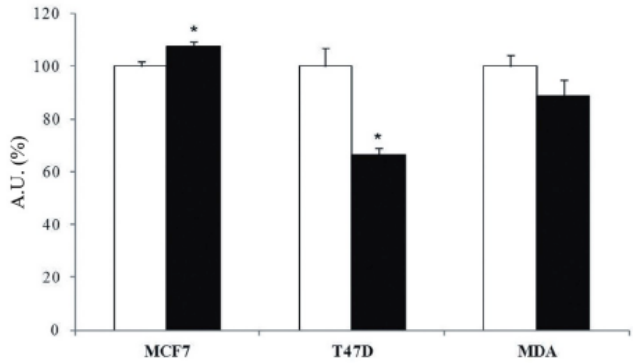

Fig. (11). Effect of genistein (GEN) on MCF-7, T47D and MDA-MB-231 cell proliferation . Cells were plated in 96-well plates and treated with GEN (1 μM) for 72h. Cell proliferation was determined by Violet Crystal staining and represented as growth percentage with respect to vehicle-treated cells (100%, treated with 0.001% DMSO). Data are represented as mean ± SEM. *Statistically significant difference between treated and vehicle-treated cells (Student's t-test; $P < 0.05$, n=6).

Some authors have reported a biphasic effect of genistein on cell growth; low concentrations (0.1-10 μM) show a stimulation of the proliferation, while high concentrations (above 10 μM) inhibit cell growth by blocking cell cycle at the G2/M phase, through a down-regulation of cyclin B [3,358,361]. In general, it is believed that the proliferative action of isoflavones at low concentrations can be inhibited by antiestrogen[362] indicating that it is an estrogen

receptor-mediated mechanism. However, the inhibition of cell growth at high concentrations is not prevented by estrogen or anti-estrogen agent indicating cell growth inhibition is not through ER-mediated mechanism or it may be due to genistein cytotoxic concentrations [362]. Actually, recent experimental researches in our laboratory agree with previous studies showing a dose-dependent cell growth regulation. In MCF-7 cell line, while low concentrations of genistein (1 nM – 10 μM) induce cell proliferation, a high dose of this isoflavone (100 μM) has a cytotoxic effect. On the other hand, in T47D cells, with predominance of ERβ, it can be observed that relative low doses of genistein (0.1 – 10 μM) inhibit cell proliferation and high doses (over 10 μM) produce cytotoxic effects in this cell line (Fig. **11**).

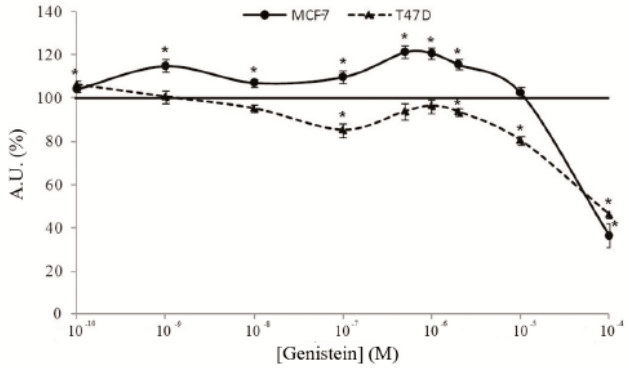

Fig. (12). Effect of genistein (GEN) on MCF-7and T47D cell proliferation . Cells were plated in 96-well plates and treated with GEN (0.0001, 0.001, 0.01, 0.1, 0.5, 1, 2, 10, 100 μM) for 48h. Cell proliferation was determined by Violet Crystal staining and represented as growth percentage with respect to vehicle-treated cells (100% solid line, treated with 0.001% DMSO). Data are represented as mean ± SEM. *Statistically significant difference between treated and vehicle-treated cells (Student's t-test; $P < 0.05$, n=6).

From another point of view, mitochondrial dysfunction has been reportedto be both the cause and outcome of cell injury, and have also been associated with cancer [363]. Increased mitochondrial biogenesis is related to the recovery of mitochondrial and cellular functions [350]. Preliminary studies performed in our laboratory suggest that genistein treatment could worsen the mitochondrial functionality in MCF-7 breast cancer cell line and maynot promote mitochondrial exchange, leading to a damaged mitochondrial pool accumulation. In contrast, genistein treatment could improve the mitochondrial functionality in T47D breast cancer cell line accompanied by a lower activation of mitochondrial biogenesis, and doingno necessary the mitochondrial exchange (Table **1**).

6.1.2. Isoflavonoids - Coumestans

Coumestans have been reported to exert several functions on certain cancer types, some of them mutually contradictory. Some studies suggest that coumestrol may exert chemoprotective effects against breast cancer. For example, in a study with T47D and MCF-7 breast cancer cells, coumestrol was found to inhibit serum-stimulated growth [364]. Moreover, coumestrol exerts a potent inhibitory effect on breast cancer cell line MDA-MB-231 invasion [365]. Otherwise, coumestrol has been shown to be able toinhibit the enzyme AKR1C3 which produces proliferative steroid hormones and prostaglandins [366,367].

On the contrary, there are several studies suggesting that coumestrol increases breast cancer risk. This compound stimulates MCF-7 cells growth but not apoptosis [57] and has the ability to inhibit apoptosis of this cell line in the presence of 17β-estradiol [368]. Thus, coumestrol could have potentially adverse mitogenic effects on tumor cells [369]. In fact, this compound shows molecular properties which are very similar to an estrogen receptor agonist like 17β-estradiol [370] and it has beenshown that coumestrol is a potent estrogen *in vitro* and acts through ER mediated mechanism [371].Therefore, itssuggested chemopreventive action in estrogen-related cancers must be mediated through other mechanisms [371].

At any rate, the current focus on the role of coumestrol in cancer prevention must take into account the biphasic effects observed in a study with MCF-7 cells which shows inhibition of DNA synthesis at high concentrations of coumestrol but induction at concentrations close to probable levels in humans [372].

6.1.3. Flavones

The properties of flavones, such as apigenin and luteolin, categorize them as a class of beneficial compounds which possess disease-preventing and health-promoting dietary effects [76].

Flavones have been shown to possess anti-estrogenic activity [373] and to promote apoptosis by inhibiting PI3K and Akt pathway in MCF-7 human breast cancer cells [374,375]. Moreover, flavones inhibit aromatase in MCF-7 cells at 1μM [376,377]. Apigenin can also induce apoptosis in MDA-MB-231 human breast cancer cells and in MDA-MB-231 xenografts through its ability to inhibit the activity of fatty acid synthase, a key metabolic enzyme expressed in neoplasic and malignant cells, and the activation of caspases 3 and 7, and PARP cleavage [378,379]. In addition, flavones have been shown to modulate cell cycle inducing G2/M cell

Table 1. ATPasa/COX ratio, PGC1*α*, TFAM, Fis1 and Mfn1 protein levels in MCF-7, T47D and MDA-MB-231 human breast cell lines after genistein treatment for 48h.

	MCF-7		T47D		MDA-MB-231	
	Control	**Genistein**	**Control**	**Genistein**	**Control**	**Genistein**
ATPasa/COX ratio (AU)	100 ± 10	**117 ± 7***	100 ± 13	**67.9 ± 8.2***	100 ± 17	85.2 ± 7.6
PGC1α (AU)	100 ± 11	82.5 ± 18.5	100 ± 16	**22.5 ± 4.7****	100 ± 2	**44.1 ± 16.0****
TFAM (AU)	100 ± 23	64.6 ± 11.9	100 ± 10	118 ± 9	100 ± 15	**131 ± 14***
Fis1 (AU)	100 ± 7	**83.4 ± 3.4***	100 ± 8	**81.3 ± 7.0***	100 ± 12	116 ± 10
Mfn1 (AU)	100 ± 12	95.8 ± 18.2	100 ± 11	**79.2 ± 6.6***	100 ± 15	75.4 ± 11.3

Data represent the means ± SEM (n=6). Values of control (vehicle-treated) MCF-7, T47D and MDA-MB-231 cell lines were set at 100. AU: arbitrary units; ATPasa, ATP synthase subunit alpha; COX, citochrome c oxidase; Fis1, mitochondrial fission 1 protein; Mfn1, mitofusin 1; PGC1α: peroxisome proliferator-activated receptor-gamma coactivator-1alpha; TFAM, mitochondrial transcription factor A.

** Significant difference between genistein-treated and vehicle-treated cell lines (Student's t-test; $P \leq 0.05$).

* Significant difference between genistein-treated and vehicle-treated cell lines (Student's t-test; $P \leq 0.1$).

cycle phase arrest in MCF-7 and MDA-MB-231 cells [380,381]. There are studies demonstrating that flavones could exert their functions through their interaction with ERs [382,383], but other studies have shown that flavones do not join to the ERs [384,385]. Seo*et al*. have demonstrated that apigenin stimulate the proliferation of ERα-positive breast cancer cells, MCF-7 and T47D, but do not stimulate the proliferation of ERα-negative breast cancer cells [361]. Regarding to invasiveness, flavones have been shown to inhibit tumor cell invasion in a dose-dependent manner in breast cancer cells [386,387]. Furthermore, luteolin has been shown to reduce tumor size and number in induced mammary carcinogenesis in rats [388].

6.1.4. Flavonols

The action of flavonols on breast cancer cells is ER dependent, and these compounds exert potent growth inhibitory effects on MCF-7 human breast cancer cell line [389,390]. Flavonols, both quercetin and kaempferol, have a higher affinity and activation for ERβ than ERα [391,392]. Specially, quercetin, even at picomolar concentrations, reduces proliferation of human breast cancer cells [393]. However, other authors show that a treatment with 150μM concentration of quercetin is necessary to induce mitochondrial cell death in MCF-7 cells [394]. The antiproliferative effects of kaempferol in breast cancer cells may be mediated by the inhibition of the MAPK/ERK pathway [36]. Moreover, several authors have observed a cell cycle arrest in G2/M phase in breast cancer cells after flavonol treatment [395,396]. Relatively low doses of quercetin (1-10μM) also induce cell cycle arrest at the G0/G1 phase in breast cancer cells but not in normal breast cancer cells, suggesting that quercetin has a cancer-specific antiproliferative effect [397]. The biphasic effects of flavonols on cell proliferation have been observed in breast cancer cell lines. The balance between the antioxidant and the pro-oxidant activity of flavonols is concentration dependent. Breast cancer cells treated with different flavonol concentrations showed that low concentrations of flavonols induce cell proliferation and flavonols might behave as powerful antioxidants. On the other hand, a high flavonol concentration induces cell death and may be oxidized, generating ROS which induce cytotoxicity [392,398]. Regarding to anti-invasive effects of flavonols, it has been shown that quercetin inhibits the *in vitro* invasion of MDA-MB-231 cells in a concentration-dependent fashion [399]. Moreover, the invasive activity of MCF-7 cells is blocked by quercetin by reducing the expression of MMP-9 and blocking the activation of the protein kinase C (PKC)/ERK/AP-1 signaling cascade [400]. The administration of quercetin suppresses tumor growth and invasion in rat mammary carcinoma *in vivo*[401]. On the other hand, kaempferol has been shown to down-regulate HIF-1 expression, a key regulator in carcinogenesis [402].

SIRT1 activation can lead to a reduction of oxidative stress and, consequently, to a lower risk of cancer [403]. In 2010, Chung *et al.* reviewed that SIRT1 is increased by quercetin[404]. The relationship between quercetin and SIRT1 has been studied recently by Hong *et al.* In this paper the authors demonstrate that quercetin can up-regulate SIRT1 [405]. Moreover, SIRT3 is increased by kaempferol treatment and this could have an important role in mitochondrial apoptosis [406].

6.1.5. Flavanones

Breast cancer chemopreventive activity has been attributed toflavanones through cell proliferation inhibition [106,108,407], apoptosis induction [106,108,408-412], aromatase activity inhibition [106,116,413], anti-angiogenic effects [108] and estrogen metabolism attenuation [106,115].

8-Prenylnaringenin (8PN) inhibits ERα mediated cell growth in MCF-7 breast cancer cells [408] and a study with the same cell line shows that 8PN specifically inhibits proliferation by targeting phosphatidylinositol-3-OH kinase activity [108]. On the contrary, experimental evidences in our laboratory showed that MCF-7 cell line treated with 8PN from 0.001 to 20 μM experiments a slightly proliferation increase with 1 and 5 μM and has no effect at the other tested concentrations[414]. Nevertheless, 8PN treatment on T47D cell line decreased proliferation or, in any case, had no effect (Fig. **12**).

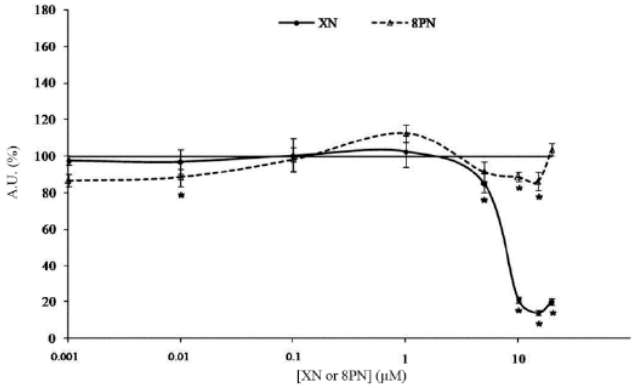

Fig. (13). Influence of xanthohumol (XN) and 8-prenylnaringenin (8PN) on T47D cell proliferation. Cells were plated in 96-well plates and treated with XN or 8PN (0.001, 0.01, 0.1, 1, 5, 10, 15 and 20 μM) for 48 h. Cell viability was assessed by DNA staining with Hoechst 33342 and represented as growing percentage with respect to vehicle-treated cells (100% solid line, treated with 0.001% of DMSO). Data are represented as mean ± SEM. *Statistically significant difference between treated and vehicle-treated cells (Student's t-test; $P < 0.05$, n=6).

Moreover, 8PN induces apoptosis in MCF-7 breast cancer cells [108,408] and SkBR3breast cancer cell line [106]. Otherwise, 8PN has the ability to inhibit the activity of aromatase in human placental microsomes, in human primary breast fibroblasts [116] and in SkBR3breast cancer cell line [106,413]. Furthermore, 8PN possesses cancer chemopreventive activity through attenuation of estrogen metabolism [115] and it is able to inhibit estrogen formation on SkBR3breast cancer cell line [106]. 8PN also exerts anti-angiogenic effects on MCF-7 breast cancer cells[108]. In our laboratory, the effects of 8PN on ROS production were assessed and it was shown that 8PN has no effect or slightly decreases ROS production in T47D cell (Fig. **13**).

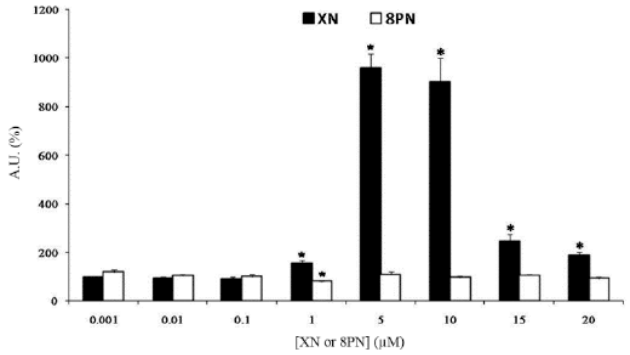

Fig. (14). Effect of xanthohumol (XN) and 8-prenylnaringenin (8PN) on T47D cell line ROS production. Cells were plated in 96-well plates and treated with XN or 8PN (0.001, 0.01, 0.1, 1, 5, 10, 15 and 20 μM) for 48 h. ROS production was determined by DCFDA and represented as ROS production percentage with respect to vehicle-treated cells (100%, treated with 0.001% DMSO). Data are represented as mean ± SEM. *Statistically significant difference between treated and vehicle-treated cells (Student's t-test; $P < 0.05$, n=6).

Otherwise, isoxanthohumol also has antiproliferative activity in MCF-7 human breast cancer cell line [407] and naryngenin induces apoptosis in different cancer types (e.g., colon, breast, and uterus cancer cell lines) [409-412].

In a recent publication, our group studied the effect of 8PN on MCF-7 cell line mitochondrial function. Treatments with 8PN at 0.01 and 1 μM led to a decrease in ROS production along with an increase of OXPHOS and sirtuin expression. These results suggest that 8PN presumably improve mitochondrial functionality[414].

6.1.6. Chalcones

It has been reported several times that chalcones have potential chemopreventive activity against breast cancer in humans. In fact, xanthohumol (XN) decreases *in vitro* MCF-7 cell proliferation in a dose-dependent manner [407,415], reduces breast cancer cell line SkBR3 proliferation [106] and inhibits DNA synthesis [407]. Actually, recent experimental researches in our laboratory agree with previous studies showing a dose-dependent cell growth regulation effect of XN. We treated MCF-7 cells with different doses of XN and significant proliferation induction was observed from 0.001 to 1 μM; on the contrary, from 10 to 20 μM cell number markedly decreased[414]. Similarly, XN from 0.001 to 1 μM had no effect on T47D proliferation but doses from 5 to 20 μM caused a significant reduction of cell number (Fig. **12**).

Moreover, XN has the ability to induce apoptosis in MCF-7 and T47D cells [141] as well as in SkBR3breast cancer cell line [106]. Otherwise, XN is able to inhibit aromatase activity on SkBR3breast cancer cell line and,therefore, estrogen formation [106,413]. XN also shows anti-invasive activity in MCF-7 and T47D cell lines through up-regulation of E-cadherin/catenin complex and inhibition of invasion *in vitro*[141]. In a study with breast and colon cancer cells, XN abolished cell invasion [138] and oral administration of XN to nude mice inoculated with MCF7 cells resulted in decreased microvessel density [415].

Furthermore, XN has antioxidant properties and is also an efficient inhibitor of lipid peroxidation and a protector against the degradation of antioxidant enzymes induced by intoxication [416]. Despite its antioxidant properties, XN is also able to trigger the generation of superoxide radicals in mitochondria, a process that subsequently leads to an increase in ROS levels, loss of GSH and ultimately cancer cell death by apoptosis. In fact, the results obtained in our laboratory show that XN form 0.001 to 0.1 μM has no effect or reduces ROS production on T47D cell line, whereas XN from 1 to 20 μM causes a significant induction of ROS production (Fig. **13**). Specifically, XN seemsto act in and against mitochondria of cells with a disturbed redox balance, such as certain cancer cells. This fact provides promising new leads to target selectively certain cancer cells with this disturbed redox balance [417].

We recently studied the effect of XN on MCF-7 mitochondrial function and it was observed that this phytoestrogen exerts a dual effect depending on the dose. Treatment with XN 0.01μM led to a decrease in ROS production along with an increase of OXPHOS and sirtuin expression; in contrast, XN 5 μM gave rise to an increase of ROS production accompanied by a decrease in OXPHOS and sirtuin expression. These results suggest that XN in low dose presumably improve mitochondrial function, whereas a high dose of XN impairs mitochondrial function[414].

6.2. Lignans

Chemopreventive actions of lignans against mammary cancer have been demonstrated in experimental models [418,419]. They are potentially mediated through mechanisms such as modulation of estrogen action and by possessing anti-angiogenic, pro-apoptotic and antioxidant properties [419]. High dietary intakes of plant lignans and high exposure to enterolignans were associated with reduced risks of ER- and PR-positive postmenopausal breast cancer in a Western population that does not consume a diet rich in soy [155]. McCann *et al.* showed no associationbetween lignans intakes and postmenopausal breast cancer. Their results suggest that dietary lignans may be important in the etiology of breast cancer, particularly among premenopausal woman [420]. Numerous *in vitro* cell culture studies and *in vivo* animal experiments demonstrated that phytoestrogens can inhibit breast tumor growth [22,152,421]. The two mammalian lignans, especially enterolactone, are inhibitors of several steroid metabolizing enzymes, such as aromatase, 5α-reductase, 7α-hydrolase and 17β-hydroxysteroid dehydrogenase [142]. Moreover phytoestrogens have received considerable attention as potential cancer-preventing agents via their antiestrogenic activity [22,422]. In fact, enterolactone inhibits E2-induced proliferation of MCF-7 breast cancer cells [142] and inhibits the growth of human colon tumor cells through regulation of the Wnt/β-catenin signaling pathway [423].

6.3. Stilbenes

Stilbenes have been shown to inhibit the activity of ROS [424]. *In vitro* studies, comparing pterostilbene and resveratrol showed that pterostilbene has more biological activity than resveratrol [425,426]. Stilbenes can exert antiestrogenic effects[427,428]. Moreover, resveratrol presents chemopreventive and chemotherapeutic effects on some estrogen-dependent tumors [429-431], especially on breast cancer [432]. ERα and ERβ play important and different roles in the effects of stilbenes, which could serve as useful tools in assessing the effectiveness of stilbenes treatment as well as in screening the anti-estrogenic drugs applied to some cancers [175]. Pterostilbene has been shown to induce apoptosis and to inhibit cell viability in both estrogen-receptor positive and negative breast cancer cell lines [174,433] and has an additive effect when combined with tamoxifen[434]. Stilbens also inhibit invasion in an *in vitro* breast cancer metastasis model [435]. Wang *et al.* showed that resveratrol inhibits proliferation, and induces cell cycle arrest and apoptosis, which are associated with decreased expression of cyclin D3 and phosphorylated retinoblastome protein [436]. Evidence that resveratrol suppresses breast carcinogenesis has also been obtained using animal studies, showing a 60% reduction in tumor formation when the animals were concurrently given resveratrol [437]. Resveratrol has also been claimed to be an activator of SIRT1 [404,438], with the consequently reduction of oxidative stress and the lower risk of cancer [403].

7. CONCLUSIONS AND PERSPECTIVES

In summary, the relationship between phytoestrogens and mitochondrial biogenesis and dynamics in breast cancer is very complex. There are some reports in support of the positive effects of phytoestrogens on breast cancer, but also exist other evidences showing negative effects that seems to be conflictive. Thus, some of the positive effects of phytoestrogens in breast cancer have been related to a greater activation of mitochondrial biogenesis and antioxidant systems. However, some authors have demonstrated that elevated concentrations of some phytoestrogens have pro-oxidant effects which could damage mitochondria and alter their function and dynamics, and therefore could contribute to the cancer development.

In our studies, weobserved different effects of some phytoestrogens (genistein, xanthohumol, 8-prenylnaringenin and resveratrol) on mitochondria in different cancer cell lines (colon, breast and prostate). Our results suggest that phytoestrogen effects depend on the doses (acting as antioxidants and improving the mitochondrial biogenesis at low doses and as pro-oxidants and worsening mitochondrial function at high doses) and the ERα/ERβ ratio (in low ratio cell lines phytoestrogens promote better mitochondrial function and antioxidant response than in the high ratio ones).

Moreover, it is worth to note that the effects of phytoestrogens can be different at the beginning or ending of the cancer disease. Thus moderating phytoestrogens ingestion diminishes breast cancer

incidence, but the same ingestion during cancer treatment could avoid the cytotoxic effects of some of the treatments based on ROS generation, as phytoestrogens decrease oxidative stress, improving mitochondrial function and antioxidant response. Nevertheless, adjuvant administration of phytoestrogens at elevated doses could increase anticancer treatment efficacy, because of the reduction of mitochondrial function and the antioxidant response.

Finally, it is important to highlight that the behavior of phytoestrogens in the life-cycle may depend on factors such as: a) timing of exposure; b) digestion, absorption and individual metabolism; and c) hormonal status (estrogen receptor ratio). For all these reasons, more experiments are necessary to explain the effects of phytoestrogens, at least some of them, on mitochondrial biogenesis and dynamics; and for design new therapy targets for cancer treatment.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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REFERENCES

[1] Farnsworth NR, Bingel AS, Cordell GA, Crane FA, Fong HS. Potential value of plants as sources of new antifertility agents II. J Pharm Sci 1975; 64: 717-54.

[2] Kurzer MS, Xu X. Dietary phytoestrogens. Annu Rev Nutr 1997; 17: 353-81.

[3] Mense SM, Hei TK, Ganju RK, Bhat HK. Phytoestrogens and breast cancer prevention: possible mechanisms of action. Environ Health Perspect 2008; 116: 426-33.

[4] Messina MJ, Loprinzi CL. Soy for breast cancer survivors: a critical review of the literature. J Nutr 2001; 131: 3095S-108S.

[5] Orlando L, Schiavone P, Cinieri S. Genistein: the future of prevention and treatment of breast cancer? Cancer Biol Ther 2011; 11: 918-20.

[6] Dixon RA. Phytoestrogens. Annu Rev Plant Biol 2004; 55: 225-61.

[7] Adlercreutz H. Phyto-oestrogens and cancer. Lancet Oncol 2002; 3: 364-73.

[8] Cotter A, Cashman KD. Genistein appears to prevent early postmenopausal bone loss as effectively as hormone replacement therapy. Nutr Rev 2003; 61: 346-51.

[9] van der Schouw YT, de Kleijn MJ, Peeters PH, Grobbee DE. Phyto-oestrogens and cardiovascular disease risk. Nutr Metab Cardiovasc Dis 2000; 10: 154-67.

[10] Anderson JJ, Anthony MS, Cline JM, Washburn SA, Garner SC. Health potential of soy isoflavones for menopausal women. Public Health Nutr 1999; 2: 489-504.

[11] Vincent A, Fitzpatrick LA. Soy isoflavones: are they useful in menopause? Mayo Clin Proc 2000; 75: 1174-84.

[12] Middleton E, Jr., Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev 2000; 52: 673-751.

[13] Setchell KD, Brown NM, Zhao X, *et al.* Soy isoflavone phase II metabolism differs between rodents and humans: implications for the effect on breast cancer risk. Am J Clin Nutr 2011; 94: 1284-94.

[14] Messina M, Wu AH. Perspectives on the soy-breast cancer relation. Am J Clin Nutr 2009; 89: 1673S-9S.

[15] Shu XO, Jin F, Dai Q, *et al.* Soyfood intake during adolescence and subsequent risk of breast cancer among Chinese women. Cancer Epidemiol Biomarkers Prev 2001; 10: 483-8.

[16] Chen J, Chen J, Cammarata PR, *et al.* Regulation of mitochondrial respiratory chain biogenesis by estrogens/estrogen receptors and physiological, pathological and pharmacological implications. Biochimica et biophysica acta 2009; 1793: 1540-70.

[17] Galluzzi L, Morselli E, Kepp O, *et al.* Mitochondrial gateways to cancer. Mol Aspects Med 2010; 31: 1-20.

[18] Neuzil J, Dong LF, Rohlena J, Truksa J, Ralph SJ. Classification of mitocans, anti-cancer drugs acting on mitochondria. Mitochondrion 2012.

[19] Rohlena J, Dong LF, Neuzil J. Targeting the Mitochondrial Electron Transport Chain Complexes for the Induction of Apoptosis and Cancer Treatment. Curr Pharm Biotechnol 2012.

[20] Anderson LN, Cotterchio M, Boucher BA, Kreiger N. Phytoestrogen intake from foods, during adolescence and adulthood, and risk of breast cancer by estrogen and progesterone receptor (ERPR) tumour subgroup among Ontario women. International journal of cancer Journal international du cancer 2012.

[21] de Cremoux P, This P, Leclercq G, Jacquot Y. Controversies concerning the use of phytoestrogens in menopause management: bioavailability and metabolism. Maturitas 2010; 65: 334-9.

[22] Adlercreutz H. Phytoestrogens: epidemiology and a possible role in cancer protection. Environ Health Perspect 1995; 103 Suppl 7: 103-12.

[23] Perkin AG, Newbury FG. The colouring matters contained in Dyer's broom (*Genista tinctoria*) and heather (*Calluna vulgaris*). J Chem Soc 1899; 75: 830-9.

[24] Banerjee S, Li Y, Wang Z, Sarkar FH. Multi-targeted therapy of cancer by genistein. Cancer Lett 2008; 269: 226-42.

[25] Marini H, Minutoli L, Polito F, *et al.* Effects of the phytoestrogen genistein on bone metabolism in osteopenic postmenopausal women: a randomized trial. Ann Intern Med 2007; 146: 839-47.

[26] Si H, Liu D. Phytochemical genistein in the regulation of vascular function: new insights. Curr Med Chem 2007; 14: 2581-9.

[27] Akiyama T, Ishida J, Nakagawa S, *et al.* Genistein, a specific inhibitor of tyrosine-specific protein kinases. J Biol Chem 1987; 262: 5592-5.

[28] Ruiz-Larrea MB, Mohan AR, Paganga G, Miller NJ, Bolwell GP, Rice-Evans CA. Antioxidant activity of phytoestrogenic isoflavones. Free Radic Res 1997; 26: 63-70.

[29] Ouyang G, Yao L, Ruan K, Song G, Mao Y, Bao S. Genistein induces G2/M cell cycle arrest and apoptosis of human ovarian cancer cells via activation of DNA damage checkpoint pathways. Cell Biol Int 2009; 33: 1237-44.

[30] Peng JH, Zhu JD, Mi MT, *et al.* Prepubertal genistein exposure affects erbB2/Akt signal and reduces rat mammary tumorigenesis. Eur J Cancer Prev 2010; 19: 110-9.

[31] Pavese JM, Farmer RL, Bergan RC. Inhibition of cancer cell invasion and metastasis by genistein. Cancer Metastasis Rev 2010; 29: 465-82.

[32] Messina M, McCaskill-Stevens W, Lampe JW. Addressing the soy and breast cancer relationship: review, commentary, and workshop proceedings. J Natl Cancer Inst 2006; 98: 1275-84.

[33] Messina M, Hilakivi-Clarke L. Early intake appears to be the key to the proposed protective effects of soy intake against breast cancer. Nutr Cancer 2009; 61: 792-8.

[34] Liggins J, Mulligan A, Runswick S, Bingham SA. Daidzein and genistein content of cereals. Eur J Clin Nutr 2002; 56: 961-6.

[35] Setchell KD, Brown NM, Lydeking-Olsen E. The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones. J Nutr 2002; 132: 3577-84.

[36] Choi EJ, Kim GH. Daidzein causes cell cycle arrest at the G1 and G2/M phases in human breast cancer MCF-7 and MDA-MB-453 cells. Phytomedicine 2008; 15: 683-90.

[37] Jin S, Zhang QY, Kang XM, Wang JX, Zhao WH. Daidzein induces MCF-7 breast cancer cell apoptosis via the mitochondrial pathway. Ann Oncol 2010; 21: 263-8.

[38] Marrian GF, Haslewood GA. Equol, a new inactive phenol isolated from the ketohydroxyoestrin fraction of mares' urine. Biochem J 1932; 26: 1227-32.

[39] Setchell KD, Clerici C. Equol: history, chemistry, and formation. J Nutr 2010; 140: 1355S-62S.

[40] Setchell KD, Clerici C. Equol: pharmacokinetics and biological actions. J Nutr 2010; 140: 1363S-8S.

[41] Matthies A, Loh G, Blaut M, Braune A. Daidzein and genistein are converted to equol and 5-hydroxy-equol by human intestinal Slackia isoflavoniconvertens in gnotobiotic rats. J Nutr 2012; 142: 40-6.

[42] Song TT, Hendrich S, Murphy PA. Estrogenic activity of glycitein, a soy isoflavone. J Agric Food Chem 1999; 47: 1607-10.

[43] Shinkaruk S, Durand M, Lamothe V, *et al*. Bioavailability of glycitein relatively to other soy isoflavones in healthy young Caucasian men. Food Chem 2012; 135: 1104-11.

[44] Park HJ, Park HJ, Moon JO, *et al*. Isoflavone glycosides from the flowers of *Pueraria thunbergiana*. Phytochemistry 1999; 51: 147–51.

[45] Winzer M, Rauner M, Pietschmann P. Glycitein decreases the generation of murine osteoclasts and increases apoptosis. Wien Med Wochenschr 2010; 160: 446-51.

[46] Lee EJ, Kim SY, Hyun JW, Min SW, Kim DH, Kim HS. Glycitein inhibits glioma cell invasion through down-regulation of MMP-3 and MMP-9 gene expression. Chem Biol Interact 2010; 185: 18-24.

[47] Kang KA, Zhang R, Piao MJ, *et al*. Inhibitory effects of glycitein on hydrogen peroxide induced cell damage by scavenging reactive oxygen species and inhibiting c-Jun N-terminal kinase. Free Radic Res 2007; 41: 720-9.

[48] Lee SO, Renouf M, Ye Z, Murphy PA, Hendrich S. Isoflavone glycitein diminished plasma cholesterol in female golden Syrian hamsters. J Agric Food Chem 2007; 55: 11063-7.

[49] Pope GS, Elcoate PV, Simpson SA, Andrews DG. Isolation of an oestrogenic isoflavone (biochanin A) from red clover. Chem Indus 1953; 1092.

[50] Booth NL, Piersen CE, Banuvar S, Geller SE, Shulman LP, Farnsworth NR. Clinical studies of red clover (Trifolium pratense) dietary supplements in menopause: a literature review. Menopause 2006; 13: 251-64.

[51] Wang Y, Man Gho W, Chan FL, Chen S, Leung LK. The red clover (Trifolium pratense) isoflavone biochanin A inhibits aromatase activity and expression. Br J Nutr 2008; 99: 303-10.

[52] Kole L, Giri B, Manna SK, Pal B, Ghosh S. Biochanin-A, an isoflavon, showed anti-proliferative and anti-inflammatory activities through the inhibition of iNOS expression, p38-MAPK and ATF-2 phosphorylation and blocking NFkappaB nuclear translocation. Eur J Pharmacol 2011; 653: 8-15.

[53] Chang CJ, Geahlen RL. Protein-tyrosine kinase inhibition: mechanism-based discovery of antitumor agents. J Nat Prod 1992; 55: 1529-60.

[54] Ndebele K, Graham B, Tchounwou PB. Estrogenic activity of coumestrol, DDT, and TCDD in human cervical cancer cells. Int J Environ Res Public Health 2010; 7: 2045-56.

[55] Canal Castro C, Pagnussat AS, Orlandi L, *et al*. Coumestrol has neuroprotective effects before and after global cerebral ischemia in female rats. Brain Res 2012; 1474: 82-90.

[56] Hoerger CC, Wettstein FE, Bachmann HJ, Hungerbuhler K, Bucheli TD. Occurrence and mass balance of isoflavones on an experimental grassland field. Environ Sci Technol 2011; 45: 6752-60.

[57] Sakamoto T, Horiguchi H, Oguma E, Kayama F. Effects of diverse dietary phytoestrogens on cell growth, cell cycle and apoptosis in estrogen-receptor-positive breast cancer cells. J Nutr Biochem 2010; 21: 856-64.

[58] Clarke DB, Bailey V, Lloyd AS. Determination of phytoestrogens in dietary supplements by LC-MS/MS. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 2008; 25: 534-47.

[59] Wu CT, Tzeng JN, Lai JN, Tsan SH, Wang JD. Prescription profile of Chinese herbal products containing coumestrol, genestein, and/or daidzein among female users: an analysis of national health insurance data in Taiwan between 1997 and 2007. Chin Med 2012; 7: 22.

[60] Jeon HY, Seo DB, Shin HJ, Lee SJ. Effect of Aspergillus oryzae-challenged germination on soybean isoflavone content and antioxidant activity. J Agric Food Chem 2012; 60: 2807-14.

[61] Mlynarczuk J, Wrobel MH, Kotwica J. Adverse influence of coumestrol on secretory function of bovine luteal cells in the first trimester of pregnancy. Environ Toxicol 2011.

[62] Nogowski L. Effects of phytoestrogen-coumestrol on lipid and carbohydrate metabolism in young ovariectomized rats may be independent of its estrogenicity. J Nutr Biochem 1999; 10: 664-9.

[63] Domon OE, McGarrity LJ, Bishop M, Yoshioka M, Chen JJ, Morris SM. Evaluation of the genotoxicity of the phytoestrogen, coumestrol, in AHH-1 TK(+/-) human lymphoblastoid cells. Mutat Res 2001; 474: 129-37.

[64] Kim JE, Son JE, Jang YJ, *et al*. Luteolin, a novel natural inhibitor of tumor progression locus 2 serine/threonine kinase, inhibits tumor necrosis factor-alpha-induced cyclooxygenase-2 expression in JB6 mouse epidermis cells. J Pharmacol Exp Ther 2011; 338: 1013-22.

[65] Shimoi K, Okada H, Furugori M, *et al*. Intestinal absorption of luteolin and luteolin 7-O-beta-glucoside in rats and humans. FEBS Lett 1998; 438: 220-4.

[66] Shimoi K, Saka N, Nozawa R, *et al*. Deglucuronidation of a flavonoid, luteolin monoglucuronide, during inflammation. Drug Metab Dispos 2001; 29: 1521-4.

[67] Lopez-Lazaro M. Distribution and biological activities of the flavonoid luteolin. Mini Rev Med Chem 2009; 9: 31-59.

[68] Lefort EC, Blay J. Apigenin and its impact on gastrointestinal cancers. Mol Nutr Food Res 2013; 57: 126-44.

[69] Chung CS, Jiang Y, Cheng D, Birt DF. Impact of adenomatous polyposis coli (APC) tumor supressor gene in human colon cancer cell lines on cell cycle arrest by apigenin. Mol Carcinog 2007; 46: 773-82.

[70] Gates MA, Tworoger SS, Hecht JL, De Vivo I, Rosner B, Hankinson SE. A prospective study of dietary flavonoid intake and incidence of epithelial ovarian cancer. Int J Cancer 2007; 121: 2225-32.

[71] Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. Am J Clin Nutr 2004; 79: 727-47.

[72] McKay DL, Blumberg JB. A review of the bioactivity and potential health benefits of chamomile tea (Matricaria recutita L.). Phytother Res 2006; 20: 519-30.

[73] Srivastava JK, Gupta S. Antiproliferative and apoptotic effects of chamomile extract in various human cancer cells. J Agric Food Chem 2007; 55: 9470-8.

[74] Bevilacqua L, Buiarelli F, Coccioli F, Jasionowska R. Identification of compounds in wine by HPLC-tandem mass spectrometry. Ann Chim 2004; 94: 679-89.

[75] Gerhauser C. Beer constituents as potential cancer chemopreventive agents. Eur J Cancer 2005; 41: 1941-54.

[76] Shukla S, Gupta S. Apigenin: a promising molecule for cancer prevention. Pharm Res 2010; 27: 962-78.

[77] Yang CS, Landau JM, Huang MT, Newmark HL. Inhibition of carcinogenesis by dietary polyphenolic compounds. Annu Rev Nutr 2001; 21: 381-406.

[78] Russo M, Spagnuolo C, Tedesco I, Bilotto S, Russo GL. The flavonoid quercetin in disease prevention and therapy: facts and fancies. Biochem Pharmacol 2012; 83: 6-15.

[79] Harwood M, Danielewska-Nikiel B, Borzelleca JF, Flamm GW, Williams GM, Lines TC. A critical review of the data related to the safety of quercetin and lack of evidence of *in vivo* toxicity, including lack of genotoxic/carcinogenic properties. Food Chem Toxicol 2007; 45: 2179-205.

[80] Hollman PC, de Vries JH, van Leeuwen SD, Mengelers MJ, Katan MB. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. Am J Clin Nutr 1995; 62: 1276-82.

[81] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100: 57-70.

[82] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-74.

[83] Calderon-Montano JM, Burgos-Moron E, Perez-Guerrero C, Lopez-Lazaro M. A review on the dietary flavonoid kaempferol. Mini Rev Med Chem 2011; 11: 298-344.

[84] Crespy V, Morand C, Besson C, *et al*. The splanchnic metabolism of flavonoids highly differed according to the nature of the compound. Am J Physiol Gastrointest Liver Physiol 2003; 284: G980-8.

[85] Lehtonen HM, Lehtinen O, Suomela JP, Viitanen M, Kallio H. Flavonol glycosides of sea buckthorn (Hippophae rhamnoides ssp. sinensis) and lingonberry (Vaccinium vitis-idaea) are bioavailable in humans and monoglucuronidated for excretion. J Agric Food Chem 2010; 58: 620-7.

[86] Yodogawa S, Arakawa T, Sugihara N, Furuno K. Glucurono- and sulfo-conjugation of kaempferol in rat liver subcellular preparations and cultured hepatocytes. Biol Pharm Bull 2003; 26: 1120-4.

[87] de Vries JH, Hollman PC, Meyboom S, *et al*. Plasma concentrations and urinary excretion of the antioxidant flavonols quercetin and kaempferol as biomarkers for dietary intake. Am J Clin Nutr 1998; 68: 60-5.

[88] Knekt P, Kumpulainen J, Jarvinen R, *et al*. Flavonoid intake and risk of chronic diseases. Am J Clin Nutr 2002; 76: 560-8.

[89] Andrade Cetto A, Wiedenfeld H, Revilla MC, Sergio IA. Hypoglycemic effect of Equisetum myriochaetum aerial parts on streptozotocin diabetic rats. J Ethnopharmacol 2000; 72: 129-33.

[90] Jorge AP, Horst H, de Sousa E, Pizzolatti MG, Silva FR. Insulinomimetic effects of kaempferitrin on glycaemia and on 14C-glucose uptake in rat soleus muscle. Chem Biol Interact 2004; 149: 89-96.

[91] Izzo G, Soder O, Svechnikov K. The prenylflavonoid phytoestrogens 8-prenylnaringenin and isoxanthohumol diferentially suppress steroidogenesis in rat Leydig cells in ontogenesis. J Appl Toxicol 2010.

[92] Fu ML, Wang W, Chen F, *et al*. Production of 8-prenylnaringenin from isoxanthohumol through biotransformation by fungi cells. J Agric Food Chem 2011; 59: 7419-26.

[93] Stevens JF, Taylor AW, Clawson JE, Deinzer ML. Fate of xanthohumol and related prenylflavonoids from hops to beer. J Agric Food Chem 1999; 47: 2421-8.

[94] Dhooghe L, Naessens T, Heyerick A, *et al*. Quantification of xanthohumol, isoxanthohumol, 8-prenylnaringenin, and 6-prenylnaringenin in hop extracts and derived capsules using secondary standards. Talanta 2010; 83: 448-56.

[95] Wyns C, Derycke L, Soenen B, *et al*. Production of monoclonal antibodies against hop-derived (Humulus lupulus L.) prenylflavonoids and the development of immunoassays. Talanta 2011; 85: 197-205.

[96] Stevens JF, Page JE. Xanthohumol and related prenylflavonoids from hops and beer: to your good health! Phytochemistry 2004; 65: 1317-30.

[97] Possemiers S, Rabot S, Espin JC, *et al*. Eubacterium limosum activates isoxanthohumol from hops (Humulus lupulus L.) into the potent phytoestrogen 8-prenylnaringenin *in vitro* and in rat intestine. J Nutr 2008; 138: 1310-6.

[98] Possemiers S, Bolca S, Grootaert C, *et al*. The prenylflavonoid isoxanthohumol from hops (Humulus lupulus L.) is activated into the potent phytoestrogen 8-prenylnaringenin *in vitro* and in the human intestine. J Nutr 2006; 136: 1862-7.

[99] Zanoli P, Zavatti M. Pharmacognostic and pharmacological profile of Humulus lupulus L. J Ethnopharmacol 2008; 116: 383-96.

[100] Lauro G, Masullo M, Piacente S, Riccio R, Bifulco G. Inverse Virtual Screening allows the discovery of the biological activity of natural compounds. Bioorg Med Chem 2012; 20: 3596-602.

[101] Negrao R, Costa R, Duarte D, Taveira *et al*. Angiogenesis and inflammation signaling are targets of beer polyphenols on vascular cells. J Cell Biochem 2010; 111: 1270-9.

[102] Bertl E, Becker H, Eicher T, *et al*. Inhibition of endothelial cell functions by novel potential cancer chemopreventive agents. Biochem Biophys Res Commun 2004; 325: 287-95.

[103] Mojzis J, Varinska L, Mojzisova G, Kostova I, Mirossay L. Antiangiogenic effects of flavonoids and chalcones. Pharmacol Res 2008; 57: 259-65.

[104] Serwe A, Rudolph K, Anke T, Erkel G. Inhibition of TGF-beta signaling, vasculogenic mimicry and proinflammatory gene expression by isoxanthohumol. Invest New Drugs 2012; 30: 898-915.

[105] Delmulle L, Vanden Berghe T, Keukeleire DD, Vandenabeele P. Treatment of PC-3 and DU145 prostate cancer cells by prenylflavonoids from hop (Humulus lupulus L.) induces a caspase-independent form of cell death. Phytother Res 2008; 22: 197-203.

[106] Monteiro R, Faria A, Azevedo I, Calhau C. Modulation of breast cancer cell survival by aromatase inhibiting hop (Humulus lupulus L.) flavonoids. J Steroid Biochem Mol Biol 2007; 105: 124-30.

[107] Aniol M, Swiderska A, Stompor M, Zolnierczyk AK. Antiproliferative activity and synthesis of 8-prenylnaringenin derivatives by demethylation of 7-O- and 4'-O-substituted isoxanthohumols. Med Chem Res 2012; 21: 4230-8.

[108] Brunelli E, Pinton G, Chianale F, Graziani A, Appendino G, Moro L. 8-Prenylnaringenin inhibits epidermal growth factor-induced MCF-7 breast cancer cell proliferation by targeting phosphatidylinositol-3-OH kinase activity. J Steroid Biochem Mol Biol 2009; 113: 163-70.

[109] Mukai R, Horikawa H, Fujikura Y, *et al*. Prevention of disuse muscle atrophy by dietary ingestion of 8-prenylnaringenin in denervated mice. PLoS One 2012; 7: e45048.

[110] Di Vito C, Bertoni A, Nalin M, Sampietro S, Zanfa M, Sinigaglia F. The phytoestrogen 8-prenylnaringenin inhibits agonist-dependent activation of human platelets. Biochim Biophys Acta 2012; 1820: 1724-33.

[111] Ming LG, Ge BF, Wang MG, Chen KM. Comparison between 8-prenylnarigenin and narigenin concerning their activities on promotion of rat bone marrow stromal cells' osteogenic differentiation *in vitro*. Cell Prolif 2012; 45: 508-15.

[112] Sehmisch S, Hammer F, Christoffel J, *et al*. Comparison of the phytohormones genistein, resveratrol and 8-prenylnaringenin as agents for preventing osteoporosis. Planta Med 2008; 74: 794-801.

[113] Bowe J, Li XF, Kinsey-Jones J, *et al*. The hop phytoestrogen, 8-prenylnaringenin, reverses the ovariectomy-induced rise in skin temperature in an animal model of menopausal hot flushes. J Endocrinol 2006; 191: 399-405.

[114] Gasiorowska J, Teisseyre A, Uryga A, Michalak K. The influence of 8-prenylnaringenin on the activity of voltage-gated Kv1.3 potassium channels in human Jurkat T cells. Cell Mol Biol Lett 2012; 17: 559-70.

[115] Hemachandra LP, Madhubhani P, Chandrasena R, *et al*. Hops (Humulus lupulus) inhibits oxidative estrogen metabolism and estrogen-induced malignant transformation in human mammary epithelial cells (MCF-10A). Cancer Prev Res (Phila) 2012; 5: 73-81.

[116] van Meeuwen JA, Nijmeijer S, Mutarapat T, *et al*. Aromatase inhibition by synthetic lactones and flavonoids in human placental microsomes and breast fibroblasts--a comparative study. Toxicol Appl Pharmacol 2008; 228: 269-76.

[117] Kawaii S, Tomono Y, Katase E, Ogawa K, Yano M. Quantitation of flavonoid constituents in citrus fruits. J Agric Food Chem 1999; 47: 3565-71.

[118] Mershiba SD, Dassprakash MV, Saraswathy SD. Protective effect of naringenin on hepatic and renal dysfunction and oxidative stress in arsenic intoxicated rats. Mol Biol Rep 2013.

[119] Wesolowska O, Wisniewski J, Sroda-Pomianek K, *et al*. Multidrug Resistance Reversal and Apoptosis Induction in Human Colon Cancer Cells by Some Flavonoids Present in Citrus Plants. J Nat Prod 2012.

[120] Kanno S, Tomizawa A, Hiura T, *et al*. Inhibitory effects of naringenin on tumor growth in human cancer cell lines and sarcoma S-180-implanted mice. Biol Pharm Bull 2005; 28: 527-30.

[121] Bear WL, Teel RW. Effects of citrus flavonoids on the mutagenicity of heterocyclic amines and on cytochrome P450 1A2 activity. Anticancer Res 2000; 20: 3609-14.

[122] Hirai S, Kim YI, Goto T, *et al*. Inhibitory effect of naringenin chalcone on inflammatory changes in the interaction between adipocytes and macrophages. Life Sci 2007; 81: 1272-9.

[123] Hamalainen M, Nieminen R, Vuorela P, Heinonen M, Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-kappaB activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-kappaB activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm 2007; 2007: 45673.

[124] Raza SS, Khan MM, Ahmad A, *et al*. Neuroprotective effect of naringenin is mediated through suppression of NF-kappaB signaling pathway in experimental stroke. Neuroscience 2012.

[125] Pietta PG. Flavonoids as antioxidants. J Nat Prod 2000; 63: 1035-42.

[126] Kapoor R, Rizvi F, Kakkar P. Naringenin prevents high glucose-induced mitochondria-mediated apoptosis involving AIF, Endo-G and caspases. Apoptosis 2012.

[127] Rajadurai M, Prince PS. Preventive effect of naringin on isoproterenol-induced cardiotoxicity in Wistar rats: an *in vivo* and *in vitro* study. Toxicology 2007; 232: 216-25.

[128] Kannappan S, Anuradha CV. Naringenin enhances insulin-stimulated tyrosine phosphorylation and improves the cellular actions of insulin in a dietary model of metabolic syndrome. Eur J Nutr 2010; 49: 101-9.

[129] Goldwasser J, Cohen PY, Yang E, Balaguer P, Yarmush ML, Nahmias Y. Transcriptional regulation of human and rat hepatic lipid metabolism by the grapefruit flavonoid naringenin: role of PPARalpha, PPARgamma and LXRalpha. PLoS One 2010; 5: e12399.

[130] Youdim KA, Qaiser MZ, Begley DJ, Rice-Evans CA, Abbott NJ. Flavonoid permeability across an in situ model of the blood-brain barrier. Free Radic Biol Med 2004; 36: 592-604.

[131] Kanaze FI, Bounartzi MI, Georgarakis M, Niopas I. Pharmacokinetics of the citrus flavanone aglycones hesperetin and naringenin after single oral administration in human subjects. Eur J Clin Nutr 2007; 61: 472-7.

[132] Cavia-Saiz M, Busto MD, Pilar-Izquierdo MC, Ortega N, Perez-Mateos M, Muniz P. Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. J Sci Food Agric 2010; 90: 1238-44.

[133] Weng CJ, Yen GC. Flavonoids, a ubiquitous dietary phenolic subclass, exert extensive *in vitro* anti-invasive and *in vivo* anti-metastatic activities. Cancer Metastasis Rev 2012; 31: 323-51.

[134] Botta B, Vitali A, Menendez P, Misiti D, Delle Monache G. Prenylated flavonoids: pharmacology and biotechnology. Curr Med Chem 2005; 12: 717-39.

[135] Stevens JF, Taylor AW, Nickerson GB, *et al.* Prenylflavonoid variation in Humulus lupulus: distribution and taxonomic significance of xanthogalenol and 4'-O-methylxanthohumol. Phytochemistry 2000; 53: 759-75.

[136] Stracke D, Schulz T, Prehm P. Inhibitors of hyaluronan export from hops prevent osteoarthritic reactions. Mol Nutr Food Res 2011; 55: 485-94.

[137] Cho YC, You SK, Kim HJ, Cho CW, Lee IS, Kang BY. Xanthohumol inhibits IL-12 production and reduces chronic allergic contact dermatitis. Int Immunopharmacol 2010; 10: 556-61.

[138] Wang Y, Chen Y, Wang J, *et al*. Xanthohumol, a prenylated chalcone derived from hops, suppresses cancer cell invasion through inhibiting the expression of CXCR4 chemokine receptor. Curr Mol Med 2012; 12: 153-62.

[139] Colgate EC, Miranda CL, Stevens JF, Bray TM, Ho E. Xanthohumol, a prenylflavonoid derived from hops induces apoptosis and inhibits NF-kappaB activation in prostate epithelial cells. Cancer Lett 2007; 246: 201-9.

[140] Lust S, Vanhoecke B, Janssens A, Philippe J, Bracke M, Offner F. Xanthohumol kills B-chronic lymphocytic leukemia cells by an apoptotic mechanism. Mol Nutr Food Res 2005; 49: 844-50.

[141] Vanhoecke B, Derycke L, Van Marck V, Depypere H, De Keukeleire D, Bracke M. Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (Humulus lupulus L.) and beer. Int J Cancer 2005; 117: 889-95.

[142] Wang LQ. Mammalian phytoestrogens: enterodiol and enterolactone. J Chromatogr B Analyt Technol Biomed Life Sci 2002; 777: 289-309.

[143] Wang LQ, Meselhy MR, Li Y, Qin GW, Hattori M. Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. Chem Pharm Bull (Tokyo) 2000; 48: 1606-10.

[144] Clavel T, Henderson G, Engst W, Dore J, Blaut M. Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. FEMS Microbiol Ecol 2006; 55: 471-8.

[145] Ayella A, Lim S, Jiang Y, *et al.* Cytostatic inhibition of cancer cell growth by lignan secoisolariciresinol diglucoside. Nutr Res 2010; 30: 762-9.

[146] Pellegrini N, Valtuena S, Ardigo D, *et al.* Intake of the plant lignans matairesinol, secoisolariciresinol, pinoresinol, and lariciresinol in relation to vascular inflammation and endothelial dysfunction in middle age-elderly men and post-menopausal women living in Northern Italy. Nutr Metab Cardiovasc Dis 2010; 20: 64-71.

[147] Mazur W. Phytoestrogen content in foods. Baillieres Clin Endocrinol Metab 1998; 12: 729-42.

[148] Niemeyer HB, Honig DM, Kulling SE, Metzler M. Studies on the metabolism of the plant lignans secoisolariciresinol and matairesinol. J Agric Food Chem 2003; 51: 6317-25.

[149] Setchell KD, Lawson AM, Borriello SP, *et al.* Lignan formation in man--microbial involvement and possible roles in relation to cancer. Lancet 1981; 2: 4-7.

[150] Borriello SP, Setchell KD, Axelson M, Lawson AM. Production and metabolism of lignans by the human faecal flora. J Appl Bacteriol 1985; 58: 37-43.

[151] Lee B, Kim KH, Jung HJ, Kwon HJ. Matairesinol inhibits angiogenesis via suppression of mitochondrial reactive oxygen species. Biochem Biophys Res Commun 2012; 421: 76-80.

[152] Abarzua S, Serikawa T, Szewczyk M, Richter DU, Piechulla B, Briese V. Antiproliferative activity of lignans against the breast carcinoma cell lines MCF 7 and BT 20. Arch Gynecol Obstet 2012; 285: 1145-51.

[153] Carreau C, Flouriot G, Bennetau-Pelissero C, Potier M. Enterodiol and enterolactone, two major diet-derived polyphenol metabolites have different impact on ERalpha transcriptional activation in human breast cancer cells. J Steroid Biochem Mol Biol 2008; 110: 176-85.

[154] Corsini E, Dell'Agli M, Facchi A, *et al*. Enterodiol and enterolactone modulate the immune response by acting on nuclear factor-kappaB (NF-kappaB) signaling. J Agric Food Chem 2010; 58: 6678-84.

[155] Touillaud MS, Thiebaut AC, Fournier A, Niravong M, Boutron-Ruault MC, Clavel-Chapelon F. Dietary lignan intake and postmenopausal breast cancer risk by estrogen and progesterone receptor status. J Natl Cancer Inst 2007; 99: 475-86.

[156] Hiroto Y, Tadokoro K, Tsuda T, *et al.* Resveratrol, a phytoestrogen found in red wine, down-regulates protein S expression in HepG2 cells. Thromb Res 2011; 127: e1-7.

[157] Das DK, Maulik N. Resveratrol in cardioprotection: a therapeutic promise of alternative medicine. Mol Interv 2006; 6: 36-47.

[158] Pervaiz S, Holme AL. Resveratrol: its biologic targets and functional activity. Antioxid Redox Signal 2009; 11: 2851-97.

[159] Bishayee A. Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials. Cancer Prev Res (Phila) 2009; 2: 409-18.

[160] Szkudelska K, Szkudelski T. Resveratrol, obesity and diabetes. Eur J Pharmacol 2010; 635: 1-8.

[161] Das S, Alagappan VK, Bagchi D, Sharma HS, Maulik N, Das DK. Coordinated induction of iNOS-VEGF-KDR-eNOS after resveratrol consumption: a potential mechanism for resveratrol preconditioning of the heart. Vascul Pharmacol 2005; 42: 281-9.

[162] Atten MJ, Attar BM, Milson T, Holian O. Resveratrol-induced inactivation of human gastric adenocarcinoma cells through a protein kinase C-mediated mechanism. Biochem Pharmacol 2001; 62: 1423-32.

[163] El-Mowafy AM, Alkhalaf M. Resveratrol activates adenylyl-cyclase in human breast cancer cells: a novel, estrogen receptor-independent cytostatic mechanism. Carcinogenesis 2003; 24: 869-73.

[164] de la Lastra CA, Villegas I. Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. Biochem Soc Trans 2007; 35: 1156-60.

[165] Mikula-Pietrasik J, Kuczmarska A, Rubis B, *et al.* Resveratrol delays replicative senescence of human mesothelial cells via mobilization of antioxidative and DNA repair mechanisms. Free Radic Biol Med 2012; 52: 2234-45.

[166] Howitz KT, Bitterman KJ, Cohen HY, *et al.* Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature 2003; 425: 191-6.

[167] Marques FZ, Markus MA, Morris BJ. Resveratrol: cellular actions of a potent natural chemical that confers a diversity of health benefits. Int J Biochem Cell Biol 2009; 41: 2125-8.

[168] Roupe KA, Remsberg CM, Yanez JA, Davies NM. Pharmacometrics of stilbenes: seguing towards the clinic. Curr Clin Pharmacol 2006; 1: 81-101.

[169] Lin HS, Yue BD, Ho PC. Determination of pterostilbene in rat plasma by a simple HPLC-UV method and its application in preclinical pharmacokinetic study. Biomed Chromatogr 2009; 23: 1308-15.

[170] Remsberg CM, Yanez JA, Ohgami Y, Vega-Villa KR, Rimando AM, Davies NM. Pharmacometrics of pterostilbene: preclinical pharmacokinetics and metabolism, anticancer, antiinflammatory, antioxidant and analgesic activity. Phytother Res 2008; 22: 169-79.

[171] Asensi M, Medina I, Ortega A, *et al.* Inhibition of cancer growth by resveratrol is related to its low bioavailability. Free Radic Biol Med 2002; 33: 387-98.

[172] Zhang L, Cui L, Zhou G, Jing H, Guo Y, Sun W. Pterostilbene, a natural small-molecular compound, promotes cytoprotective macroautophagy in vascular endothelial cells. J Nutr Biochem 2012.

[173] Moon D, McCormack D, McDonald D, McFadden D. Pterostilbene induces mitochondrially derived apoptosis in breast cancer cells *in vitro*. J Surg Res 2012.

[174] Alosi JA, McDonald DE, Schneider JS, Privette AR, McFadden DW. Pterostilbene inhibits breast cancer *in vitro* through mitochondrial depolarization and induction of caspase-dependent apoptosis. J Surg Res 2010; 161: 195-201.

[175] Wang Y, Ding L, Wang X, *et al.* Pterostilbene simultaneously induces apoptosis, cell cycle arrest and cyto-protective autophagy in breast cancer cells. Am J Transl Res 2012; 4: 44-51.

[176] Chen RJ, Ho CT, Wang YJ. Pterostilbene induces autophagy and apoptosis in sensitive and chemoresistant human bladder cancer cells. Mol Nutr Food Res 2010; 54: 1819-32.
[177] Paul S, Rimando AM, Lee HJ, Ji Y, Reddy BS, Suh N. Anti-inflammatory action of pterostilbene is mediated through the p38 mitogen-activated protein kinase pathway in colon cancer cells. Cancer Prev Res (Phila) 2009; 2: 650-7.
[178] Braschi E, McBride HM. Mitochondria and the culture of the Borg: understanding the integration of mitochondrial function within the reticulum, the cell, and the organism. Bioessays 2010; 32: 958-66.
[179] Gonzalez DH, Bonnard G, Grienenberger JM. A gene involved in the biogenesis of c-type cytochromes is co-transcribed with a ribosomal protein gene in wheat mitochondria [corrected]. Curr Genet 1993; 24: 248-55.
[180] Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature 1961; 191: 144-8.
[181] Nicholls DG, Ferguson FJ, editors. Bioenergetics: An Introduction to the Chemiosmotic Theory. New York, NY, USA, : Academic Press; 1992.
[182] Brand MD. The proton leak across the mitochondrial inner membrane. Biochim Biophys Acta 1990; 1018: 128-33.
[183] Valle A, Oliver J, Roca P. Role of Uncoupling Proteins in Cancer. Cancers 2010; 2: 567-91.
[184] Klingenberg M. Uncoupling protein--a useful energy dissipator. J Bioenerg Biomembr 1999; 31: 419-30.
[185] Lin CS, Klingenberg M. Characteristics of the isolated purine nucleotide binding protein from brown fat mitochondria. Biochemistry 1982; 21: 2950-6.
[186] Diehl AM, Hoek JB. Mitochondrial uncoupling: role of uncoupling protein anion carriers and relationship to thermogenesis and weight control "the benefits of losing control". Journal of Bioenergetics and Biomembranes 1999; 31: 493-506.
[187] Echtay KS. Mitochondrial uncoupling proteins--what is their physiological role? Free Radic Biol Med 2007; 43: 1351-71.
[188] Goglia F, Skulachev VP. A function for novel uncoupling proteins: antioxidant defense of mitochondrial matrix by translocating fatty acid peroxides from the inner to the outer membrane leaflet. FASEB J 2003; 17: 1585-91.
[189] Lombardi A, Busiello RA, Napolitano L, *et al*. UCP3 translocates lipid hydroperoxide and mediates lipid hydroperoxide-dependent mitochondrial uncoupling. J Biol Chem 2010; 285: 16599-605.
[190] Samudio I, Fiegl M, Andreeff M. Mitochondrial uncoupling and the Warburg effect: molecular basis for the reprogramming of cancer cell metabolism. Cancer Res 2009; 69: 2163-6.
[191] Lenaz G. The mitochondrial production of reactive oxygen species: mechanisms and implications in human pathology. IUBMB Life 2001; 52: 159-64.
[192] Chen Q, Vazquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. Production of reactive oxygen species by mitochondria: central role of complex III. J Biol Chem 2003; 278: 36027-31.
[193] Bartosz G. Reactive oxygen species: destroyers or messengers? Biochem Pharmacol 2009; 77: 1303-15.
[194] Zorov DB, Bannikova SY, Belousov VV, *et al*. Reactive oxygen and nitrogen species: friends or foes? Biochemistry (Mosc) 2005; 70: 215-21.
[195] Valle A, Roca P. Mitochondria at the crossroads of cancer and oxidative stress. In: Reyes AM, Contreras CD, editors. Handbook on Oxidative Stress New Research. New York: Nova Science Publisher, Inc.; 2012; pp. 229-56.
[196] Andreyev AY, Kushnareva YE, Starkov AA. Mitochondrial metabolism of reactive oxygen species. Biochemistry (Mosc) 2005; 70: 200-14.
[197] Murphy MP. How mitochondria produce reactive oxygen species. Biochem J 2009; 417: 1-13.
[198] St-Pierre J, Buckingham JA, Roebuck SJ, Brand MD. Topology of superoxide production from different sites in the mitochondrial electron transport chain. J Biol Chem 2002; 277: 44784-90.
[199] Fariss MW, Chan CB, Patel M, Van Houten B, Orrenius S. Role of mitochondria in toxic oxidative stress. Mol Interv 2005; 5: 94-111.
[200] Kongara S, Karantza V. The interplay between autophagy and ROS in tumorigenesis. Front Oncol 2012; 2: 171.
[201] Barrera G. Oxidative stress and lipid peroxidation products in cancer progression and therapy. ISRN Oncol 2012; 2012: 137289.
[202] Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. World Allergy Organ J 2012; 5: 9-19.
[203] Pani G, Galeotti T, Chiarugi P. Metastasis: cancer cell's escape from oxidative stress. Cancer Metastasis Rev 2010; 29: 351-78.
[204] Lu W, Ogasawara MA, Huang P. Models of reactive oxygen species in cancer. Drug Discov Today Dis Models 2007; 4: 67-73.
[205] Echtay KS, Roussel D, St-Pierre J, *et al*. Superoxide activates mitochondrial uncoupling proteins. Nature 2002; 415: 96-9.
[206] Santandreu FM, Valle A, Fernandez de Mattos S, Roca P, Oliver J. Hydrogen peroxide regulates the mitochondrial content of uncoupling protein 5 in colon cancer cells. Cell Physiol Biochem 2009; 24: 379-90.
[207] Santandreu FM, Roca P, Oliver J. Uncoupling protein-2 knockdown mediates the cytotoxic effects of cisplatin. Free Radic Biol Med 2010; 49: 658-66.
[208] Negre-Salvayre A, Hirtz C, Carrera G, *et al*. A role for uncoupling protein-2 as a regulator of mitochondrial hydrogen peroxide generation. FASEB Journal 1997; 11: 809-15.
[209] Talbot DA, Lambert AJ, Brand MD. Production of endogenous matrix superoxide from mitochondrial complex I leads to activation of uncoupling protein 3. FEBS Letters 2004; 556: 111-5.
[210] Echtay KS, Esteves TC, Pakay JL, *et al*. A signalling role for 4-hydroxy-2-nonenal in regulation of mitochondrial uncoupling. EMBO Journal 2003; 22: 4103-10.
[211] Addison R, McCormick DB. Biogenesis of flavoprotein and cytochrome components in hepatic mitochondria from riboflavin-deficient rats. Biochem Biophys Res Commun 1978; 81: 133-8.
[212] Lu F. Reactive oxygen species in cancer, too much or too little? Med Hypotheses 2007; 69: 1293-8.
[213] Laurent A, Nicco C, Chereau C, *et al*. Controlling tumor growth by modulating endogenous production of reactive oxygen species. Cancer Res 2005; 65: 948-56.
[214] Cejas P, Casado E, Belda-Iniesta C, *et al*. Implications of oxidative stress and cell membrane lipid peroxidation in human cancer (Spain). Cancer Causes Control 2004; 15: 707-19.
[215] Martindale JL, Holbrook NJ. Cellular response to oxidative stress: signaling for suicide and survival. J Cell Physiol 2002; 192: 1-15.
[216] Benhar M, Engelberg D, Levitzki A. ROS, stress-activated kinases and stress signaling in cancer. EMBO Rep 2002; 3: 420-5.
[217] Sauer H, Wartenberg M, Hescheler J. Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell Physiol Biochem 2001; 11: 173-86.
[218] Chen JQ, Yager JD, Russo J. Regulation of mitochondrial respiratory chain structure and function by estrogens/estrogen receptors and potential physiological/pathophysiological implications. Biochim Biophys Acta 2005; 1746: 1-17.
[219] Amutha B, Gordon DM, Gu Y, Lyver ER, Dancis A, Pain D. GTP is required for iron-sulfur cluster biogenesis in mitochondria. J Biol Chem 2008; 283: 1362-71.
[220] Goffart S, Wiesner RJ. Regulation and co-ordination of nuclear gene expression during mitochondrial biogenesis. Exp Physiol 2003; 88: 33-40.
[221] Menassa R, El-Rouby N, Brown GG. An open reading frame for a protein involved in cytochrome c biogenesis is split into two parts in Brassica mitochondria. Curr Genet 1997; 31: 70-9.
[222] Klingenspor M, Ivemeyer M, Wiesinger H, Haas K, Heldmaier G, Wiesner RJ. Biogenesis of thermogenic mitochondria in brown adipose tissue of Djungarian hamsters during cold adaptation. Biochem J 1996; 316 (Pt 2): 607-13.
[223] Addya S, Anandatheerthavarada HK, Biswas G, Bhagwat SV, Mullick J, Avadhani NG. Targeting of NH2-terminal-processed microsomal protein to mitochondria: a novel pathway for the biogenesis of hepatic mitochondrial P450MT2. J Cell Biol 1997; 139: 589-99.
[224] Schuster W. The highly edited orf206 in Oenothera mitochondria may encode a component of a heme transporter involved in cytochrome c biogenesis. Plant Mol Biol 1994; 25: 33-42.
[225] Ventura-Clapier R, Garnier A, Veksler V. Transcriptional control of mitochondrial biogenesis: the central role of PGC-1alpha. Cardiovasc Res 2008; 79: 208-17.
[226] Garesse R, Vallejo CG. Animal mitochondrial biogenesis and function: a regulatory cross-talk between two genomes. Gene 2001; 263: 1-16.
[227] Giege P, Grienenberger JM, Bonnard G. Cytochrome c biogenesis in mitochondria. Mitochondrion 2008; 8: 61-73.

[228] Mansour AM, Nass S. *In vivo* cortisol action on RNA synthesis in rat liver nuclei and mitochondria. Nature 1970; 228: 665-7.
[229] Cornwall GA, Orgebin-Crist MC, Hann SR. Differential expression of the mouse mitochondrial genes and the mitochondrial RNA-processing endoribonuclease RNA by androgens. Mol Endocrinol 1992; 6: 1032-42.
[230] Scheller K, Sekeris CE, Krohne G, Hock R, Hansen IA, Scheer U. Localization of glucocorticoid hormone receptors in mitochondria of human cells. Eur J Cell Biol 2000; 79: 299-307.
[231] Kessler MA, Lamm L, Jarnagin K, DeLuca HF. 1,25-Dihydroxyvitamin D3-stimulated mRNAs in rat small intestine. Arch Biochem Biophys 1986; 251: 403-12.
[232] Wrutniak-Cabello C, Casas F, Cabello G. Thyroid hormone action in mitochondria. J Mol Endocrinol 2001; 26: 67-77.
[233] Chen JQ, Delannoy M, Cooke C, Yager JD. Mitochondrial localization of ERalpha and ERbeta in human MCF7 cells. Am J Physiol Endocrinol Metab 2004; 286: E1011-22.
[234] Casas F, Domenjoud L, Rochard P, *et al*. A 45 kDa protein related to PPARgamma2, induced by peroxisome proliferators, is located in the mitochondrial matrix. FEBS Lett 2000; 478: 4-8.
[235] Palmer CS, Osellame LD, Stojanovski D, Ryan MT. The regulation of mitochondrial morphology: intricate mechanisms and dynamic machinery. Cell Signal 2011; 23: 1534-45.
[236] Cereghetti GM, Scorrano L. The many shapes of mitochondrial death. Oncogene 2006; 25: 4717-24.
[237] Shaw JM, Winge DR. Shaping the mitochondrion: mitochondrial biogenesis, dynamics and dysfunction. Conference on Mitochondrial Assembly and Dynamics in Health and Disease. EMBO Rep 2009; 10: 1301-5.
[238] Justo R, Boada J, Frontera M, Oliver J, Bermudez J, Gianotti M. Gender dimorphism in rat liver mitochondrial oxidative metabolism and biogenesis. Am J Physiol Cell Physiol 2005; 289: C372-8.
[239] Liesa M, Palacin M, Zorzano A. Mitochondrial dynamics in mammalian health and disease. Physiol Rev 2009; 89: 799-845.
[240] Youle RJ, Narendra DP. Mechanisms of mitophagy. Nat Rev Mol Cell Biol 2011; 12: 9-14.
[241] Okamoto K, Kondo-Okamoto N. Mitochondria and autophagy: critical interplay between the two homeostats. Biochim Biophys Acta 2012; 1820: 595-600.
[242] Karbowski M, Youle RJ. Dynamics of mitochondrial morphology in healthy cells and during apoptosis. Cell Death Differ 2003; 10: 870-80.
[243] Chen H, Detmer SA, Ewald AJ, Griffin EE, Fraser SE, Chan DC. Mitofusins Mfn1 and Mfn2 coordinately regulate mitochondrial fusion and are essential for embryonic development. J Cell Biol 2003; 160: 189-200.
[244] Rojo M, Legros F, Chateau D, Lombes A. Membrane topology and mitochondrial targeting of mitofusins, ubiquitous mammalian homologs of the transmembrane GTPase Fzo. J Cell Sci 2002; 115: 1663-74.
[245] Frezza C, Cipolat S, Martins de Brito O, *et al*. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. Cell 2006; 126: 177-89.
[246] Cipolat S, Rudka T, Hartmann D, *et al*. Mitochondrial rhomboid PARL regulates cytochrome c release during apoptosis via OPA1-dependent cristae remodeling. Cell 2006; 126: 163-75.
[247] Herzig S, Martinou JC. Mitochondrial dynamics: to be in good shape to survive. Curr Mol Med 2008; 8: 131-7.
[248] Twig G, Elorza A, Molina AJ, *et al*. Fission and selective fusion govern mitochondrial segregation and elimination by autophagy. EMBO J 2008; 27: 433-46.
[249] Rambold AS, Kostelecky B, Lippincott-Schwartz J. Together we are stronger: fusion protects mitochondria from autophagosomal degradation. Autophagy 2011; 7: 1568-9.
[250] Lee YJ, Jeong SY, Karbowski M, Smith CL, Youle RJ. Roles of the mammalian mitochondrial fission and fusion mediators Fis1, Drp1, and Opa1 in apoptosis. Mol Biol Cell 2004; 15: 5001-11.
[251] James DI, Parone PA, Mattenberger Y, Martinou JC. hFis1, a novel component of the mammalian mitochondrial fission machinery. J Biol Chem 2003; 278: 36373-9.
[252] Brooks C, Cho SG, Wang CY, Yang T, Dong Z. Fragmented mitochondria are sensitized to Bax insertion and activation during apoptosis. Am J Physiol Cell Physiol 2011; 300: C447-55.
[253] Karbowski M, Norris KL, Cleland MM, Jeong SY, Youle RJ. Role of Bax and Bak in mitochondrial morphogenesis. Nature 2006; 443: 658-62.
[254] Hackenbrock CR. Ultrastructural bases for metabolically linked mechanical activity in mitochondria. I. Reversible ultrastructural changes with change in metabolic steady state in isolated liver mitochondria. J Cell Biol 1966; 30: 269-97.
[255] Grandemange S, Herzig S, Martinou JC. Mitochondrial dynamics and cancer. Semin Cancer Biol 2009; 19: 50-6.
[256] Yu M, Zhou Y, Shi Y, *et al*. Reduced mitochondrial DNA copy number is correlated with tumor progression and prognosis in Chinese breast cancer patients. IUBMB Life 2007; 59: 450-7.
[257] Naito A, Carcel-Trullols J, Xie CH, Evans TT, Mizumachi T, Higuchi M. Induction of acquired resistance to antiestrogen by reversible mitochondrial DNA depletion in breast cancer cell line. Int J Cancer 2008; 122: 1506-11.
[258] Warburg O, Posener K, Negelein E. Über den Stoffwechsel der Tumoren. Biochem Z 1924; 152: 319-44.
[259] Warburg O. On the origin of cancer cells. Science 1956; 123: 309-14.
[260] Bartrons R, Caro J. Hypoxia, glucose metabolism and the Warburg's effect. J Bioenerg Biomembr 2007; 39: 223-9.
[261] Demetrius LA, Coy JF, Tuszynski JA. Cancer proliferation and therapy: the Warburg effect and quantum metabolism. Theor Biol Med Model 2010; 7: 2.
[262] Pedersen PL. Warburg, me and Hexokinase 2: Multiple discoveries of key molecular events underlying one of cancers' most common phenotypes, the "Warburg Effect", i.e., elevated glycolysis in the presence of oxygen. J Bioenerg Biomembr 2007; 39: 211-22.
[263] Lunt SY, Vander Heiden MG. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol 2011; 27: 441-64.
[264] Upadhyay M, Samal J, Kandpal M, Singh OV, Vivekanandan P. The Warburg effect: Insights from the past decade. Pharmacol Ther 2012.
[265] Finkel T, Deng CX, Mostoslavsky R. Recent progress in the biology and physiology of sirtuins. Nature 2009; 460: 587-91.
[266] Zhong L, Mostoslavsky R. Fine tuning our cellular factories: sirtuins in mitochondrial biology. Cell Metab 2011; 13: 621-6.
[267] Lomb DJ, Laurent G, Haigis MC. Sirtuins regulate key aspects of lipid metabolism. Biochim Biophys Acta 2010; 1804: 1652-7.
[268] Kume S, Uzu T, Horiike K, *et al*. Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. J Clin Invest 2010; 120: 1043-55.
[269] Zhong L, D'Urso A, Toiber D, *et al*. The histone deacetylase Sirt6 regulates glucose homeostasis via Hif1alpha. Cell 2010; 140: 280-93.
[270] Sebastian C, Zwaans BM, Silberman DM, *et al*. The histone deacetylase SIRT6 is a tumor suppressor that controls cancer metabolism. Cell 2012; 151: 1185-99.
[271] Martinez-Pastor B, Mostoslavsky R. Sirtuins, metabolism, and cancer. Front Pharmacol 2012; 3: 22.
[272] Kim HS, Patel K, Muldoon-Jacobs K, *et al*. SIRT3 is a mitochondria-localized tumor suppressor required for maintenance of mitochondrial integrity and metabolism during stress. Cancer Cell 2010; 17: 41-52.
[273] Deng CX. SIRT1, is it a tumor promoter or tumor suppressor? Int J Biol Sci 2009; 5: 147-52.
[274] Finley LW, Carracedo A, Lee J, *et al*. SIRT3 opposes reprogramming of cancer cell metabolism through HIF1alpha destabilization. Cancer Cell 2011; 19: 416-28.
[275] Ashraf N, Zino S, Macintyre A, *et al*. Altered sirtuin expression is associated with node-positive breast cancer. Br J Cancer 2006; 95: 1056-61.
[276] Hockel M, Vaupel P. Biological consequences of tumor hypoxia. Semin Oncol 2001; 28: 36-41.
[277] Santandreu FM, Brell M, Gene AL, *et al*. Differences in Mitochondrial Function and Antioxidant Systems between Regions of Human Glioma. Cell Physiol Biochem 2008; 22: 757-68.
[278] Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 2006; 3: 177-85.
[279] Papandreou I, Cairns RA, Fontana L, Lim AL, Denko NC. HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab 2006; 3: 187-97.
[280] Semenza GL. HIF-1: upstream and downstream of cancer metabolism. Curr Opin Genet Dev 2010; 20: 51-6.

[281] Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 2008; 8: 705-13.
[282] Guzy RD, Schumacker PT. Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. Exp Physiol 2006; 91: 807-19.
[283] Gerald D, Berra E, Frapart YM, *et al*. JunD reduces tumor angiogenesis by protecting cells from oxidative stress. Cell 2004; 118: 781-94.
[284] Yasinska IM, Sumbayev VV. S-nitrosation of Cys-800 of HIF-1alpha protein activates its interaction with p300 and stimulates its transcriptional activity. FEBS Lett 2003; 549: 105-9.
[285] Halliwell B. Oxidative stress and cancer: have we moved forward? Biochemical Journal 2007; 401: 1-11.
[286] Sena LA, Chandel NS. Physiological roles of mitochondrial reactive oxygen species. Mol Cell 2012; 48: 158-67.
[287] Burdon RH. Superoxide and hydrogen peroxide in relation to mammalian cell proliferation. Free Radic Biol Med 1995; 18: 775-94.
[288] Kamata H, Hirata H. Redox regulation of cellular signalling. Cellular Signalling 1999; 11: 1-14.
[289] Obbink DJ, Spithill TW, Maxwell RJ, Linnane AW. Biogenesis of mitochondria 48: mikamycin resistance in Saccharomyces cerevisiae--a mitochondrial mutation conferring resistance to an antimycin A-like contaminant in mikamycin. Mol Gen Genet 1977; 151: 127-36.
[290] Harkness TA, Nargang FE, van der Klei I, Neupert W, Lill R. A crucial role of the mitochondrial protein import receptor MOM19 for the biogenesis of mitochondria. J Cell Biol 1994; 124: 637-48.
[291] Sanders LM, Henderson CE, Hong MY, *et al*. An increase in reactive oxygen species by dietary fish oil coupled with the attenuation of antioxidant defenses by dietary pectin enhances rat colonocyte apoptosis. J Nutr 2004; 134: 3233-8.
[292] Janssen-Heininger YM, Mossman BT, Heintz NH, *et al*. Redox-based regulation of signal transduction: principles, pitfalls, and promises. Free Radic Biol Med 2008; 45: 1-17.
[293] Paget MS, Buttner MJ. Thiol-based regulatory switches. Annu Rev Genet 2003; 37: 91-121.
[294] Chiarugi P, Cirri P. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. Trends Biochem Sci 2003; 28: 509-14.
[295] Ferlay J, Autier P, Boniol M, Heanue M, Colombet M, Boyle P. Estimates of the cancer incidence and mortality in Europe in 2006. Ann Oncol 2007; 18: 581-92.
[296] Youlden DR, Cramb SM, Dunn NA, Muller JM, Pyke CM, Baade PD. The descriptive epidemiology of female breast cancer: an international comparison of screening, incidence, survival and mortality. Cancer Epidemiol 2012; 36: 237-48.
[297] Clemons M, Goss P. Estrogen and the risk of breast cancer. N Engl J Med 2001; 344: 276-85.
[298] Henderson BE, Ross R, Bernstein L. Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. Cancer Res 1988; 48: 246-53.
[299] Roy D, Palangat M, Chen CW, *et al*. Biochemical and molecular changes at the cellular level in response to exposure to environmental estrogen-like chemicals. J Toxicol Environ Health 1997; 50: 1-29.
[300] Roy D, Cai Q, Felty Q, Narayan S. Estrogen-induced generation of reactive oxygen and nitrogen species, gene damage, and estrogen-dependent cancers. J Toxicol Environ Health B Crit Rev 2007; 10: 235-57.
[301] Okoh V, Deoraj A, Roy D. Estrogen-induced reactive oxygen species-mediated signalings contribute to breast cancer. Biochim Biophys Acta 2011; 1815: 115-33.
[302] Bernstein L, Ross RK. Endogenous hormones and breast cancer risk. Epidemiol Rev 1993; 15: 48-65.
[303] Kelsey JL, Gammon MD, John EM. Reproductive factors and breast cancer. Epidemiol Rev 1993; 15: 36-47.
[304] Key TJ, Verkasalo PK, Banks E. Epidemiology of breast cancer. Lancet Oncol 2001; 2: 133-40.
[305] Xu WH, Xiang YB, Ruan ZX, *et al*. Menstrual and reproductive factors and endometrial cancer risk: Results from a population-based case-control study in urban Shanghai. Int J Cancer 2004; 108: 613-9.
[306] Santen RJ, Brodie H, Simpson ER, Siiteri PK, Brodie A. History of aromatase: saga of an important biological mediator and therapeutic target. Endocr Rev 2009; 30: 343-75.
[307] Katzenellenbogen BS. Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol Reprod 1996; 54: 287-93.
[308] Zhao C, Dahlman-Wright K, Gustafsson JA. Estrogen signaling via estrogen receptor {beta}. J Biol Chem 2010; 285: 39575-9.
[309] Warner M, Gustafsson JA. The role of estrogen receptor beta (ER-beta) in malignant diseases--a new potential target for antiproliferative drugs in prevention and treatment of cancer. Biochem Biophys Res Commun 2010; 396: 63-6.
[310] Heldring N, Pike A, Andersson S, *et al*. Estrogen receptors: how do they signal and what are their targets. Physiol Rev 2007; 87: 905-31.
[311] Kuiper GG, Carlsson B, Grandien K, *et al*. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 1997; 138: 863-70.
[312] Kuiper GG, Lemmen JG, Carlsson B, *et al*. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 1998; 139: 4252-63.
[313] Morani A, Warner M, Gustafsson JA. Biological functions and clinical implications of oestrogen receptors alfa and beta in epithelial tissues. J Intern Med 2008; 264: 128-42.
[314] Saji S, Jensen EV, Nilsson S, Rylander T, Warner M, Gustafsson JA. Estrogen receptors alpha and beta in the rodent mammary gland. Proc Natl Acad Sci U S A 2000; 97: 337-42.
[315] Saville B, Wormke M, Wang F, *et al*. Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J Biol Chem 2000; 275: 5379-87.
[316] Cheng G, Weihua Z, Warner M, Gustafsson JA. Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. Proc Natl Acad Sci U S A 2004; 101: 3739-46.
[317] Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci U S A 2004; 101: 1566-71.
[318] Clarke RB. Human breast cell proliferation and its relationship to steroid receptor expression. Climacteric 2004; 7: 129-37.
[319] Sugiyama N, Barros RP, Warner M, Gustafsson JA. ERbeta: recent understanding of estrogen signaling. Trends Endocrinol Metab 2010; 21: 545-52.
[320] Pearce ST, Jordan VC. The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol 2004; 50: 3-22.
[321] Chang EC, Charn TH, Park SH, *et al*. Estrogen Receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding. Mol Endocrinol 2008; 22: 1032-43.
[322] Jensen EV, Jacobson HI, Walf AA, Frye CA. Estrogen action: a historic perspective on the implications of considering alternative approaches. Physiol Behav 2010; 99: 151-62.
[323] Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM. Functional domains of the human glucocorticoid receptor. Cell 1986; 46: 645-52.
[324] Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P. Functional domains of the human estrogen receptor. Cell 1987; 51: 941-51.
[325] Nilsson S, Makela S, Treuter E, *et al*. Mechanisms of estrogen action. Physiol Rev 2001; 81: 1535-65.
[326] Leung YK, Lee MT, Lam HM, Tarapore P, Ho SM. Estrogen receptor-beta and breast cancer: translating biology into clinical practice. Steroids 2012; 77: 727-37.
[327] Smith CL, Nawaz Z, O'Malley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 1997; 11: 657-66.
[328] Webb P, Nguyen P, Kushner PJ. Differential SERM effects on corepressor binding dictate ERalpha activity *in vivo*. J Biol Chem 2003; 278: 6912-20.
[329] Garcia-Roves P, Huss JM, Han DH, *et al*. Raising plasma fatty acid concentration induces increased biogenesis of mitochondria in skeletal muscle. Proc Natl Acad Sci U S A 2007; 104: 10709-13.
[330] Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS. Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: distinct and common target genes for these receptors. Endocrinology 2004; 145: 3473-86.
[331] Adam AC, Bornhovd C, Prokisch H, Neupert W, Hell K. The Nfs1 interacting protein Isd11 has an essential role in Fe/S cluster biogenesis in mitochondria. EMBO J 2006; 25: 174-83.

[332] Power KA, Thompson LU. Ligand-induced regulation of ERalpha and ERbeta is indicative of human breast cancer cell proliferation. Breast Cancer Res Treat 2003; 81: 209-21.

[333] Sastre-Serra J, Nadal-Serrano M, Gabriel Pons D, *et al*. The oxidative stress in breast tumors of postmenopausal women is ERalpha/ERbeta Ratio dependent. Free Radic Biol Med 2013; 61: 11-7.

[334] Sotoca AM, Ratman D, van der Saag P, *et al*. Phytoestrogen-mediated inhibition of proliferation of the human T47D breast cancer cells depends on the ERalpha/ERbeta ratio. J Steroid Biochem Mol Biol 2008; 112: 171-8.

[335] Quaedackers ME, Van Den Brink CE, Wissink S, *et al*. 4-hydroxytamoxifen trans-represses nuclear factor-kappa B activity in human osteoblastic U2-OS cells through estrogen receptor (ER)alpha, and not through ER beta. Endocrinology 2001; 142: 1156-66.

[336] Lee YR, Park J, Yu HN, Kim JS, Youn HJ, Jung SH. Up-regulation of PI3K/Akt signaling by 17beta-estradiol through activation of estrogen receptor-alpha, but not estrogen receptor-beta, and stimulates cell growth in breast cancer cells. Biochem Biophys Res Commun 2005; 336: 1221-6.

[337] Sogl B, Gellissen G, Wiesner RJ. Biogenesis of giant mitochondria during insect flight muscle development in the locust, Locusta migratoria (L.). Transcription, translation and copy number of mitochondrial DNA. Eur J Biochem 2000; 267: 11-7.

[338] Amutha B, Gordon DM, Dancis A, Pain D. Chapter 14 Nucleotide-dependent iron-sulfur cluster biogenesis of endogenous and imported apoproteins in isolated intact mitochondria. Methods Enzymol 2009; 456: 247-66.

[339] Usmanova N, Tomilin N, Zhivotovsky B, Kropotov A. Transcription factor GABP/NRF-2 controlling biogenesis of mitochondria regulates basal expression of peroxiredoxin V but the mitochondrial function of peroxiredoxin V is dispensable in the dog. Biochimie 2011; 93: 306-13.

[340] Pedram A, Razandi M, Wallace DC, Levin ER. Functional estrogen receptors in the mitochondria of breast cancer cells. Mol Biol Cell 2006; 17: 2125-37.

[341] Russo J, Hasan Lareef M, Balogh G, Guo S, Russo IH. Estrogen and its metabolites are carcinogenic agents in human breast epithelial cells. J Steroid Biochem Mol Biol 2003; 87: 1-25.

[342] Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med 2006; 354: 270-82.

[343] Valle A, Catala-Niell A, Colom B, Garcia-Palmer FJ, Oliver J, Roca P. Sex-related differences in energy balance in response to caloric restriction. Am J Physiol Endocrinol Metab 2005; 289: E15-22.

[344] Colom B, Oliver J, Roca P, Garcia-Palmer FJ. Caloric restriction and gender modulate cardiac muscle mitochondrial H2O2 production and oxidative damage. Cardiovasc Res 2007; 74: 456-65.

[345] Colom B, Alcolea MP, Valle A, Oliver J, Roca P, Garcia-Palmer FJ. Skeletal muscle of female rats exhibit higher mitochondrial mass and oxidative-phosphorylative capacities compared to males. Cell Physiol Biochem 2007; 19: 205-12.

[346] Valle A, Guevara R, Garcia-Palmer FJ, Roca P, Oliver J. Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions. Am J Physiol Cell Physiol 2007; 293: C1302-8.

[347] Guevara R, Santandreu FM, Valle A, Gianotti M, Oliver J, Roca P. Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress. Free Radic Biol Med 2009.

[348] Valle A, Guevara R, Garcia-Palmer FJ, Roca P, Oliver J. Caloric restriction retards the age-related decline in mitochondrial function of brown adipose tissue. Rejuvenation Res 2008; 11: 597-604.

[349] Nadal-Serrano M, Sastre-Serra J, Pons DG, Miro AM, Oliver J, Roca P. The ERalpha/ERbeta ratio determines oxidative stress in breast cancer cell lines in response to 17beta-estradiol. J Cell Biochem 2012; 113: 3178-85.

[350] Sastre-Serra J, Nadal-Serrano M, Pons DG, Valle A, Oliver J, Roca P. The effects of 17beta-estradiol on mitochondrial biogenesis and function in breast cancer cell lines are dependent on the ERalpha/ERbeta ratio. Cell Physiol Biochem 2012; 29: 261-8.

[351] Houstek J, Kopecky J, Baudysova M, Janikova D, Pavelka S, Klement P. Differentiation of brown adipose tissue and biogenesis of thermogenic mitochondria in situ and in cell culture. Biochim Biophys Acta 1990; 1018: 243-7.

[352] Adlercreutz H. Western diet and Western diseases: some hormonal and biochemical mechanisms and associations. Scand J Clin Lab Invest Suppl 1990; 201: 3-23.

[353] Sastre-Serra J, Valle A, Company MM, Garau I, Oliver J, Roca P. Estrogen down-regulates uncoupling proteins and increases oxidative stress in breast cancer. Free Radic Biol Med 2010; 48: 506-12.

[354] Folman Y, Pope GS. The interaction in the immature mouse of potent oestrogens with coumestrol, genistein and other uterovaginotrophic compounds of low potency. J Endocrinol 1966; 34: 215-25.

[355] Folman Y, Pope GS. Effect of norethisterone acetate, dimethylstilboestrol, genistein and coumestrol on uptake of [3H]oestradiol by uterus, vagina and skeletal muscle of immature mice. J Endocrinol 1969; 44: 213-8.

[356] An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N, Leitman DC. Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. J Biol Chem 2001; 276: 17808-14.

[357] Kostelac D, Rechkemmer G, Briviba K. Phytoestrogens modulate binding response of estrogen receptors alpha and beta to the estrogen response element. J Agric Food Chem 2003; 51: 7632-5.

[358] Matsumura A, Ghosh A, Pope GS, Darbre PD. Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells. J Steroid Biochem Mol Biol 2005; 94: 431-43.

[359] Hsieh CY, Santell RC, Haslam SZ, Helferich WG. Estrogenic effects of genistein on the growth of estrogen receptor-positive human breast cancer (MCF-7) cells *in vitro* and *in vivo*. Cancer Res 1998; 58: 3833-8.

[360] Ju YH, Allred CD, Allred KF, Karko KL, Doerge DR, Helferich WG. Physiological concentrations of dietary genistein dose-dependently stimulate growth of estrogen-dependent human breast cancer (MCF-7) tumors implanted in athymic nude mice. J Nutr 2001; 131: 2957-62.

[361] Seo HS, DeNardo DG, Jacquot Y, *et al*. Stimulatory effect of genistein and apigenin on the growth of breast cancer cells correlates with their ability to activate ER alpha. Breast Cancer Res Treat 2006; 99: 121-34.

[362] Maggiolini M, Bonofiglio D, Marsico S, *et al*. Estrogen receptor alpha mediates the proliferative but not the cytotoxic dose-dependent effects of two major phytoestrogens on human breast cancer cells. Mol Pharmacol 2001; 60: 595-602.

[363] Kroemer G. Mitochondria in cancer. Oncogene 2006; 25: 4630-2.

[364] Dixon-Shanies D, Shaikh N. Growth inhibition of human breast cancer cells by herbs and phytoestrogens. Oncol Rep 1999; 6: 1383-7.

[365] Magee PJ, McGlynn H, Rowland IR. Differential effects of isoflavones and lignans on invasiveness of MDA-MB-231 breast cancer cells *in vitro*. Cancer Lett 2004; 208: 35-41.

[366] Gobec S, Brozic P, Rizner TL. Nonsteroidal anti-inflammatory drugs and their analogues as inhibitors of aldo-keto reductase AKR1C3: new lead compounds for the development of anticancer agents. Bioorg Med Chem Lett 2005; 15: 5170-5.

[367] Skarydova L, Zivna L, Xiong G, Maser E, Wsol V. AKR1C3 as a potential target for the inhibitory effect of dietary flavonoids. Chem Biol Interact 2009; 178: 138-44.

[368] Schmidt S, Michna H, Diel P. Combinatory effects of phytoestrogens and 17beta-estradiol on proliferation and apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 2005; 94: 445-9.

[369] Limer JL, Parkes AT, Speirs V. Differential response to phytoestrogens in endocrine sensitive and resistant breast cancer cells *in vitro*. Int J Cancer 2006; 119: 515-21.

[370] Diel P, Olff S, Schmidt S, Michna H. Molecular identification of potential selective estrogen receptor modulator (SERM) like properties of phytoestrogens in the human breast cancer cell line MCF-7. Planta Med 2001; 67: 510-4.

[371] Makela S, Davis VL, Tally WC, *et al*. Dietary Estrogens Act through Estrogen Receptor-Mediated Processes and Show No Antiestrogenicity in Cultured Breast Cancer Cells. Environ Health Perspect 1994; 102: 572-8.

[372] Wang C, Kurzer MS. Phytoestrogen concentration determines effects on DNA synthesis in human breast cancer cells. Nutr Cancer 1997; 28: 236-47.

[373] Wang LM, Xie KP, Huo HN, Shang F, Zou W, Xie MJ. Luteolin inhibits proliferation induced by IGF-1 pathway dependent ERalpha in human breast cancer MCF-7 cells. Asian Pac J Cancer Prev 2012; 13: 1431-7.

[374] Weldon CB, McKee A, Collins-Burow BM, *et al.* PKC-mediated survival signaling in breast carcinoma cells: a role for MEK1-AP1 signaling. Int J Oncol 2005; 26: 763-8.

[375] Jeon YW, Suh YJ. Synergistic apoptotic effect of celecoxib and luteolin on breast cancer cells. Oncol Rep 2013; 29: 819-25.

[376] van Meeuwen JA, Korthagen N, de Jong PC, Piersma AH, van den Berg M. (Anti)estrogenic effects of phytochemicals on human primary mammary fibroblasts, MCF-7 cells and their co-culture. Toxicol Appl Pharmacol 2007; 221: 372-83.

[377] Li F, Ye L, Lin SM, Leung LK. Dietary flavones and flavonones display differential effects on aromatase (CYP19) transcription in the breast cancer cells MCF-7. Mol Cell Endocrinol 2011; 344: 51-8.

[378] Chen T, Li LP, Lu XY, Jiang HD, Zeng S. Absorption and excretion of luteolin and apigenin in rats after oral administration of Chrysanthemum morifolium extract. J Agric Food Chem 2007; 55: 273-7.

[379] Brusselmans K, Vrolix R, Verhoeven G, Swinnen JV. Induction of cancer cell apoptosis by flavonoids is associated with their ability to inhibit fatty acid synthase activity. J Biol Chem 2005; 280: 5636-45.

[380] Lee EJ, Oh SY, Sung MK. Luteolin exerts anti-tumor activity through the suppression of epidermal growth factor receptor-mediated pathway in MDA-MB-231 ER-negative breast cancer cells. Food Chem Toxicol 2012; 50: 4136-43.

[381] Yin F, Giuliano AE, Law RE, Van Herle AJ. Apigenin inhibits growth and induces G2/M arrest by modulating cyclin-CDK regulators and ERK MAP kinase activation in breast carcinoma cells. Anticancer Res 2001; 21: 413-20.

[382] Han DH, Denison MS, Tachibana H, Yamada K. Relationship between estrogen receptor-binding and estrogenic activities of environmental estrogens and suppression by flavonoids. Biosci Biotechnol Biochem 2002; 66: 1479-87.

[383] Shanle EK, Hawse JR, Xu W. Generation of stable reporter breast cancer cell lines for the identification of ER subtype selective ligands. Biochem Pharmacol 2011; 82: 1940-9.

[384] Morrissey C, O'Neill A, Spengler B, Christoffel V, Fitzpatrick JM, Watson RW. Apigenin drives the production of reactive oxygen species and initiates a mitochondrial mediated cell death pathway in prostate epithelial cells. Prostate 2005; 63: 131-42.

[385] Rocha A, Wang L, Penichet M, Martins-Green M. Pomegranate juice and specific components inhibit cell and molecular processes critical for metastasis of breast cancer. Breast Cancer Res Treat 2012; 136: 647-58.

[386] Lindenmeyer F, Li H, Menashi S, Soria C, Lu H. Apigenin acts on the tumor cell invasion process and regulates protease production. Nutr Cancer 2001; 39: 139-47.

[387] Attoub S, Hassan AH, Vanhoecke B, *et al.* Inhibition of cell survival, invasion, tumor growth and histone deacetylase activity by the dietary flavonoid luteolin in human epithelioid cancer cells. Eur J Pharmacol 2011; 651: 18-25.

[388] Samy RP, Gopalakrishnakone P, Ignacimuthu S. Anti-tumor promoting potential of luteolin against 7,12-dimethylbenz(a) anthracene-induced mammary tumors in rats. Chem Biol Interact 2006; 164: 1-14.

[389] Huang YT, Hwang JJ, Lee PP, *et al.* Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor. Br J Pharmacol 1999; 128: 999-1010.

[390] Smith JA, Poteet-Smith CE, Xu Y, Errington TM, Hecht SM, Lannigan DA. Identification of the first specific inhibitor of p90 ribosomal S6 kinase (RSK) reveals an unexpected role for RSK in cancer cell proliferation. Cancer Res 2005; 65: 1027-34.

[391] van der Woude H, Ter Veld MG, Jacobs N, van der Saag PT, Murk AJ, Rietjens IM. The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor. Mol Nutr Food Res 2005; 49: 763-71.

[392] Kim BW, Lee ER, Min HM, *et al.* Sustained ERK activation is involved in the kaempferol-induced apoptosis of breast cancer cells and is more evident under 3-D culture condition. Cancer Biol Ther 2008; 7: 1080-9.

[393] Le Marchand L. Cancer preventive effects of flavonoids--a review. Biomed Pharmacother 2002; 56: 296-301.

[394] Chou CC, Yang JS, Lu HF, *et al.* Quercetin-mediated cell cycle arrest and apoptosis involving activation of a caspase cascade through the mitochondrial pathway in human breast cancer MCF-7 cells. Arch Pharm Res 2010; 33: 1181-91.

[395] Avila MA, Velasco JA, Cansado J, Notario V. Quercetin mediates the down-regulation of mutant p53 in the human breast cancer cell line MDA-MB468. Cancer Res 1994; 54: 2424-8.

[396] Kang GY, Lee ER, Kim JH, *et al.* Downregulation of PLK-1 expression in kaempferol-induced apoptosis of MCF-7 cells. Eur J Pharmacol 2009; 611: 17-21.

[397] Jeong JH, An JY, Kwon YT, Rhee JG, Lee YJ. Effects of low dose quercetin: cancer cell-specific inhibition of cell cycle progression. J Cell Biochem 2009; 106: 73-82.

[398] van der Woude H, Gliszczynska-Swiglo A, Struijs K, Smeets A, Alink GM, Rietjens IM. Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans. Cancer Lett 2003; 200: 41-7.

[399] Phromnoi K, Yodkeeree S, Anuchapreeda S, Limtrakul P. Inhibition of MMP-3 activity and invasion of the MDA-MB-231 human invasive breast carcinoma cell line by bioflavonoids. Acta Pharmacol Sin 2009; 30: 1169-76.

[400] Lin CW, Hou WC, Shen SC, *et al.* Quercetin inhibition of tumor invasion via suppressing PKC delta/ERK/AP-1-dependent matrix metalloproteinase-9 activation in breast carcinoma cells. Carcinogenesis 2008; 29: 1807-15.

[401] Devipriya S, Ganapathy V, Shyamaladevi CS. Suppression of tumor growth and invasion in 9,10 dimethyl benz(a) anthracene induced mammary carcinoma by the plant bioflavonoid quercetin. Chem Biol Interact 2006; 162: 106-13.

[402] Luo H, Rankin GO, Liu L, Daddysman MK, Jiang BH, Chen YC. Kaempferol inhibits angiogenesis and VEGF expression through both HIF dependent and independent pathways in human ovarian cancer cells. Nutr Cancer 2009; 61: 554-63.

[403] Mendoza EE, Burd R. Quercetin as a systemic chemopreventative agent: structural and functional mechanisms. Mini Rev Med Chem 2011; 11: 1216-21.

[404] Chung S, Yao H, Caito S, Hwang JW, Arunachalam G, Rahman I. Regulation of SIRT1 in cellular functions: role of polyphenols. Arch Biochem Biophys 2010; 501: 79-90.

[405] Hong KS, Park JI, Kim MJ, *et al.* Involvement of SIRT1 in hypoxic down-regulation of c-Myc and beta-catenin and hypoxic preconditioning effect of polyphenols. Toxicol Appl Pharmacol 2012; 259: 210-8.

[406] Marfe G, Tafani M, Indelicato M, *et al.* Kaempferol induces apoptosis in two different cell lines via Akt inactivation, Bax and SIRT3 activation, and mitochondrial dysfunction. J Cell Biochem 2009; 106: 643-50.

[407] Miranda CL, Stevens JF, Helmrich A, *et al.* Antiproliferative and cytotoxic effects of prenylated flavonoids from hops (Humulus lupulus) in human cancer cell lines. Food Chem Toxicol 1999; 37: 271-85.

[408] Brunelli E, Minassi A, Appendino G, Moro L. 8-Prenylnaringenin, inhibits estrogen receptor-alpha mediated cell growth and induces apoptosis in MCF-7 breast cancer cells. J Steroid Biochem Mol Biol 2007; 107: 140-8.

[409] Totta P, Acconcia F, Leone S, Cardillo I, Marino M. Mechanisms of naringenin-induced apoptotic cascade in cancer cells: involvement of estrogen receptor alpha and beta signalling. IUBMB Life 2004; 56: 491-9.

[410] Galluzzo P, Ascenzi P, Bulzomi P, Marino M. The nutritional flavanone naringenin triggers antiestrogenic effects by regulating estrogen receptor alpha-palmitoylation. Endocrinology 2008; 149: 2567-75.

[411] Galluzzo P, Martini C, Bulzomi P, *et al.* Quercetin-induced apoptotic cascade in cancer cells: antioxidant versus estrogen receptor alpha-dependent mechanisms. Mol Nutr Food Res 2009; 53: 699-708.

[412] Bulzomi P, Bolli A, Galluzzo P, Acconcia F, Ascenzi P, Marino M. The naringenin-induced proapoptotic effect in breast cancer cell lines holds out against a high bisphenol a background. IUBMB Life 2012; 64: 690-6.

[413] Monteiro R, Becker H, Azevedo I, Calhau C. Effect of hop (Humulus lupulus L.) flavonoids on aromatase (estrogen synthase) activity. J Agric Food Chem 2006; 54: 2938-43.

[414] Blanquer-Rosselló MM, Oliver J, Valle A, Roca P. Effect of xanthohumol and 8-prenylnaringenin on MCF-7 breast cancer cells oxidative stress and mitochondrial complexes expression. Submitted manuscript 2013; 114: 2785-94.

[415] Monteiro R, Calhau C, Silva AO, *et al*. Xanthohumol inhibits inflammatory factor production and angiogenesis in breast cancer xenografts. J Cell Biochem 2008; 104: 1699-707.
[416] Pinto C, Duque AL, Rodriguez-Galdon B, Cestero JJ, Macias P. Xanthohumol prevents carbon tetrachloride-induced acute liver injury in rats. Food Chem Toxicol 2012; 50: 3405-12.
[417] Strathmann J, Klimo K, Sauer SW, Okun JG, Prehn JH, Gerhauser C. Xanthohumol-induced transient superoxide anion radical formation triggers cancer cells into apoptosis via a mitochondria-mediated mechanism. FASEB J 2010; 24: 2938-50.
[418] Power KA, Saarinen NM, Chen JM, Thompson LU. Mammalian lignans enterolactone and enterodiol, alone and in combination with the isoflavone genistein, do not promote the growth of MCF-7 xenografts in ovariectomized athymic nude mice. Int J Cancer 2006; 118: 1316-20.
[419] Saarinen NM, Warri A, Airio M, Smeds A, Makela S. Role of dietary lignans in the reduction of breast cancer risk. Mol Nutr Food Res 2007; 51: 857-66.
[420] McCann SE, Muti P, Vito D, Edge SB, Trevisan M, Freudenheim JL. Dietary lignan intakes and risk of pre- and postmenopausal breast cancer. Int J Cancer 2004; 111: 440-3.
[421] Zhou JR, Gugger ET, Tanaka T, Guo Y, Blackburn GL, Clinton SK. Soybean phytochemicals inhibit the growth of transplantable human prostate carcinoma and tumor angiogenesis in mice. J Nutr 1999; 129: 1628-35.
[422] Pereira MA, Grubbs CJ, Barnes LH, *et al*. Effects of the phytochemicals, curcumin and quercetin, upon azoxymethane-induced colon cancer and 7,12-dimethylbenz[a]anthracene-induced mammary cancer in rats. Carcinogenesis 1996; 17: 1305-11.
[423] Yoo JH, Lee HJ, Kang K, *et al*. Lignans inhibit cell growth via regulation of Wnt/beta-catenin signaling. Food Chem Toxicol 2010; 48: 2247-52.
[424] Chakraborty A, Gupta N, Ghosh K, Roy P. *In vitro* evaluation of the cytotoxic, anti-proliferative and anti-oxidant properties of pterostilbene isolated from Pterocarpus marsupium. Toxicol *In vitro* 2010; 24: 1215-28.
[425] Macickova T, Pecivova J, Harmatha J, Svitekova K, Nosal R. Effect of stilbene derivative on superoxide generation and enzyme release from human neutrophils *in vitro*. Interdiscip Toxicol 2012; 5: 71-5.
[426] Chiou YS, Tsai ML, Nagabhushanam K, *et al*. Pterostilbene is more potent than resveratrol in preventing azoxymethane (AOM)-induced colon tumorigenesis via activation of the NF-E2-related factor 2 (Nrf2)-mediated antioxidant signaling pathway. J Agric Food Chem 2011; 59: 2725-33.
[427] Bhat KP, Lantvit D, Christov K, Mehta RG, Moon RC, Pezzuto JM. Estrogenic and antiestrogenic properties of resveratrol in mammary tumor models. Cancer Res 2001; 61: 7456-63.
[428] Azios NG, Dharmawardhane SF. Resveratrol and estradiol exert disparate effects on cell migration, cell surface actin structures, and focal adhesion assembly in MDA-MB-231 human breast cancer cells. Neoplasia 2005; 7: 128-40.
[429] Le Corre L, Chalabi N, Delort L, Bignon YJ, Bernard-Gallon DJ. Resveratrol and breast cancer chemoprevention: molecular mechanisms. Mol Nutr Food Res 2005; 49: 462-71.
[430] Harper CE, Patel BB, Wang J, Arabshahi A, Eltoum IA, Lamartiniere CA. Resveratrol suppresses prostate cancer progression in transgenic mice. Carcinogenesis 2007; 28: 1946-53.
[431] Wong DH, Villanueva JA, Cress AB, Duleba AJ. Effects of resveratrol on proliferation and apoptosis in rat ovarian theca-interstitial cells. Mol Hum Reprod 2010; 16: 251-9.
[432] Lu R, Serrero G. Resveratrol, a natural product derived from grape, exhibits antiestrogenic activity and inhibits the growth of human breast cancer cells. J Cell Physiol 1999; 179: 297-304.
[433] McCormack D, Schneider J, McDonald D, McFadden D. The anti-proliferative effects of pterostilbene on breast cancer *in vitro* are via inhibition of constitutive and leptin-induced Janus kinase/signal transducer and activator of transcription activation. Am J Surg 2011; 202: 541-4.
[434] Mannal P, McDonald D, McFadden D. Pterostilbene and tamoxifen show an additive effect against breast cancer *in vitro*. Am J Surg 2010; 200: 577-80.
[435] Pan MH, Lin YT, Lin CL, Wei CS, Ho CT, Chen WJ. Suppression of Heregulin-beta1/HER2-Modulated Invasive and Aggressive Phenotype of Breast Carcinoma by Pterostilbene via Inhibition of Matrix Metalloproteinase-9, p38 Kinase Cascade and Akt Activation. Evid Based Complement Alternat Med 2011; 2011: 562187.
[436] Wang C, Hu ZQ, Chu M, *et al*. Resveratrol inhibited GH3 cell growth and decreased prolactin level via estrogen receptors. Clin Neurol Neurosurg 2012; 114: 241-8.
[437] Hsieh TC, Wu JM. Resveratrol: Biological and pharmaceutical properties as anticancer molecule. Biofactors 2010; 36: 360-9.
[438] Alcain FJ, Villalba JM. Sirtuin activators. Expert Opin Ther Pat 2009; 19: 403-14.

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