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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;

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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

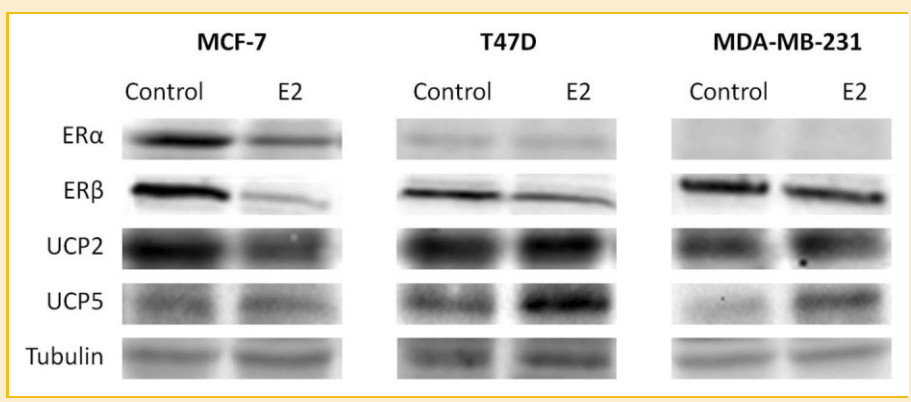

Fig. 1. Representative bands of Western blot are shown. ERα: estrogen receptor alpha; ERβ: estrogen receptor beta; UCP2: uncoupling protein 2; UCP5: uncoupling protein 5 and Tubulina as a housekeeping protein.

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.

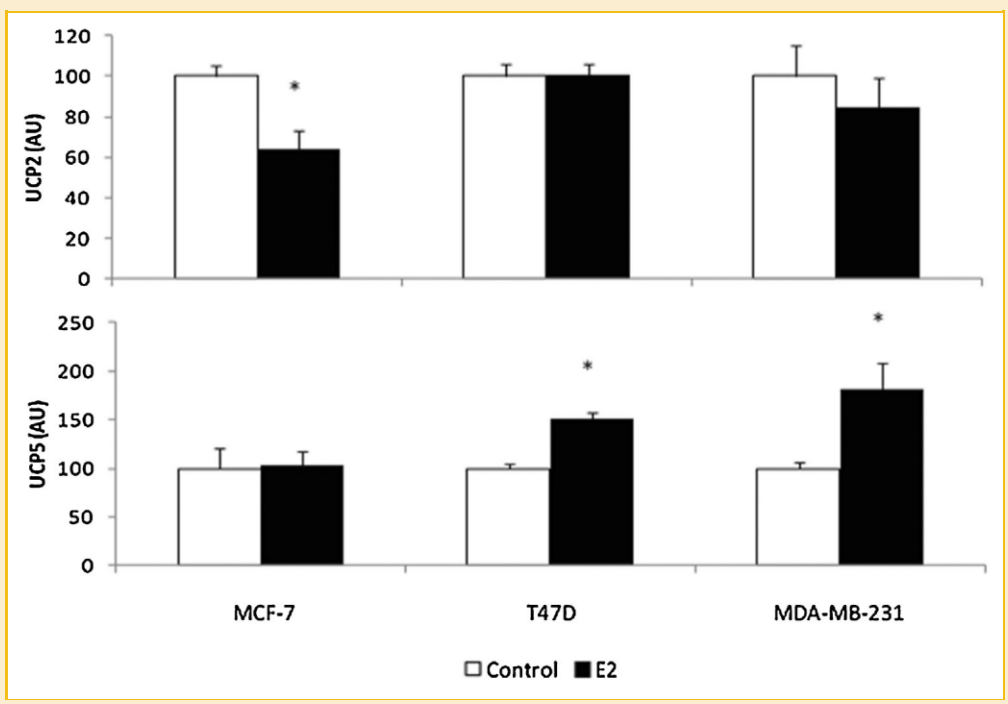

Fig. 2. UCP2 and UCP5 protein levels in response to E2-treatment in MCF-7, T47D, and MDA-MB-231 cell lines. Levels of uncoupling proteins were measured by Western blot as described in the Materials and Methods Section. AU: arbitrary units; UCP2 and UCP5: uncoupling protein 2 and uncoupling protein 5. 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.

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