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Tesis doctoral:
Doctoral thesis:

FUNCIÓN MITOCONDRIAL Y ESTRÉS OXIDATIVO EN EL ENVEJECIMIENTO DEL CEREBRO DE RATA. DIFERENCIAS ENTRE SEXOS.

MITOCHONDRIAL FUNCTION AND OXIDATIVE STRESS IN RAT BRAIN AGING. SEXUAL DIFFERENCES.

Programa de Doctorado Interuniversitario de Nutrición Humana

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Rocío Guevara de Bonis

A toda mi familia, que no es poca, presente y ausente,
porque la quiero y estoy orgullosa de ella.

To all my family which is not small, both those present and absent,
because I love you and I am proud of you.

AGRADECIMIENTOS / ***ACKNOWLEDGEMENTS***

A todas las personas que me han ayudado y apoyado,
sinceramente gracias.

To everybody who has helped and supported me,
thank you very much indeed.

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ABREVIATURAS / *ABBREVIATIONS*

ADNmt / *mtDNA*	ADN mitocondrial / *mitochondrial DNA*
ADNn / *nDNA*	ADN nuclear / *nuclear DNA*
ADP	difosfato de adenosina / a*denosine diphosphate*
ATP	trifosfato de adenosina / a*denosine triphosphate*
COX	citocromo c oxidasa / *cytochrome c oxidase*
CRM / *MRC*	cadena respiratoria mitocondrial / *mitochondrial respiratory chain*
Cu,Zn-SOD	cobre, zinc SOD / *copper, zinc SOD*
FAD	dinucleótido de flavina y adenina / *flavin adenine dinucleotide*
GDP	difosfato de guanosina / *guanosine diphosphate*
GPx	glutatión peroxidasa / *glutathione peroxidase*
GR	glutatión reductasa / *glutathione reductase*
GSH	glutatión reducido / *reduced glutathione*
GSSG	glutatión oxidado / *oxidized glutathione*
Mn-SOD	manganeso SOD / manganese SOD
NADH	dinucleótido de nicotinamida y adenina / *nicotinamide adenine dinucleotide*
P_i	Fosfato inorgánico / *inorganic phosphate*
RCR	tasa de control de la respiración / *respiratory control ratio*
ROS	radical libre de oxígeno / *reactive oxygen species*
SNC / *SNC*	sistema nervioso central / *central nervous system*
SOD	superóxido dismutasa / superoxide dismutase
UCP	proteína desacoplante / *uncoupling protein*

1. RESUMEN

ABSTRACT

RESUMEN

Durante el proceso de envejecimiento se produce el declive de la función celular y mitocondrial. El cerebro tiene una elevada tasa metabólica por lo que necesita grandes cantidades de ATP generados por el metabolismo oxidativo mitocondrial. Así, la función y la supervivencia neuronal son muy sensibles a la disfunción mitocondrial que se produce con el envejecimiento.

El objetivo general de la presente tesis fue estudiar los cambios producidos en la función y el estrés oxidativo mitocondriales, así como los daños oxidativos acumulados, en el cerebro de rata en respuesta al envejecimiento, centrándonos en las diferencias de sexo.

Durante el envejecimiento se produjo un aumento en la actividad oxidativa y fosforilativa de la cadena respiratoria mitocondrial en el conjunto del cerebro, paralelamente al aumento del número de mitocondrias. Si bien, por unidad mitocondrial, estas perdieron densidad proteica y funcionalidad con el tiempo. En consecuencia aumentó la producción de ROS y los niveles de las defensas antioxidantes inducibles por estrés oxidativo, sin que se llegase a compensar el estrés oxidativo generado, dando como resultado una acumulación del daño oxidativo.

Las mitocondrias de las ratas hembra mantuvieron mejor su funcionalidad durante el envejecimiento, lo que se reflejó en un mayor aumento de la actividad de la cadena respiratoria mitocondrial a nivel tisular, con igual producción de ROS, mayores niveles de defensas antioxidantes inducibles por estrés oxidativo, entre las que se encontraba la UCP5, y como resultado, menor daño oxidativo celular.

En conclusión, los resultados obtenidos en la presente tesis demuestran una progresiva disfunción mitocondrial con la edad, causa y consecuencia del empeoramiento del balance oxidativo en el cerebro de las ratas, situación contrarrestada por el aumento del número de mitocondrias. Este declive cerebral fue menor en las ratas hembra, por lo que con el envejecimiento, el dimorfismos sexual fue aumentando gradualmente.

ABSTRACT

A decline of cell and mitochondrial functions takes place during the aging process. Brain has a very high metabolic rate, needing large amounts of ATP generated by mitochondrial oxidative metabolism. Thus, neuronal function and survival are highly sensitive to mitochondrial dysfunction caused by aging.

The general aim of the present thesis was to study both the changes occurring in mitochondrial function and oxidative stress, and the oxidative damage accumulated in rat brain owing to aging, focusing on sex differences.

During aging, oxidative and phosphorylative activities from mitochondrial respiratory chain rose in the whole brain, in line with a mitochondrial number increase. However, each of the mitochondria lost protein density and functionality with age. Consequently, ROS production and levels of antioxidant defences – those that may be up-regulated by oxidative stress – rose, although the generated oxidative stress could not be counteracted. As a result, oxidative damage accumulation took place.

Female rat mitochondria better maintained their functionality during aging, which was reflected by a greater increase of mitochondrial respiratory chain activity in the tissue, with similar ROS production, higher levels of antioxidant defences – those that may be up-regulated by oxidative stress – UCP5 among these and, as a consequence, lesser cellular oxidative damage.

In conclusion, the results obtained in the present thesis prove a progressive mitochondrial dysfunction with age, cause and consequence of oxidative balance deterioration in rat brain, which is counteracted by an increase in mitochondrial number. This brain decline was less pronounced in female rats, thus, the sexual dimorphism rose gradually throughout aging.

2. LISTADO DE PUBLICACIONES

LIST OF PUBLICATIONS

LISTADO DE PUBLICACIONES / *LIST OF PUBLICATIONS*

La presente tesis se basa en las siguientes publicaciones / *This thesis is based on the following papers:*

I. Guevara R, Santandreu FM, Valle A, Gianotti M, Oliver J, Roca P. Sex-dependent differences in old rat brain mitochondrial function and oxidative stress. Free Radical Biology and Medicine 2009 Jan 15;46(2):169-75. Epub 2008 Oct 17.

II. Guevara R, Gianotti M, Roca P, Oliver J. Age and sex-related changes in rat brain mitochondrial function. Cellular Physiology and Biochemistry; accepted for publication. 2010.

III. Guevara R, Gianotti M, Oliver J, Roca P. Age and sex-related changes in rat brain mitochondrial oxidative status. Manuscript.

Además, durante la realización de la tesis, la doctoranda ha colaborado en la realización de otros estudios relacionados con el proyecto de la tesis, que han dado lugar a las publicaciones que se presentan en el anexo. / *Moreover, during this thesis, the PhD student has also been involved in complementary projects that have led to the publication of the articles presented in the appendix.*

IV. Valle A, Guevara R, García-Palmer FJ, Roca P, Oliver J. Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions. American Journal of Physiology-Cell Physiology. 2007 Oct;293(4):C1302-8. Epub 2007 Jul 25.

V. Guevara R, Valle A, Gianotti M, Roca P, Oliver J. Gender-dependent differences in serum profiles of insulin and leptin in caloric restricted rats. Hormone and Metabolic Research. 2008 Jan;40(1):38-43. Epub 2008 Jan 7.

VI. Valle A, Guevara R, García-Palmer FJ, Roca P, Oliver J. Caloric restriction retards the age-related decline in mitochondrial function of brown adipose tissue. Rejuvenation Research 2008 Jun;1(Ames):597-604.

VII. Santandreu FM, Brell M, Gene AH, Guevara R, Oliver J, Couce ME, Roca P. Differences in mitochondrial function and antioxidant systems between regions of human glioma. Cellular Physiology and Biochemistry. 2008;22(5-6):757-68. Epub 2008 Dec 9.

VIII. Paisán-Ruiz C, Guevara R, Federoff M, Hanagasi H, Sina F, Elahe E, Schneider SA, Schwingenschuh P, Bajaj N, Emre M, Singleton AB, Hardy J, Bhatia KP, Brandner S, Lees AJ, Houlden H. Early-onset L-dopa-responsive parkinsonism with pyramidal signs due to ATP13A2, PLA2G6, FBXO7 and spatacsin mutations. Movement Disorders. 2010 Sep 15;25(12):1791-800.

3. INTRODUCCIÓN

INTRODUCTION

3.1. El envejecimiento

El envejecimiento es un proceso biológico caracterizado por un declive fisiológico generalizado y un aumento de la susceptibilidad a las enfermedades. Según la teoría del "soma desechable", los organismos destinarían los recursos metabólicos necesarios al mantenimiento del soma, con objeto de conservar el organismo en buenas condiciones físicas, mientras tengan razonables oportunidades de sobrevivir y reproducirse (Kirkwood, 1977). Dado que en la naturaleza, la muerte de los animales sobreviene generalmente por causas diferentes al envejecimiento, la selección natural podría haber permitido la selección favorable de genes pleiotrópicos, con efectos beneficiosos a edades tempranas aunque nocivos a edades tardías (Williams, 1957) o simplemente la acumulación de mutaciones con efectos deletéreos que son detectables sólo en el declive de la vida (Martin et al., 1996). Lo que sí está claro es que son muchos los genes que influyen en el proceso de envejecimiento, siendo éste la etapa final de la ontogénesis.

En el envejecimiento, se ha observado un amplio espectro de alteraciones celulares en los tejidos somáticos, especialmente en las mitocondrias y su ADN. La mitocondria es el principal orgánulo que provee a la célula de la energía, en forma de ATP, necesaria para su funcionamiento. Además, la mitocondria también juega un papel esencial en la homeostasis intracelular del Ca^{2+}, el metabolismo intermediario, las vías de señalización y regulación intracelular, la síntesis de esteroides, la generación de radicales libres de oxígeno (ROS) y la muerte celular por apoptosis. De modo que, la disfunción mitocondrial tiene efectos devastadores en la integridad de la célula y por tanto está implicada en el envejecimiento y en las enfermedades degenerativas (Kann et al., 2007).

En el envejecimiento de los organismos, la celularidad decrece debido al predominio de la apoptosis sobre la proliferación. Así, la principal diferencia entre organismos jóvenes y viejos sería dicha celularidad, es decir, el número de células funcionales en el organismo o tejido más que la calidad de estas células. (Severin et al., 2009).

3.2. La mitocondria

La membrana mitocondrial interna contiene los complejos de la cadena respiratoria mitocondrial (CRM), o cadena de transporte electrónico (Figura 1). Dichos complejos son proteínas integrales de membrana con grupos prostéticos, que funcionan como centros redox, capaces de catalizar la transferencia de electrones de un complejo a otro. El último componente es la ATP sintasa encargada de la formación del ATP.

Los complejos de la CRM, a excepción del complejo II (succinato-ubiquinona oxidorreductasa o succinato deshidrogenasa), además de su función transportadora de electrones, tienen la capacidad de translocar protones desde la matriz mitocondrial al espacio intermembranal. Dicha función puede verse anulada por inhibidores específicos de cada complejo como la rotenona, inhibidor específico del complejo I (NADH-ubiquinona oxidorreductasa o NADH deshidrogenasa), la antimicina, inhibidor específico del complejo III (ubiquinol-citocromo c oxidorreductasa), o el cianuro y el monóxido de carbono, inhibidores específicos del complejo IV (citocromo c-O_2 oxidorreductasa, citocromo c oxidasa o COX). La transferencia de electrones entre los complejos la llevan a cabo moléculas de menor tamaño y mayor movilidad como la ubiquinona lipídica coenzima Q, que la realiza desde los complejos I y II al complejo III, y el citocromo c, proteína periférica de membrana, desde el complejo III al complejo IV, aceptor final de los electrones con los que reduce el O_2 a H_2O. La ATP sintasa, también llamada complejo V, constituye el canal por el que retornan los protones a la matriz, pudiendo ser inhibida específicamente por la oligomicina (Walker et al., 1995; Boyer, 1998).

La estequiometría de los complejos de la CRM presenta una ratio molar simple, lo que implica que los transportadores y complejos de la cadena se encuentran asociados con interacciones específicas proteína-proteína, que hacen más efectiva la transferencia de electrones al limitar las distancias intermoleculares (Chance et al., 1956).

La mitocondria desempeña un papel central en el metabolismo energético ya que es el orgánulo encargado de suministrar la mayor parte de la energía necesaria para la actividad celular. La mitocondria utiliza aproximadamente el 90% del oxígeno consumido por las células en la fosforilación oxidativa, sistema mediante el cual se produce energía en forma de moléculas altamente energéticas: ATP, constituyendo la

fuente de la mayoría del ATP utilizado por la célula. La fosforilación oxidativa conlleva la oxidación de los nutrientes a CO_2 y H_2O. Acoplada a la oxidación de los sustratos, se produce la reducción del dinucleótido de nicotinamida y adenina (NADH) y del dinucleótido de flavina y adenina (FAD), convirtiéndose en los dadores iniciales de electrones, en el complejo I y el complejo II respectivamente, los cuales fluyen así a lo largo de la CRM de la membrana interna, de acuerdo al potencial de oxidación de sus componentes.

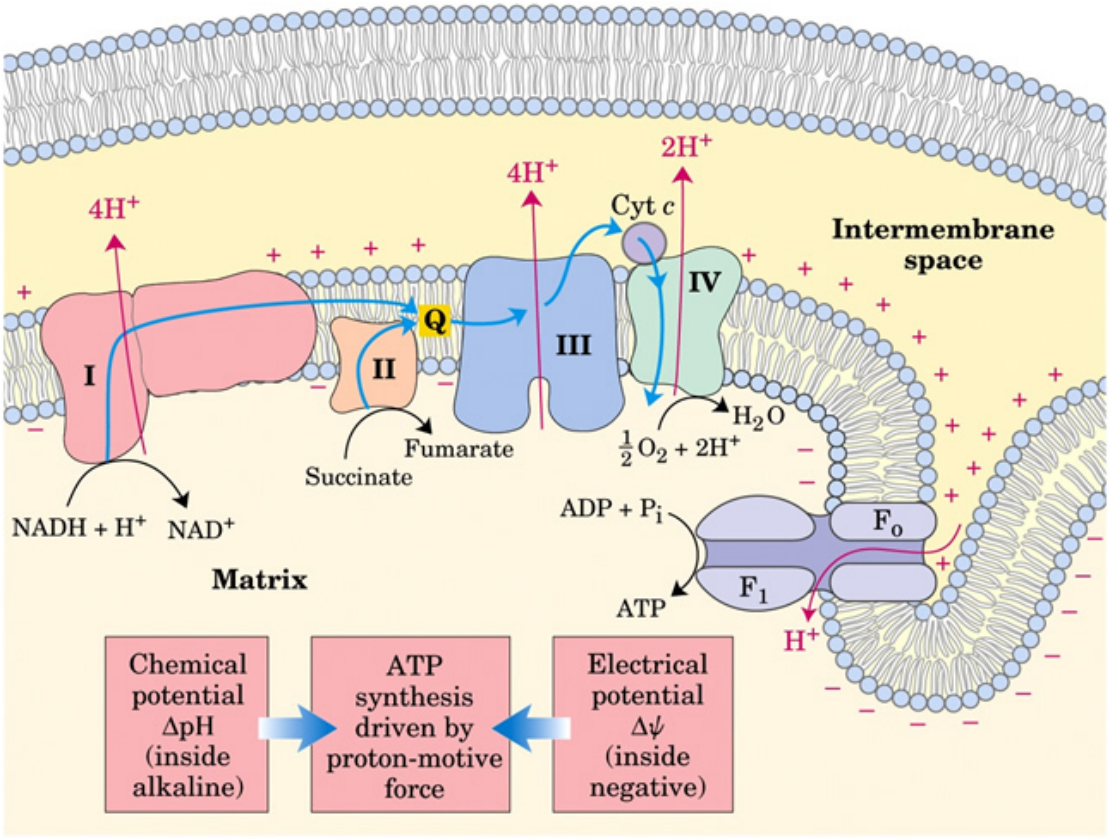

Figura 1: Cadena Respiratoria Mitocondrial (CRM). Translocación de protones a través de los complejos I, III y IV y formación de ATP en el complejo V o ATP sintasa. Q: ubiquinona o coenzima Q. Cyt c: citocromo c. Fuente: Lehninger et al.

La diferencia en el potencial redox de los transportadores de electrones define las reacciones que son suficientemente exergónicas como para proveer de la energía libre necesaria para el proceso acoplado de bombeo de protones desde la matriz. Gracias a esta translocación de protones, la matriz adquiere carga negativa y el espacio intermembranal carga positiva, dando como resultado un gradiente electroquímico, que genera una fuerza protonmotriz capaz de impulsar la síntesis de ATP, al retornar los protones a la matriz mitocondrial a través del complejo de la ATP sintasa.

La velocidad de la transferencia de electrones y de la síntesis de ATP está determinada por las necesidades energéticas de la célula. Cuando la demanda energética aumenta, se incrementa la ruptura del ATP a ADP y P_i. El aumento de disponibilidad de ADP incrementa la velocidad de la transferencia de electrones, generándose ATP. Generalmente, la capacidad de fosforilación está finamente regulada y fluctúa sólo ligeramente en la mayoría de los tejidos, si bien es cierto que la fosforilación oxidativa nunca está completamente acoplada en la mitocondria. El control de la CRM y los estados metabólicos mitocondriales fueron definidos por primera vez en 1956 (Chance et al., 1956). El Estado 4 o estado de respiración en reposo, se caracteriza por una gran disponibilidad de sustratos pero no de ADP, ocasionando una baja velocidad de transferencia de electrones, lo que conlleva una alta producción de ROS, alrededor de 1 nmol H_2O_2/min·mg de proteína. En el Estado 3 o estado de respiración activa, la mitocondria se encuentra con plena disponibilidad de sustratos y de ADP, produciéndose una alta velocidad de transferencia electrónica, alcanzando a la velocidad fisiológica máxima de producción de ATP y consumo de O_2 y mostrando una velocidad de producción de ROS muy baja en estas condiciones, alrededor de 0,1 nmol H_2O_2/min·mg de proteína (Boveris et al., 2000). La velocidad de transferencia de electrones en mitocondrias aisladas es de 3 a 8 veces más rápida en el Estado 3 que en el 4, por tanto la tasa de control de la respiración se halla en este intervalo (Chance et al., 1956). Se ha estimado que las mitocondrias de mamíferos, en condiciones fisiológicas, se encuentran principalmente en Estado 4, alrededor de un 60-70%, y sólo el 30-40% restante están en Estado 3 (Boveris et al., 1999).

3.3. La producción de ROS

Los radicales libres son especies químicas que tienen un electrón desapareado en un orbital externo, lo que les confiere una alta inestabilidad y en consecuencia reactividad, especialmente los de bajo peso molecular. Durante la transferencia de electrones mitocondrial, los principales ROS que se producen son el radical superóxido ($O_2^{\cdot -}$), el radical hidroxilo ($OH^{\cdot}$), y el peróxido de hidrógeno (H_2O_2), que si bien no es un verdadero radical libre, se considera como tal debido a su alta reactividad.

Las mitocondrias son la principal fuente de oxidantes de la célula. Aproximadamente el 1-2 % del flujo de electrones a través de la CRM tiene como resultado la generación del radical superóxido (Beckman et al., 1998), que ocurre cuando uno de los electrones es transferido al O_2 en vez de al complejo siguiente de la CRM. Las condiciones propicias para esta desviación de electrones son un elevado potencial de membrana y una baja demanda de ATP. De hecho, se ha probado que la magnitud del gradiente protónico está directamente correlacionada con la producción de ROS por la CRM (Korshunov et al., 1997). Los sitios predominantes de producción de ROS son el complejo I y el complejo III (Figura 1), aunque no son los únicos ya que, dentro de la CRM, la población de ubiquinonas podría ser también productora de ROS (Boveris et al., 1999) dado que la forma ubisemiquinona puede funcionar como reductor, tanto del oxígeno como del peróxido de hidrógeno, formándose el anión superóxido y radicales hidroxilos. Además, la membrana plasmática también es una fuente de producción de ROS, a causa de oxidasas integrales de membrana (Christman et al., 1985; Storz et al., 1990; Faulkner et al., 1993; Rhee, 1999; Thannickal et al., 2000).

3.4. El daño oxidativo

Los ROS atacan centros con alta densidad electrónica, tales como el átomo de nitrógeno y el de oxígeno, presentes en proteínas y ácidos nucleicos, y dobles enlaces de carbono, presentes en ácidos grasos poliinsaturados. Así se inicia una serie de reacciones autocatalíticas que van produciendo intermediarios reactivos, hasta que finaliza la reacción en cadena, dejando a su paso un sinfín de moléculas oxidadas.

La peroxidación lipídica comporta la formación de lipoperóxidos, que pueden reaccionar con otros lípidos y proteínas de membrana, lo que conduce a la formación de enlaces cruzados entre lípido-lípido, lípido-proteína, proteína-proteína, o también se puede producir la descomposición de estos lipoperóxidos, generándose subproductos de bajo peso molecular como el malondialdehido (Esterbauer et al., 1991). Esta oxidación provoca una disminución de la fluidez y un incremento de la permeabilidad de las membranas. Por otro lado, la oxidación de proteínas tiene como consecuencia una acumulación de grupos carbonilos cuyo resultado son enzimas funcionalmente inactivas, convirtiéndose en dianas para su degradación proteolítica (Stadtman, 1992). Por último, el ADN, tanto el nuclear como el mitocondrial, también se ve afectado por la oxidación, rindiendo derivados nucleotídicos como por ejemplo la 8-oxo-

deoxiguanosina (Ames, 1989), modificaciones que pueden generar mutaciones, que a pesar de los sistemas de reparación, van acumulándose con la edad (Stadtman, 1992).

El ADN mitocondrial es particularmente propenso a las mutaciones, estimándose que es unas 10 veces más susceptible que el ADN nuclear (Ames, 1989; Richter et al., 1997). Esta predisposición es debida a su localización cerca de la membrana interna mitocondrial, donde tiene lugar la fosforilación oxidativa, expuesto a los radicales formados por la normal respiración mitocondrial, a la ausencia de proteínas protectoras como las histonas y a la ausencia de sistemas eficientes de reparación del ADN (Barja et al., 2000). Las mutaciones del ADN mitocondrial conducen a alteraciones en el funcionamiento de la CRM. Disfunción que a su vez, altera el funcionamiento general de la mitocondria, disminuyendo la producción de ATP, alterándose la homeostasis del Ca^{2+} y aumentando la producción de ROS. Estos producirán posteriores daños, incluyendo más oxidación del ADN mitocondrial, proteínas y lípidos, volviendo a comenzar el círculo vicioso con más alteraciones en el funcionamiento de la CRM, generándose una escalada de daños que van aumentando y acumulándose con la edad, siendo la principal causa de envejecimiento y pudiendo comprometer la supervivencia de la célula a largo plazo (Halliwell, 1999).

3.5. Las defensas antioxidantes

Frente a esta producción de radicales libres, los seres vivos han ido desarrollando, a lo largo de la evolución, sistemas de defensa antioxidante (Halliwell, 1999). Estos sistemas actúan previniendo su formación o capturando y neutralizando los radicales ya formados, ya que son capaces de ceder un electrón al radical libre, quedando ellos mismos como radical pero de muy baja reactividad, incapaces de iniciar o continuar una reacción en cadena. Un antioxidante puede definirse como cualquier sustancia que, presente a bajas concentraciones comparadas con las del sustrato susceptible de oxidación, lo que incluye todo tipo de biomoléculas: glúcidos, lípidos, proteínas y ADN, retrasa significativamente o previene la oxidación de dicho sustrato (Halliwell, 1990). Cada antioxidante posee afinidad hacia un determinado tipo de radical. Tradicionalmente, las defensas antioxidantes se han dividido en dos grandes grupos: las enzimáticas y las no enzimáticas (Sies, 1997).

Las enzimas antioxidantes, sintetizadas en el organismo, protegen frente a los ROS ya producidos durante el metabolismo. Entre estos sistemas defensivos se encuentran tres importantes enzimas (Figura 2): la superóxido dismutasa (SOD), la catalasa, y la glutatión peroxidasa (GPx). La SOD cataliza la dismutación del anión superóxido, rindiendo peróxido de hidrógeno. Existen distintas formas dependiendo del metal del centro catalítico y de su ubicación: Cu,Zn-SOD citosólica, la Mn-SOD mitocondrial y la Cu,Zn-SOD extracelular (Fridovich, 1975). La catalasa es una enzima tetramérica que cataliza la descomposición del peróxido de hidrógeno en agua. Está presente en la mayoría de las células eucariotas, localizándose a nivel de los peroxisomas (Di Giulio, 1991). Por último, está la GPx, localizada tanto a nivel del citosol como de las mitocondrias (Blum et al., 1984), que también contribuye a la eliminación del peróxido de hidrógeno, pero a diferencia de la catalasa que usa el peróxido de hidrógeno como dador de electrones, ésta utiliza el glutatión reducido (GSH). Además, el sistema se completa con la glutatión reductasa (GR), que se encarga de regenerar el glutatión que ha quedado oxidado (GSSG) (Rall et al., 1952), manteniendo equilibrada la proporción GSH/GSSG.

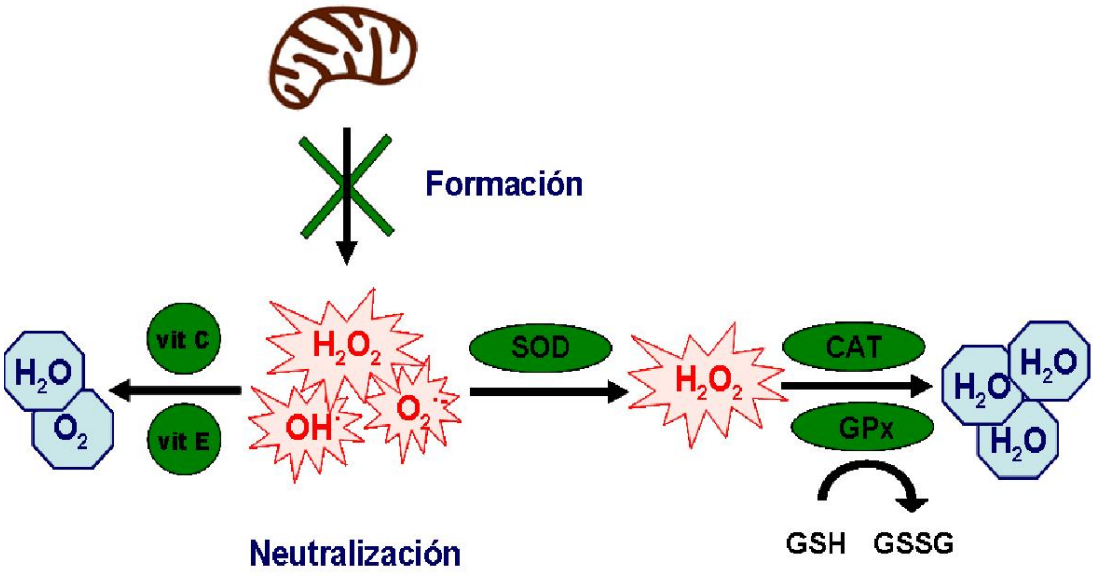

Figura 2: Formación de radicales por la mitocondria y eliminación de los mismos por los sistemas de defensa antioxidantes. vit C: vitamina C. vit E: vitamina E. SOD: superoxido dismutasa. Cat: catalasa. GPx: glutatión peroxidada. GSH: glutatión reducido. GSSG: glutatión oxidado.

Los antioxidantes no enzimáticos son sustancias capaces de neutralizar un único radical libre por molécula, es decir, son cazadores estequiométricos, por lo que sólo actúan a concentraciones elevadas, cumpliendo un papel mínimo en comparación con los antioxidantes enzimáticos. Un primer grupo de antioxidantes no enzimáticos lo

constituyen pequeñas moléculas neutralizadoras como el α-tocoferol o vitamina E (Traber et al., 1996), que captura radicales hidroxilo y aniones superóxido y neutraliza peróxidos; o el ácido ascórbico o vitamina C, que también captura radicales hidroxilo y aniones superóxidos y regenera la forma oxidada de la vitamina E (Briviba et al., 1994). En este grupo también se encuentran los carotenoides, como por ejemplo el α-caroteno o provitamina A y el glutatión (Sies et al., 1995).

Un segundo grupo de antioxidantes no enzimáticos son determinadas proteínas quelantes catiónicas, que son capaces de retener en su estructura metales de transición como el cobre y sobre todo el hierro, disminuyendo así su disponibilidad, necesaria para la formación del radical hidroxilo. Entre éstas se encuentra la ferritina, la transferrina, la lactoferrina, la metalotioneína o la ceruloplasmina (Sies, 1997).

3.6. Las proteínas desacoplantes

Las proteínas desacoplantes de la cadena respiratoria mitocondrial (UCP) podrían ser otro de los sistemas antioxidantes de prevención de formación de ROS (Negre-Salvayre et al., 1997), si bien su papel es controvertido. Reciben su nombre por el hecho de que desacoplan la cadena de transporte electrónico de la síntesis de ATP al usar el gradiente electroquímico sin síntesis de ATP, es decir, el desacoplamiento resulta de la fuga de protones que regresan a la matriz, disipando la fuerza protón motriz y reduciendo el número de protones que fluyen a través de la ATP sintasa (Figura 3). Estas proteínas pertenecen a una familia de translocadores protónicos localizados en la membrana mitocondrial interna (Pecqueur et al., 2001), compuesta por varios miembros conocidos hasta el momento UCP1-UCP5. Las UCP se expresan de forma específica en función del tejido, lo que sugiere diferentes funciones y regulación en las diferentes localizaciones. Así, la UCP1 se expresa en las células del tejido adiposo marrón (Tam et al.), siendo activada por el frío, dando como resultando el desacoplamiento mitocondrial que ocasiona la producción de calor suficiente para mantener la temperatura corporal, por lo que su función es termogénica (Pecqueur et al., 2001; Argiles et al., 2002). Las restantes UCP se hallan a niveles mucho menores en los tejidos en los que se expresan, comparadas con la UCP1 en el tejido adiposo marrón, sugiriendo que la función de estas isoformas no sería la termogénesis (Kim-Han et al., 2005). La UCP2 se expresa en múltiples tejidos, incluyendo ciertas regiones del cerebro, (Andrews et al., 2005a; Andrews et al., 2005b). La UCP3 se expresa principalmente en las células del músculo esquelético y

cardiaco (Dulloo et al., 2001). La UCP4 y la UCP5, también conocida esta última como BMCP1, se expresan principalmente en el cerebro (Sanchis et al., 1998; Mao et al., 1999), siendo la UCP4 exclusiva del cerebro (Mao et al., 1999), mientras que la UCP5 se expresa también en otros tejidos (Sanchis et al., 1998).

Se ha postulado que la función translocadora de protones de las UCP tendría como consecuencia una moderada reducción del potencial de membrana, disminuyendo así la fuga de electrones de la CRM y en consecuencia, reduciendo la producción de ROS (Negre-Salvayre et al., 1997). De hecho, se ha descrito que incluso un leve desacoplamiento reduce la producción de ROS de forma significativa (Korshunov et al., 1997; Miwa et al., 2003; Starkov et al., 2003). Una disminución del 10% del potencial de membrana resulta en una disminución del 55% en la producción de H_2O_2, mientras que un pequeño incremento en dicho potencial puede causar una importante producción de $O_2^{\cdot -}$ y H_2O_2 (Korshunov et al., 1997). Por tanto, el efecto neto del aumento del desacoplamiento causado por las UCP conllevaría una disminución del estrés oxidativo.

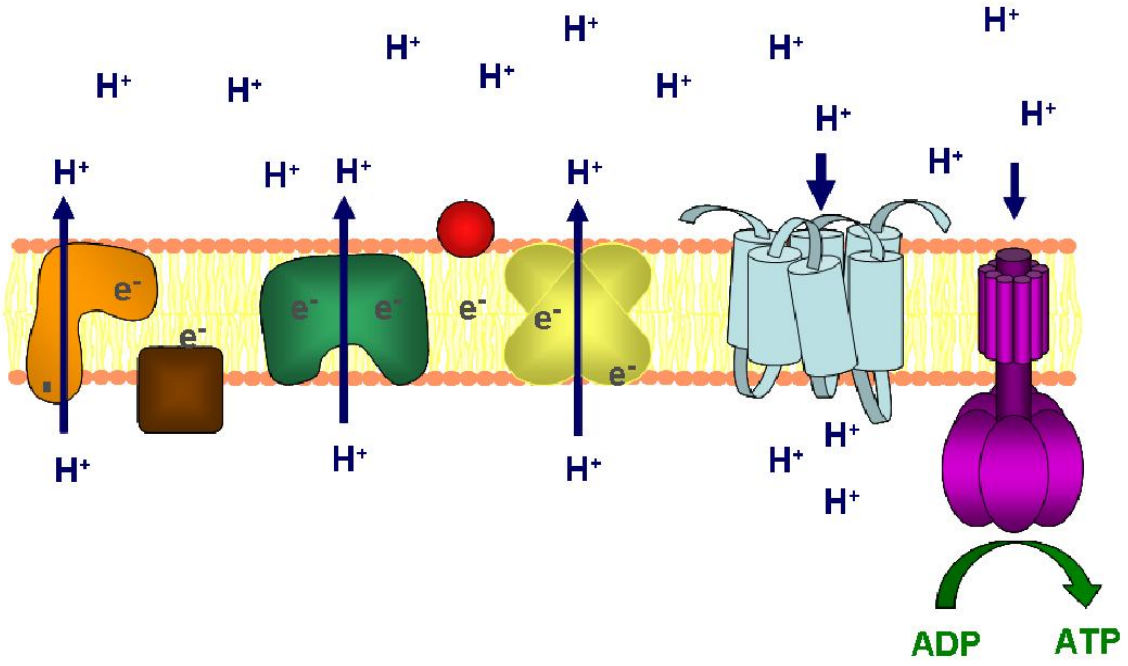

Figura 3: Cadena respiratoria mitocondrial (CRM). Translocación de protones a través de los complejos I, III y IV y retorno a la matriz de los mismos a través de las UCP.

Aunque el mecanismo detallado de la acción de las UCP en la regulación del metabolismo energético y la producción de ROS no se conoce, evidencias experimentales han demostrado que la actividad desacoplante de las UCP puede ser

activada por el estrés oxidativo (Echtay et al., 2002; Echtay et al., 2003) y por los ácidos grasos libres (Hagen et al., 2000), e inhibida por los nucleótidos de purina: GDP y ADP (Nicholls et al., 1984).

3.7. El estrés oxidativo en el envejecimiento

El estrés oxidativo es el desequilibrio existente entre la producción de radicales libres y los niveles de defensas antioxidantes (Sies, 1985). En este sentido, una situación de estrés oxidativo sería aquella en la cual se produce un aumento de la generación de ROS o una disminución de los sistemas de defensa antioxidante, o ambas circunstancias simultáneamente, desplazándose el equilibrio hacia una mayor concentración de ROS. El estrés oxidativo se ha postulado como una de las principales causas del envejecimiento y de muchas patologías degenerativas que lo acompañan como son las enfermedades neurodegenerativas (Kim-Han et al., 2001; Lindholm et al., 2004).

La teoría del envejecimiento por radicales libres fue propuesta por primera vez por Gerschmann y colaboradores. y Harman en los años 50 (Gerschman et al., 1954; Harman, 1956). En ella se postulaba que el envejecimiento y las enfermedades degenerativas asociadas a él, se debían básicamente a los ataques deletéreos de los radicales libres sobre los constituyentes celulares y el tejido conectivo. En 1972, Harman propuso que las mitocondrias podrían tener un papel central en el proceso de envejecimiento (Harman, 1972). De acuerdo con esta teoría, los radicales libres generados por el metabolismo mitocondrial podrían actuar como causa de disfunción y muerte celular.

Existen muchas evidencias que apoyan esta teoría. Por un lado, los daños oxidativos a nivel molecular en el ADN y las proteínas, aumentan exponencialmente con la edad y, concomitantemente, está incrementada la tasa mitocondrial de producción de $O_2^{\cdot -}$ y de H_2O_2 (Calabrese et al., 2001). Por otro lado, las manipulaciones experimentales que alargan la duración de la vida, tales como la restricción calórica en mamíferos y la reducción de la tasa metabólica en insectos, disminuyen la acumulación de daño oxidativo (Calabrese et al., 2001). Además, la sobreexpresión de los genes que codifican para las enzimas antioxidantes SOD y catalasa prolonga la duración de la vida en las especies estudiadas, retardando la acumulación de daños oxidativos asociada a la edad (Orr et al., 1993).

Generalmente se ha asumido que una tasa alta de consumo de oxígeno estaba asociada a una corta esperanza de vida. Sin embargo, se ha descrito que la longevidad de las especies, en mamíferos, está claramente relacionada con la producción de radicales en la mitocondria (Ku et al., 1993). En este sentido, sería más determinante en una elevada esperanza de vida una baja producción de radicales libres por unidad de oxígeno consumido, aunque la tasa de consumo de oxígeno fuese elevada (Barja et al., 1994b). Por tanto, existirá también una correlación negativa entre la esperanza de vida de una especie y su potencial antioxidante (Perez-Campo et al., 1998). Animales de corta esperanza de vida, debido a su alta tasa de generación de ROS, necesitan la presencia continua de altos niveles de antioxidantes en sus tejidos (Barja et al., 1994a; Barja et al., 1994b). A la inversa, los animales longevos no necesitan niveles constitutivos altos de antioxidantes debido a su baja tasa basal de generación de ROS, produciéndose una inducción de las defensas antioxidantes cuando son expuestos a un aumento transitorio de la producción de ROS (Barja et al., 1994a; Barja et al., 1994b).

3.8. El cerebro

El cerebro de un mamífero adulto representa un 0,5-2% del peso corporal total, recibiendo, en el caso de los humanos, el 14% del flujo cardiaco (McHenry et al., 1978) y consumiendo el 20% del oxígeno total del cuerpo (Ginsberg et al., 1992; Silver et al., 1998). Esta alta tasa metabólica, junto con el alto contenido de sustratos fácilmente oxidables como los ácidos grasos poliinsaturados, así como el elevado contenido en hierro en diversas áreas del cerebro tales como *globus pallidus* y *substantia nigra* (Calabrese et al., 2001), confieren al sistema nervioso central (SNC) un gran riesgo de daño oxidativo (Halliwell, 1999). Sin embargo, comparado con otros tejidos, presenta bajos niveles de antioxidantes tales como el glutatión, la vitamina E y los enzimas antioxidantes GPx, catalasa y SOD y, sobre todo, teniendo en cuenta que el SNC contiene células neuronales no replicativas que, una vez dañadas, pueden mantener su disfuncionalidad de forma permanente o comprometerse en un programa de muerte celular por apoptosis (Calabrese et al., 2001). Por el contrario, se ha descrito la presencia de las cinco isoformas de UCP (UCP1 - UCP5) en cerebro (Klingenberg et al., 2001; Li et al., 2001; Jezek et al., 2004; Lengacher et al., 2004), lo que podría suplir los bajos niveles de otros sistemas antioxidantes, ya que, como se ha postulado, estarían implicadas en la reducción del potencial de membrana y en consecuencia de

la producción de ROS. Este papel en el caso de las UCP neuronales podría ser crucial en la reducción del estrés oxidativo, lo que podría explicar el efecto neuroprotector de las UCP en patologías neurodegenerativas (Andrews et al., 2005a).

Además, en el sistema nervioso, se ha demostrado que el desacoplamiento mitocondrial es capaz de promover la biogénesis mitocondrial, evitando por tanto, una disminución de la capacidad celular de formación de ATP, al mismo tiempo que se produce una reducción de la producción de ROS a niveles que pueden ser neutralizados por los mecanismos antioxidantes intracelulares. En consecuencia, se ha propuesto que las UCP reducirían la producción de ROS sin comprometer la producción de ATP (Andrews et al., 2005a).

3.9. Las diferencias de sexo en el envejecimiento

En muchas especies de mamíferos, incluida la humana, las hembras viven más tiempo que los machos. Como ya se ha comentado anteriormente, según la teoría del envejecimiento por radicales libres, el principal factor influyente en la variación de la duración de la vida es la producción de ROS, especialmente en la mitocondria. En consecuencia, la diferencia entre sexos en la esperanza de vida se ha atribuido a que esta producción en las mitocondrias de las hembras es menor que en las de los machos (Viña et al., 2005). Esta divergencia en la producción de ROS podría obedecer a diferencias mitocondriales más profundas. En este sentido, se ha atribuido a los estrógenos un efecto importante en la morfología y biogénesis mitocondriales, observándose mitocondrias mayores y con crestas mejor definidas en hembras (Rodriguez-Cuenca et al., 2002; Chen et al., 2005). Además, el 17β-estradiol, incrementa la transcripción de genes que codifican para proteínas de la CRM y su ensamblaje en los complejos, lo que aumenta la función de la CRM y la fosforilación oxidativa (Chen et al., 2005). Paralelamente, el 17β-estradiol muestra un efecto compensatorio estimulando la expresión de genes que codifican para enzimas antioxidantes como la Mn-SOD y la GPx y estimulando también sus actividades (Chen et al., 2005; Viña et al., 2005), mostrando así, las hembras, el doble de expresión y de función de dichas enzimas que los machos (Strehlow et al., 2003; Viña et al., 2005).

Estudios realizados en el tejido adiposo marrón comparando ambos sexos han confirmado que las ratas hembras poseen mitocondrias de mayor tamaño y más funcionales, además de mayor capacidad termogénica que los machos (Rodriguez et

al., 2001; Rodriguez-Cuenca et al., 2002). También se han observado mitocondrias con más proteína, es decir, más diferenciadas, en el tejido hepático de ratas hembra (Valle et al., 2007). Igualmente, se ha publicado mayor capacidad oxidativa y fosforilativa y mayor actividad de la enzima GPx en el tejido adiposo marrón de las hembras (Justo et al., 2005; Valle et al., 2007) y también de la SOD (Pinto et al., 1969; Borras et al., 2003), así como menores niveles de ADN oxidado (Borras et al., 2003). El músculo esquelético también presenta mayor contenido de mitocondrias, capacidad oxidativa y fosforilativa y actividad GPx (Colom et al., 2007a). En el músculo cardiaco, también se ha puesto de manifiesto menor contenido de mitocondrias pero con mayor eficiencia fosforilativa, indicando un mayor nivel de diferenciación mitocondrial en las ratas hembra, así como una menor producción de ROS y en consecuencia menor daño oxidativo (Colom et al., 2007b).

Por otro lado, los estrógenos tienen efectos neuroprotectores (McEwen, 2001; Behl, 2002b; Nilsen et al., 2004). Así, se ha descrito que una terapia de reemplazo de estrógenos retrasa la aparición de enfermedades neurodegenerativas (Behl, 2002a; Czlonkowska et al., 2003). Por tanto, la bien conocida actividad neuroprotectora de los estrógenos podría estar relacionada con la diferente prevalencia entre géneros de determinadas enfermedades neurodegenerativas.

1. Aging

Aging is a biological process characterized by a generalized physiological decline and an increase in susceptibility to diseases. According to the 'disposable soma' theory, organisms would use needed metabolic resources for effective somatic maintenance in order to keep the organism in sound physiological condition for as long as they have a reasonable chance of survival and reproduction (Kirkwood, 1977). Given that, in the wild, animal death generally happens due to different causes other than aging, natural selection would have favoured pleiotropic genes with good early effects even if these genes had bad effects at later ages (Williams, 1957) or would simply have allowed mutations with late-acting deleterious effects to accumulate (Martin et al., 1996). So, it is clear that there are many genes influencing the aging process, which is the final step of ontogenesis.

A wide range of cellular alterations in somatic tissues has been observed in aging, especially in mitochondria and their DNA. Mitochondria are the main organelles providing the host cell with ATP as energy needed for its operation. Mitochondria also play a central role in intracellular Ca^{2+} homeostasis, intermediary metabolism, intracellular signalling and regulation pathways, steroid synthesis, free radical oxygen species (ROS) production and apoptotic cell death. As a consequence, mitochondrial dysfunction has devastating effects on cell integrity and is therefore involved in aging and degenerative diseases (Kann et al., 2007).

In aging organisms, cellularity decreases due to the prevalence of apoptosis over proliferation. Thus, cellularity would be the main difference between young and old organisms, that is, the number of functional cells in organs or tissues, rather than their quality (Severin et al., 2009).

2. Mitochondria

The mitochondrial inner membrane contains the mitochondrial respiratory chain (MRC) complexes, or electron transport chain complexes (Figure 1). These complexes are integral membrane proteins with prosthetic groups that act as redox centres

capable of catalyzing electron transfer from one complex to the next. The final component is ATP synthase, responsible for ATP formation.

MRC complexes, except for Complex II (succinate-ubiquinone oxidoreductase or succinate dehydrogenase) have, as well as their electron transport function, the capacity of proton translocation from the mitochondrial matrix into the intermembrane space. This function can be prevented by complex inhibitors such as rotenone, specific inhibitor of Complex I (NADH-ubiquinone oxidoreductase or NADH dehydrogenase), antimycin, specific inhibitor of Complex III (ubiquinol-cytochrome c oxidoreductase) or cyanide and carbon monoxide, specific inhibitors of Complex IV (cytochrome c-O_2 oxidoreductase, cytochrome c oxidase or COX). The electron shuttle between complexes is carried out by smaller, more diffusible, molecules such as the lipidic ubiquinone coenzyme Q, from complex I and II to complex III, and cytochrome c, peripheral membrane protein, which shuttles electrons from complex III to complex IV - the final electron acceptor - which reduces O_2 to H_2O. ATP synthase, also known as complex V, is the channel through which protons go back into the matrix and can be specifically inhibited by oligomycin (Walker et al., 1995; Boyer, 1998).

Stoichiometry of MRC complexes shows a simple molar ratio, which means respiratory carriers and complexes are arranged in assemblies with specific protein-protein interactions, which are effective for rapid electron transfer by limiting intermolecular distances (Chance et al., 1956).

Mitochondria play a key role in energy metabolism since they are the organelles responsible for supplying most of the energy needed for cellular activity. Mitochondria consume approximately the 90% of the oxygen arriving at cell in oxidative phosphorylation, a mechanism that produces energy as high energy molecules: ATP, which constitutes the source of most ATP used by cell. Oxidative phosphorylation entails nutrient oxidation to CO_2 and H_2O. Together with substrate oxidation, reduction of nicotinamide adenine dinucleotide (NADH) in complex I and flavin adenine dinucleotide (FAD) in complex II takes place, thus becoming the initial donors of electrons, which then flow throw the MRC of the inner membrane following the oxidation potential of their components.

The differences in redox potential of the electron carriers define the reactions that are exergonic enough to provide the free energy required for the coupled H^+ pumping from the matrix into the intermembrane space. This H^+ translocation causes the matrix to become negatively charged and the intermembrane space positively

charged, producing an electrochemical gradient that generates a proton-motive force great enough to drive ATP synthesis when protons go back into the matrix through the ATP synthase complex.

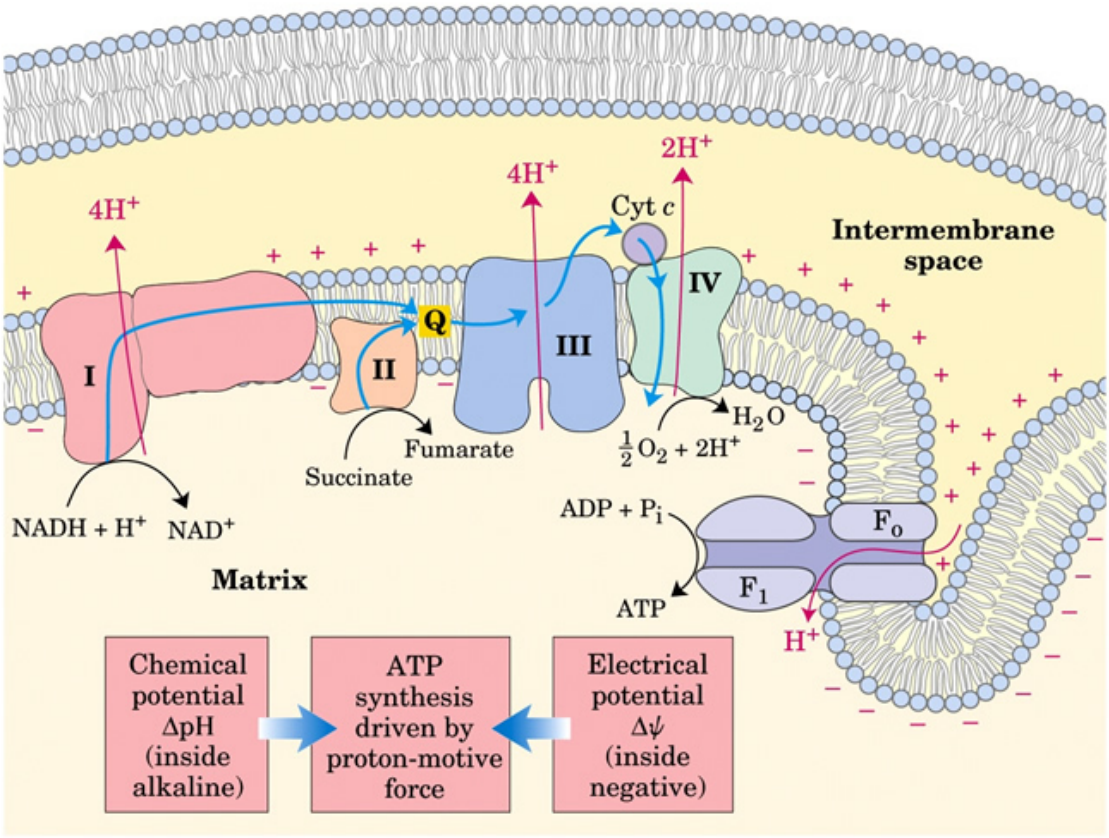

Figura 1: Mitochondrial Respiratory Chain (MRC). Proton translocation through complex I, III and IV and ATP generation in ATP synthase or complex V. Q: ubiquinone. Cyt c: Cytochrome c. Source: Lehninger et al.

The rate of mitochondrial electron transfer and ATP synthesis depend on cell energy requirements. When energy needs increase, ATP breakdown into ADP and Pi also rises. The increase in ADP availability speeds up the electron transfer generating ATP. Normally, phosphorylation capacity is tightly regulated, fluctuating only slightly in most tissues, although it is quite clear that oxidative phosphorylation is never completely coupled in mitochondria. MRC control and mitochondrial metabolic states were first defined in 1956 (Chance et al., 1956). State 4 or resting respiration is characterized by a great substrate but limited ADP availability, which causes a slow electron transfer rate leading to high ROS production, around 1 nmol H_2O_2/min·mg of

protein. In State 3 or phosphorylating state, there are plenty of substrates and ADP in mitochondria, which causes a high electron transfer speed reaching the maximal physiologic rate of ATP production and O_2 consumption and showing very low ROS production rate, around 0.1 nmol H_2O_2/min·mg of protein (Boveris et al., 2000). The electron transfer rate is between 3 and 8 times faster in State 3 than in State 4 in isolated mitochondria, thus the respiratory control rate is within this range (Chance et al., 1956). Mammalian mitochondria in physiological conditions have been estimated to be mainly in State 4, around 60-70%, and only the remaining 30-40% in State 3 (Boveris et al., 1999).

3. ROS production

Free radicals are chemical species with an unpaired electron on an open shell configuration, which confers them high instability and as a result high chemical reactivity, especially those of low molecular weight. The main ROS produced in mitochondrial electron transfer are superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2), which is not a true free radical but is considered as such because of its high chemical reactivity.

Mitochondria are the main source of cell oxidants. Approximately, 1-2% of electrons flowing through MRC result in superoxide radical generation (Beckman et al., 1998) which happens when an electron is transferred to O_2 instead of to the next MRC complex. A high membrane potential and low ATP demand are the favourable conditions for this electron diversion. In fact, proton gradient magnitude has been proven to be directly correlated with MRC ROS production (Korshunov et al., 1997). Predominant places for ROS production are complex I and complex II (Figure 1), although they are not the only ones since the ubiquinone pool within the MRC could also be a ROS producer (Boveris et al., 1999) given that the ubisemiquinone species can work as reducing agent for both oxygen and hydrogen peroxide generating superoxide anions and hydroxyl radicals. Moreover, plasma membrane is also a source of ROS because of integral membrane oxidases (Christman et al., 1985; Storz et al., 1990; Faulkner et al., 1993; Rhee, 1999; Thannickal et al., 2000).

4. Oxidative damage

ROS attack high electron density regions such as nitrogen and oxygen atoms present in proteins and nucleic acids, and carbon double bonds present in polyunsaturated fatty acids. This way autocatalytic reaction sequences are started, which produces intermediate reactive product and leaves a great many oxidized molecules until the chain reaction finishes.

Lipid peroxidation entails formation of lipoperoxides that can react with other membrane lipids and proteins. This leads to formation of cross bonds between lipid-lipid, lipid-protein and protein-protein, as well as to these lipoperoxide breakdown generating low molecular weight by-products such as malondialdehyde (Esterbauer et al., 1991). This oxidation causes a decrease in membrane fluidity and increase in permeability. On the other hand, protein oxidation results in a carbonyl group accumulation that entails functionally inactive enzymes, which become targets for their proteolytic degradation (Stadtman, 1992). Finally, nuclear and mitochondrial DNA also undergo oxidation, generating nucleotide derivatives such as 8-oxo-deoxyguanosine (Ames, 1989). Those alterations can generate mutations that accumulate with aging despite the repair systems (Stadtman, 1992).

Mitochondrial DNA is especially prone to mutations having been estimated to be ten times more susceptible than nuclear DNA (Ames, 1989; Richter et al., 1997). This predisposition is due to its location close to the mitochondrial inner membrane where oxidative phosphorylation takes place and the mtDNA is itself exposed to radicals generated by normal mitochondrial respiration, to a lack of protective proteins like histones and to the absence of efficient DNA repair systems (Barja et al., 2000). MtDNA mutations lead to alterations in MRC function which in turn affect general mitochondrial running, which decreases ATP production, alters Ca^{2+} homeostasis and increases ROS production. These will then produce damage including more mtDNA, proteins and lipids oxidation, which again begins the vicious circle causing more alterations in MRC function. Thus, a damage escalation that grows and accumulates with age is generated, which is the main cause of aging and may compromise cell survival in the long term (Halliwell, 1999).

5. Antioxidante defences

Living beings have developed antioxidant defence systems against free radical production throughout evolution (Halliwell, 1999). These systems act by preventing their formation or by scavenging and neutralizing the already generated radicals because of their capacity of donating an electron to the free radical themselves remaining as a radical but with a very low reactivity unable to start or further any chain reaction. An antioxidant can be defined as any substance that importantly slows down or prevents oxidation of substrates, including all kinds of biomolecules such as carbohydrates, lipids, proteins and DNA, which is found in low concentration related to the substrate susceptible of oxidation (Halliwell, 1990). Each antioxidant has an affinity for a certain sort of radical. Traditionally, antioxidant defences have been classified in two main groups: enzymatic and non enzymatic ones (Sies, 1997).

Antioxidant enzymes, those synthesized within the organism, protect against ROS produced during metabolism. Among these defence systems there are three important enzymes (Figure 2): superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx). SOD catalyzes the dismutation of the superoxide anion into hydrogen peroxide. There are several forms of SOD depending on the metal in the catalytic centre and their location: cytoplasmatic Cu,Zn-SOD, mitochondrial Mn-SOD and extracellular Cu,Zn-SOD (Fridovich, 1975). Catalase is a tetrameric enzyme catalyzing hydrogen peroxide breakdown into water. This enzyme is present in most eukaryotic cells within peroxisomes (Di Giulio, 1991). Finally, GPx is located in both cytosol and mitochondria (Blum et al., 1984) contributing also to hydrogen peroxide elimination using reduced glutathione (GSH) as electron donor, unlike catalase which uses hydrogen peroxide as electron donor. Besides, the system is completed by glutathione reductase (GR) which takes care of regenerating the glutathione that has been oxidized (GSSG) (Rall et al., 1952) keeping the GSH/GSSG proportion balanced.

Non enzymatic antioxidants are substances able to neutralize only one free radical per molecule, that is, they are stoichiometric scavengers, so they act only in a high concentration and accomplish a minimal role compared with enzymatic antioxidants. A first group of non enzymatic antioxidants is made up of small neutralizing molecules such as α-tocopherol or vitamin E (Traber et al., 1996), which scavenge hydroxyl radicals and superoxide anions and neutralizes peroxides; and ascorbic acid or vitamin C, which also captures hydroxyl radicals and superoxide anions and regenerates the oxidized vitamin E species (Briviba et al., 1994).

Carotenoids are also included in this group, α-carotene or provitamin A and glutathione as examples (Sies et al., 1995).

A second group of non enzymatic antioxidants is composed of certain cationic chelating proteins able to keep in transition metals such as copper and above all iron, thus diminishing their availability which is necessary for hydroxyl radical formation. Among these proteins are ferritin, transferrin, lactoferrin, metallothionein and ceruloplasmin (Sies, 1997).

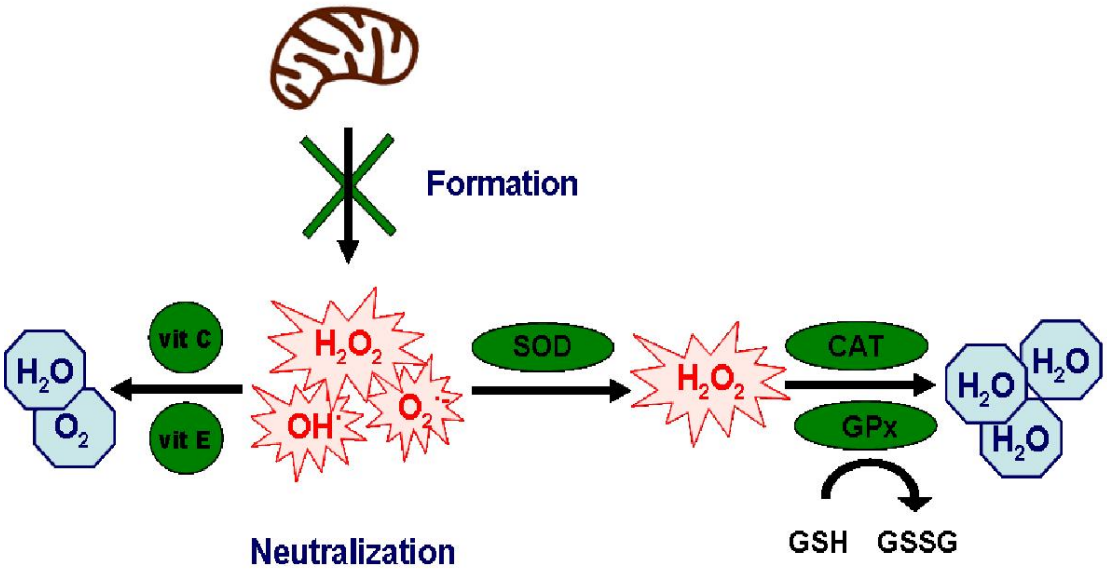

Figure 2: Radical formation by mitochondria and removing them by antioxidant defence systems. vit C: vitamin C. vit E: vitamin E. SOD: superoxide dismutase. Cat: catalase. GPx: glutathion peroxidase. GSH: reduced glutathione. GSSG: oxidized glutathione.

6. Uncoupling proteins

Uncoupling proteins (UCPs) in the mitochondrial respiratory chain could be another of the antioxidant systems preventing ROS formation (Negre-Salvayre et al., 1997), although their role is controversial. They are so called because they uncouple ATP synthesis from the electron transport chain by using electrochemical gradient without ATP synthesis, that is, the uncoupling is the result of the proton leak into the matrix dissipating the proton-motive force and then slowing down the number of

protons that flow through ATP synthase (Figure 3). These proteins belong to a proton carrier family located in the mitochondrial inner membrane (Pecqueur et al., 2001) consisting of several members known to date: UCP1 – UCP5. UCPs are expressed depending on the tissue, which suggests they have different functions and regulations depending on their location. That is, UCP1 is expressed in brown adipose tissue cells and is activated by cold which results in mitochondrial uncoupling that leads to enough heat production so as to maintain body temperature, thus they have a thermogenic function (Pecqueur et al., 2001; Argiles et al., 2002). The remaining UCPs are present at much lower levels in the tissues in which they are expressed, compared with UCP1 in brown adipose tissue, suggesting that the function of theses isoforms would not be thermogenesis (Kim-Han et al., 2005). UCP2 is expressed in many tissues including several brain regions (Andrews et al., 2005a; Andrews et al., 2005b). UCP3 is mainly expressed in skeletal and cardiac muscle cells (Dulloo et al., 2001). UCP4 and UCP5 - the latter is also known as BMCP1 - are mainly expressed in brain (Sanchis et al., 1998); UCP4 is exclusive to brain (Mao et al., 1999) whereas UCP5 is also expressed in other tissues (Sanchis et al., 1998).

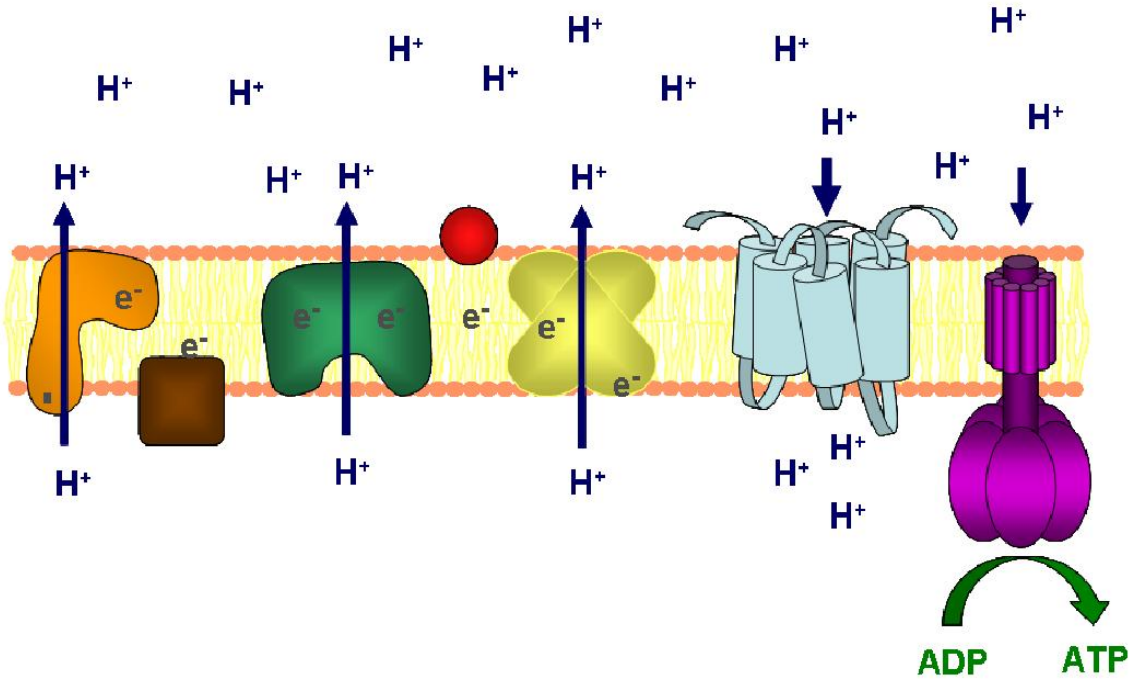

Figure 3: Mitochondrial respiratory chain (MRC). Proton translocation through complex I, III and IV, and return to matrix through UCPs.

UCPs proton translocation function has been postulated to result in a mild reduction of mitochondrial membrane potential, in this way diminishing the MRC electron leak and as result reducing ROS production (Negre-Salvayre et al., 1997). In fact, even mild uncoupling significantly reduces mitochondrial ROS production, as has been described (Korshunov et al., 1997; Miwa et al., 2003; Starkov et al., 2003). A 10% drop in the membrane potential causes a 55% reduction in H_2O_2 production, whereas a slight increase in this potential may cause an important $O_2^{\cdot -}$ and H_2O_2 production (Korshunov et al., 1997). Therefore, uncoupling increase by UCPs has a net effect toward a drop in oxidative stress.

Despite detailed mechanism of UCPs action on energetic metabolism regulation and ROS production is not known, there are experimental evidences that prove UCPs uncoupling activity can be stimulated by both oxidative stress (Echtay et al., 2002; Echtay et al., 2003) and free fatty acids (Hagen et al., 2000) and can be inhibited by purine nucleotides: GDP and ADP (Nicholls et al., 1984).

7. Oxidative stress in aging

Oxidative stress is the imbalance between free radical production and antioxidant defence level (Sies, 1985). In this sense, a situation of oxidative stress would be one in which there is increased ROS production or decreased antioxidant defence levels or both circumstances at the same time, causing a balance shifting toward greater ROS concentration. Oxidative stress has been said to be one of the main causes of aging and many degenerative pathologies accompanying it such as neurodegenerative diseases (Kim-Han et al., 2001; Lindholm et al., 2004).

The free radical theory of aging was first propounded by Gerschmann and Harman in the fifties (Gerschman et al., 1954; Harman, 1956). This theory postulated that aging and the degenerative diseases associated with it were attributed basically to the deleterious side attacks of free radicals on cell constituents and on the connective tissues. Harman, in 1972, propounded that mitochondria could play a central role in the aging process (Harman, 1972). According to this theory, free radicals generated by mitochondrial metabolism could act by causing cell dysfunction and death.

This theory is supported by much evidence. First of all, DNA and protein molecular oxidative damage exponentially rise with age and concomitantly

mitochondrial production rate of $O_2^{\cdot-}$ and H_2O_2 increase as well (Calabrese et al., 2001). Secondly, life span prolonged by experimental interventions such as caloric restriction in mammals and decrease of metabolic rate in insects reduce oxidative damage accumulation (Calabrese et al., 2001). Finally, overexpression of the genes encoding SOD and catalase antioxidant enzymes prolong life span and slow down oxidative damage accumulation related to age, in studied species (Orr et al., 1993).

Generally, it was assumed that a high oxygen consumption rate correlated wit a short life expectancy. However, it has been described that longevity of mammal species is clearly correlated with radical production in mitochondria (Ku et al., 1993). In this sense, a low free radical production per unit of consumed oxygen would be more decisive for a long life expectancy even though the oxygen consumption rate were high (Barja et al., 1994b). Therefore, the life expectancy of a species will have a negative correlation with its antioxidant potential (Perez-Campo et al., 1998). Short-life-span animals need high antioxidant levels in their tissues in a constant way due to their high rate of ROS production (Barja et al., 1994a; Barja et al., 1994b). Conversely, long-life-span animals do not need high constitutive antioxidant levels due to their low basal rate of ROS production, and antioxidant defences will only be stimulated when exposed to a transient increase of ROS production (Barja et al., 1994a; Barja et al., 1994b).

8. Brain

Brain from adult mammals represents 0.5-2% of total body weight, receives 14% of the cardiac output (McHenry et al., 1978) and consumes 20% of the total body oxygen (Ginsberg et al., 1992; Silver et al., 1998). This high metabolic demand together with both the high content of easily oxidizable substrates such as polyunsaturated fatty acids and the high iron content in several brain regions such as *globus pallidus* and *substantia nigra* (Calabrese et al., 2001) confer a great oxidative damage risk to the central nervous system (CNS) (Halliwell, 1999). However, brain has low levels of antioxidants such as glutathione, vitamin E and antioxidant enzymes, GPx, catalase and SOD, compared with other tissues, even more so taking into account that CNS has non-dividing neurons which, when become damaged, can maintain their dysfunctionality forever or can start a cellular death program by apoptosis (Calabrese et al., 2001). Conversely, the five UCP isoforms (UCP1 - UCP5) are present in brain (Klingenberg et al., 2001; Li et al., 2001; Jezek et al., 2004; Lengacher et al., 2004), which could supplement the low levels of other antioxidant

systems since they have been said to be involved in membrane potential reduction and, thus, in ROS production. This neuronal UCP role could be crucial for oxidative stress reduction which could explain the neuroprotector effect of UCPs in neurodegenerative diseases (Andrews et al., 2005a).

Furthermore, mitochondrial uncoupling in the nervous system has been proven to be able to promote mitochondrial biogenesis and this way, to prevent a decrease in cellular capacity of ATP formation. Biogenesis also produces a fall of ROS production to levels that can be neutralized by intracellular antioxidant mechanisms. As a result, UCPs have been propounded to reduce ROS production without compromising ATP generation (Andrews et al., 2005a).

9. Sex differences in aging

Females live longer than males in many mammalian species, including humans. The main factor influencing life span variation is ROS production, especially in mitochondria, according to the free radicals theory of aging as previously mentioned. Consequently, differences between sexes concerning life expectancy has been attributed to the fact that female mitochondria produce fewer ROS than male ones (Viña et al., 2005). This difference in ROS production could be based on deeper mitochondrial differences. In this sense, estrogens have been attributed an important effect on mitochondrial morphology and biogenesis, hence, greater mitochondria and with better defined cristae have been observed in females (Rodriguez-Cuenca et al., 2002; Chen et al., 2005). Moreover, 17β-estradiol increases transcription of genes that code for CRM proteins and their assembly in the complexes, which increases MRC function and oxidative phosphoritalion (Chen et al., 2005). In line with this, 17β-estradiol shows a compensatory effect by stimulating both the expression of genes that code for antioxidant enzymes such as Mn-SOD and GPx and their activities (Chen et al., 2005; Viña et al., 2005), thus females show two fold expression and function of theses enzymes than males (Strehlow et al., 2003; Chen et al., 2005; Viña et al., 2005).

Studies carried out in brown adipose tissue comparing both sexes have confirmed that female rats own larger sized, more functional mitochondria with a greater thermogenic capacity than males (Rodriguez et al., 2001; Rodriguez-Cuenca et al., 2002). What is more, mitochondria with more protein, hat is, more highly

differentiated mitochondria, have been observed in female rat hepatic tissue (Valle et al., 2007b). Likewise, greater oxidative and phosphorylative capacity and higher GPx activity have been reported in female brown adipose tissue (Justo et al., 2005; Valle et al., 2007a), as well as greater SOD activity (Pinto et al., 1969; Borras et al., 2003) and lower oxidized DNA levels (Borras et al., 2003). Skeletal muscle also presents a higher mitochondria content, greater oxidative and phosphorylative capacities and higher GPx activity in female rats (Colom et al., 2007a). Finally, lower mitochondrial content but with greater phosphorylative efficiency, which indicates a greater degree of mitochondrial differentiation has also been highlighted in female rat cardiac muscle, as well as a lower ROS production and, as a result, lesser oxidative damage (Colom et al., 2007b).

On the other hand, estrogens have neuroprotective effects (McEwen, 2001; Behl, 2002b; Nilsen et al., 2004). Replacement estrogen therapy has been described to delay neurodegenerative disease onset (Behl, 2002a; Czlonkowska et al., 2003). Therefore, the well-known neuroprotective activity of estrogen could be related to the different prevalence of several neurodegenerative diseases between genders.

4. OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

OBJECTIVES AND EXPERIMENTAL DESIGN

OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

La presente tesis se enmarca dentro de los proyectos de investigación "Influencia del género en la función y biogénesis mitocondrial inducida por estrés oxidativo" financiado por el Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo (PI060293) y "Envejecimiento saludable: factores implicados en la mejora de la calidad de vida" financiado por la *Conselleria d'Economia, Hisenda i Innovació*, *Govern de les Illes Balears* (Programa para el fomento de la investigación interinstitucional. Proyectos de R+D+I para grupos emergentes y competitivos de las Islas Baleares). Estos proyectos se han llevado a cabo en el Departamento de Biología Fundamental y Ciencias de la Salud de la Universidad de las Islas Baleares, por el Grupo de Metabolismo Energético y Nutrición.

El objetivo general de la presente tesis fue estudiar los cambios producidos en la bioenergética, la función y el estrés oxidativo mitocondriales, así como los daños oxidativos acumulados, en el cerebro de rata, en respuesta al envejecimiento, centrándose en las diferencias de sexo.

Las hembras de muchas especies, incluida la humana, presentan una mayor esperanza de vida que los machos y una menor incidencia de diversas patologías neurodegenerativas asociadas al estrés oxidativo. Estudios previos realizados en nuestro laboratorio habían puesto de manifiesto la existencia de diferencias morfofuncionales a nivel mitocondrial entre ambos sexos, en tejidos como el hígado, el músculo esquelético y cardiaco y el adiposo marrón y blanco. Dichas diferencias se habían observado tanto a nivel morfológico, de contenido y tamaño mitocondriales, como de funcionalidad, capacidad oxidativa y fosforilativa, grado de diferenciación y eficiencia mitocondriales. Además, se había puesto de manifiesto un dimorfismo sexual en estos tejidos, con diferentes niveles de defensas antioxidantes y de producción de ROS, y en consecuencia, diferencias en el nivel de estrés oxidativo (Rodriguez, Monjo et al. 2001; Rodriguez-Cuenca, Pujol et al. 2002; Justo, Boada et al. 2005; Justo, Frontera et al. 2005; Colom, Alcolea et al. 2007; Colom, Oliver et al. 2007; Valle, Garcia-Palmer et al. 2007; Valle, Guevara et al. 2007). En base a estos trabajos previos, se consideró de gran interés realizar un estudio comparativo entre las mitocondrias del cerebro de ratas macho y hembra. Así, el primer objetivo planteado fue investigar la existencia de diferencias entre sexos, en la bioenergética y balance oxidativo en mitocondrias de cerebro y, dado que el daño oxidativo se acumula con la

edad, se decidió realizar el estudio en ratas macho y hembra de 24 meses de edad. Los resultados obtenidos y las conclusiones de este estudio han quedado reflejados en el **Manuscrito I**.

Las diferencias de sexo que se observaron en el cerebro de las ratas viejas: menor contenido mitocondrial pero con mayor grado de diferenciación en las hembras dada su mayor función oxidativa y fosforilativa, nos animó a seguir profundizando en las diferencias de sexo que podrían producirse en el cerebro a lo largo del envejecimiento. Para llevar a cabo este objetivo decidimos realizar un estudio más detallado de la función mitocondrial en el cerebro de ratas machos y hembras de diferentes edades, 6, 12, 18 y 24 meses, analizando el contenido proteico y de ADN a nivel celular y mitocondrial y la función oxidativa y fosforilativa mitocondriales. Los resultados obtenidos y las conclusiones de este estudio se detallan en el **Manuscrito II**.

Los resultados obtenidos en este segundo trabajo que mostraban un aumento del número de mitocondrias con la edad pero menos diferenciadas, y que las hembras mantenían mejor la funcionalidad que los machos, confirmaban los resultados observados en las ratas de 24 meses de edad. Por ello, nos planteamos si este dimorfismo en la función mitocondrial estaría influyendo en el mejor balance oxidativo que se observó en las hembras viejas, reflejado por una mayor función mitocondrial con similar producción de ROS, mayor capacidad antioxidante debida tanto a una mayor actividad enzimática como a una mayor presencia de UCP y en consecuencia menor daño oxidativo. Para abordar este nuevo objetivo decidimos estudiar los cambios producidos durante el envejecimiento en el estado oxidativo mitocondrial, en los mismos grupos experimentales estudiados en el trabajo precedente. Los resultados y las conclusiones obtenidos a partir de este estudio se presentan en el **Manuscrito III**.

Para el desarrollo del proyecto de la presente tesis la doctoranda obtuvo una beca predoctoral del Plan de Formación de Personal Investigador de la *Conselleria d'Innovació, Interior i Justícia* del *Govern de les Illes Balears* (convocatoria 2005).

Además, la doctoranda ha realizado una estancia científica en el año 2008 en el *Department of Molecular Neuroscience del Institute of Neurology* de la *University College London* en el Reino Unido, bajo la supervisión científica del profesor Dr. John Hardy.

La metodología utilizada para la consecución de los objetivos planteados en los distintos trabajos realizados queda detallada a continuación:

- Determinaciones biométricas de peso de los animales y tejido objeto de experimentación.

- Aislamiento mitocondrial por centrifugación diferencial.

- Determinación del contenido en proteína total y mitocondrial.

- Determinación del contenido en lípidos totales.

- Cuantificación del contenido en ADN total, como indicador del número de células, del ADNmt como indicador del número de mitocondrias y de la actividad enzimática de la citrato sintasa (EC 2.3.3.1) como marcador de masa mitocondrial.

- Valoración de la capacidad oxidativa mitocondrial mediante el análisis del consumo de oxígeno por la CRM y la actividad de la citocromo c oxidasa (EC 1.9.3.1).

- Valoración de la capacidad fosforilativa mitocondrial mediante la actividad de la ATP sintasa (EC 3.6.1.3).

- Evaluación de la capacidad antioxidante mitocondrial mediante la determinación de la actividad de las enzimas superóxido dismutasa (EC 1.15.1.1), glutatión peroxidada (EC 1.11.1.9) y catalasa (EC 1.11.1.6) y mediante estimación de los niveles proteicos de las UCP.

- Estudio de la producción mitocondrial de ROS mediante el análisis de los niveles de H_2O_2 generados en el estado 3 y el estado 4 de la respiración mitocondrial, en presencia de inhibidores de la CRM y en presencia de un inhibidor del desacoplamiento mitocondrial.

- Determinación del daño oxidativo mitocondrial y celular mediante la cuantificación de las proteínas oxidadas, los lipoperóxidos y la oxidación del ADN.

- Estudio del estatus hormonal mediante la determinación de los niveles circulantes de las hormonas sexuales 17β-estradiol, progesterona y testosterona.

OBJECTIVES AND EXPERIMENTAL DESIGN

The present thesis is part of the research projects: “gender influence on mitochondrial function and biogenesis induced by oxidative stress” funded by *Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Ministerio de Sanidad y Consumo* j and “Healthy aging: factors involved in quality of life improvement” funded by *Conselleria d'Economia, Hisenda i Innovació, Govern de les Illes Balears* (Program for interinstitutional research promotion. R+D+I projects for emerging and competitive groups from Balearic Islands). Theses projects have been carried out in the Department of *Biología Fundamental y Ciencias de la Salud* at the *Universidad de las Islas Baleares*, by the group of *Metabolismo Energético y Nutrición*.

The general aim of the present thesis was to study the changes produced in both bioenergetics and mitochondrial function and oxidative stress, as well as to study the accumulated oxidative damage in rat brain in response to aging, focusing on sex differences.

Females show longer life expectancy than males and lower incidence of several neurodegenerative diseases related to oxidative stress in many species, including humans. Previous studies carried out in our laboratory had highlighted the existence of mitochondrial morphologic and functional differences between sexes, in tissues such as liver, skeletal and cardiac muscle, and brown and white adipose. These differences had been observed in both morphology, that is, mitochondrial content and size, and functionality, that is, oxidative and phosphorylative capacity, differentiation degree and mitochondrial efficiency. Furthermore, a sexual dimorphism had been highlighted in these tissues which showed different mitochondrial levels of antioxidant defences and ROS production, and as a consequence, differences in cellular oxidative stress level (Rodriguez, Monjo et al. 2001; Rodriguez-Cuenca, Pujol et al. 2002; Justo, Boada et al. 2005; Justo, Frontera et al. 2005; Colom, Alcolea et al. 2007; Colom, Oliver et al. 2007; Valle, Garcia-Palmer et al. 2007; Valle, Guevara et al. 2007). On the basis of these previous works, we considered it highly interesting to carry out a comparative study between male and female rat brain mitochondria. Thus, the first planned aim was to investigate the sex-difference existence in brain mitochondria bioenergetics and oxidative balance. Given that oxidative damage mounts up with age, we decided to conduct the study with 24-month old male and female rats. The results and conclusions obtained from this work have been expressed in **Manuscript I**.

Sex-differences observed in old rat brain -- lower mitochondrial content but with greater differentiation degree in females given their greater oxidative and phosphorylative function – encouraged us to further study in depth sex-differences that could take place in brain throughout aging. To tackle this goal, we decided to carry out a more detailed study on brain mitochondrial function from 6, 12, 18 and 24 months old male and female rats, analysing protein and DNA content in cellular and mitochondrial fractions and mitochondrial oxidative and phosphorylative function. This study results and conclusions are detailed in **Manuscript II**.

Results obtained from this second work – which showed an increase in mitochondria number with age although they were less differentiated, and a better female maintenance of mitochondrial functionality than males – confirmed the results observed in 24 month old rats. Because of this, we wondered whether this mitochondrial function dimorphism would influence the better oxidative balance observed in old females, which is reflected by greater mitochondrial function but similar ROS production and greater antioxidant capacity due to both higher enzymatic activity and higher UCPs levels, and as a result, lesser oxidative damage. In order to tackle this new aim, we decided to study the changes produced in mitochondrial oxidative status during aging in the same experimental groups studied in the previous work. The results and conclusions drawn from this study are presented in **Manuscript III**.

The PhD student obtained a research scholarship so as to carry out the project of this doctoral thesis, from the Plan for research fellowship training of *Conselleria d'Innovació, Interior I Justicia* of *Govern de les Illes Balears* (official announcement 2005).

Moreover, the PhD student has made a research stay at the Department of Molecular Neuroscience of the Institute of Neurology of the University College London, United Kingdom, in 2008, under scientific supervision of Professor Dr. John Hardy.

Methodology used to achieve the planned goals in the different works carried out is detailed below:

- Biometric determinations of weights from animals and tissues subject of experimentation.
- Mitochondrial isolation by differential centrifugation.
- Total and mitochondrial protein content assessment.
- Total lipid content assessment.
- Quantification of total DNA content as cell number indicator, of mtDNA as mitochondria number indicator and of citrate synthase (EC 2.3.3.1) enzymatic activity as mitochondrial mass marker.
- Mitochondrial oxidative capacity estimation by both MRC oxygen consumption and cytochrome-c oxidase (EC 1.9.3.1) activity assessment.
- Mitochondrial phosphorylative capacity estimation by ATP synthase (EC 3.6.1.3) activity.
- Mitochondrial antioxidant capacity estimation by both activities assessment of superoxide dismutase (EC 1.15.1.1) glutathione peroxidase (EC 1.11.1.9) and catalase (EC 1.11.1.6) and determination of UCPs protein levels.
- Study of mitochondrial ROS production by analysing H_2O_2 levels generated in several conditions: mitochondrial respiration State 3 and State 4, in the presence of MRC inhibitors and in the presence of a mitochondrial uncoupling inhibitor.
- Cellular and mitochondrial oxidative damage assessment by oxidized protein, lipoperoxide and oxidized DNA quantification.
- Hormonal status study by determination of circulating levels of sex hormone 17β-estradiol, progesterone and testosterone.

5. RESULTADOS Y DISCUSIÓN

RESULTS AND DISCUSSION

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Original Contribution

Sex-dependent differences in aged rat brain mitochondrial function and oxidative stress

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ABSTRACT

Females show lower incidences of several neurodegenerative diseases related to oxidative stress and mitochondrial dysfunction than males. In addition, female rats show more differentiated mitochondria than males in several tissues. The aim of this work was to investigate the existence of sex-dependent differences in brain mitochondrial bioenergetics and oxidative balance in aged rats. Results showed that aged female rat brain had a lower mitochondria content than aged male brain but with a greater differentiation degree given the higher mitochondrial protein content and mitochondrial complex activities in females. Female rat brain also showed a better oxidative balance than that of males, reflected by the fact that higher mitochondrial respiratory chain function is accompanied by a similar ROS production and greater antioxidant enzyme activities, which could be responsible for the lesser oxidative damage observed in proteins and lipids in this sex. Interestingly, levels of UCP4 and UCP5—proteins related to a decrease in ROS production—were also higher in females. In conclusion, aged female rat brain had more differentiated mitochondria than male brain and showed a better control of oxidative stress balance, which could be due, in part, to the neuroprotective effect of UCPs.

Introduction

Mitochondria and peroxisomes are the main source of free radical species, and mitochondria are a target for their damaging effects. These oxidative lesions accumulate with age and play a major role in causing mitochondrial dysfunction [1,2], which has deleterious effects on the integrity of cells and may thus be critically involved in aging [3] and degenerative diseases [4].

The central nervous system has a very high metabolic rate, as it consumes about 20% of the oxygen inspired at rest, while accounting for only 0.5–2% of the body weight [5]. This high metabolic demand exists because neurons are highly differentiated cells needing large amounts of ATP for maintenance of ionic gradients across the cell membranes and for neurotransmission. Because most neuronal ATP is generated by oxidative metabolism, neurons critically depend on mitochondrial function and oxygen supply [6,7]. Thus, neuronal function and survival are very sensitive to mitochondrial dysfunction [8,9].

Females live longer than males in many mammalian species, including rats and humans [10,11], and show lower incidences of several neurodegenerative diseases [12] related to oxidative stress and mitochondrial dysfunction. Specifically, in Wistar rats, the experimental animals used in this work, a gender dimorphism in life span has been described, with a higher longevity in females compared to males [13], 27 and 23 months, respectively, although the magnitude of this difference varies with the strain [14]. Thus, several studies have reported that female rats show more differentiated mitochondria, meaning a greater mitochondrial machinery, and, as a result, the mitochondria show a higher capacity and efficiency of substrate oxidation than those of males in liver [15,16], brown adipose tissue [17–19], cardiac muscle [20], and skeletal muscle [21].

Uncoupling proteins (UCPs)[1] belong to a family of ion carriers located in the inner mitochondrial membrane [22] and all of which are found in the brain [23]. The protonphoric action of UCPs results in reduced mitochondrial membrane potential and production of reactive oxygen species (ROS) [24]. The uncoupling activity of UCPs can also be activated by oxidative stress [25,26] and free fatty acids [27] and inhibited by purine nucleotides [28]. UCP4 and UCP5 are expressed mainly in the nervous system [29,30] and have been reported to play an important role in reduction of ROS production [31,32].

Abbreviations: ATPase, ATP synthase; COX, ferrocytochrome-c:oxygen oxidoreductase; Ct, crossing point; DNP-hydrazone, 2,4-dinitrophenylhydrazone; GPx, glutathione peroxidase; MnSOD, manganese superoxide dismutase; MRC, mitochondrial respiratory chain; mtDNA, mitochondrial DNA; RCR, respiratory control rate; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; UCP, uncoupling proteins.

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Taking the above into account and given that oxidative damage is cumulative with aging, the aim of this work was to investigate the existence of sex-dependent differences in brain mitochondrial bioenergetics and oxidative balance in aged rats. For this purpose, we studied mitochondrial function parameters such as mitochondrial DNA (mtDNA), protein content, enzymatic activities, oxygen consumption, hydrogen peroxide (H_2O_2) production, antioxidant systems, UCP4 and UCP5 protein levels, and oxidative damage in 24-month-old male and female rats.

Materials and methods

Chemicals

Routine chemicals were supplied by Sigma–Aldrich (St. Louis, MO, USA), Panreac (Barcelona, Spain), Amersham Pharmacia Biotech (Little Chalfont, UK), and Chemicon International (Temecula, CA, USA). Antibodies against UCP4 and UCP5 were obtained from Alpha Diagnostic International (San Antonio, TX, USA). Real-time PCR reagents and oligonucleotide primers were supplied by Roche Diagnostics (Basel, Switzerland), Amplex red reagent, by Molecular Probes (Paisley, UK) Oxyblot Protein Oxidation Detection Kit, by Chemicon–Millipore (Billerica, MA, USA) malondialdehyde assay, by Northwest Life Science Specialties (Vancouver, BC, Canada); and nitrocellulose filter, sensitive film, and protein molecular mass standard, by Bio-Rad (Hercules, CA, USA).

Animals, sacrifice, and mitochondrial isolation

Animals were treated in accordance with the University Bioethical Committee guidelines for animal care and the European Union regulations (86/609/EEC).

Twenty-four-month-old Wistar rats, six males and six females, supplied by Charles River (Barcelona, Spain) were housed individually and were acclimated in our animal facility at 22°C with a 12-h light/dark cycle. Animals were fed ad libitum with pelleted standard diet (A04 Panlab, Barcelona, Spain).

All animals were weighed and sacrificed by decapitation at the start of the light cycle. After sacrifice animals underwent a visual internal inspection to exclude aged animals with any pathological signal. Brain was quickly removed and weighed. Homogenization and mitochondrial isolation were performed according to the previously described procedures [33]. Briefly, brain was homogenized in 35 ml of cold isolation medium (0.25 M sucrose, 10 mM Tris–HCl, and 0.5 mM EDTA-K^+, pH 7.4) in a manual homogenizer with a glass pestle with a total clearance of 0.1 mm. An aliquot of the homogenate was stored at −20°C for posterior analyses and the remainder was used to perform biochemical assays and for mitochondrial isolation. Nuclei and cell debris were twice sedimented by differential centrifugation at 2000*g* for 3 min and the pellets were discarded. Supernatant was subjected to a further centrifugation at 12,500*g* for 8 min and the crude mitochondrial pellet obtained was resuspended with 0.8 ml of isolation medium. Protein concentration was determined by Bradford's method [34].

Enzymatic activities

Citrate synthase (EC 2.3.3.1) [35], ferrocytochrome-c:oxygen oxidoreductase (COX or complex IV; EC 1.9.3.1) [36], and ATP synthase (ATPase or complex V; EC 3.6.1.3) [37] activities were determined in resuspended mitochondrial pellet. Catalase (EC 1.11.1.6.) [38] in homogenate and mitochondrial antioxidant activities, manganese superoxide dismutase (MnSOD; EC 1.15.1.1) [39], and glutathione peroxidase (GPx; EC 1.11.1.9) [40] were also assayed. COX activity was also measured in brain homogenate to calculate mitochondrial recovery.

Quantification of mitochondrial DNA by real-time PCR

mtDNA extraction and semiquantification were carried out as previously described [41]. Briefly, real-time PCR was performed to amplify a 162-nt region of the mitochondrial NADH dehydrogenase subunit 4 gene, which is present only in mtDNA. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche) using a total volume of 10 μl containing each primer at 0.375 μM, 3 mM $MgCl_2$, 1 μl LightCycler FastStart DNA Master SYBR Green I, and 2.5 μl of pretreated mitochondrial sample. The PCRs were cycled 35 times after initial denaturation (95°C, 10 min). Primers and conditions are shown in Table 1.

Mitochondrial oxygen consumption

Oxygen consumption of brain mitochondria was measured by a thermostatically controlled Clark-type O_2 electrode (Oxygraph, Hansatech, UK). Reaction conditions were 1 mg of mitochondrial protein in up to 0.5 ml of respiration buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.1% BSA, fatty acid free, pH 7.4) at 37°C. The substrate used was pyruvate/malate (5 and 2.5 mM, respectively) in the presence (State 3, phosphorylating) and in the absence (State 4, resting) of 500 μM ADP. Mitochondrial viability was checked by the respiratory control rate (RCR= State 3/State 4).

Mitochondrial hydrogen peroxide production

H_2O_2 production from mitochondria was measured fluorimetrically using the Amplex red reagent (530 nm excitation, 590 nm emission) in the presence of horseradish peroxidase. Assays were performed at 37°C for 20 min in a 96-well microplate fluorimeter (Biotek Instruments, Winooski, VT, USA). Mitochondria (0.4 mg protein/ml) were added to the same medium used for respiration and supplemented with 0.1 U/ml horseradish peroxidase and 50 μM Amplex red reagent. The assays were performed in the presence of pyruvate/malate as substrates (5 and 2.5 mM, respectively) in State 4

Table 1
Oligonucleotide primer sequences and amplification conditions

Fragment	Primer sequence (5′–3′)	Denaturation temp °C (time, s)	Annealing temp °C (time, s)	Extension temp °C (time, s)	Melting temp °C
mtDNA quantification	F: TACACGATGAGGCAACCAAA R: GGTAGGGGGTGTGTTGTGAG	95 (10)	60 (12)	72 (12)	80.6
Large fragment (LF) mtDNA[a]	F: CCTGAAACTTCAATGCCAAA R: CGAAGAGGTAAGATCATCTGGT	95 (10)	58 (12)	72 (12)	78.6
Small fragment (SF) mtDNA[a]	F: CAGCACAAAGTCCTGTGGAA R: CGAAGAGGTAAGATCATCTGGT	95 (10)	58 (12)	72 (12)	74.7

F, forward; R, reverse.
[a] LF and SF were coamplified.

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Table 2
Biometric parameters, total protein content, and mitochondrial features in aged male and female rat brain

	Males	Females	*P*
Body weight (g)	707±41	396±16*	0.000
Brain weight (g)	2.20±0.05	2.01±0.04*	0.008
%BW[a]	0.312±0.017	0.510±0.016*	0.000
Total protein (mg/g tissue)	65.1±2.6	77.2±1.6*	0.002
Mitochondrial protein (mg/g tissue)	34.4±2.0	45.5±4.1*	0.021
Mitochondrial DNA (a.u./g tissue)[b]	100±12.2	77±5	0.056
Mitochondrial protein/mtDNA (mg/a.u.)	0.374±0.063	0.594±0.027*	0.011
Citrate synthase activity (μIU/g tissue)[c]	22.1±2.5	29.1±1.1*	0.018
Catalase activity (mIU/g tissue)	0.273±0.029	0.248±0.034	0.235

Values are means ± SEM from six animals per group.
[a] Percentage that brain weight represents with regard to body weight.
[b] a.u., arbitrary units; levels found in male rats were set at 100%.
[c] IU, International Units.
* Significant difference between male and female rats ($p < 0.05$).

of mitochondrial respiration, or in the presence of these substrates plus 2 μM rotenone as inhibitor of complex I, or plus 500 μM GDP as inhibitor of mitochondrial chain uncoupling.

Tissue oxidative damage

Protein carbonyl group content of brain homogenate and isolated mitochondria was measured by an Oxyblot Protein Oxidation Detection Kit using a Bio-Dot SF apparatus (Bio-Rad). The carbonyl groups from 5 μg of homogenate protein and 0.5 μg of mitochondrial protein were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine. The DNP-derivatized samples were dot blotted onto a nitrocellulose filter, which was incubated with primary antibody specific to the DNP moiety of the proteins. Incubation with appropriate secondary antibody–horseradish peroxidase, treatment with enhanced chemiluminescent reagent, exposure to sensitive film, and quantification by photodensitometric analysis (Kodak 1D Image Analysis Software, New York, NY, USA) were then performed.

Malondialdehyde, a low-molecular-weight end-product of lipid peroxidation decomposition used as an index of lipid peroxidation, was measured as thiobarbituric acid-reactive substances (TBARS) content using a quantitative kit in homogenates by the previously described method [42].

Many mtDNA lesions are located in the mitochondrial displacement loop region (D-loop) and in particular in a polycytidine stretch (C-tract). The D-loop is highly susceptible to mutations because of its vulnerability to DNA damage and inefficient repair mechanisms. Moreover, there are large differences in the susceptibility to damage of various regions of mtDNA. The purpose of this assay was to examine the ROS-induced DNA damage in the mitochondrial genome, based on previous methods [43–46]. Two different-sized fragments located in the same mtDNA region, a short fragment (SF; 100 bp) and a long fragment (LF; 188 bp) containing the SF, were coamplified by real-time PCR. The LF, which includes the C-tract region, is more sensitive to oxidative damage than the SF; in previous experiments, we observed that its amplification was delayed compared with the SF after exposure to DNA-damaging agents (H_2O_2) in a dose-dependent manner. Pretreatment of mitochondrial pellet suspensions to obtain mtDNA was carried out as described above for the quantification of mtDNA. After the amplification of mitochondrial-specific regions, the LF/SF ratio for each sample was calculated by dividing the crossing point (Ct) of its long fragment by the Ct of its short fragment. There was a positive correlation between the increase in this ratio and mtDNA oxidative damage in the analyzed sample. All oligonucleotide primer sequences were obtained from Primer3 and tested with IDT OligoAnalyser 3.0. Finally, a basic local alignment search tool (NCBI Blast) revealed that the primer sequence homology was obtained only for the target genes. Primers and conditions are shown in Table 1.

Western blot of UCP4 and UCP5

Forty micrograms of total protein was electrophoresed on 10% v/v polyacrylamide SDS–PAGE gels. Proteins were electrotransferred onto a nitrocellulose filter. Ponceau S staining was performed to check correct loading and electrophoretic transfer. Blots were incubated with the corresponding antibodies against UCP4 and UCP5. Immunoblot development was performed using an enhanced chemiluminescence Western blotting analysis system. Film blots were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software, New York, NY, USA). Apparent molecular weights of proteins were estimated using a protein molecular-mass standard.

Sex steroid circulating levels

Blood samples were allowed to clot and were centrifuged at 1000 *g* for 10 min to obtain serum. Samples were stored at −70°C until use. Serum testosterone, progesterone, and 17β-estradiol levels were measured using competitive immunoenzymatic colorimetric kits according to the manufacturer's protocols (Diametra, Milano, Italy).

Statistics

All statistical analyses were performed with the Statistical Program for the Social Sciences software for Windows (SPSS version 15.0). Values were expressed as means ± SEM. Differences between groups were assessed by Student's *t* test. Statistical significance was set at $p<0.05$.

Results

Body weight and brain composition

Body and brain weights were higher in male rats than in females; however, the brain was proportionally larger in females taking into account the brain-to-body relative percentage (Table 2). A higher content of total and mitochondrial protein was found in female rats. In contrast, brain mtDNA content, an indicator of mitochondria number [47,48], was slightly lower in females than in males. Thus, the mitochondrial-protein/mtDNA ratio was higher in female rats,

Table 3
Mitochondrial COX and ATPase activities and O_2 consumption in aged male and female rat brain

	Males	Females	*p*
COX activity			
(a.u./mg mt protein)[a]	100±6	85.8±6.7	0.082
(a.u./g tissue)[a]	100±2	112±4*	0.014
ATPase activity			
(IU/mg mt protein)[b]	0.341±0.037	0.305±0.010	0.185
(IU/g tissue)	10.8±0.1	13.7±0.8*	0.011
O_2 consumption S3			
(nmol/min mg mt protein)	134±9	97±14*	0.027
(μmol/min g tissue)	4.44±0.03	4.25±0.50	0.361
O_2 consumption S4			
(nmol/min mg mt protein)	20.3±2.1	16.4±1.8	0.098
(μmol/min g tissue)	0.63±0.03	0.73±0.07	0.150
RCR	6.74±0.39	5.86±0.50	0.102

Measurements of O_2 consumption in both State 3 (S3) with ADP and State 4 (S4) without ADP were carried out with pyruvate/malate as substrates. RCR, respiratory control rate, State 3/State 4. Values are means ± SEM from six animals per group.
[a] a.u., arbitrary units; levels found in male rats were set at 100%.
[b] IU, International Units.
* Significant difference between male and female rats ($p < 0.05$).

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indicating greater protein content per mitochondrion in this sex. Furthermore, female rats had greater citrate synthase activity—a marker of mitochondrial mass. Catalase activity was similar in both genders in whole tissue.

Most of the parameters analyzed have been expressed both per milligram of mitochondrial protein and per gram of tissue when required. Expression per gram of tissue is considered to have a more physiological relevance owing to the higher amount of mitochondrial protein described in females with respect to males (Table 2).

Mitochondrial oxidative function

Female mitochondria showed lower COX and ATPase activities expressed per milligram of mitochondrial protein, although differences were not statistically significant. However, when these activities were expressed per gram of tissue, they did show statistically significant higher values in females than in males (Table 3). In contrast, oxygen consumption using pyruvate/malate as substrate both in the presence (State 3) and in the absence (State 4) of ADP was lower in female brain mitochondria than in those of males when expressed per milligram of mitochondrial protein, but was significant only in State 3. Nevertheless, when expressed per gram of tissue, oxygen consumption showed similar levels between sexes. RCR (State 3/State 4) was also lower in female mitochondria, although without reaching statistical significance.

Mitochondrial oxidative stress

Table 4 shows brain mitochondrial H_2O_2 production with pyruvate/malate as substrate in State 4 and also in the presence of rotenone as an inhibitor of complex and antioxidant enzyme activities. Mitochondria from female rats showed lower H_2O_2 production per milligram of mitochondrial protein in State 4, but it did not reach statistical significance, owing to the great variability of values from males. Maximum H_2O_2 production capacity per milligram of mitochondrial protein (in the presence of rotenone) was lower in female mitochondria than in those of males. Remarkably, female mitochondrial H_2O_2 production per gram of tissue equaled, without exceeding, the levels of males under both conditions, State 4 and in the presence of rotenone. The activities of the antioxidant enzymes GPx and SOD were similar in both sexes when expressed per milligram of mitochondrial protein. However, these activities expressed per gram of tissue were higher in females, although only GPx reached statistically significant difference.

Table 4
Mitochondrial H_2O_2 production and antioxidant enzyme activities in aged male and female rat brain

	Males	Females	*p*
H_2O_2 production S4			
(pmol/min mg mt protein)	8.76±2.98	6.45±0.72	0.215
(nmol/min g tissue)	0.306±0.116	0.297±0.046	0.470
H_2O_2 production Rot			
(pmol/min mg mt protein)	39.8±1.44	32.8±1.54*	0.005
(nmol/min g tissue)	1.37±0.085	1.49±0.141	0.238
GPx activity			
(mIU/mg mt protein)[a]	19.4±1.7	19.9±1.3	0.424
(IU/g tissue)	0.657±0.036	0.888±0.045	0.002
SOD activity			
(mIU/mg mt protein)	8.92±1.80	8.82±2.20	0.487
(IU/g tissue)	0.348±0.030	0.442±0.081	0.159

Measurements of H_2O_2 production in State 4 of respiration (S4) and in the presence of rotenone (Rot) were carried out with pyruvate/malate as substrates. Values are means ± SEM from six animals per group.
[a] IU, International Units.
* Significant difference between male and female rats ($p < 0.05$).

Table 5
Oxidative damage in aged male and female rat brain

	Males	Females	*p*
Total protein oxidation			
(a.u./mg total protein)[a]	100±2	80.5±4.4*	0.003
(a.u./g tissue)[a]	100±4	96.0±3.4	0.261
Mitochondrial protein oxidation			
(a.u./mg mt protein)[a]	100±2	86.6±2.9*	0.010
(a.u./g tissue)[a]	100±5	98.3±4.6	0.408
TBARS			
(nmol/mg total protein)	9.97±0.20	9.33±0.23*	0.031
(nmol/g tissue)	649±30	653±19	0.463
mtDNA oxidation index (Ct_{LF}/Ct_{SF})[b]	1.15±0.003	1.13±0.01	0.103

Values are means ± SEM from six animals per group.
[a] a.u., arbitrary units; levels found in male rats were set at 100%.
[b] See Materials and methods.
* Significant difference between male and female rats ($p < 0.05$).

Oxidation of total and mitochondrial protein, measured as protein carbonyl group levels, and total lipid peroxidation, measured as TBARS levels, expressed per protein content of corresponding sample, were significantly lower in female rats compared to males (Table 5), but these differences disappear when the results are expressed per gram of tissue. There were no differences between genders in the mtDNA oxidation index.

UCP4 and UCP5 protein levels

UCP4 and UCP5 levels, expressed per milligram of total protein, were higher in female rats than in males, but reached statistical significance only for UCP5 (Fig. 1). Given the higher total protein in female brain, differences between sexes were more evident when the levels were expressed per gram of tissue, with females reaching 55 and 102% more than males for UCP4 and UCP5, respectively.

Sex steroid hormone serum levels

17β-Estradiol and progesterone were higher in female rats than in males, whereas testosterone was higher in males than in females (Table 6). In all cases these differences were statistically significant.

Discussion

This study has demonstrated that aged female rat brain has lower mitochondrial content than aged male brain but with a greater differentiation degree given the higher oxidative and phosphorylative mitochondrial function and the greater antioxidant capacity.

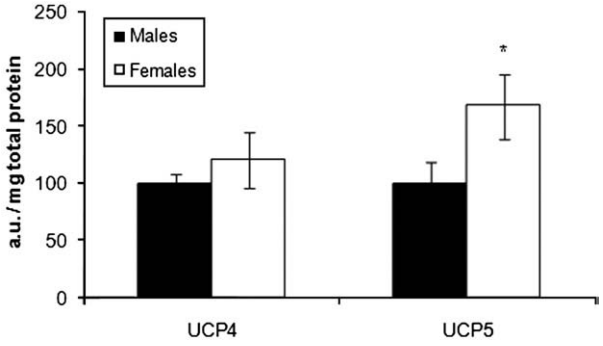

Fig. 1. UCP4 and UCP5 protein levels in aged male and female rat brain. Values are expressed as means ± SEM from six animals per group. a.u., arbitrary units; levels found in male rats were set at 100%. *Significant difference between male and female rats ($p < 0.05$).

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Female rat brain had greater mitochondrial functionality than male brain according to both the higher mitochondrial protein content and the citrate synthase activity—markers of mitochondrial function. In fact, the higher total protein content per gram of tissue in female brain compared with males (12.1 mg total protein/g of tissue more in females) could be attributed to the mitochondrial protein, because the difference in mitochondrial content between males and females was practically the same as the above (11.1 mg mitochondrial protein/g of tissue more in females). In contrast, taking into account that in a given tissue a direct relationship between mtDNA number copies and mitochondria number has been described [48,49] and, moreover, mtDNA per mitochondrion is maintained at a constant level, it is thus assumed that mtDNA levels are a good indicator of mitochondrial number [47]. In this work, female rat brain had lower mitochondrial content, as shown by mtDNA. Therefore, taking into account mitochondrial protein and mtDNA and considering their ratio as a mitochondrial differentiation marker [15,16], the female brain thus showed a greater mitochondrial differentiation degree than that of males, which would be related to the lower mitochondrial content observed in this sex. More differentiated mitochondria in females has been described in other tissues such as liver [15,16], brown adipose tissue [17,18], cardiac muscle [20], and skeletal muscle [21]. In agreement with the greater mitochondrial machinery that reflects the greater degree of differentiation, female rat brain showed greater oxidative and phosphorylative mitochondrial capacities than that of males, whereas oxygen consumption reached the same levels in both sexes. These results are also in concordance with the greater oxidative and phosphorylative mitochondrial capacities observed in the above-mentioned tissues [15,19–21].

Regarding these sex-dependent mitochondria differences, the sex hormones 17β-estradiol and progesterone have been proved to be involved in the enhanced expression of nuclear and mitochondrial genes encoding proteins from the mitochondrial respiratory chain (MRC) [50] and involved in enhanced MRC function [51]. Although sex hormone levels have been described extensively as declining toward the end of life, especially in females, when they become acyclic [52], females still had much higher 17β-estradiol and progesterone levels than males in the studied 24-month-old rats.

The lower H_2O_2 production per milligram of mitochondrial protein in female rats was in agreement with other studies in cardiac muscle [20], liver, and brain [53], a pattern that has been attributed to their higher estrogen levels [54]. Nevertheless, the higher MRC activity in female brain was not accompanied by a higher H_2O_2 production per gram of tissue. In contrast, some authors have also attributed an upregulating effect on the activities of mitochondrial antioxidant enzymes such as MnSOD and GPx to estrogens [54–56]. The notion of an estrogen influence on antioxidant enzyme activity is supported by the study by Pinto and Bartley, which describes no differences in liver between male and female rats until sexual maturity, at which time, in females, the activity increased abruptly [57]. In our study, even though no differences between sexes were found in the antioxidant enzyme activities per milligram of mitochondrial protein, when the activity was expressed per gram of tissue, which could be considered to better reflect the functional capacity, 24-month-old female brain showed a higher GPx activity and a tendency to greater MnSOD activity, suggesting that the neuroprotective effect of estrogens [54] is maintained in aged female rats. In fact, a suppression of brain mitochondrial stress by estrogen treatment has been recently described in both cultured cells and in gonadectomized female and male rats [56].

Table 6
Serum levels of sex hormones in aged male and female rats

	Males	Females	*p*
17β-Estradiol (pg/ml)	15.7±4.5	27.3±3.9*	0.002
Progesterone (ng/ml)	1.28±0.37	23.2±6.7*	0.015
Testosterone (ng/ml)	0.401±0.063	0.094±0.023*	0.001

Values are means ± SEM from six animals per group.
* Significant difference between male and female rats ($p < 0.05$).

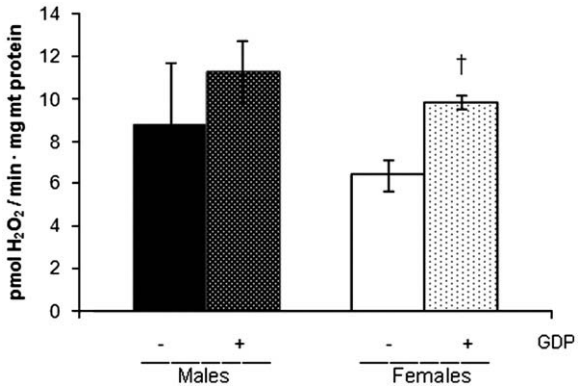

Fig. 2. GDP effect on mitochondrial H_2O_2 production in State 4 in aged male and female rat brain. Substrates were pyruvate/malate in all cases. Values are expressed as means ± SEM from six animals per group. †Significant difference between State 4 without GDP and with GDP ($p < 0.05$).

As a result of the oxidative balance, less oxidative damage per milligram of protein in female than in male rats has been described in liver and brain [54] and in cardiac muscle [20]. In the present study, 24-month-old female rats continued showing less brain oxidative damage per milligram of mitochondrial protein than males, expressed per protein content of corresponding sample, reflecting the sex differences in oxidative stress balance.

Studies have described that antioxidant enzymes are overexpressed in females only in species in which females live longer [53,58]; however, in mice, in which the males live longer than the females, this does not occur [59].

UCP4 and UCP5 have been described as downregulators of brain mitochondrial ROS production [31,32], due to their dissipating effect on the proton gradient [24,31,32], in which even mild uncoupling significantly reduces the ROS production [60]. The higher UCP4 and UCP5 levels observed in aged female rats with respect to males would contribute to the lower H_2O_2 production in this sex. To confirm the role of these uncoupling proteins in the sex-dependent oxidative balance differences, we analyzed respiratory State 4 H_2O_2 production in the absence and presence of GDP, a UCP function inhibitor [28] (see Fig. 2). GDP addition caused a rise in H_2O_2 production in both sexes, but the increase was higher in female brain mitochondria (53%) than in those of males (24%), probably reflecting an important UCP role in diminishing H_2O_2 production in females.

In summary, this study has demonstrated that aged female rat brain had lower mitochondrial content but with a greater differentiation degree than male brain. In accordance with this, female rat brain showed a better oxidative balance than that of males, reflected by a higher MRC function, similar ROS production, and greater antioxidant enzyme activities, which could be responsible for the lesser oxidative damage in proteins and lipids observed in this sex. In addition, this study has shown that UCP4 and UCP5 could also be involved in the better oxidative homeostasis maintenance of females, leading to greater neuroprotection and contributing to a lower incidence and progression of neurological disease in females.

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MANUSCRITO II / *MANUSCRIPT II*

Age and sex-related changes in rat brain mitochondrial function.

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AGE AND SEX-RELATED CHANGES IN RAT BRAIN MITOCHONDRIAL FUNCTION

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ABSTRACT

Aging is responsible for the decline in the function of mitochondria and their increase in size and number - adaptive mechanism to restore mitochondrial function. Estrogens increase mitochondrial function, specially in female rats. The aim of this study was to determine the age-related changes in rat brain mitochondrial function focusing on sex differences. Mitochondria protein content diminished during aging shifting toward lesser mitochondrial differentiation and the mitochondria number increased. A sex dimorphism was determined, with female rat brain showing more highly differentiated mitochondria than males. These sex differences gradually increased during aging, possibly due in part to the sex-hormonal milieu.

Keywords: Aging, sex differences, mitochondrial function, estrogens, brain.

Abbreviations: mitochondrial DNA (mtDNA), nuclear DNA (nDNA), mitochondrial protein (mt protein), Citrate synthase (Kann et al.), ferrocytochrome-c:oxygen oxidoreductase (COX), ATP synthase (ATPase), active respiration (state 3), resting respiration (State 4).

1. INTRODUCTION

Aging is characterized by a general decline of physiological function, determined by cellular dysfunction and mitochondria impairment, with a more marked effect in the functions that depend on the central nervous system (Forster et al., 1996; Navarro et al., 2007). Many studies have reported a decline in mitochondrial function upon aging consisting of a decrease in electron transfer activity and ATP production (Shigenaga et al., 1994; Navarro et al., 2007) whereas other authors have described an increase of mtDNA copy number in senescent tissues (Gadaleta et al., 1992) and age-dependent increased mitochondrial size (Sastre et al., 1998), supporting the idea of an adaptive mechanism designed to restore mitochondrial function.

Brain has a very high metabolic rate, as it consumes about 20% of the oxygen inspired at rest, while accounting for only 0.5-2% of the body weight (Silver et al., 1998). This high metabolic demand is because neurons are highly differentiated cells needing large amounts of ATP for maintenance of ionic gradients across the cell membranes and for neurotransmission. Since most neuronal ATP is generated by oxidative metabolism, neurons critically depend on mitochondrial function and oxygen supply (Ames, 2000; Erecinska et al., 2004).

Sex hormones, specifically estrogens, have profound neuro-protective effects, having been reported to increase mitochondrial respiration efficiency and mitochondrial functions (Nilsen et al., 2004). Several studies have reported that female rats show more highly differentiated mitochondria than males in liver (Justo et al., 2005; Valle et al., 2007), brown adipose tissue (Rodriguez-Cuenca et al., 2002; Justo et al., 2005; Valle et al., 2007), cardiac (Colom et al., 2007) and skeletal muscle (Colom et al., 2007), and aged brain (Guevara et al., 2009), meaning greater mitochondrial machinery and, as a result, higher capacity and efficiency.

Taking all these precedents into account, the aim of this study was to determine the age-related changes in rat brain mitochondrial function focusing on sex differences. To tackle this aim we analyzed both cellular and mitochondrial protein and DNA content, and mitochondrial oxidative and phosphorylative function in male and female rat brain from four different age groups: 6, 12, 18 and 24 months old.

2. MATERIALS AND METHODS

2.1. Chemicals

Routine chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), Roche Diagnostics (Basel, Switzerland), Bio-Rad (Hercules, CA, USA) and Panreac (Barcelona, Spain). Antibodies against COX IV subunit and COX II subunit were obtained from MitoSciences (Eugene, OR, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively.

2.2. Animals, sacrifice and mitochondrial isolation

Animals were treated in accordance with the University Bioethical Committee guidelines for animal care and European Union regulations (2003/65/CE).

Wistar rats, supplied by Charles River (Barcelona, Spain), were housed individually at 22 °C with a 12-h light/dark cycle and fed *ad libitum* with pelleted standard diet (A04 Panlab, Barcelona, Spain). Eight groups were made: males and females of four different ages, 6 months old (young rats), 12 months old (adult rats), 18 months old (old rats) and 24 months old (senescent rats), including 6-8 animals in each group.

All animals were weighed and sacrificed by decapitation at the start of the light cycle. After sacrifice animals underwent a visual internal inspection in order to exclude old animals with any pathological signal. Brain was quickly removed and weighed. Homogenization and mitochondrial isolation were performed according to previously described procedure (Lai et al., 1979). Briefly, brain was homogenized in 35 mL of cold isolation medium (0.25 M sucrose, 10 mM tris-HCl and 0.5 mM EDTA-K^+, pH 7.4) in a manual homogenizer with a glass pestle, total clearance 0.1 mm. An aliquot of the homogenate was stored at -20° C for posterior analyses and the remainder was used to perform biochemical assays and for mitochondrial isolation. Nuclei and cell debris were twice sedimented by differential centrifugation at 2000xg for 3 min and the pellets were discarded. Supernatant was subjected to a further centrifugation at 12500xg for 8 min and the crude mitochondrial pellet obtained was resuspended with 0.8 mL of isolation medium. Protein concentration was determined in both homogenate and mitochondrial fraction by Bradford's method (Bradford, 1976). Total DNA content was tested in homogenate as previously described (Thomas et al., 1978).

2.3. Quantification of mitochondrial DNA by real-time PCR

Mitochondrial DNA (mtDNA) extraction and semiquantification were carried out as previously described (Justo et al., 2005). Briefly, real-time PCR was performed to amplify a 162-nt region of the mitochondrial NADH dehydrogenase subunit 4 gene, which is exclusive to mtDNA. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche) using a total volume of 10 µl containing each primer at 0.375 µM, 3 mM MgCl2, 1 µl LightCycler FastStart DNA Master SYBR Green I, and 2.5 µl of pretreated mitochondrial sample. The PCRs were cycled 35 times after initial denaturation (95°C, 10 min). The primer sequences were 5'-TACACGATGAGGCAACCAAA-3' and 5'-GGTAGGGGGTGTGTTGTGAG3'.

2.4. Enzymatic activities

Citrate synthase (CS, EC 2.3.3.1) (Nakano et al., 2005), ferrocytochrome-c:oxygen oxidoreductase (COX, EC 1.9.3.1) (Chrzanowska-Lightowlers et al., 1993) and ATP synthase (ATPase, EC 3.6.1.3) (Ragan et al., 1987) activities were determined in resuspended mitochondrial pellet.

2.5. Mitochondrial oxygen consumption

Oxygen consumption of brain mitochondria was measured by a thermostatically controlled Clark-type O_2 electrode (Oxygraph, Hansatech, UK). Reaction conditions were 1 mg of mitochondrial protein in up to 0.5 mL of respiration buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA and 0.1% BSA fatty acid free, pH 7.4) at 37º C. The substrate used was pyruvate/malate (5 mM and 2.5 mM respectively) in the presence (State 3, phosphorylating) and absence (State 4, resting) of 500 µM ADP. Mitochondrial viability was checked by the respiratory control rate (RCR = state 3 / state 4).

2.6. Western blot of COX IV subunit and COX II subunit

10 µg of total protein in the case of COX IV and 40 µg in the case of COX II were electrophoresed on 15% v/v polyacrilamide SDS-PAGE gels. Proteins were electrotransferred onto a nitrocellulose membrane and Ponceau S staining was performed to check the correct loading and electrophoretic transfer. Membranes were incubated with

the corresponding antibodies against COX IV subunit and COX II subunit; immunoreactive bands were developed by chemiluminescence, detected by a ChemiDoc XRS system (BIO-Rad, CA, USA) and quantified by the image analysis program Quantity One© (BIO-Rad, CA, USA). Apparent molecular weights of proteins were estimated using a protein molecular-mass standard.

2.7. Statistics

All statistical analyses were performed with the Statistical Program for Social Sciences software SPSS version 17.0 for Windows (Chicago, IL, USA). Data are expressed as mean ± SEM. Differences between groups were assessed by two-way analysis of variance (ANOVA) to determine the effect of age and sex, and Student's t-test for post-hoc comparisons. Statistical significance was set at $p<0.05$.

3. RESULTS AND DISCUSSION

3.1. Body weight and brain composition

Table 1 shows biometric parameters and protein and DNA content in male and female rat brain during aging process (6, 12, 18 and 24-mo old rats). Body and brain weights gradually increased with age, but were always higher in male than in female rats. A slight increase with age until 18-mo was observed in brain total protein content in both genders and a marked sex dimorphism, with an increase in females and no change in males between 18-mo to 24-mo, was detected. Total DNA content in rat brain presented no changes until 18-mo old and a decrease at 24-mo, similar to previously published results (Haripriya et al., 2005). This decrease was more evident in female rats than in males (-47% females and -31% in males); suggesting that the decrease in brain cell number was accompanied by a slight, gradual increase in protein content from 6-mo to 18-mo old rats in both sexes, and showing a marked increase in 24-mo only in females. The total protein/total DNA ratio reflected this pattern and showed a sex dimorphism, with a greater increase in brain cell protein density in females compared to males at senescence (+116% females vs. +51% males).

3.2. Mitochondrial DNA, protein and citrate synthase

mtDNA content (Table 2), a parameter that could reflect the mitochondria number (Djouadi et al., 1994; Koekemoer et al., 2001), showed a marked, yet gradual increase from 6-mo to 24-mo of age, which was higher in females than males (+237% females vs. +147% males). An increase in the amount of mtDNA with age has previously been described in other tissues such us liver (Dinardo et al., 2003), brown adipose tissue (Valle et al., 2008), heart (Gadaleta et al., 1992), spleen and kidney (Masuyama et al., 2005). The mtDNA/total DNA ratio, which reflects the mitochondrial content per cell, showed a sharp increase, more marked from old age to senescence. This pattern is more evident in females than in male rats (6.3 vs. 3.6 fold respectively, when 6-mo to 24-mo ages were compared). Mitochondrial protein (mt protein), indicator of mitochondrial functionality, underwent a progressive increase with age in both sexes and this increase was again greater in females than in males, reaching sex differences at 24-mo old. These results highlight the fact that the increase in brain mt protein content rose at a lower rate than the mtDNA content did, thus, the mt protein/mtDNA ratio showed that protein content per mitochondrion decreased with age in both sexes, with females showing higher protein content per mitochondrion at any age, indicative of a sex dimorphism. Likewise, citrate synthase – a Krebs cycle key enzyme - activity was also increased during aging with a similar profile to mt protein, again showing a sex dimorphism, with a greater increase in females with respect to males (+90% females vs. +48% males). Considering mitochondrial differentiation as an increase in the functional capabilities of pre-existing mitochondria in order to acquire the ultrastructure and functional features of mature mitochondria (Koekemoer et al., 2001; Justo et al., 2002), the mt protein/mtDNA ratio, which reflects the mitochondrial machinery per mitochondria (Quevedo et al., 1998; Rodriguez et al., 2002), could be taken as a good mitochondrial differentiation marker. Our results show that rat brain mitochondria lost functional capacity with age in both sexes, especially from old age; however, females maintained mitochondrial functionality with a greater degree of differentiation than males at any age.

3.3. Mitochondrial oxidative and phosphorylative function

Indicative parameters of mitochondrial oxidative and phosphorylative function in both sexes during aging are shown in Table 3. These data are expressed per gram of tissue to determine the total functional capacity of the studied animal brain. COX activity was measured to assess the oxidative mitochondrial capacity, since it catalyzes a rate-controlling step of mitochondrial respiration. Rat brain COX activity was maintained with

age in both male and female rats; however, female COX activity was higher than that of males at any analysed age. Other authors report a decline of COX activity in old rats but only in males (Navarro et al., 2004). ATPase activity was increased with age in both sexes, showing the same sex-dependent difference as the other mitochondrial parameters analyzed, that is, the increase was again more marked in female rat brain than in males, reaching higher levels at 24-mo of age. Oxygen consumption using pyruvate/malate as substrate, both in the presence (State 3) and the absence (State 4) of ADP, showed a gradual increase during aging, with similar levels between sexes.

These mitochondrial function results are in agreement with the aforementioned increase in mt protein with age in both sexes, showing only a clear sex dimorphism in ATPase activity of the oldest rats. On the other hand, COX activity showed a different pattern, maintaining its levels during aging; nevertheless, the sex dimorphism was present at all ages.

3.4. COX content

Because of the different pattern followed by COX activity compared with the other mitochondrial features and taking into account that enzymatic activity can be related to enzymatic content, we decided to quantify the protein levels of the nuclear DNA (nDNA)-encoded COX IV and the mtDNA-encoded COX II subunits by western blot (Fig. 1). COX IV subunit content remained practically unchanged with age in both sexes, whereas COX II subunit content underwent a pronounced increase with age, greater in females than in males, leading to a marked difference between sexes at the end of the studied period. In a given cell or tissue, the total mitochondrial function, depending on mitochondrial biogenesis, is the result of both the proliferation process, which defines mitochondrial population, and the differentiation process, which defines mitochondrial functional capabilities (Justo et al., 2005; Alcolea et al., 2008). Mitochondrial protein content is the result of the coordinated expression of both mtDNA and nDNA genes (Garesse et al., 2001). In this context, our results could indicate that although the mtDNA-encoded COX II subunit content increased with age in parallel to the increase in mtDNA, the nDNA-encoded COX IV subunit content remained unchanged with age, which agrees with the unchanged profile of COX activity.

However, the sex dimorphism in COX activity, with higher levels in females with respect to males could not be explained by COX IV subunit content since they were very similar in both sexes. This fact could suggest a COX activity up-regulation in female rat

brain, which could be related to the different hormonal milieu as has been described in other tissues (Colom et al., 2007; Valle et al., 2007). In this sense, 17β-estradiol and progesterone have been proved to be involved in the enhancement of both the function (Chen et al., 2004) and expression of nuclear and mitochondrial genes encoding proteins from the mitochondrial respiratory chain (Chen et al., 2005). Although sex hormone levels, which show a marked sex dimorphism, have been described extensively as declining toward the end of life, males and females still had higher levels of corresponding sex-hormones than the other sex at senescence (Guevara et al., 2008). This could explain, at least in part, the sex-dependent differences found in some mitochondrial features and function.

3.5. Conclusions

To sum up, this study has demonstrated that rat brain mitochondria decreased their protein content during aging, especially between old age and senescence, indicating a shift toward lesser mitochondrial differentiation linked with age, which could be counteracted by increasing the mitochondria number. As in other tissues, a sex dimorphism in mitochondrial function has been determined, with female rat brain showing more highly differentiated mitochondria than male ones. These sex differences gradually increased during aging, possibly due in part to the sex-hormonal milieu, which could lead to lesser mitochondrial dysfunction induced by aging and thus a greater neuroprotection of female rat brain.

Table 1. Biometric parameters and brain protein and DNA content in male and female rats during aging process.

		6 months	12 months	18 months	24 months	ANOVA
Body weight (g)	M	484 ± 13	601 ± 15[a]	579 ± 17[b]	707 ± 32[cef]	A, S, AxS
	F	264 ± 6*	310 ± 9*[a]	353 ± 14*[bd]	396 ± 16*[cef]	
Brain weight (g)	M	2.00 ± 0.04	2.15 ± 0.01	2.11 ± 0.03	2.20 ± 0.05	A_{abc}, S
	F	1.82 ± 0.02*	1.95 ± 0.02*	2.02 ± 0.02*	2.01 ± 0.04*	
Brain total protein (mg/g tissue)	M	51.8 ± 1.1	52.4 ± 0.3	55.4 ± 0.9	53.3 ± 3.0	A_{bcef}, AxS
	F	52.0 ± 0.6	52.8 ± 1.4	54.2 ± 1.2	61.2 ± 2.6*	
Brain total DNA (mg/g tissue)	M	1.00 ± 0.03	1.09 ± 0.05	1.13 ± 0.05	0.69 ± 0.04	A_{cef}, AxS
	F	1.15 ± 0.05*	1.08 ± 0.04	1.03 ± 0.04	0.61 ± 0.01	
Total protein/total DNA (mg/mg)	M	51.9 ± 1.2	48.6 ± 2.2	49.4 ± 2.2	78.4 ± 7.2[cef]	A, S, AxS
	F	46.7 ± 1.0	49.2 ± 2.6	53.2 ± 2.1[b]	100.8 ± 3.0*[cef]	

Values are mean ± SEM; n = 6 - 8 animals per group. ANOVA ($p<0.05$). A: age effect; S: sex effect; AxS: age and sex interaction; NS: no significant differences. Student's t-test post hoc analysis ($p<0.05$). [a] 12-mo old vs. 6-mo old; [b] 18-mo old vs. 6-mo old; [c] 24-mo old vs. 6-mo old; [d] 18-mo old vs. 12-mo old; [e] 24-mo old vs. 12-mo old; [f] 24-mo old vs. 18-mo old. * females vs. males.
M: males; F: females.

Table 2. Mitochondrial DNA, protein and citrate synthase in male and female rat brain during aging process.

		6 months	12 months	18 months	24 months	ANOVA
mtDNA (a.u./g tissue)	M	100 ± 6	149 ± 9	188 ± 23	247 ± 17	A_{abcdef}, S
	F	65 ± 8*	132 ± 3	143 ± 23	219 ± 16	
mtDNA/totalDNA (a.u./ug)	M	12.9 ± 0.9	17.4 ± 0.8[a]	21.6 ± 2.9[bd]	46.1 ± 4.4[e]	A_{abcef}
	F	7.4 ± 0.9*	15.7 ± 0.8	18.0 ± 3.1[bd]	46.1 ± 3.2[ce]	
mt protein (mg/g tissue)	M	28.7 ± 1.6	34.4 ± 1.3	39.2 ± 1.3	42.1 ± 3.0	A_{abcdef}, S
	F	29.5 ± 1.2	40.7 ± 1.7*	42.6 ± 2.9	49.2 ± 0.9*	
mt protein/mtDNA (mg/a.u.)	M	2.32 ± 0.19	1.74 ± 0.13	1.82 ± 0.19	1.23 ± 0.06	A_{abcef}, S
	F	3.88 ± 0.43*	2.59 ± 0.21*	2.55 ± 0.48	1.71 ± 0.20*	
Citrate synthase activity (mIU/g tissue)	M	63.8 ± 3.1	58.2 ± 2.2	84.6 ± 3.7[bd]	94.1 ± 8.6[ce]	A, S, AxS
	F	59.4 ± 2.6	69.7 ± 2.7*[a]	86.3 ± 5.0[bd]	113.0 ± 6.2[cef]	

Values are mean ± SEM; n = 6-8 animals per group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post hoc analysis (LSD test, $p<0.05$): [a] 6-mo old vs. 12-mo old; [b] 6-mo old vs. 18-mo old; [c] 6-mo old vs. 24-mo old; [d] 12-mo old vs. 18-mo old; [e] 12-mo old vs. 24-mo old; [f] 18-mo old vs. 24-mo old. Post hoc analysis (Student's t-test, $p<0.05$): * females vs. males. M: males; F: females. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%. mt DNA: mitochondrial DNA; mt protein: mitochondrial protein.

Table 3. Mitochondrial oxidative and phosphorylative function in male and female rat brain during aging process.

.

		6 months	12 months	18 months	24 months	ANOVA
COX activity (a.u./g tissue)	M	100 ± 8	100 ± 1	97 ± 2	77 ± 17	S
	F	143 ± 8*	122 ± 7*	118 ± 2*	131 ± 12*	
ATPase activity (IU/g tissue)	M	7.78 ± 0.44	7.44 ± 0.53	10.07 ± 0.53	13.26 ± 0.97	A_{bcdef}, S
	F	7.70 ± 0.32	9.50 ± 0.53*	10.85 ± 0.98	16.30 ± 0.73*	
O2 consumption S3 (nmol/min·g tissue)	M	201 ± 16	241 ± 16	272 ± 25	360 ± 40	A_{abcef}
	F	213 ± 13	271 ± 15	257 ± 8	385 ± 22	
O2 consumption S4 (nmol/min·g tissue)	M	32.7 ± 1.4	39.3 ± 4.0	42.9 ± 3.2	53.4 ± 4.7	A_{abcef}
	F	34.6 ± 2.1	43.2 ± 4.0	43.0 ± 0.6	57.3 ± 6.6	

Values are mean ± SEM; n = 6-8 animals per Group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post hoc analysis (LSD test, $p<0.05$): [a] 6-mo old vs. 12-mo old; [b] 6-mo old vs. 18-mo old; [c] 6-mo old vs. 24-mo old; [d] 12-mo old vs. 18-mo old; [e] 12-mo old vs. 24-mo old; [f] 18-mo old vs. 24-mo old. Post hoc analysis (Student's t-test, $p<0.05$): * females vs. males. M: males; F: females. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%. COX: ferrocytochrome-c:oxygen oxidoreductase; ATPase: ATP synthase. S3: respiration state 3; S4: respiration state 4.

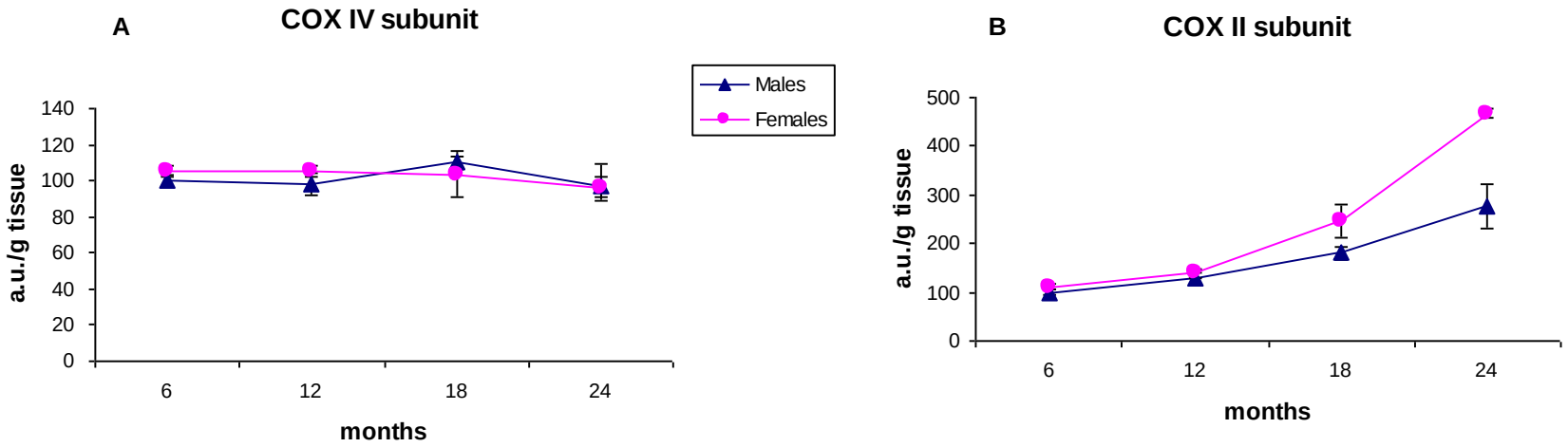

Figure 1. Protein content of COX IV subunit and COX II subunit in male and female rat brain during aging process.
Values are mean ± SEM; n = 6-8 animals per group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post hoc analysis (LSD test, $p<0.05$): [a] 6-mo old vs. 12-mo old; [b] 6-mo old vs. 18-mo old; [c] 6-mo old vs. 24-mo old; [d] 12-mo old vs. 18-mo old; [e] 12-mo old vs. 24-mo old; [f] 18-mo old vs. 24-mo old. Post hoc analysis (Student's t-test, $p<0.05$): * females vs. males. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%.

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MANUSCRITO III / *MANUSCRIPT III*

Age and sex-related changes in rat brain mitochondrial oxidative status.

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AGE AND SEX-RELATED CHANGES IN RAT BRAIN MITOCHONDRIAL OXIDATIVE STATUS

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ABSTRACT

Mitochondria are the main source of free radical species and the most direct target for their damaging effects, which especially affects the brain mitochondrial function, which is better maintained by females than males. The aim of this work was to investigate the age-related changes in rat brain mitochondrial oxidative status focusing on sex differences. Male and female rat brain from four different age groups (6, 12, 18 and 24 months old) were analyzed. Oxidative damage accumulates in rat brain throughout aging, related to the increasing MRC activity and failure of several antioxidant defences. The aging effect was less marked in females, which accumulated lesser oxidative damage than males due in part to the greater antioxidant capacity, such us higher GPx activity and UCP5 expression level. This sexual dimorphism gradually increased during aging.

Keywords: Aging, sex differences, mitochondria, oxidative balance, oxidative damage, uncoupling proteins, brain.

Abbreviations: Mitochondrial respiratory chain (MRC), active respiration (state 3), resting respiration (State 4), ferrocytochrome-c:oxygen oxidoreductase (COX), ATP synthase (ATPase), reactive oxygen species (ROS), glutathione peroxidase (GPx), superoxide dismutase SOD, uncoupling protein (UCP), 8-oxo-deoxyguanosine (8-oxo-dG), thiobarbituric acid-reactive substances (TBARS).

INTRODUCTION

Mitochondria are the main source of free radical species and the most direct target for their damaging effects. Oxidative lesions accumulated with age play a major role in causing mitochondrial dysfunction (Harman, 1972; Mecocci et al., 1993) and cellular impairment, which determines general decline of physiological function, which is critically involved in aging (Miquel et al., 1980) and degenerative diseases (Kann et al., 2007).

The brain is especially affected by mitochondrial dysfunction because of its very high metabolic demand for energy, mainly obtained from mitochondrial oxidative metabolism. Besides, the brain is believed to be particularly vulnerable to oxidative stress due to a relatively high rate of oxygen free radical generation without commensurate levels of antioxidant defences (Sohal et al., 1990; Halliwell, 1992). Thus, oxidative damage is considered a likely cause of age-associated brain dysfunction. Indeed, there is a progressive increase in the steady-state concentration of oxidatively modified DNA and proteins in the brain during aging (Stadtman, 1992; Sohal et al., 1994).

Females live longer than males in many mammalian species including humans (Schroots et al., 1999; Borras et al., 2007) and show lower incidence of several neurodegenerative diseases (Baldereschi et al., 2000). Several studies have reported that female rats show more highly differentiated mitochondria than males in liver (Justo et al., 2005; Valle et al., 2007), brown adipose tissue (Rodriguez-Cuenca et al., 2002; Justo et al., 2005; Valle et al., 2007), cardiac (Colom et al., 2007) and skeletal muscle (Colom et al., 2007), and brain (Guevara et al., 2009), which has been associated with greater mitochondrial functionality and, as a result, increased mitochondrial capacity and efficiency.

Previous studies carried out in our laboratory have demonstrated that rat brain mitochondria decrease their protein content during aging, indicating a shift toward lesser mitochondrial differentiation, which could be counteracted by increasing the mitochondria number (Guevara et al., 2011). Furthermore, a sexual dimorphism in mitochondrial function has been determined, with female rat brain better maintaining their mitochondrial function and differentiation degree than males. These sex differences gradually increase during aging, which lead to lesser mitochondrial dysfunction induced by aging in female rats (Guevara et al., 2011).

Uncoupling proteins (UCPs) belong to a family of ion carriers located in the inner mitochondrial membrane (Pecqueur et al., 2001). The protonphoric action of UCPs results in diminished mitochondrial membrane potential and reactive oxygen species (ROS) production (Negre-Salvayre et al., 1997). The uncoupling activity of UCPs can also be activated by oxidative stress (Echtay et al., 2002; Echtay et al., 2003) and inhibited by purine nucleotides (Nicholls et al., 1984). UCP4 and UCP5 are expressed mainly in the brain (Sanchis et al., 1998; Mao et al., 1999) and have been reported to play a role in reduction of ROS production (Kim-Han et al., 2001; Liu et al., 2006).

Taking this background into account, the aim of this work was to investigate the age-related changes in rat brain mitochondrial oxidative balance focusing on sex differences. To tackle this aim we studied mitochondrial function parameters such as oxygen consumption, hydrogen peroxide (H_2O_2) production, antioxidant system activities, UCP4 and UCP5 protein levels and oxidative damage in male and female rat brain from four different age groups: 6, 12, 18 and 24 months old.

MATERIALS AND METHODS

Chemicals

Routine chemicals were supplied by Sigma-Aldrich (St. Louis, MO, USA), Roche Diagnostics (Basel, Switzerland), Bio-Rad (Hercules, CA, USA) and Panreac (Barcelona, Spain). Antibodies against UCP4 and UCP5 were obtained from Alpha Diagnostic International (San Antonio, TX, USA) and antibody against 8-oxo-deoxyguanosine (8-oxo-dG) from Trevigen (Gaithersburg, MD, USA). Amplex Red reagent, by Molecular Probes (Paisley, UK); Oxyblot^{TM} Protein Oxidation Detection Kit, by Chemicon-Millipore (Billerica, MA, USA) and Malondialdehyde Assay, by Northwest Life Science Specialties (Vancouver, Canada).

Animals, sacrifice and mitochondrial isolation

Animals were treated in accordance with the University Bioethical Committee guidelines for animal care and the European Union regulations (86/609/EEC and 2003/65/CE).

Wistar rats, supplied by Charles River (Barcelona, Spain), were housed individually at 22 °C with a 12-h light/dark cycle and fed *ad libitum* with pelleted standard diet (A04 Panlab, Barcelona, Spain). Eight groups were made: males and females of four different ages, young-adult rats (6 months old), adult rats (12 months old), old rats (18 months old) and senescent rats (24 months old), including 6-8 animals in each group.

All animals were weighed and sacrificed by decapitation at the start of the light cycle. After sacrifice animals underwent a visual internal inspection in order to exclude aged animals with any pathological signal. Brain was quickly removed and weighed. Homogenization and mitochondrial isolation were performed according to the previously described procedure (Lai et al., 1979). Briefly, brain was homogenized in 35 mL of cold isolation medium (0.25 M sucrose, 10 mM tris-HCl and 0.5 mM EDTA-K^+, pH 7.4) in a manual homogenizer with a glass pestle, total clearance 0.1 mm. An aliquot of the homogenate was stored at -20°C for posterior analyses and the remainder was used to perform biochemical assays and for mitochondrial isolation. Nuclei and cell debris were twice sedimented by differential centrifugation at 2000xg for 3 min and the pellets were discarded. Supernatant was subjected to a further centrifugation at 12500xg for 8 min and the crude mitochondrial pellet obtained was resuspended with 0.8 mL of isolation medium. Protein concentration was determined in both homogenate and mitochondrial fraction by Bradford's method (Bradford, 1976).

Mitochondrial oxygen consumption

Oxygen consumption of brain mitochondria was measured by a thermostatically controlled Clark-type O_2 electrode (Oxygraph, Hansatech, UK). Reaction conditions were 0.5 mg of mitochondrial protein in up to 0.5 mL of respiration buffer (145 mM KCl, 30 mM Hepes, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA and 0.1% BSA fatty acid free, pH 7.4) at 37 ºC. The substrate used was succinate (10 mM) in the presence (State 3, phosphorylating) and absence (State 4, resting) of 500 µM ADP. Mitochondrial viability was checked by the respiratory control rate (RCR = state 3 / state 4).

Mitochondrial hydrogen peroxide production

H_2O_2 production from mitochondria was measured fluorimetrically by Amplex Red reagent (530 nm excitation, 590 nm emission) in the presence of horseradish peroxidase. Assays were performed at 37 °C for 20 min in a 96-well microplate fluorimeter (Biotek instruments, Winooski, VT, USA). Mitochondria (0.4 mg protein/mL) were added to the same medium used for respiration and supplemented with 0.1 U/mL horseradish peroxidase and 50 µM Amplex Red reagent. The assays were performed in the presence of 10 mM succinate as substrate, in the State 4 of mitochondria respiration, or in the presence of this substrate plus 2 µM rotenone ─ as inhibitor of complex I ─ and 2 µM antimycin ─ as inhibitor of complex III, or plus 500 µM GDP as inhibitor of mitochondrial respiratory chain uncoupling.

Tissue oxidative damage

Protein carbonyl group content of brain homogenate was measured by an Oxyblot™ Protein Oxidation Detection Kit using a Bio-Dot SF apparatus (Bio-Rad, Hercules, CA, USA). The carbonyl groups from 5 µg of homogenate protein were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized samples were dot blotted onto a nitrocellulose filter, which was incubated with primary antibody specific to the DNP moiety of the proteins. Incubation with appropriate secondary antibody-horseradish peroxidase, treatment with enhanced chemiluminescent reagent, detection by a ChemiDoc XRS system (BIO-Rad, CA, USA) and quantification by photodensitometry with the image analysis program Quantity One© (BIO-Rad, CA, USA) were then performed.

Malondialdehyde (MDA), a low-molecular-weight end-product of lipid peroxidation decomposition used as an index of lipid peroxidation, was measured as thiobarbituric acid-reactive substances (TBARS) content, using a quantitative kit in homogenate samples by the previously described method (Slater et al., 1971).

DNA oxidation was measured in brain homogenate as previously described (Musarrat et al., 1994) with several modifications. Pure genomic DNA was isolated by a GenElute Mammalian Genomic DNA Miniprep Kit following the manufacturer's instructions from Sigma-Aldrich (St. Louis, MO, USA); afterwards it was quantified, digested by restriction enzyme *Bcl* I (Promega Corporation, Madison, WI, USA) at 50 ºC

for 4 hours and denatured at 92 ºC for 15 min. Five µg of DNA from each sample were loaded onto a positively charged nylon membrane (Roche Diagnostics, Basel, Switzerland) using a Bio-Dot SF apparatus (Bio-Rad, Hercules, CA, USA). Membrane blots were fixed by 80 ºC for 1 hour. Then, the membran was blocked with 5% BSA, incubated with primary antibody against 8-oxo-dG, next with appropriate secondary antibody-horseradish peroxidise and treated with enhanced chemiluminescent reagent. Finally, membrane blots were detected by a ChemiDoc XRS system and quantified by photodensitometry with the image analysis program Quantity One.

Enzymatic activities

Mitochondrial antioxidant activities, manganese-superoxide dismutase (MnSOD, EC 1.15.1.1) (Quick et al., 2000) and glutathione peroxidase (GPx, EC 1.11.1.9) (Smith et al., 2001) were determined in mitochondrial fraction. Catalase (EC 1.11.1.6) (Aebi, 1984) was determined in homogenate.

Western blot of UCP4 and UCP5

Forty micrograms of total protein were eletrophoresed on 12% v/v polyacrilamide SDS-PAGE gels. Proteins were electrotransferred onto a nitrocellulose membrane and Ponceau S staining was performed to check the correct loading and electrophoretic transfer. Membranes were incubated with the corresponding antibodies against UCP4 and UCP5 and secondary antibody-horseradish peroxidase, and then treated with enhanced chemiluminescent reagent. Membrane bands were detected by a ChemiDoc XRS system and quantified by the image analysis program Quantity One. Apparent molecular weights of proteins were estimated using a protein molecular-mass standard.

Statistics

All statistical analyses were performed with the Statistical Program for the Social Sciences SPSS software version 17.0 for Windows (Chicago, IL, USA). Data are expressed as mean ± SEM. Differences between groups were assessed by two-way analysis of variance (ANOVA) to determine the effect of age and sex, and Student's t-test for post-hoc comparisons. Statistical significance was set at $p<0.05$.

RESULTS

Mitochondrial oxygen consumption and H_2O_2 production

Aging induced an increase in rat brain mitochondrial oxygen consumption (Figure 1), using succinate as substrate in the presence of ADP (State 3), moderate until old age (18 months) and more marked at senescence (24 months), without showing significant differences between sexes. When measurements were made in the absence of ADP (State 4), the oxygen consumption showed a gradual increase throughout all the period studied, females reaching higher levels at 24 months.

Brain mitochondrial H_2O_2 production was measured using succinate as substrate and in the presence of rotenone and antimycin, inhibitors of complex I and complex III, respectively (Figure 1). H_2O_2 production in these conditions, which represents the maximum production capacity, progressively increased until old age, remaining unchanged up to 24 months, without significant differences between sexes.

Oxidative damage

Table 1 shows protein, lipid and DNA oxidation. Protein oxidation, measured as protein carbonyl group content per mg of total protein, rose with age in both sexes but, whereas females showed a gradual increase, in males the increase was more marked at 24 months.

Lipid peroxidation, measured as TBARS levels per µg of total lipid, did not show any statistical difference either with age or with sex.

DNA oxidation, measured as 8-oxo-dG presence per µg of total DNA, again showed an increase in both sexes but more pronounced in male rats, and DNA oxidation level was always lower in female than in male rat brain.

Antioxidant enzyme activities

Table 2 shows SOD, GPx and Catalase activities. SOD activity gradually decreased with age, showing no sex differences. GPx activity presented an opposite pattern; this enzyme showed a progressive increase with age in both sexes, and was greater in females than in male rats, leading to a sexual dimorphism that reached

statistical significance at 24 months old. Catalase activity, like SOD activity, decreased with age. This activity was higher in males than in females at 6 months, but without statistical significance, and diminished with a greater rate in males, thus reaching similar level as females at 24 months.

UCP protein levels

UCP levels are shown in table 3. Brain UCP4 content progressively decreased in male rats with aging, whereas female rats maintained UCP4 levels throughout the whole period studied. Conversely, UCP5 showed a marked increase in females at senescence whereas males maintained their levels with age.

GDP effect on mitochondrial H_2O_2 production

GDP effect inhibiting UCP activity (Nicholls et al., 1984) was determined, using succinate as substrate, comparing mitochondrial H_2O_2 production in respiratory State 4 without GDP – when UCPs carry out their protonphoric action - and with GDP – when inhibited UCPs are coupling oxidative phosphorilation (Figure 2). State 4 H_2O_2 production increased with age in both sexes, and when UCPs were inhibited by GDP, H_2O_2 production increased at every age but to a greater extent in females than in males.

DISCUSSION

On the whole, our results show an oxidative damage accumulation in rat brain throughout aging, related to the increasing mitochondrial respiratory chain (MRC) activity and failure of several antioxidant defences. Nevertheless, the aging effect was less marked in females, which accumulated lesser oxidative damage than males due to greater MRC efficiency and greater antioxidant presence, such us higher GPx activity and UCP5 level.

Aging effect

Oxidative damage rose with aging as a result of oxidative stress in agreement with previous studies that have described an increase in protein and DNA oxidation by

oxidative stress effect (Ames, 1989; Stadtman, 1992; Mecocci et al., 1993). This oxidative damage accumulation occurred in parallel with the growing mitochondrial oxidative function, reflected by oxygen consumption and maximum H_2O_2 production capacity, and agrees with the overall mitochondrial protein increase and mitochondria number rise with age previously described (Guevara et al., 2011).

On the other hand, this damage accumulation agrees with the fall of some antioxidant defences such as SOD and Catalase activities through aging. However, GPx activity and UCP5 level increased, possibly as a consequence of an up-regulation by oxidative stress (Barja et al., 1994; Echtay et al., 2002).

UCP4 and UCP5 have been described as possible downregulators of brain mitochondrial ROS production, due to their diminishing effect on the proton gradient (Negre-Salvayre et al., 1997; Kim-Han et al., 2001; Liu et al., 2006), in which even mild uncoupling significantly reduces the ROS production (Korshunov et al., 1997). This UCP downregulator function was confirmed by the GDP effect which caused an increase in H_2O_2 production highlighting the UCP involvement in this process. The different behaviour of two UCPs with age, UCP4 diminishing its level whereas UCP5 increasing it throughout aging, suggestes that UCP5 rather than UCP4 could be playing a role in preventing ROS production in rat brain, which could be due to the greater presence of UCP5 in brain with respect to UCP4 (Kim-Han et al., 2005).

Sex differences

The oxygen consumption in State 3, when MRC works under greater energetic request and the rate of ROS production is the lowest, was similar in both sexes, but it has been reported that only about 30% of mitochondria are in this state (Boveris et al., 1999). The remainder of mitochondria are in State 4, under lesser energetic request and in which mitochondria carry out a major H_2O_2 production. Remarkably, female brain mitochondria showed higher State 4 oxygen consumption at senescence than males, consistent with the greater female mitochondrial protein content at this age (Guevara et al., 2009; Guevara et al., 2011). However, H_2O_2 production in this state was not higher in females, even maximum H_2O_2 production capacity was also similar in both sexes, which agrees with the better mitochondrial efficiency of females, which has been previously described (Guevara et al., 2009) and probably influenced by estrogen regulation (Chen et al., 2005).

Nevertheless, female rat brain presented lesser oxidative damage accumulation with age than male rats. This better protection against oxidative damage in female rats could be due to a greater antioxidant capacity at both levels, quenching ROS, given that oxidative damage was lesser, even though H_2O_2 production was similar, and preventing their formation, as H_2O_2 production was similar despite the higher MRC activity.

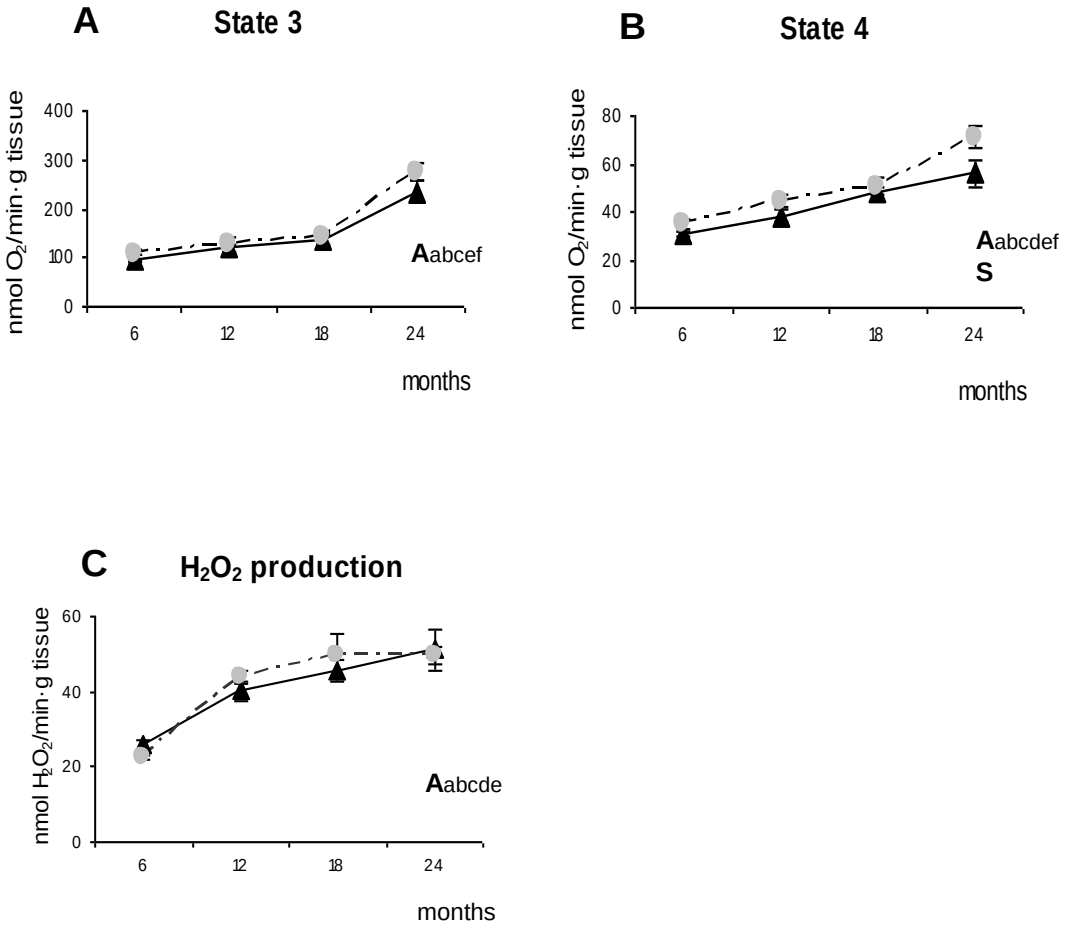
On the one hand, the GPx enzyme, an H_2O_2 quencher, increased its activity with aging, and with higher rate in females, which could lead to the better oxidative balance in this sex. In this sense, several studies have described that antioxidant enzymes are overexpressed in females only in species in which females live longer than males (Borras et al., 2003; Viña et al., 2003). In fact, higher antioxidant enzyme expression and activity have been reported in females due to the hormonal regulation exerted by estrogens (Viña et al., 2005).

On the other hand, UCP5, an ROS production inhibitor, increased its level with a higher rate in female rats with respect to males, which could be preventing H_2O_2 generation in a more effective way in this sex. The opposite pattern between sexes throughout aging observed in H_2O_2 production when UCPs are inhibited by GDP, also agrees with the sexual dimorphism, since this GDP effect decreased in males with aging correlating with the total UCP level drop, whereas in females, the GDP effect rose with age correlating with the total UCP increase.

Therefore, the better maintenance of female mitochondrial function (Guevara et al., 2011) together with this greater antioxidant capacity shown by females could lead to the better oxidative balance in this sex and, consequently, the lower oxidative damage accumulation.

To sum up, this study has demonstrated that the aging process causes lower ROS production and, in turn, lesser oxidative damage in female rat brain than in males. The better oxidative homeostasis balance of females with aging could be due to the better mitochondrial functionality maintenance by females previously described, and the greater antioxidant capacity of female rat brain shown in this study. This sexual dimorphism gradually increased during aging, leading to lesser mitochondrial dysfunction (Guevara et al., 2011) and greater neuroprotection and possibly contributing to a lower incidence and progression of neurological disease in female rat brain than in males with age.

Figure 1. Aging effect on oxygen consumption and H_2O_2 production in male and female rat brain.



Measurements of O_2 consumption in both State 3 (with ADP) (**A**) and State 4 (without ADP) (**B**) of respiration were carried out with succinate as substrate. Measurements of H_2O_2 production were carried out with succinate as substrate in the presence of rotenone plus antimicine (**C**).
Values are means ± SEM from 6 - 8 animals per group. ANOVA ($p<0.05$). A: age effect; S: sex effect. Post-hoc analysis between age groups (LSD test, $p<0.05$): [a] 12-mo old vs. 6-mo old; [b] 18-mo old vs. 6-mo old; [c] 24-mo old vs. 6-mo old; [d] 18-mo old vs. 12-mo old; [e] 24-mo old vs. 12-mo old; [f] 24-mo old vs. 18-mo old. Post hoc analysis between sexes (Student's t-test, $p<0.05$): * females vs. males. ▲: males; ●: females.

Table 1. Aging effect on oxidative damage in male and female rat brain.

		6 months	12 months	18 months	24 months	ANOVA
Total protein oxidation						
(a.u./mg total protein)	M	100 ± 10	128 ± 33	111 ± 14	280 ± 38^{cef}	A, S, AxS
	F	64 ± 9*	77 ± 22	138 ± 16^{bd}	171 ± 6*^{ce}	
TBARS						
(nmol/µg total lipid)	M	8.71 ± 0.74	8.92 ± 0.34	7.87 ± 1.10	7.26 ± 1.00	NS
	F	7.89 ± 0.70	7.69 ± 0.52	8.20 ± 1.08	6.74 ± 0.40	
Total DNA oxidation						
(a.u./µg total DNA)	M	100 ± 5	97 ± 6	121 ± 7	197 ± 8	A_{bcdef}, S
	F	84 ± 9	93 ± 11	102 ± 7	143 ± 13	

Values are means ± SEM from 6 - 8 animals per group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post-hoc analysis between age groups (LSD test, $p<0.05$): [a] 6-mo old vs. 12-mo old; [b] 6-mo old vs. 18-mo old; [c] 6-mo old vs. 24-mo old; [d] 12-mo old vs. 18-mo old; [e] 12-mo old vs. 24-mo old; [f] 18-mo old vs. 24-mo old. Post hoc analysis between sexes (Student's t-test, $p<0.05$): * females vs. males. M: males; F: females. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%.

Table 2. Aging effect on antioxidant enzyme activities in male and female rat brain.

		6 months	12 months	18 months	24 months	ANOVA
SOD activity (IU/g tissue)	M	1.72 ± 0.16	0.99 ± 0.08	0.96 ± 0.05	0.51 ± 0.06	A_{abcef}
	F	1.73 ± 0.14	1.18 ± 0.10	0.86 ± 0.03	0.50 ± 0.04	
GPx activity (mIU/g tissue)	M	0.685 ± 0.045	0.639 ± 0.029	0.929 ± 0.043^{bd}	1.075 ± 0.098^{cef}	A, S, AxS
	F	0.653 ± 0.025	0.762 ± 0.068^{a}	0.953 ± 0.019^{bd}	1.327 ± 0.033*^{cef}	
Catalase activity (a.u./g tissue)	M	100 ± 5.5	71.7 ± 5.1	82.4 ± 4.0	64.6 ± 4.9	A_{abcdf}, S
	F	82.7 ± 4.8	60.2 ± 5.5	69.1 ± 4.1	61.0 ± 2.9	

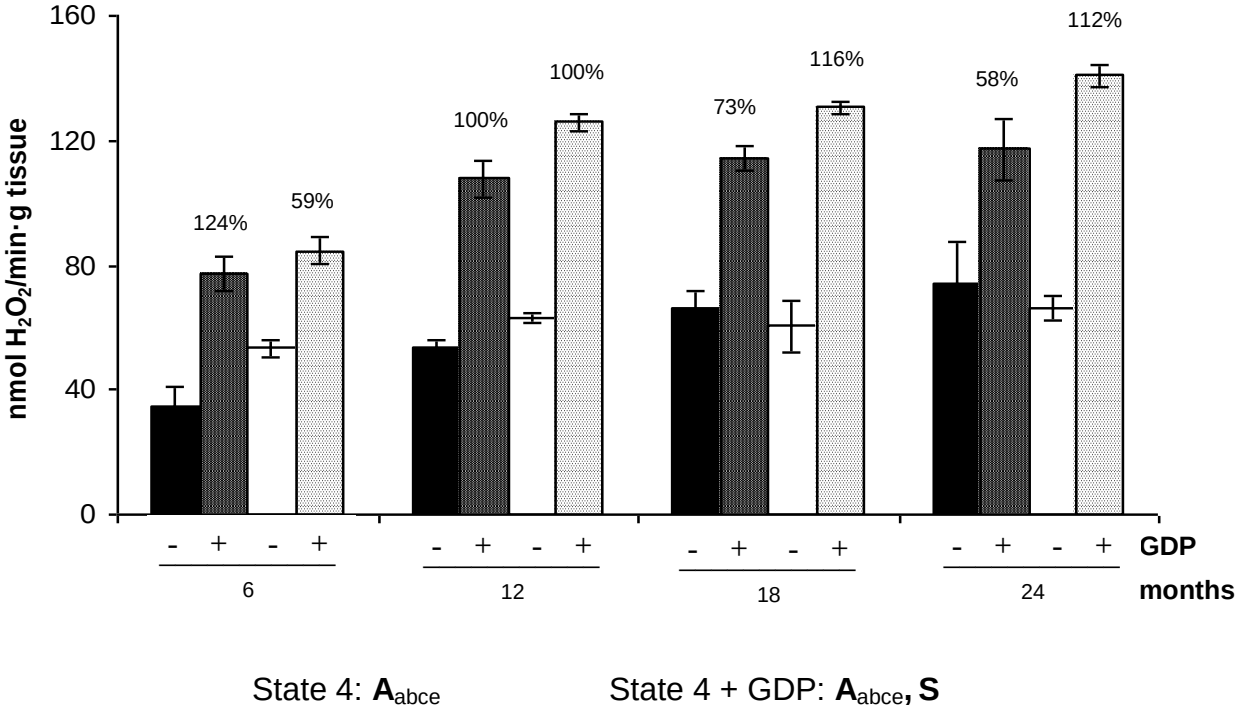
Values are means ± SEM from 6 - 8 animals per group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction; NS: no significant differences. Post-hoc analysis between age groups (LSD test, $p<0.05$): [a] 6-mo old vs. 12-mo old; [b] 6-mo old vs. 18-mo old; [c] 6-mo old vs. 24-mo old; [d] 12-mo old vs. 18-mo old; [e] 12-mo old vs. 24-mo old; [f] 18-mo old vs. 24-mo old. Post hoc analysis between sexes (Student's t-test, $p<0.05$): * females vs. males. M: males; F: females. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%.

Table 3. Aging effect on uncoupling protein levels in male and female rat brain.

		6 months	12 months	18 months	24 months	ANOVA
UCP4 (a.u./g tissue)	M	100 ± 10	95 ± 11	80 ± 6	69 ± 6	A_{bc}, S
	F	117 ± 12	103 ± 9	88 ± 5	102 ± 4	
UCP5 (a.u./g tissue)	M	100 ± 14	102 ± 10	109 ± 13	117 ± 7	A, S, AxS
	F	119 ± 16	104 ± 13	116 ± 11	188 ± 19^{*cef}	

Values are means ± SEM from 6 - 8 animals per group. ANOVA ($p<0.05$): A: age effect; S: gender effect; AxS: age and gender interaction. Post-hoc analysis between age groups (LSD test, $p<0.05$): [a] 6-mo old vs. 12-mo old; [b] 6-mo old vs. 18-mo old; [c] 6-mo old vs. 24-mo old; [d] 12-mo old vs. 18-mo old; [e] 12-mo old vs. 24-mo old; [f] 18-mo old vs. 24-mo old. Post hoc analysis between sexes (Student's t-test, $p<0.05$): * females vs. males. M: males; F: females. a.u., arbitrary units; levels found in 6-month-old male rats were set as 100%.

Figure 2..GDP effect on mitochondrial H_2O_2 production in State 4 in male and female rat brain during aging process.



Measurements of H_2O_2 production were carried out in the absence (S4) and presence of GDP with succinate as substrate. ■ males, S4; ▩ males, S4+GDP; □ females, S4; ⁘ females, S4+GDP. %: increase percentage in the H_2O_2 production without and with GDP.
Values are means ± SEM from 6 - 8 animals per group. ANOVA ($p<0.05$). A: age effect; S: sex effect. Post-hoc analysis between age groups (LSD test, $p<0.05$): [a] 6-mo old vs. 12-mo old; [b] 6-mo old vs. 18-mo old; [c] 6-mo old vs. 24-mo old; [d] 12-mo old vs. 18-mo old; [e] 12-mo old vs. 24-mo old; [f] 18-mo old vs. 24-mo old.

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6. RECAPITULACIÓN

SUMMARY

RECAPITULACIÓN

El proceso de envejecimiento se caracteriza por un declive de la función celular y mitocondrial con marcados efectos en el balance oxidativo, mostrando un dimorfismo sexual cada vez mayor con el paso del tiempo. El cerebro tiene una elevada tasa metabólica debido a que las neuronas son células altamente diferenciadas que necesitan grandes cantidades de ATP generados por el metabolismo oxidativo mitocondrial. Por tanto, la función y la supervivencia neuronal son muy sensibles a la disfunción mitocondrial que se produce con el envejecimiento.

Efecto del envejecimiento

Durante el proceso de envejecimiento se produjo una disminución del número de células del cerebro de las ratas Wistar, reducción ya descrita en diversas regiones cerebrales humanas (Haripriya et al., 2005), acompañada de un incremento de su densidad proteica. Además, el número de mitocondrias, tanto por célula como por gramo de tejido, aumentó con la edad, hecho observado también en otros tejidos como el hígado (Dinardo et al., 2003), el adiposo marrón (Valle et al., 2008), corazón (Gadaleta et al., 1992), bazo y riñón (Masuyama et al., 2005). Así mismo, aumentó la proteína mitocondrial, lo que se reflejaba en el aumento de la función oxidativa y fosforilativa mitocondriales tisulares, el consumo de oxígeno y la actividad ATPasa. Sin embargo, el contenido de proteína mitocondrial aumentó en menor proporción que el número de mitocondrias, por lo que el contenido de proteína por mitocondria, indicador de diferenciación mitocondrial (Justo et al., 2005; Valle et al., 2007b), fue reduciéndose con el paso del tiempo. Así, nuestros resultados mostraban una pérdida de capacidad funcional de las mitocondrias del cerebro de las ratas con la edad y por tanto menor grado de diferenciación.

No obstante, la actividad COX tisular, enzima catalizadora de uno de los pasos limitantes de la respiración mitocondrial, se mantuvo estable con la edad, a diferencia del resto de parámetros mitocondriales que aumentaban. Hecho que podría explicarse ya que el contenido proteico mitocondrial es el resultado de la expresión coordinada de genes del ADN nuclear y el ADNmt (Garesse and Vallejo, 2001) y, mientras que el contenido de la subunidad II de la COX, codificada por el ADNmt, sufrió un pronunciado aumento con la edad paralelo al incremento del ADNmt, el contenido de

la subunidad IV de la COX, codificada por el ADN nuclear permaneció estable durante el tiempo.

La descompensación entre el aumento de la función oxidativa con la edad y la estabilidad de la actividad COX podría ser una de las causas del aumento de la producción de ROS, que junto con la disminución tisular de las enzimas antioxidantes SOD y catalasa, provocaba la acumulación del daño oxidativo observado durante el envejecimiento en las ratas. Daño oxidativo que habría producido alteraciones en el funcionamiento de la CRM, que habrían conducido a su vez a una mayor generación de ROS. Nuestros resultados concordaban con otros autores que también han descrito un incremento en la oxidación de proteínas y ADN con el envejecimiento como resultado de un aumento del estrés oxidativo (Ames, 1989; Mecocci et al., 1993; Stadtman, 1992).

Frente a este aumento de la producción de ROS en el cerebro a lo largo del envejecimiento, se observó una respuesta creciente de la actividad enzimática antioxidante de la GPx y un aumento de los niveles de UCP5, debido al efecto estimulador del estrés oxidativo sobre la expresión de estas proteínas (Barja et al., 1994; Echtay et al., 2002). A la UCP4 y UCP5 se les atribuye una acción preventiva de la producción de ROS mitocondrial debido a su efecto disipador del gradiente protónico (Kim-Han et al., 2001; Liu et al., 2006; Negre-Salvayre et al., 1997), en el cual, incluso un ligero desacoplamiento reduce significativamente la producción de ROS (Korshunov et al., 1997). Esta función reguladora de las UCP se confirmó por el efecto del GDP, inhibidor de la función desacoplante de las UCP (Nicholls and Locke, 1984). Al inhibir las UCP con GDP aumentaba el acoplamiento de la CRM y en consecuencia aumentaba también, y de forma proporcional, la producción de H_2O_2, evidenciando la implicación de las UCP en este proceso. El diferente comportamiento de las dos UCP con la edad, la UCP4 disminuyendo sus niveles mientras la UCP5 incrementándolos, sugería que esta última, podía estar jugando un mayor papel en la prevención de la producción de ROS en el cerebro, resultado que concuerda con la mayor presencia de UCP5 respecto a UCP4 en este tejido (Kim-Han and Dugan, 2005).

En consecuencia, el aumento de la producción de ROS con la edad provocó una acumulación de daño oxidativo en las mitocondrias, causando su progresiva disfunción, situación frente a la cual, la célula respondió intentando contrarrestarla aumentando el número de mitocondrias.

Diferencias de sexo

El cerebro de las ratas Wistar mostraba un dimorfismo sexual en muchos aspectos. La densidad proteica de las células del cerebro aumentó con el tiempo más en las hembras. También se observó que el cerebro de las ratas hembra presentaba un menor número de mitocondrias por gramo de tejido que los machos, aunque el número de mitocondrias por célula era similar en ambos sexos en la senectud. Por todo ello, las mitocondrias de las hembras presentaban mayor capacidad funcional en todas las edades estudiadas, dado el mayor contenido proteico por mitocondria y la mayor actividad COX, diferencias que fueron acrecentándose con la edad debido al mayor aumento en las hembras de la función oxidativa. Por tanto, las mitocondrias de las hembras presentaban mayor grado de diferenciación y mantenían mejor su funcionalidad en la vejez, a pesar del declive mitocondrial. Estos resultados están de acuerdo con estudios previos realizados en nuestro laboratorio que describían en las hembras mitocondrias más diferenciadas y con mayor capacidad oxidativa y fosforilativa en tejidos como el hígado (Justo et al., 2005; Valle et al., 2007b), el adiposo marrón (Rodriguez-Cuenca et al., 2002; Valle et al., 2007a), el músculo cardiaco (Colom et al., 2007b), y el esquelético (Colom et al., 2007a).

Este dimorfismo sexual sería indicador de una estimulación de las actividades de la CRM en el cerebro de las ratas hembra que, como se ha descrito previamente en otros tejidos, podría estar relacionada con el diferente entorno hormonal (Colom et al., 2007a; Valle et al., 2007b). En este sentido, se ha demostrado que el 17β-estradiol y la progesterona están implicadas en el aumento tanto de la expresión (Chen et al., 2005) de genes nucleares y mitocondriales que codifican para proteínas de la CRM como de su función (Chen and Yager, 2004). Los nivel de las hormonas sexuales disminuyen al final de la vida, especialmente en las hembras, cuando se vuelven acíclicas (Huang and Meites, 1975), aunque las hembras aún presentan niveles mucho mayores de 17β-estradiol y progesterona que los machos en la vejez, lo que podía explicar, al menos en parte, las diferencias de sexo encontradas en la función mitocondrial.

La mayor actividad de la CRM en el cerebro de las ratas hembras, dio como resultado una producción tisular de H_2O_2 similar en ambos sexos, y aún así, las hembras presentaron menor acumulación de daño oxidativo que los machos, circunstancia previamente descrita en cerebro y otros tejidos como hígado (Viña et al., 2005) y músculo cardiaco (Colom et al., 2007b). Esta mejor protección de las hembras frente al daño oxidativo podía explicarse en parte por una mayor respuesta

antioxidante tanto a nivel de la eliminación de los ROS formados como a nivel de la prevención de su formación.

Por un lado, la enzima GPx, aumentó su actividad con la edad más en las hembras, lo que contribuía al mejor balance oxidativo en este sexo. Resultados que estaban de acuerdo con estudios previos de otros autores que han atribuido también a los estrógenos un efecto estimulador de las actividades de las enzimas antioxidantes mitocondriales tales como la SOD-Mn y la GPx (Razmara et al., 2007; Strehlow et al., 2003; Viña et al., 2005).

Por otro lado, los mayores niveles de UCP observados en el cerebro de las ratas hembras con respecto a los machos, podían estar contribuyendo a la prevención de la generación de H_2O_2 de un modo más eficiente en este sexo. Mediante el estudio del efecto inhibidor del GDP sobre las UCP quedó patente el hecho de que a mayor presencia de UCP, se daba un mayor efecto preventivo en la formación de ROS, por su función desacoplante. Por todo ello, el divergente patrón entre ambos sexos detectado en la presencia y función de las UCP, remarcaba el creciente dimorfismo sexual observado a lo largo del envejecimiento.

En resumen, el mejor mantenimiento de la función mitocondrial con la edad en el cerebro de las ratas hembra respecto a los machos, junto con el mayor aumento de las defensas antioxidantes, podrían haber llevado al mejor balance oxidativo en este sexo y consecuentemente, a la menor acumulación de daño oxidativo. Estas diferencias entre sexos fueron aumentando gradualmente durante el envejecimiento, conduciendo a la menor disfunción mitocondrial en las hembras viejas.

Recapitulaciones

The aging process is characterized by a cellular and mitochondrial function decline which has a marked effect on oxidative balance and shows greater and greater sexual dimorphism as time passes. Brain has a high metabolic rate because neurons are highly differentiated cells that need a large amount of ATP generated by mitochondrial oxidative metabolism. Therefore, neuronal function and survival are highly sensitive to mitochondrial dysfunction produced during aging.

Aging effect

Wistar rat brain cell number fell throughout the aging process, which has been described in several human brain regions (Haripriya, Sangeetha et al. 2005), and was accompanied by a slight, gradual increase in protein content. Moreover, mitochondrial number rose both per cell and per gram of tissue with age, which had also been observed in other tissues such as liver (Dinardo, Musicco et al. 2003), brown adipose (Valle, 2008 #160), heart (Gadaleta, 1992 #157), spleen and kidney (Masuyama, 2005 #159). Likewise, mitochondrial protein rose, which was reflected in the tissue increase of mitochondrial oxidative and phosphorylative function, oxygen consumption and ATPase activity. However, mitochondrial protein content rose slower than mitochondria number, so protein content per mitochondria, an indicator of mitochondrial differentiation (Justo, 2005 #20; Valle, 2007 #17), diminished over time. Thus, our results show a loss of functional capacity by rat brain mitochondria with age and, as a result, a lesser degree of differentiation.

Nevertheless, tissue COX activity, an enzyme catalyzing one of the mitochondrial respiration limiting steps, remained stable with age, unlike the other mitochondrial parameters that rose. This fact could be explained since mitochondrial protein content is the result of coordinated expression of nDNA and mtDNA genes (Garesse, 2001 #184). MtDNA encoded COX II subunit content experienced a sharp rise with age in line with mtDNA increase whereas nDNA encoded COX IV subunit content remained without change throughout time.

Decompensation between oxidative function increase with age and COX activity stability could be one of the causes of the rise in ROS production that, together with antioxidant enzyme SOD and catalase tissue decrease, causes the observed oxidative damage accumulation in rat brain during aging. This oxidative damage would have produced alterations in MRC function which would in turn have led to greater ROS

production. Our results agree with other authors who have also described an oxidized protein and DNA increase with age as a consequence of a rise in oxidative stress (Ames, 1989 #224; Mecocci, 1993 #80; Stadtman, 1992 #225).

Facing this brain ROS production increase throughout aging, both a growing response of the GPx enzyme antioxidant activity and a UCP5 level rise was observed due to the oxidative stress upregulation effect on these protein expressions (Barja, 1994 #91; Echtay, 2002 #55). UCP4 and UCP5 have been claimed to have a preventive action against mitochondrial ROS production due to their dissipating effect on proton gradient (Kim-Han, 2001 #65; Liu, 2006 #8; Negre-Salvayre, 1997 #48), in which even slight uncoupling significantly reduces ROS production (Korshunov, 1997 #49). This UCP regulating function was confirmed by the effect of GDP – UCP uncoupling function inhibitor (Nicholls, 1984 #57). MRC coupling increases when UCPs are inhibited by GDP and as a result ROS production also increases in a proportional way which highlights UCP involvement in this process. The different behaviour between the two UCPs with age, that is, UCP4 diminishing its levels whereas UCP5 increases them, suggests that UCP5 might be playing a greater role in preventing brain ROS production. This result agrees with the greater UCP5 presence related to UCP4 in this tissue (Kim-Han, 2005 #135).

Consequently, ROS production increase with age induced a mitochondrial oxidative damage accumulation that caused their progressive dysfunction. Facing thist, the cells' response was to try to counteract this situation by raising mitochondrial number.

Sex differences

Wistar rat brain showed a sexual dimorphism in many aspects. Brain cell protein density increased more in females with age. Female rat brain also presented lower mitochondrial number per gram of tissue than males, although mitochondrial number per cell was similar in both sexes in senescence. Therefore, female mitochondria possessed greater functional capacity at any studied age, given the higher protein content per mitochondria and greater COX activity. These differences grew with age due to the greater increase of oxidative function in females. Thus, female mitochondria showed a greater degree of differentiation and better functionality maintenance in senescence despite mitochondrial decline. These results agree with previous studies carried out in our laboratory that describe more differentiated mitochondria and greater oxidative and phosphorylative capacity in females in tissues

such as liver (Justo, 2005 #20; Valle, 2007 #17), brown adipose (Rodriguez-Cuenca, 2002 #21; Valle, 2007 #18), cardiac muscle (Colom, 2007 #15), and skeletal muscle (Colom, 2007 #16).(Colom, 2007 #16).

This sex dimorphism would indicate an MRC activity up-regulation in female rat brain that could be related to the different hormonal milieu as previously described in other tissues (Colom, 2007 #16; Valle, 2007 #17). In this sense, 17β-estradiol and progesterone have been proved to be involved in raising both expression of nuclear and mitochondrial genes coding for MRC proteins (Chen, 2005 #118) and their function (Chen, 2004 #138). Sex hormone levels diminish to the end of life especially in females when they become acyclic (Huang, 1975 #141). However, females still present much higher 17β-estradiol and progesterone levels than males in old age, which could explain, at least in part, the sex differences fond in mitochondrial function.

Higher MRC activity in female rat brain resulted in similar tissue H_2O_2 production in both sexes and even then females showed lesser oxidative damage accumulation than males, which has been previously described in brain and other tissues such as liver (Viña, 2005 #25) and cardiac muscle (Colom, 2007 #15). This better female protection against oxidative damage could partly be explained by a greater antioxidant response at both levels: the elimination of already generated ROS and the prevention of their formation.

On the one hand, the higher UCP levels observed in female rat brain with regard to males might be contributing to prevention of H_2O_2 production in a more efficient way in this sex. The higher the UCP presence the greater the preventive effect on ROS generation thanks to their uncoupling function, which was clear by studying GDP inhibitor effect on UCP. Thus, the diverging pattern from females to males observed in UCP level and function emphasizes the growing sex dimorphism observed throughout aging.

To sum up, the better maintenance upon aging of rat brain mitochondrial function in females with regard to males, together with the greater increase of antioxidant defences also in females could have led to the better oxidative balance in this sex and, as a result, to the lesser oxidative damage accumulation. These sex differences rose gradually throughout aging, which led to the lesser mitochondrial dysfunction observed in females.

SUMMARY

The aging process is characterized by a cellular and mitochondrial function decline which has a marked effect on oxidative balance and shows greater and greater sexual dimorphism as time passes. Brain has a high metabolic rate because neurons are highly differentiated cells that need a large amount of ATP generated by mitochondrial oxidative metabolism. Therefore, neuronal function and survival are highly sensitive to mitochondrial dysfunction produced during aging.

Aging effect

Wistar rat brain cell number fell throughout the aging process, which has been described in several human brain regions (Haripriya et al., 2005), and was accompanied by a slight, gradual increase in protein content. Moreover, mitochondrial number rose both per cell and per gram of tissue with age, which had also been observed in other tissues such as liver (Dinardo et al., 2003), brown adipose (Valle et al., 2008), heart (Gadaleta et al., 1992), spleen and kidney (Masuyama et al., 2005). Likewise, mitochondrial protein rose, which was reflected in the tissue increase of mitochondrial oxidative and phosphorylative function, oxygen consumption and ATPase activity. However, mitochondrial protein content rose slower than mitochondria number, so protein content per mitochondria, an indicator of mitochondrial differentiation (Justo et al., 2005; Valle et al., 2007b), diminished over time. Thus, our results show a loss of functional capacity by rat brain mitochondria with age and, as a result, a lesser degree of differentiation.

Nevertheless, tissue COX activity, an enzyme catalyzing one of the mitochondrial respiration limiting steps, remained stable with age, unlike the other mitochondrial parameters that rose. This fact could be explained since mitochondrial protein content is the result of coordinated expression of nDNA and mtDNA genes (Garesse et al., 2001). MtDNA encoded COX II subunit content experienced a sharp rise with age in line with mtDNA increase whereas nDNA encoded COX IV subunit content remained without change throughout time.

Decompensation between oxidative function increase with age and COX activity stability could be one of the causes of the rise in ROS production that, together with antioxidant enzyme SOD and catalase tissue decrease, causes the observed oxidative damage accumulation in rat brain during aging. This oxidative damage would have produced alterations in MRC function which would in turn have led to greater ROS production. Our results agree with other authors who have also described an oxidized protein and DNA increase with age as a consequence of a rise in oxidative stress (Ames, 1989; Stadtman, 1992; Mecocci et al., 1993).

Facing this brain ROS production increase throughout aging, both a growing response of the GPx enzyme antioxidant activity and a UCP5 level rise was observed due to the oxidative stress upregulation effect on these protein expressions (Barja et al., 1994; Echtay et al., 2002). UCP4 and UCP5 have been claimed to have a preventive action against mitochondrial ROS production due to their dissipating effect on proton gradient (Negre-Salvayre et al., 1997; Kim-Han et al., 2001; Liu et al., 2006), in which even slight uncoupling significantly reduces ROS production (Korshunov et al., 1997). This UCP regulating function was confirmed by the effect of GDP – UCP uncoupling function inhibitor (Nicholls et al., 1984). MRC coupling increases when UCPs are inhibited by GDP and as a result ROS production also increases in a proportional way which highlights UCP involvement in this process. The different behaviour between the two UCPs with age, that is, UCP4 diminishing its levels whereas UCP5 increases them, suggests that UCP5 might be playing a greater role in preventing brain ROS production. This result agrees with the greater UCP5 presence related to UCP4 in this tissue (Kim-Han et al., 2005).

Consequently, ROS production increase with age induced a mitochondrial oxidative damage accumulation that caused their progressive dysfunction. Facing this, the cells' response was to try to counteract this situation by raising mitochondrial number.

Sex differences

Wistar rat brain showed a sexual dimorphism in many aspects. Brain cell protein density increased more in females with age. Female rat brain also presented lower mitochondrial number per gram of tissue than males, although mitochondrial number per cell was similar in both sexes in senescence. Therefore, female mitochondria possessed greater functional capacity at any studied age, given the

higher protein content per mitochondria and greater COX activity. These differences grew with age due to the greater increase of oxidative function in females. Thus, female mitochondria showed a greater degree of differentiation and better functionality maintenance in senescence despite mitochondrial decline. These results agree with previous studies carried out in our laboratory that describe more differentiated mitochondria and greater oxidative and phosphorylative capacity in females in tissues such as liver (Justo et al., 2005; Valle et al., 2007b), brown adipose (Rodriguez-Cuenca et al., 2002; Valle et al., 2007a), cardiac muscle (Colom et al., 2007b), and skeletal muscle (Colom et al., 2007a).

This sex dimorphism would indicate an MRC activity up-regulation in female rat brain that could be related to the different hormonal milieu as previously described in other tissues (Colom et al., 2007a; Valle et al., 2007b). In this sense, 17β-estradiol and progesterone have been proved to be involved in raising both expression of nuclear and mitochondrial genes coding for MRC proteins (Chen et al., 2005) and their function (Chen et al., 2004). Sex hormone levels diminish to the end of life especially in females when they become acyclic (Huang et al., 1975). However, females still present much higher 17β-estradiol and progesterone levels than males in old age, which could explain, at least in part, the sex differences fond in mitochondrial function.

Higher MRC activity in female rat brain resulted in similar tissue H_2O_2 production in both sexes and even then females showed lesser oxidative damage accumulation than males, which has been previously described in brain and other tissues such as liver (Viña et al., 2005) and cardiac muscle (Colom et al., 2007b). This better female protection against oxidative damage could partly be explained by a greater antioxidant response at both levels: the elimination of already generated ROS and the prevention of their formation.

On the one hand, the higher UCP levels observed in female rat brain with regard to males might be contributing to prevention of H_2O_2 production in a more efficient way in this sex. The higher the UCP presence the greater the preventive effect on ROS generation thanks to their uncoupling function, which was clear by studying GDP inhibitor effect on UCP. Thus, the diverging pattern from females to males observed in UCP level and function emphasizes the growing sex dimorphism observed throughout aging.

To sum up, the better maintenance upon aging of rat brain mitochondrial function in females with regard to males, together with the greater increase of antioxidant defences also in females could have led to the better oxidative balance in this sex and, as a result, to the lesser oxidative damage accumulation. These sex differences rose gradually throughout aging, which led to the lesser mitochondrial dysfunction observed in females.

7. CONCLUSIONES

CONCLUSIONS

CONCLUSIONES

1. Durante el envejecimiento se produce una pérdida de funcionalidad de las mitocondrias del cerebro de las ratas, que se intenta contrarrestar aumentando el número de las mismas.

2. Los niveles de daño oxidativo mitocondrial y celular en el cerebro de las ratas van acumulándose con la edad, a pesar del incremento de la actividad de las enzimas antioxidantes inducibles por estrés oxidativo, que no es capaz de eliminar la creciente cantidad de ROS producidos como consecuencia de la mayor actividad de la CRM y la pérdida de funcionalidad mitocondrial.

3. Las UCP juegan un importante papel en la prevención de la producción de ROS en el cerebro, contribuyendo la UCP5 en mayor medida que la UCP4.

4. Las mitocondrias del cerebro de las ratas hembra mantienen mejor la capacidad funcional durante el envejecimiento y muestran mayor grado de diferenciación que los machos en todas las edades estudiadas.

5. Las ratas hembra presentan mayor actividad que los machos, de las enzimas antioxidantes reguladas por estrés oxidativo, en todas las edades estudiadas.

6. Las hembras, en comparación con los machos, presentan un mayor aumento de los niveles de expresión de la UCP5 con la edad, lo que puede contribuir a la disminución de la producción de ROS de un modo más eficiente y, en consecuencia, al menor daño oxidativo observado en las hembras.

7. El daño oxidativo que va acumulándose durante el envejecimiento es menor en el cerebro de las hembras que en el de los machos, hecho que podría atribuirse tanto al mejor funcionamiento mitocondrial como a su mayor capacidad antioxidante total.

8. El dimorfismo sexual observado tanto en el grado de diferenciación mitocondrial como en la actividad de las enzimas antioxidantes y niveles de UCP, puede ser debido al entorno hormonal.

CONCLUSIOS

1. Throughout aging, rat brain mitochondria lose functionality, fact that cells try to counteract by increasing their number.

2. Rat brain mitochondrial and cellular oxidative damage levels accumulate with age despite the increase in antioxidant enzyme activities – those that may be up-regulated by oxidative stress – which is not able to eliminate the growing quantity of ROS produced as a consequence of the greater MRC activity and the loss of mitochondrial functionality.

3. UCPs play an important role in prevention of brain ROS production, specifically UCP5 contributes in a greater level than UCP4.

4. Female rat brain mitochondria better maintain functional capacity during aging and show greater degree of differentiation than those of males at any studied age.

5. Female rats present higher antioxidant enzyme activity – those that may be up-regulated by oxidative stress - than males, at any studied age.

6. Females show higher increase in UCP5 expression level throughout aging with regard to males, which can contribute to the ROS production fall in a more efficient way and consequently to the lesser oxidative damage observed in females.

7. The accumulation of oxidative damage that tale place throughout aging is lower in female brain than in male one. This fact could be attributed to both the better mitochondrial function and the greater total antioxidant capacity.

8. Sexual dimorphism observed in mitochondrial degree of differentiation, antioxidant enzyme activity and UCP levels can be due to hormonal milieu.

8. BIBLIOGRAFÍA

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9. ANEXO

APPENDIX

MANUSCRITO IV / ***MANUSCRIPT IV***

Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions.

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Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions

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Valle A, Guevara R, García-Palmer FJ, Roca P, Oliver J. Sexual dimorphism in liver mitochondrial oxidative capacity is conserved under caloric restriction conditions. *Am J Physiol Cell Physiol* 293: C1302–C1308, 2007. First published July 25, 2007; doi:10.1152/ajpcell.00203.2007.—Caloric restriction (CR) without malnutrition has been shown to increase maximal life span and delay the rate of aging in a wide range of species. It has been proposed that reduction in energy expenditure and oxidative damage may explain the life-extending effect of CR. Sex-related differences also have been shown to influence longevity and energy expenditure in many mammalian species. The aim of the present study was to determine the sex-related differences in rat liver mitochondrial machinery, bioenergetics, and oxidative balance in response to short-term CR. Mitochondria were isolated from 6-mo-old male and female Wistar rats fed ad libitum or subjected to 40% CR for 3 mo. Mitochondrial O_2 consumption, activities of the oxidative phosphorylation system (complexes I, III, IV, and V), antioxidative activities [MnSOD, glutathione peroxidase (GPx)], mitochondrial DNA and protein content, mitochondrial H_2O_2 production, and markers of oxidative damage, as well as cytochrome C oxidase and mitochondrial transcription factor A levels, were measured. Female rats showed a higher oxidative capacity and GPx activity than males. This sexual dimorphism was not modified by CR. Restricted rats showed slightly increased oxygen consumption, complex III activity, and GPx antioxidant activity together with lower levels of oxidative damage. In conclusion, the sexual dimorphism in liver mitochondrial oxidative capacity was unaffected by CR, with females showing higher mitochondrial functionality and ROS protection than males.

oxidative phosphorylation; free radicals; antioxidant enzymes; mitochondrial transcription factor A

CALORIC RESTRICTION (CR) without malnutrition is the only known experimental intervention that has been shown to extend life span and to delay the onset of age-related diseases in mammals (34, 49). Although the benefits of CR are well established, the mechanisms underlying its effects remain unclear. During the last decades increasing evidence has suggested that oxidative damage to proteins, lipids, and DNA may be responsible for normal aging, and CR may operate by decreasing the accumulation of this damage to macromolecules (10, 45). Since electron transport chain is the main source of free radical production in the cell, mitochondria are likely to play a central role in the life-extending mechanism underlying CR.

Naturally occurring episodes of CR in animal populations are common due to adverse climatic or biological changes such as drought, cold, or plagues. The need to postpone reproduction until more energy-favorable periods seems to be the selective pressure responsible for the development of these anti-aging mechanisms (21). However, some authors have argued that because of their higher relative importance for reproduction and the survival of the species, females have been subjected to more severe selection pressures to be more resistant to CR than males (19, 51). In fact, several studies on rodents have shown that CR has a greater and more permanent effect on physical growth in male than in female rats (8, 18). Recently, in our laboratory (47, 48), CR was described to produce a higher deactivation of brown adipose tissue by means of a loss of mitochondrial recruitment in female rats, which contributes to a large extent to overall energy saving.

During nutritional interventions, the liver is one of the most affected tissues, since it orchestrates the supply of energy substrates to different tissues. Although CR has been shown to decrease oxidative stress in liver mitochondria (15, 16), the influence of sex in this response has not been explored to date. Previous works have demonstrated that liver from female rats shows highly differentiated mitochondria with greater machinery per mitochondrion (20). On the other hand, mitochondria from female rats exhibit higher antioxidant gene expression (4), which seems to be induced by estrogens (3).

Taking this into account, the aim of this study was to determine the sex-related differences in rat liver mitochondrial machinery, bioenergetics, and oxidative balance in response to short-term CR. To tackle this aim, we measured mitochondrial O_2 consumption, oxidative phosphorylation (OXPHOS) activities, protein content, and the levels of mitochondrial transcription factor A (TFAM) in male and female rats maintained on a 40% CR diet for 3 mo. H_2O_2 production, antioxidative activities, and markers of protein and lipid oxidative damage were also measured.

MATERIALS AND METHODS

Materials. Routine chemicals were supplied by Sigma-Aldrich (St. Louis, MO), Panreac (Barcelona, Spain), and Amersham Pharmacia Biotech (Little Chalfont, UK). Real-time PCR reagents and oligonucleotide primer sequences were supplied by Roche Diagnostics (Basel, Switzerland).

Animals and diets. All animals were treated in accordance with the university bioethical committee guidelines for animal care and Euro-

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Table 1. *Effect of sex and CR on biometric parameters and liver composition*

	Male		Female		
	AL	CR	AL	CR	ANOVA
Body weight, g	482±14	354±8†	261±8*	194±4*†	S, R, S×R
Liver weight, g	13.7±0.7	9.86±0.49†	7.17±0.39*	5.63±0.20†	S, R, S×R
%Body weight	2.84±0.12	2.78±0.12	2.75±0.12	2.91±0.13	NS
Protein content, mg/g tissue	148±6	145±5	163±8	146±9	NS
DNA content, mg/g tissue	6.70±0.40	7.03±0.33	7.07±0.48	7.75±0.32	NS
Triglycerides, mg/g tissue	532±38	546±60	493±60	611±54	NS

Data are means ± SE of 6 animals per group. ANOVA: S, effect of sex ($P < 0.05$); R, effect of diet ($P < 0.05$); S×R, interactive effect ($P < 0.05$); NS, not significant. Student's *t*-test: *$P < 0.05$, male vs. female. †$P < 0.05$, control vs. restricted. AL, ad libitum feeding; CR, 40% caloric restriction.

pean Union regulations (86/609/EEC) and were approved by the ethics committee. Male and female Wistar rats ages 3 mo were purchased from Charles River (Barcelona, Spain) and housed individually in wire-bottom cages at 22°C with 12 h light-dark cycle. To study the effects of CR in both sexes, 12 males and 12 female rats were randomly divided into four groups ($n = 6$): control male and control female groups fed ad libitum (AL) with standard chow pellets (A04; supplied by Panlab, Barcelona, Spain) and restricted male and restricted female groups subjected to 40% food restriction (CR) for 3 mo. In restricted animals, food was supplied on a daily basis at the beginning of the dark cycle and was weekly updated compared with ad libitum rats to correct for growth requirements.

Death and mitochondria isolation. Animals were killed by decapitation, and livers were removed rapidly, weighed, and placed in ice-cold isolation buffer (250 mM sucrose, 5 mM Tris·HCl, and 2 mM EGTA, pH 7.4). Liver was finely chopped and rinsed in the isolation buffer to remove excess blood. Liver samples (5 g) were homogenized in 35 ml of ice-cold isolation buffer with a Teflon/glass homogenizer. Aliquots were stored at −80°C for determination of total DNA (46), triglycerides (43), and protein content (5). The rest of the homogenate was used for isolation of mitochondria by differential centrifugation. Briefly, nuclei and cell debris were removed by centrifugation at 500 *g* for 10 min, and supernatants were centrifuged at 8,000 *g* to yield the mitochondrial pellet. Pellets were washed once by resuspension and centrifuged (8,000 *g*), and the final pellets were resuspended in the same buffer. Mitochondrial protein was measured using the Bradford method (5).

Measurement of mitochondrial O_2 consumption. Liver mitochondrial O_2 consumption was measured polarographically as described previously (31) with minor modifications. Mitochondria were incubated in a water-thermostatically regulated chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph; Hansatech, Norfolk, UK) at a concentration of 1 mg/ml mitochondrial protein in respiration buffer (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.1% BSA, pH 7.4 at 37 °C). Glutamate/malate (2.5 mM/2.5 mM) or succinate (5 mM) was used as substrate in the absence (state 4) and in the presence (state 3) of 500 μM ADP. Mitochondrial viability was checked by the respiratory control ratio (state 3/state 4).

Mitochondrial activities. The measurement of the specific activities of the OXPHOS complex I (NADH:ubiquinone oxidoreductase; EC 1.6.99.5) (39), complex III (ubiquinol:cytochrome *c* reductase; EC 1.10.2.2) (24), and complex IV or COX (cytochrome *c* oxidase; EC 1.9.3.1) were performed as described and adapted to microtiter plate assay with some modifications. Mitochondrial antioxidant superoxide dismutase (MnSOD) (38) and glutathione peroxidase (GPx) (44) activities were also assayed. The assays were performed in a 200-μl final volume with 1–5 μg of mitochondrial proteins. COX activity was also measured in homogenates to calculate mitochondrial recovery.

Detection of H_2O_2 production in mitochondria. The rate of H_2O_2 production in mitochondria was determined by using the oxidation of the fluorogenic indicator Amplex red (Molecular Probes, Paisley, UK) in the presence of horseradish peroxidase. Mitochondria (0.25 mg protein/ml) were incubated at 37°C in respiration buffer containing 0.1 U/ml horseradish peroxidase and 50 μM Amplex red. H_2O_2 production was initiated in mitochondria by adding succinate (5 mM) as substrate. Background fluorescence was measured in parallel in wells containing all reactants except substrate. Fluorescence was recorded in a microplate reader (FLx800; Bio-Tek Instruments, Winooski, VT) with 530-nm excitation and 590-nm emission wavelengths. Levels of H_2O_2 were expressed as fluorescence minus background (pmol·mg protein^{-1}·min^{-1}). Rates were determined by converting fluorescence readings, using standard curves generated over a range of H_2O_2 concentrations.

Extraction and quantification of mitochondrial DNA. Mitochondrial DNA (mtDNA) was extracted by digestion with proteinase K (100 μg/μl) in a buffer containing 50 mM KCl, 10 mM Tris·HCl, 2.5 mM $MgCl_2$, and 0.5% Tween 20. Mitochondria samples were incubated overnight at 37°C and then boiled for 5 min to inactivate the enzyme. Mitochondrial DNA was linearized by digestion with *Bcl*I restriction enzyme for 3 h at 50°C and boiled for 5 min. Samples were centrifuged at 7,000 *g* for 5 min, and the resulting supernatant was used for amplification. A quantitative PCR assay was adapted to the

Table 2. *Effect of sex and CR on mtDNA, mitochondrial protein, TFAM, and COX II protein levels in liver*

	Male		Female		
	AL	CR	AL	CR	ANOVA
Mitochondrial protein, mg/g tissue	39.3±5.2	44.9±4.7	41.1±6.3	40.2±7.7	NS
mtDNA, au/g tissue	4.36±0.99	5.89±0.84	1.79±0.41*	1.80±0.48*	S
mtDNA, au/mg DNA	1.36±0.23	1.74±0.27	0.51±0.09*	0.45±0.10*	S
Mitochondrial protein/mtDNA, mg/au	13.1±3.0	9.59±1.71	23.3±4.5*	30.6±10.3*	S
TFAM, au/mg mitochondrial protein	100±16	73±16	136±19	126±19	S
COX II, au/g tissue	100±28	161±39	169±30	167±17	NS

Data are means ± SE of 6 animals per group. ANOVA: S ($P < 0.05$). Student's *t*-test: *$P < 0.05$, male vs. female. mtDNA, mitochondrial DNA; TFAM, mitochondrial transcription factor A; COX, cytochrome *c* oxidase; au, arbitrary units.

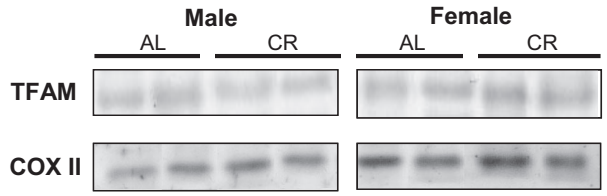

Fig. 1. Western blot of mitochondrial transcription factor A (TFAM) and cytochrome *c* oxidase (COX II) in liver of male and female rats. Total protein (40 μg) for TFAM and COX II (30 μg) were fractioned in 12% SDS-PAGE. Ponceau S staining was used to provide visual evidence of correct loading and electrophoretic transfer of proteins to nitrocellulose filter. Representative bands from 2 animals of each group are shown. AL, ad libitum; CR, 40% caloric restriction.

LightCycler technology from Koekemoer et al. (22). PCR was performed to amplify a 162-nt fragment of the mitochondrial NADH dehydrogenase subunit 4 gene. The primer sequences were 5′-TACACGATGAGGCAACCAAA-3′ and 5′-GGTAGGGGGTGTGTTGTGAG-3′. The concentration of the purified template was determined spectrophotometrically. Increasing amounts of template were amplified in parallel reactions to obtain a standard curve. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche) using a total volume of 10 μl containing 0.375 μM of each primer, 3 mM $MgCl_2$, 1 μl of LightCycler FastStart DNA Master SYBR green I (Roche), and 2.5 μl of sample prepared as described above. The PCR reactions were cycled 35 times after initial denaturation (95°C, 10 min), with the following parameters: denaturation at 95°C for 10 s, annealing at 60°C for 12 s, and extension at 72°C for 12 s.

TFAM and COX II Western blotting. For TFAM and COX II, 40 μg of mitochondrial and 30 μg of homogenate protein, respectively, were fractioned by SDS-PAGE (12% polyacrylamide gel) and electrotransferred onto nitrocellulose filters. Ponceau S staining was used to provide visual evidence of correct loading and electrophoretic transfer of proteins to nitrocellulose filter. Membranes were incubated overnight at 4–6°C in a blocking solution of 5% nonfat powdered milk in Tris-buffered saline (20 mM Tris·HCl, 0.13 mM NaCl, and 0.1% Tween 20). Developments of the immunoblots were performed using an enhanced chemiluminescence Western blotting analysis system (Amersham). Bands in films were analyzed using scanner photodensitometry and quantified using Kodak 1D Image Analysis software.

Measurement of carbonyl content. Carbonyl groups were quantified using the Oxyblot protein oxidation detection kit (Chemicon, Chandlers Ford, UK). 2,4-Dinitrophenylhydrazine (DNPH) derivatization was carried out for 15 min on 15 μg of homogenate protein following the manufacturer's instructions. Proteins were transferred to nitrocellulose filters by means of a slot-blot system (Bio-Rad, Hercules, CA). After incubation with anti-DNP antibody, blots were developed using a chemiluminescence detection system (Amersham). Bands in films were analyzed using scanner photodensitometry and quantified using Kodak 1D Image Analysis software. To determine 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 to the DNPH derivatization.

Measurement of thiobarbituric acid-reactive substances. Lipid peroxidation levels or thiobarbituric acid-reactive substances (TBARS) were determined as malondialdehyde-thiobarbituric acid adducts according to Buege et al. (6). Peroxidation levels were measured spectrophotometrically at 532 nm, using a molar extinction coefficient of 1.56×10^5 M^{-1}/cm, and expressed as nanomoles of TBARS per milligram of protein.

Statistics. Results are means ± SE. Statistical analysis was carried out using the Statistical Program for the Social Sciences software (SPSS 14.0). Statistical significance of the data was assessed using two-way ANOVA. The statistical factors analyzed were restriction diet (R) and sex (S). Student's *t*-test was used to determine the differences between the groups involved. Statistical significance was set at $P < 0.05$.

RESULTS

Liver mass and composition. The effects of CR and sex on liver mass and composition are summarized in Table 1. As expected, body and liver mass were significantly lower in CR compared with AL-fed rats of both sexes. Liver mass loss in restricted male rats seemed to be slightly greater compared that in female rats (28 vs. 21%). No significant differences were found in total protein, DNA, or triglyceride content.

Liver mitochondrial content. The levels of several mitochondrial markers such as mitochondrial protein, mtDNA, or COX II content are compiled in Table 2. No differences were observed in mitochondrial protein content per gram of tissue in any of the studied groups. Nevertheless, mtDNA levels per gram of tissue were significantly higher in males compared with females in both control and restricted rats. Thus the protein/mtDNA ratio was higher in female rats, indicating a higher protein content per mitochondria in that sex.

This greater amount in mitochondrial protein adjusted to mtDNA is in agreement with the higher levels of TFAM found in females (representative Western blots are shown in Fig. 1). COX subunit II also showed higher levels in female compared with male rats fed under AL conditions. No CR effects were found on these parameters.

O_2 consumption. The rate of mitochondrial O_2 consumption was measured with glutamate/malate or succinate as substrates in states 4 and 3. As shown in Fig. 2, female rats showed higher

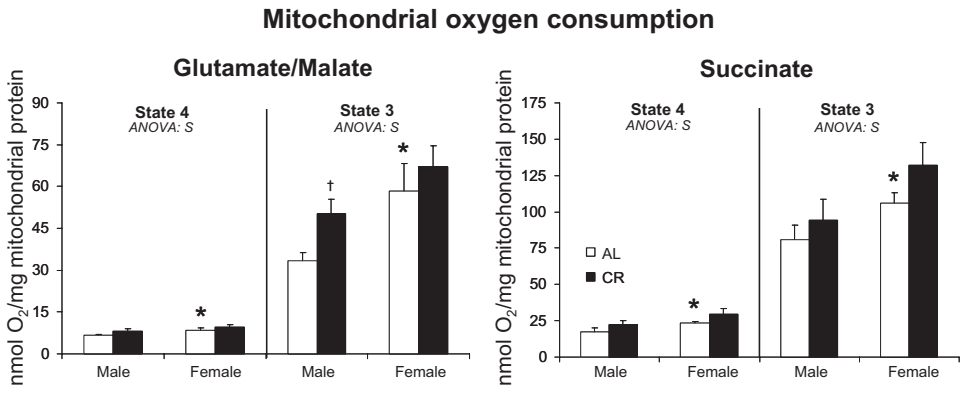

Fig. 2. Effect of sex and CR on liver mitochondrial O_2 consumption. The substrate/ADP titration protocol is described in detail in MATERIALS AND METHODS. Bars represent means ± SE of 6 animals per group. ANOVA: S, effect of sex ($P < 0.05$). Student's *t*-test: *$P < 0.05$, male vs. female. †$P < 0.05$, control (AL) vs. restricted (CR).

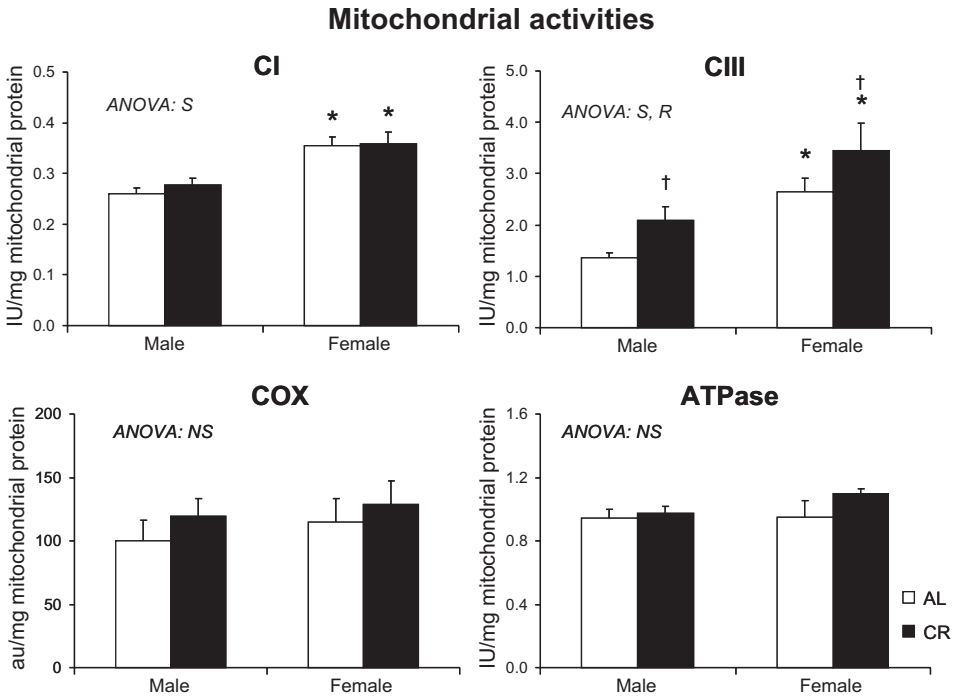

Fig. 3. Effect of sex and CR on activity of mitochondrial oxidative phosphorylation (OXPHOS) complexes (CI, complex I; CIII, complex III) in liver mitochondria. Bars represent means ± SE of 6 animals per group (au, arbitrary units). ANOVA: S ($P < 0.05$); R, effect of diet ($P < 0.05$); NS, not significant. Student's *t*-test: *$P < 0.05$, male vs. female. †$P < 0.05$ control vs. restricted.

oxidative capacity than male rats independently of group, state, or substrate used.

Under CR conditions, O_2 consumption showed a trend for increase in both sexes that only marginally failed to reach significance ($P = 0.063$). Nevertheless, this increase was statistically significant in male liver mitochondria when respiring in state 3 with glutamate/malate as substrate.

Mitochondrial activities. Figure 3 shows the activities of several electron transport chain complexes key in oxidative and phosphorylative capacities of mitochondria. Complex I and III activities were significantly higher in female rats in both dietary conditions. CR had no effects on complex I activity, whereas complex III activity was significantly increased by CR in both sexes. Complex IV or COX activity was unaffected by sex or CR. With regard to ATPase, phosphorylative capacity of liver mitochondria was unchanged in either sex or dietary intervention.

Mitochondrial H_2O_2 production. In view of the greater activity of some mitochondrial complexes in female rats and the increasing tendency induced by CR in these parameters, we measured the production of H_2O_2 in mitochondria using succinate as substrate (Fig. 4). The rate of H_2O_2 production of liver mitochondria from female AL-fed rats was significantly higher than that of males. This sex-related difference in H_2O_2 production disappeared under CR by decreasing female H_2O_2 production rate.

Markers of oxidative damage. To estimate the profile of oxidative damage, we measured the protein carbonyl and TBARS content in the rat liver of the studied groups (Fig. 5). For protein carbonyls, no sex-related differences were observed between AL groups, whereas CR induced a decrease in carbonyl content that was statistically significant in female rats. Lipid peroxidation measured as TBARS content was also found to be reduced in both sexes by CR.

Mitochondrial antioxidative activities. Figure 6 shows the specific activity of the main antioxidant enzymes MnSOD and GPx in mitochondrial H_2O_2 scavenging. MnSOD activity was similar between sexes but was slightly increased by CR in female rats ($P < 0.1$). GPx activity in female rats was twice that in male rats, and CR showed a trend to increase this activity in both sexes ($P < 0.1$).

DISCUSSION

Sexual dimorphism in liver mitochondria. Several studies have demonstrated that there is a sexual dimorphism in mitochondrial metabolism in rodents, especially affecting tissues with an important involvement in the energy metabolism such as liver, muscle, or brown adipose tissue (7, 20, 43). Our laboratory (20) previously reported that female rats exhibit functional and morphological differences in liver mitochondria showing greater machinery and differentiation degree than males. In the present study, comparisons between mitochondria

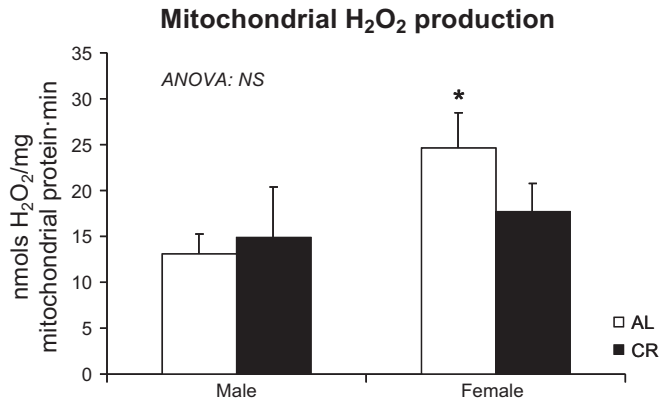

Fig. 4. Effect of sex and CR on H_2O_2 production in liver mitochondria. Mitochondria were incubated with succinate as substrate in the presence of Amplex red as probe for H_2O_2 production as described in MATERIALS AND METHODS. Bars represent means ± SE of 6 animals per group. ANOVA: NS. Student's *t*-test: *$P < 0.05$, male vs. female.

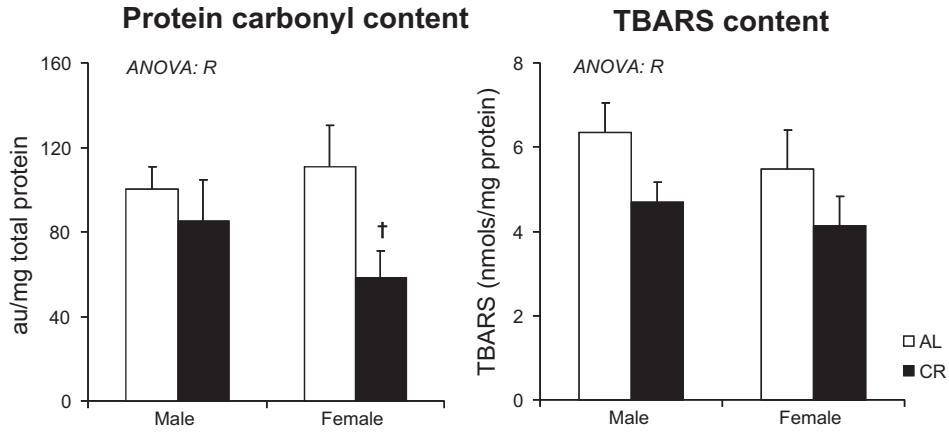

Fig. 5. Effect of sex and CR on markers of oxidative stress. Protein carbonyls and thiobarbituric acid-reactive substances (TBARS) were determined as described in MATERIALS AND METHODS. Bars represent means ± SE of 6 animals per group. ANOVA: R ($P < 0.05$). Student's *t*-test: †$P < 0.05$, control vs. restricted.

from control male and female rats confirmed our previous findings but also highlighted differences in liver mitochondrial number between sexes. Considering that mtDNA content is indicative of number of mitochondria (9, 23), female rats showed a lower mitochondrial content per cell compared with males. However, the content of protein per mitochondria, and its specific O_2 consumption, indicates a greater machinery or differentiation state of these mitochondria in agreement with the higher membrane potential previously described in female liver mitochondria (20). Although female rats presented a lower number of mitochondria, their total levels of COX II exceeded that of males (see Table 2). This suggests that the higher maturation degree of mitochondria from female rats counteracts their lower number of mitochondria, even exceeding the total oxidative capacity of males as interpreted by O_2 consumption data. Nevertheless, although COX II protein levels and mitochondrial respiration rate were higher in female compared with male rats, mitochondrial COX activity was found not to be significantly different between sexes, indicating that additional factors may be modulating the activity of this flux-generating complex in mitochondria (28).

The aforementioned differences in mitochondrial characteristics imply variations in the proliferation and differentiation patterns. In this sense, TFAM plays a key role in mitochondrial biogenesis, because it is basic to the initiation of both replication and expression of mtDNA, which codes for part of the mitochondrial proteins such as COX II (11, 37). It has been postulated that small amounts of TFAM are necessary to initiate mtDNA replication, whereas expression of mtDNA is activated only at high concentrations of this factor (13, 33). Our data agree with this idea, since mitochondria from female rats showed higher TFAM levels in concert with their higher differentiation state, whereas the lower levels of males agree with their greater mitochondria number as interpreted by their mtDNA content.

It has been shown that increased mitochondrial membrane potential and/or elevated O_2 consumption may result in elevated reactive oxygen species (ROS) (36). In our study, the higher hydrogen peroxide production observed in mitochondria from female rats is consistent with this notion. However, these results differ from other studies, which found a lower hydrogen peroxide production in mitochondria from female rats (3, 4). These studies consider that the higher antioxidative activities such as GPx in female rat mitochondria are responsible for the lower ROS production. Our study and those previously mentioned agree in the finding of higher antioxidant activity in female rats but differ in the involvement of these enzymes in the net ROS production in the in vitro assay conditions. In this way, end products of oxidative damage such as carbonyl content or TBARS could be better markers of oxidative stress, since they are the result of the balance among total peroxide production, antioxidant defenses, and repairing systems. No sex-related differences were found in these markers of oxidative damage, indicating that the higher GPx activity

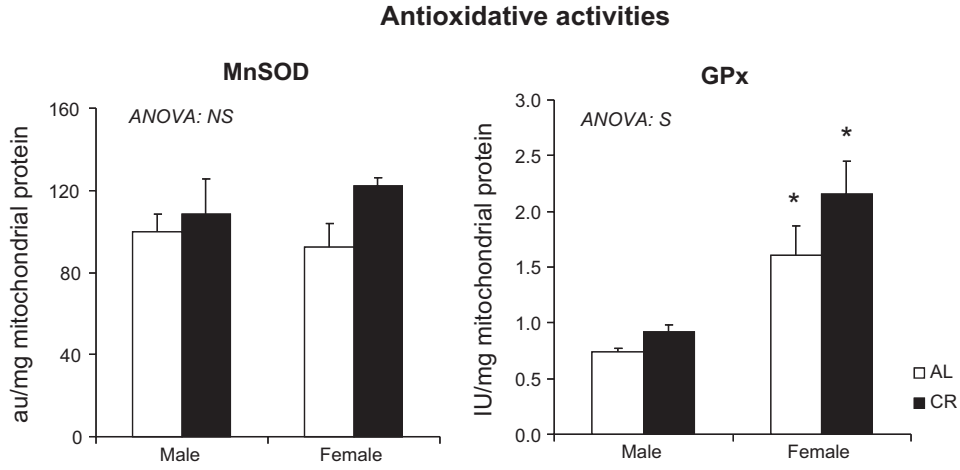

Fig. 6. Effect of sex and CR on liver mitochondrial antioxidative activities. Mitochondrial antioxidant superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) activity was determined as described in MATERIALS AND METHODS. Bars represent means ± SE of 6 animals per group. ANOVA: S ($P < 0.05$); NS, not significant. Student's *t*-test: *$P < 0.05$, male vs. female.

observed in the current study in mitochondria from female rats, or any other repairing system, may counteract the higher peroxide production.

Effect of sex on liver mitochondrial response to short-term CR. Taking into account the sexual dimorphism observed in liver mitochondria bioenergetics, our purpose in this study was to determine whether the CR effects on mitochondria are dependent on sex. To our knowledge, this is the first work that looks for sex-related differences in the liver mitochondrial phenotype in response to CR. Previous works in male rats have found that CR does not modify O_2 consumption or metabolic rate in mitochondria and hepatocytes isolated from liver of rats fed under CR conditions (26, 50). In contrast, a recent study reported that hepatocytes isolated from CR male rats reduce their total O_2 consumption but increase their mitochondria pool (30). Our results are in agreement with the former set of reports, with CR not exerting significant changes in mitochondrial O_2 consumption or activities. However, in the current study we noticed that CR induced a significant increase in complex III activity, with a similar trend in both COX activity ($P = 0.067$) and mitochondrial respiration rate ($P = 0.063$). This increase in mitochondrial respiration rate was even statistically significant in mitochondria of restricted male rats respiring in state 3 with glutamate/malate. These findings are in agreement with a previous work showing a time-dependent increment of mitochondrial oxidative capacity in liver of male rats subjected to moderate CR (26%) (2). This tendency to increase mitochondrial oxidative capacity could be related to a higher energy demand due to the role the liver plays in the adaptation of the organism to nutritional changes. Thus, in the case of CR, animals are subjected to intermittent and more prolonged fasting periods, increasing insulin sensitivity, liver β-oxidation, and ketone bodies synthesis (17, 32), functions that could be affecting liver energy demands. Interestingly, intermittent fasting (diets with reduced meal frequencies such as every-other-day fasting) also have been shown to increase life span similarly to CR, even when there is little or no overall decrease in calorie intake (1, 14). Further studies are necessary to clarify whether the benefits of CR come from the reduction in calories or from prolonging fasting periods.

With regard to oxidative stress, previous works in male rats have demonstrated that long-term CR (>12 mo) attenuates oxidative damage to macromolecules by lowering H_2O_2 production (15, 16, 31). However, when animals are subjected to short-term CR (<6 mo), a reduction or no change has been reported depending on the study (15, 25, 41). In our 3-mo CR assessment, we did not find a statistically significant reduction in H_2O_2 production, although females showed a trend toward a reduction ($P = 0.08$), achieving levels similar to that in males. The levels of markers of protein and lipid oxidation were also decreased by CR in agreement with previous reports (12, 27). No sex-related effects were found in these parameters in response to CR, although carbonyls showed a profile in accordance with higher CR effects in females. It is worth noting that females started from a higher oxidative capacity in liver mitochondria that was increased by CR. However, as shown in Fig. 5, the levels of oxidative damage were reduced, showing levels similar to that in males. This fact may be explained on the basis of the higher GPx activity found in the liver mitochondria from female rats. Nevertheless, the likelihood of other mechanisms operating in this attenuation of oxidative damage, such as changes in mitochondrial efficiency, protein turnover, or other antioxidant systems, cannot be ruled out.

In relation to this, CR induced an upward trend in GPx activity in agreement with previous reports (40, 52), whereas MnSOD activity remained almost unchanged (29). However, the different studies in the literature do not support a clear-cut pattern of CR-related changes in antioxidant defenses, with reports showing controversial effects on these activities (35, 42). Differences in rodent model, strain, age of initiation, and duration of CR may be responsible for the differences between these studies.

The intrinsic sex-related differences in GPx activity were not modified by restricted feeding, with female rats showing higher activity than males. This may explain, at least in part, the aforementioned similar-sex attenuation of oxidative damage despite the higher oxidative capacity of female rats. It is worth noting that the activity of the glutathione system depends on the availability of reducing equivalents. Whether greater GPx activity in females also involves sex-related differences in the NADPH mitochondrial production system awaits further experimental exploration.

In summary, liver mitochondria from female rats show signs of a higher oxidative capacity with a greater differentiation degree than that of male rats. These mitochondrial features are consistent with the higher hydrogen peroxide production but not with elevated oxidative damage, probably in relation with the greater antioxidant protection in females. Furthermore, the results also demonstrate that CR does not alter the sexual dimorphism in liver mitochondrial oxidative capacity and antioxidant defenses.

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Gender-dependent Differences in Serum Profiles of Insulin and Leptin in Caloric Restricted Rats

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Key words
- fat adipose tissue
- body weight
- sex-hormones
- energy homeostasis
- insulin level
- leptin level

Abstract

In the present study, we have investigated whether differences between male and female rats described in response to 40% caloric restriction (CR) were influenced by circulating level variations of sex hormones and/or insulin and leptin. Body weights (BW), organ weights, and adipose depot weights (ADW) were also measured. The most affected tissues by CR were the fat depots. Metabolically active organs were the least affected, especially more in females than in males (male weight lost: 24.3% vs. female: 17.3%). Testosterone and estradiol circulating levels did not show changes by CR. Insulin levels were decreased by CR in both genders, but was more evident in female rats than males. Leptin serum levels were higher in male rats than in females, and CR caused a circulating leptin level reduction only in males. In conclusion, our results indicate that leptin and insulin could be one of the keys of the different hormonal control of energy homeostasis in response to CR between female and male rats. In this sense, leptin serum levels correlated statistically with BW and with individual ADW only in male rats, whereas insulin serum levels correlated statistically with BW and with any of the ADW studied only in females.

Introduction

Caloric restriction (CR) is the only nongenetic factor that has been shown to extend life span and to slow the aging rate and disease in mammals [1,2]. CR is a frequent condition in nature, and hence throughout the evolution organisms have evolved mechanisms to respond and adapt to this situation.

Evolution theories suggest that mammalian females could have been under greater evolutionary selection pressures than males because of their role in reproduction and species maintenance and would therefore have developed more efficient response mechanisms against CR. In fact, several studies on rodents have shown that CR has a greater and more permanent effect on physical growth in male rats than in females [3]. In female rats, the response to CR is accompanied by thermogenesis deactivation in the brown adipose tissue diminishing energy expenditure, and consequently saving energy [4,5].

These gender differences in response to CR could be caused by a different hormonal environment, specifically, by circulating levels of sex hormones, insulin, and leptin, since these hormones are key factors in energy homeostasis regulation. Likewise, this dimorphism could also be influenced by the different distribution and extent of the different adipose tissue depots in both genders, which are closely correlated with circulating levels of these hormones. In rats, sex hormones (testosterone, 17β-estradiol and progesterone) have been shown to be involved in both the control of thermogenesis and energy balance [6–9], as well as to influence the body fat distribution [8,10–12]. Besides, sex hormone serum levels are reduced by CR [13,14].

Energy balance depends on the mechanisms regulating and coordinating food intake and energy expenditure. Food intake causes a rapid increase in insulin and leptin circulating levels [15]. Insulin acts by upregulating glucose uptake and by downregulating TAG (triacylglycerol) hydrolysis [16] while leptin acts as a satiety factor and increases energy expenditure [17–19]; besides, leptin serum levels reflect fat energy reserves [17,20–23]. In this way, several authors have reported a serum level reduction of insulin in

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Table 1 Effect of 40 % calorie restriction (CR) on body weight, organ weights (OW) (heart, liver, kidneys, and brain), their sum weight (Sum OW), and organ weight as a percentage relative to body weight (%BW), in male and female rats compared with their respective ad libitum (AL) fed animals

		Males		Females		
		AL	40 % CR	AL	40 % CR	ANOVA
body weight	Weight g	482 ± 14	354 ± 8†	261 ± 8*	194 ± 4†*	S, D, SxD
heart	OW g	0.99 ± 0.02	0. 80 ± 0.02	0.68 ± 0.03	0.55 ± 0.01	S, D
	% BW	0.21 ± 0.01	0.23 ± 0.01	0.27 ± 0.01	0.29 ± 0.01	S, D
liver	OW g	13.7 ± 0.7	9.86 ± 0.49†	7.17 ± 0.39*	5.63 ± 0.20†*	S, D, SxD
	% BW	2.84 ± 0.12	2.78 ± 0.11	2.75 ± 0.12	2.91 ± 0.13	NS
kidneys	OW g	2.42 ± 0.10	1.90 ± 0.03†	1.41 ± 0.05*	1.18 ± 0.04†*	S, D, SxD
	% BW	0.50 ± 0.02	0.54 ± 0.01	0.54 ± 0.02	0.61 ± 0.02	S, D
brain	OW g	1.73 ± 0.05	1.73 ± 0.11	1.61 ± 0.08	1.66 ± 0.06	NS
	% BW	0.36 ± 0.01	0.49 ± 0.03	0.62 ± 0.04	0.85 ± 0.02	S, D
sum OW	OW g	18.9 ± 0.9	14.3 ± 0.6†	10.9 ± 0.5*	9.02 ± 0.15†*	S, D, SxD
	% BW	3.91 ± 0.13	4.03 ± 0.12	4.25 ± 0.12	4.66 ± 0.12	S, D

Values are the mean ± SEM; n = 6 animals per group. ANOVA ($p < 0.05$): S: gender effect; D: diet effect; SxD: interaction of gender and diet; NS: no significant difference. Student's t-test post hoc analysis ($p < 0.05$)

†Restricted vs. ad libitum

* Males vs. females

response to CR in male and female rats [24, 25], and of leptin in males [26].

The aim of this study was to determine whether differences between male and female rats in the control of energy homeostasis in response to 40 % CR were influenced by circulating level variations of sex hormones and/or insulin and leptin.

Materials and Methods

All animals were treated in accordance with the University Bioethical Committee guidelines for animal care and EU regulations (86/609/EEC).

Ten-week-old Wistar rats, 12 males and 12 females (supplied by Charles River, Barcelona, Spain) were housed individually in wire-bottomed cages to prevent coprophagia and were acclimatized in our animal facility (22 °C, 12 h light/dark cycle, 10:00 AM/10:00 PM). Animals were randomized into four experimental groups of six animals each: ad libitum (AL) males, AL females, CR males and CR females. AL animals were fed with standard chow pellets (A04, Panlab, Barcelona, Spain). Restricted animals were fed for 100 days at the start of the dark cycle with 60 % of the mean amount of diet consumed by the same gender AL group the previous week.

One hundred days after the beginning of CR, all animals were sacrificed by decapitation at the start of the light cycle. Heart, liver, kidneys, brain, and the main white adipose tissue depots (inguinal, retroperitoneal, mesenteric, and gonadal: epididymal from males or periovarian from females) were dissected, weighed, and collected.

Serum was collected and stored at −70 °C until analysis. Serum 17β-estradiol and testosterone concentrations were measured with competitive immunoenzymatic colorimetric kits (DiaMetra, Milano, Italy); leptin, insulin, and glucose concentrations were measured with a Quantikine Mouse Leptin Immunoassay (R & D Systems, Minneapolis, USA), a rat insulin ELISA Enzyme Immunoassay (Mercodia, Upsala, Sweden), and a R-Biopharm d-Glucose Enzymatic BioAnalyse UV-test (Roche Diagnostics), respectively. Hormone and glucose measurements were made following the manufacturer's instructions.

In order not to distort the ad libitum condition, animals were not under fasting conditions at the moment of sacrifice. For this reason, we calculated the insulin/glucose ratio in order to normalize the differences between groups by removing intake effect from the insulin serum level values.

The Statistical Program for the Social Sciences software for Windows (SPSS, Version 14.0) was employed for all statistical analyses. Data are presented as mean ± SEM. Differences between groups were analyzed by two-way analysis of variance (ANOVA) to assess the effects of diet and gender, and Student's t-test for post hoc comparisons. Statistical significance was set at $p < 0.05$. Bivariate correlations were assessed by Pearson's correlation coefficients (with one-tailed test of significance).

Results

Organ weights

Table 1 shows the body weight (BW), the single organ weights (OW) (heart, liver, kidneys, and brain), their sum weight (Sum OW), and the percentage representing these weights with regard to body weight (%BW) at the end of the experiment, in each experimental group.

In general, the organs of the males were heavier than those of females, except for brain that showed similar weight. However, regarding %BW, females had proportionally larger organs. This situation was observed in both AL (total OW males: 3.91 vs. females: 4.25 %BW) and CR animals (total OW males: 4.03 vs. females: 4.66 %BW).

Total and single OW were lower in CR than in AL animals, in both genders. Nevertheless, the %BW of most of the organs was higher in CR groups.

Adipose tissue depot weights

Table 2 shows single adipose depot weights (ADW) (inguinal, retroperitoneal, mesenteric, and gonadal), their sum weight (Sum ADW), and the percentage representing these weights with regard to body weight (%BW) at the end of the experiment, in each experimental group.

Males had a greater quantity of Sum ADW and this represented a higher %BW than females in both AL (males: 6.03 vs. females:

Table 2 Effect of 40 % calorie restriction (CR) on adipose depot weights (ADW) (inguinal, retroperitoneal, mesenteric, and gonadal), their sum weight (Sum ADW) and on tissue weight as a percentage relative to body weight (%BW), in male and female rats compared with their respective ad libitum (AL) fed animals

		Males		Females		
		A L	40 % CR	A L	40 % CR	ANOVA
inguinal	ADW g	7.36 ± 0.79	2.97 ± 0.29†	2.74 ± 0.43*	1.06 ± 0.13†*	S, D, SxD
	% BW	1.52 ± 0.15	0.85 ± 0.10	1.05 ± 0.16	0.55 ± 0.07	S, D
retroperitoneal	ADW g	8.28 ± 0.93	1.83 ± 0.53†	3.01 ± 0.91*	0.55 ± 0.16†*	S, D, SxD
	% BW	1.71 ± 0.17	0.53 ± 0.17	1.15 ± 0.35	0.28 ± 0.08	S, D
mesenteric	ADW g	5.79 ± 0.46	1.74 ± 0.32†	2.99 ± 0.63*	0.72 ± 0.08†*	S, D, SxD
	% BW	1.20 ± 0.08	0.50 ± 0.10	0.93 ± 0.13	0.37 ± 0.04	D
gonadal	ADW g	9.32 ± 0.81	3.11 ± 0.41	5.19 ± 1.03	1.08 ± 0.29	S, D
	% BW	1.92 ± 0.12	0.89 ± 0.13	1.99 ± 0.40	0.56 ± 0.15	D
sum ADW	weight g	29.2 ± 2.8	9.65 ± 1.46†	10.6 ± 1.5*	3.41 ± 0.63†*	S, D, SxD
	% BW	6.03 ± 0.42	2.76 ± 0.46	4.08 ± 0.56	1.76 ± 0.32	S, D

Values are the mean ± SEM; n = 6 animals per group. ANOVA ($p < 0.05$): S: gender effect; D: diet effect; SxD: interaction of gender and diet; NS: no significant difference. Student's t-test post hoc analysis ($p < 0.05$)

†Restricted vs. ad libitum

* Males vs. females

Table 3 Effect of 40 % calorie restriction (CR) on hormones (estradiol, testosterone, leptin, and insulin) and glucose serum concentrations compared with ad libitum (AL) feeding

	Males		Females		
	A L	40 % CR	A L	40 % CR	ANOVA
estradiol (pg/ml)	2.94 ± 0.49	3.20 ± 0.53	6.32 ± 0.91*	5.57 ± 1.03*	S
testosterone(pg/ml)	526 ± 246	497 ± 227	26 ± 6*	36 ± 9*	S
leptin (ng/ml)	6.33 ± 1.06	2.43 ± 0.27†	3.49 ± 0.40*	3.13 ± 0.29	D, SxD
insulin (pg/ml)	596 ± 124	491 ± 109	492 ± 60	241 ± 33†*	S, D
glucose (mM)	7.30 ± 0.41	5.65 ± 0.33†	9.49 ± 0.56*	5.85 ± 0.48†	S, D, SxD
insulin/glucose	81.5 ± 10.6	85.9 ± 15.9	51.4 ± 5.0*	42.0 ± 5.5*	S

Values are means ± SEM; n = 6 animals per group. ANOVA ($p < 0.05$): S: gender effect; D: diet effect; SxD: interaction of gender and diet; NS: no significant difference. Student's t-test analysis ($p < 0.05$)

†Restricted vs. ad libitum

* Males vs. females

4.08 %BW) and CR animals (males: 2.76 vs. females: 1.76 %BW). The greater fat mass was in the gonadal depot in both genders. CR caused a decrease not only in all single and Sum ADW, but also in the %BW of each depot.

Hormone and glucose serum levels

Table 3 shows the circulating levels of testosterone, estradiol, insulin, leptin, glucose, and the insulin/glucose ratio at the end of the experiment. As expected, testosterone serum levels were higher in male rats whereas estradiol serum levels were higher in females. Neither hormone showed significant variations by CR effect.

Serum leptin levels were higher in AL male rats than in females. CR caused a significant fall in their circulating levels in males whereas they were maintained in females. Female rats showed lower insulin circulating levels than males, but not statistically significant. Despite this effect, CR caused a drop in circulating levels, which was greater in females.

Serum glucose levels were higher in AL females than in males, with a greater fall taking place in CR females down to the same level as in CR males. This drop followed a similar tendency to insulin level variations. Nevertheless, taking into account the insulin/glucose ratio, which would reflect insulin levels independently of status intake, this ratio was lower in female rats than in males and CR caused a tendency to decrease whereas in males it was maintained.

The correlation of leptin and insulin serum levels with the sum ADW from male and female rats is presented in **Fig. 1**. Leptin showed a statistically significant correlation with Sum ADW only in males, whereas insulin correlated with Sum ADW only in females.

Table 4 shows the correlations of insulin and leptin with the body weight, inguinal, retroperitoneal, mesenteric and gonadal weights, visceral as the sum of mesenteric plus gonadal ADW, visceral + retroperitoneal ADW (V+R), and the Sum ADW. Leptin serum levels correlated statistically with the BW and also with individual ADW, but only in males. Conversely, in females, it was the insulin serum levels which correlate with BW and most of ADW.

Discussion and Conclusion

Forty per cent CR for 100 days in young, growing rats caused a deceleration in their general growth (26%), which affected tissues and organs in a different way. The most affected tissues were the fat depots, in which not only did CR produce weight reduction (67.4%), but also fat mobilization relative to AL-fed animals. Metabolically active organs were the least affected – the most protected – more so in females than males (male weight lost: 24.3% vs. female: 17.3%), which is in agreement with other authors [27,28]. These results were also in line with

Table 4 Correlations of insulin and leptin with the body weight, inguinal, retroperitoneal, mesenteric and gonadal weights, visceral as the sum of mesenteric plus gonadal ADW, visceral + retroperitoneal ADW (V + R), and the Sum ADW

	Males		Females	
Weights/hormones	**Leptin**	**Insulin**	**Leptin**	**Insulin**
body weight	0.838**	0.056	0.488	0.843**
inguinal	0.862**	0.062	0.469	0.643*
retroperitoneal	0.893**	0.025	0.542	0.546
mesenteric	0.885**	0.150	0.650*	0.769**
gonadal	0.910**	0.135	0.090	0.368
visceral	0.903**	0.142	0.268	0.729*
V + R	0.904**	0.096	0.270	0.723*
sum ADW	0.904**	0.090	0.245	0.716*

Values are Pearson correlation coefficients. Level of significance (1-tailed)

* $p < 0.05$

** $p < 0.01$

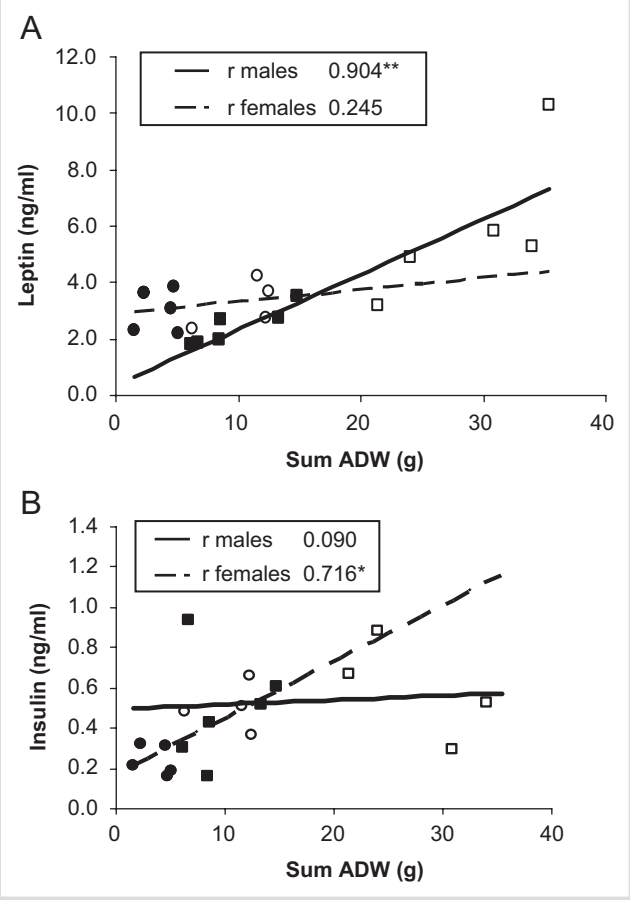

Fig. 1 Correlation of leptin (**A**) and insulin (**B**) serum levels with the sum of adipose depot weights (Sum ADW). Bivariate linear regression analysis was made. Regression lines for male and female rats (6 animals per group) are shown. For each gender, ad libitum (AL) and restricted (RC) animals were pooled. Open and closed symbols represent AL and RC animals, respectively; squares represent males and circles females. Continuous and discontinuous lines represent fit line for males and females, respectively.

the prior work from our research group, which reported that CR produces brown adipose tissue thermogenesis deactivation only in female rats, thus decreasing their energy expenditure [4], and explaining the better maintenance of metabolically active organs in females.

In order to analyze the different response between genders to CR, we decided to study whether hormonal environment also reflected this gender dimorphism. We specifically analyzed circulating levels of sex hormones, leptin, and insulin.

After 100 days of CR, testosterone and estradiol circulating levels did not show changes. Other authors, however, have reported a testosterone serum level reduction [13, 14], but in younger male rats with a shorter period of CR. Testosterone has been described as a thermogenesis inhibitor and estradiol as an activator in cultured brown adipocytes [6]; however, when administered in vivo testosterone lacks effects and estradiol has an inhibitory role [29, 30]. Since testosterone and estradiol maintained their circulating levels in this situation, the energetic metabolism changes and diminishing female rat thermogenesis could not be attributed to level variations of circulating sex hormones.

CR caused insulin circulating level reduction in both genders, as described in rodents by other authors [24, 25], but was more evident in female rats than males. This fall in insulin serum levels could be attributed to change in food schedule, since, during CR, pellets were supplied at dark cycle onset and the food was consumed over a short time, which would be equivalent to daily limited intermittent fasting. Nevertheless, food schedule change fails to explain the different insulin serum level drop between male and female rats, given that, the alimentary habit change was similar in both genders. In fact, insulin/glucose ratio values, which makes it possible to remove the intake effect from insulin circulating level values, were lower in females than males and showed a slight fall due to CR only in females.

Leptin serum levels were higher in male rats than in females, as other authors have already reported [31–34]. CR caused a circulating leptin level reduction only in males in spite of the significant reduction in body fat percentage of both genders and the fact that leptin has been considered a good body fat mass indicator. Research carried out by Landt et al. [28], in which they state that male rats generally have higher leptin serum concentrations in relation to body fat content than female rats with a much more apparent distinction at a percentage of body fat of >7%, could explain this fact. Hence, a correlation test of leptin serum levels with Sum ADW (the sum of inguinal, retroperitoneal, mesenteric, and gonadal depot weights) was carried out (see **Fig. 1A**), showing a statistically significant correlation in males ($r = 0.904$), but not in females ($r = 0.245$). Likewise, in human beings, 25% greater body fat percentage has been reported to be needed for leptin serum levels to start to differ between genders, with these leptin levels rising 3 times faster in

women than in men [35]. This idea of a threshold could be happening also in the case of rats. Thus, it would be necessary for leptin level to pass a threshold in order to reflect the body fat content. Therefore, the low %BW represented by Sum ADW in females (AL: 4.08 and CR: 1.76%) could explain the lack of significant correlation between total adipose depot weight and serum leptin observed.

On the other hand, insulin secretion has also been reported in direct proportion to BMI [36], which reflects body fat level. Therefore, the correlation between insulin serum levels and Sum ADW (see **Fig. 1B**) was calculated, and an opposite relationship was observed with regards to leptin. In male rats there was no correlation (r=0.090), whereas in females there was a statistically significant correlation between insulin levels and total adipose depot weight (r=0.716).

Both leptin and insulin serum levels were correlated with body weight and individual adipose depot weights (see **Table 4**), in accordance with the above mentioned results. In males, leptin serum levels correlated statistically with the BW and also with individual ADW (r greater than 0.838), whereas in females there was no correlation with either BW or single ADW (r less than 0.542), except for mesenteric depot. Conversely, insulin serum levels in males, as with Sum ADW, did not correlate with either BW or any of the ADW studied (r lower than 0.150); whereas in females these correlations did exist with BW and most of ADW (r greater than 0.643), except for retroperitoneal and gonadal.

As our research group has previously described in this model, the response to CR in female rats, in contrast to male ones, is accompanied by thermogenesis deactivation in the brown adipose tissue diminishing energy expenditure, and consequently saving energy [4,5]. Accordingly, in female rats, which had lower adiposity than males, leptin could not play such a relevant role in the control of either energy metabolism or thermogenesis. Given that in females leptin levels were maintained but adipose depot weight and thermogenesis dropped, leptin levels and adipose depot weight fell in male rats while the thermogenesis was maintained. In this context, insulin could be the hormone which plays a more relevant role in energy metabolism control in female rats. The preferential action of leptin vs. insulin and vice versa on the control of the energy metabolism could be influenced by the sex-hormone environment. It is worth pointing out that sex hormones are responsible, at least in part, for the different body fat distribution between genders [11,12], as well as for their different sensitivity to insulin and leptin [10,37], since these hormones share both the same signaling pathway activation [38,39] and metabolical functions [40].

The gender differences described in the preferential signaling of insulin versus leptin in female rats could be determined by the greater evolutionary selection pressures undergone by females due to their more relevant role in reproduction and species maintenance. This fact has conferred female rats with a better adaptation capacity to adverse conditions such as CR, greater survival capacity and, consequently, a longer life span.

In summary, leptin and insulin could be one of the keys of the different hormonal control of energy homeostasis in response to CR between genders and the predominance of one hormonal signal or the other could be conditioned by adiposity level and distribution, which in turn could be conditioned by sex hormones. Nevertheless, further experiments would be necessary in order to analyze in depth the role of these hormones in energy homeostasis control.

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Caloric Restriction Retards the Age-Related Decline in Mitochondrial Function of Brown Adipose Tissue

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Abstract

Caloric restriction (CR) has been shown to prevent the age-associated loss of mitochondrial function and biogenesis in several tissues such as liver, heart, and skeletal muscle. However, little is known about the effects of CR on a tissue in which the mitochondria have no adenosine triphosphate (ATP)-producing purpose but show a high degree of uncoupling, namely brown adipose tissue (BAT). Hence, the aim of the present study was to analyze the effect of long-term CR on BAT mitochondrial function and biogenesis. BAT mitochondria obtained from 24-month-old male and female rats previously subjected to 40% CR for 12 months were compared with mitochondria from old (24 months) and young (6 months) ad libitum fed rats. Old restricted rats compared to old ad libitum fed ones showed a reduction in BAT size with respect to fat content and adipocyte number. Mitochondrial DNA content in BAT increased with age and even more so in restricted rats, indicating a summative effect of age and CR on mitochondrial proliferation. CR induced resistance to lose total and mitochondrial protein, COX activity, and uncoupling capacity with advancing age, in relation with a lower decrease of mitochondrial transcription factor A (TFAM). In summary, our results demonstrate CR prevents the age-associated decline in mitochondrial function in BAT, probably in relation with a lower impairment of mitochondrial biogenesis.

Introduction

Thermogenesis in brown adipose tissue (BAT) is a means to dissipate energy as heat in order to counteract changing environmental conditions, such as cold or excessive caloric intake.[1] BAT thermogenesis is dependent on the activity of its specific uncoupling protein 1 (UCP1), an inner-membrane mitochondrial protein that is able to short circuit the proton gradient generated by the respiratory chain and that is selectively inhibited by nucleotides diphosphate such as guanosine diphosphate (GDP).[2] Unlike white adipocytes, each brown adipocyte contains a high number of mitochondria with a huge UCP1 content, contributing, as a whole, to the great heat-producing capacity of the tissue.[3]

The ability to regulate body temperature diminishes with age both in human and rodents.[4,5] This decline in thermoregulation often results in a decrease in cold tolerance and in a lower control of body weight, promoting the age-related increase in adiposity. In rodent models, the age-associated decline in thermoregulation has been related to BAT atrophy,[6] which is manifested by great lipid accumulation, a decrease in adipocyte number, and also losses of functional protein and UCP1 activity (reviewed in Cannon and Nedergaard[3]). Moreover, this age-related decline in BAT functionality has been shown to be influenced by gender, with females showing a lower loss of thermogenic capacity with advancing age.[7,8]

Several studies have suggested that mitochondria play a crucial role in the aging process since they are the main source of free radicals and, at the same time, are the most immediate target of the oxidative damage. Impaired mitochondrial function would compromise the capacity to produce ATP and, therefore, the normal animal physiologic activities.[9] Caloric restriction (CR) is the only intervention known to be able to increase lifespan and delay the rate of aging in many species, including rodents.[10] Although the underlying mechanisms of CR are not completely understood, previous studies in rodent liver, heart, and skeletal muscle have revealed that CR reduces the generation of free radicals by mitochondria,[11–14] prevents the age-associated decline in mitochondrial function[15] and promotes mitochondrial biogenesis.[13,15,16] This improvement in mitochondrial

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functionality may prevent the age-associated decrease in adenosine triphosphate (ATP) production and, therefore, may be a reasonable mechanism in the amelioration of organ function and the extension of lifespan in old restricted animals. To date, nevertheless, no studies have investigated the effect of CR in the age-associated functional decline of BAT that, as mentioned, is a mitochondria-rich tissue, but in which the mitochondria have no ATP-producing purpose, and whose function, although beneficial for homeostasis, has great costs for the organism that would be counter-productive during low energy availability. In fact, in a previous report we demonstrated that short-term CR decreases BAT thermogenic capacity in young female rats, by decreasing UCP1 content and mitochondrial differentiation.[17] Thus, in order to extend the knowledge of the antiaging mechanisms underlying CR, our objective was to determine what effects long-term CR has on the decline in BAT mitochondrial function in old rats. To tackle this aim, we analyzed parameters of BAT thermogenic capacity in young (6 months) and old (24 months) rats of both genders and compare these with that of age-matched old rats subjected to 12 months 40% CR. Paradoxically, our study demonstrates that BAT thermogenic machinery is preserved by CR in old rats.

Materials and Methods

Animals and diets

All animals were treated in accordance with the university bioethical committee guidelines for animal care and EU regulations (86/609/EEC). One-year old Wistar rats (Charles River, Barcelona, Spain), seven males and seven females, were subjected to 40% caloric restriction for 1 year (old restricted rats, OR). The same number of age-matched rats were allowed ad libitum acces to a pelleted standard diet (Old rats, O) until the moment of sacrifice. The amount of food offered to OR rats was updated weekly according to the intake of old rats. Additionally, another group of ad libitum fed-rats was included in the study and sacrificed at 6 months of age (young rats, Y, $n = 8$). The rats were housed individually in wire-bottomed cages to prevent coprophagia in a temperature controlled room (22°C) with a 12-hour light-dark cycle and free access to water.

Sacrifice and isolation of BAT mitochondria

Animals were killed by decapitation at the start of the light phase. BAT was removed from the interscapular region and carefully dissected in order to prevent any contaminating muscle, connective or white adipose tissue. The tissue was weighed and homogenized in isolation buffer (250 mM sucrose, 5 mM Tris-HCl, 2 mM EGTA, pH 7.2, 4°C) in a Teflon/glass homogenizer. The homogenate was filtered through a layer of gauze. Aliquots were used for determination of total protein and triglyceride content as described elsewhere[18,19] and also complex IV activity and Western blotting measurements (see below). The rest of the homogenate was used for isolation of mitochondria by differential centrifugation. The nuclei and cell debris were removed by centrifugation at 500*g* for 10 min. The supernatant was centrifuged at 8000*g* to yield the mitochondrial pellet. The pellet was washed once by resuspension, centrifuged (8000*g*), and the final pellet was resuspended in the same buffer. The gravitational force used was chosen in order to achieve a representative fraction of whole mitochondrial population preserving integrity.[20] Mitochondrial protein was measured by the Bradford method.[18]

Mitochondrial oxygen consumption

Mitochondria were incubated in a water-thermostatically regulated chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph; Hansatech, Norfolk, UK) at a concentration of 0.15 mg mitochondrial protein per milliliter in buffer containing 145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM ethyleneglycoltetraacetic acid (EGTA) and 0.1 % bovine serum albumin (BSA; pH 7.4 at 37°C). Mitochondrial respiration rate was measured with glycerol-3-phosphate (10 mM) as substrate in the absence and in the presence of 500 μM GDP in order to inhibit UCP1.

Complex IV activity

Complex IV or COX (cytochrome c oxidase; EC 1.9.3.1) was measured using a spectrophotometric method.[21] Briefly, mitochondria were incubated in 0.1 M $NaPO_4H_2$, pH 7, in the presence of 2 μg/mL catalase and 5 mM substrate DAB (3,3′diaminebenzidine-tetrachloride). After 30 seconds, 100 μM reduced cytochrome c was added to start the reaction, and absorbance variation was followed over 15 min at 450 nm. COX activity was also measured in homogenate in order to calculate the total activity.

Total and mitochondrial DNA

Total DNA was measured in homogenates using the diaminobenzoic acid method.[22] Mitochondrial DNA was measured in pre-treated homogenates by real-time PCR as previously described[20] (primers sequence: 5′-TACACGATGAGGCAACCAAA-3′; 5′-GGTAGGGGGTGTGTTGTGAG-3′). Mitochondrial DNA was corrected by milligram of total DNA to provide an estimation of the mitochondrial content per adipocyte.

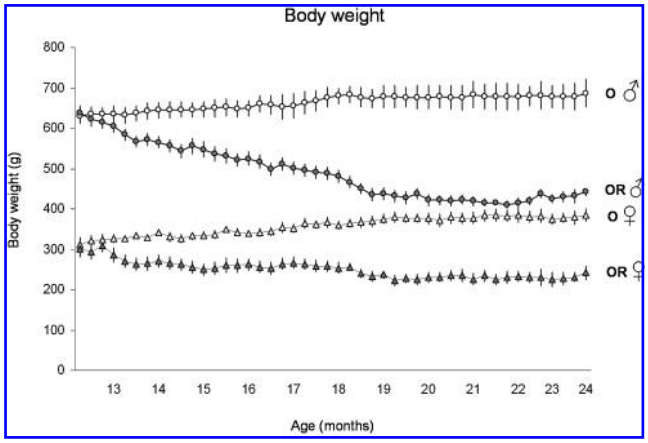

FIG. 1. Evolution of body weight in old and old restricted rats. Figure shows the evolution of body weight of old ad libitum fed rats (closed symbols) and old retricted rats (open symbols) throughout the period of CR (from 12 to 24 months of age). Circles and triangles represent males (♂) and females (♀) respectively. O, old rats; OR, old restricted rats. Values are mean ± standard error of the mean (SEM; $n = 7$).

Table 1. Effect of Age and CR on BAT Mass and Composition

	Male			*Female*				
	Young	*Old*	*Old restricted*	*Young*	*Old*	*Old restricted*	*ANOVA I*	*ANOVA II*
Body weight (g)	484 ± 13	687 ± 37	442 ± 9[b]	263 ± 6	395 ± 16[c]	242 ± 7[b,c]	A, G	G, R, GxF
BAT weight (g)	0.665 ± 0.027	1.051 ± 0.141	0.428 ± 0.040	0.327 ± 0.019	0.817 ± 0.073	0.288 ± 0.026	A, G	G, R
% BAT/Bw	0.138 ± 0.008	0.151 ± 0.015	0.097 ± 0.010	0.124 ± 0.010	0.202 ± 0.012[a,c]	0.119 ± 0.006	A, AxG	G, R
DNA/depot (mg)	1.53 ± 0.04	1.09 ± 0.24	0.69 ± 0.11	1.61 ± 0.13	0.91 ± 0.06	0.42 ± 0.04	A	R
Protein mg/mg DNA	20.1 ± 1.2	13.1 ± 1.2	22.6 ± 1.5	22.2 ± 1.4	15.5 ± 1.1	22.9 ± 1.3	A	R
Triglycerides mg/mg DNA	86.8 ± 8.3	246 ± 18	158 ± 18	79.03 ± 9.7	258 ± 31	178 ± 42	A	R

ANOVA, analysis of variance; BAT, brown adipose tissue; CR, caloric restriction; SEM, standard error of the mean.

ANOVA I, two-way ANOVA to assess the effects of aging and gender between young and old rats; ANOVA II, two-way ANOVA to assess the effects of restriction and gender between old and old restricted rats. Values are mean ± SEM ($n = 8$ for young, $n = 7$ for old and old restricted rats). For ANOVA ($p < 0.05$), A indicates aging effect, G indicates gender effect, R indicates restriction effect and GxR and AxG indicate the corresponding interactive effects. Student's *t*-test ($p < 0.05$).

[a]Y vs O.

[b]O vs OR.

[c]Males vs. females.

Western blot for UCP1 and TFAM

As described elsewhere,[23,24] 15 μg mitochondrial protein or 30 μg homogenate protein was fractioned by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; 12% polyacrylamide) and electrotransferred onto nitrocellulose filters. Ponceu S staining was used to provide visual evidence of correct loading and electrophoretic transfer of proteins to nitrocellulose filter. Membranes were incubated overnight at 4°C in a blocking solution of 5% non-fat powdered milk in T-phosphate-buffered saline (PBS; pH 7.5, containing 0.1% Tween 20). Antisera against UCP1 (UCP12-A, Alpha Diagnostics International, San Antonio, TX) and mitochondrial transcription factor A (TFAM, provided by Dr. Hidetoshi Inagaki) were used as primary antibodies. Developments of the immunoblots were performed using an enhanced chemiluminiscence Western blotting analysis system (Amersham Pharmacia Biotech, Piscataway, NJ). Bands in films were analysed by scanner photodensitometry and quantified using Kodak 1D Image Analysis Software.

Statistics

The Statistical Program for the Social Sciences software for Windows (SPSS, version 14.0; SPSS Inc., Chicago, IL) was used for all statistical analyses. Data are presented as means ± standard error of the mean (SEM). Statistical differences between young and old rats were analyzed by two-way analysis of variance (ANOVA) to assess the effects of aging and gender (ANOVA I). The effects of CR and gender were assessed in the same way but between old and old restricted rats (ANOVA II). Student's *t* test was performed when an interactive effect of aging and gender or CR and gender was shown. Statistical significance was set at $p < 0.05$ level.

Results and Discussion

Many experiments have established that CR markedly decreases body weight and fat mass in mammals.[10] As illustrated by Figure 1, our OR rats showed approximately 35%–38% body weight reduction that was achieved after approximately 7 months of CR and remained relatively stable until the age of sacrifice. In contrast to CR, it is known that aging has opposite effects on the lipid content of adipose tissues, increasing adiposity index with age. To avoid the dilutive/concentrative effect of fat when any parameter is expressed per gram of tissue, we preferred to adjust in our study all parameters to DNA content, since it is proportional to adipocyte number.

Aged rodents show diminished ability to regulate body temperature,[4,5] this impairment being related, among others, to an inadequate capacity for thermogenesis in BAT.[3] BAT atrophy is characterized by a great loss in functional protein as well as increased fat accumulation, with brown adipocytes acquiring a white adipocyte-like phenotype.[25] These qualitative changes lead to an increase in BAT mass, however, on the contrary to what occurs under cold exposure or high caloric intake, this increase in mass is not concomitant with an increase in thermogenic function. As shown in Table 1, CR prevented this age-associated increase in BAT mass, even when BAT mass was normalized per body weight. This effect takes place in part by means of attenuation of lipid accumulation but also by a decrease in adipocyte number (total DNA per depot). Similar results were previously observed for young female rats with CR decreasing fat and adipocyte content in BAT.[17] Nevertheless, in contrast with this report, in our study CR completely prevented the age-dependent loss of protein (Table 1). On one hand these results suggest that old restricted rats may lose thermogenic capacity by decreasing adipocyte number in BAT, as hap-

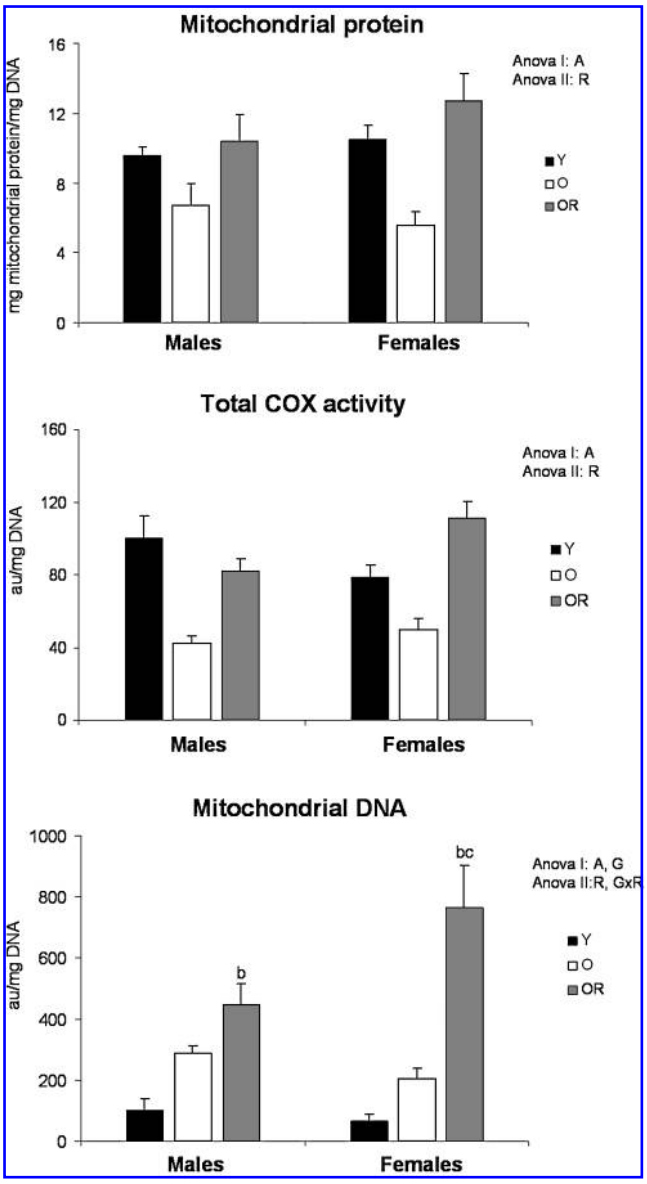

FIG. 2. Effect of aging and caloric restriction (CR) on mitochondrial protein, COX activity and mitochondrial DNA (mtDNA) content. Bars represent mitochondrial protein, COX activity and mtDNA expressed per milligram of total DNA. ANOVA I, two-way analysis of variance (ANOVA) to assess the effects of aging and gender between young (Y) and old (O) rats; ANOVA II, two-way ANOVA to assess the effects of CR and gender between O and old-restricted (OR) rats. Values are mean ± standard error of the mean (SEM; $n = 8$ for Y, $n = 7$ for O and OR). For ANOVA ($p < 0.05$), A indicates aging effect, G indicates gender effect, R indicates restriction effect and GxR represent the corresponding interactive effect. Student's *t* test ($p < 0.05$): [b]O versus OR, [c]males versus females.

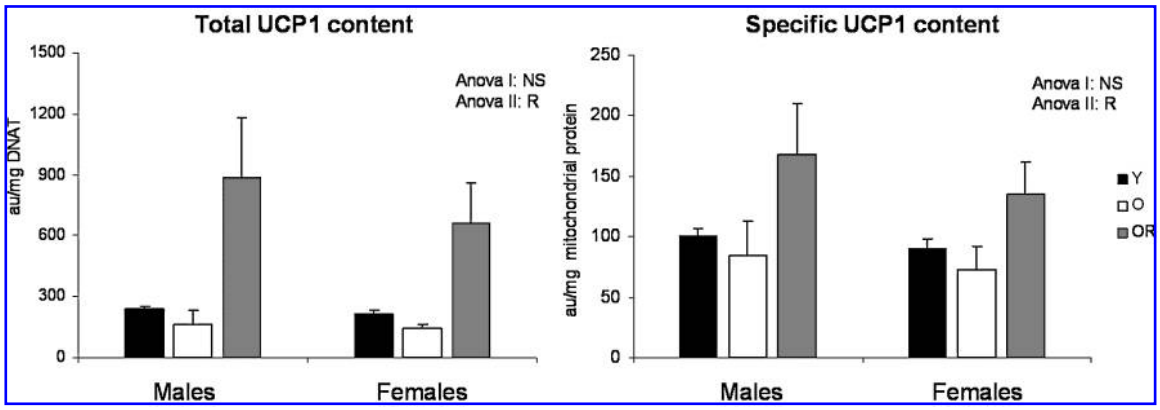

FIG. 3. Effect of aging and caloric restriction (CR) on uncoupling protein 1 (UCP1) content. Bars represent UCP1 content measured in homogenates (left) and in mitochondrial fraction (right) isolated as described in Materials and Methods. ANOVA I, two-way analysis of variance (ANOVA) to assess the effects of aging and gender between young (Y) and old (O) rats; ANOVA II, two-way ANOVA to assess the effects of CR and gender between O and old restricted (OR) rats. Values are mean ± standard error of the mean (SEM; $n = 8$ for Y, $n = 7$ for O and OR). For ANOVA ($p < 0.05$), A indicates aging effect and R indicates restriction effect.

pens in young female rats,[17] however, on the other hand, CR improves functional capacity of the tissue, as interpreted by the preservation of protein content and the prevention of fat accumulation.

In order to study the effect of CR on BAT thermogenic capacity, we measured several markers of mitochondrial content in the adipocyte, such as mitochondrial protein, COX activity and mitochondrial DNA (mtDNA). As shown in Figure 2, mitochondrial protein content and COX activity followed a similar profile, showing that CR prevents the age-associated decreases in these markers of mitochondrial machinery. On the other hand, mitochondrial DNA (mtDNA), which is a suitable marker of mitochondria number,[26,27] was significantly increased with age and even more so with CR, suggesting an increase in mitochondrial proliferation with aging and CR. Similar increases in the amount of mtDNA with age have been described previously in a wide variety of rodent tissues including heart,[28] liver,[29] cerebellum,[29] spleen,[30] kidney,[30] as well as in human skeletal muscle[31,32] and lung.[33] This increase in mitochondrial number with advancing age has been proposed to be a compensatory mechanism for the loss of mitochondrial function.[32]

BAT thermogenic activity relies on mitochondrial function and, particularly, on its tissue-specific UCP1 protein. As shown in Figure 3, age had no significant effects on total or specific UCP1 content. This is in agreement with previous reports showing that the loss of thermogenic capacity with aging is related with a decrease in UCP1 activity but not in UCP1 protein.[34,35] In contrast, CR was found to increase specific UCP1 protein levels in mitochondria, resulting in a threefold to fourfold higher total levels in OR compared to old rats. This great difference in total UCP1 content is probably a result of the CR effects on both UCP1 expression and mitochondrial proliferation. Considering the discrepancy between UCP1 activity and protein levels reported in old rats, we decided to measure mitochondrial oxygen consumption both in the presence and in the absence of GDP, which is a useful tool to detect UCP1 activity. As shown in Figure 4, CR prevented the age-related decline in mitochondrial oxygen consumption and preserved the inhibitory ability of GDP, which reflects the conservation of UCP1 activity in old restricted rats compared to old ad libitum fed ones. These findings suggest that CR protects UCP1 function and oxidative capacity in mitochondria of old rats.

The observed changes found in this study in mitochondrial machinery and functionality indicate that CR modifies the normal age-associated changes in the mitochondrial biogenetic program. Mitochondrial biogenesis depends on the combination of both proliferation and differentiation processes. Proliferation is an increase in the mitochondrial

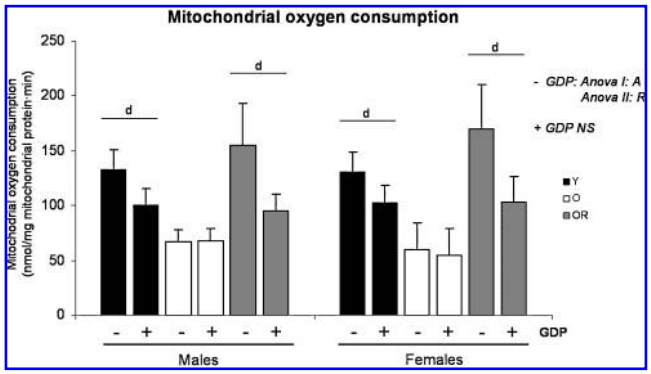

FIG. 4. Effect of aging and caloric restriction (CR) on mitochondrial oxygen consumption. Bars represent mitochondrial oxygen consumption expressed as nmols $O_2 \cdot$ mg mitochondrial protein$^{-1} \cdot$ min^{-1} using glycerol-3-phosphate as substrate. ANOVA I, two-way analysis of variance (ANOVA) to assess the effects of aging and gender between young (Y) and old (O) rats; ANOVA II, two-way ANOVA to assess the effects of CR and gender between O and old restricted (OR) rats. Values are mean ± SEM ($n = 8$ for Y, $n = 7$ for O and OR). Oxygen consumption was measured in the absence (-) and in the presence (+) of 500 μM guanosine diphosphate (GDP) in order to inhibit uncoupling protein 1 (UCP1)-dependent oxygen consumption. For ANOVA ($p < 0.05$), A indicates aging effect, *R* indicates restriction effect, and NS indicates not significant. [d]GDP effect was analyzed by Student's *t* test ($p < 0.05$).

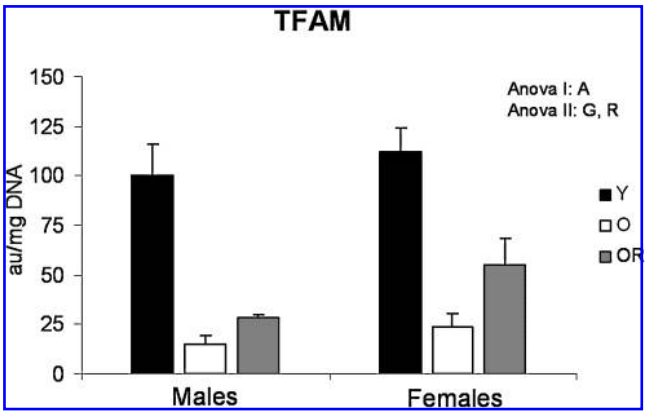

FIG. 5. Effect of aging and caloric restriction (CR) on transcription factor A (TFAM) levels. Bars represent TFAM protein levels measured by Western blot in brown adipose tissue homogenates and adjusted to milligram of of DNA. ANOVA I, two-way analysis of variance (ANOVA) to assess the effects of aging and gender between young (Y) and old (O) rats; ANOVA II, two-way ANOVA to assess the effects of CR and gender between O and old restricted (OR) rats. Values are mean ± standard error of the mean (SEM; $n = 8$ for Y, $n = 7$ for O and OR). For ANOVA ($p < 0.05$), A indicates aging effect, G indicates gender effect and R indicates restriction effect.

population, while differentiation can be defined as an improvement of the functional capabilities of pre-existing mitochondria.[20,36,37] Although CR prevented the age-associated loss in mitochondrial protein, and, therefore, appeared to maintain the mitochondrial differentiation process, mtDNA data suggest that proliferation probably exceeds mitochondrial differentiation. This is in agreement with the pattern found for the levels of mitochondrial transcription factor A (TFAM; Fig. 5). TFAM is a member of the high mobility group domain family which is essential for mtDNA transcription and replication.[38,39] Although the precise mechanism by which TFAM controls mtDNA replication and transcription are not fully understood, some reports have pointed out that mtDNA replication can be initiated by small amounts of TFAM, whereas expression of mtDNA, which is related to mitochondrial differentiation, is activated only at high concentrations of this factor.[40–43] In accordance with this idea, the increase in mtDNA observed in old and old restricted rats could be related with the low TFAM levels in these animals compared to the young ones. In the case of OR rats, the slightly higher levels of TFAM compared to old ad libitum fed animals, might be responsible for promoting higher mtDNA replication, and also differentiation.

In a previous work, we found that CR in young rats affected BAT thermogenic capacity in a gender-dependent way, with female rats showing a great deactivation of BAT thermogenic capacity[17,44] whereas males failed to shown signs of such deactivation. There is certain controversy regarding the effects of short-term CR in BAT of male rats, with reports showing increase[45] or no effects[17,44] on thermogenic capacity. These gender differences in the response to energy constraints may be determined by the cost that reproduction means for females.[46] The effect on males is probably less severe, and may be influenced by variations in the experimental protocols such as the duration of restriction, the species of rodent used, the feeding schedule or the housing temperature. Nevertheless, here we report consistently that long-term CR in old rats prevents the age-related decline in BAT mitochondrial thermogenic capacity in both genders. Gender differences were marginally observed only for mtDNA, with females showing a higher CR-induced increase in mtDNA content. The loss of fertility in the old rats, and its consequences in the females' metabolism, may account for the lack of gender differences in the response of BAT to CR when compared with the response of young rats.

Recently, other authors have shown that CR increases mitochondrial proliferation in rat liver[47] and human skeletal muscle.[48] In these studies, CR seems to promote the proliferation of a more energy efficient mitochondria that maintains ATP production but produces less reactive oxygen species, which would be in relation with the life-extending effects of CR. Here we show that CR also protects mitochondrial function and promotes mitochondrial proliferation in an uncoupled tissue in which the main function is not ATP production. Although further studies are necessary to understand why restricted rats conserve mitochondrial capacity in several tissues, probably in BAT it may be determined by an increase in thermogenic demand since restricted animals are smaller than controls and housing temperature is below thermoneutrality.

To our knowledge, this is the first time that the effects of long-term CR on the uncoupled mitochondria characteristic of BAT are explored. In broad terms, we observed that old restricted animals compared to old ad libitum fed ones have a reduced BAT size, even normalized per body weight, which probably is due to a lower fat content and adipocyte number in the tissue. Nevertheless, CR rats consistently showed resistance to lose total and mitochondrial protein, COX activity and uncoupling capacity in brown adipocytes with advancing age. As happens in liver or skeletal muscle, CR was also shown to promote mitochondrial biogenesis in BAT. On the whole, this conservation of BAT thermogenic capacity may confer several advantages, such as a higher ability to respond to cold exposure or to control body weight when food supply is restored, and, therefore, be part of the rejuvenation mechanisms underlying the life-span extension induced by CR.

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Differences in Mitochondrial Function and Antioxidant Systems between Regions of Human Glioma

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Key Words

Reactive oxygen species • Human glioma • Redox behaviour • Oxidative metabolism • Manganese-superoxide dismutase • Fresh tissue • Intratumoral regions • Isolated mitochondria

Abstract

Metabolic features and oxidative stress have been extensively studied in cancer cells. However, comparative studies between cancer cell populations that coexist in human neoplastic tissue are not frequently available. The aim of the present study was to characterize markers of oxidative status and mitochondrial function in center vs. periphery of human fresh glioma samples; therefore, antioxidant systems, oxidative stress and mitochondrial parameters were assessed in gross total resections of gliomas. Mitochondrial protein and mitochondrial DNA content, enzymatic activities of mitochondrial oxidative and phosphorylative system, antioxidant mechanisms, mitochondrial H_2O_2 production, oxygen consumption and cellular oxidative damage were measured in human gliomas. Concentric regions of human glioma tissue showed similar mitochondrial structural markers; conversely, the functionality of their isolated mitochondria was significantly different. In this way, the tumor periphery exhibited higher respiratory rate and fewer antioxidant systems than tumor center. Our results have expanded previous investigations, which report the presence of cell populations with different oxidative susceptibility in human brain tumor samples. This is, to our knowledge, the first study to investigate metabolic differences in concentric regions of gross total resections of glioma. Interestingly, the cancer cell population that exhibits an increased antioxidant capacity within the tumor mass might be responsible for tumor resistance to chemotherapy and radiotherapy.

Introduction

Mitochondria are involved in a strikingly diverse range of disease processes. In this sense, mitochondrial dysfunction is increasingly recognized as a contributor to common diseases, such as cancer; they have multifactorial pathogenesis and a clear relationship to oxidative stress of mitochondrial origin. The mitochondrion is the main source of cellular ATP but it is much more than this; this organelle is intimately involved in the life and death of the cell, capable of integrating pro- and anti-apoptotic

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signals and committing the cell to apoptosis. Mitochondrial research has contributed to two paradigm shifts in oncology: the first was the pioneering research by Warburg showing that cancer cells often rely heavily on glycolytic metabolism, even in the presence of an adequate oxygen supply [1]; the second paradigm is the appreciation that the ability of cancer cells to avoid apoptosis contributes to their limitless replicative potential and limits the efficacy of cancer chemotherapy [2]. On the other hand, this organelle is the only one apart from the nucleus to contain DNA. Mitochondrial mutations and profound mitochondrial ultrastructure alterations have been reported to exist in gliomas [3, 4]. Besides, primary mutations in mitochondrial housekeeping genes such as fumarate hydratase and succinate dehydrogenase have been described to play an important role in cancer pathogenesis [5, 6]. Additionally, recent studies have shown that tumors frequently bear homoplasmic mitochondrial DNA (mtDNA) mutations that may contribute to tumor promotion.

The ability of certain tumor cells to sustain either metabolic alterations, chronic hypoxia or chronically elevated oxidative stress are factors that help define the hallmarks of cancer [7, 8].

Although tumor invasion has been intensely investigated, many aspects of cancer biology remain poorly understood [9]. In this way, tumor growth is a complex process dependent on tumor cells proliferating and spreading in host tissues. Interestingly, it has been described that cell growth in human cancer is limited to the tumor border [10].

During cellular metabolism reactive oxygen species are produced by mitochondrial electron transport, by the cellular redox system and by immune responses. At concentrations actually compatible with the physiology of the cell, reactive oxygen species (ROS) appear able to exert, as intracellular messengers, a large variety of biochemical effects which may contribute to modulation of cell viability and function [11]. However, long-term exposure of a human cell to ROS may elicit serious consequences of enhanced oxidative damage and cell death in affected tissues [12].

It is worth noting that most primary brain tumors derive from glial cells and are collectively called gliomas. Malignant gliomas are rapidly growing tumors. The ones with highest WHO grade (grade IV) generally have vascular proliferation with malformed vessels and/or areas of necrosis and hypoxia. These tumors are among the most challenging cancers to treat and the high grade ones have a particularly poor prognosis due to their exceptional ability to infiltrate normal brain [13]. This feature makes complete surgical resection virtually impossible [14]; and even after complete surgical excision, high grade gliomas almost always grow back. However, it has been reported that an aggressive surgical procedure for this primary brain cancer can result in increased survival for selected groups of patients [15]. Treatment for most brain tumors depends on the location and the grade and is often a combined approach, using surgery, radiation therapy, and chemotherapy. On the other hand, radiotherapy and anticancer drugs increase reactive oxygen species in cancer cells or can lead to an inhibition of antioxidant enzymatic activities [16]. Furthermore, cancer cells may respond to the oxidative stimuli and allow biochemical adaptations to oxidative stress. In this sense, the increase in antioxidant systems has been postulated as a mechanism by which tumoral cells become resistant to antineoplastic agents.

Taking this into account, the specific aim of this study was to compare in fresh tissues the mitochondrial metabolism features and oxidative status between cells in the tumor center and tumor periphery from maximal surgical resections of human gliomas.

Materials and Methods

Materials

Routine chemicals used were supplied by Sigma-Aldrich (St. Louis, USA), Panreac (Barcelona, Spain) and Amersham Pharmacia Biotech (Little Chalfont, UK). Real-time PCR reagents and oligonucleotide primer sequences were supplied by Roche Diagnostics (Basel, Switzerland) and Amplex Red reagent by Molecular Probes (Paisley, UK).

Subjects and human brain tumor samples

The study protocol was approved by the Ethics Committee of the Son Dureta University Hospital and all subjects gave their written consent to participate in the present study. All human tumors evaluated had a signed patient consent form on file for the study. These tumor specimens were surgically removed in one block and evaluated for intraoperative diagnosis. After the diagnosis was established, concentric tissue samples were removed, therefore representative sections from central and peripheral regions were taken fresh for mitochondrial and oxidative studies. Another part was frozen, and fixed in buffered formalin for histological evaluation; the purity of each area was estimated by using hematoxylin and eosin staining to ensure that each region contained > 65% of neoplastic cells. This was carried out by a pathologist (MEC) at Son Dureta University Hospital (Palma de Mallorca, Spain). Maximal surgical resections of gliomas were obtained from 6 patients aged 20-79 years (4 men and 2 women) as shown in Table 1. Mitochondria from the 12 glioma samples were rapidly isolated after

surgical procedure. Specifically, mitochondrial isolation was started at the hospital 15 minutes after neurosurgery and was completed within 35 minutes of tumor removal.

Mitochondrial isolation

Isolation of glioma mitochondria was performed at 4 ºC using a procedure based on differential centrifugation. After neurosurgery, gross total resection specimens were immediately transported, on ice, to the pathology lab and were rapidly dissected on ice-cold surface. Each region of the tumor was then weighed, finely minced, and diluted 20 times with ice-cold isolation medium (250 mM sucrose, 10 mM Tris-HCl, 0.5 mM EDTA-K^+; pH 7.4). Each sample was homogenized using 24 up-and-down strokes in a manual homogenizer with a glass pestle (clearance 0.1 mm). An aliquot of homogenate from each region was stored at 4 ºC until functional measurements were carried out. The remaining homogenate was centrifuged for 3 min at 2000xg*;* then, the supernatant was collected and centrifuged again for 3 min at 2000xg*;* the supernatant obtained at this time was centrifuged for 8 min at 12,500xg; finally, the supernatant was discarded and the pellet was resuspended in ice-cold isolation buffer at a final concentration of 5-10 mg mitochondrial protein per milliliter, determined by Bradford's method [17]. The crude mitochondrial fractions were stored at 4 ºC until mitochondrial functionality was assayed (H_2O_2 production, respiration and enzymatic activities). The homogenate was used to determine lipid peroxidation, glutathione levels, lactate dehydrogenase (LDH) activity, total protein content and also total citrate synthase (CS) and total cytochrome *c* oxidase (COX) activities.

Enzymatic activities

All spectrophotometric and fluorimetric determinations were assayed in microtiter plate and were performed as previously described protocols with some modifications. LDH activity was an adaptation of Vassault method [18]. The CS assay was based on the technique described by Nakano et al. [19]. CS activity was measured in homogenates and mitochondrial fractions in order to calculate the mitochondrial recovery. Oxidative phosphorylation system (OXPHOS) activities were assessed in resuspended mitochondrial pellet. COX activity was determined by Chrzanowska-Lightowlers et al. protocol [20]. Complex V or F1F0-ATP synthase (ATPase) was assayed by an adaptation of Ragan et al. method [21]. Manganese-superoxide dismutase (MnSOD) activity was assayed by Quick et al. method [22]. Glutathione peroxidase (GPx) and glutathione reductase (GRd) were assayed by modified Smith et al. method [23] and Wen et al. method [24], respectively. Antioxidant enzymatic analysis (GPx, GRd, MnSOD and total superoxide dismutase activities) were performed in the supernatant of samples previously disrupted by sonication (10,000 g for 10 min).

Measurement of mitochondrial respiration

Mitochondrial respiration was measured polarographically, with minor modifications as described previously by Lopez-Torres et al. assay [25]. Briefly, mitochondria were incubated in a water-thermostatically regulated chamber with a computer-controlled Clark-type O_2 electrode (Oxygraph; Hansatech, Norfolk, UK) at a concentration of 0.5-1 mg mitochondrial protein/ml in respiration buffer containing 145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, and 0.1% bovine serum albumin free fatty acids (pH 7.4 at 37 °C). State 4 respiration (absence of ADP) was measured using pyruvate-malate (5 mM/2.5 mM) and succinate (5 mM) as substrates. State 3 respiration was initiated by adding ADP (500 μM). The ratio [state 3 rate]:[state 4 rate] of mitochondrial preparations was also checked; this respiratory control index (RCR) indicates the tightness of the coupling between respiration and phosphorylation.

Surgical cases	Sex	Age	Diagnosis	Glioma recurrence
1	F	69	glioblastoma (astrocytoma grade IV)	no
2	M	75	glioblastoma (astrocytoma grade IV)	no
6	M	60	anaplastic oligodendroglioma (grade III)	yes
7	F	79	glioblastoma (astrocytoma grade IV)	no
9	M	23	astrocytoma grade II	no
10	M	20	astrocytoma grade III/IV	no

Table 1. Human brain tumor samples

Detection of mitochondrial H_2O_2 production capacity

The rate of H_2O_2 production in mitochondria was determined by the fluorescent probe Amplex Red as described previously [26]. Mitochondria (0.4 mg protein/ml) were incubated at 37 ºC in respiration buffer containing 0.1 U/ml horseradish peroxidase and 50 μM Amplex Red. Modulation of H_2O_2 production capacity by rotenone (2 μM), antimycin (5 μM), ADP (500 μM) or GDP (500 μM) was also assessed. H_2O_2 production was initiated by adding the substrate (succinate (5 mM) or pyruvate-malate (5 mM/2.5 mM)); background fluorescence was measured in parallel in wells containing all reactants except substrate. Fluorescence was measured in a microplate reader (FLx800; Bio-Tek Instruments, Winooski, VT) with 530 nm excitation and 590 nm emission wavelengths. Fluorescence rate was expressed as H_2O_2 production using a standard curve generated with known concentrations of H_2O_2 stabilized solution.

Glutathione assay

Glutathione levels were measured in the supernatant of deproteinized tissue lysates by a modified 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) - GSSG reductase recycling assay [27]. Reduced-/oxidized glutathione ratio (GSH/GSSG) was calculated.

Quantification of human mitochondrial DNA

mtDNA was extracted by digestion with proteinase K (100 μg/μl) in a buffer containing 50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$ and 0.5% Tween 20. Mitochondrial samples were incubated overnight at 37 °C and then boiled for 5 min in order to inactivate the enzyme. mtDNA was linearised by digestion with Bcl I restriction enzyme for 3 h at 50 °C and then boiled for 5 min. Samples were centrifuged at 10,000 rpm for 5 min and the resulting supernatant was used for amplification. A quantitative PCR assay was adapted to the LightCycler technology from previous methods [28, 29]. PCR was performed to amplify

Table 2. Biochemical parameters and mitochondrial markers. Values are the means ± s.e.m. from 5-6 regions per group; data are expressed per gram of fresh tissue. No significant differences were found between central and peripheral regions of gliomas (P< 0.05, Student's paired test). a The low LDH activity in mitochondrial fractions indicates the purity of isolated mitochondria.

	Center	Periphery	*n*	*p*
Total protein (mg/g)	54.2 ± 8.3	54.5 ± 8.9	6	0.466
Mitochondrial DNA (a.u./g)	21.8 ± 9.8	20.0 ± 8.8	5	0.184
Mitochondrial protein (mg/g)	23.2 ± 3.3	22.9 ± 2.9	6	0.362
Mitochondrial protein/mtDNA (a.u.)	8.20 ± 3.81	9.69 ± 4.36	6	0.342
Total COX activity (a.u./min·g)	11.3 ± 1.8	12.2 ± 1.8	5	0.307
Total CS activity (IU/g)	2.59 ± 0.68	2.87 ± 0.67	6	0.319
Total LDH activity (IU/g)	23.9 ± 3.0	22.3 ± 4.0	6	0.324
Mitochondrial LDH activity [a] (IU/g)	0.71 ± 0.13	0.78 ± 0.15	6	0.284

Table 3. OXPHOS activities. Values are the means ± s.e.m. from 6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas (P< 0.05, Student's paired test).

	Center	Periphery
COX activity (a.u/min•mg mt protein)	455 ± 106*	596 ± 129
ATPase activity/COX activity	0.85 ± 0.21*	0.66 ± 0.18
ATPase activity (mIU/mg mt protein)	318 ± 44	324 ± 39
ATPase activity (IU/g)	8.19 ± 1.67	10.0 ± 2.75

	Center	Periphery
H_2O_2 S4 succinate (pmol/min·mg)	15.8 ± 11.1	21.4 ± 9.0
H_2O_2 S4 succinate (pmol/min·g)	456 ± 269	603 ± 220
H_2O_2 S4 GDP+succinate (pmol/min·mg)	15.4 ± 9.9	32.3 ± 16.6
H_2O_2 S4 GDP+succinate (pmol/min·g)	442 ± 243	911 ± 373
H_2O_2 S4 succinate+R+AA (pmol/min·mg)	20.9 ± 8.9	22.2 ± 5.8
H_2O_2 S4 succinate+R+AA (pmol/min·g)	551 ± 196	625 ± 139
H_2O_2 S4 pyr-mal+R (pmol/min·mg)	7.76 ± 3.35	17.2 ± 5.4
H_2O_2 S4 pyr-mal+R (pmol/min·g)	264 ± 100	525 ± 147

Table 4. Mitochondrial H2O2 production capacity was measured in absence of ADP (S4). Succinate 5 mM was used as substrate with or without GDP 500 µM, rotenone 2 µM and antimycin A 5 µM; rate of H2O2 production with pyruvate-malate (5 mM/2.5 mM) was measured in the presence of rotenone 2 µM. Values are the means ± s.e.m. from 4 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas (P< 0.05, Student's paired test). R, rotenone; AA, antimycin A; pyr-mal, pyruvate-malate; S4,state 4.

	Center	Periphery
O_2 consumption S4 pyr-mal (nmol/min•mg)	4.29 ± 1.25	5.21 ± 0.79
O_2 consumption S4 pyr-mal (nmol/min•g)	77.4 ± 27.8	106 ± 26
O_2 consumption S3 pyr-mal (nmol/min•mg)	9.29 ± 5.83	11.2 ± 5.49
O_2 consumption S3 pyr-mal (nmol/min•g)	189 ± 137	250 ± 141
O_2 consumption S4 succinate (nmol/min•mg)	8.19 ± 2.82*	9.35 ± 2.55
O_2 consumption S4 succinate (nmol/min•g)	165 ± 47	193 ± 40
O_2 consumption S3 succinate (nmol/min•mg)	13.0 ± 5.7	14.9 ± 4.6
O_2 consumption S3 succinate (nmol/min•g)	269 ± 123	320 ± 107

Table 5. Mitochondrial oxygen consumption. Mitochondrial oxygen consumption was assessed under state 4 (absence of ADP) or state 3 (500 µM ADP); succinate 5 mM or pyruvate-malate (5 mM/2.5 mM) were used as substrates. Values are the means ± s.e.m. from 4-5 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas (P< 0.05, Student's paired test). S3, state 3; S4, state 4; pyr-mal, pyruvate-malate.

a 192-nts fragment of the human mitochondrial NADH dehydrogenase subunit 4 gene. The primer sequences were 5'-CCT GAC TCC TAC CCC TCA CA-3' and 5'-ATC GGG TGA TGA TAG CCA AG-3'. The concentration of the purified template was determined spectrophotometrically. Increasing amounts of template were amplified in parallel reactions to obtain a standard curve. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche, Switzerland) using a total volume of 10 µl containing 0.375 µM of each primer, 3 mM $MgCl_2$, 1 µl LightCycler-FastStart DNA Master SYBR™ Green I and 2.5 µl of sample prepared as described above. The PCR reactions were cycled 32 times after initial denaturation (95 °C, 10 min), with the following parameters: denaturation at 95 °C for 10 s, annealing at 68 °C for 12 s, extension at 72 °C for 12 s.

Quantification of human mtDNA oxidative damage by realtime PCR

Many mtDNA lesions are located in the mitochondrial displacement loop region (D-loop) and in particular in a polycytidine stretch (C-tract). There are large differences in the susceptibility to damage of various regions of mtDNA. Moreover, the D-loop is highly susceptible to mutations because of its vulnerability to DNA damage and inefficient repair mechanisms. The purpose of this assay was to examine the ROS-induced DNA damage in the mitochondrial genome, based on previous methods [30-33]. The human mtDNA primer sequences were: 5'-AGT GCA TAC CGC CAA AAG AT-3' and 5'-GGC CAC AGC ACT TAA ACA-3' for a 99-nts fragment that we called sF; 5'-AGT GCA TAC CGC CAA AAG AT-3' and 5'-AGC CAC TTT CCA CAC AGA CA-3' for a 162-nts fragment that we called lF. The large fragment (lF), which includes the C-tract region, is more sensitive to oxidative damage than the small fragment (sF); in previous experiments, we observed that its amplification was delayed when compared with sF after exposure to DNA-damaging agents (H_2O_2) in a dose-dependent manner. Pre-treatment of mitochondrial pellet suspensions to obtain mtDNA was carried out as described above for the quantification of mtDNA. Amplification was performed in a LightCycler rapid thermal cycler system using a total volume of 10 µL containing 0.375 µM of each primer, 3 mM $MgCl_2$, 1 µl LightCycler-FastStart DNA Master SYBRTM Green I and 2.5 µL of sample. PCR reactions were cycled 32 times after initial denaturation (95 °C, 10 min), with the following parameters: 95 °C for 10 s, annealing at 59 °C for 12 s, extension at 72 °C for 12 s. After the amplification of mitochondrial specific regions,

lF/sF ratio for each sample was calculated by dividing the Ct of its large fragment by the Ct of its small fragment. There is a positive correlation between the increase in this ratio and mtDNA oxidative damage in the analysed sample. All oligonucleotide primer sequences were obtained from Primer3 and tested with IDT OligoAnalyser 3.0. Finally, a basic local alignment search tool (NCBI Blast) revealed that the primer sequence homology was obtained only for the target genes.

Measurement of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation was determined in homogenates as malondialdehyde-thiobarbituric acid adducts according to Buege et al. [34]. The assay was performed spectrophotometrically at 532 nm, using a molar extinction coefficient of 1.56 x 10^5 $M^{-1}\cdot cm^{-1}$.

Protein carbonyl derivates determination

Protein carbonyl groups were measured by an adaptation of previous methods [35, 36] using the precipitates of deproteinized samples. Protein pellets were resuspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at room temperature. Then, 20% trichloroacetic acid was added, and samples were centrifuged 10 min at 10,000 g at 4 ºC. After the final wash, protein pellets were resuspended in 300 µl of 3 M guanidine hydrochloride in 2 mM phosphate buffer (pH 2.3) by vortexing. Finally, the absorbance of the supernatant was measured at 360 nm. The molar absorption of 22,000 $M^{-1}\cdot cm^{-1}$ was used to quantify total protein carbonyl levels. Samples were analyzed against a blank of guanidine solution and protein content was determined after the washing steps.

Statistics

Data were expressed as mean ± s.e.m. (n = 6 regions/group). Intratumoral differences were analyzed by paired Student's t-test using the Statistical Program for the Social Sciences software for Windows (SPSS, Version 15.0). Statistical significance was set at $P < 0.05$.

Results

Glioma tissue composition and mitochondrial content

The isolation procedure was chosen due to the need for a rapid method to obtain sufficient volume of crude mitochondrial pellet to immediately assess ROS production and oxygen consumption, as well as to ensure the same mitochondrial recovery between the tumor regions studied (center (32.5 ± 5.0 %) vs. periphery (33.9 ± 5.1 %). All glioma tissues were highly vascular in both regions; similar cellularity was observed in both areas by using hematoxylin and eosin staining of contiguous sections. Tissue protein expressed as milligram of total protein per gram of fresh tissue was almost the same in the central and peripheral regions (54.2 ± 8.3 vs. 54.5 ± 8.9, respectively). As shown in Table 2, no differences were observed per gram of fresh tissue in regards to mitochondrial protein content, mtDNA levels, total COX activity, total CS activity (all of which are markers of mitochondrial abundance) or in protein content per mitochondrion; the mitochondrial protein adjusted to mtDNA levels was the parameter assessed that gave the closest approximation to mitochondrial mass.

To further exclude the possible influence of the number of mitochondria, mitochondrial function measurements (expressed per gram of fresh tissue) were corrected for CS recovery. It is important to highlight that DNA content is not a good marker of cell number in glioma cells due to the presence of aneuploidy, thus, we expressed our results per protein content, an index of cellular mass.

	Center	Periphery
TBARS content (nmol/mg total protein)	390 ± 92	263 ± 47
TBARS content (µmol/g)	23.4 ± 6.7	14.5 ± 3.1
Oxidized mtDNA (a.u/mg mt protein)	1.51 ± 0.01	1.52 ± 0.00
Oxidized mtDNA (a.u./g)	35.1 ± 4.9	34.8 ± 4.4
Protein carbonyl groups (µmol/mg total protein)	250 ± 95	221 ± 39
Protein carbonyl groups (µmol/g)	6.26 ± 2.76	5.33 ± 1.27

Table 6. Oxidative damage markers. Values are the means ± s.e.m. from 6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas (P< 0.05, Student's paired test).

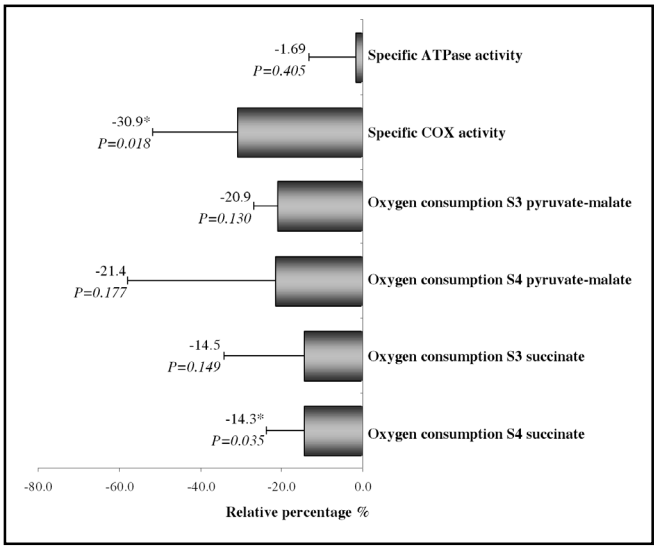
	Center	Periphery
GSH/GSSG	5.23 ± 1.23*	2.55 ± 0.61
GSSG (nmol/mg total protein)	1.42 ± 0.41	1.46 ± 0.33
GSSG (nmol/g)	86.8 ± 26.9	89.7 ± 25.5
GSH (nmol/mg total protein)	5.84 ± 1.68	3.35 ± 0.84
GSH (nmol/g)	333 ± 104 *	181 ± 53

Table 7. Glutathione content. Values are the means ± s.e.m. from 6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas (P< 0.05, Student's paired test).

	Center	Periphery
MnSOD activity (mIU/mg mt protein)	10.9 ± 3.7*	8.61 ± 3.65
MnSOD activity (mIU/g)	306 ± 136*	242 ± 126
GPx activity (µIU/mg mt protein)	28.6 ± 12.8	21.9 ± 8.2
GPx activity (µIU/g)	695 ± 373	487 ± 219
GRd activity (µIU/mg mt protein)	5.93 ± 0.69	5.74 ± 0.43
GRd activity (µIU/g)	130 ± 25	123 ± 19
Total SOD activity (mIU/mg total protein)	10.1 ± 2.8	7.03 ± 1.90
Total SOD activity (mIU/g)	532 ± 176	343 ± 95

Table 8. Antioxidant activities. Values are the means ± s.e.m. from 5-6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas (P< 0.05, Student's paired test).

Fig. 1. Mitochondrial functionality. Oxygen consumption and OXPHOS activities of tumor periphery compared to tumor center. Data show relative percentage (central values compared to peripheral values). A negative percentage represents higher mitochondrial function in peripheral region than in central one. Mitochondrial oxygen consumption was assessed under state 4 (S4; absence of ADP) or state 3 (S3; 500 µM ADP); succinate 5 mM or pyruvate-malate (5 mM/2.5 mM) were used as substrates. n = 4-6. Mean difference between center and periphery, *significant differences P< 0.05, Student's paired test.



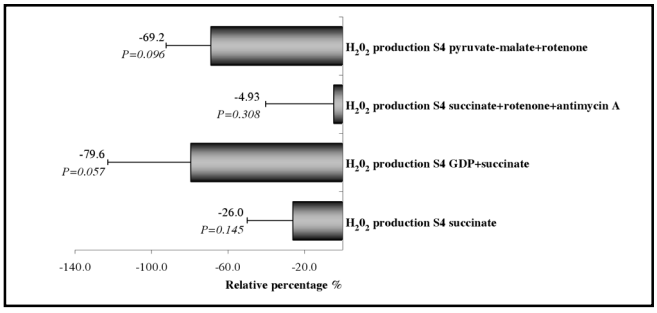


Fig. 2. Mitochondrial pro-oxidant production capacity. Bars show relative percentage (central values compared to peripheral values). A negative percentage represents higher mitochondrial capacity to produce H2O2 in peripheral region than in central region in absence of ADP. Succinate 5 mM was used as substrate with or without GDP 500 µM, rotenone 2 µM and antimycin A 5µM; rate of H2O2 production with pyruvate-malate (5 mM/2.5 mM) as substrates was measured in the presence of rotenone 2 µM. n = 4. Mean difference between center and periphery, *significant differences P< 0.05, Student's paired test.

Oxygen consumption

The actively respiring state is sometimes refered to as "state 3" respiration, while the slower rate after all the ADP has been phosphorylated to form ATP is refered to as "state 4". Mitochondria from the periphery consumed significantly higher amounts of oxygen in State 4 than mitochondria from the tumor center (Fig. 1, Table 5). Under energy demand (ADP presence), there was an increase in mitochondrial respiration from both tumor regions; the differences found in mitochondrial oxygen consumption were reduced under active state respiration. Although there was a considerable stimulation of oxygen consumption with substrates pyruvate-malate and succinate, the RCR observed was quite low in mitochondria from the center (1.45 ± 0.39) and in mitochondria from the periphery (1.61 ± 0.29). These RCR values could suggest poor quality of the isolated mitochondria, however, suitable mitochondrial recovery and mitochondrial purity were obtained. Mitochondrial recovery measured as CS activity and COX activity in mitochondrial fractions was about 30% with respect to homogenate (as shown in the Glioma tissue composition and mitochondrial content section). Furthermore, the low LDH activity detected in crude mitochondrial pellets from the central and peripheral regions (2.97% ± 0.41 vs. 3.63% ± 0.58, with respect to the homogenate) proved the low presence of contaminating cytosolic components (Table 2). In addition to this, when the same methodology was applied to other tissues, such as rat brain, RCR values ranged from 5 to 7 could be obtained. In fact, low RCR values were expected in glioma tisse since RCR of isolated mitochondria from rat brain is higher than RCR of isolated mitochondria from human brain (parahippocampal cortex) according to the results of Kudin et al. [37]; additionally, human glioma tissue compared to non-tumoral human brain tissue show attenuated mitochondrial bioenergetics and enhanced glycolysis dependency; thus, decreased RCR values, which are associated to mitochondrial dysfunction in other pathological conditions, might be caused by an increased basal respiration (state 4) coupled to a diminished state 3 respiration in glioma tissue.

OXPHOS activities

Cytochrome *c* oxidase or complex IV and ATPase are key electron transport chain complexes in oxidative and phosphorilative capacities, respectively. Under basal state, specific COX activity was significantly higher in mitochondrial fractions from the periphery compared to those from the center (Fig. 1). The results obtained in specific COX activity were in concert with our data on oxygen consumption. This observation points to a higher oxidative capacity of isolated mitochondria from the periphery than central ones in basal state. Whereas COX activity was significantly decreased in the central region,

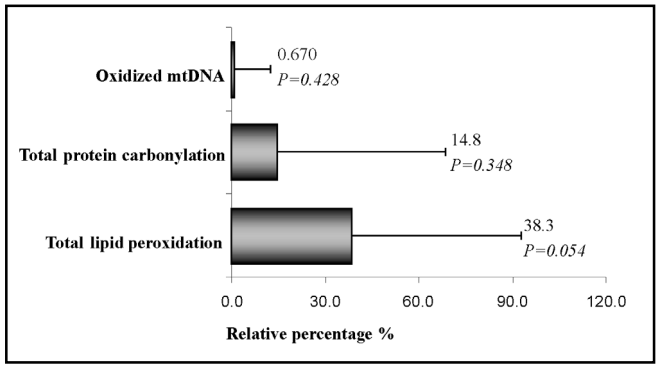


Fig. 3. Oxidative damage markers. Data show relative percentage (central values compared to peripheral values). A positive percentage represents higher oxidative injury in central region than in peripheral one. n = 6. Mean difference between center and periphery, *significant differences P< 0.05, Student's paired test.

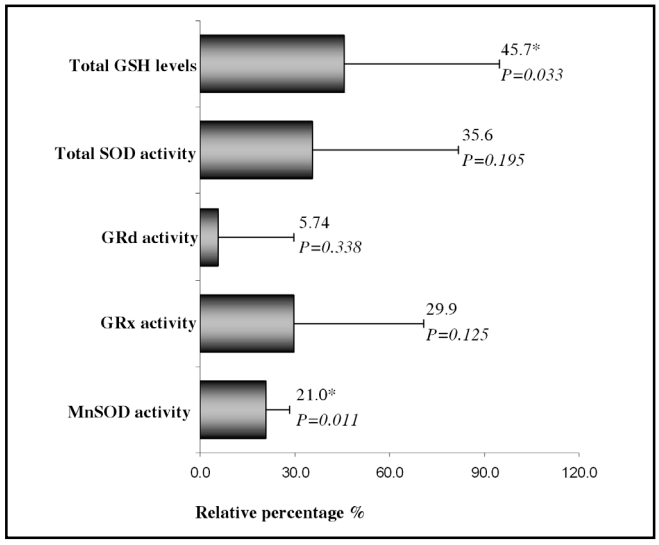


Fig. 4. Antioxidant capacity. Bars show relative percentage (central values compared to peripheral values). A positive percentage represents higher antioxidant systems in central region than in peripheral one. n = 5-6. Mean difference between center and periphery, *significant differences P< 0.05, Student's paired test.

ATPase activity was not more compromised in central areas when compared to the outer regions of the tumor (Table 3). Statistically higher ATPase/complex IV activity ratio in the central region was in accordance with a decreased COX activity in the tumor center (mean difference between central and peripheral regions was 0.186 ± 0.058; P = 0.012, Student's paired test). Given that basal respiration rate was statistically higher in mitochondria from the outer region of the tumor than in mitochondria from the tumor center, our data agree with the role of this mitochondrial complex which limits the rate of electron flow through mitochondrial electron transport chain.

Mitochondrial H_2O_2 production capacity

Superoxide anion, a product of one-electron reduction of oxygen, is the by-product of normal functioning of the mitochondrial respiratory chain. This radical has been reported to be generated by complexes I and III (CI and CIII) of the mitochondrial respiratory chain and readily converted to H_2O_2 by mitochondrial Mn-SOD. We approached this question using Amplex Red, a specific reagent to measure H_2O_2, which does not form resorufin in the presence of other reactive oxygen and nitrogen species [38]; therefore, we determined the H_2O_2 realeased by isolated mitochondria from the tumor in a spectrofluorimetric microtiter plate. Our results showed a trend towards an increase in H_2O_2 production capacity of peripheral mitochondria when compared to central mitochondria (P = 0.067). The rate of H_2O_2 production showed a differential response by adding GDP: higher in mitochondria from the periphery (Fig. 2). GDP could act as an inhibitor of uncoupling proteins, which are located in the inner mitochondrial membrane and are thought to be involved in the modulation of mitochondrial ROS production in human brain [39, 40]. In all conditions tested (Table 4), this trend was maintained, but standard error of the mean was too high to reach significance. Moreover, there was a significant decrease in rate of H_2O_2 production (data not shown) under State 3 (ADP presence) and under the presence of an hydrogen peroxide scavenger such as pyruvate [41]. While brain tumor mitochondria in the presence of pyruvate+malate alone generated only small amounts of H_2O_2, a substantial production was observed after the addition of the complex I inhibitor rotenone (7.76 ± 3.35 and 17.2 ± 5.4 nmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$ central regions vs. peripheral regions, respectively)

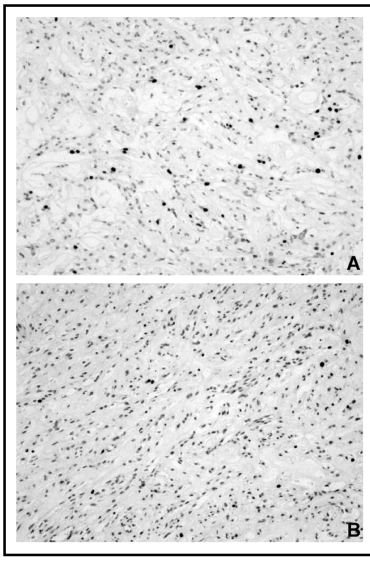


Fig. 5. Immunohistochemical stain with Ki-67 antibody. The cell proliferation index is higher at the peripheral regions of the tumors (A) compared to central regions (B)

or in the presence of the respiratory substrate succinate alone in mitochondria from the center (15.8 ± 11.1 nmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$) and in mitochondria from the periphery (21.4 ± 9.0 nmol $H_2O_2 \cdot min^{-1} \cdot mg^{-1}$). The maximal rate of H_2O_2 generation was observed in complex III under the presence of antimycin A and rotenone; the addition of both inhibitors specifically makes it possible to measure the production of H_2O_2 in complex III since rotenone inhibits H_2O_2 generation caused by reversed electron flow through complex I. High rates of production of reactive oxygen species were features of respiratory chain-inhibited mitochondria and of reversed electron flow, respectively, in isolated mitochondria from both regions of glioma tissue. Similar observations have been described in mitochondria isolated from human parahippocampal gyrus [37].

Markers of oxidative damage

In order to estimate the profile of oxidative damage as possible consequence of free radical attack and modification of subcellular components (nucleic acids, lipids and proteins), we determined lipid peroxidation (TBARS), mitochondrial DNA oxidative damage and protein carbonylation in opposite regions of glioma samples (Table 6). For mitochondrial DNA damage and protein oxidative damage no significant differences were found between center and periphery (Fig. 3). However, there was a trend towards an increase in lipid peroxidation per gram of fresh tissue in the central area in comparison with the peripheral one, which only marginally failed to reach significance ($P = 0.054$).

Glutathione content

The GSH/GSSG couple is thought to be the major redox buffer in the cell. In the present experiments, GSH/GSSG values in central region were two-fold higher than in peripheral region (5.23 ± 1.23 vs. 2.55 ± 0.61). Mean difference of GSH/GSSG ratio between central and peripheral region was 2.68 ± 0.71; $P = 0.007$, Student's paired test. There were similar GSSG levels per gram of fresh tissue (mean difference between central and peripheral region was -0.0029 ± 0.0101; $P = 0.392$, Student's paired test), however, a statistically significant rise in GSH levels per gram of fresh tissue was found in the tumor center (Fig. 4, Table 7).

Mitochondrial antioxidant activities

There was a higher specific activity of manganese superoxide dismutase in mitochondria from tumor center compared to mitochondria from periphery in all the cases subjected to study (Fig. 4, Table 8). Moreover, GPx activity showed a trend towards an increase in mitochondria from central region in comparison with mitochondria from periphery. Conversely, glutathione reductase activity was only slightly higher in the tumor center compared to tumor marginal zone of resection.

Discussion

Gross total *en bloc* resections of primary brain tumors are not a common practice worldwide. The rapid availability of these rare samples at our Institution gave us the unique possibility of studying the relationship between functional characteristics (bioenergetics and oxidative status) of cancer cell populations and their spatial distribution within a solid tumor. Measurements were achieved in central and peripheral regions of fresh tissue samples from patients with brain tumors. It is important to point out that our data were not affected by a possible contaminating effect of residual non-tumoral tissue in the periphery compared to the center. Histological analysis reveals that glioma cells grow within the healthy brain parenchima, therefore, there is residual non-tumoral tissue both at the central and the peripheral regions. However, in all the cases subjected to study the percentage of non-neoplastic brain tissue was minimal at both locations. Our data show phenotypic heterogeneity between cancer cell populations, given that similar density of neoplastic cells was identified in each region of the tumor. In accordance to our results, other studies have proved that there is a correlation between the karyotypic pattern and certain phenotypic features of cancer cell populations from human glioma [42]. In the present experiments glioma cell populations inside the neoplastic tissue exhibited differences in mitochondrial oxidative capacity as well as in the activation of their cellular and mitochondrial antioxidant mechanisms. This fact is in concert with other studies in human glioma that show heterogeneity in basal oxidative activity of their cell populations [43]. Keeping these observations in mind, our study in human glioma tissue corroborates the phenotypic heterogeneity observed in malignant glioma cells and suggests that functional nonuniformity may be related to particular regions of the tumor.

As has been reported, these functional differences could be related to an increase in tumor size: malignant cells are often exposed to heterogeneous environment, with some regions of solid tumors containing microenvironmental niches that create a gradient of criti-

cal metabolites, including oxygen, glucose, other nutrients and growth factors [44]. Additionally, histological and magnetic resonance imaging studies have described that brain tumors show a structural heterogeneity, with different zones from the periphery, which is thought to be the most proliferative area of the tumor, to the center, which could be necrotic in particular cases [45]. Moreover, this structural heterogeneity seems to be correlated with heterogeneous vascularization [46]. In relation to this, we determined cell proliferation in adjacent sections of these glioma samples using ki-67 immunohistochemical technique. In our study, the proliferative index was higher in the peripheral tumor sections (representative images are shown in figure 5). In our cases, immunostaining for p53 showed equal intensity in the center and in the periphery of the glioma mass (data from an on-going parallel study); the histological pattern did not seem to reveal an increased resistance to apoptosis in the tumor's center compared to the tumor's periphery. However, complementary studies in primary cultures are needed to be able to assess the possible differences in apoptosis resistance between both regions under specific stimuli.

Brain tumors suffer from reduced respiratory capacity coupled to increased glycolysis and lactic acid production [47]. Given that our results showed a higher respiratory rate in isolated mitochondria from the outer regions of the tumor compared to central regions, we thought that this could be related to the utilization of a preferential pathway to obtain energy. Nevertheless, we did not find significant differences between both regions in cellular LDH activity, a key enzyme of glycolytic metabolism.

We did not find statistically significant differences in mtDNA content between central and peripheral regions of the analysed samples; however, some studies report high amplification as the most common alteration in mitochondrial DNA of gliomas [3]. Considering well-established markers of mitochondrial number (mtDNA content, total CS activity and total COX activity), our results pointed to similar mitochondrial abundance between the center and periphery [4, 48, 49]. In contrast to the apparent structural homogeneity observed between mitochondria populations in central and peripheral areas of human glioma, we obtained an important heterogeneity in mitochondrial function measured in each region. In the present study, the drop in mitochondrial functionality from tumor periphery to the center consisted of diminished COX activity, decreased respiration in basal state and a lack of maximal functional capacity of CI and CIII (which was assessed as mitochondrial capacity to produce H_2O_2 from both complexes). This article describes for the first time, differences in oxidative capacity and oxidative stimuli in concentric distant regions within the same tumor mass.

It is of further interest to note that the observed decline in mitochondrial oxidative capacity in the glioma center compared to glioma periphery could be better explained on the basis of underlying greater hypoxia and oxidative stress in tumor center than alterations in mtDNA content or differential mtDNA oxidative damage between both regions. Previously, the decline in COX activity has been attributed to a peroxidative damage in mitochondrial inner membrane [50], moreover, experimental hypoxia results in significantly lower COX activity and vastly increased ROS production by this complex [51]. Interestingly, mitochondrial function is unlikely to be altered in spite of mitochondrial mutations in gliomas (which are closely linked to mtDNA oxidative damage) [52]. Hence, our data suggest that mitochondrial functional alterations between central and peripheral regions might be related to oxidative injury of the most susceptible macromolecules.

In our study, the greater hydrogen peroxide production capacity observed in mitochondria from periphery is consistent with the notion that increased oxygen consumption may result in elevated reactive oxygen species production [53]. With regards to oxidative injury in glioma cells, we did not find significant differences in total protein oxidation between both regions. On the other hand, an important trend towards a higher lipid damage per gram of fresh tissue was identified in the central regions of the glioma. This finding is interesting since end products from lipid peroxidation could be used as markers for increased oxidative stress in these regions (as a result of the balance between total peroxide production, antioxidant defenses and repairing systems).

Paradoxically, the observed tendency towards a decreased capacity of production of mitochondrial H_2O_2 was coupled to a higher cellular lipid peroxidation in the central region of the neoplasia. This fact can be explained on the basis that the rate of mitochondrial H_2O_2 production measured *in vitro* indicate the fraction of hydrogen peroxide released by isolated mitochondria, thus it reflects mitochondrial capacity to produce H_2O_2 but not the intracellular levels (or ROS variations over time) to which cancer cells are exposed. The greater ROS production capacity in tumor periphery was in agreement with its higher mitochondrial functionality. Although we could not measure intracellular ROS levels, their effects are the best markers of long-term exposure of cells to ROS [54]:

- The tendency towards an accumulation of oxidative injury in tumor center
- The impairment in mitochondrial function of tumor center
- The increase in central region of soluble antioxidants, such as glutathione, and enzymes that detoxify free radicals and reactive oxygen species to cope with high intracellular levels of ROS.

On the other hand, we have to consider cancer cell divisions: the less proliferative activity in the central region would make it easy to accumulate oxidative damage; if cells inside tumor center had a lower proliferation rate than cells in tumor periphery, energy demand in tumor mass would be restricted mainly to the periphery. In this sense, it is well known that cells under high energy demand operate a "respiratory control" by stimulation of respiration by ADP that stops after conversion of ADP into ATP; it is assumed that ROS production within the cell is low in this situation [55, 56].

The present study suggests a dysregulation of the redox homeostasis in glioma center. In all the studied tumors, we found significantly higher cellular levels of reduced glutathione (expressed per gram of fresh tissue), MnSOD activity and GSH/GSSG ratio in central glioma areas when compared to peripheral ones. In this way, the increase in antioxidant systems in central region could be considered an adaptative mechanism by which cancer cells counteract chronic oxidative stress [57-59]. Interestingly, antioxidant systems could have primary implications in management of malignant gliomas [60]. On the one hand, overexpression of MnSOD (an essential primary antioxidant enzyme that converts superoxide radical to hydrogen peroxide and molecular oxygen within the mitochondrial matrix) causes tumor suppression [61] and growth inhibition which may correlate with the intracellular redox status in glioma cells [62]. On the other hand, several studies have provided evidence for the role of increased levels of antioxidant systems in resistance to anticancer treatments. Cross-resistance to cisplatin and radiation may be mediated by increased content of detoxifying thiols such as glutathione. Other studies have also proved that in glioblastoma cell lines highly coordinated activation of antioxidant enzymes such as superoxide dismutase, catalase, GPx and GRd is related to radioresistance and cross-resistance to chemotherapeutic agents [63]. Moreover, the higher content in tumor center of reduced glutathione may improve its efficiency of drug detoxifying metabolism [64]. Sublines resistant to doxorubicin, a widely used antitumoral drug, show a decrease of cytochrome *c* oxidase activity and cytochrome *aa3* content [65]. Recently, a cellular metabolic strategy that supports the connection between mitochondrial activity and cellular response to chemotherapeutic agents has been identified [66]. Considered together, there is clearly a similarity between the presented changes in metabolic and antioxidant capacities in glioma center and the key features of drug resistance.

Ideally, regional differences in mitochondrial behaviour and antioxidant systems of cancer cell populations inside the glioma mass should be considered in therapeutic strategies. Standard therapies for malignant gliomas (such as radiation) may retard glioma growth over the short term, but they can facilitate glioma recurrence and enhance growth rate over the longer term through alterations in morphogenetic fields [67, 68]. The presence of cancer cells in the tumor mass with different susceptibility to therapy may help to explain the different response of patients to standard therapies even when a same histological grade is diagnosed. Radiotherapy and certain chemotherapeutic agents might establish a selective pressure *in vivo* by which they might select the most resistant cancer cell population.

In this study, we have proved that differences in energy metabolism and oxidative status within glioma mass are not random but follow a characteristic pattern; differences in cancer cell behaviour are associated to macroscopically distant concentric regions of human glioma tissue. The highest antioxidant capacity is localized in the tumor center whereas the greatest mitochondrial functionality is identified in the tumor periphery. Finally, our study supports the primary role of mitochondrial function and redox reactions as targets to modulate in new anticancer therapeutic strategies [69, 70] and points to the need for more profound knowledge about oxidative mitochondrial metabolism in human brain tumors. Especially, further studies about the different behaviour of cancer cell populations inside the fresh neoplastic tissue are required given that it could have primary therapeutic implications.

Abbreviations

COX (cytochrome *c* oxidase); CI (complex I or NADH-ubiquinone oxidoreductase); CIII (complex III or ubiquinol-cytochrome *c* oxidoreductase); CS (citrate synthase); LDH (lactate dehydrogenase); TBARS (thiobarbituric acid-reactive substances); ROS (reactive oxygen species); OXPHOS (oxidative phosphorylation system); mtDNA (mitochondrial DNA); mt protein (mitochondrial protein); MnSOD (manganese-superoxide

dismutase); GPx (glutathione peroxidase); GRd (glutathione reductase); GSH (reduced glutathione); GSSG (oxidized glutathione).

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Early-Onset L-dopa-Responsive Parkinsonism with Pyramidal Signs Due to *ATP13A2, PLA2G6, FBXO7* and *Spatacsin* Mutations

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Video 

Abstract: Seven autosomal recessive genes associated with juvenile and young-onset Levodopa-responsive parkinsonism have been identified. Mutations in *PRKN, DJ-1,* and *PINK1* are associated with a rather pure parkinsonian phenotype, and have a more benign course with sustained treatment response and absence of dementia. On the other hand, Kufor-Rakeb syndrome has additional signs, which distinguish it clearly from Parkinson's disease including supranuclear vertical gaze palsy, myoclonic jerks, pyramidal signs, and cognitive impairment. Neurodegeneration with brain iron accumulation type I (Hallervorden-Spatz syndrome) due to mutations in *PANK2* gene may share similar features with Kufor-Rakeb syndrome. Mutations in three other genes, *PLA2G6* (PARK14), *FBXO7* (PARK15), and Spatacsin (SPG11) also produce clinical similar phenotypes in that they presented with rapidly progressive parkinsonism, initially responsive to Levodopa treatment but later, developed additional features including cognitive decline and loss of Levodopa responsiveness. Here, using homozygosity mapping and sequence analysis in families with complex parkinsonisms, we identified genetic defects in the *ATP13A2* (1 family), *PLA2G6* (1 family) *FBXO7* (2 families), and SPG11 (1 family). The genetic heterogeneity was surprising given their initially common clinical features. On careful

Additional Supporting Information may be found in the online version of this article.

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review, we found the *FBXO7* cases to have a phenotype more similar to PRKN gene associated parkinsonism. The *ATP13A2* and *PLA2G6* cases were more seriously disabled with additional swallowing problems, dystonic features, severe in some, and usually pyramidal involvement including pyramidal weakness. These data suggest that these four genes account for many cases of Levodopa responsive parkinsonism with pyramidal signs cases formerly categorized clinically as pallido-pyramidal syndrome. © 2010 Movement Disorder Society

Key words: parkinsonism; recessive; ATP13A2; PLA2G6; FBXO7; Spatacsin

In 1954, Davison described five cases of juvenile parkinsonism with associated upper motor neuron signs. Post mortem examination revealed lesions in the pallidum, the substantia nigra, the ansa lenticularis, and the corticospinal tract, thus termed pallido-pyramidal disease. Since then some similar cases have been reported, characterized by autosomal recessive inheritance, normal neuroimaging (although usually without T2* assessment), and L-dopa responsiveness.[1–3]

Over the last 10 years numerous autosomal recessive genes causing L-dopa-responsive parkinsonism have been identified.[4–6] Parkin (*PRKN)* gene mutations are associated with hyperreflexia,[7] however, there is only one report of a pallido-pyramidal phenotype.[8] Pyramidal signs are also infrequent in *DJ-1* and *PINK1* mutations.[9] Dopa-responsive dystonia (DRD) can often mimic early-onset parkinsonism (EOPD), sensitive to low L-dopa doses, and carriers of *GTP cyclohydrolase* mutations do usually not develop dyskinesias. Tyrosine hydroxylase,[10] sepiapterin reductase deficiency,[11] and spasticity have been reported in DRD,[12] but these are clinically distinct from pallido-pyramidal disease.

Furthermore, mutations in *ATP13A2*, *PLA2G6, FBXO7,* and *SPG11* have recently been identified in cases similar to Davison's seminal report.[13–16] Here, we summarize the phenotypic and genotypic characteristics of cases with homozygous mutations in these four genes. This case series represent the cases of this syndrome, in which we have mapped the lesions by homozygosity mapping. We restrict our discussion of the literature to findings in cases with homozygous and compound heterozygous changes because only in such circumstances, we can be certain of their pathogenic nature (Table 1).

ATP13A2 (PARK9)

Homozygous and compound heterozygous *ATP13A2* (PARK9) mutations were first described in patients of Jordanian and Chilean ancestries. The main clinical features were juvenile akinetic-rigid parkinsonism, pyramidal weakness, spasticity, and Babinski signs, supranuclear gaze paresis, and cognitive impairment.[13,21,22] On clinical follow-up visual hallucinations, facial-faucial-finger mini myoclonus and oculogyric dystonic spasms were added to the phenotypic spectrum.[23] The Chilean and Japanese kindreds were clinically similar.[13,17] An apparently sporadic Brazilian patient with a single homozygous mutation with disease onset aged 12 was also reported. However, Babinski signs, supranuclear gaze paresis or dementia[18] were absent and the case closely resembled *PRKN* disease. This single case suggests that *ATP13A2* mutations may play a role in EOPD, [24] although it has to be acknowledged that the pathogenicity remains uncertain.

PLA2G6 (PARK 14)

PLA2G6 mutations have been associated with neurodegenerative disorders with increased basal ganglia iron accumulation, such as infantile neuroaxonal dystrophy (INAD) and neurodegeneration with brain iron accumulation (NBIA-type 2).[25,26] Pathologically, both, INAD and NBIA, show axonal degeneration with spheroid bodies (distended axons) throughout the central nervous system. *PLA2G6* mutations have also been found in patients without spheroids and in classical INAD.[27] INAD presents in infancy and death by age 10 is usual. Typically, NBIA presents between infancy and 30 years of age with faster disease progression in infantile and juvenile onset cases.[19,28] There is clinical heterogeneity as recently L-dopa responsive dystonia-parkinsonism cases with an onset age ranging from 10 to 26, whose main clinical features were severe akinesia and rigidity, generalized dystonia and cognitive impairment, however, with no evidence of brain iron accumulation on neuroimaging were described.[15,19] These latest findings led to a designation of *PLA2G6* as PARK14. However, the fact that identical disease-associated *PLA2G6* mutations may cause NBIA, INAD, and dystonia-parkinsonism suggests that additional unknown genetic, epigenetic, or nongenetic factors may influence the *PLA2G6*-associated phenotype.[15,19,26]

FBX07 (PARK 15)

A disease-associated variant in *FBXO7* causing p.Arg378Gly has recently been identified in an Iranian

TABLE 1. *Previously reported and novel autosomal recessive parkinsonism mutations*

	cDNA	Protein	References
ATP13A2: PARK9	c.546C>A	p.Phe182Leu	Ref. 17
	c.1103_1104insGA	p.Thr367ArgfsX29	This paper
	c.1306+5G>A	NA	Ref. 13
	c.1510G>C	p.Gly504Arg	Ref. 18
	c.1632_1653dup22	p.Leu552fsX237	Ref. 13
	c.3057delC	p.Gly1019fsX3	Ref. 13
PLA2G6: PARK14	c.109C>T	p.Arg37X	Unpublished data
	c.1078−3C>A	NA	Unpublished data
	c.1715C>T	p.Thr572Ile	Unpublished data
	c.1894C>T	p.Arg632Trp	Ref. 19
	c.2222G>A	p.Arg741Gln	Ref. 14
	c.2239C>T	p.Arg747Trp	Ref. 14
FBXO7: PARK15	c.65C>T	p.Thr22Met	Ref. 20
	c.907+1G>T	NA	Ref. 20
	c.1132C>G	p.Arg378Gly	Ref. 15
	c.1492C>T	p.Arg498X	Ref. 20
SPATACSIN: SPG11	c.704_705delAT	p.His235ArgfsX12	Ref. 16
	c.733_734delAT	p.Met245fsX2	This paper

All ATP13A2, PLA2G6, FBXO7, and Spatacsin mutations identified to date in either recessive parkinsonism or idiopathic Parkinson's disease patients. Only homozygous or compound heterozygous mutations are included because on these have strong evidence for pathogenicity.

NA, Not Applicable.

kindred which presented with spastic weakness and Babinski signs. Parkinsonism with bradykinesia and rigidity was developed as a late feature in some familial members.[15] Three novel *FBXO7* mutations, c.907+1G>T and p.Thr22Met in the compound heterozygous state and p.Arg498X in homozygous state, were later identified in Dutch and Italian families exhibiting spasticity and Babinski signs, tremor, bradykinesia, and postural instability. Dystonia was also present in the homozygous p.Arg498X mutation carriers. These families expanded the phenotypic spectrum associated with *FBXO7* mutations making it another cause of recessive EOPD (PARK15).[20]

SPATACSIN (SPG11)

Spatacsin (SPG11) is the major mutated gene in autosomal recessive spastic paraplegia with thin corpus callosum (ARHSP-TCC). To date, more than 50 different *SPG11* mutations, including nonsense, spice-site, and frameshift variants, have been reported in familial and idiopathic cases presenting with complicated HSP.[29–31] In addition, an unusual parkinsonism presenting with resting tremor, akinesia and with either weak or no L-dopa response has recently been described in two SPG11 patients from a consanguineous Turkish family. Both showed mental retardation, characteristic of the complex HSP, and bilateral Babinski signs. An ^{123}I-ioflupane SPECT scan revealed dopaminergic denervation in one of the probands. They carried a homozygous frameshift SPG11 mutation (p.His235ArgfsX12).[16]

SUBJECTS AND METHODS

Subjects

Patients from five unrelated consanguineous families with L-dopa-responsive EOPD gave informed consent to this study approved by the local ethics committee. Different cases were clinically examined by the clinicians involved in the patients' care and video footage of all cases was retrospectively reviewed by HH, KPB, and AJL. Clinical details are partly given below and summarized in Table 2. For full information see supplements. We also compare the clinical features of two previously published *PLA2G6* mutation families (Table 2) and the video of Iranian *FBX07* mutation family E. The family trees for families reported here, with the exception of family E where only one proband was available for study, are shown in Figure 1.

Family A (PLA2G6)

This patient was described by Paisan-Ruiz et al.[14] without video documentation.

In summary, onset was at age 26 with progressive cognitive decline, slow movements (video segment 1), clumsiness, progressive imbalance, hand tremor, and slurred speech, followed by the development of dystonia. There was an excellent L-dopa response. However,

TABLE 2. *Summary of the clinical details of the families reported here (families A–D)*

Table	Family A PLA2G6		Family B ATP13A2		Family C FBX07			Family D FBX07	Family E SPG11	Family DP Sina et al.[27] PLA2G6	Family 2 from Paisan-Ruiz et al.[14] PLA2G6
Family/case	1	2	1	2	1	2	3	1	1	Cases 1–3	1
Current age (yr)	35	41	41	44	22	44	22	Died 27	27	23, 25, 31	21
Age of onset (yr)/first symptom	26 Cognitive	29 Falls	16 Psychosis	18 Gait	17 Eyelid dyspraxia	24 Bradykinesia	22 Bradykinesia	17	14	21, 22, 25 Dragging feet	18 Dragging foot
Cognitive decline	++	+++	++	+++	+	+	+	−	+	++	+
Psychiatric features	+	+	++	++	+	++	++	+	(+)	+	+
Extrapyramidal signs	+++	++	++	++	++	++	++	++	++	+++	++
Pyramidal features	+	++	+	++	+	+	+	−	+++	++	++
L-dopa response	++	++	++PT	++PT	+++	++	++	++	++PT*	++	++
L-dopa-induced dyskinesias	++	++	++	++	+	++	++	++	NA	++	+
Dystonia	+++	+++	++	+	−	−	+	−	+	+++	++
Eye movement abnormalities	+	++	++	++	++	+	+	−	+	+	+
Imbalance/impaired postural reflexes	++	++	+	+	+	+	+	+	+	++	+
Dysarthria/dysphonia	+++	+++	++	++	++	++	+++	+++	+	++	+
Swallowing problems	+++/PEG	+++/PEG	++	++	+	++	++	+	−	++	+
Other	Seizures pale blue sclera	Pale blue sclera	Bleph	OA	Bleph cateracts	Cateracts	Cervical dystonia	Nicotine responsive. Dopa induced dystonia and aggression	Nil	Nil	Foot dystonia with hemiparetic gait
MRI brain	Frontal white matter	General atrophy	General/ Caudate atrophy	General/ Caudate atrophy	Normal	General atrophy	General atrophy	Normal MRI. Beta-CIT SPECT, no uptake	General atrophy, thin corpus callosum	General atrophy	General atrophy

We have previously reported two other families with PLA2G6 mutations and their clinical features are also shown for comparison. +++ = severe, ++ = moderate, + = mild, (+) = related to treatment, − = absent. PEG = Percutaneous endoscopic gastrostomy, Bleph = blepharospasm/clonus, OA = optic atrophy. PT = poorly tolerated: *, treated with ropinerole.

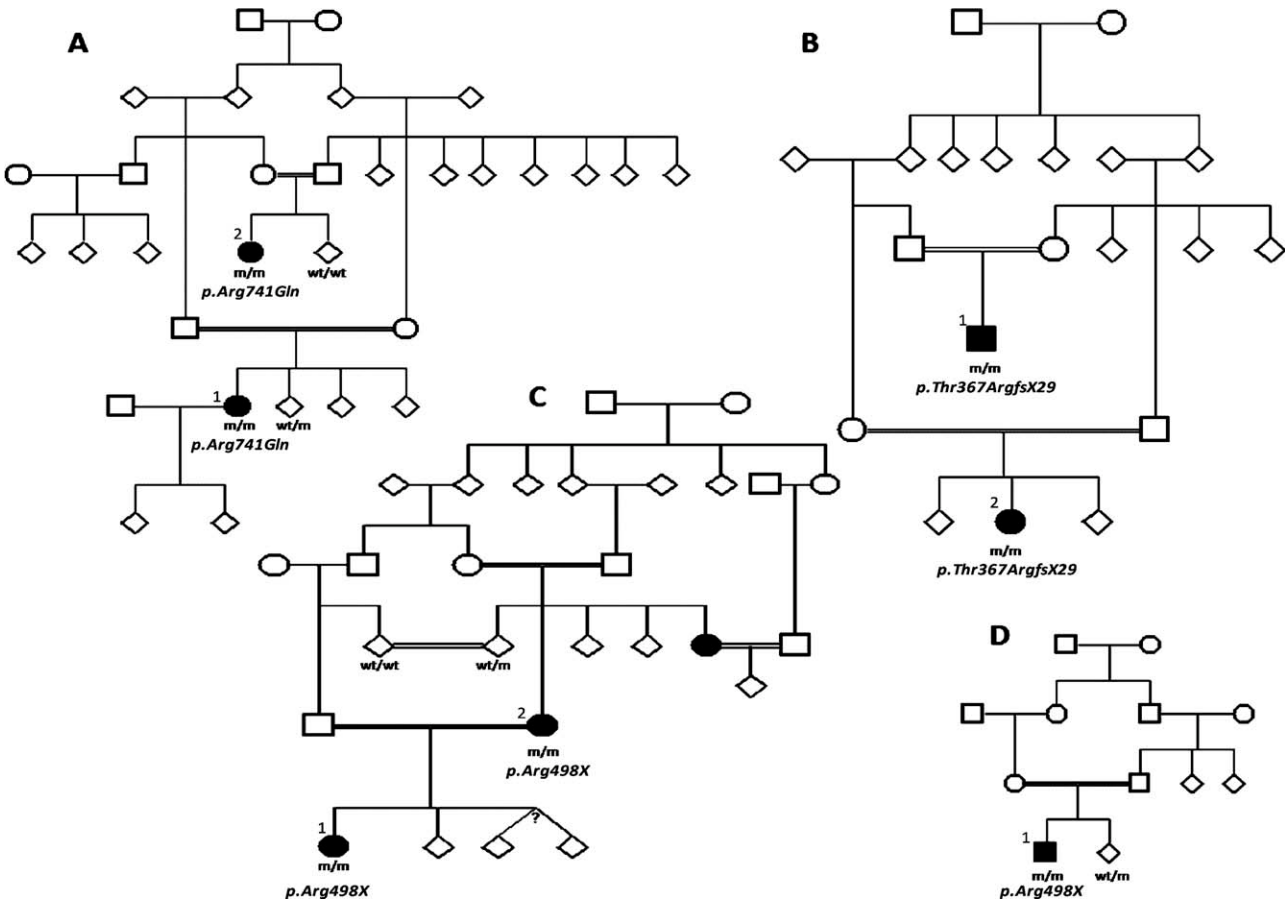

FIG. 1. Pedigrees of families reported here. Manifesting members are shown in bold. A: *PLA2G6* family, B: *ATP13A2* family, C and D: *FBXO7* families. m/m: homozygous mutation carriers, wt/m: heterozygous mutation carriers, wt/wt: homozygous carriers for the wild type sequence.

after 1 year she developed prominent dyskinesias and improvement declined considerably over the next few years. By age 34 years, she was bed-bound (Video segment 2) and started to have epileptic seizures.

Additional data is also provided now on the previously undescribed cousin, a 36-year-old North Indian female. Her cousin had a foot drag dystonia at age 10. At age 26, she developed arm and leg tremor, followed by infrequent falls from age 29. In view of the findings of ankle clonus and extensor planters on examination a diagnosis of spastic quadriplegia was initially made (Video segment 3). She later developed bradykinesia and extreme rigidity. On examination at age 33, she had a supranuclear vertical gaze palsy, eyelid opening apraxia, a positive glabellar tap sign, and facial hypomimia (Video segment 4). She had developed a pill-rolling tremor, limb bradykinesia, brisk reflexes, and bilateral Babinski signs. She was severely dysarthic with slow tongue movements. L-dopa treatment was beneficial but caused prominent early dyskinesias.

Family B (ATP13A2)

This case was first described in 1995.[32] At age 16 he developed an L-dopa-responsive akinetic rigid syndrome. He developed dyskinesias at high doses and secondary nonresponsiveness to dopaminergic therapy. Over the next 15 years he deteriorated. On examination at age 40 years he was anarthric. He had normal fundi but reduced up- and down-gaze with broken pursuit and slow saccades. There was marked dystonia, brisk reflexes, ankle clonus, and bilateral Babinski signs (Video segment 5).

Brain MRI showed general involutional change involving the cerebral cortex, basal ganglia, and cerebellum with presence of basal ganglia iron on T2* sequences[33]. A sural nerve biopsy performed at age 40 showed acute axonal degeneration, some regeneration, and a very mild chronic inflammatory response with endoneurial and epineurial T-cells. There were numerous cytoplasmic inclusion bodies (1–5 μm in diameter) within the Schwann cells, perineurial and epineurial

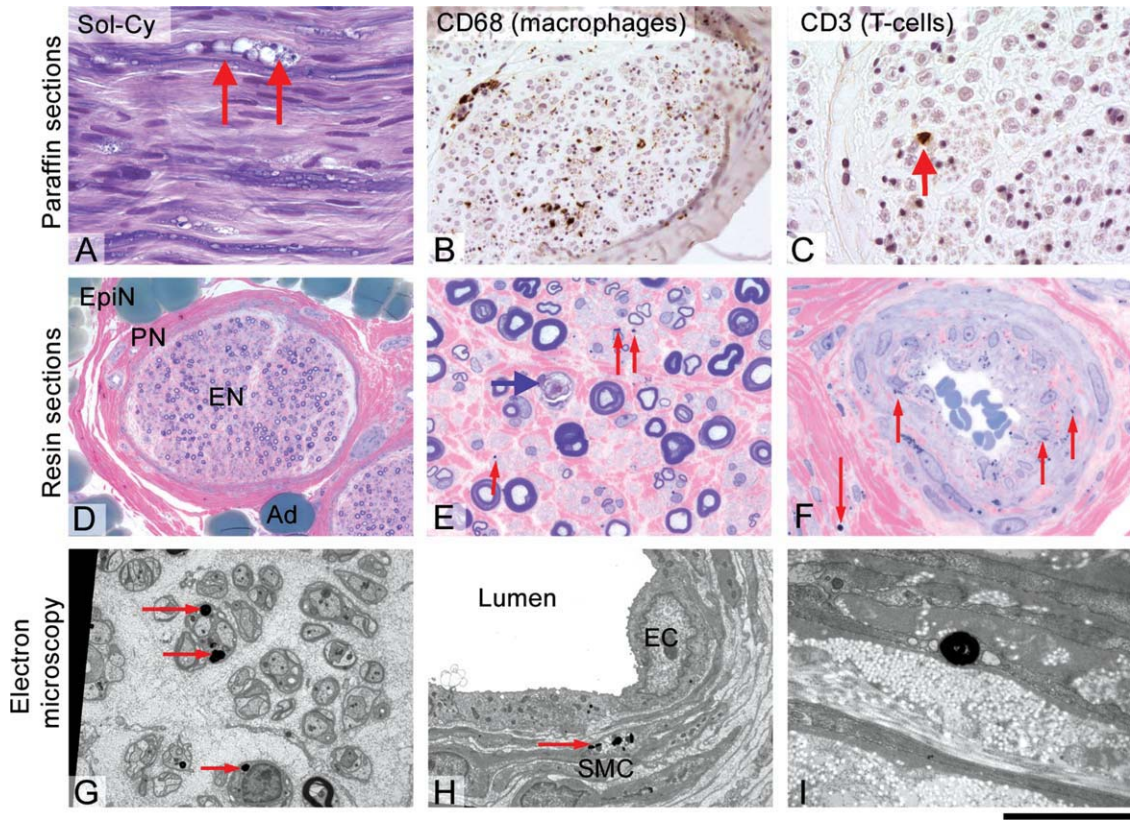

FIG. 2. Histological and ultrastructural analysis of the sural nerve biopsy of Family B (*ATP13A2)*: Paraffin sections (A, B, C) show a reduction of myelinated fibre density with frequent formation of myelin digestion chambers (arrows) (A). Immunohistochemical staining for CD68 on a transverse section shows frequent endoneurial macrophages (B), a characteristic finding in florid axonal neuropathies. Very occasionally, there were endoneurial and scattered epineurial T-cells (CD3 immunohistochemistry; C). Resin semi thin sections (D, E, F) show a mild generalized axon loss, subperineurial, and endoneurial oedema (D) and significant numbers of degenerating axons (E, blue arrow). Strikingly, there are numerous small cytoplasmic inclusion (E, red arrows and F, red arrows). These inclusions were found in the endoneurium, in smooth muscle cells of vessels and in the perineurium. Electron microscopy (G, H, I) confirms the presence of electron dense inclusions of circa 1 μm size, which are always located intracellularly, and are most frequently seen in the cytoplasm of Schwann cells (G, red arrows) and in smooth muscle cells (H, I). Scale bar 40 μm (E, F), 60 μm (A, C), 120 μm (B), 230 μm (D).

cells but not in axons. Electron microscopy showed the inclusions to be membrane-bound, irregular, and occasionally folded. Overall they resembled irregular primary lysosomes (Fig. 2).

The proband's cousin was phenotypically very similar. Onset was at age when aged 18 she developed gait difficulty with frequent falls backwards. She developed arm tremor and urine incontinence. Video segment 6 shows her at age 26 years. L-dopa treatment produced significant improvement; however, with the emergence of early drug-induced dyskinesias and the L-dopa effect reduced within 5 years.

Family C (FBX07)

The proband originating from Pakistan from a family with multiple consanguineous loops presented at age 17 years with difficulty opening her eyes, generalized stiffness and bradykinesia. Over 5 years she developed dysarthria, hypophonic speech, frequent respiratory sighs, and urinary problems.

On examination, she had cataracts, prominent apraxia of eye opening, and supranuclear gaze palsy. She had slow saccades with prominent blepharospasm. There was upper and lower limb rigidity, bradykinesia but no tremor. Reflexes were brisk and the plantars were extensor (Video segment 7).

An L-dopa challenge was strongly positive (UPDRS score 42 pre- and 20 post-treatment). For aggression and mood she later required Olanzapine.

The proband's mother was similarly affected by L-dopa-responsive parkinsonism without tremor and onset at age 24. She had difficulty with upgaze and abnormal respiration with sighs. Aged 40 she had

cataracts, was very rigid and slow, incomprehensible speech, cognitive problems, and swallowing difficulties. For details of the proband's aunt and investigational results see supporting information.

Family D (FBX07)

This family with multiple consanguineous loops originated from southeast Turkey. Clinical details and a video of the proband have previously been reported by Hanagasi et al.[34] before the gene was identified. The 26-year-old male proband developed walking difficulties at age 17, followed by L-dopa-responsive limb rigidity and marked bradykinesia. Because L-dopa caused psychosis it had to be withdrawn. See supplements for further clinical details and Ref. 33 for a video. The patient died at age 28.

The patient had four paternal cousins, who were said to have had severe gait difficulty and bradykinesia. Their symptoms had also started before the age of 20, and they had died within a few years in a bedridden state.

Family E (SPG11)

The symptoms of this 27-year-old Asian from a consanguineous family began at age 14 with postural and writing tremor. Aged 17 he developed walking difficulties with imbalance, speech problems, and slowness. His gait became progressively stiff and he complained of leg weakness and falls. Pharmacological treatment (i.e., baclofen, tizanidine, clonazepam) was either ineffective or produced intolerable side-effects. At age 24 (Video segment 8) he presented with mild gynaecomastia, facial hypomimia, laryngeal dystonia, upgaze skew deviation with slowed upward eye movements, hand dystonia and writing tremor, marked spastic paraplegia, bradykinesia, axial rigidity, and imbalance. Reflexes were brisk bilaterally with bilateral ankle clonus. Routine and genetic testing for SCAs 1,2,3,7,17, DRPLA, and SPG4 were normal. An MRI brain scan revealed generalized atrophy with a thin corpus callosum. A DaT-SPECT scan showed decreased bilateral putaminal and caudate uptake. Motor symptoms improved on ropinirole but caused confusion and hallucination.

Family F (PLA2G6)

See Ref. 27 for genetic findings and Video segment 9 for clinical features.

Molecular Analysis

Genome-wide SNP genotyping was carried out using either HumanHap240 or HumanHap317 illumina beadchips. Homozygosity mapping was performed as previously described[35,36] and using the Homozygosity detector plug-in software within the BeadStudio 3.2 program where a minimum physical size threshold of 1Mb and at least 100 adjacent markers in length were used as limiting parameters (www.illumina.com). Gene screening analyses for *ATP13A2*, *PLA2G6, FBXO7,* and *SPG11* were performed by PCR analysis using 10 picomoles of both forward and reverse primers (Supporting information 1) and FastStart Taq DNA polymerase (http://www.roche-applied-science.com). Each purified PCR product was then sequenced with Applied Biosystems BigDye terminator v3.1 sequencing chemistry as per the manufacturer's instructions; the resulting reactions were resolved on an ABI3730 XL genetic analyzer (Applied Biosystems, Foster city, CA) and analyzed by Sequencher 4.8 software (Gene Codes Corporation, Ann Arbor, MI).

RESULTS

All families presented with EOPD that was initially L-dopa responsive. Cognitive and psychiatric features were common in all except the *FBX07* mutation cases where agitation and mood problems occurred only after L-dopa treatment. Supranuclear gaze palsy, severe bulbar signs with speech and swallowing difficulties were present in all families. Pyramidal signs were perhaps most marked in the family E (*SPG11)* and were absent in the Turkish family D (*FBX07*). Dystonia was also present in the PLA2G6 and ATP13A2 families but they were not a significant feature in the *FBX07* mutation families where only one individual had cervical dystonia. MRI brain scans revealed generalized involutional change in most cases. In family E (SPG11), there was a thin corpus callosum in addition to the generalized atrophy. Details of the nerve biopsy in family B are given above in the clinical description.

Comprehensive homozygosity mapping was carried out in nine individuals (eight affected and one unaffected) belonging to four families. No homozygosity mapping was performed in family E as only the proband was available for study. In first instance, we searched for autozygous segments shared among all affected individuals to locate the pathogenic loci,[14] although the proximity of the *FBXO7* gene (5.7 Mb upstream of PLA2G6) to *PLA2G6* on chromosome 22 prevented this analysis alone from distinguishing these

loci before gene sequencing (Supporting information 2). Gene sequencing identified the lesion in each family. Similarly, the proximity of the *ATP13A2* gene to the established parkinsonism gene *PINK1* on chromosome 1 meant that homozygosity mapping alone could not delineate the lesion in this family (Supplement 2). In conclusion, Family A carried the previously reported *PLA2G6* mutation (p.Arg741Gln; c.2222G>A), Family B a novel *ATP13A2* mutation (p.Thr367ArgfsX29; c.1103-1104insGA), Families C and D the same *FBXO7* mutation (p.Arg498X; c.1492C>T), and Family E a 2bp SPG11 deletion (c.733_734delAT; p.Met245fsX2) previously reported in families presenting with complicated ARHSP-TCC (Table 1).[29]

DISCUSSION

There are now eight recessive loci, which can lead to EOPD syndromes. These are the classical recessive loci, *PRKN* (PARK2), *PINK1* (PARK6), *DJ-1* (PARK7), the four loci we present examples of here, *ATP13A2* (PARK9), *PLA2G6* (PARK14), *FBX07* (PARK15), and SPG11 and the *PANK2* genes.[16,37] It is important to be able to characterize disease causing mutations and the phenotypic features associated with the mutation in the genes for clinical purposes and, also because observed distinctions may give mechanistic insights.

Loss of function mutations at *PRKN*, *PINK1,* and *DJ-1* nearly always give rise to a pure parkinsonian phenotype which has an early onset, a benign course, sleep benefit and a good and prolonged response to L-dopa. The lifespan of mutation carriers is only marginally reduced and there have been no reports of brain iron accumulation. All three proteins have functions related to mitochondrial biology and *PRKN* mutations are usually not associated with Lewy bodies.[37]

Loss of function mutations in *PLA2G6* and *PANK2* lead to variable and overlapping clinical features of progressive parkinsonism, dystonia, ataxia, and cognitive decline. The endophenotypes range from the aggressive INAD and Hallevorden-Spatz disease with variable brain iron inclusion and death usually before the age of 20 years, through to the patients that present with predominantly EOPD and dystonia and later develop other manifestations. The pathology of these cases is likely to include extensive Lewy bodies as seen in childhood onset neuroaxonal dystrophy,[26] although the neuropathology of adult onset cases with *PLA2G6* mutations has not yet been reported. Over many years there have been reports of Hallervorden-Spatz cases (PKAN/*PANK2*) with extensive Lewy body disease. We suggest that Kufor Rakeb syndrome may also belong to this same class of diseases, as gene products of both *PLA2G6* and *PANK2* impinge on ceramide metabolism. The role of *ATP13A2* as a lysosomal pump fits with this suggestion, although its precise function is not known.[37]

Loss of function mutations in *FBX07* appears to give a phenotype intermediate between the two disease classes. In some cases the phenotype resembles *PRKN* mutation associated phenotype,[33,38] but the disease is generally less benign and has a reduced life expectancy, pyramidal signs and late cognitive problems. This overlap of phenotypes related to *FBX07* and *PRKN* mutations is consistent with the related functions of these two genes and their likely common disease pathway. Like *PRKN*, F-box proteins, such as *FBXO7*, are components of the modular E3 ubiquitin protein ligases.[39]

These findings have allowed us to dissect the pallido-pyramidal disease described by Davison[1] into at least five recessive forms of complex parkinsonism with subtle clinical differences. Although there are still typical cases of L-dopa-responsive parkinsonism with pyramidal signs where the genetic cause is yet to be identified these data suggest that these five genes account for many of these cases.

LEGENDS TO THE VIDEO

Segment 1. Family A (PLA2G6) (Video of the proband). At 29 years of age she is in a wheelchair. There is a mild postural tremor of both hands and some dystonic posturing and she is slow in her movements. On testing eye movements, pursuits are broken and there is limitation of upgaze.

Segment 2. At age 32 years she requires nasogastric feeding and has facial hypomimia, dystonic facial grimacing and spontaneous tongue protrusion (not related to Levodopa). There is severe dystonic posturing in her upper limbs and a bilateral resting tremor, greater on the left.

Segment 3. Family A (PLA2G6) (Video of the cousin). The patient is now age of 33 years and in a wheelchair. This video essentially demonstrates the bilateral brisk reflexes and the Babinski signs to show the marked pyramidal component of the syndrome.

Segment 4. She has facial hypomimia and square wave jerks on primary gaze with slowed pursuit movements but of full range. Eyelid opening apraxia and a supranuclear vertical gaze palsy is demonstrated (although in the video the patient's eyes were force-

fully retracted which can exaggerate this appearance). She is dysarthric with slow tongue movements. There are orolingual dyskinesias probably related to Levodopa treatment. There is a pill rolling rest tremor of her left hand, mild bilateral postural arm tremor (left more than right) and bilateral bradykinesia.

Segment 5. Family B (ATP13A2) (Video of the proband). The initial segment shows him at the age of 16 after he had an acute hypomanic psychotic episode associated with a phenothiazine induced akinetic rigid syndrome (June 1985), this improved after a few weeks but left him with mild parkinsonian features with cog-wheel rigidity clearly demonstrated in October 1985. The second part of the video shows him at the age of 40 when his disease had progressed. He was anarthric and confined to his bed. He has facial hypomimia and *risus sardonicus*. There is reduced upgaze with broken pursuit and slow saccades with occasional blepharospasm. There is marked dystonic posturing and increased tone in his upper and lower limbs with little movement, there was upper limb bradykinesia and the lower limb reflexes were brisk.

Segment 6. Family B (ATP13A2) (Video of the cousin). The patient showed slow, stooped, dragging gait at the age of 25 years.

Segment 7. Family C (FBX07) (Video of the proband). At the age of 22 years this girl had slow monotonous speech, facial hypomia and hirsutism. During the examination of eye movements (at the start and in between) she has blinking and difficulty opening her eyes (possible apraxia of eye opening or blepharospasm). There is slowed and limited upgaze. She has bradykinesia and a postural but no rest tremor. Planters were extensor and her gait was stooped and slow with poor arm swing.

Segment 8. Family E (SPG11). There is a stiff, spastic gait with difficulty turning. When holding the arms outstretched, there is dystonic posturing of the arms. On writing, there is micrographia and a small amplitude tremor (best seen when drawing a spiral).

Segment 9. Family F (PLA2G6) (Video of three Iranian cases). Case DP5: The patient showed stooped, slow shuffling gait with hypomimic face and drooling. Upper limb bradykinesia and poor tandem walking with a tendency to fall can also be observed. There is no speech and reduced vertical up gaze. Case DP3: The patient is confined to his bed with severe dystonic posturing of his upper and lower limbs. He has no speech and swallowing difficulties. Case DP4: The patient is confined to his bed with severe dystonic posturing of his upper and lower limbs and involuntary movements.

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Author Roles: Coro Paisán-Ruiz carried out and supervised the molecular work. Rosa Guevara and Monica Federoff assisted with the molecular work. Elaha Elahe, Susanne A. Schneider, Petra Schwingenschuh, Nin Bajaj, Murat Emre, Kailash P. Bhatia, Andrew J. Lees carried out the clinical investigations, Sebastian Brandner carried out the pathological investigation. John Hardy, Henry Houlden, Coro Paisán-Ruiz and Andrew B. Singleton obtained funding to support the work, John Hardy and Coro Paisán-Ruiz drafted the manuscript which was edited by Susanne A. Schneider, Kailash P. Bhatia, Andrew J. Lees and Henry Houlden.

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