# Implicación del estrés oxidativo asociado a la obesidad dietética en la alteración de la sensibilidad a la insulina en el músculo esquelético de rata. Diferencias entre machos y hembras

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IMPLICACIÓN DEL ESTRÉS OXIDATIVO ASOCIADO A LA OBESIDAD DIETÉTICA EN LA ALTERACIÓN DE LA SENSIBILIDAD A LA INSULINA EN EL MÚSCULO ESQUELÉTICO DE RATA. DIFERENCIAS ENTRE MACHOS Y HEMBRAS.

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Sigue el camino de baldosas amarillas

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ÍNDICE

ABREVIATURAS

ACC: acetil coenzima A carboxilasa

ACS: acil coenzima A sintasa

Acetil-CoA: acetil coenzima A

Acil-CoA: acil coenzima A

AdipoR1: receptor de adiponectina 1

AdipoR2: receptor de adiponectina 2

ADP: adenosina difosfato

Akt: proteína quinasa B

p-Akt: proteína quinasa B fosforilada

AMPK: proteína quinasa dependiente de adenosina monofosfato

p-AMPK: proteína quinasa dependiente de adenosina monofosfato fosforilada

AMPKK: proteína quinasa de la AMPK

ANT: adenina nucleótido translocasa

APPL1: proteína adaptadora

ATGL: lipasa de triacilglicéridos del adipocito

ATP: adenosina trifosfato

COX: citocromo c oxidasa

COXII: subunidad II de la citocromo c oxidasa

COXIV: subunidad IV de la citocromo c oxidasa

CPT: carnitina palmitoiltransferasa

$FADH_2$: dinucleótido de adenilflavina reducido

GLUT2: transportador de glucosa 2

GLUT4: transportador de glucosa 4

HFD: *high fat diet*; dieta de alto contenido graso

HMW adiponectin: *high molecular weight adiponectin*; adiponectina de alto peso molecular

HNE: hidroxinonenal

HOMA-β: *Homeostasis Model Assessment of insulin secretion;* modelo de evaluación de la homeostasis para estimar la secreción de insulina

HOMA-IR: *Homeostasis Model Assessment of insulin resistance;* modelo de evaluación de la homeostasis para estimar la resistencia a la insulina

HSL: lipasa sensible a hormonas

H_2O_2: peróxido de hidrógeno

IR: receptor de insulina

IR-β: subunidad beta del receptor de insulina

IRS1: sustrato 1 del receptor de insulina

IRS2: sustrato 2 del receptor de insulina

LCFA: ácido graso de cadena larga

LPL: lipoproteína lipasa

MEF2c: factor potenciador específico de miocito 2c

MTE-1: tioesterasa mitocondrial 1

NADH: nicotinamida adenina dinucleótido fosfato

NRF1: factor de respiración nuclear 1

NRF2: factor de respiración nuclear 2

PDK-1: proteína quinasa 1 dependiente de fosfoinosítidos

PGC-1α: coactivador 1α del receptor activado por proliferadores peroxisomales gamma

PI3K: proteína quinasa 3 de fosfoinosítidos

PIP_3: fosfoinositol trifosfato

PKB: proteína quinasa B

aPKC: proteína quinasa C atípica

PPARα: receptor activado por proliferadores peroxisomales α

Cu, Zn-SOD: cobre, zinc superóxido dismutasa

Mn-SOD: manganeso superóxido dismutasa

OH^-: anión hidróxido

O_2^-: anión superóxido

ROS: especies reactivas de oxígeno

TBARS: sustancias reactivas al ácido tiobarbitúrico

TCA: ciclo de los ácidos tricarboxílicos

TFAM: factor de transcripción mitocondrial A

TNF-α: factor de necrosis tumoral α

UCP1, 2 y 3: proteínas desacoplantes 1, 2 y 3

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Tesis doctoral, Yolanda Gómez Pérez, Departament de Biologia Fonamental i Ciències de la Salut, Universitat de les Illes Balears, Palma de Mallorca, España.

Resumen

La resistencia a la insulina es un importante proceso fisiopatológico subyacente a la diabetes tipo 2 y a otras complicaciones asociadas a la obesidad que tienen su origen en alteraciones del metabolismo oxidativo. Aunque los mecanismos moleculares implicados en la alteración de la sensibilidad a la insulina no están completamente establecidos, cada vez hay más evidencias que sugieren un papel clave del estrés oxidativo, así como la posible existencia de un dimorfismo sexual.

El objetivo principal de esta tesis doctoral ha sido el estudio de la implicación del estrés oxidativo en la alteración de la sensibilidad a la insulina inducida por la obesidad dietética, evaluando la influencia del sexo y de la edad. Para conseguir este objetivo, en primer lugar, hemos analizado, en ratas de ambos sexos, los efectos de la alimentación con una dieta hiperlipídica en el grado de estrés oxidativo del músculo esquelético y del páncreas y su posible relación con el desarrollo de resistencia a la insulina. En segundo lugar, hemos estudiado el efecto de la edad en la alteración de la sensibilidad a la insulina en animales de ambos sexos y en dos tipos de músculos esqueléticos con distintas características metabólicas. Finalmente, con el objetivo de profundizar en los mecanismos moleculares implicados en el dimorfismo sexual encontrado en la alteración de la sensibilidad a la insulina asociada a la obesidad dietética, hemos evaluado la posible alteración diferencial de la función mitocondrial del músculo esquelético.

Los resultados obtenidos han demostrado un dimorfismo sexual en la alteración de la sensibilidad a la insulina asociada a la obesidad dietética y al envejecimiento, que podemos atribuir, en parte, a las diferencias entre sexos en el daño oxidativo generado en el músculo esquelético y en el páncreas. Las ratas macho muestran un perfil de resistencia a la insulina más marcado, que podría relacionarse con un mayor impacto del estrés oxidativo asociado a la edad en comparación con las hembras. Éstas, en cambio, responden a la dieta hiperlipídica aumentando la actividad COX y manteniendo elevados los niveles de UCPs en el músculo esquelético y en el páncreas, que ejercerían una importante función antioxidante y contribuirían a su perfil de sensibilidad a la insulina menos alterado. Además, en una situación de obesidad dietética, las ratas hembra evitan la acumulación ectópica de lípidos en el páncreas y aumentan el tamaño de los islotes pancreáticos para asegurar el mantenimiento de la secreción de insulina.

En conjunto, los resultados obtenidos en esta tesis doctoral demuestran que las ratas hembra presentan una mayor capacidad que los machos para paliar los efectos deletéreos del estrés oxidativo asociado a la obesidad dietética y al envejecimiento sobre la sensibilidad a la insulina, lo que podría relacionarse con el dimorfismo sexual descrito en la incidencia de algunas enfermedades crónicas.

LISTADO DE PUBLICACIONES

Esta tesis se basa en los siguientes artículos:

I. Gómez-Pérez Y, Amengual-Cladera E, Català-Niell A, Thomàs-Moyà E, Gianotti M, Proenza AM, Lladó I. *Gender dimorphism in high-fat-diet-induced insulin resistance in skeletal muscle of aged rats.* Cell Physiol Biochem 22:539-48, 2008.

II. Yolanda Gómez-Pérez; Magdalena Gianotti, PhD; Isabel Lladó, PhD; Ana M Proenza, PhD. *Gender dependent effects of high fat diet feeding on rat pancreas oxidative stress.* Pancreas. In press, accepted manuscript.

III. Yolanda Gómez-Pérez; Magdalena Gianotti, Ana M Proenza and Isabel Lladó. *Age-related decline of skeletal muscle insulin sensitivity in rats: Effect of sex and muscle type.* DOI: 10.1089/rej.2010.1107. Rejuvenation Research. In press, accepted manuscript.

IV. Yolanda Gómez-Perez, Gabriela Capllonch-Amer, Magdalena Gianotti, Isabel Lladó and Ana M. Proenza. *Long-term high-fat-diet feeding induces skeletal muscle mitocondrial biogenesis in rats in a sex-dependent and muscle-type specific manner.* Manuscript.

Además, durante la realización de la presente tesis, la doctoranda ha colaborado en la realización de otros estudios, que han dado lugar a los siguientes artículos que se presentan en el anexo 3.

V. Thomàs-Moyà E, Gómez-Pérez Y, Fiol M, Gianotti M, Lladó I, Proenza AM. *Gender related differences in paraoxonase 1 response to high-fat diet-induced oxidative stress.* Obesity (Silver Spring) 10:2232-8, 2008.

VI. Emilia Amengual-Cladera, Antònia Nadal-Casellas, Yolanda Gómez-Pérez, Isabel Gomila, Rafael M. Prieto, Ana María Proenza, Isabel Lladó. *The antioxidant effects of*

quercetin in a rat model of hyperoxaluria involve serum paraoxonase 1 activation.
Manuscript.

1. INTRODUCCIÓN

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1.1. Mitocondria y estrés oxidativo

Las especies reactivas de oxígeno (ROS), entre las que destacan el peróxido de hidrógeno, el anión superóxido o el radical hidroxilo, son moléculas de naturaleza altamente reactiva que resultan de reacciones de óxido-reducción celular (Vincent et al. 2007). Aunque en la célula tienen lugar distintas reacciones de generación de ROS, su principal fuente es la cadena respiratoria mitocondrial como resultado del metabolismo oxidativo celular (Poli et al. 2004; Lambert et al. 2009). Las ROS participan en la regulación de las funciones celulares y en una gran variedad de vías de señalización que regulan funciones fisiológicas importantes (Forman et al. 2002; Veal et al. 2007). Sin embargo, el exceso de ROS produce daño celular al alterar la estructura de lípidos, proteínas y ADN (Vincent et al. 2007), lo que repercute negativamente en el estado de óxido-reducción celular, en los procesos de señalización y en la función de los tejidos.

El organismo se protege de los efectos lesivos de las ROS mediante sistemas antioxidantes que están constituidos principalmente por sistemas enzimáticos (catalasa, superóxido dismutasa, glutatión peroxidasa y glutatión reductasa) y por moléculas antioxidantes de bajo peso molecular (ácido lipoico, glutatión y las vitaminas C y E). La superóxido dismutasa se localiza en el citoplasma celular (Cu, Zn-SOD) y en la matriz mitocondrial (Mn-SOD) y cataliza la reacción de conversión del anión superóxido a peróxido de hidrógeno. La catalasa, localizada en los peroxisomas, y la glutatión peroxidasa y la glutatión reductasa, localizadas en el citoplasma, en la mitocondria y en el núcleo, están implicadas en la conversión del peróxido de hidrógeno en agua. Cuando estos sistemas antioxidantes no son capaces de neutralizar la producción de ROS, se produce una situación de estrés oxidativo que conduce al desarrollo de patologías como consecuencia de la disfunción celular (Maritim et al. 2003; Vincent et al. 2007).

La mitocondria es el orgánulo celular responsable de generar la energía necesaria para mantener las funciones celulares. La obtención de energía en forma de ATP tiene lugar mediante los procesos de respiración y fosforilación oxidativa. El paso de los electrones a través de la cadena respiratoria crea un gradiente electroquímico de protones que es aprovechado por la ATPasa para obtener energía en forma de ATP. Durante este proceso se producen ROS, de forma que entre el 0,2 y el 2% del oxígeno

consumido por las mitocondrias genera ROS (Harper et al. 2004; Johannsen et al. 2009).

La formación de ROS se da mayoritariamente a nivel de los complejos I y III de la cadena respiratoria (ver figura 1), donde la oxidación incompleta del oxígeno da lugar al anión superóxido (Lambert et al. 2009). La producción de ROS es directamente proporcional al gradiente protónico a través de la membrana mitocondrial interna y al potencial de membrana. Cuando estos dos parámetros son elevados, se produce un enlentecimiento del flujo electrónico a través de la cadena respiratoria, lo que favorece la reducción incompleta del oxígeno y la consiguiente formación de ROS (Starkov et al. 2003).
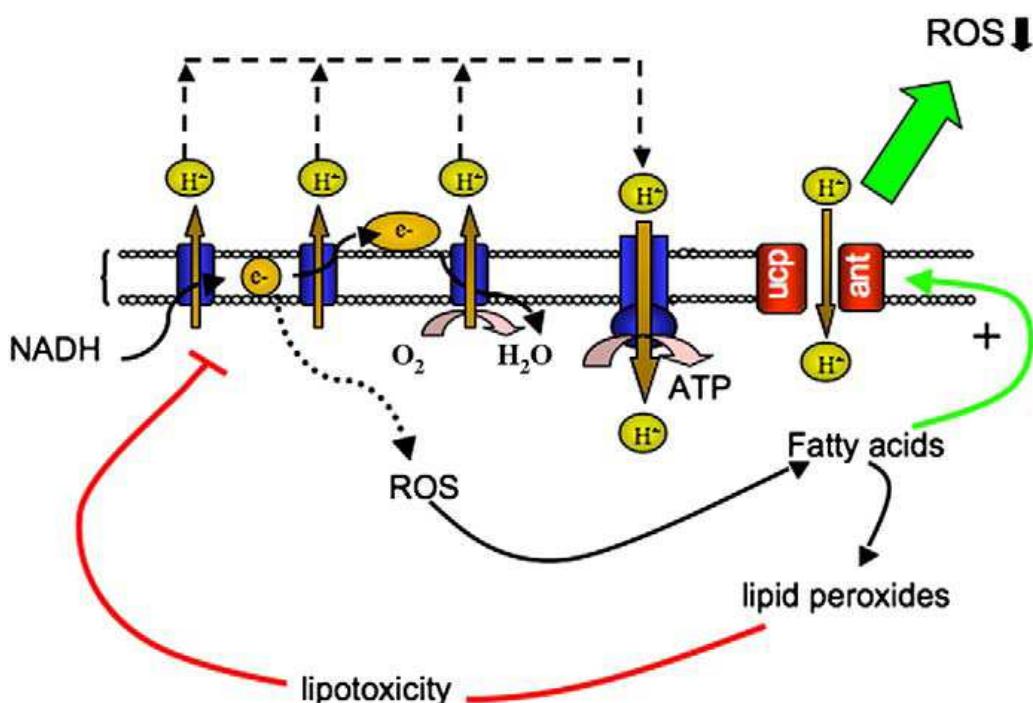
Figura 1. Representación esquemática de la producción mitocondrial de ROS y del papel de las UCPs en su atenuación (Schrauwen et al. 2010). NADH, nicotinamida adenina dinucleótido fosfato; ANT, adenina nucleótido translocasa; UCP, proteína desacoplante; ROS, especies reactivas de oxígeno.

Además, la producción de ROS depende del estado energético de las mitocondrias. En estado 4 (ausencia de ADP), los complejos de la cadena respiratoria están en estado reducido, la cadena respiratoria está inhibida y la producción del anión superóxido es elevada. Por el contrario, en estado 3 (presencia de ADP), la respiración aumenta, disminuye el estado de reducción de las moléculas encargadas del transporte de electrones y, en consecuencia, también disminuye la producción de ROS (Barja 1999).

El desacoplamiento del potencial protónico, mediante la difusión de protones a través de la membrana mitocondrial interna de forma no asociada a la síntesis de ATP, favorece la disminución del potencial de membrana y de la producción de ROS. En este proceso parecen tener un papel importante las proteínas desacoplantes (UCPs) de la cadena respiratoria mitocondrial (Echtay et al. 2003; Bezaire et al. 2007). Se ha propuesto que el exceso de ROS y de peróxidos lipídicos podría activar el desacoplamiento mitocondrial mediado por las UCPs, disminuyendo el potencial de membrana y, en consecuencia, atenuando la producción de ROS (ver figura 1).

1.1.1. La obesidad dietética como inductora de estrés oxidativo

Durante los últimos años se está produciendo un aumento de la prevalencia de enfermedades crónicas, debido principalmente a la elevada incidencia de obesidad en los países desarrollados (Misra et al. 2008). El sobrepeso y la obesidad se asocian a numerosas alteraciones metabólicas como son la disminución de la tolerancia a la glucosa, la hiperlipidemia, la hipertensión arterial y la aterosclerosis, complicaciones que se engloban bajo la denominación común de síndrome metabólico y cuyo punto de conexión parece ser la resistencia a la insulina (Duvnjak et al. 2009). Muchas de estas alteraciones parecen tener como denominador común el estrés oxidativo (Roberts et al. 2009). Así, la obesidad se ha considerado una situación fisiopatológica generadora de estrés oxidativo debido tanto al exceso crónico de nutrientes como al incremento de las reservas grasas del organismo (Morrow 2003; Rudich et al. 2007).

Las dietas muy ricas en calorías y en grasas, que son las consumidas mayoritariamente en los países desarrollados, constituyen un factor inductor de estrés oxidativo (Zhang et al. 2009). La elevada disponibilidad de sustratos oxidables (principalmente ácidos grasos) asociada al consumo de este tipo de dietas acelera el metabolismo oxidativo e incrementa la producción mitocondrial de ROS, lo que contribuye, junto a la menor expresión y/o funcionamiento de los sistemas antioxidantes, al establecimiento de una situación de estrés oxidativo (Fridlyand et al. 2006). Cuando el exceso de nutrientes sobrepasa la capacidad de almacenamiento del tejido adiposo, el organismo deriva la acumulación de lípidos hacia otros tejidos, entre ellos el músculo esquelético, el hígado y el páncreas, donde pueden convertirse en especies tóxicas comprometiendo el funcionamiento celular. Esta lipotoxicidad, junto con el estrés oxidativo, tienen como consecuencia el desarrollo de diversos procesos

patológicos asociados al síndrome metabólico, como la resistencia a la insulina (Unger et al. 2010; Virtue et al. 2010).

1.1.2. Estrés oxidativo asociado al envejecimiento

Una de las teorías más aceptadas sobre las bases moleculares del envejecimiento es la propuesta por Harman en los años 50 (Harman 1956), según la cual el envejecimiento se debe a la generación de ROS durante el metabolismo oxidativo que, junto a la disminución de los sistemas antioxidantes con el paso del tiempo, son responsables de la acumulación progresiva e irreversible de daño oxidativo en las estructuras celulares y contribuyen a la alteración de los procesos celulares (Salmon et al. 2010).

Hasta la fecha, numerosos estudios han demostrado que el estrés oxidativo y la disfunción mitocondrial aumentan con el envejecimiento en diferentes tipos de organismos (Wei et al. 2009). De hecho, la diferente esperanza de vida entre las distintas especies se ha relacionado con diferencias en la producción de ROS y en la acumulación de daño oxidativo, estableciéndose una conexión entre el estrés oxidativo y el ritmo de envejecimiento (Sohal et al. 1996; Barja 1999).

Debido a la importancia de las mitocondrias en la obtención de energía para la realización de las funciones celulares, las deficiencias o defectos en las mismas con el paso del tiempo se han asociado a diversas patologías. La disfunción mitocondrial aparece como consecuencia de la alteración de la expresión y función de los complejos de la cadena respiratoria mitocondrial y de la acumulación de mutaciones en el ADN mitocondrial y está implicada en la pérdida progresiva de la función de los tejidos y en el desarrollo de enfermedades asociadas al envejecimiento (Johannsen et al. 2009; Wei et al. 2009).

1.1.3. Biogénesis mitocondrial

Las mitocondrias muestran variaciones en el tamaño, la forma, la abundancia y la capacidad funcional en función de la demanda energética y frente a diferentes condiciones fisiológicas (Williams et al. 1986; Renis et al. 1989; Wiesner et al. 1992; Koves et al. 2005; Alcolea et al. 2007). La biogénesis mitocondrial o mitocondriogénesis engloba tanto la proliferación (aumento del número de mitocondrias por célula) como la diferenciación (aumento de la capacidad funcional de las mitocondrias preexistentes) mitocondriales (Ostronoff et al. 1996). El ADN

mitocondrial codifica para 13 polipéptidos (7 subunidades del complejo I, 3 subunidades del complejo IV, 2 subunidades de la F1-Fo ATPasa, y el citocromo b), dos ARNr ribosómicos (12S y 16S) y todos los ARN de transferencia necesarios para su traducción (Marin-Garcia 2010). El resto de las proteínas mitocondriales están codificadas por el ADN nuclear, por lo que se requiere la coordinación entre los dos genomas para asegurar el buen funcionamiento de la mitocondria (Fernandez-Silva et al. 2003; Marin-Garcia 2010).

En el control de la biogénesis mitocondrial participan numerosos factores reguladores de la transcripción y replicación mitocondriales, reguladores nucleares de la expresión de genes relacionados con la función mitocondrial y coactivadores transcripcionales. El PGC-1α (coactivador 1α del receptor activado por proliferadores peroxisomales gamma) juega un papel central en el control del proceso de biogénesis mitocondrial, regulando la expresión de diferentes factores de transcripción en respuesta a los requerimientos energéticos celulares (Scarpulla 2008). Entre ellos se encuentran los factores de respiración nucleares 1 y 2 (NRF1 y NRF2), que se unen directamente al ADN nuclear para activar la expresión de genes que codifican para los cinco complejos de la cadena respiratoria mitocondrial, de genes nucleares implicados en la transcripción del ADN mitocondrial, como el factor de transcripción mitocondrial A (TFAM) y de otras proteínas necesarias para el funcionamiento mitocondrial (Scarpulla 2008).

1.2. El músculo esquelético

El músculo esquelético es el tejido más abundante del organismo (representa el 40-50% del peso corporal) y, al ser el responsable del movimiento, desempeña un papel crucial en el metabolismo energético corporal (Matsakas et al. 2009). Es por ello que el tejido muscular presenta un elevado contenido de mitocondrias, cuya función es producir la energía necesaria para la contracción muscular (Johannsen et al. 2009).

El músculo esquelético utiliza glucosa o ácidos grasos libres como combustibles para la obtención de energía. En una situación de alimentación, el aumento de los niveles séricos de insulina estimula la captación de glucosa en el músculo esquelético y la activación de enzimas clave del metabolismo de la glucosa, favoreciéndose su oxidación. Por otro lado, en el ayuno, el músculo disminuye la captación de glucosa y utiliza los ácidos grasos libres como principal combustible metabólico (Abdul-Ghani et al. 2010). Esta habilidad que presenta el músculo esquelético para cambiar de

combustible en función del estado fisiológico se conoce como flexibilidad metabólica (Kiens 2006) y parece estar relacionada con las características metabólicas que presentan los diferentes tipos de fibras musculares. Así, las fibras musculares se clasifican en fibras de contracción lenta (tipo I), que obtienen la energía mediante el metabolismo oxidativo y son muy resistentes a la fatiga, y en fibras glucolíticas de contracción rápida (tipo IIa y IIb), que obtienen la energía de procesos anaeróbicos (Zierath et al. 2004; Janovska et al. 2008). Los músculos oxidativos están compuestos mayoritariamente de fibras tipo I, que contienen un elevado número de mitocondrias, mientras que los músculos glucolíticos contienen principalmente fibras tipo IIa y IIb y presentan un menor contenido en mitocondrias (Janovska et al. 2008).

Tal y como se ha apuntado anteriormente, el músculo esquelético es un tejido con una elevada capacidad de adaptación a cambios fisiológicos y ambientales, de forma que es capaz de responder a los estímulos externos, como el ejercicio o los cambios nutricionales, modificando su tamaño y composición a través de cambios de sus propiedades metabólicas y de expresión génica (Pette et al. 2001; Zierath et al. 2004; Matsakas et al. 2009). Así, por ejemplo, estudios realizados en deportistas han demostrado un incremento en la proporción de fibras oxidativas en respuesta a un entrenamiento de resistencia (Zierath et al. 2004). Otros estudios en roedores han demostrado que la ingesta de una dieta rica en grasas incrementa el contenido de fibras tipo I y de los complejos de la cadena respiratoria mitocondrial en el músculo cuádriceps, favoreciendo el metabolismo oxidativo (de Wilde et al. 2008).

1.2.1. Sensibilidad a la insulina en el músculo esquelético

El músculo esquelético, debido a su masa, es el principal tejido responsable de la captación de glucosa dependiente de insulina, lo que hace que el músculo sea el tejido que más contribuye a la resistencia a la insulina en la diabetes tipo 2 (Stump et al. 2006).

El GLUT4 es la isoforma de las proteínas transportadoras de glucosa que predomina en el músculo y en el tejido adiposo, y media el transporte de glucosa estimulado por insulina a favor de gradiente desde el torrente sanguíneo (Watson et al. 2001). La capacidad de la insulina para incrementar el transporte de glucosa en el músculo esquelético depende de la translocación de este transportador desde compartimentos intracelulares hasta la membrana plasmática (McCarthy et al. 2007).

Los efectos de la insulina sobre la translocación del GLUT4 están mediados por una cascada de señales intracelulares (ver figura 2). La insulina se une a su receptor (IR), que pertenece a una familia de receptores con actividad tirosina quinasa, y le provoca un cambio conformacional que conduce a la autofosforilación del receptor y a la fosforilación de los sustratos del receptor de la insulina IRS1 e IRS2. Éstos activan a la proteína quinasa 3 de fosfoinosítidos (PI3K) que genera fosfatidilinositol 3,4,5-trifosfato (PIP$_3$), responsable de la activación de la proteína quinasa dependiente de fosfoinosítidos (PDK-1) y, subsecuentemente, de la proteína quinasa C atípica (aPKC) y de la proteína quinasa B (PKB, también conocida como Akt). La fosforilación de la Akt en residuos de serina y treonina está implicada en la translocación de las vesículas intracelulares que contienen el GLUT4 hacia la membrana plasmática con el consiguiente aumento del número de transportadores (Thong et al. 2005). Una insuficiente translocación de GLUT4 a la membrana plasmática, debido a la desactivación de esta vía de señalización (McCarthy et al. 2007), supone una disminución de la captación de glucosa característica de la resistencia muscular a la insulina.
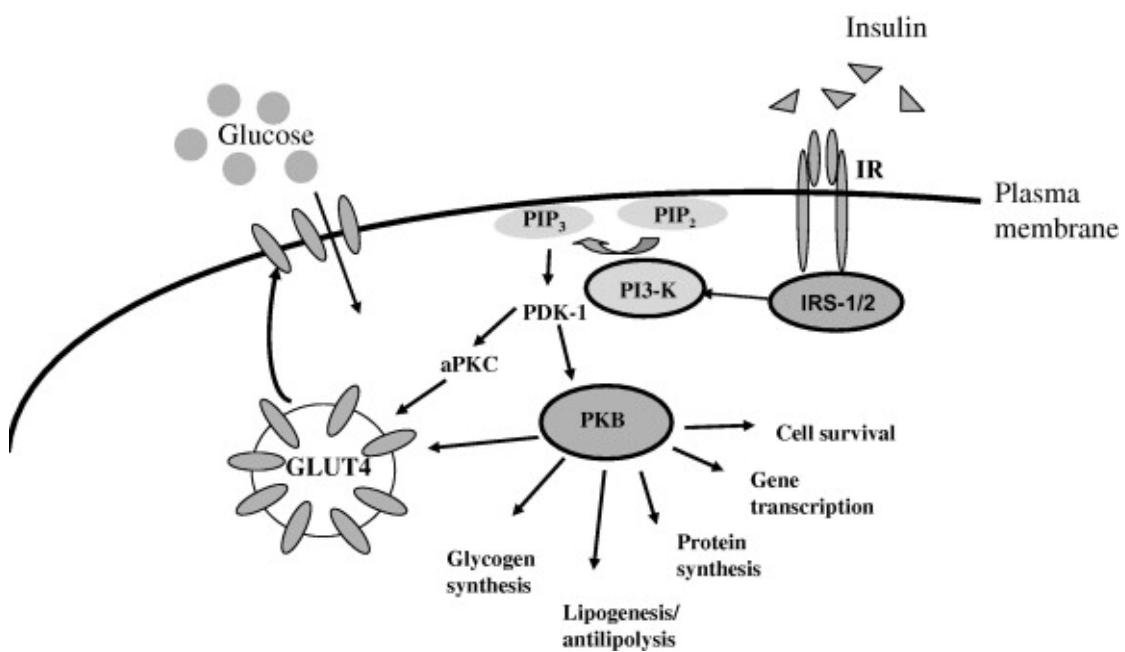
Figura 2. Esquema de la vía de activación del transporte de glucosa dependiente de insulina en el músculo esquelético (Eriksson 2007). IR, receptor de insulina; IRS-1/2, sustratos del receptor de insulina 1 y 2; PI3K, proteína quinasa 3 de fosfoinosítidos; PIP$_3$, fosfatidilinositol 3,4,5-trifosfato; PIP$_2$, fosfatidilinositol 3,4,5-difosfato; PDK-1, proteína quinasa dependiente de fosfoinosítidos; aPKC, proteína quinasa C atípica; PKB, proteína quinasa B; GLUT4, proteína transportadora de glucosa 4.

1.2.2. Resistencia a la insulina y estrés oxidativo asociado a la obesidad en el músculo esquelético

La resistencia a la insulina es un importante proceso fisiopatológico subyacente a la diabetes tipo 2 y a otras complicaciones asociadas a la obesidad y al estilo de vida sedentario, como son la dislipidemia, la hipertensión y la aterosclerosis. El origen de esta resistencia se encuentra en procesos proinflamatorios y prooxidantes (Duvnjak et al. 2009).

Aunque inicialmente el estudio de la resistencia a la insulina se centró en el metabolismo de carbohidratos, en las últimas décadas se ha producido un cambio hacia el estudio del metabolismo de ácidos grasos como principal promotor de esta complicación. De este modo, numerosos estudios han descrito que la acumulación de lípidos en el músculo, ya sea debido a un aporte excesivo en la dieta o a una alteración en su oxidación, actúa interfiriendo en la señalización de la insulina y conduciendo a una desensibilización del músculo a las acciones de la insulina (Abdul-Ghani et al. 2010).

El exceso de ácidos grasos circulantes en el estado obeso se ha asociado a una disminución de la capacidad oxidativa del músculo esquelético. En este sentido, algunos estudios han propuesto que el incremento de los niveles circulantes de ácidos grasos disminuye la expresión de genes implicados en la función mitocondrial tanto en roedores (Sparks et al. 2005) como en humanos (Tunstall et al. 2005). No obstante, otros estudios posteriores muestran efectos contrarios (Turner et al. 2007; Hancock et al. 2008; Hoeks et al. 2008), y sugieren un aumento de la capacidad de oxidar ácidos grasos como una respuesta homeostática para compensar la elevada disponibilidad de sustratos lipídicos. La disfunción mitocondrial aparece cuando el músculo pierde esta capacidad, y se ha relacionado con exposiciones prolongadas a ácidos grasos asociadas a situaciones de resistencia a la insulina (Bonnard et al. 2008). En esta situación, los metabolitos derivados de ácidos grasos intracelulares, como el diacilglicerol, los acil-CoA y las ceramidas, y también las ROS actúan como moléculas de señalización celular activando múltiples cascadas de quinasas de serinas y treoninas que inhiben la captación de glucosa dependiente de insulina en el músculo (Dyck et al. 2006; Mlinar et al. 2007; Abdul-Ghani et al. 2010).

Existen evidencias que sugieren que el vínculo que relaciona obesidad y resistencia a la insulina es el incremento de la producción de ROS. Así, algunos estudios han demostrado que el tratamiento con antioxidantes mejora el estado de resistencia a la insulina en roedores y humanos, lo que sugiere que el estrés oxidativo es un factor clave en el desarrollo de la resistencia a la insulina (Eriksson 2007; Qatanani et al. 2007). De hecho, se ha postulado que la resistencia a la insulina constituiría un mecanismo de protección frente al estrés oxidativo, ya que disminuye la captación de glucosa y ácidos grasos y el funcionamiento de la cadena respiratoria mitocondrial, conduciendo a una menor producción de ROS (Fridlyand et al. 2006; Eriksson 2007).

1.2.3. Papel de la adiponectina en la resistencia muscular a la insulina asociada a la obesidad

El exceso de adiposidad característico de la obesidad provoca la sobreproducción de factores solubles secretados por el adipocito (adipoquinas) que participan en importantes funciones metabólicas y que, cuando se altera su producción, ejercen efectos proinflamatorios y prooxidantes que comprometen la sensibilidad a la insulina (ver figura 3) (Dandona et al. 2004; Dyck et al. 2006).
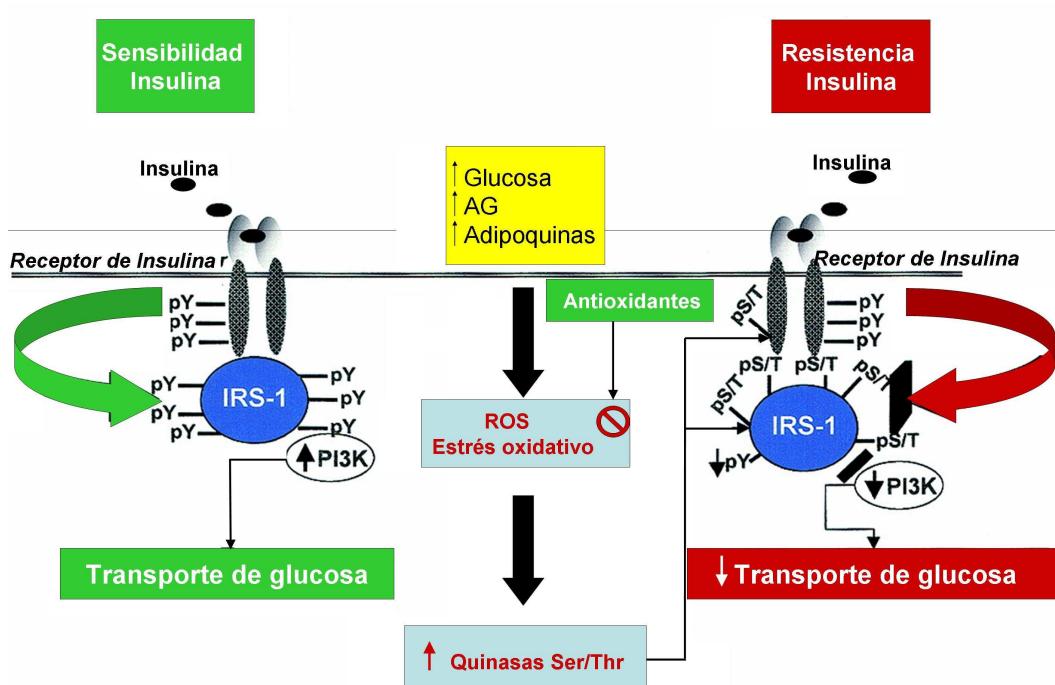
**Figura 3. Efecto de la obesidad y del estrés oxidativo sobre la captación de glucosa dependiente de insulina en el músculo (modificada de Evans 2007).** IRS-1, sustrato del receptor de insulina 1; PI3K, proteína quinasa 3 de fosfoinosítidos; pY, residuos de fosforilación en tirosinas, pS/T, residuos de fosforilación en serinas y treoninas; AG, ácidos grasos; ROS, especies reactivas de oxígeno; Ser, serina; Thr, treonina.

Algunas adipoquinas, como el el factor de necrosis tumoral α (TNF-α) y la resistina, disminuyen la acción de la insulina en el músculo esquelético inhibiendo la vía de señalización de la PI3K, mientras que otras, como la adiponectina, actúan aumentando la sensibilidad a la insulina (Dyck 2009).

La adiponectina es una adipoquina de 30kDa que se expresa mayoritariamente en el tejido adiposo, pero también en el músculo esquelético (Bonnard et al. 2008). Circula en forma de trímeros, hexámeros y multímeros de alto peso molecular (HMW), siendo esta última estructura la que presenta mayores efectos sobre la sensibilidad a la insulina, favoreciendo la oxidación de ácidos grasos en el músculo esquelético (Yamauchi et al. 2008). Estudios realizados en ratones diabéticos han demostrado que la administración exógena de adiponectina mejora el estado de resistencia a la insulina (Yamauchi et al. 2001). Además, los ratones deficientes en adiponectina desarrollan características propias del síndrome metabólico, como la aterosclerosis y la resistencia a la insulina (Kubota et al. 2002; Maeda et al. 2002).

Aunque el mecanismo de acción de la adiponectina sobre la sensibilidad a la insulina no se conoce con exactitud, sus efectos se asocian a un aumento de la biogénesis mitocondrial (ver figura 4), de la oxidación de ácidos grasos y de la captación de glucosa en el músculo esquelético y parecen estar mediados por la activación de la proteína quinasa dependiente de AMP (AMPK) (Civitarese et al. 2007; Yamauchi et al. 2008).

La adiponectina se une a los receptores AdipoR1 y AdipoR2, que se expresan de manera ubicua (Civitarese et al. 2004; Kadowaki et al. 2005). En roedores, el AdipoR1 es mayoritario en el músculo esquelético y el AdipoR2 en el hígado (Kadowaki et al. 2005), mientras que en humanos las dos formas del receptor se expresan por igual en el músculo esquelético (Civitarese et al. 2004). La unión de la adiponectina a sus receptores activa a la AMPK, que inactiva a la acetil-CoA carboxilasa (ACC) e induce la expresión del receptor activado por proliferadores peroxisomales α (PPARα), favoreciendo la oxidación de los ácidos grasos y la disminución del contenido de triglicéridos. El descenso de los niveles de ácidos grasos mejora la sensibilidad a la insulina a nivel sistémico (Yamauchi et al. 2008). Además, la AMPK activa al PGC-1α, que está implicado en el aumento de la masa y la capacidad oxidativa mitocondrial y de la expresión de la Mn-SOD, favoreciendo la disminución del estrés oxidativo (Civitarese et al. 2007).
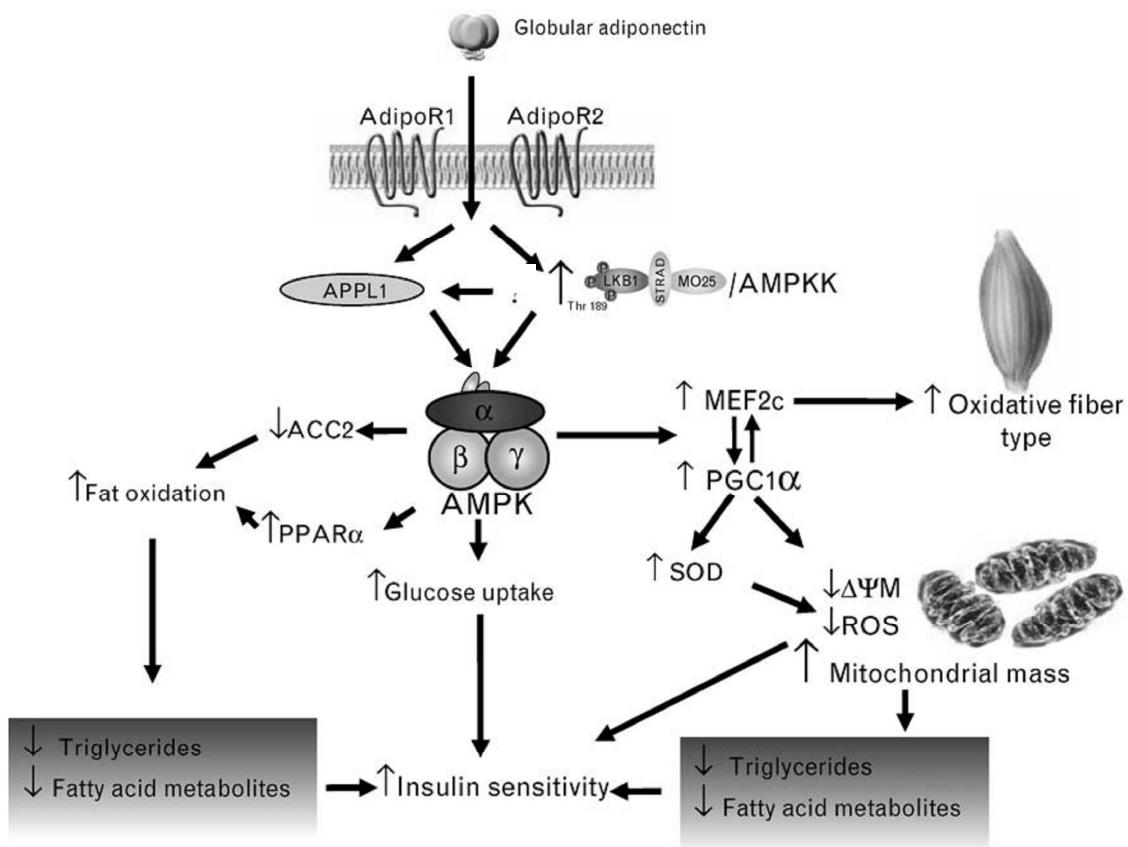
Figura 4. Esquema del papel de la adiponectina en la regulación de la sensibilidad a la insulina (modificado de Civitarese et al. 2007). APPL1, proteína adaptadora; LKB1/STRAD/MO25, complejo de proteínas quinasas; AMPKK, proteína quinasa de la AMPK; ACC2, acetil-CoA carboxilasa tipo 2; MEF2c, factor potenciador específico de miocito 2c; $\Delta\Psi$M, potencial de membrana.

En situaciones de obesidad y de resistencia a la insulina disminuye la capacidad del músculo esquelético para responder a los efectos de la adiponectina, lo que contribuye a la disminución de la sensibilidad a la insulina (Dyck 2009). Sin embargo, existe cierta controversia sobre el efecto de la obesidad sobre los niveles de adiponectina circulante, ya que aunque inicialmente la obesidad se asoció a una disminución de su niveles (Yamauchi et al. 2001), algunos estudios han demostrado que la obesidad y la resistencia a la insulina no siempre se acompaña de cambios en los niveles de adiponectina circulante y han atribuido la incapacidad del músculo esquelético para responder a sus efectos a un estado de resistencia a la adiponectina (Barnea et al. 2006; Rodriguez et al. 2008).

1.2.4. UCP3 y regulación de la producción mitocondrial de ROS en el músculo esquelético

La UCP3 pertenece a la familia de proteínas desacoplantes de la cadena respiratoria mitocondrial (ver figura 5) y se expresa principalmente en el músculo esquelético, a diferencia de su proteína homóloga UCP1 que se expresa casi exclusivamente en el tejido adiposo marrón en el que es responsable de la termogénesis adaptativa. Aunque la elevada homología entre la UCP3 y la UCP1 apuntaba en un principio hacia un posible papel de la primera en la termogénesis adaptativa, la falta de evidencias claras en este sentido ha sugerido que la principal función de la UCP3 no sería la disipación de energía (Bezaire et al. 2007).
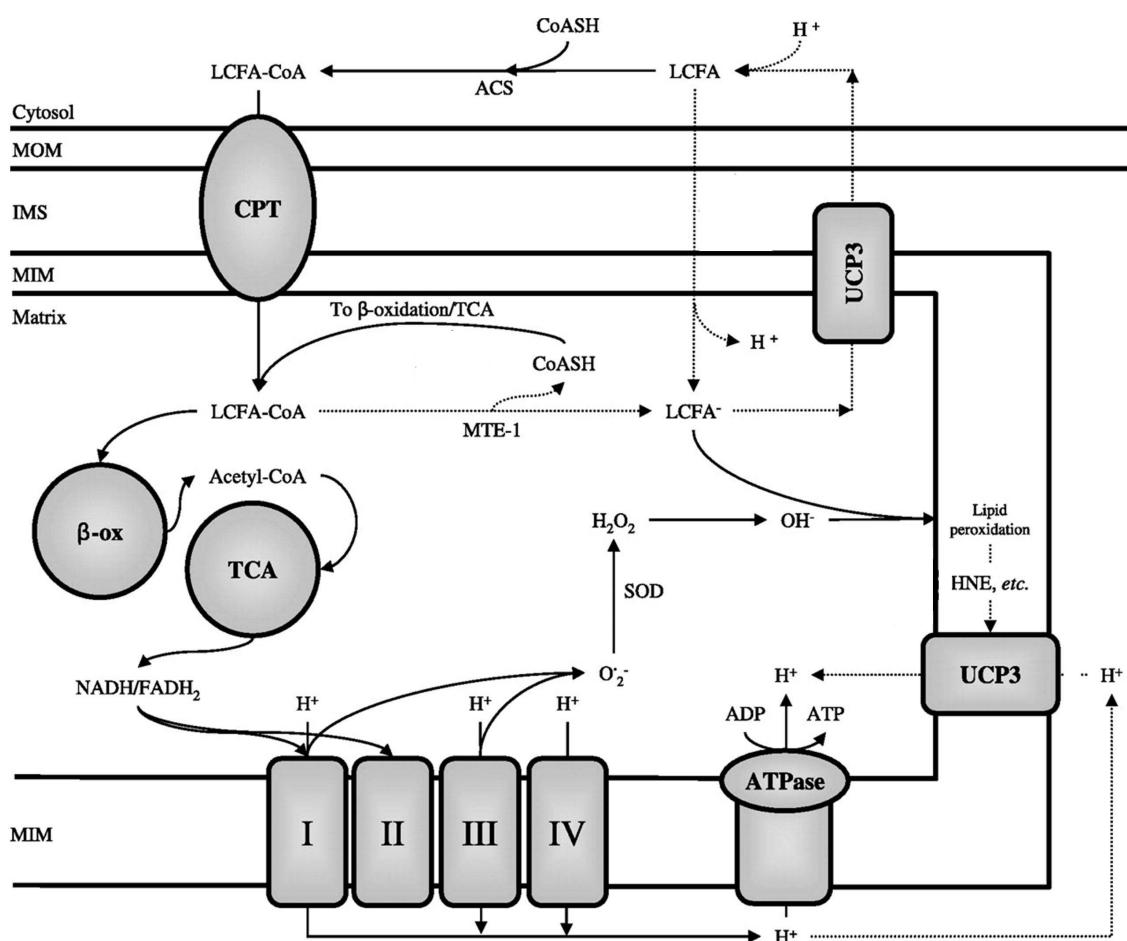
Figura 5. Modelo sobre el papel de la UCP3 en la regulación de la producción mitocondrial de ROS (modificado de Bezaire et al. 2007). MOM, membrana mitocondrial externa; IMS, espacio intermembrana; MIM, membrana mitocondrial interna; CPT, carnitina palmitoiltransferasa; LCFA, ácido graso de cadena larga, $LCFA^-$, anión ácido graso de cadena larga; ACS, acil-coenzima A sintasa; β-ox, beta-oxidación; TCA, ciclo de los ácidos tricarboxílicos; MTE-1, tioesterasa mitocondrial 1; HNE, hidroxinonenal; H_2O_2, peróxido de hidrógeno; SOD, superóxido dismutasa; O_2^-, anión superóxido; OH^-, anión hidróxido; NADH, nicotinamida adenina dinucleótido fosfato; $FADH_2$, dinucleótido de adenilflavina reducido; I-IV, complejos proteicos de la cadena de transporte de electrones.

El incremento de los niveles de UCP3 observado en situaciones de elevada disponibilidad de ácidos grasos, como el ayuno o la obesidad dietética (Millet et al. 1997; Samec et al. 1998; Hesselink et al. 2003), ha sugerido un posible papel de la UCP3 en la regulación del metabolismo de los ácidos grasos (Schrauwen et al. 2004). Se ha propuesto que, en situaciones en las que el suministro de ácidos grasos supera la capacidad oxidativa mitocondrial, la UCP3 podría actuar como transportador, desde la matriz mitocondrial al citosol, de ácidos grasos que no pueden ser oxidados (ver figura 5). Esto favorecería el mantenimiento de la capacidad oxidativa mitocondrial y evitaría la acumulación de peróxidos lipídicos potencialmente tóxicos (Schrauwen et al. 2004; Bezaire et al. 2007).

Por otra parte, el exceso de ROS y de peróxidos lipídicos podría activar el desacoplamiento mitocondrial mediado por la UCP3, disminuyendo el potencial de membrana y, en consecuencia, atenuando la producción de ROS. Así, la UCP3 podría tener una función antioxidante en el músculo esquelético, de manera que el incremento de su expresión constituiría un mecanismo protector frente al estrés oxidativo (Echtay et al. 2003; Bezaire et al. 2007). Algunos estudios han propuesto que la sobreexpresión de UCP3 en el estado obeso podría mejorar la sensibilidad a la insulina en el músculo esquelético, mediante la activación de la translocación de GLUT4 dependiente de insulina a la superficie de la membrana (Bezaire et al. 2007). No obstante, todavía no se conoce con exactitud el mecanismo molecular que relacionaría ambos procesos.

1.2.5. Estrés oxidativo en el músculo esquelético asociado al envejecimiento

El envejecimiento se caracteriza por una disminución de la masa, fuerza y calidad muscular, conocida como sarcopenia, que resulta de la reducción del tamaño de las fibras musculares y, eventualmente, de su pérdida. Se ha descrito que la sarcopenia afecta en mayor medida a las fibras de tipo II que a las de tipo I, por lo que los músculos glucolíticos son más susceptibles a los efectos de la edad que los músculos oxidativos (Lexell 1995).

Existen evidencias de la disminución de la función mitocondrial del músculo esquelético con el envejecimiento, si bien todavía no está claro si este efecto se debe al envejecimiento *per se* o a cambios en el estilo de vida asociados a éste (Lanza et al. 2009). Diversos estudios han demostrado que, con la edad, en el músculo esquelético se produce una disminución de la capacidad oxidativa mitocondrial (Drew et al. 2003;

Short et al. 2005), de los niveles de proteínas mitocondriales (Short et al. 2005) y del contenido de ADN mitocondrial (Barazzoni et al. 2000; Short et al. 2005; Menshikova et al. 2006) y un incremento del daño oxidativo en el ADN mitocondrial (Melov et al. 1995). Además, estudios realizados en ratones deficientes para el enzima antioxidante Cu, Zn-SOD muestran una pérdida acelerada de masa muscular con el envejecimiento (Muller et al. 2004), sugiriendo que la pérdida de la capacidad antioxidante con la edad podría estar implicada en la pérdida de la masa y función musculares.

Uno de los efectos más importantes del envejecimiento en el músculo esquelético es la disminución de la sensibilidad a la insulina, de forma que la edad aumenta la incidencia de diabetes tipo 2. A nivel molecular, la disminución de la sensibilidad a la insulina se asocia a la alteración de la vía de señalización de la insulina en el músculo esquelético. Así, diversos estudios han demostrado una disminución de la actividad y/o niveles de proteínas clave de la vía de señalización de la insulina con el envejecimiento (Carvalho et al. 2000; Arias et al. 2001; Gupte et al. 2008; Serrano et al. 2009), aunque no se conocen con exactitud los mecanismos moleculares responsables de la resistencia a la insulina asociada al envejecimiento, en la que podría estar implicado el incremento del estrés oxidativo asociado a esta situación fisiológica (Gupte et al. 2008; Chanseaume et al. 2009; Jackson 2009). El envejecimiento se asocia también a un incremento de los depósitos grasos en el organismo que se acompaña de cambios en los niveles de adipoquinas circulantes y de un aumento de la acumulación de lípidos en el músculo esquelético que contribuyen al desarrollo de resistencia a la insulina (ver apartado 1.2.2).

1.3. El páncreas

El páncreas es un órgano con una doble función, exocrina y endocrina. El páncreas exocrino está constituido por células acinares que se encargan de sintetizar, almacenar y secretar diversas enzimas digestivas. El páncreas endocrino está formado por unas pequeñas asociaciones de células endocrinas especializadas que están organizadas en islotes pancreáticos o islotes de *Langerhans*. Estas agrupaciones fueron descubiertas en 1869 por Paul Langerhans (Weir et al. 1990).

El tamaño de los islotes puede variar considerablemente según en la región pancreática en la que se encuentren (Elayat et al. 1995). A pesar de estas variaciones, la estructura interna de los islotes de Langerhans en mamíferos es característica. Se han

identificado 4 tipos celulares distintos (Weir et al. 1990; Atkinson et al. 1993): células β, especializadas en la síntesis y secreción de insulina; células α, que secretan glucagón; células δ, que secretan somatostatina y células PP, secretoras del polipéptido pancreático. Las células β ocupan la parte central del islote y son las mayoritarias, representando aproximadamente el 60% del mismo. Las células α (10-20%) se localizan en la periferia, las células δ (5-10%) se distribuyen de forma variable en el islote y las células PP (1-2%) normalmente se localizan en la periferia del islote, pero a veces también se encuentran en el parénquima exocrino (Atkinson et al. 1993).

Los islotes de Langerhans ejercen una función importante en el control de la homeostasis de la glucosa del organismo. Es por ello que están muy vascularizados e irrigados, de forma que pueden responder a los estímulos metabólicos que continuamente se producen en el organismo (Nyman et al. 2008).

1.3.1. Regulación de la secreción de insulina por glucosa y ácidos grasos

La glucosa es la principal fuente de energía utilizada por la célula. En los mamíferos, la regulación de la concentración de glucosa en sangre es esencial para el organismo, ya que asegura su disponibilidad a los tejidos dependientes de glucosa. Esta regulación se realiza principalmente mediante la secreción coordinada de insulina y glucagón por parte del páncreas, que controlan el equilibrio entre el flujo de glucosa dentro y fuera del espacio extracelular (Unger 1991).

Tras la ingesta de alimentos, el incremento de los niveles de glucosa en el suero induce la secreción de insulina por parte de las células β del páncreas. El aumento de la glucemia y de la insulinemia estimula la captación de glucosa por los tejidos periféricos (músculo y tejido adiposo) e inhibe la producción hepática de glucosa. Durante el ayuno, el mantenimiento de la glucemia se consigue gracias a la acción del glucagón y de otros factores hormonales y nerviosos que favorecen la producción de glucosa en el hígado, en el que aumenta la gluconeogénesis y la glucogenolisis. Alteraciones funcionales a nivel de la célula β pueden conducir a trastornos en la homeostasis de la glucosa, al desarrollo de intolerancia a la glucosa, o incluso, a la aparición de la diabetes tipo 2 (DeFronzo 2004).

Las células β utilizan el catabolismo de la glucosa para obtener la energía necesaria para el metabolismo celular, a través del cual generan la señal que induce la secreción

de insulina. La integración de estas dos funciones requiere un suministro energético importante (Lenzen 2008). La oxidación de la glucosa en la célula β provoca la despolarización de la membrana celular, que desencadena una serie de acontecimientos que finalizan con la secreción de insulina. Así, el incremento de la relación ATP/ADP induce el cierre de los canales de K^+ sensibles a ATP que conduce a la despolarización de la membrana celular. Esto provoca la apertura de los canales de Ca^{2+} dependientes de voltaje, favoreciendo el incremento de los niveles de Ca^{2+} intracelulares que desencadena la exocitosis de las vesículas de insulina (Ashcroft et al. 1994).

Los lípidos desempeñan también un papel importante en la regulación de la secreción de insulina dependiente de glucosa. Un suministro adecuado de ácidos grasos libres procedentes de la circulación y de los depósitos de triglicéridos es esencial para el funcionamiento normal de las células β (Pappan et al. 2005; Nolan et al. 2006; Winzell et al. 2006), de forma que algunos intermediarios del metabolismo lipídico, como los acil-CoA de cadena larga y el diacilglicerol, actúan como moléculas señalizadoras favoreciendo la secreción de insulina. Sin embargo, la exposición prolongada a ácidos grasos incrementa la acumulación de lípidos en el páncreas y disminuye su capacidad para secretar insulina, condición conocida como lipotoxicidad, que incluye la alteración del metabolismo de la glucosa y la apoptosis de las células β y que puede conducir al desarrollo de diabetes tipo 2 (Pinnick et al. 2008). Aunque no se conocen con exactitud los mecanismos a través de los cuales se produce esta lipotoxicidad, evidencias experimentales apuntan que sus efectos tóxicos están mediados por metabolitos lipídicos derivados de la esterificación de los ácidos grasos (Robertson et al. 2004; Nolan et al. 2006).

El contenido de triglicéridos en las células β depende de la acción de las lipasas intracelulares que se expresan en los islotes pancreáticos. La lipoproteína lipasa (LPL) regula la entrada de los triglicéridos circulantes al páncreas (Pappan et al. 2005), mientras que la lipasa sensible a hormonas (HSL) está implicada en la hidrólisis de los triglicéridos acumulados en el interior celular (Winzell et al. 2006; Fex et al. 2008). Además, se ha detectado la expresión de otras lipasas en el páncreas, como la lipasa de triglicéridos del adipocito (Fex et al. 2008).

Diversos estudios han propuesto que la acción de estas lipasas podría estar implicada en la regulación de la secreción de insulina dependiente de glucosa a través de la formación de intermediarios del metabolismo lipídico. Así, la inhibición

farmacológica de la lipólisis en islotes de rata disminuye la secreción de insulina dependiente de glucosa, lo que sugiere que la movilización de lípidos almacenados en el interior celular tiene un papel fundamental en la secreción de insulina (Mulder et al. 2004). Por otro lado, la sobreexpresión de la LPL y de la HSL en islotes pancreáticos ha mostrado efectos negativos en la función de las células β, que podrían ser debidos al exceso de metabolitos lipotóxicos generados como consecuencia de una elevada actividad lipolítica (Winzell et al. 2003; Pappan et al. 2005).

1.3.2. Páncreas y estrés oxidativo

En una situación de exceso de nutrientes y de resistencia a la insulina, los islotes pancreáticos responden incrementando la secreción de insulina para mantener la glucemia. Esta respuesta se acompaña de un aumento de la masa de los islotes pancreáticos para mantener la capacidad de sintetizar y secretar insulina y del metabolismo oxidativo, que favorece la formación de ATP y la activación de la secreción de insulina inducida por glucosa (Prentki et al. 2006). Sin embargo, el aumento del flujo metabólico a través de la cadena respiratoria mitocondrial incrementa la producción de ROS, comprometiendo el funcionamiento del páncreas. El páncreas es un tejido muy sensible a los efectos del daño oxidativo, ya que las células β presentan una escasa defensa antioxidante, debido a la expresión particularmente baja de los principales enzimas antioxidantes en comparación con otros tejidos (Lenzen et al. 1996; Tiedge et al. 1997). En este sentido, la proteína desacoplante UCP2 que se expresa en el páncreas parece estar implicada, al igual que ocurre con su homóloga UCP3 en el músculo esquelético, en la disminución de la producción mitocondrial de ROS (Brand et al. 2005), de forma que podría tener un papel protector frente al daño oxidativo. De hecho, recientemente se ha propuesto que la ausencia crónica de UCP2 en islotes pancreáticos induce una situación de estrés oxidativo (Pi et al. 2009). Sin embargo, y de forma contradictoria, hay evidencias que apuntan hacia un posible papel de la UCP2 en la disminución de la sensibilidad a la insulina dependiente de glucosa, contribuyendo a la disfunción de la célula β y a la patogénesis de la diabetes tipo 2 (Affourtit et al. 2008).

Se ha propuesto que la UCP2 podría tener un papel importante en la respuesta de las células β a los cambios nutricionales. En una situación de ayuno, la oxidación de los ácidos grasos favorece la producción de anión superóxido que induce la expresión y

actividad de la UCP2. El desacoplamiento del gradiente protónico disminuye la relación ATP/ADP y, en consecuencia, la secreción de insulina, evitando la hipoglucemia (Brand et al. 2004; Chan et al. 2006). Por otro lado, tras la ingesta de alimento, el incremento de los niveles circulantes de glucosa y lípidos requiere la activación de la secreción de insulina. En estas condiciones, la activación de la UCP2 favorece el buen funcionamiento del ciclo de los ácidos tricarboxílicos y la formación de intermediarios lipídicos que actúan como moduladores de la secreción de insulina. Sin embargo, en una situación de hiperglucemia e hiperinsulinemia provocada por una exposición crónica a niveles elevados de nutrientes, la activación persistente de la UCP2 asociada al exceso de ROS disminuye la secreción de insulina dependiente de glucosa (Newsholme et al. 2007; Affourtit et al. 2008).

1.4. Diferencias de sexo en el estrés oxidativo y la función mitocondrial

Se ha descrito un dimorfismo sexual en el metabolismo energético y en la función mitocondrial en diferentes tejidos de rata, como el hígado, músculo, corazón, tejido adiposo marrón y cerebro (Rodriguez-Cuenca et al. 2002; Justo et al. 2005a; Justo et al. 2005b; Colom et al. 2007a; Colom et al. 2007b; Valle et al. 2007a; Valle et al. 2007b; Catala-Niell et al. 2008; Guevara et al. 2009). Las ratas hembra presentan una mayor capacidad oxidativa que los machos que se asocia a una mayor biogénesis mitocondrial y a diferencias morfológicas consistentes en mitocondrias más grandes y mayor densidad y tamaño de sus crestas.

Además, las hembras muestran una mayor protección frente a la producción de ROS, que se atribuye a una mayor actividad de los enzimas antioxidantes, en la que las hormonas sexuales parecen tener una función importante, ya que los estrógenos están implicados en la regulación de la expresión de los principales enzimas antioxidantes (Borras et al. 2005).

En el músculo esquelético, las diferencias de sexo en la capacidad oxidativa mitocondrial y en el estado de estrés oxidativo se dan tanto en una situación de alimentación con dieta estándar (Colom et al. 2007a) como en respuesta a la alimentación con una dieta rica en grasa (Catala-Niell et al. 2008). Además existen evidencias de que el músculo esquelético de las hembras presenta una mayor capacidad de adaptación al ejercicio que los machos (Tiidus 2000) y una mayor protección frente a los efectos perjudiciales de los lípidos sobre la acción de la insulina (Hevener et al.

2002) y frente al perfil de disminución de la masa muscular con el envejecimiento (Paturi et al. 2010), así como una menor disminución de la capacidad antioxidante con la edad (Ko et al. 2010).

La mayor protección que las hembras de diferentes especies, incluidos los humanos, presentan frente al estrés oxidativo se ha relacionado con una menor incidencia de desórdenes metabólicos asociados a la obesidad como la hipertensión y la enfermedad cardiovascular (Dantas et al. 2004) y con una mayor esperanza de vida (Viña et al. 2006) y se refleja en un perfil de sensibilidad a la insulina más favorable en comparación con los machos (Geer et al. 2009).

2. OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

2. OBJETIVOS Y PLANTEAMIENTO EXPERIMENTAL

El trabajo desarrollado en la presente tesis doctoral forma parte de una línea de investigación más amplia que pretende identificar los mecanismos moleculares implicados en las diferencias encontradas entre sexos en la incidencia de determinadas patologías crónicas frecuentes en las sociedades desarrolladas, y que tienen su origen en alteraciones del metabolismo oxidativo causadas por la ingesta excesiva de nutrientes, como la resistencia a la insulina. El objetivo principal de esta tesis doctoral ha sido el estudio de la implicación del estrés oxidativo en la alteración de la sensibilidad a la insulina inducida por la obesidad dietética, evaluando la influencia del sexo y de la edad.

Los objetivos específicos se describen a continuación:

1. Determinar si existen diferencias entre sexos en el grado de estrés oxidativo generado por la obesidad dietética en el músculo esquelético y en el páncreas de rata, y estudiar su posible relación con el desarrollo de resistencia a la insulina.

2. Estudiar el efecto de la edad en la alteración de la sensibilidad a la insulina en animales de ambos sexos y en dos tipos de músculos esqueléticos con distintas características metabólicas.

3. Dilucidar si las diferencias de sexo encontradas en la alteración de la sensibilidad a la insulina asociada a la obesidad dietética se deben a una alteración diferencial de la función mitocondrial del músculo esquelético.

Estudios previos realizados por nuestro grupo de investigación habían puesto de manifiesto la existencia de un claro dimorfismo sexual en la función mitocondrial y en el grado de estrés oxidativo en distintos tejidos de rata, tanto en condiciones de alimentación estándar y bajo contenido graso (Rodriguez-Cuenca et al. 2002; Justo et al. 2005a; Justo et al. 2005b; Colom et al. 2007a; Colom et al. 2007b), como en respuesta a la restricción calórica (Colom et al. 2007b; Valle et al. 2007a; Valle et al. 2007b) y a la alimentación con una dieta rica en grasas (Catala-Niell et al. 2008). Considerando este dimorfismo sexual y la relación existente entre la disfunción mitocondrial y la alteración de la sensibilidad a la insulina, nos planteamos, en primer lugar, relacionar las posibles diferencias entre sexos en el grado de estrés oxidativo en el músculo esquelético con diferencias en el desarrollo de resistencia a la insulina inducida por la alimentación con una dieta hiperlipídica e hipercalórica. Para ello, se utilizaron ratas Wistar de ambos

sexos y 15 meses de edad que fueron alimentadas *ad libitum* con una dieta estándar (3% de grasa) o con una dieta hiperlipídica (32% de grasa) durante 14 semanas. Como modelo de dieta rica en grasa se utilizó la denominada *dieta de cafetería*, de características similares a las de las dietas de consumo humano, que induce hiperfagia voluntaria y un considerable incremento del peso corporal en roedores (Sclafani et al. 1976). Durante la intervención dietética se realizó un seguimiento del peso corporal y de la ingesta. Con el objetivo de evaluar el perfil de sensibilidad sistémica a la insulina, una semana antes del sacrificio de los animales se realizó un test de tolerancia a la glucosa. Los niveles séricos de glucosa, insulina, adiponectina, resistina y leptina se determinaron como indicadores de resistencia a la insulina y de inflamación asociada a la obesidad. Se evaluó el estado de estrés oxidativo en el músculo esquelético gastrocnemius mediante la medida de la producción mitocondrial de ROS, los niveles de marcadores de daño oxidativo (peróxidos lipídicos y grupos carbonilos en proteínas), la actividad de los principales enzimas antioxidantes y los niveles de la proteína desacoplante UCP3. Para evaluar la capacidad oxidativa del tejido se midieron, en las mitocondrias aisladas, el consumo de oxígeno y la actividad citocromo c oxidasa. Se determinaron también los niveles tisulares de GLUT4 como indicadores de la sensibilidad a la insulina del músculo esquelético y los de triglicéridos. Los resultados obtenidos y las conclusiones más relevantes se detallan en el ***Manuscrito I.***

Los resultados obtenidos en este primer estudio indicaban la existencia de un dimorfismo sexual en la disminución de la sensibilidad a la insulina asociada a la ingesta de una dieta hiperlipídica. Las ratas hembra, a pesar de haber alcanzado un mayor exceso de peso corporal, mostraban un perfil de sensibilidad a la insulina menos alterado que las ratas macho, reflejado, entre otros factores, por los menores niveles de insulina circulante y del índice de resistencia a la insulina HOMA-IR y por el perfil de las curvas de tolerancia a la glucosa. Dado que el páncreas es el órgano responsable de la síntesis y secreción de insulina, y considerando las diferencias entre sexos en el grado de estrés oxidativo encontradas en otros tejidos, nos preguntamos si existiría también un dimorfismo sexual en el estado redox del páncreas por efecto del tratamiento dietético. Así, nos propusimos comparar el estado de estrés oxidativo asociado a la dieta del páncreas de ratas macho y hembra como determinante de una posible disfunción de este órgano. Para evaluar la integridad del páncreas, en la medida en que se asocia a su función, se realizó un análisis morfométrico de los islotes pancreáticos en cortes de

tejido incubados con anticuerpo contra la insulina por técnicas de inmunohistoquímica. Se determinaron los niveles de los principales marcadores de daño oxidativo, la actividad de los principales enzimas antioxidantes y los niveles de UCP2. Los niveles de LPL se determinaron como indicadores de la entrada al páncreas de lípidos derivados de la dieta, mientras que los niveles de perilipina y de HSL se determinaron como marcadores de la capacidad lipolítica del tejido. Los resultados obtenidos se recogen y discuten en el *Manuscrito II.*

Los resultados recopilados en el Manuscrito I, que habían sido obtenidos en ratas de 18 meses de edad, habían puesto de manifiesto un perfil de sensibilidad a la insulina más alterado en las ratas macho que en las hembras que, dada la asociación existente entre envejecimiento y disminución de la capacidad de respuesta a la insulina, podría atribuirse a un efecto más deletéreo de la edad en los machos en comparación con las hembras. Por este motivo nos preguntamos si las diferencias entre sexos observadas en el perfil de sensibilidad a la insulina en ratas de esta edad, que podríamos considerar avanzada, podían ser atribuidas a diferencias en la forma en que evoluciona con la edad la alteración de la vía de señalización de la insulina en el músculo esquelético. Nos interesaba estudiar, además, si la alteración variaba en función de las características metabólicas del músculo. Para ello se utilizaron el músculo gastrocnemius, típicamente glucolítico y que genera la mayor parte del ATP a través de la glucólisis, y el músculo soleus, más oxidativo, que obtiene la energía principalmente a través de la oxidación de los lípidos y glúcidos y que es más sensible a los efectos de la insulina. Se utilizaron ratas Wistar macho y hembra de 3, 9 y 18 meses de edad con el fin de dilucidar el momento en el que se empezarían a poner de manifiesto las diferencias entre machos y hembras en la alteración de la vía de señalización de la insulina que se observa en edades más avanzadas. Se realizó un test de tolerancia a la glucosa y se determinaron los niveles séricos de glucosa, insulina y adiponectina. Se determinaron los principales marcadores de daño oxidativo y los niveles de UCP3 en los músculos soleus y gastrocnemius. Para evaluar las posibles alteraciones en la vía de señalización de la insulina se determinaron los niveles de las proteínas clave de la ruta: IRβ, IRS-1, Akt, en sus formas total y fosforilada, y GLUT4. Los resultados obtenidos y las principales conclusiones se recogen en el *Manuscrito III.*

La alteración de la sensibilidad a la insulina asociada a la obesidad se ha relacionado con una disminución de la función mitocondrial del músculo esquelético (Bonnard et al.

2008). La adiponectina es una hormona secretada por los adipocitos con efectos sensibilizantes a la acción de la insulina, que favorece la biogénesis y la función mitocondriales y la actividad de los sistemas antioxidantes del músculo esquelético (Civitarese et al. 2007). Las diferencias de sexo en el perfil de sensibilidad a la insulina, en la capacidad oxidativa y en el estado de estrés oxidativo del músculo esquelético, descritas en el Manuscrito I, nos hicieron plantearnos el investigar si este dimorfismo sexual se constataba también a nivel de la vía de señalización de la adiponectina y de los principales marcadores de biogénesis mitocondrial del músculo esquelético. Se utilizaron nuevamente el músculo gastrocnemius, típicamente glucolítico, y el músculo soleus, más oxidativo, dada la relación existente entre las características metabólicas y la funcionalidad mitocondrial. El modelo experimental utilizado para la obtención de los resultados que aparecen recogidos en los manuscritos I y II (animales de 15 meses de edad) no había conseguido inducir una obesidad manifiesta en las ratas macho (únicamente 16% de ganancia de peso tras 14 semanas de tratamiento). Con el objetivo de conseguir un mayor grado de obesidad en las ratas macho, se utilizaron animales más jóvenes (de 8 semanas de edad) y se prolongó el tratamiento con la misma dieta que en el primer modelo experimental, hasta las 26 semanas, momento en que se consiguió en los machos un 20% de exceso de peso corporal. Se evaluó el perfil de sensibilidad a la insulina mediante la determinación de los parámetros sistémicos habituales y de los niveles de las formas total y de alto peso molecular de la adiponectina. Como indicadores de la capacidad oxidativa mitocondrial, se determinaron los niveles de las subunidades II (codificada por el ADN mitocondrial) y IV (codificada por el ADN nuclear) de la COX en los músculos soleus y gastrocnemius. Los niveles de las proteínas PGC-1α y TFAM se cuantificaron como marcadores de biogénesis mitocondrial. El estado de estrés oxidativo del músculo esquelético se evaluó mediante la determinación de los niveles de los enzimas antioxidantes Mn-SOD (mitocondrial) y Cu-SOD (citosólica), los marcadores habituales de daño oxidativo en lípidos y proteínas y los niveles de UCP3. Para evaluar las posibles alteraciones en la vía de señalización de la adiponectina se determinaron los niveles del receptor de adiponectina, AdipoR1, mayoritario en el músculo esquelético de roedores, y de las formas fosforilada y no fosforilada de la proteína AMPK en los dos tipos de músculo. Los resultados obtenidos se recogen y discuten en el *Manuscrito IV.*

3. RESULTADOS Y DISCUSIÓN

Manuscrito I.

Gender dimorphism in high-fat-diet-induced insulin resistance in skeletal muscle of aged rats.

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Gender Dimorphism in High-Fat-Diet-Induced Insulin Resistance in Skeletal Muscle of Aged Rats

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Abstract
Muscle resistance to insulin plays a key role in the metabolic dysregulation associated to obesity. A pro-inflammatory and pro-oxidant status has been proposed to be the link between dietary obesity and insulin resistance. Given the gender differences previously found in mitochondrial function and oxidative stress, the aim of the present study was to investigate whether this gender dimorphism leads to differences in the development of high-fat-diet-induced insulin resistance in rat skeletal muscle. Male and female rats of 15 months of age were fed with a high-fat-diet (32% fat) for 14 weeks. Control male rats showed a more marked insulin resistance status compared to females, as indicated by the glucose tolerance curve profile and the serum insulin, resistin and adiponectin levels. High-fat-diet feeding induced an excess of body weight of 16.2% in males and 38.4% in females, an increase in both muscle mitochondrial hydrogen peroxide production and in oxidative damage, together with a decrease in the Mn-superoxide dismutase activity in both genders. However, high-fat-diet fed female rats showed a less marked insulin resistance profile than males, higher mitochondrial oxygen consumption and cytochrome c oxidase activity, and a better capacity to counteract the oxidative-stress-dependent insulin resistance through an overexpression of both muscle UCP3 and GLUT4 proteins. These results point to a gender dimorphism in the insulin resistance status and in the response of skeletal muscle to high-fat-diet feeding which could be related to a more detrimental effect of age in male rats.

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Introduction

Skeletal muscle constitutes the main insulin-sensitive tissue in the body and is the primary site for insulin-stimulated glucose utilization. Muscle resistance to insulin plays a key role in the metabolic dysregulation associated to obesity contributing to the development of the metabolic syndrome [1].

A pro-inflammatory and pro-oxidant status has been proposed to be the link between obesity associated to

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excessive nutrient and energy intake and insulin resistance [2, 3]. High-fat-diet intake increases muscle fatty acid uptake and oxidation leading to increased mitochondrial reactive oxygen species (ROS) production in this tissue [4]. Moreover, an impairment of antioxidant defences has also been reported in diet-induced obesity [5]. The excessive ROS production, not counteracted by the antioxidant defences, leads to an oxidative stress status and to mitochondrial damage. Furthermore, ROS-induced mitochondrial dysfunction can lead to disruptions of the lipid metabolism, by increasing the intracellular lipid content and contributing to lipid-dependent insulin resistance in muscle cells. Insulin resistance has been considered a compensatory mechanism to protect the cells from further insulin-stimulated glucose and fatty acid uptake and therefore from oxidative damage [4, 6].

Several studies in rats have reported gender differences in the development of obesity-associated to high fat diet feeding, in which males showed a greater resistance to increase body weight than females [7, 8]. However, females seem to be more protected from obesity-associated disorders, such as hypertension [7] and sucrose-induced insulin resistance [9], and this protection has been attributed to the sex hormone milieu [7]. Gender differences have also been observed in mitochondrial function and in oxidative stress parameters in different tissues such as muscle [10, 11], liver [12-14] and brown adipose tissue . Sex dimorphism in oxidative capacity, antioxidant enzyme activities and hydrogen peroxide production points to a higher protection against mitochondrial oxidative damage in female rats.

Taking into account the reported role of oxidative stress in the development of obesity-associated insulin sensitivity impairment, the aim of the present study was to investigate whether the gender differences found in mitochondrial function and oxidative stress lead to differences in the development of high-fat-diet-induced insulin resistance in rat skeletal muscle.

Materials and Methods

Animals and diets
Animal experiments were performed in accordance with general guidelines approved by our institutional ethics committee and EU regulations (86/609/EEC). Male and female *Rattus norvegicus* Wistar rats of 15 months of age (Charles River, Barcelona, Spain) were housed two per cage with free access to food and water and were kept at 22°C under a 12-hour light-dark cycle. Both male and female rats were divided into two groups (n=6) with a similar mean body weight (568 ±

27 g for male rats and 355 ± 10 g for female rats) and were fed a control pelleted diet (3,385 Kcal/Kg diet; 2.9% fat by weight) or a high fat diet (HFD) (4,516 Kcal/Kg diet; 32% of fat by weight) for 14 weeks. The HFD (namely cafeteria diet) components were cookies, pork liver pâté, fresh bacon, fairy cakes, chocolate, carrots and ensaïmada (a typical Majorcan pastry). The energy composition of the HFD was 8% protein, 34% carbohydrate and 64% lipid, and of the control pelleted diet (A04, Panlab, Barcelona) 19% protein, 73% carbohydrate and 8% lipid. Animal body weights were assessed weekly and food intake fortnightly throughout the dietary treatment. The rats were introduced into metabolic cages (Panlab, Barcelona) for 24 hours, to measure their food intake (HFD and/or standard diet). All the components of the HFD were presented in several small pieces and in gross excess so as to allow the recovery the following day of at least part of all the components offered. The amount of each component consumed by each animal was calculated from the difference between the amount offered and the amount recovered the next day. Rats were sacrificed by decapitation after a 12-hours period of fasting. Serum was collected and gastrocnemius skeletal muscle was rapidly dissected and weighed. Serum samples and a piece of the muscle were frozen in liquid N_2 and stored at -70 °C until analysed, the rest of the tissue was immediately processed.

Materials
Acutrend® GCT-meter and glucose test strips were supplied by Roche Diagnostics (Basel, Switzerland). Rat insulin enzyme immunoassay kit was purchased from Mercodia (Uppsala, Sweden) and enzyme immunoassays for measurement of rat adiponectin, leptin and resistin were from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). The kit for measurement of triglycerides was acquired from Linear Chemicals SL (Barcelona, Spain). Amplex Red kit was obtained from Invitrogen (Carlsbad, CA, USA). Oxyblot™ Protein Oxidation Detection Kit was purchased from Chemicon International (Temecula, CA, USA). Rabbit polyclonal antibodies to rat UCP3 (Cat. Num. AB4036) and GLUT4 (Cat. Num. sc-7938) were obtained from Chemicon International (Temecula, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively. Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO, USA).

Oral Glucose Tolerance Test
The test was performed on the week before the sacrifice. Rats were fasted for 12 hours and then glucose (2 g/Kg body weight) was given orally. Blood was collected from the saphena vein before glucose administration and after 15, 30, 60, 90 and 120 min, and glucose concentrations were measured using the Accutrend® system. The analysis of the area under the curve (AUC) of the blood glucose response was calculated as described previously [17].

Serum glucose, insulin and adipokine levels
Serum glucose levels were measured by using the Accutrend® system and serum insulin, adiponectin, leptin and resistin levels were measured by enzyme immunoassay kits.

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	Male		Female		
	Control	HFD	Control	HFD	ANOVA
Food intake (g/Kg day)	30.8±1.1	112±12	47.9±3.0	130±13	D
Energy intake (Kcal/Kg day)	92.5±10.5	404±67	143±16	458±73	D
Body weight gain (g)	12.8±8.5	129±17	19.7±10.2	169±18	D
Energetic efficiency (g weight gain/Kg body weight 100Kcal)	0.325±0.203	0.676±0.091	0.978±0.497	1.45±0.22	G
Muscle weight (g)	4.98±0.14	5.40±0.41	3.48±0.07	3.94±0.08	G, D
Relative muscle weight (g/100g)	0.817±0.047	0.809±0.028	0.895±0.044	0.797±0.053	NS
Triglyceride (mg/g tissue)	1.83±0.05	1.85±0.01	1.22±0.06†	1.84±0.02*	G, D,GxD
Protein (mg/g tissue)	102±6	107±8	102±6	99.0±7.6	NS
DNA (mg/g tissue)	1.37±0.06	1.46±0.22	1.17±0.05	1.53±0.07	D

Table 1. Energy intake, body weight gain and muscle composition. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): G indicates gender effect, D indicates diet effect, GxD indicates gender and HFD interactive effect and NS stands for non significant. Student's t-test (p<0.05): * diet *vs* control, † females *vs* males.

Homeostasis Model Assesment HOMA-β and HOMA-IR were used to estimate insulin secretion and insulin resistance, respectively [18]. HOMA-β was calculated as [20 x fasting insulin (μU/mL)]/[fasting glucose (mM) - 3.5] and HOMA-IR as [fasting glucose (mM) x fasting insulin (μU/mL)]/22.5.

Muscle triglyceride content
Tissue triglycerides were extracted from 15 mg of muscle in 2 mL of 2:1 chloroform-methanol [19]. Triglycerides were determined spectrophotometrically with a commercial kit.

Muscle mitochondria isolation
Mitochondria were isolated from muscle as previously described [20]. Briefly, tissue samples were minced at 4°C and then incubated in constant stirring for a total of 8 minutes at 4°C with buffer A (0.1 M KCl, 0.05 M Tris HCl, 2 mM EGTA, 1 mM ATP, 5 mM $MgCl_2$, 0.5% free fatty acid BSA, 0.15 g/L type VIII collagenase, pH 7.4) in a proportion of 10 mL per gram of tissue. After the first 4 minutes of incubation, tissue was homogenized with a Potter-Elvehjem homogenizer and then incubated again until the end of the aforementioned 8 minutes. To remove nuclei and cell debris the homogenate was centrifuged at 500xg for 10 minutes at 4°C. The resulting supernatant was filtered through a nylon mesh and centrifuged at 10,000xg for 10 minutes at 4°C. The pellet was resuspended with buffer B (0.1 M KCl, 0.05 M Tris HCl, 2 mM EGTA, pH 7.4) and again centrifuged at 10,000xg for 10 minutes at 4°C. Finally, the resulting pellet was resuspended with buffer B and centrifuged at 4,000xg for 10 minutes at 4°C. The mitochondria pellet was resuspended with 1 mL of buffer B.

Muscle mitochondrial oxygen consumption and cytochrome c oxidase activity
Mitochondrial oxygen consumption was measured polarographically as described previously [21], with minor modifications. Isolated mitochondria (0.5 mg protein/mL) were incubated in a water-thermostatically regulated chamber with a computer-controlled oxygen Clark-type electrode Oxygraph (Hansatech, Norfolk, UK) in respiratory buffer (145 mM KCl, 30 mM HEPES, 5 mM KH_2PO_4, 3 mM $MgCl_2$, 0.1 mM EGTA, 0.1% BSA pH 7.4, at 37°C). Mitochondrial respiratory states 4 and 3 were assayed with pyruvate/malate (5 mM/5 mM) in the absence (state 4) and in the presence (state 3) of 500 μM ADP. Mitochondrial cytochrome c oxidase (COX) activity was determined as previously described [22].

Muscle mitochondrial hydrogen peroxide production
Hydrogen peroxide production was assayed in isolated mitochondria by measuring the increase in fluorescence (530 nm excitation, 590 nm emission) due to the reaction of Amplex Red reagent with hydrogen peroxide in the presence of horseradish peroxidase [23]. Assays were performed at 37°C for 20 min in a 96-well microplate fluorimeter FLx800 (Bio-tek instruments, Winooski, VT, USA). Mitochondria (0.2 mg protein/mL) were added to the same medium used for respiration supplemented with 0.1 U/mL horseradish peroxidase and 50 μM Amplex Red reagent. Maximal hydrogen peroxide generation rates of complex III were measured as previously described [24], using succinate (5 mM), antimycin A (5 μM) and rotenone (2 μM). The rate of hydrogen peroxide production was calculated using a standard curve of hydrogen peroxide generated by addition of β-D(+)glucose 14 mM in the presence of glucose oxidase.

Measurements of muscle thiobarbituric acid-reactive substances (TBARS) and protein carbonyl groups
TBARS levels were measured as previously described [25] and used as index of serum lipid peroxidation. Protein carbonyl groups were determined as index of protein oxidation by immunoblot detection by using the OxyBlot™ Protein Oxidation Detection Kit according to the manufacturer's protocol.

Muscle antioxidant enzyme activity measurements
Catalase activity was determined in homogenate [26] and activities of glutathione peroxidase, glutathione reductase and Mn-superoxide dismutase were measured in isolated mitochondria as previously described [27-29].

Fig. 1. Oral Glucose Tolerance Curves. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): D indicates diet effect. Values of area under the blood glucose curve were 3,889 in control males, 5,383 in HFD males, 2,558 in control females and 3,315 in HFD females rats.
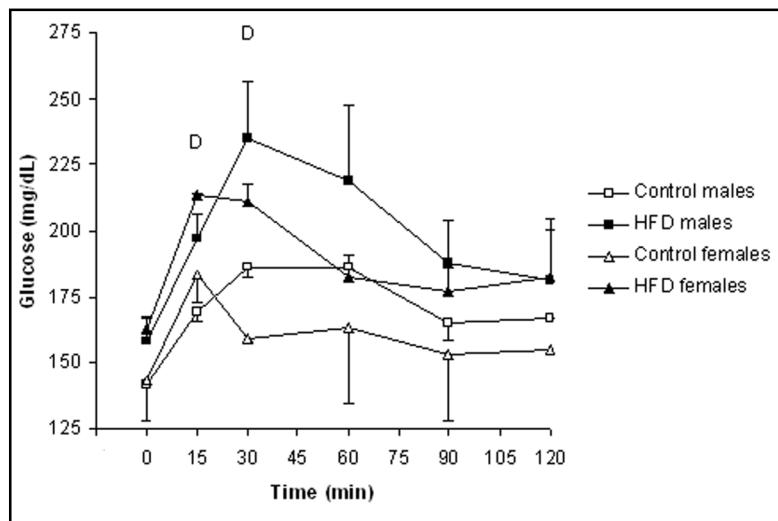
Western blot analysis of muscle UCP3 and GLUT4 protein levels

30 μg (UCP3) or 50 μg (GLUT4) of muscle protein were fractionated on 15% SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Rabbit polyclonal antibodies against rat UCP3 and GLUT4 were used as primary antibodies. Anti-rabbit IgG-alkaline phosphatase antibodies were used as secondary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands in films were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). Autoradiograms revealed an apparent molecular mass of 34 kDa and 45 kDa for UCP3 and GLUT4, respectively.

Statistical analysis

All data are expressed as mean values ± SEM of 6 animals per group. Statistical analyses were performed by using a statistical software package (SPSS 13.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups were analysed by two-way analysis of variance (ANOVA). Student's t-test, as post-hoc comparison, was performed when an interactive effect of gender and diet was shown. A p-value of less than 0.05 was considered statistically significant.

Results

Energy intake, body weight gain and muscle composition

Both food and energy intake of the HFD animals was about three times higher than those of the control rats, and the body weight gain was almost ten times higher (Table 1). No significant differences between genders were found either in food and energy intake or in body weight gain. Energetic efficiency was higher in female rats than in males and increased due to the consumption
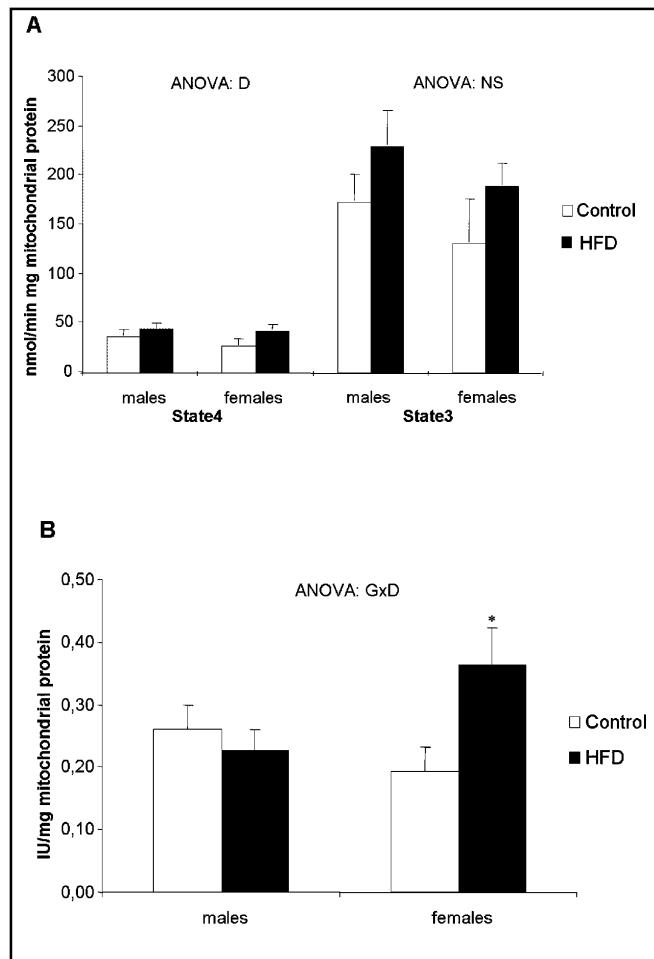
Fig. 2. Muscle mitochondrial oxygen consumption (A) and COX activity (B). Mitochondrial respiratory states 4 and 3 were assayed with pyruvate/malate (5 mM/5 mM). Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): D indicates diet, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test (p<0.05): *diet *vs* control.

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Table 2. Serum glucose, insulin and adipokine levels. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect and NS stands for non significant. Student's t-test (p<0.05): * diet *vs* control, † females *vs* males.

| | Male | | Female | | |
	Control	HFD	Control	HFD	ANOVA
Glucose (mM)	8.01 ± 0.20	7.87 ± 0.65	7.76 ± 0.28	7.99 ± 0.14	NS
Insulin (μU/mL)	68.3 ± 5.8	$261 \pm 6.8*$	28.6 ± 3.4	$88.7 \pm 5.4*^†$	G,D, GxD
HOMA-IR	24.4 ± 4.7	$93.5 \pm 5.9*$	$9.86 \pm 1.89^†$	$31.3 \pm 4.8*^†$	G,D, GxD
HOMA-β	304 ± 55	$1213 \pm 208*$	$138 \pm 25^†$	$401 \pm 59*^†$	G,D, GxD
Resistin (ng/mL)	52.2 ± 4.1	51.7 ± 3.3	26.6 ± 1.8	32.3 ± 3.5	G
Adiponectin (μg/mL)	7.90 ± 0.90	11.1 ± 1.9	$24.4 \pm 4.3^†$	$13.9 \pm 1.2*$	G, GxD
Leptin (ng/mL)	9.10 ± 2.40	18.7 ± 0.9	6.80 ± 1.60	14.2 ± 1.6	D

| | Male | | Female | | |
	Control	HFD	Control	HFD	ANOVA
Hydrogen peroxide production (pmolH$_2$O$_2$/min mg protein)	83.1 ± 9.3	281 ± 55	205 ± 56	357 ± 23	G, D
TBARS (nmol/mg protein)	1.52 ± 0.11	1.35 ± 0.24	$1.20 \pm 0.07^†$	$1.75 \pm 0.17*$	GxD
Protein carbonyl groups (%)	100 ± 34	204 ± 58	130 ± 35	265 ± 77	D

Table 3. Muscle mitochondrial hydrogen peroxide production, lipid peroxide levels and protein carbonyl groups. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): G indicates gender effect, D indicates diet effect and NS stands for non significant. Student's t-test (p<0.05): * diet *vs* control, †females *vs* males. Hydrogen peroxide production was determined in isolated mitochondria, lipid peroxide levels (TBARS) and protein carbonyl groups were determined in homogenate. Protein carbonyl groups were assessed by immunoblot detection and value of control male rats was set as 100%.

of the HFD, although it did not reach statistical significance. Relative muscle weight was not affected by gender or HFD feeding. Muscle triglyceride content was higher in control male rats compared to females and increased with HFD feeding only in female rats. Muscle DNA levels increased with the HFD but did not change with gender. No significant gender or diet effects were found in protein levels.

Oral Glucose Tolerance Test

HFD feeding increased blood glucose levels at 15 and 30 minutes after glucose load (Fig. 1). For both control and HFD female rats, the glucose level peaked at 15 minutes, and dropped thereafter, while for both groups of male rats the glucose level peaked at 30 minutes.

Serum glucose, insulin and adipokine levels

Serum insulin levels, HOMA-IR and HOMA-β ratios were higher in control male rats than in females (Table 2) and were significantly increased in both genders with HFD feeding (four times in male rats and three times in females). Resistin levels were higher in male rats in comparison to females and no differences were found with HFD feeding. Adiponectin levels, which were 3-fold higher in female control rats compared to males,

strongly decreased (43%) in HFD female rats reaching the same value as males. In both genders leptin levels significantly increased in response to HFD feeding. No significant gender or diet effects were found in serum glucose levels.

Mitochondrial oxygen consumption and cytochrome c oxidase activity

The intake of the HFD increased muscle mitochondrial oxygen consumption in state 4 (Fig. 2A). This increase was 58.2% in female rats and only 22.6% in males, although no significant gender effect was found. This is in agreement with the higher COX activity shown by HFD female rats (Fig. 2B).

Muscle mitochondrial hydrogen peroxide production, lipid peroxide levels and protein carbonyl groups

Mitochondrial hydrogen peroxide production was higher in control female rats than in males (Table 3). The lipid peroxide levels (TBARS) were lower in female rats compared to males and no significant gender differences were found in protein carbonyl groups. In both genders, HFD feeding increased the mitochondria hydrogen peroxide production, to a higher degree in males (238%)

Table 4. Muscle antioxidant enzyme activities. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): G indicates gender effect, D indicates diet effect and NS stands for non significant.

	Male		Female		
	Control	HFD	Control	HFD	ANOVA
Catalase (nKat/mg protein)	308±37	414±81	377±61	375±75	NS
Glutathione peroxidase (nKat/mg protein)	1.06±0.24	0.689±0.120	1.09±0.27	0.988±0.130	NS
Glutathione reductase (nKat/mg protein)	0.329±0.036	0.363±0.031	0.422±0.042	0.522±0.058	G
Mn-Superoxide dismutase (mIU/mg protein)	185±51	95.5±16.4	234±76	82.3±10.8	D

than in females (74%), and the protein carbonyl groups. The TBARS levels increased with HFD feeding only in female rats.

Muscle antioxidant enzymes activities

Glutathione reductase activity was higher in female rats than in males and no significant differences between genders were found in catalase, glutathione peroxidase and Mn-superoxide dismutase (Table 4). In both genders, HFD feeding significantly decreased the activity of Mn-superoxide dismutase.

Muscle UCP3 and GLUT4 protein levels

The consumption of the HFD strongly increased both UCP3 and GLUT4 protein levels in female rats (Fig. 3). In control rats, no significant differences between genders were found in these parameters.

Discussion

In this study, we found a gender dimorphism in the insulin resistance profile of old rats in both the control situation and in response to high-fat-diet feeding, with female rats showing a lower impairment of insulin sensitivity profile as well as a higher muscle oxidative function and ability to counteract the oxidative stress dependent insulin resistance.

Control male rats of 18 months of age showed a marked insulin resistance state compared to females, as suggested by insulin resistance markers - serum insulin levels, HOMA-IR and HOMA-β indexes and glucose tolerance curve - in accordance with that previously reported in both rodents [30] and humans [19]. Moreover, when the present results are compared with those obtained in a previous study using younger rats [31], we observe an age related increase of both serum insulin levels and HOMA-IR index in both genders that is two-fold higher for male rats than females. This difference in the
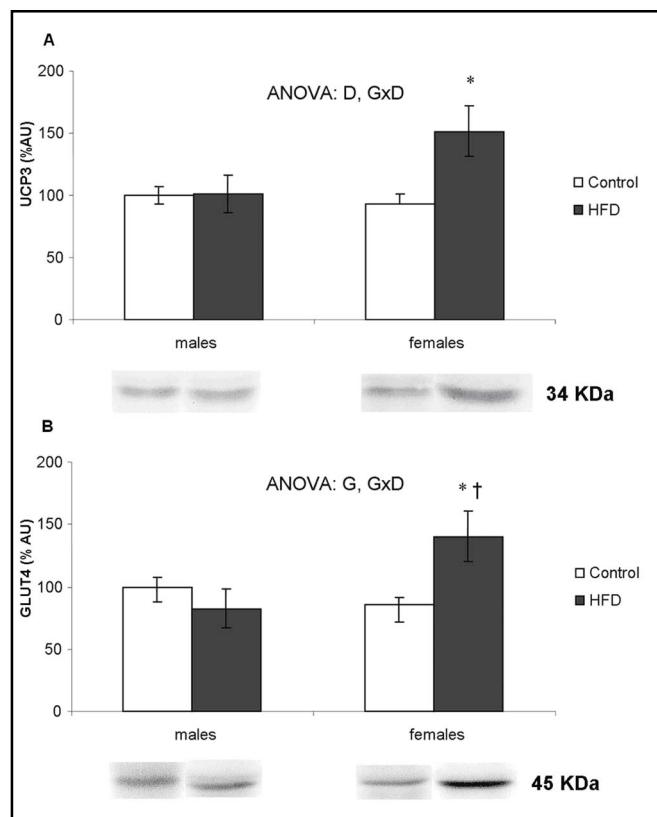
Fig. 3. Muscle UCP3 (A) and GLUT4 (B) protein levels. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): D indicates diet effect and GxD indicates gender and diet interactive effect. Student's t-test (p<0.05): *diet *vs* control, † females *vs* males. Levels of control male rats were set as 100%.

magnitude of the increase from similar values in male and female young rats points to the aging process as the main cause for the impairment of insulin sensitivity showed by control male rats.

The gender differences observed in both serum resistin and adiponectin levels also reflect the insulin resistance profile shown by control male rats. The elevated serum adiponectin levels in female rats have

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been reported to be consistent with their improved insulin sensitivity compared with their male counterparts [19, 30] which agrees with the lower resistin levels shown by female rats. In this way, the intramuscular accumulation of lipids observed in male rats together with its reported deleterious effect on insulin action [32-34] further supports this idea. This sex dimorphism could point towards a higher tendency of male rats to undergo metabolic syndrome manifestations associated to age-related pro-oxidant state.

Although female rat mitochondria produced more hydrogen peroxide than those from males, the muscle oxidative damage was not enhanced which could be explained by a slight increase in the muscle antioxidant protection and by the lower levels of triglycerides accumulated in this tissue. All in all, 18-month-old male rats showed a more marked insulin resistance profile compared with their female counterparts which could be attributable in part to the different hormonal milieu to which they have been exposed during their fertile period [35-37].

HFD feeding generated a pro-oxidant status as reported previous studies [3, 38-40], represented by increases in both muscle mitochondrial hydrogen peroxide production and oxidative damage and by the loss of the SOD activity. The consumption of the HFD also entailed a pro-inflammatory profile with altered adipokine levels and an impairment of insulin sensitivity, as has been previously reported to occur in association with the obese state [41, 42]. In our study, male rats showed a higher resistance to increase body weight in response to HFD feeding than females (the excess of body weight was 38.4% for females and only 16.2% for males). However, in spite of this higher body weight gain, HFD female rats showed a less marked insulin resistance profile than males. The excess of body weight induced in female rats by the dietary treatment caused an insulin resistant state similar to that exhibited by both control and overweight males at 18 months of age. This could be understood as a more deleterious effect of the aging process on males than on females.

The consumption of the HFD - through the increase of the fat supply to muscle - provokes a triglyceride accumulation in female rats that matches male rat levels, in spite of their increased mitochondrial oxidative capacity. Although an impairment of muscle fatty acid oxidation has been proposed to play a key role in the intramuscular accumulation of lipids and their deleterious effects on insulin action [4], Turner and collaborators [43, 44] have recently suggested that the intramuscular lipid

accumulation and insulin resistance in rodents can occur along with an increase in the fatty acid oxidative capacity, such as we have also observed in our study, and can be understood as an homeostatic response to attempt to compensate for the elevated availability of lipids.

Interestingly, HFD female rats show an increase in oxidative damage probably due to the higher pro-oxidant situation they underwent. Nevertheless, in spite of the high excess of body weight, HFD female rats seem to have the ability to induce mechanisms for attenuating the deleterious consequences of the HFD. It can be suggested that the increase in lipid peroxides shown by HFD female rats could be responsible, in part, for the strong increase of muscle UCP3 protein levels found in this gender aimed at counteracting the increased hydrogen peroxide production associated to the obese state. This higher UCP3 protein levels in HFD females does not seem to be attributable to the increase of mitochondrial proteins levels associated to HFD feeding, since the increase occurs in both genders (data not shown). It has been hypothesized that during fatty acid oxidation, UCP3 is specifically activated by free radical species or lipid peroxides, leading to a mild uncoupling that reduces membrane potential and mitigates free radical species production to protect against oxidative damage [45, 46]. In contrast, in male rats HFD feeding did not achieve the UCP3 overexpression, unlike that reported in other studies performed with younger male rats [43, 44, 47]. This apparent controversy could be explained by an age-related decrease in muscle UCP3 expression which has been previously reported [48]. In fact, a similar effect of age decreasing UCP1 levels has also been described in brown adipose tissue [49, 50]. These results reinforce the postulated impaired ability of 18-month-old male rats to counteract the effects of HFD feeding.

Moreover, it could not be discarded an additional role for this UCP3 induction as a mechanism of adiposity control in situations of high fuel availability, as it has been recently reported in human UCP3 transgenic mice [51].

Since UCP3 has been related with an improvement of insulin resistance through an increase in the muscle GLUT4 traslocation [52], gender dimorphism in muscle UCP3 levels could be linked to the gender differences found in the insulin resistance status. In fact, the positive correlation between UCP3 and GLUT4 protein levels (R=0.786, p=0.000) further reinforces this idea. The reported effects of HFD diets on GLUT4 expression in skeletal muscle are variable and seem to be dependent on the quality of the diet and the age of the animals [53, 54]. However, the enhanced GLUT4 levels did not

prevent the insulin resistance profile in obese female rats, but could attenuate it, thus resulting in a better profile than that of the otherwise less obese HFD males. GLUT4 levels do not necessary reflect changes in GLUT4 traslocation. In fact, a deficient insulin-induced GLUT4 traslocation in the presence of an unaltered GLUT4 expression has been detected in skeletal muscle of different animal models of obesity or type 2 diabetes [55, 56]. This could explain that control male rats showed a higher impaired insulin sensitivity compared to control females, in spite of having similar UCP3 and GLUT4 levels.

In summary, our results point to a gender dimorphism in the response of skeletal muscle to high-fat-diet feeding. Female rats, in spite of showing a higher excess of body weight than males, have a better oxidative and inflammatory profile, and could have a higher ability to counteract the oxidative-stress-associated insulin resistance, in part through an overexpression of both UCP3 and GLUT4. Moreover, the more marked insulin resistant state of control male rats could be attributed to a more detrimental effect of age in this gender.

Acknowledgements

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Manuscrito II.

Gender dependent effects of high fat diet feeding on rat pancreas oxidative stress.

Yolanda Gómez-Pérez; Magdalena Gianotti, PhD; Isabel Lladó, PhD; Ana M Proenza, PhD.

20 Dic 2010

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Gender dependent effects of high fat diet feeding on rat pancreas oxidative stress

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Running head: Gender dimorphism in pancreas oxidative stress

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ABSTRACT

Objectives: To investigate whether gender differences in oxidative stress-associated insulin resistance previously reported in rats could be attributed to a possible gender dimorphism in pancreas redox status.

Methods: 15 month-old male and female Wistar rats were fed a control diet or a high fat diet (HFD) for 14 weeks. Serum glucose, lipids, and hormone levels were measured. Insulin immunohistochemistry and morphometric analysis of islets were performed. Pancreas triglyceride content, oxidative damage and antioxidant enzymatic activities were determined. Lipoprotein lipase, hormone sensitive lipase and uncoupling protein 2 (UCP2) levels were also measured.

Results: Male rats showed a more marked insulin resistance profile than females. In control female rats, pancreas Mn-superoxide dismutase activity and UCP2 levels were higher and oxidative damage was lower compared to males. HFD feeding decreased pancreas triglyceride content in female rats and UCP2 levels in male rats. HFD female rats showed larger islets than both their control and gender counterparts.

Conclusions: These results confirm the existence of a gender dimorphism in pancreas oxidative status in both control and HFD feeding situations, with female rats showing higher protection against oxidative stress, thus maintaining pancreatic function and contributing to a lower risk of insulin resistance.

Keywords: obesity, oxidative damage, antioxidant defence, pancreas, UCP2.

INTRODUCTION

The consumption of high-fat diets, which represents a chronic exposure of body tissues to fatty acids, induces overweight and insulin resistance [1, 2] and increases lipid accumulation in non-adipose tissues. In pancreas, the ectopic fat impairs the function of insulin secreting cells, leading to the development of type 2 diabetes [3]. Pancreatic hormone-sensitive lipase (HSL) and lipoprotein lipase (LPL) play important roles in the regulation of the intracellular pool of triglycerides in β-cells. In fact, free fatty acids obtained from the circulation and from stored triglycerides are essential for normal glucose-stimulated insulin secretion [4, 5]. However, the excess of fatty acids released from lipids accumulated by the pancreas may derive towards toxic pathways, leading to β-cell dysfunction and apoptosis [3, 6].

Recently, it has been suggested that reactive oxygen species (ROS) could be involved in obesity-associated β-cell dysfunction [7]; because of the relatively low expression of antioxidant enzymes, β cells may be rather sensitive to ROS attack, emerging as targets of oxidative stress-mediated tissue damage [8]. Evidence is accumulating in favour of a role of uncoupling protein 2 (UCP2) - which is the only uncoupling protein present in pancreatic β cells - in reducing the production of ROS by mitochondria [9] and protecting against the consequent tissue damage. In fact, a recent report has showed that the chronic absence of UCP2 in pancreatic islets causes oxidative stress [10]. In addition, UCP2 overexpression has been associated with the inhibition of glucose-stimulated insulin secretion [11].

Gender dimorphism has been described in mitochondrial function and oxidative stress parameters in different tissues such as muscle [1, 12-14], liver [15-17], brown adipose tissue [18, 19] and brain [20], which points to better protection against oxidative stress in female rats. However, not many studies have been conducted concerning gender differences in the redox status of pancreas, in spite of the more insulin-sensitive environment described in women compared to men [21].

In a previous study using the same experimental model [1], we reported a gender dimorphism in the insulin sensitivity of skeletal muscle of HFD fed rats, with male rats undergoing more detrimental consequences of age and HFD feeding-induced oxidative stress. Given the results obtained, the possible existence of gender differences in the pancreatic redox status, which would be related with the insulin resistance profile shown by male and female rats, might be considered. Thus, the aim of the present study was to compare the pancreas oxidative stress induced by a high-fat diet (HFD) in male

and female rats in relation with the gender related differences found in insulin sensitivity.

MATERIALS AND METHODS

Animals and diets

Animal experiments were performed in accordance with general guidelines approved by ethics committee of University of Balearic Islands and EU regulations (86/609/CEE and 2003/65/CE). Male and nullipara female Wistar rats of 15 months of age were housed two per cage with free access to food and water. All animals were kept at 22ºC under a 12-hour light-dark cycle. Both male and female rats were divided into two groups (n=6) with a similar mean body weight (568±27g for male rats and 355±10g for female rats) and were fed a control pelleted diet (3,385Kcal/Kg diet; 2.9% fat by weight) or a high fat diet (HFD) (4,516Kcal/Kg diet; 32% of fat by weight) for 14 weeks. The HFD (namely cafeteria diet) components were cookies, pork liver pâté, fresh bacon, fairy cakes, chocolate, carrots and ensaïmada (a typical Majorcan pastry). The energy composition of the HFD was 8% protein, 34% carbohydrate and 64% lipid, and of the control pelleted diet (A04, Panlab, Barcelona) 19% protein, 73% carbohydrate and 8% lipid. Animal body weights were assessed weekly and food intake fortnightly throughout the dietary treatment (body weight gain reached by rats was 12.8±8.5g in control males, 129±17g in HFD fed males, 19.7±10.2g in control females, and 169±18g in HFD fed females. ANOVA: diet effect). Rats were sacrificed by decapitation after a 12-hour period of fasting. Blood was collected and pancreas was rapidly dissected and weighed. A piece of pancreas was immediately homogenized in 1/10 (w/v) phosphate-buffered saline to determine the Mn-superoxide dismutase activity and another piece of pancreas was fixed for immunohistochemistry. The rest of the tissue and serum samples were frozen and stored at −70 °C until analysed.

Materials

Acutrend® GCT-meter and glucose test strips were supplied by Roche Diagnostics (Basel, Switzerland). Enzyme immunoassays kits were used for measurement of rat serum insulin (Mercodia, Uppsala, Sweden), total adiponectin and resistin (Phoenix Pharmaceuticals Inc., Belmont, CA, USA) and 17β-estradiol (Diametra, Milano, Italy).

Non esterified fatty acid assay kit was from Wako Chemicals (Chuo-ku, Osaka, Japan). The kit for measurement of triglycerides was acquired from Linear Chemicals SL (Barcelona, Spain). Oxyblot^{TM}. Protein Oxidation Detection Kit was purchased from Chemicon International (Temecula, CA, USA). HSL rabbit polyclonal antibody was kindly provided by Dr. F. Kraemer. UCP2 (Cat. Num. UCP22A) and GLUT2 (Cat. Num. AB1342) rabbit polyclonal antibodies were obtained from Alpha Diagnostics (San Antonio, TX, USA) and Chemicon International (Temecula, CA, USA), respectively. Perilipin A (Cat. Num. ab60269) antibody was obtained from Abcam (Cambridge, UK). LPL chicken polyclonal antibody was developed in our laboratory [22]. Chemiluminescence kit (ECL) for immunoblot development was purchased from Amersham (Little Chalfont, UK). Normal goat serum, biotinylated anti-rabbit IgG and Vectastain ® ABC kit for immunohistochemistry were from Vector Laboratories Inc. (Burlingame, CA, USA). Rabbit insulin antibody (Cat. Num. sc-9168) was provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA) and diaminobenzidine was from Sigma-Aldrich (St. Louis, MO, USA). Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO, USA).

Serum glucose, lipids and hormone levels
Serum glucose and triglyceride levels were measured by using the Accutrend$^®$ system. Serum insulin, resistin, adiponectin and 17β-estradiol levels were measured by enzyme immunoassay kits. Non esterified free fatty acids levels were measured by using a spectrophotometric assay kit. Homeostasis Model Assessment HOMA-β and HOMA-IR were used to estimate insulin secretion and insulin resistance, respectively [23]. HOMA-β was calculated as [20 x fasting insulin (μU/mL)]/[fasting glucose (mM)-3.5] and HOMA-IR as [fasting glucose (mM) x fasting insulin (μU/mL)]/22.5.

Immunohistochemistry and morphometric analysis of islets
Pancreatic tissue fragments of three animals from each experimental group were fixed in 10% neutral buffered formaldehyde dehydrated, embedded in paraffin wax and sectioned at 4μm thickness. Histological sections were stained with haematoxylin and eosin or with primary antibody against insulin (working dilution 1:200) by the avidin-peroxidase method (Vectastain® ABC kit). Visualization was performed using 3,3'-

diaminobenzidine tetrahydrochloride and all sections were counterstained with haematoxylin-eosin. Immunohistochemical analyses were generally performed on two sections of each tissue from individual animals and repeated in at least three distinct animals. Sections were examined with an optical microscope and photographed with a digital camera linked to the microscope. We randomly chose 30 Langerhans islets from each animal and measured the area of each islet using the Scion Image software.

Pancreas triglyceride content

Tissue triglycerides were extracted from 30 mg of pancreas in 9 mL of 2:1 chloroform-methanol [24]. Triglycerides were determined spectrophotometrically with a commercial kit.

Pancreas enzyme activities measurements

Mn-superoxide dismutase (Mn-SOD) was determined freshly in homogenized samples, as previously reported [25]. Citrate synthase, catalase, glutathione peroxidase and glutathione reductase activities were determined as previously described [26-28] in frozen homogenized samples which were sonicated and then centrifuged at 20,000g for 15 min [29] before performing analysis.

Measurement of pancreas thiobarbituric acid-reactive substances (TBARS) and protein carbonyl groups

TBARS levels were measured as previously described [30] and used as an index of lipid peroxidation. Protein carbonyl groups were determined as an index of protein oxidation by Dot-Blot detection using the OxyBlot^{TM} Protein Oxidation Detection Kit according to the manufacturer's protocol with several modifications [20].

Western blot analysis

50 μg (UCP2, GLUT2), 75 μg (perilipin A), 100 μg (LPL) and 150 μg (HSL) of pancreas protein were fractionated on 15% or 10% SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Rabbit polyclonal antibodies UCP2, GLUT2 and HSL, goat polyclonal antibody against perilipin A and chicken polyclonal antibody to LPL were used as primary antibodies. Anti-rabbit, anti-goat and anti-chicken IgG-alkaline phosphatase antibodies were used as secondary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence

kit. Bands were visualized with the ChemiDoc XRS system (Bio-Rad, CA, USA) and analyzed with the image analysis program Quantity one© (Bio-Rad, CA, USA). Bands revealed an apparent molecular mass of 34 kDa, 45 kDa, 57 kDa, 65 kDa and 85 kDa for UCP2, GLUT2, perilipin A, LPL and HSL, respectively.

Statistical analysis

All data are expressed as mean values ± SEM of 6 animals per group. Statistical analyses were performed using a statistical software package (SPSS 17.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups were analysed by two-way analysis of variance (ANOVA). Student's t-test was performed as a post-hoc comparison when an interactive effect of gender and diet was shown. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Body, white fat and pancreas weights, pancreas triglyceride content and islet area
HFD feeding increased body weight in both genders (Table 1). White fat depot weight was higher in control male rats than in females, while adiposity index was higher in control female rats. Both parameters increased in both genders with HFD feeding, although to a greater degree in females. Relative pancreas weight was higher in female rats compared to males but did not change with HFD feeding. Pancreas triglyceride levels were about two times higher in control female rats than in males and decreased with HFD feeding in females. No significant gender effect in islet area of control animals was found. HFD female rats showed larger islets than both their control and gender counterparts (Figure 1). Moreover, HFD fed males showed more irregularly shaped islets. The percentage of irregularly shaped islets was 20.4% for control male rats, 51.6% for HFD male rats, 14.3% for control female rats and 28.7% for HFD female rats.

Table 1. Body, white fat and pancreas weights, pancreas triglyceride content and islet area.

	Male		Female		
	Control	*HFD*	*Control*	*HFD*	*ANOVA*
Body weight (g)	580 ± 30	674 ± 42	376 ± 4	521 ± 34	G, D
Body weight gain (g)	12.8 ± 8.5	129 ± 17	19.7 ± 10.2	169 ± 18	D
White fat depot weight (g)	62.7 ± 3.9	75.3 ± 3.6[*]	42.6 ± 4.8[†]	80.2 ± 5.6[*]	D, GxD
Adiposity index (g/100g BW)	10.0 ± 0.4	11.6 ± 0.5[*]	11.0 ± 0.8[†]	18.1 ± 0.7[*,†]	G, D, GxD
Pancreas					
Weight (g)	0.812 ± 0.075	0.995 ± 0.150	0.783 ± 0.098	0.959 ± 0.104	NS
g/100g body weight	0.136 ± 0.020	0.147 ± 0.014	0.202 ± 0.028	0.188 ± 0.007	G
Triglyceride (mg/g tissue)	4.74 ± 0.20	6.17 ± 0.85	8.58 ± 0.55[†]	4.07 ± 1.11[*]	G, G*D
Islets area(μm^2)	17813 ± 2929	12402 ± 2217	14575 ± 2161	20874 ± 2979[*,†]	GxD

BW, body weight. White fat depot weight is the sum of inguinal, gonadal, mesenteric and lumbar fat depot weights. Adiposity index is the white fat depot weight relative to 100 of body weight. Values are expressed as the mean ± S.E.M of 6 animals per group. Islets area is expressed as the mean ± S.E.M of 30 islets from each animal. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect whereas NS stands for non significant. Student's t-test as a post-hoc comparison ($p<0.05$): [*]diet *vs* control, [†]females *vs* males.

Serum glucose, lipids and hormone levels

Serum insulin levels, HOMA-IR and HOMA-β ratios were higher in control male rats than in females (Table 2) and were significantly increased in both genders with HFD feeding (four times in male rats and three times in females). Serum free fatty acids decreased in both genders with HFD feeding but no significant gender or diet effects were found in serum glucose or triglyceride levels. Resistin levels were higher in male rats compared to females but no differences were found with HFD feeding. Adiponectin levels, which were 3-fold higher in female control rats compared to males, strongly decreased (43%) in HFD female rats reaching similar values to males. 17β-estradiol levels were higher in female rats than in males in both control and HFD feeding rats.

Table 2. Serum glucose, lipid and hormone levels.

	Male		Female		
	Control	*HFD*	*Control*	*HFD*	*ANOVA*
Glucose (g/L)	1.44 ± 0.04	1.42 ± 0.12	1.40 ± 0.05	1.44 ± 0.03	NS
Insulin (µg/L)	2.35 ± 0.44	$9.02 \pm 0.8^{*}$	0.99 ± 0.18	$3.06 \pm 0.46^{*,\dagger}$	G, D, GxD
HOMA-β	304 ± 55	$1213 \pm 208^{*}$	$138 \pm 25^{\dagger}$	$401 \pm 59^{*,\dagger}$	G, D, GxD
HOMA-IR	24.4 ± 4.7	$93.5 \pm 5.9^{*}$	$9.86 \pm 1.89^{\dagger}$	$31.3 \pm 4.8^{*,\dagger}$	G,D, GxD
Resistin (µg/L)	52.2 ± 4.1	51.7 ± 3.3	26.6 ± 1.8	32.3 ± 3.5	G
Adiponectin (mg/L)	7.91 ± 0.9	11.1 ± 2.0	$24.4 \pm 4.3^{\dagger}$	$13.9 \pm 1.2^{*}$	G, GxD
17β-estradiol (pmol/mL)	35.3 ± 1.89	32.9 ± 2.9	39.7 ± 1.8	39.8 ± 0.9	G
Free fatty acids (g/L)	0.267 ± 0.017	0.248 ± 0.029	0.293 ± 0.010	0.223 ± 0.014	D
Triglyceride (g/L)	3.44 ± 0.30	2.62 ± 0.80	2.63 ± 0.42	1.92 ± 0.15	NS

Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): G indicates gender effect, D indicates diet effect, GxD indicates gender and diet interactive effect whereas NS stands for non significant. Student's t-test as post-hoc comparison (p<0.05): *diet *vs* control, †females *vs* males. Homeostasis Model Assessment HOMA-IR and HOMA-β were used to estimate insulin resistance and insulin secretion, respectively. HOMA-β was calculated as [20 x fasting insulin (µU/mL)] / [fasting glucose (mM) - 3.5] and HOMA-IR as [fasting glucose (mM) x fasting insulin (µU/mL)]/22.5.

Pancreas enzyme activities and oxidative damage

There was a significant gender effect on citrate synthase, Mn-SOD and glutathione reductase activities (Table 3). Mn-SOD activities were greater in female rats compared to males, while citrate synthase and glutathione reductase activity showed the opposite profile. No significant differences between genders were found in catalase, or glutathione peroxidase activities. HFD feeding increased catalase activity in both genders but no effect was found in the other enzyme activities. Lipid peroxide levels (TBARS) and protein carbonyl groups were lower in female rats compared to males. No significant effect of dietary treatment was seen in these parameters, although a tendency to increase protein oxidation levels was observed in HFD male rats (p=0.096).

Table 3. Pancreas enzyme activities and oxidative damage.

	Male		Female		
	Control	*HFD*	*Control*	*HFD*	*ANOVA*
Citrate synthase (IU/mg protein)	36.0 ± 1.8	33.2 ± 2.2	31.2 ± 3.2	27.9 ± 1.0	G
Catalase (nKat/mg protein)	343 ± 19	360 ± 44	325 ± 17	461 ± 40	D
Glutathione peroxidase (nKat/mg protein)	0.320 ± 0.011	0.238 ± 0.027	0.306 ± 0.06	0.275 ± 0.067	NS
Glutathione reductase (nKat/mg protein)	0.497 ± 0.055	0.544 ± 0.025	0.394 ± 0.030	0.378 ± 0.032	G
Mn-Superoxide dismutase (mIU/mg protein)	28.5 ± 7.8	15.6 ± 2.6	39.6 ± 5.5	38.4 ± 9.3	G
TBARS (nmol/mg protein)	2.18 ± 0.20	2.21 ± 0.33	1.51 ± 0.09	1.46 ± 0.06	G
Protein carbonyl groups (%)	100 ± 15	194 ± 45	83 ± 31	87 ± 28	G

Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA ($p<0.05$): G indicates gender effect, D indicates diet effect whereas NS stands for non significant. Protein carbonyl groups were assessed by immunoblot detection and value of control male rats was set as 100%.
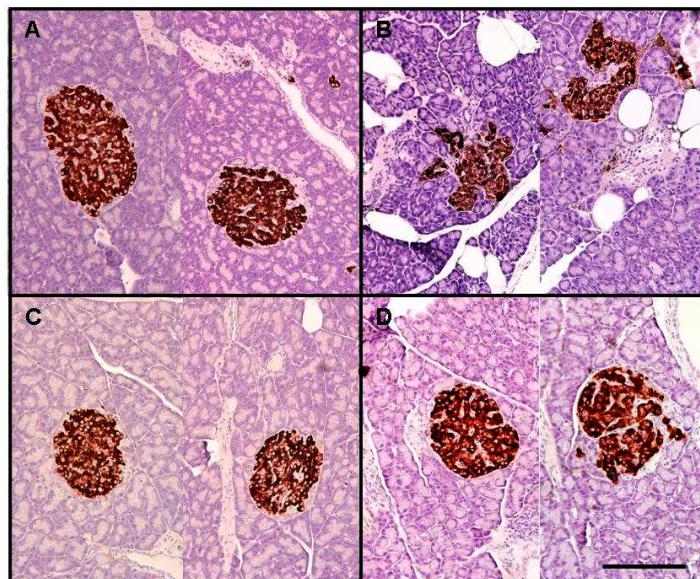
Figure 1. Immunohistochemistry and morphometric analysis of islets. Histological sections of pancreas tissue fragments were stained with primary antibody against insulin and counterstained with haematoxylin-eosin. Sections were examined with an optical microscope and photographed with a digital camera linked to the microscope. **A**, Control male rats; **B**, HFD fed male rats; **C**, Control female rats; and **D**, HFD fed female rats. Scale bar, 100 μm.

Pancreas UCP2, LPL, HSL, Perilipin A and GLUT2 protein levels

UCP2 protein levels (Figure 2) were higher in female rats compared to males, and decreased with HFD feeding in males but not in females. HSL protein levels decreased in both genders (Table 4), and the HSL to LPL ratio was lower in female rats than in males. No significant effects of gender or diet were found either in perilipin A, LPL, or GLUT2 protein levels.
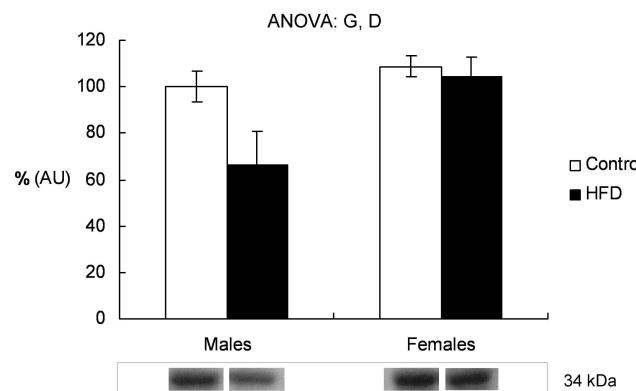
Figure 2. Pancreas UCP2 protein levels. Values are expressed as the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): D indicates diet effect and G indicates gender effect. Levels of control male rats were set as 100%.

Table 4. Pancreas HSL, LPL, Perilipin A and GLUT2 protein levels.

| | *Male* | | *Female* | | |
	Control	*HFD*	*Control*	*HFD*	*ANOVA*
HSL	100 ± 9	85.6 ± 5.1	88.1 ± 11.6	64.0 ± 2.9	D
LPL	100 ± 7	87.4 ± 5.1	97.6 ± 6.5	84.8 ± 3.9	NS
HSL/LPL ratio	100 ± 4	98.2 ± 1.7	90.4 ± 13.6	76.5 ± 6.2	G
Perilipin A	100 ± 7	70.8 ± 16.0	78.3 ± 14.7	74.2 ± 5.5	NS
GLUT2	100 ± 11	93.6 ± 19.6	98.2 ± 25.1	90.9 ± 8.7	NS

Values are expressed as % AU (arbitrary units), where levels of control male rats were set as 100%. Data are the mean ± S.E.M of 6 animals per group. ANOVA (p<0.05): G indicates gender effect, D indicates diet effect whereas NS stands for non significant.

DISCUSSION

We found a gender dimorphism in insulin resistance profile of rats fed a high-fat diet, which might be attributable to differences in whole body lipid storage capacity, and pancreas islet mass, and oxidative stress status.

In this study, male rats showed a more marked insulin resistance profile compared with their female counterparts, as indicated by the higher serum values of resistin, insulin and both HOMA-IR and HOMA-β indexes, as well as the lower levels of adiponectin. This serum profile is in agreement with the impairment of the serum glucose tolerance showed by these animals and it has previously been discussed elsewhere [1]. These gender differences are amplified in HFD groups and could be associated, in part, to differences at pancreas level. Likewise, the altered islet morphology shown by HFD male rats in comparison with their female counterparts suggests an impairment of pancreatic function. Female rats would maintain the ability to respond to obesity-associated HFD feeding by increasing pancreatic islet mass and, probably, insulin secretion. Along these lines, the increase of beta cell mass has been considered an initial compensatory strategy when facing obesity-associated insulin resistance since the onset of type 2 diabetes in both humans and rodent models has been reported to be accompanied by a progressive decrease in beta cell mass [31]. Thus, islet area data further confirm that, despite reaching a greater degree of obesity, female rats show a milder insulin resistance status than male rats.

HFD feeding induced a more pronounced increase of adiposity in female rats compared to males, suggesting greater adipose tissue expandability of the former in response to a positive energy balance (the increase of white fat weight with HFD feeding was 88% for females and 20% for males), thus avoiding the detrimental effects of the ectopic deposition of reactive lipid species in non adipose tissues [32]. Triglyceride accumulation in non-adipose tissues, such as skeletal muscle and liver, may constitute a safer storage of excess fat, thus avoiding free fatty acid tissue accumulation that could be derived towards toxic metabolic pathways [32, 33]. However, there is increasing evidence that triglyceride accumulation in pancreas leads directly to beta cell dysfunction [32]. In this sense, the decreased pancreas triglyceride levels in HFD female rats would be in agreement with a higher adipose tissue expandability and with an hypothetical greater capacity to protect the pancreas from lipotoxicity, thus maintaining better pancreatic function. Although the HFD associated decrease of HSL to LPL ratio in female rats would point to higher triglyceride storage, protein levels do not

necessarily reflect enzyme activities. Moreover, the involvement of other lipases in the pancreas lipid metabolism could not be ruled out [34].

Gender differences in insulin resistance and pancreatic function could also be related to the redox status of the tissue induced by HFD feeding. Interestingly, the animals used in this study were aged rats, and there is evidence that oxidative damage increases with aging [33]. Moreover, we have recently reported a more detrimental effect of the aging process in male rats compared to females [1]. In addition, high-fat diet-induced obesity is associated with a low degree pro-inflammatory state, in which impairments in both oxidative stress and antioxidant mechanisms would be involved [35]. Since β-cells are known to be particularly susceptible to ROS damage due to their relatively low expression of antioxidant enzymes compared to other tissues [36, 37], a defensive role of pancreas UCP2 against ROS production has been proposed to be required [38]. In this sense, the decrease of UCP2 protein levels found in HFD male rats would impair their capacity to counteract oxidative stress. The decrease in Mn-SOD activity also observed in this experimental group further reinforces this idea and would result in the higher pancreas oxidative damage they showed compared to their female counterparts.

Our results suggest a decreased ability of male rats to keep the mechanisms for attenuating mitochondrial ROS production active. In contrast, female rats are able to maintain their antioxidant response capacity in spite of the HFD feeding. Moreover, we have recently described in this same experimental model a similar picture in skeletal muscle, in which the increase of UCP3 protein levels showed by HFD female rats, and not observed in males, would contribute to counteract the enhanced mitochondrial ROS production associated with HFD feeding in female rats [1].

In addition, the higher UCP2 protein levels of HFD female rats compared to their male counterparts might also contribute to a better regulation of insulin secretion. A role of pancreas UCP2 in the control of insulin secretion and glucose sensing by ß-cells in both rodents and humans has been proposed [39]. Besides, in periods of fasting, UCP2 expression and activity is stimulated, resulting in a decrease of insulin secretion to prevent hypoglycaemia [11, 40]. Moreover, UCP2 overexpression has been associated with impaired glucose-induced insulin secretion and diabetes [11]. Thus, the decrease observed in UCP2 protein levels only in HFD males further reinforces the key role that has been proposed for UCP2 in glucose homeostasis, and agrees with the altered ability of male rats to respond to insulin in HFD feeding conditions. Nevertheless, in spite of the three-month HFD feeding period, neither male nor female rats reached a diabetic

state since they were still able to maintain glycaemia. We suggest that the fact that UCP2 did not undergo up-regulation in HFD animals could in the short term protect them from the development of diabetes.

In summary, the gender dimorphism found in body fat storage capacity, in pancreatic islet mass and in pancreas redox status results in an improved capacity of female rats to prevent the hyperglycaemia, lipotoxicity and pancreas oxidative stress associated with HFD feeding. This may confer female rats greater protection against the diet-associated impairment of pancreatic function, which would contribute to a lower risk of type 2 diabetes in females.

ACKNOWLEDGEMENTS

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Manuscrito III.

Age-related decline of skeletal muscle insulina sensitivity in rats: Effect of sex and muscle type.

Age-Related Decline of Skeletal Muscle Insulin Sensitivity in Rats: Effect of Sex and Muscle Type

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Abstract

Aging is associated with a progressive decline of skeletal muscle function and insulin sensitivity. Sex differences in the insulin response to different physiological situations have been found, leading to the development of type 2 diabetes. The aim of this study was to investigate the changes in insulin sensitivity with age in male and female rats and to elucidate whether there are sex differences in the alteration profiles of systemic insulin sensitivity parameters, adiposity, skeletal muscle oxidative damage, and the insulin signaling pathway. The gastrocnemius and soleus muscles of male and female rats of 3, 9, and 18 months of age were used. The decrease of insulin sensitivity with age was higher in female than in male rats. However, the increase of both serum insulin levels and adiposity with age shows a different profile in both sexes and suggests an earlier onset of age-related impairment of insulin sensitivity in male than in female rats. Sex differences in insulin signaling key protein levels were found mainly in the most aged rats, suggesting that sex differences in these proteins would be manifested at more advanced ages than differences in the insulin-sensitivity serum profile. In addition, the gastrocnemius muscle showed more age-associated oxidative damage and insulin resistance impact than the soleus in both sexes. These results suggest the sex differences found in the impairment of insulin sensitivity of aged rats would not be attributable to differences between sexes in the time course of the levels of key proteins of the skeletal muscle insulin signaling pathway, at least in the first 18 months of life.

Introduction

SKELETAL MUSCLE REPRESENTS THE main tissue of insulin-stimulated glucose uptake, playing a crucial role in whole-body energy metabolism. This tissue can easily switch between glucose and fat oxidation according to physiological changes and the metabolic and biochemical characteristics of the different fiber types.[1,2] Oxidative muscles have a high proportion of slow-twitch or slow-oxidative fibers, are very resistant to fatigue, and obtain energy by oxidative metabolic processes, whereas glycolytic muscles are mainly composed of fast-twitch or fast-glycolytic fibers and generate energy by anaerobic metabolic processes.[2]

Insulin binds to its receptor, which belongs to a family of tyrosine kinase receptors and provokes the phosphorylation of insulin receptor substrate 1 (IRS-1) and, subsequently, the protein kinase B (Akt), finally resulting in the activation of glucose transporter 4 (GLUT4) translocation and glucose uptake.[3] It has been shown that insulin action is greater in oxidative than in glycolytic skeletal muscles.[4,5]

Aging is associated with a progressive decline of muscle mass and function due to a reduction in the size and number of muscle fibers.[6] This condition is accompanied by a decrease of insulin sensitivity that has been attributed to chronological age itself and/or to a variety of secondary factors associated with the aging process, such as an increase in body fat and/or in central adiposity, and a reduction in spontaneous physical activity.[7,8] The decline of insulin action occurs through an impairment of the insulin signaling pathway,[9] leading to the development of type 2 diabetes.

Although the underlying mechanism for age-related insulin resistance is not completely elucidated, increased oxidative stress and decreased antioxidant defenses are likely to be involved in the impairment of muscle insulin signaling.[4,10,11] Moreover, the increase of adiposity associated with aging is accompanied by changes in the levels of circulating adipokines, some of which are negative regulators of insulin signaling contributing to the development of insulin resistance.[12] Most studies in rats on aging-associated impairment of skeletal insulin sensitivity have shown a decline of the insulin signaling pathway beyond 24 months of age,[4,9,13] and some evidence points to an effect at earlier ages, at 12[14] and 20[15] months. However, the time point that may be considered the beginning of this impairment remains unclear and

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◀AU1

whether it is different between sexes. Thus, we selected male and female rats of both sexes and of three groups of age (3, 9, and 18 months) to investigate the time course of the alterations of insulin sensitivity found in aged rats (over 30 months of age).

Previous studies in our laboratory showed sex differences in mitochondrial oxidative function and oxidative stress in different tissues of rat, such as liver,[16–18] brown adipose tissue,[19,20] brain,[21] pancreas (unpublished results), and skeletal muscle,[22–24] in which female rats showed a higher mitochondrial oxidative capacity and a more effective antioxidant protection than males. Moreover, female rats have been reported to be protected from lipid-induced reductions in insulin action compared to male rats[25] as well as to show a better response of skeletal muscle insulin sensitivity to high-fat diet feeding than males.[24] In addition, sex-related differences in the pattern of muscle mass loss with age have been described.[26] However, as far as we know, there are no studies comparing the progression of skeletal muscle insulin sensitivity between sexes that would allow us to understand the molecular mechanisms underlying the different incidence of type 2 diabetes found in humans.[27]

The aim of this study was to investigate whether the differences between sexes found in the systemic insulin sensitivity of aged rats could be due to differences in the time course of the impairment of insulin signaling pathway. We hypothesized that the changes in insulin signaling key intermediates that occur with age may show a different profile in both sexes and in different muscle types. To test this hypothesis, we investigated the progression with age of the levels of key proteins of the insulin signaling pathway in two skeletal muscles with different metabolic characteristics, one oxidative (soleus) and another glycolytic (gastrocnemius), as well as the possible sex differences in the change over time of systemic insulin-sensitivity parameters, adiposity, and skeletal muscle oxidative damage in male and female rats. The understanding of the molecular mechanisms underlying the sex differences in the progression of insulin sensitivity with age may be useful in designing strategies to prevent the development of type 2 diabetes.

Methods

Animals and diets

Animal experiments were performed in accordance with the general guidelines approved by our institutional ethics committee and European Union (EU) regulations (86/609/CEE and 2003/65/CE). Eight male and 8 female Wistar rats (Charles River, Barcelona, Spain) 3, 9, and 18 months of age, respectively, were housed 2 per cage with free access to a pelleted diet (A04, Panlab, Barcelona) and water. They were kept at 22°C under a 12-h light–dark cycle. Rats were sacrificed by decapitation after a 12-h period of fasting. Blood was collected, and serum was separated by centrifugation at $900 \times g$ for 20 min at 4°C. Adipose tissue depots were extracted, and the adiposity index was calculated from the sum of inguinal, gonadal, mesenteric, and retroperitoneal depot weights relative to 100 g of body weight.

Soleus and gastrocnemius skeletal muscles were dissected rapidly, weighed, frozen in liquid nitrogen, and stored at −80°C until processed. Pieces of muscle were homogenized

at 4°C in a proportion of 1 g of muscle in 10 mL of buffer (50 mM HEPES, 100 mM NaF, 10 mM EDTA, 1 mM Na_3VO_4, 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride [PMSF], 10 μg/mL aprotinin, 10 μg/mL leupeptin, pH 7.4).

Materials

The Accutrend® glucose cholesterol triglyceride (GCT) meter and glucose test strips were supplied by Roche Diagnostics (Basel, Switzerland). Enzyme immunoassay kits were used for measurement of rat serum insulin (Mercodia, Uppsala, Sweden), and total and high-molecular-weight adiponectin (Phoenix Pharmaceuticals Inc., Belmont, CA, and Biovendor, Heidelberg, Germany, respectively). The Oxyblot™ Protein Oxidation Detection kit and rabbit polyclonal antibodies to rat UCP3 (cat. no. AB4036) and GLUT4 (cat. no. sc-7938) were purchased from Chemicon International (Temecula, CA). Insulin receptor-β (IR-β) antibody (cat. no. 611277) was from BD Biosciences (San Jose, CA). IRS-1 (cat. no. 2382); Akt (cat. no. 9272) and phosphorylated-Akt (p-Akt, cat. no. 9271) antibodies were obtained from Cell Signaling Technology (Danvers, MA). The chemiluminescence kit (ECL) for immunoblot development was purchased from Bio-Rad (Hercules, CA). Routine chemicals were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain), and Sigma-Aldrich (St. Louis, MO).

Oral glucose tolerance test

The test was performed on the week before the sacrifice. Rats were fasted for 12 h and then glucose (2 g/kg body weight) was given orally. Blood was collected from the tail vein just before glucose administration and after 15, 30, 60, 90, and 120 min, and glucose concentrations were measured using the Accutrend® system.

Serum glucose, insulin, and adiponectin levels. Serum glucose levels were measured by using the Accutrend® system, and serum insulin and adiponectin levels were measured by enzyme immunoassay kits. Homeostasis model assessment (HOMA) was used to estimate insulin resistance (IR)[28] and was calculated as [fasting glucose (mM)×fasting insulin (μU/mL)]/22.5.

Measurements of skeletal muscle thiobarbituric acid-reactive substances (TBARS) and protein carbonyl groups. TBARS levels were measured in sample homogenates as previously described[29] and used as an index of lipid peroxidation. Protein carbonyl groups were determined as an index of protein oxidation by dot-blot detection using the OxyBlot™ Protein Oxidation Detection kit according to the manufacturer's protocol with several modifications.[21]

Western blot analysis. Homogenized samples were centrifuged for 20 min at 13,000×g at 4°C, and supernatants were collected, as previously reported.[30] Then 50 μg of soleus or gastrocnemius muscle protein were fractionated on 12% or 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and electrotransferred onto a nitrocellulose filter. At least one sample from each experimental group was loaded in every gel, and gels were run simultaneously. Rabbit polyclonal antibodies to rat IR-β, IRS-1, Akt, p-Akt, UCP3, and GLUT4 were used as primary antibodies.

Anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase antibody was used as a secondary antibody. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands were visualized with the ChemiDoc XRS system (Bio-Rad, Hercules, CA) and analyzed with the image analysis program Quantity One© (Bio-Rad, Hercules, CA). Bands revealed an apparent molecular mass of 92 kDa (IR-β), 180 kD (IRS-1), 60 kD (Akt and p-Akt), 34 kD (UCP3), and 45 kD (GLUT4).

Statistical analysis. All data are expressed as mean values ± standard error of the mean (SEM) of 8 animals per group. Statistical analyses were performed using a statistical software package (SPSS 17.0 for Windows, Inc., Chicago, IL). Statistical differences between experimental groups were analyzed by two-way analysis of variance (ANOVA) followed by least significance difference (LSD) *post hoc* test and the Student *t*-test. A *p* value of less than 0.05 was considered statistically significant.

Results

Body and skeletal muscle tissue weights and adiposity index

T1 ► Body weight and adiposity index increased with age in both sexes (Table 1). Adiposity index was lower in 9-month-old female rats than in their male counterparts, but no differences between sexes at 3 and 18 months of age were found. Gastrocnemius muscle weight increased with age in both sexes and was higher in male rats than in females. Soleus muscle weight was not modified with age and was also higher in males. Soleus and gastrocnemius relative weights decreased with age in both sexes and, as a whole, were higher in female rats than in males.

Serum glucose, insulin and adiponectin levels

Serum glucose and insulin levels and HOMA-IR values were higher in male rats than in females (Table 2) and increased with age in both sexes. When serum glucose and insulin levels were expressed as a percentage with respect to the values shown by 3-month-old animals (Fig. 1), the higher increase of serum insulin was observed between 3 and 9 months of age in males (2.3 times), but between 9 and 18 months of age in females (2.2 times), with both sexes reaching a similar percentage increase at 18 months. Both sexes showed a significant increase of glycemia at 18 months of age. Serum adiponectin levels (Table 2) rose in males between 3 and 9 months but dropped in both sexes at 18 months of age. ◄ T2 ◄ F1

Oral glucose tolerance test

In both sexes, the time-course glucose levels increased at 18 months of age (Fig. 2), showing an impaired profile of glucose tolerance compared to rats of 3 and 9 months of age. ◄ F2

Skeletal muscle oxidative damage and UCP3 levels

In gastrocnemius muscle, 18-month-old male rats showed higher TBARS levels and protein carbonyl groups than their female counterparts (Table 3), but no differences between sexes at 3 and 9 months of age were found. Age increased gastrocnemius TBARS levels and protein carbonyl groups in both sexes. Gastrocnemius UCP3 protein levels were higher in female rats than in males, although the sex effect did not reach statistical significance (*p* = 0.051). ◄ T3

In soleus, male rats of 3 and 18 months of age showed higher protein carbonyl group levels than their female counterparts. In male rats, soleus muscle protein carbonyl levels increased at the age of 18 months, whereas in females they increased

TABLE 1. BODY AND SKELETAL MUSCLE TISSUE WEIGHTS AND ADIPOSITY INDEX

	3 months	9 months	18 months	ANOVA
Body weight (g)				
Males	364 ± 14	547 ± 8	606 ± 25[a,b]	S, A, S*A
Females	199 ± 3[c]	289 ± 4[c]	391 ± 13[a,b,c]	
Adiposity index (g/100g)				
Males	5.44 ± 0.36	9.37 ± 0.69[a]	9.56 ± 0.56[a]	A, S*A
Females	4.65 ± 0.25	6.96 ± 0.61[a,c]	10.9 ± 1.1[a,b]	
Tissue weight (g)				
Gastrocnemius				
Males	4.29 ± 0.22	5.14 ± 0.16[a]	4.98 ± 0.14[a]	S, A
Females	2.57 ± 0.07[c]	3.20 ± 0.121[a,c]	3.51 ± 0.07[a,c]	
Soleus				
Males	0.248 ± 0.017	0.244 ± 0.018	0.300 ± 0.044	S
Females	0.168 ± 0.005[c]	0.152 ± 0.018[c]	0.178 ± 0.036[c]	
Relative tissue weight (g/Kg)				
Gastrocnemius				
Males	12.1 ± 0.2	9.41 ± 0.34[a]	8.31 ± 0.38[a,b]	S, A
Females	12.9 ± 0.3	11.1 ± 0.4[a,c]	8.95 ± 0.39[a,b]	
Soleus				
Males	0.679 ± 0.027	0.447 ± 0.037[a]	0.508 ± 0.081[a]	S, A
Females	0.847 ± 0.027[c]	0.525 ± 0.065[a]	0.554 ± 0.065[a]	

Adiposity index is the sum of inguinal, gonadal, mesenteric, and retroperitoneal depot weights relative to 100 g of body weight. Values are expressed as the mean ± the standard error of the mean (SEM) of 8 animals per group. Analysis of variance (ANOVA) (*p* < 0.05): S indicates sex effect, A indicates aging effect, and S*A indicates sex and aging interactive effect. *Post hoc* analysis of age (least significant difference [LSD] test; *p* < 0.05): a indicates 9- and 18-month-old rats versus 3-month-old rats; b indicates 18-month-old rats versus 9 month-old rats. Student *t*-test (*p* < 0.05): c indicates female rats versus male rats.

<p align="center">TABLE 2. SERUM GLUCOSE, INSULIN, AND ADIPONECTIN LEVELS</p>

	3 months	9 months	18 months	ANOVA
Glucose (mM)				
Males	6.61 ± 0.26	6.76 ± 0.23	$8.01 \pm 0.20^{a,b}$	S, A
Females	5.90 ± 0.21^{c}	5.69 ± 0.27^{c}	$7.76 \pm 0.28^{a,b}$	
Insulin (?U/mL)				
Males	22.5 ± 1.4	51.8 ± 10.5^{a}	68.3 ± 12.8^{a}	S, A
Females	8.36 ± 1.79^{c}	12.8 ± 3.6^{c}	$28.6 \pm 5.1^{a,b,c}$	
HOMA-IR				
Males	6.96 ± 0.50	13.6 ± 3.4^{a}	$24.4 \pm 4.7^{a,b}$	S, A
Females	2.60 ± 0.60^{c}	4.14 ± 1.72^{c}	$9.86 \pm 1.89^{a,b,c}$	
Adiponectin (ng/?L)				
Males	4.85 ± 0.34	6.52 ± 0.29^{a}	$0.399 \pm 0.051^{a,b}$	A
Females	5.38 ± 0.49	5.51 ± 0.40^{c}	$0.514 \pm 0.059^{a,b}$	

AU3 ▶ (Insulin row)
AU3 ▶ (Adiponectin row)

Homeostasis model assesment of insulin resistance (HOMA-IR) was used to estimate insulin resistance and was calculated as [fasting glucose (mM)×fasting insulin (μU/mL)]/22.5. Values are expressed as the mean $\pm$ standard error of the mean (SEM) of 8 animals per group. Analysis of variance (ANOVA) ($p < 0.05$): S indicates sex effect and A indicates aging effect. *Post hoc* analysis of age (least significant difference [LSD] test; $p < 0.05$): a indicates 9- and 18-month-old rats versus 3-month-old rats, b indicates 18-month-old rats versus 9-month-old rats. Student *t*-test ($p < 0.05$): c indicates female rats versus male rats.

earlier, at the age of 9 months, and decreased at 18 months, reaching 3-month-old rat levels. No significant sex or age effects were found in soleus TBARS or UCP3 protein levels.

Skeletal muscle insulin signaling key protein levels

Gastrocnemius IR-β protein levels were higher in 3-month-old female rats compared to males and decreased with age, whereas male rats showed an age-related increase F3 ▶ at 18 months (Fig. 3a). In soleus, age decreased IR-β protein levels in 18-month-old rats of both sexes (Fig. 3b). Gastrocnemius and soleus IRS-1 protein levels decreased with age in both genders (Fig. 3). In male rats, gastrocnemius IRS-1 protein levels decreased at 9 months of age (Fig. 3a) and soleus IRS-1 at 18 months (Fig. 3b), whereas in females IRS-1 protein levels decreased at 9 months of age in both muscles. Gastrocnemius Akt protein levels were higher in 9- and 18-month-old female rats compared to their male counterparts F4 ▶ (Fig. 4a), whereas the pAkt/Akt ratio was higher in males than in females at 3 and 9 months of age. Soleus Akt protein

levels were higher in 3-month-old female rats than in their male counterparts (Fig. 4b). In both sexes, total Akt decreased at 18 months of age, whereas p-Akt and the p-Akt/Akt ratio decreased in 9-month-old rats and then increased in 18-month-old rats, with 18 month-old female rats showing a higher ratio value than their male counterparts. Three-month-old female rats showed lower gastrocnemius GLUT4 protein levels than males (Fig. 3a). GLUT4 levels decreased with aging in both sexes. No significant sex or age effects were found in soleus GLUT4 protein levels (Fig. 3b).

Discussion

Age implies a loss of insulin sensitivity that leads to an increase of serum insulin levels to maintain glucose homeostasis. We have found notable sex differences in the profile of the increase of serum insulin levels that occurs with age. Thus, at 9 months of age, male rats need to increase their insulin levels more than females to maintain glycemia, whereas in female rats this increase occurs at 18 months of
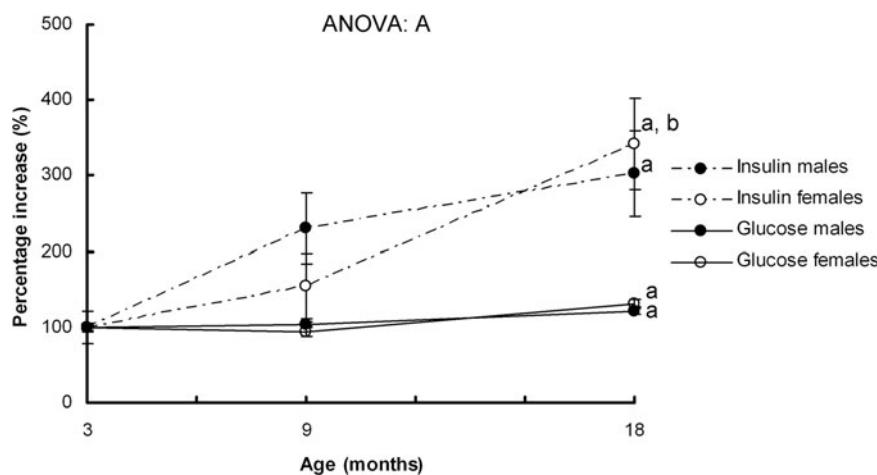
FIG. 1. Serum insulin and glucose increase with age. Values are expressed as percentages and the levels of 3-month-old male rats were set as 100%. Data are the mean $\pm$ standard error of the mean (SEM) of 8 animals per group. Analysis of variance (ANOVA) ($p < 0.05$): A indicates aging effect. *Post hoc* analysis of age (least significance difference [LSD] test; $p < 0.05$). **a** represents 9- and 18-month-old rats versus 3-month-old rats. **b** represents 18-month-old rats versus 9-month-old rats.
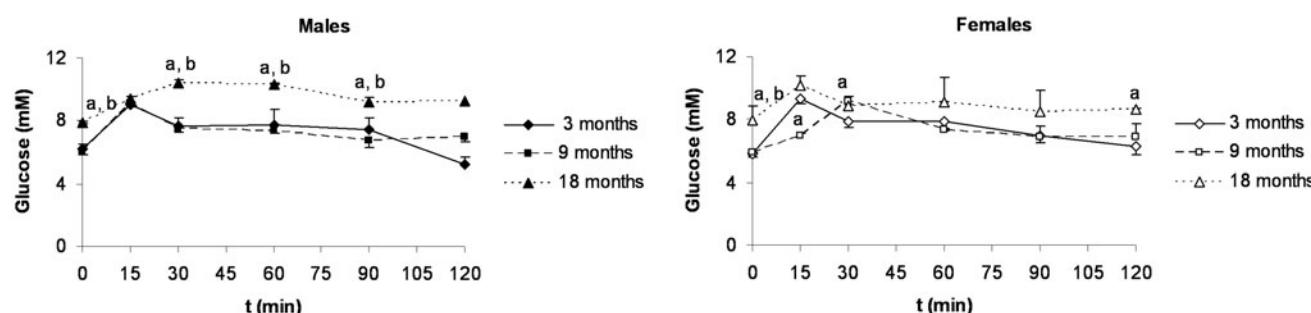
FIG. 2. Oral glucose tolerance curves. **a** and **b** represent glucose tolerance curves in male and female rats, respectively. Values are expressed as the mean ± standard error of the mean (SEM) of 8 animals per group. Analysis of variance (ANOVA) ($p < 0.05$): A indicates aging effect, S*A indicates sex, and aging interactive effect. *Post hoc* analysis of age (least significance difference [LSD] test; $p < 0.05$): **a** represents 9- and 18-month-old rats versus 3-month-old rats. **b** represents 18-month-old rats versus 9-month-old rats. Student *t*-test ($p < 0.05$). (**c**) Female rats versus male rats.

age. These results suggest that the age-associated impairment of insulin sensitivity may develop earlier in male rats than in females. Despite 18-month-old female rats reaching a similar percentage of serum insulin increase than their male counterparts, male rats maintain a serum profile of lower insulin sensitivity than females in all the age groups, as has been previously reported in both rodents[24,31] and humans.[32]

Although the underlying mechanism for age-related insulin resistance remains poorly defined, it has been proposed that increased oxidative stress and impaired antioxidant defenses are likely to be involved.[4,10,11] In our study, we found an increase of skeletal muscle oxidative damage with age, which takes place to a greater extent in male rats than in females and which is more enhanced in gastrocnemius muscle than in soleus, as levels of damage of both lipids and proteins suggested. In male rats, we observed a gradual in-

crease of oxidative damage with age, whereas in females, the increase occurs between 3 and 9 months of age and tends to decrease at 18 months of age, suggesting that female rats may develop mechanisms to counteract the age-associated increase of oxidative damage.

Since a defensive role of UCP3 against skeletal muscle oxidative stress has been described,[33] sex differences in UCP3 level variations with age reinforce the idea that female rats have a higher protection against oxidative damage than males. Moreover, the sexual dimorphism found in the increase of skeletal muscle oxidative damage associated with age may also be attributed to differences in other components of the antioxidant defenses. In fact, a greater decrease of some mitochondrial antioxidant systems with age, such as glutathione and manganese superoxide dismutase (Mn-SOD) activity, has been recently reported in male mice

TABLE 3. SKELETAL MUSCLE OXIDATIVE DAMAGE AND UCP3 PROTEIN LEVELS

	3 months	9 months	18 months	ANOVA
TBARS (nmol/mg protein)				
Gastrocnemius				
Males	0.307 ± 0.050	2.32 ± 0.23^a	2.19 ± 0.21^a	S, A, S*A
Females	0.296 ± 0.038	2.18 ± 0.13^a	$1.35 \pm 0.16^{a,b,c}$	
Soleus				
Males	4.04 ± 0.68	4.48 ± 0.59	3.93 ± 0.38	N.S.
Females	3.76 ± 0.27	3.84 ± 0.24	3.13 ± 0.20	
Protein carbonyl groups (%)				
Gastrocnemius				
Males	100 ± 25	639 ± 161^a	$1311 \pm 291^{a,b}$	S, A, S*A
Females	128 ± 33	756 ± 81^a	$662 \pm 159^{a,c}$	
Soleus				
Males	100 ± 9	78 ± 7^a	$157 \pm 19^{a,b}$	S, S*A
Females	59 ± 7^c	87 ± 10^a	$54 \pm 11^{b,c}$	
UCP3 protein levels (%)				
Gastrocnemius				
Males	100 ± 12	97.6 ± 6.5	111 ± 11	N.S.
Females	126 ± 7	106 ± 13	129 ± 10	
Soleus				
Males	100 ± 5	84.0 ± 6.6	96.5 ± 5.8	N.S.
Females	89.1 ± 6.2	79.9 ± 11.3	101.8 ± 3.1	

Values of protein carbonyl groups and UCP3 protein levels are expressed as %AU (arbitrary units), where levels of 3-month-old male rats were set as 100%. Data are the mean ± standard error of the mean (SEM) of 8 animals per group. Analysis of variance (ANOVA) ($p < 0.05$): S indicates sex effect, A indicates aging effect, and S*A indicates sex and aging interactive effect. *Post hoc* analysis of age Least significant difference [LSD] test, $p < 0.05$: a indicates 9- and 18-month-old rats versus 3-month-old rats, b indicates 18-month-old rats versus 9-month-old rats. Student *t*-test ($p < 0.05$): c indicates female rats versus male rats.
TBARS, thiobarbituric acid-reactive substances; N.S., not significant.
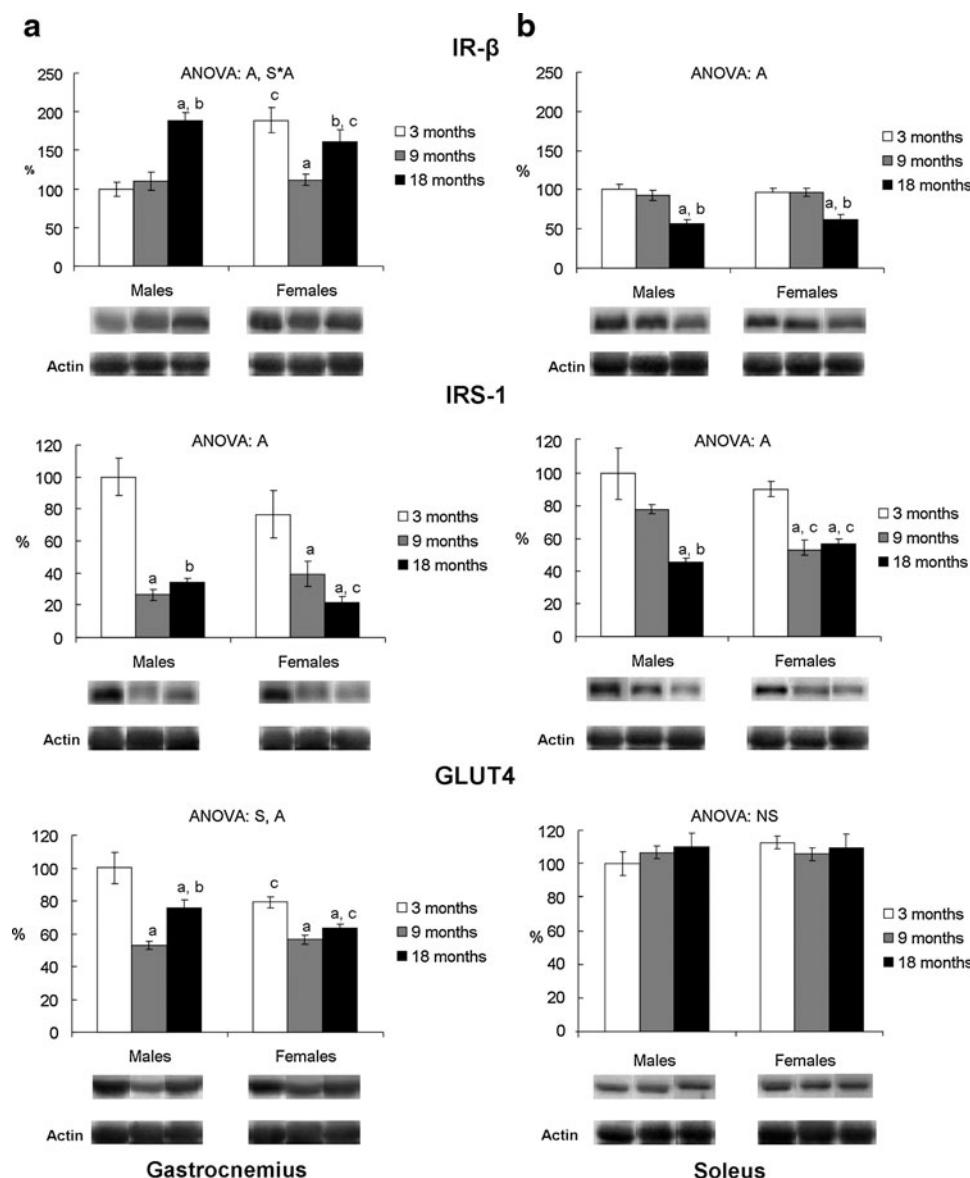
FIG. 3. Gastrocnemius (**a**) and soleus (**b**) insulin receptor-β (IR-β), insulin receptor substrate 1 (IRS-1), and glucose transporter 4 (GLUT4) protein levels. Values are expressed as %AU (arbitrary units), where levels of 3-month-old male rats were set as 100%. Data are the mean ± standard error of the mean (SEM) of 8 animals per group. Analysis of variance (ANOVA) ($p < 0.05$): S indicates sex effect, A indicates aging effect, and S*A indicates sex and aging interactive effect. *Post hoc* analysis of age (least significance difference [LSD] test; $p < 0.05$): **a** represents 9- and 18-month-old rats versus 3-month-old rats. **b** represents 18-month-old rats versus 9-month-old rats. Student *t*-test ($p < 0.05$). **c** represents female rats versus male rats.

compared to females.[34] Moreover, we have previously reported sex differences in skeletal muscle mitochondrial antioxidant enzyme capacity in rats of 6[22] and 18 months of age,[24] reinforcing the idea of a greater ability of female rats to maintain the efficiency of antioxidant enzymes compared to males.

The aforementioned higher age-associated increase of oxidative damage found in gastrocnemius muscle of both sexes compared to soleus points to a greater impact of age on glycolytic muscles compared to oxidative ones and agrees with previous studies.[35] However, neither gastrocnemius nor soleus oxidative damage was accompanied by the age-associated decline of muscle mass recently described in 30- and 36-month-old rats,[26,35] probably due to the high muscle plasticity to adapt to physiological changes and because

muscle loss may appear at more advanced ages, when this plasticity would begin to fail.

Age-related insulin resistance may also be associated to the increase of adiposity that occurs with age, which is in turn linked to physical inactivity.[7,8] The increase of adiposity is accompanied by a decrease of serum adiponectin levels, which have been reported to be negative factors of insulin-stimulated glucose uptake.[12,36] In addition, the excess of adiposity is associated to the activation of inflammatory signaling pathways, such as IκB kinase complex (IKK-β) and c-jun N-terminal kinase (JNK), which inhibit the insulin signaling pathway in skeletal muscle and contribute to age-related insulin resistance.[37–39] Interestingly, we found sex differences in the profile of age-increased adiposity, which
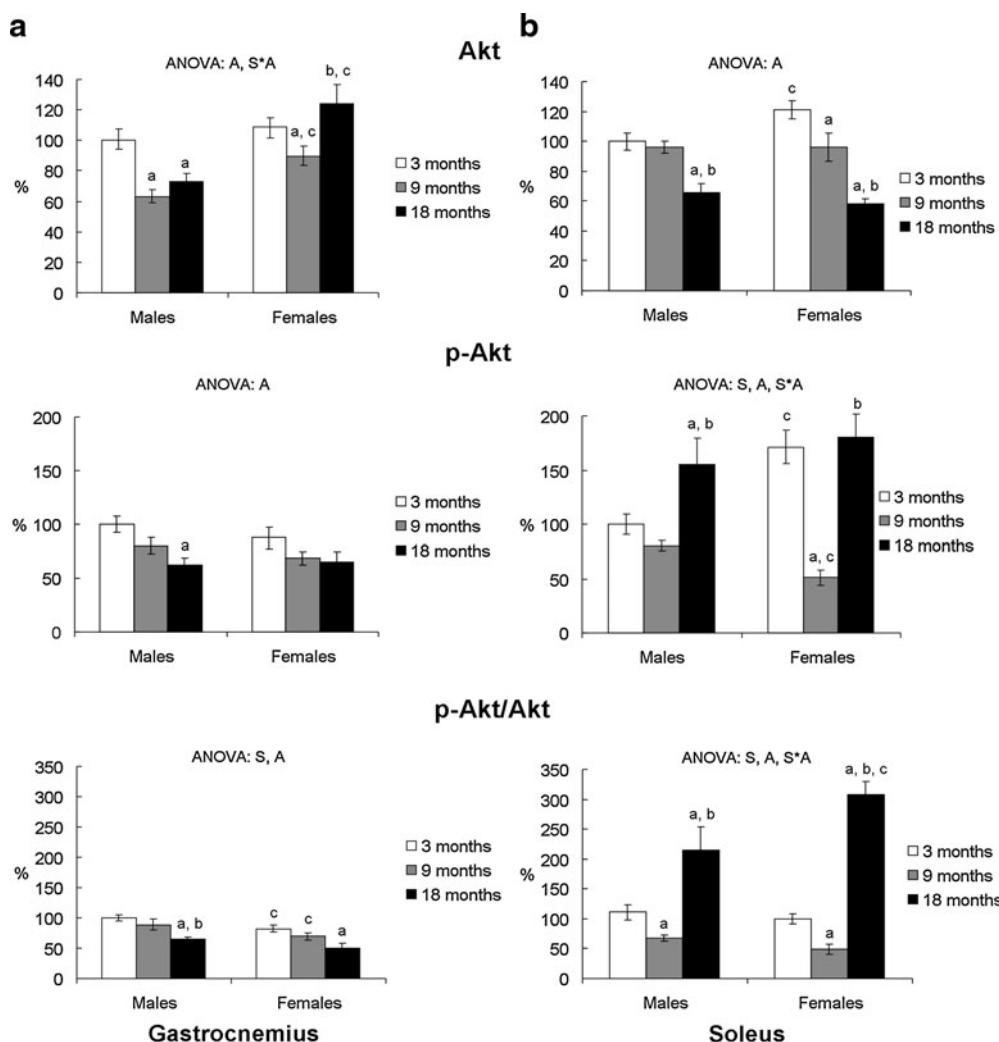
FIG. 4. Gastrocnemius (**a**) and soleus (**b**) protein kinase B (Akt) and p-Akt protein levels and p-Akt/Akt ratio. Values are expressed as %AU (arbitrary units), where levels of 3-month-old male rats were set as 100%. Data are the mean ± standard error of the mean (SEM) of 8 animals per group. Analysis of variance (ANOVA) ($p < 0.05$): S indicates sex effect, A indicates aging effect, and S*A indicates sex and aging interactive effect. *Post hoc* analysis of age (least significance difference [LSD] test; $p < 0.05$): **a** represents 9- and 18-month-old rats versus 3-month-old rats. **b** represents 18-month-old rats *vs* 9-month-old rats. Student *t*-test ($p < 0.05$). **c** represents female rats versus male rats.

could be involved in the sexual dimorphism found in age-associated impairment of insulin sensitivity.

The adiposity index increases with age following a similar profile to that of serum insulin levels, with male rats showing the greatest increase of adiposity at 9 months of age (72% increase in males and 50% increase in females with respect to their 3-month-old counterparts), whereas females exhibit it at 18 months (2% increase in males and 57% increase in females with respect to their 9-month-old counterparts). Because it has been shown in rats that age-associated impairment of insulin-stimulated glucose uptake occurs earlier in adipose tissue, at 8 months of age, than in skeletal muscle, at 24 months of age,[12] the greater increase of adiposity shown by male rats at 9 months of age would reinforce the idea of an earlier impairment of insulin sensitivity in male rats than in females.

Although tissues such as liver and white adipose tissue also contribute to the impairment of systemic insulin sensitivity, skeletal muscle is considered the most important. In our study, sex differences in the profile of insulin sensitivity with age do not exactly match the time course of the levels of key proteins of the skeletal muscle insulin signaling pathway, above all in the early ages. At 18 months of age, the higher gastrocnemius Akt protein levels and soleus Akt activation shown by female rats could be a response to attempt to maintain GLUT4 levels and may explain their higher capacity to counteract insulin resistance. This result is in concordance with other studies[26,40] in which Akt phosphorylation in 26- and 30-month-old female rats is associated with the maintenance of muscle mass with aging.

Thus, sexual dimorphism in insulin sensitivity could be related to changes in insulin signaling key protein levels, mainly in the most aged rats, suggesting that sex differences in these proteins would be manifested at more advanced ages than differences in the insulin-sensitivity serum profile probably, because both sexes may develop different strategies to attempt to counteract aging-associated insulin resistance. Nevertheless, an earlier impairment of white adipose

tissue insulin sensitivity prior to that of skeletal muscle could also contribute to the alterations of the systemic insulin resistance markers.[9]

Soleus and gastrocnemius muscles showed a different response to the effect of age on the insulin signaling pathway. Soleus muscle seemed to be better adapted than gastrocnemius to counteract age-associated changes in insulin sensitivity, as shown by levels of insulin signaling protein intermediates. Age brought about an impairment of gastrocnemius insulin signaling in both sexes, as indicated by the decrease of IRS-1, p-Akt/Akt ratio, and GLUT4 protein levels. However, IR-β levels did not decrease with age, supporting the idea that insulin signaling impairment with age could occur downstream of the insulin receptor, in agreement with a previous study.[13]

The increase of IR-β levels observed only in the most aged rats could be understood as an attempt to counteract the impairment of insulin signaling by increasing their muscle insulin receptor levels. In soleus, although age decreased levels of IR-β, IRS-1, and total Akt proteins, insulin sensitivity could not have been compromised, as the maintenance of GLUT4 levels suggests, although the glucose uptake actually depends on GLUT4 translocation. Unfortunately, direct measurement of skeletal muscle glucose uptake has not been performed, which constitutes a limitation of our study. The enhanced Akt activation shown by the soleus muscle of the oldest group could be a response to counteract the decrease of the other insulin signaling intermediates. The different response that soleus and gastrocnemius muscles showed to the effect of age on insulin signaling was in agreement with a previous study reporting a greater age-associated insulin resistance impact in glycolytic than in oxidative muscle in rats of 24 months of age.[4]

These data support the idea that insulin signaling seems to be more compromised in muscles composed primarily of glycolytic than oxidative fibers,[4,5] probably due to the preferential atrophy of fast-twitch fibers with age[4,6,9,41] and the greater decrease in expression of insulin signaling intermediates compared to slow-twitch fibers.[4] We found this different response to age of gastrocnemius and soleus muscles in up to 18-month-old rats, suggesting that differences found in the impact of age on the insulin signaling pathway may be evident prior to aging.

In summary, we have studied the changes in insulin sensitivity with age in male and female rats to discriminate whether the differences between sexes in the profile of age-associated variations of adiposity, skeletal muscle oxidative damage, and/or levels of insulin signaling pathway components may explain the sex differences in systemic insulin sensitivity previously found in aged rats. Overall, insulin sensitivity decreases with age in both sexes, and sex differences in both age-related increase of insulinemia and adiposity suggest an earlier impairment of insulin sensitivity in male rats than in females. However, sex differences in skeletal muscle oxidative damage and insulin signaling key protein levels were found only in the most aged rats. Thus, 18-month-old female rats showed a greater ability to respond to the impairment of the insulin signaling pathway than their male counterparts. In addition, gastrocnemius muscle showed more age-associated oxidative damage and insulin resistance impact than soleus. These results suggest that sex differences found in the age-associated impairment of insulin

sensitivity would not be attributable to a different profile of age-associated alterations in the skeletal muscle insulin signaling pathway, probably because sex differences in the decline of insulin signaling proteins are manifested at more advanced ages.

Author Disclosure Statement

No competing financial interests exist.

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Manuscrito IV.

Long-term high-fat-diet feeding induces skeletal muscle mitocondrial biogenesis in rats in a sex-dependent and muscle-type specific manner

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Long-term high-fat-diet feeding induces skeletal muscle mitochondrial biogenesis in rats in a sex-dependent and muscle-type specific manner

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Running title: High-fat-diet induces muscle mitochondrial biogenesis

ABSTRACT

Mitochondrial dysfunction is thought to play a crucial role in the etiology of insulin resistance, in which skeletal muscle is the main tissue contributor. Sex differences in skeletal muscle insulin and antioxidant responses to high fat diet (HFD) feeding have been described. The aim of this study was to elucidate whether there is a sex dimorphism in the effects of HFD feeding on skeletal muscle mitochondrial biogenesis and on the adiponectin signaling pathway, as well as the influence of the muscle type (oxidative or glycolytic). Gastrocnemius and soleus muscles of male and female Wistar rats of 2 months of age fed with a HFD or a low fat diet for 26 weeks were used. Mitochondrial biogenesis and oxidative damage markers, oxidative capacity and antioxidant defences were analyzed. Serum insulin sensitivity parameters and the levels of proteins involved in adiponectin signaling pathway were also determined. HFD feeding induced mitochondrial biogenesis in both sexes, but to a higher degree in male rats. Although HFD female rats showed greater antioxidant protection and maintained a better insulin sensitivity profile than their male counterparts, both sexes showed an impaired response to adiponectin, which was more evident in gastrocnemius muscle. We conclude that HFD rats may induce skeletal muscle mitochondrial biogenesis as an attempt to compensate the deleterious consequences of adiponectin and insulin resistance on oxidative metabolism, and that the effects of HFD feeding are sex-dependent and muscle-type specific.

KEY WORDS:

Gastrocnemius, soleus, insulin sensitivity, adiponectin, PGC-1 α, TFAM, UCP3.

INTRODUCTION

Insulin resistance is a major risk factor for developing type 2 diabetes, which is caused by the inability of insulin-target tissues to respond properly to insulin (Choi et al. 2010), and in whose etiology mitochondrial dysfunction is thought to play a crucial role (Johannsen et al. 2009; van den Broek et al. 2010). Skeletal muscle is the main tissue responsible for the insulin-stimulated disposal of glucose and is the main contributor to the development of insulin resistance in type 2 diabetes (Stump et al. 2006).

Skeletal muscle is a heterogeneous tissue made up of different contractile fibre types, in which the relative importance of glycolysis and mitochondrial oxidative phosphorylation for energy production varies. Glycolytic muscles are mainly composed of fast twitch or fast glycolytic fibres and generate energy by means of anaerobic metabolic processes, whereas oxidative muscles have a high proportion of slow twitch or slow oxidative fibres, are very resistant to fatigue and obtain energy through oxidative metabolic processes (Zierath et al. 2004; Matsakas et al. 2009). Under normal feeding conditions, glycolytic muscles use either glucose or fatty acids as a substrate, whereas oxidative muscles are highly dependent upon lipids (Samec et al. 1998). Because of the differences between muscle types in energy demand and reliance on mitochondrial oxidative activity, differences in mitochondrial function can not be ruled out.

Skeletal muscle oxidative capacity is mainly determined by mitochondrial function and biogenesis (Chanseaume et al. 2009). Mitochondrial biogenesis involves both proliferation and differentiation processes, which imply an increase in mitochondrial content and an improvement of the functional capabilities of pre-existing mitochondria, respectively (Ostronoff et al. 1996). Mitochondrial biogenesis requires the coordinate participation of both mitochondrial and nuclear genomes (Fernandez-Silva et al. 2003) through numerous transcription factors. Peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) coactivates different transcription factors in response to energy requirements resulting in the activation of nuclear genes involved in mitochondrial biogenesis. Among them, mitochondrial transcription factor A (TFAM) is one of the regulatory factors needed for proper transcription of mtDNA and of the genes encoding subunits of respiratory complexes (Gleyzer et al. 2005; Scarpulla 2006). Mitochondrial dysfunction has been proposed to be involved in the alteration of oxidative metabolism associated to insulin resistance. However, the cause-and-effect relationship between mitochondrial dysfunction and the development of insulin

resistance remains unclear (Johannsen et al. 2009; Abdul-Ghani et al. 2010; van den Broek et al. 2010).

High-fat diet feeding (HFD) leads to obesity and insulin resistance (Schrauwen 2007). Adiponectin is a hormone secreted by adipocytes that circulates in high concentrations in serum and plays an important role in the regulation of mitochondrial biogenesis and insulin sensitivity (Civitarese et al. 2007; Yamauchi et al. 2008). Adiponectin binds to its receptors (AdipoR1, the most abundantly expressed in skeletal muscle, and AdipoR2) activating 5'-AMP-activated protein kinase (AMPK), which finally leads to the stimulation of glucose uptake and fatty acid oxidation. AMPK has also been implicated in the regulation of PGC-1α, the master regulator of mitochondrial biogenesis (Civitarese et al. 2007; Yamauchi et al. 2008).

Sex differences have been previously described in mitochondrial biogenesis of skeletal muscle (Colom et al. 2007a) and of other tissues, such as liver (Justo et al. 2005a; Nadal-Casellas et al. 2010), brain (Guevara et al. 2009), heart (Colom et al. 2007b) and brown adipose tissue (Rodriguez-Cuenca et al. 2002; Justo et al. 2005b). Moreover, we have also reported a higher skeletal muscle antioxidant capacity and a better insulin sensitivity profile in response to high fat diet (HFD) feeding in female rats compared to males (Gomez-Perez et al. 2008). This sex dimorphism led us to extend our study to mitochondrial biogenesis as a possible origin of these differences. Thus, the aim of the present study was to elucidate whether there is a sex dimorphism in the effects of HFD feeding on muscle mitochondrial biogenesis and the adiponectin signaling pathway, and whether these effects are dependent on muscle type.

METHODS AND MATERIALS

Animals and diets

Animal experiments were performed in accordance with the general guidelines approved by EU regulations (86/609/EEC and 2003/65/CE) and our institutional ethics committee. Male and female Wistar rats of 2 months of age (Charles River, Barcelona, Spain) were housed two per cage with free access to food and water and were kept at 22ºC under a 12-hour light-dark cycle. Both male and female rats were divided into two groups (8-10 rats per group) with a similar body weight (333 ± 4g for male rats and 215 ± 4g for female rats) and were fed a low fat diet (3,385 Kcal/Kg diet; 2.9% fat by

weight; A04, Panlab, Barcelona) or a high fat diet (HFD, 3,876 Kcal/Kg diet; 26% fat by weight) for 26 weeks. The HFD (namely cafeteria diet) components were cookies, pork liver pâté, fresh bacon, chocolate and ensaïmada (a typical Majorcan pastry). The energy composition of the HFD was 13% protein, 33% carbohydrate and 54% lipid, whereas the low fat diet (A04, Panlab, Barcelona, Spain) was 19% protein, 73% carbohydrate and 8% lipid. Animal body weights were assessed weekly and food intake fortnightly throughout the dietary treatment (animal final body weights were: 546 ±9g for control male rats, 675 ± 25g for HFD male rats, 295 ± 7g for control female rats and 462 ± 24g for HFD female rats). Rats were sacrificed by decapitation after a 12-hour-period of fasting. Blood was collected and soleus and gastrocnemius skeletal muscles were rapidly dissected and weighed. Serum samples and a piece of each muscle were frozen in liquid N_2 and stored at −80°C until analyzed; the rest of the tissues were immediately processed. Pieces of muscle were homogenized at 4ºC in a proportion of 1g of muscle in 10ml of buffer (50mM HEPES, 100mM NaF, 10mM EDTA, 1mM Na_3VO_4, 1% Triton X-100, 2mM PMSF, 10μg/ml aprotinin, 10μg/ml leupeptin, pH 7.4).

Materials

Accutrend® GCT-meter and glucose and triglyceride test strips were supplied by Roche Diagnostics (Basel, Switzerland). Enzyme immunoassay kits were used for measurement of rat serum insulin (Mercodia, Uppsala, Sweden), and total and high molecular weight adiponectin (Phoenix Pharmaceuticals Inc., Belmont, CA, USA and Biovendor, Heidelberg, Germany, respectively). The kit for measurement of triglycerides was acquired from Linear Chemicals SL (Barcelona, Spain). Oxyblot™ Protein Oxidation Detection kit and antibodies to rat UCP3 (Cat. Num. AB4036) and PGC-1α (Cat. Num AB3242) were purchased from Chemicon International (Temecula, CA, USA). AdipoR1 antibody (Cat. Num. ADIPOR12-A) was from Alpha Diagnostic International (San Antonio, TX, USA). AMPKα (Cat. Num. 2532) and p-AMPKα (Cat. Num. 2531) antibodies were from Cell Signaling Technology (Danvers, MA, USA). TFAM antibody was kindly provided by Dr. H. Inagaki (Inagaki et al. 2000). COXII antibody (Cat. Num sc-23984) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and COXIV antibody (Cat. Num MS407) was from MitoSciences (Eugene, OR, USA). Mn-SOD (Cat. Num 574596) and Cu-SOD (Cat. Num 574597) antibodies were obtained from Calbiochem (San Diego, CA, USA). Chemiluminescence kit (ECL) for

immunoblot development was purchased from BioRad (Hercules, CA, USA). Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain) and Sigma-Aldrich (St. Louis, MO, USA).

Serum glucose, insulin, adiponectin and triglyceride levels

Serum parameters were measured using the Accutrend® system (glucose and triglyceride levels) and enzyme immunoassay kits (insulin and adiponectin levels). Homeostasis Model Assessment HOMA-IR was used to estimate insulin resistance (Pickavance et al. 2007) and was calculated as [fasting glucose (mM) x fasting insulin (μU/mL)]/22.5.

Skeletal muscle composition

Total protein was determined in homogenates as previously described (Bradford 1976). Triglycerides were measured spectrophotometrically in homogenates with a commercial kit.

Measurements of skeletal muscle thiobarbituric acid-reactive substances (TBARS) and protein carbonyl groups

TBARS levels were measured as previously described (Slater 1971) and used as an index of lipid peroxidation. Protein carbonyl groups were determined as index of protein oxidation by Dot-Blot detection using the OxyBlot™ Protein Oxidation Detection Kit according to the manufacturer's protocol with several modifications (Guevara et al. 2009).

Western blot analysis

Homogenized samples were centrifuged for 20 min at 13,000xg and at 4ºC and supernatants were collected as previously reported (Sloniger et al. 2005). Fifty μg of soleus and gastrocnemius homogenate protein was fractionated on 10 or 12% SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Rabbit polyclonal antibodies to rat AdipoR1, AMPKα, p-AMPKα, UCP3, PGC-1α and TFAM, goat polyclonal antibody against COXII and mouse monoclonal antibody against COXIV were used as primary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands were visualized with the ChemiDoc XRS

system (Bio-Rad, CA, USA) and analyzed with the image analysis program Quantity one© (Bio-Rad, CA, USA). Bands revealed an apparent molecular mass of 18kDa (COXIV), 25kDa (TFAM), 27.5kDa (COXII), 37kDa (UCP3), 42kDa (AdipoR1), 62kDa (AMPKα and p-AMPKα) and 92kDa (PGC-1α).

Statistical analysis

All data are expressed as mean values ± SEM of 8-10 animals per group. Statistical analyses were performed using a statistical software package (SPSS 19.0 for Windows, Inc., Chicago, IL, USA). Statistical differences between experimental groups were analyzed by two-way analysis of variance (ANOVA) followed by Student's t-test as a post-hoc comparison. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Energy intake, biometrical parameters and skeletal muscle composition
Control female rats showed a higher energy intake and lower body weight gain than males (Table 1). HFD feeding increased energy intake and body weight in both sexes and the body weight increase was higher in female rats than in males. Gastrocnemius and soleus muscle weights (Table 2) were higher in male rats compared to females and increased with HFD feeding in female rats. Relative gastrocnemius weight was higher in control female rats than in their male counterparts and decreased with HFD feeding in both sexes. Non significant sex and HFD effects were found in the relative weight of soleus muscle. Soleus triglyceride content was higher in male rats than in females and increased with HFD feeding in female rats. Non significant sex and HFD effects were found in gastrocnemius triglyceride levels. Soleus protein content decreased in male rats with HFD feeding and their values were lower than those of their female counterparts. Non significant sex and HFD effects were found in gastrocnemius protein content.

Table 1. Energy intake and body weight gain

	Control	HFD	ANOVA
Energy intake (Kcal/Kg day)			
male	169 ± 7	269 ± 11^a	S, D
female	294 ± 18^b	332 ± 40	
Body weight gain (%)			
male	64.1 ± 2.2	107 ± 7^a	S*D
female	40.0 ± 2.0^b	112 ± 11^a	

Body weight gain is calculated relative to body weight at the beginning of the treatment. Values are expressed as the mean ± S.E.M of eight animals per group. ANOVA (p<0.05): S sex effect, D HFD effect, S*D sex and HFD interactive effect and NS non significant. Student's t-test (p<0.05): [a] HFD *vs* control, [b] female *vs* male.

Table 2. Skeletal muscle weight and composition

	Gastrocnemius			Soleus		
	Control	HFD	ANOVA	Control	HFD	ANOVA
Tissue weight (g)						
male	5.14 ± 0.16	5.23 ± 0.08	S	0.244 ± 0.018	0.262 ± 0.024	S, D
female	3.20 ± 0.12^b	$3.59 \pm 0.11^{a, b}$		0.152 ± 0.018^b	0.226 ± 0.014^a	
Relative tissue weight (g/Kg)						
male	9.27 ± 0.33	8.01 ± 0.29^a	S, D, S*D	0.440 ± 0.035	0.401 ± 0.039	NS
female	11.1 ± 0.4^b	7.88 ± 0.37^a		0.525 ± 0.065	0.497 ± 0.035	
Triglyceride (mg/g tissue)						
male	6.88 ± 0.89	8.79 ± 1.77	NS	22.2 ± 3.2	19.1 ± 1.5	S, S*D
female	7.78 ± 1.85	10.9 ± 1.3		12.5 ± 1.6^b	18.7 ± 1.0^a	
Protein (mg/g tissue)						
male	81.6 ± 2.4	84.7 ± 3.8	NS	76.3 ± 1.9	60.8 ± 1.4^a	D
female	81.5 ± 1.8	78.7 ± 4.7		75.2 ± 4.0	69.8 ± 2.1^b	

Values are expressed as the mean ± S.E.M of eight animals per group. ANOVA (p<0.05): S sex effect, D HFD effect, S*D sex and HFD interactive effect and NS non significant. Student's t-test (p<0.05): [a] HFD *vs* control, [b] female *vs* male.

Serum parameters

Serum insulin, triglyceride and total adiponectin levels and HOMA-IR values were higher in control male rats than in females (Table 3), but increased with HFD feeding only in female rats. Serum high molecular weight (HMW) adiponectin levels decreased with HFD feeding in male rats, and HFD female rats showed higher levels than HFD males. HMW adiponectin/total adiponectin ratio, which represents the proportion of the most active form of adiponectin for insulin sensitizing effects (Yamauchi et al. 2008), decreased with HFD feeding only in males and, as a consequence, values of HFD male rats were lower than those of their female counterparts.

Table 3. Serum parameters

	Control	*HFD*	*ANOVA*
Glucose (mM)			
male	6.76 ± 0.23	6.34 ± 0.20	NS
female	5.95 ± 0.37	6.37 ± 0.36	
Insulin (µg/L)			
male	1.79 ± 0.36	1.92 ± 0.47	S
female	0.442 ± 0.124^{b}	$0.976 \pm 0.151^{a, b}$	
HOMA-IR			
male	14.6 ± 2.4	11.4 ± 2.2	S, S*D
female	2.45 ± 0.64^{b}	$7.01 \pm 0.70^{a,b}$	
Total Adiponectin (ng/µL)			
male	5.86 ± 0.20	5.97 ± 0.46	D, S*D
female	5.06 ± 0.39^{b}	6.49 ± 0.31^{a}	
HMW Adiponectin (ng/ µL)			
male	3.86 ± 0.53	2.39 ± 0.32^{a}	S*D
female	3.63 ± 0.36	4.11 ± 0.40^{b}	
HMW Adiponectin/ Total Adiponectin			
male	0.655 ± 0.078	0.405 ± 0.054^{a}	S, D
female	0.773 ± 0.121	0.636 ± 0.053^{b}	
Triglyceride (g/L)			
male	2.58 ± 0.25	2.43 ± 0.20	D, S*D
female	1.67 ± 0.14^{b}	2.68 ± 0.21^{a}	

HOMA-IR was calculated as [fasting glucose (mM) x fasting insulin (µU/mL)]/22.5. Values are expressed as the mean ± S.E.M of ten animals per group. ANOVA (p<0.05): S sex effect, D HFD effect, S*D sex and HFD interactive effect and NS non significant. Student's t-test (p<0.05): [a] HFD *vs* control, [b] female *vs* male.

Gastrocnemius and soleus AdipoR1, AMPK and p-AMPK protein levels

Gastrocnemius and soleus muscle AdipoR1 protein levels (Table 4) increased with HFD feeding in both sexes, and no significant differences between sexes were found. In gastrocnemius muscle, HFD feeding increased AMPK levels, to a higher degree in female rats (237%) than in males (143%). The p-AMPK/AMPK ratio, which represents the proportion of active AMPK, decreased in both sexes (the decrease was 54% in male rats and 73% in females). No differences between sexes in these parameters were found in control rats. In soleus muscle, control male rats showed a higher p-AMPK/AMPK ratio than females. In response to HFD feeding, male rats increased AMPK and p-AMPK protein levels and maintained p-AMPK/AMPK ratio, which is lower in female rats, unaltered.

Table 4. Skeletal muscle AdipoR1, AMPK and p-AMPK protein levels

	Gastrocnemius			*Soleus*		
	Control	*HFD*	*ANOVA*	*Control*	*HFD*	*ANOVA*
AdipoR1						
male	100 ± 20	223 ± 12[a]	D	100 ± 9	185 ± 15[a]	D
female	76.1 ± 23.0	238 ± 14[a]		82.1 ± 8.1	169 ± 20[a]	
AMPK						
male	100 ± 13	243 ± 32[a]	D	100 ± 8	146 ± 11[a]	S, D
female	100 ± 16	337 ± 24[a, b]		145 ± 12[b]	164 ± 14	
p-AMPK						
male	100 ± 7	118 ± 20	NS	100 ± 14	133 ± 13[a]	D
female	122 ± 21	127 ± 23		99.1 ± 4.4	113 ± 6[a]	
p-AMPK/AMPK						
male	100 ± 11	46.0 ± 5.5[a]	D	100 ± 9	95.4 ± 13.3	S
female	132 ± 27	35.5 ± 5.8[a]		70.3 ± 5.7[b]	73.7 ± 9.1	

Values of protein levels are expressed as %AU (arbitrary units), where control male rats were set as 100%. Data are expressed as the mean ± S.E.M of eight animals per group. ANOVA ($p<0.05$): S sex effect, D HFD effect and NS non significant. Student's t-test ($p<0.05$): [a] HFD *vs* control, [b] female *vs* male.

Gastrocnemius and soleus muscles mitochondrial biogenesis markers

In gastrocnemius, control female rats showed higher COXIV protein and mitochondrial DNA levels than their male counterparts (Table 5), whereas no differences between sexes were found in PGC-1α, TFAM or COXII protein levels. HFD feeding increased TFAM levels in both sexes, PGC-1α levels in male rats and COXII protein levels in female rats. In contrast, HFD feeding decreased COXIV protein levels in female rats.

In soleus of control rats, TFAM protein levels were higher in females than in males, but no differences between sexes were found in PGC-1α, COXII or COXIV protein levels. HFD feeding increased PGC-1α and TFAM levels in both sexes and COXIV levels in male rats. No statistically significant differences were found in COXII protein levels.

Table 5. Skeletal muscle PGC-1, TFAM, COXII and COXIV protein levels

	Gastrocnemius			*Soleus*		
	Control	*HFD*	*ANOVA*	*Control*	*HFD*	*ANOVA*
PGC-1						
male	100 ± 31	294 ± 74^a	D	100 ± 7	199 ± 50^a	D
female	97.6 ± 31.3	167 ± 41		85.4 ± 12.3	301 ± 65^a	
TFAM						
male	100 ± 15	304 ± 45^a	S, S*D	100 ± 10	159 ± 17^a	D
female	103 ± 14	$198 \pm 15^{a,b}$		128 ± 8^b	190 ± 32^a	
COXII						
male	100 ± 9	114 ± 7	D	100 ± 7	114 ± 4	NS
female	102 ± 4	$128 \pm 5^{a,\ b}$		100 ± 11	111 ± 8	
COXIV						
male	100 ± 6	97 ± 12	D, S*D	100 ± 8	158 ± 19^a	D
female	126 ± 10^b	$64 \pm 6^{a,\ b}$		113 ± 11	133 ± 12	

Values of protein levels are expressed as %AU (arbitrary units), where control male rats were set as 100%. Values are expressed as the mean ± S.E.M of eight animals per group. ANOVA (p<0.05): S sex effect, D HFD effect, S*D sex and HFD interactive effect and NS non significant. Student's t-test (p<0.05): [a] HFD *vs* control, [b] female *vs* male.

Gastrocnemius and soleus oxidative damage and UCP3 levels

In gastrocnemius muscle, Mn-SOD (Table 6) was higher in control female rats than in their male counterparts. No differences between sexes were found in TBARS, protein carbonyl groups, Cu-SOD or UCP3 levels in control rats. HFD feeding increased UCP3 levels in both sexes and protein carbonyl groups in male rats. In contrast, HFD feeding decreased Mn-SOD protein levels in both sexes — with HFD male rats showing lower levels than their female counterparts ─ and TBARS levels in female rats.

In soleus muscle, control female rats showed higher UCP3 protein levels and lower TBARS levels than their male counterparts. HFD feeding increased protein carbonyl groups in both sexes, but to a higher degree in male rats (376% vs 35%), and UCP3, Cu-SOD and TBARS levels in male rats. In contrast, HFD feeding decreased TBARS levels in female rats.

Table 6. Skeletal muscle TBARS levels, protein carbonyl groups and Mn-SOD and Cu-SOD protein levels

	Gastrocnemius			*Soleus*		
	Control	*HFD*	*ANOVA*	*Control*	*HFD*	*ANOVA*
TBARS						
male	2.41 ± 0.3	2.11 ± 0.28	S	3.27 ± 0.25	4.47 ± 0.14^a	S, S*D
female	1.86 ± 0.30	$1.10 \pm 0.15^{a, b}$		2.28 ± 0.28^b	$1.40 \pm 0.16^{a,b}$	
Carbonyl groups						
male	100 ± 12	169 ± 32^a	D	100 ± 9	476 ± 101^a	S, D, S*D
female	94 ± 19	125 ± 33		100 ± 10	$135 \pm 13^{a,b}$	
Mn-SOD						
male	100 ± 6	76.2 ± 8.3^a	S, D	100 ± 14	109 ± 15	NS
female	124 ± 5^b	$91.0 \pm 8.1^{a, b}$		92 ± 11	107 ± 8	
Cu-SOD						
male	100 ± 5	80.8 ± 5.9^a	S	100 ± 10	144 ± 10^a	D
female	101 ± 5	108 ± 10^b		112 ± 12	129 ± 8	
UCP3						
male	100 ± 17	244 ± 19^a	D	100 ± 8	156 ± 17^a	S*D
female	136 ± 44	270 ± 27^a		148 ± 22^b	118 ± 18	

TBARS levels are expressed in nmol/mg protein. Protein carbonyl groups, Mn-SOD, Cu-SOD and UCP3 protein levels are expressed as %AU (arbitrary units), where control male rats were set as 100%. Values are expressed as the mean $\pm$ S.E.M of eight animals per group. ANOVA (p<0.05): S sex effect, D HFD effect, S*D sex and HFD interactive effect and NS non significant. Student's t-test (p<0.05): [a] HFD *vs* control, [b] female *vs* male.

DISCUSSION

HFD feeding induces skeletal muscle mitochondrial biogenesis in both sexes, as the increase of both PGC-1α and TFAM protein levels suggest. Increased levels of PGC-1α point to the induction of skeletal muscle oxidative capacity, since PGC-1α has been identified as a master regulator of mitochondrial biogenesis in skeletal muscle (Olesen et al. 2010). This response could be understood as an adaptation aimed to counteract the elevated amount of substrate available. It is in agreement with previous studies developed in male rats, in which HFD feeding induced skeletal muscle oxidative capacity by increasing the expression of mitochondrial PGC-1α and respiratory chain units (Turner et al. 2007) or mitochondria number (van den Broek et al. 2010), although these studies did not compare differences between sexes.

In gastrocnemius muscle, the effect of HFD feeding on mitochondrial biogenesis is sex-dependent, with male rats showing more marked increases of both PGC-1α and TFAM levels compared to females (194 vs 71% and 204 vs 92%, respectively). These results point to enhanced mitochondrial biogenesis in male rats in order to increase gastrocnemius muscle oxidative capacity. The HFD-feeding-induced mitochondrial biogenesis is more attenuated in female rats, and could be related to their greater capacity to expand the white adipose tissue, to prevent ectopic fat deposition in other tissues and, subsequently, tissue dysfunction (Virtue et al. 2010). The higher adiposity index that HFD female rats show compared to their male counterparts (Nadal-Casellas et al. 2010) points to a greater lipid storage capacity of adipose tissue that would protect skeletal muscle from deposition of toxic lipids. The lower skeletal muscle TBARS levels shown by HFD female rats compared to males further supports this idea. This greater adipose tissue expandability would make the development of strategies to avoid the detrimental effects of lipotoxicity less necessary in female rats. Moreover, it would likely to be associated with their reproductive function. During evolution, mammalian females have developed mechanisms to handle their energy resources more efficiently than males to facilitate their progeny's survival and their own (Hoyenga et al. 1982).

Oxidative muscles are more prone to accumulate lipids than glycolytic ones (van den Broek et al. 2010). In this sense, in our study, soleus muscle accumulated more lipids than gastrocnemius. We also found a sex dimorphism in skeletal muscle oxidative damage as an effect of HFD feeding, especially in soleus muscle, with male rats showing greater levels of the oxidative damage main parameters.

In both sexes, the marked increase of UCP3 levels found in gastrocnemius muscle rats in response to HFD feeding accompanies the aforementioned induction of mitochondrial differentiation and could be aimed at compensating the decreased levels of antioxidant enzymes. These results support the proposed role of PGC-1α, which is also increased, in the improvement of skeletal muscle antioxidant defence by regulating the expression of UCP3 (Olesen et al. 2010), which plays an important role in the protection of mitochondria against increased ROS production derived from enhanced fat oxidation (Bezaire et al. 2007). A similar effect of HFD feeding on gastrocnemius muscle enhancing UCP3 expression has been previously reported in 6 month-old male and female rats (Catala-Niell et al. 2008) and in 18-month-old female rats, but interestingly not in the male rats (Gomez-Perez et al. 2008). Taken together, both the present (performed in 9-month-old rats) and the aforementioned studies suggest the existence of sex differences in the capacity to induce UCP3 expression in response to HFD feeding with age. Thus, female rats are able to increase the expression of UCP3 at 6 (Catala-Niell et al. 2008), 9 and 18 months of age in response to HFD feeding (Gomez-Perez et al. 2008), whereas male rats increase UCP3 protein levels at 6 (Catala-Niell et al. 2008) and 9 months of age, but not at 18 months (Gomez-Perez et al. 2008). Our results suggest that, given the proposed role of UCP3 in the regulation of insulin sensitivity (Senese et al. 2010), male rats would decrease their capacity to induce gastrocnemius UCP3 expression in response to HFD feeding between 9 and 18 months of age, in accordance with their earlier impairment of insulin sensitivity with age compared to females (Gomez-Perez et al. 2011).

In soleus muscle, the increase of UCP3 levels in response to HFD feeding turns out to be more attenuated than in the gastrocnemius one, in agreement with previous studies performed in male rats (Hoeks et al. 2003), which showed a higher induction of UCP3 in glycolytic muscles than in oxidative ones. In our study, differences between muscle types only appear in male rats, suggesting that differences in the capacity to induce UCP3 expression may also be dependent on muscle type. Although this response of male rats to HFD feeding is accompanied by an increase of soleus Cu-SOD levels, it may not be enough to compensate the increase of oxidative stress, as its enhanced oxidative damage indicated. All in all, these results point to a more detrimental effect of HFD feeding on both skeletal muscles of male rats compared to females.

HFD feeding also impairs insulin sensitivity in a sex-dependent manner. Thus, female rats maintain a better profile of serum markers of insulin sensitivity than their male

counterparts, despite showing a greater increase of body weight induced by HFD feeding. This better serum profile of HFD female rats is reflected by lower insulin levels and HOMA-IR index values, as well as by the unchanged serum HMW adiponectin to total adiponectin ratio, a marker of the insulin-sensitizing effect of the adipokine (Yamauchi et al. 2008). In contrast, HFD male rats show a more marked insulin resistance status, as pointed out by both decreased serum HMW adiponectin levels and HMW adiponectin to total adiponectin ratio.

In spite of the sex dimorphism found in serum adiponectin levels, we did not detect sex differences in the effect of HFD feeding on skeletal muscle adiponectin signaling pathway. In skeletal muscles of both sexes, HFD feeding resulted in an impaired adiponectin response or "adiponectin resistance" as the increase of AdipoR1 protein levels and the decrease of p-AMPK/AMPK ratio indicate. This dysregulation of adiponectin-AMPK signaling has been proposed to contribute to the impairment of insulin sensitivity, through an alteration in fatty acid metabolism that increases lipid accumulation in skeletal muscle which, ultimately, leads to the development of insulin resistance (Barnea et al. 2006; Rodriguez et al. 2008; Mullen et al. 2009). Increased levels of AdipoR1 may reflect a defective compensatory mechanism to overcome this adiponectin resistance, in agreement with previous studies showing a similar response in animal models with features of the metabolic syndrome (Barnea et al. 2006; Rodriguez et al. 2008). Moreover, our results suggest that the effect of HFD feeding on the response of skeletal muscle to adiponectin action is sex-independent. Thus, the sex differences found in the response of mitochondrial biogenesis to HFD feeding can not be attributed to differences in the response of the adiponectin signaling pathway.

As regards the involvement of muscle type in the effect of HFD feeding on adiponectin signaling pathway, gastrocnemius muscle shows a more marked response than soleus, by increasing AdipoR1 levels and decreasing the activation of AMPK. In fact, these parameters would point to greater adiponectin resistance in gastrocnemius muscle than in soleus, which, otherwise, maintains the activation of AMPK unaltered. Given these findings and the above mentioned relationship between adiponectin and insulin resistance, our results suggest that gastrocnemius muscle may contribute to the obesity-associated onset of insulin resistance to a greater extent than soleus, despite having a metabolism less dependent on insulin (Song et al. 1999).

Although previous studies have reported that adiponectin resistance contributes to the impairment of skeletal muscle oxidative metabolism in HFD fed rodents (Barnea et al.

2006; Mullen et al. 2009), we found that, in response to chronic HFD feeding, adiponectin resistance is accompanied by an enhanced oxidative capacity, which is reflected by an increase of mitochondrial biogenesis. Our results are in agreement with a previous study which reveals an increase of mitochondrial content as a consequence of HFD feeding to maintain normal oxidative capacity during later stages of insulin resistance (van den Broek et al. 2010). We hypothesise that the increase of mitochondrial biogenesis may be an adaptation to chronic HFD feeding as an attempt to compensate the deleterious consequences of adiponectin and insulin resistance on skeletal muscle oxidative metabolism.

In summary, we found a sex dimorphism in the effects of HFD feeding on skeletal muscle mitochondrial biogenesis that is more enhanced in male rats than in females, which could be attributed to a compensatory response to counteract the more marked increase of lipotoxicity and oxidative damage associated to HFD feeding in male rats. HFD female rats are more protected from lipotoxicity and maintain a better insulin sensitivity profile than their male counterparts. However, there are no sex differences in the response of skeletal muscle adiponectin signaling pathway to chronic HFD feeding, with both sexes showing a similar profile of adiponectin resistance. The response to HFD feeding is more evident in gastrocnemius muscle and may lead HFD rats to increase gastrocnemius mitochondrial biogenesis in order to counteract the deleterious consequences of adiponectin and insulin resistance on skeletal muscle oxidative metabolism. Our results suggest that HFD feeding has a skeletal muscle-type specific effect on adiponectin signaling pathway and a sex-dependent effect on the induction of mitochondrial biogenesis.

ACKNOWLEDGEMENTS

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4. RECAPITULACIÓN

4. Recapitulación

Los resultados obtenidos en la presente tesis doctoral ponen de manifiesto la existencia en la rata de un dimorfismo sexual en la alteración de la sensibilidad a la insulina, a nivel circulante y del músculo esquelético, en distintas situaciones que implican un aumento del estrés oxidativo, como son la alimentación crónica con una dieta hiperlipídica y el envejecimiento. La obesidad se considera un estado inflamatorio crónico de bajo grado que genera estrés oxidativo y que coexiste con el síndrome metabólico, mientras que el envejecimiento es un proceso biológico natural que se produce como consecuencia del deterioro progresivo de la función de los tejidos del organismo con el paso del tiempo, debido a la acumulación de daño oxidativo en las estructuras celulares.

Hemos observado, tanto en una situación de obesidad dietética como con la edad, claras diferencias entre sexos en los niveles circulantes de marcadores de sensibilidad a la insulina, que se ponen de manifiesto incluso en los animales control. Las ratas macho presentan un perfil sistémico de sensibilidad a la insulina más alterado por efecto de la obesidad dietética y de la edad que las ratas hembra, como indican las curvas de tolerancia a la glucosa, los niveles séricos de insulina y adiponectina y el índice de resistencia a la insulina HOMA-IR, lo que sugiere una mayor predisposición de los machos a desarrollar manifestaciones del síndrome metabólico, como la resistencia a la insulina, en respuesta a situaciones que implican un incremento del grado de estrés oxidativo.

La disminución de la sensibilidad a la insulina por efecto de la edad que se observa a nivel circulante se manifiesta también en el músculo esquelético y es diferente según las características metabólicas del mismo, mostrando el músculo gastrocnemius un mayor impacto de la edad que el soleus. Estas diferencias entre músculos podrían atribuirse a una mayor susceptibilidad del gastrocnemius a los efectos del estrés oxidativo, como indica la mayor acumulación de daño oxidativo que se produce con la edad.

Aunque la evolución de los marcadores circulantes de resistencia a la insulina sugiere un efecto más temprano del envejecimiento sobre la sensibilidad a la insulina en las ratas macho que en las hembras, no se observan diferencias entre sexos en los niveles de las principales proteínas intermediarias de la vía de señalización de la

insulina en el músculo esquelético. En este sentido, en roedores se ha descrito que la resistencia a la insulina podría manifestarse antes en el tejido adiposo blanco que en el músculo esquelético (Escriva et al. 2007; Serrano et al. 2009), de manera que las diferencias observadas a nivel circulante podrían ser el reflejo de diferencias entre sexos en el tejido adiposo blanco. No obstante, este dimorfismo no ha sido comprobado todavía.

Tanto la ingesta crónica de dietas ricas en grasa como el envejecimiento se asocian a un aumento de la deposición de grasa corporal y tisular, lo que compromete la sensibilidad tisular a la insulina. En los animales alimentados con dieta hiperlipídica, el exceso de lípidos procedentes de la dieta repercute negativamente sobre la integridad del páncreas y la sensibilidad a la insulina del músculo esquelético, si bien se observa un mayor impacto en los machos que en las hembras, que podría relacionarse con la menor capacidad de los machos para expandir el tejido adiposo blanco, que limita la asimilación de ácidos grasos, derivando el exceso de lípidos hacia otros tejidos.

El origen del dimorfismo sexual observado en la alteración muscular de la sensibilidad a la insulina podría estar en la mayor acumulación de daño oxidativo que presentan las ratas macho en el músculo esquelético y en el páncreas, y que resultaría de una menor capacidad para mantener el funcionamiento óptimo de las defensas antioxidantes, como indican la menor actividad Mn-SOD en el páncreas y los menores niveles de UCP2 en el páncreas y de UCP3 en el músculo gastrocnemius de los machos en comparación con las hembras. En este sentido, es interesante destacar que, en respuesta al incremento de estrés oxidativo asociado al consumo crónico de una dieta hiperlipídica, la inducción de la expresión de las UCPs tendría un papel más importante en la protección antioxidante que el de los principales enzimas antioxidantes.

La acumulación de lípidos y de daño oxidativo en el páncreas, por efecto de la obesidad dietética, podría comprometer la capacidad de las ratas macho de sintetizar y secretar insulina, tal y como sugiere la morfología alterada de los islotes pancreáticos, y contribuiría a la alteración del perfil de sensibilidad a la insulina. En cambio, las ratas hembra responden a la disminución de la sensibilidad muscular a la insulina, aumentando el tamaño de los islotes pancreáticos para asegurar el mantenimiento de la secreción de insulina. Es interesante destacar que la evolución de los niveles séricos de insulina con la edad, junto con el mayor daño oxidativo que alcanzan las ratas macho en el páncreas a los 18 meses de edad, sugieren un efecto más perjudicial de la edad sobre

la función del páncreas en los machos, que se refleja en una menor tolerancia a la glucosa, y que compromete la captación de glucosa por parte del músculo esquelético. Por otra parte, las ratas macho muestran una mayor acumulación de lípidos en el músculo esquelético, que son susceptibles de ser transformados en especies tóxicas que contribuyen a la alteración de la sensibilidad a la insulina. Estos resultados confirman el estado de sensibilidad a la insulina más alterado en las ratas macho, que ya sugería la evolución de los parámetros sistémicos con la edad, y apuntan hacia una mayor predisposición de las ratas macho a desarrollar resistencia a la insulina en respuesta a situaciones que incrementan el estrés oxidativo, como la obesidad dietética.

El dimorfismo sexual observado en la alteración de la sensibilidad a la insulina del músculo esquelético por efecto de la obesidad dietética en los animales de 18 meses de edad, no se detecta a los 9 meses. A esta edad, las ratas de ambos sexos alimentadas con dieta hiperlipídica muestran una acumulación similar de lípidos en el músculo esquelético y un perfil parecido de resistencia a los efectos de la adiponectina, hormona implicada en la regulación de la secreción de insulina. Estos resultados sugieren que el dimorfismo sexual en la alteración de la sensibilidad a la insulina aparece en edades más avanzadas, en las que también aparecen diferencias entre sexos en la alteración de los niveles de las principales proteínas intermediarias de la vía de señalización de la insulina.

Considerando la implicación del estrés oxidativo en la alteración de la sensibilidad a la insulina con la edad, el dimorfismo sexual observado en la respuesta de los animales más viejos a la dieta hiperlipídica podría ser consecuencia del diferente perfil de incremento de daño oxidativo que muestran machos y hembras con la edad. Así, la mayor acumulación de daño oxidativo en el músculo y en el páncreas de los machos de 18 meses de edad sugiere una alteración de los sistemas antioxidantes, que podría ser determinante en la pérdida progresiva de sensibilidad tisular a la insulina y en la disminución de la capacidad de responder al estrés oxidativo asociado a la obesidad dietética. En cambio, en edades avanzadas, las ratas hembra consiguen mantener un nivel de sensibilidad muscular a la insulina más óptimo que los machos en respuesta a la obesidad dietética, a través de la inducción de la expresión de proteínas clave de la vía de señalización de la insulina, como la Akt y el GLUT4. Esta mayor protección de las hembras podría atribuirse a una mayor capacidad para mantener activas las defensas antioxidantes, entre ellas, la UCP2 y la UCP3, que ejercerían un papel importante en el

mantenimiento de la sensibilidad tisular a la insulina. Además, los estrógenos, que parecen estar implicados en la mayor longevidad que suelen presentar las hembras en comparación con los machos (Borras et al. 2005), regularían la expresión de los principales enzimas antioxidantes, de forma que podrían contribuir al mejor estado de sensibilidad a la insulina de las hembras.

Existe una cierta controversia acerca del efecto de la alimentación crónica con una dieta rica en grasas sobre el metabolismo oxidativo del músculo esquelético. Algunos estudios desarrollados en roedores han demostrado que la alimentación con este tipo de dietas provoca una disfunción en las mitocondrias del músculo esquelético (Sparks et al. 2005; Bonnard et al. 2008), mientras que otros estudios muestran una inducción de la función mitocondrial, a pesar de que los animales desarrollan resistencia a la insulina (Turner et al. 2007; van den Broek et al. 2010). Aunque la razón de esta discrepancia no está clara, parece ser que los efectos de la dieta hiperlipídica sobre el funcionamiento mitocondrial podría depender de la composición de la dieta y de la duración del tratamiento. Los resultados que hemos obtenido indican que, en ambos sexos, la dieta hiperlipídica induce un aumento de la capacidad oxidativa mitocondrial del músculo esquelético, posiblemente con la finalidad de compensar el desequilibrio entre la disponibilidad de ácidos grasos en las mitocondrias y su oxidación. No obstante, esta respuesta resulta insuficiente, lo que deriva en la acumulación de lípidos y de daño oxidativo en el músculo esquelético, y en consecuencia, en el desarrollo de resistencia a la insulina. Esta respuesta del músculo podría constituir una adaptación para mantener inalterado el funcionamiento del metabolismo oxidativo, a pesar de la resistencia a la adiponectina y a la insulina asociadas a la dieta hiperlipídica.

En animales más jóvenes, el tratamiento dietético provoca una inducción de la capacidad oxidativa mitocondrial mayor en el músculo gastrocnemius, de naturaleza glucolítica, que en el soleus, más oxidativo, como indican los parámetros de diferenciación mitocondrial PGC-1α y TFAM, y posiblemente refleja la necesidad de adaptar las características metabólicas del músculo gastrocnemius al exceso de sustratos lipídicos suministrados por la dieta. El aumento de la capacidad oxidativa mitocondrial del músculo gastrocnemius en respuesta a la dieta es mayor en las ratas macho que en las hembras, y es indicativo de la mayor necesidad en los machos de oxidar el exceso de lípidos que llega al músculo para evitar los efectos tóxicos que puede tener la acumulación de los mismos. La menor respuesta de las hembras podría estar relacionada

con su mayor capacidad para almacenar lípidos en el tejido adiposo, que protege el músculo esquelético de la acumulación de lípidos ectópicos y hace que sea menos necesaria la inducción del metabolismo oxidativo para contrarrestar el estado de resistencia a la adiponectina y a la insulina.

El dimorfismo sexual observado en la inducción de la capacidad oxidativa mitocondrial por efecto de la dieta cambia de sentido en los animales de más edad. Para contrarrestar el incremento del estrés oxidativo y de la resistencia a la insulina asociadas a la obesidad, las ratas hembra aumentan la actividad COX y los niveles de UCP3 y de GLUT4 del músculo esquelético. Además, mantienen más elevadas la actividad Mn-SOD y los niveles de UCP2 en el páncreas y aumentan el tamaño de los islotes pancreáticos para asegurar el mantenimiento de la secreción de insulina. Estos resultados indican que, en edades avanzadas, las hembras estarían más preparadas que los machos para enfrentarse a los efectos perjudiciales de la obesidad sobre la sensibilidad a la insulina, lo que se traduciría en la menor alteración que muestran con la edad.

En conjunto, los resultados obtenidos en esta tesis doctoral han demostrado que existen diferencias importantes entre sexos en la alteración de la sensibilidad a la insulina por efecto del estrés oxidativo asociado a la obesidad dietética y a la edad. La mayor susceptibilidad que presentan las ratas macho a los efectos deletéreos de la obesidad podría tener su origen en el mayor impacto del estrés oxidativo inducido por la dieta y que se ve influenciado por la edad. Las ratas hembra, en cambio, presentan una mayor capacidad que los machos para adaptarse a los cambios inducidos por la obesidad dietética y por el envejecimiento, así como de contrarrestar los efectos deletéreos del estrés oxidativo sobre la sensibilidad a la insulina. Esta mayor habilidad de las hembras para paliar la resistencia a la insulina podría implicar una mayor capacidad para prevenir el desarrollo de diabetes tipo 2 en comparación con los machos.

5. CONCLUSIONES

5. CONCLUSIONES

I. Existe un claro dimorfismo sexual en el grado de alteración de la sensibilidad a la insulina en ratas, en situaciones que se asocian a un incremento del estrés oxidativo como la obesidad dietética y el envejecimiento. Este dimorfismo consiste en una mayor predisposición de las ratas macho a manifestar resistencia a la insulina, a nivel circulante y del músculo esquelético.

II. Tanto con la edad como en respuesta a la alimentación con una dieta hiperlipídica, el mayor incremento de adiposidad que experimentan las ratas hembra, y que es indicativo de una mayor capacidad de expansión del tejido adiposo, evitaría la acumulación ectópica de lípidos y la alteración del perfil circulante de sensibilidad a la insulina, contribuyendo al dimorfismo sexual observado.

III. Las diferencias entre sexos en la acumulación de daño oxidativo en el músculo esquelético y en el páncreas por efecto de la obesidad dietética y de la edad podrían ser responsables, en parte, del dimorfismo sexual en la alteración de la sensibilidad muscular a la insulina y ponen de manifiesto un mayor impacto del estrés oxidativo en las ratas macho, que repercute negativamente en la capacidad oxidativa del músculo esquelético y en su sensibilidad a la insulina.

IV. En las ratas de ambos sexos, la inducción de la biogénesis mitocondrial en el músculo esquelético, en respuesta a la alimentación crónica con una dieta hiperlipídica, constituiría un mecanismo para intentar compensar la alteración del metabolismo oxidativo, consecuencia de la resistencia a los efectos de la adiponectina y de la insulina. Cuando la edad es más avanzada, la intensidad de esta respuesta a la dieta no es tan marcada en las ratas macho, lo que contribuye a su mayor resistencia a la insulina en comparación con las hembras, que estarían más preparadas para hacer frente a los efectos prejudiciales de la obesidad sobre la sensibilidad a la insulina.

V. En las ratas hembra, la inducción de las UCPs en el músculo y en el páncreas se perfila como un mecanismo de prevención para hacer frente al estrés oxidativo asociado a la dieta hiperlipídica, lo que sugiere la existencia de un dimorfismo sexual en la

naturaleza de la respuesta antioxidante desencadenada para contrarrestar el incremento de estrés oxidativo y la resistencia a la insulina.

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7. ANEXO 1.
METODOLOGÍA ADICIONAL UTILIZADA DURANTE LA TESIS

ANEXO 1. METODOLOGÍA ADICIONAL UTILIZADA DURANTE LA TESIS

A raíz de la constatación de la existencia de un dimorfismo sexual en la alteración de la integridad del páncreas por efecto del estrés oxidativo asociado a la obesidad dietética, nos planteamos determinar si estas diferencias aparecían también a nivel de la funcionalidad de este órgano. Para ello, nos propusimos analizar los efectos del estrés oxidativo asociado a la obesidad dietética sobre la secreción de insulina en islotes aislados de páncreas de ratas macho y hembra.

Diseño experimental

Se utilizaron ratas Wistar macho y hembra de 9 meses de edad, que habían sido alimentadas durante 26 semanas con la misma dieta hiperlipídica descrita en el apartado *2. Objetivos y planteamiento experimental* de la presente tesis doctoral. Los animales fueron sacrificados tras 12 horas de ayuno e, inmediatamente después, se procedió a la perfusión del páncreas para facilitar su extracción y el aislamiento de los islotes pancreáticos (ver detalles en el apartado correspondiente). Una vez aislados, los islotes fueron incubados en presencia de glucosa y/o peróxido de hidrógeno (H_2O_2), y se tomaron alícuotas del medio de incubación, que fueron guardadas a -20ºC para la posterior determinación de la secreción de insulina. Además, se recogieron los islotes pancreáticos incubados y se guardaron a -20ºC y a -80ºC para la determinación del contenido total de insulina y para la extracción de ARN, respectivamente.

Reactivos

- Colagenasa P (2,64U/mg) (Roche Diagnostics, Basilea, Suiza).
- Solución Hanks: NaCl 137mM, KCl 5,36mM, $MgSO_4 \cdot 7H_2O$ 0,81mM, $Na_2HPO_4 \cdot 2H_2O$ 0,34mM, KH_2PO_4 0,44mM, $CaCl_2 \cdot 2H_2O$ 1,26mM y $NaHCO_3$ 4,17mM, a 4ºC y pH 7,4.
- Disoluciones de glucosa 2,8mM y 16,7mM (D-(+)-glucosa monohidrato, Merck, Darmstadt, Alemania) en solución Hanks suplementada con albúmina sérica bovina al 0,1% (BSA, Sigma-Aldrich, St. Louis, MO, USA).
- Disolución 200mM de H_2O_2 estabilizado 30% p/v (100 vol.).
- Reactivo Tripure (Roche Diagnostics, Basilea, Suiza).

Los reactivos fueron suministrados por Panreac (Barcelona), excepto cuando se especifica lo contrario.

Aislamiento de islotes pancreáticos

Para la puesta a punto de la técnica, la doctoranda realizó una breve estancia en el laboratorio del grupo de la Dra. Carmen Álvarez del departamento de Bioquímica y Biología Molecular II de la Facultad de Farmacia de la Universidad Complutense de Madrid. El método utilizado consiste en la perfusión del páncreas de rata con solución Hanks, la digestión mecánica y enzimática del tejido y la separación de los islotes pancreáticos del tejido exocrino (Malaisse-Lagae y Malaisse 1984). El aislamiento de los islotes pancreáticos se realizaba a partir del páncreas de una rata, en el caso de los machos, o bien de los páncreas de dos ratas, en el caso de las hembras.

Después del sacrificio y de abrir la cavidad abdominal del animal, se apartaba el hígado y se localizaban el páncreas y el conducto colédoco. A continuación, se realizaba un clampaje del conducto colédoco en la zona próxima al intestino y, con ayuda de unas pinzas de punta curva, se limpiaba de grasa el conducto. Inmediatamente después, se introducía un catéter de polietileno (0,95mm de diámetro externo y 0,58mm de diámetro interno) en el conducto colédoco. El catéter estaba conectado a una jeringa de 10mL, que contenía medio Hanks, previamente gaseado con gas carbógeno [O_2 (95%)-CO_2 (5%)] para asegurar la disponibilidad de oxígeno en los islotes. A través del catéter, se introducía lentamente solución Hanks en los conductos pancreáticos hasta llenar completamente de líquido el páncreas. A continuación, con ayuda de pinzas y tijeras de punta curva, se procedía a extraer el páncreas, separando primero la zona adherida al intestino grueso y a parte del intestino delgado y, después, las zonas unidas al estómago y a la última porción del intestino delgado.

Una vez extraído el páncreas, se depositaba en una placa de Petri con solución Hanks y se limpiaba de sangre, grasa y ganglios. Seguidamente, se eliminaba el exceso de solución Hanks del páncreas, se introducía el tejido en un vaso de precipitados de 15mL y se troceaba con unas tijeras de forma enérgica durante 10 minutos. A continuación, se realizaban tres lavados, que consistían en la adición de solución Hanks hasta llenar el vaso de precipitados y la sedimentación de los fragmentos de tejido durante 5 minutos. El sobrenadante se descartaba con ayuda de una pipeta Pasteur.

Seguidamente, se vertía el páncreas troceado en un tubo de centrífuga, se dejaba sedimentar durante 5 minutos y se eliminaba el exceso de solución Hanks.

Una vez el páncreas estaba limpio y troceado, se procedía a su digestión, añadiendo 2,5mg de colagenasa (por páncreas) directamente sobre el tejido en el tubo y se mezclaba suavemente. Inmediatamente después, la suspensión se gaseaba durante 10 minutos con gas carbógeno en un baño a 37ºC. Seguidamente, se retiraba el sistema de gas carbógeno y el tubo se agitaba vigorosamente en posición horizontal a 37ºC durante 2 minutos. Posteriormente, se agitaba a temperatura ambiente durante 6 minutos, sosteniendo el tubo con la mano y describiendo un ángulo de 180º con movimientos vigorosos de extensión y flexión del antebrazo en posición horizontal. Finalmente, la digestión se detenía vertiendo la suspensión en un cristalizador que contenía solución Hanks (aproximadamente 20mL).

A continuación, se procedía a la separación de los islotes pancreáticos del tejido exocrino no digerido, haciendo pasar varias veces la suspensión a través de una pipeta Pasteur de vidrio. Seguidamente, se realizaban sucesivos lavados de los islotes aislados, añadiendo medio Hanks al cristalizador, dejando sedimentar los islotes durante 5, 4, 2 y 1 minutos y aspirando muy lentamente el sobrenadante con una bomba de succión, para evitar la pérdida de islotes. El objetivo de estos lavados era eliminar el tejido exocrino no digerido, que sedimenta más lentamente que los islotes pancreáticos aislados. Finalmente, éstos eran visualizados con la lupa y recogidos, con una pipeta automática, para su posterior utilización en diferentes tratamientos.

Para la determinación del contenido total de insulina de los islotes pancreáticos, previamente a la incubación, se recogían 20 islotes de cada grupo experimental y se guardaban a -20ºC. Por otro lado, se incubaban 6 islotes (por triplicado) en 1mL de solución Hanks suplementada con BSA 0,1% y glucosa 2,8mM en un baño a 37ºC con agitación suave durante 30 minutos. Pasado este tiempo, se recogía el medio de incubación y se guardaba a -20ºC para la determinación de la secreción de insulina. A continuación, los islotes se incubaban a 37ºC durante 90 minutos en 1mL de solución Hanks suplementada con glucosa 2,8mM o 16,7mM, en presencia o ausencia de H_2O_2 200µM. Transcurridos los 90 minutos de incubación, se tomaba una alícuota del medio de incubación de cada tubo y se guardaba a -20ºC para la determinación de los niveles de insulina. Finalmente, se recuperaban y se reunían los 6 islotes de cada triplicado en

un único tubo, al que se añadía 1mL de reactivo Tripure, y se congelaba a -80ºC para la posterior extracción de ARN.

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8. ANEXO 2.
PAPEL DE LA AMPK EN EL CONTROL DE LA SECRECIÓN DE INSULINA POR LA ADIPONECTINA

ANEXO 2. PAPEL DE LA AMPK EN EL CONTROL DE LA SECRECIÓN DE INSULINA POR LA ADIPONECTINA

Durante la tesis doctoral, la doctoranda colaboró en un proyecto de investigación que desarrollaba el grupo del *Professor Guy A. Rutter* en el *Department of Cell Biology, Division of Medicine, Faculty of Medicine* del *Imperial College* de Londres. Esta colaboración tuvo lugar durante la estancia de tres meses que la doctoranda realizó en dicho centro de investigación y fue posible gracias a una ayuda concedida por el *Govern de les Illes Balears*.

Objetivo

Estudiar, en islotes pancreáticos de ratón, la posible implicación de la AMPK en el efecto de la adiponectina sobre la secreción de insulina. Para alcanzar este objetivo se aislaron islotes pancreáticos de ratones de la cepa CD1, se incubaron en presencia de adiponectina y se evaluó la secreción de insulina en el medio de incubación y la actividad AMPK en los islotes.

Reactivos

- Colagenasa tipo V (1U/mg) (SERVA, Heidelberg, Alemania)
- Medio de cultivo RPMI 1640 (BioWhittaker™/Cambrex, Verviers, Bélgica), sin suplementar y suplementado con glucosa 10mM, compuesto de suero fetal bovino al 10% inactivado por calor, L-glutamina 2mM, penicilina 100UI/mL y estreptomicina 100g/mL.
- Histopaque 1,119g/L; 1,083g/L y 1,077g/L (Sigma-Aldrich, St. Louis, MO, USA).
- Medio Krebs Ringer: NaCl 120mM, KCl 4,8mM, $CaCl_2$ 2,5mM, $MgCl_2$ 1,2mM y $NaHCO_3$ 24mM, a 4ºC y pH 7,4.
- Disoluciones de glucosa 3mM, 5,6mM y 16,7mM en medio Krebs Ringer.
- Disolución de adiponectina 10μg/mL (Sigma-Aldrich, St. Louis, MO, USA) en tampón PBS con BSA 1%.
- Tampón de lisis: Tris·HCl 50mM (pH 7,4 a 4ºC), sacarosa 250mM, NaF 50mM, pirofosfato de sodio 1mM, EDTA 1mM, EGTA 1mM, DTT 1mM, Triton X-100 1% (vol/vol) y una mezcla comercial de inhibidores de proteasas (Complet Protease Inhibitor Cocktail Tablet, Roche Diagnostics, Basilea, Suiza).

- Kit de radioinmunoensayo para la detección de insulina (Linco Research, St. Charles, MO, USA).
- Reactivos para el ensayo de la actividad AMPK: Tampón HEPES 50mM (pH 7,4), péptido SAMS (péptido sintético HMRSAMSGLHLVKRR) 200µM, ^{32}P-ATP 200µM, ATP 200µM, AMP 200µM, MgCl$_2$ 5mM, ácido fosfórico 1%.

Aislamiento de islotes pancreáticos de ratón

Los aislamientos se realizaron mediante perfusión intraductal del páncreas y digestión con colagenasa (Ravier et al. 2005). Todo el proceso se realizaba en condiciones de esterilidad para evitar la contaminación de los islotes pancreáticos. Una vez localizados el páncreas y el conducto colédoco del animal, se realizaba un clampaje del conducto y, seguidamente, se le introducía un catéter conectado a una jeringa de 10mL. El páncreas se perfundía con 5mL de una disolución de colagenasa en medio RPMI 1640, previamente filtrada a través de un filtro de 0,20µm. A continuación, el páncreas se extraía, se introducía en un tubo de centrífuga y se incubaba en un baño a 37ºC durante 10 minutos, para activar la colagenasa e iniciar la digestión enzimática del tejido pancreático. Pasado este tiempo, se realizaba un lavado para detener la actividad enzimática, que consistía en la adición de 20mL de medio RPMI 1640 al tubo y en la posterior centrifugación a 1000xg durante 1 minuto. A continuación, se iniciaba la digestión mecánica: se agitaba manualmente el producto de la digestión hasta conseguir disgregar los fragmentos de tejido y, posteriormente, se realizaban 3 lavados en medio RPMI 1640 por centrifugación a 1000xg durante 1 minuto. Seguidamente, se procedía a la separación de los islotes pancreáticos del tejido exocrino, utilizando un gradiente de concentración comercial (Histopaque, 3mL de 1,119g/L; 3mL de 1,083g/L; 3mL de 1,077g/L y 3mL de medio RPMI 1640) y centrifugando a 2500xg durante 20 minutos. A continuación, se recogían los islotes que quedaban en la interfase entre el gradiente de concentración y el medio RPMI 1640, y se realizaba un último lavado en medio RPMI 1640 para eliminar el exceso de solución Histopaque. Finalmente, los islotes eran depositados en una placa de Petri con medio RPMI 1640 para ser visualizados con la lupa. Los islotes funcionales se seleccionaban según el tamaño y la integridad de los mismos, y se mantenían en cultivo, en medio suplementado con glucosa 10mM, suero fetal de ternera inactivado por calor al 10%, L-glutamina 2mM, penicilina 100UI/mL y

estreptomicina 100g/mL, a 37ºC en una atmósfera con un 5% de CO_2 durante 24-48 horas.

Análisis de los efectos de la adiponectina sobre la secreción de insulina en los islotes pancreáticos

Se incubaban 6 islotes en medio Krebs Ringer, previamente gaseado con O_2/CO_2 y suplementado con 1mg/mL de BSA y con glucosa de concentración 3mM, en un baño a 37ºC durante 30 minutos. A continuación, se eliminaba el medio y se incubaban los islotes a 37ºC durante 60 minutos con medio Krebs Ringer suplementado con glucosa 5,6mM o 16,7mM, en presencia o ausencia de adiponectina 10μg/mL. Posteriormente, se tomaba una alícuota del medio de incubación y se guardaba a -20ºC, para la determinación de los niveles de insulina.

Una vez eliminado el medio de incubación, los islotes pancreáticos se resuspendían en tampón de lisis, se sonicaban y se centrifugaban a 13000xg durante 2 minutos. Seguidamente, se recogía el sobrenadante y se guardaba a -20ºC, para la determinación del contenido total de insulina de los islotes. Las medidas de secreción de insulina y del contenido total de insulina de los islotes se determinaron por radioinmunoensayo, utilizando un kit comercial y un contador de centelleo (Ravier et al. 2005).

Análisis de los efectos de la adiponectina sobre la actividad AMPK de los islotes pancreáticos

La determinación de la actividad AMPK se realizaba en 150 islotes pancreáticos aislados, previamente incubados en medio Krebs Ringer suplementado con glucosa 5,6mM o 16,7mM en presencia o ausencia de adiponectina 10μg/mL, utilizando el mismo procedimiento anterior. Los islotes se sonicaban y se centrifugaban. Posteriormente, se tomaba el sobrenadante y se guardaba a -20ºC para la determinación de la actividad AMPK. Las medidas de la actividad AMPK se realizaron mediante un método radioactivo descrito previamente (da Silva Xavier et al. 2000).

Resultados preliminares

En presencia de adiponectina 10μg/mL, la secreción de insulina aumentaba en condiciones de baja concentración de glucosa (Figura 1), lo que coincidía con otros

resultados previamente publicados (Okamoto et al. 2008). La secreción de insulina se expresa como la relación entre los niveles de insulina en el medio de incubación y los niveles totales de insulina en los islotes.
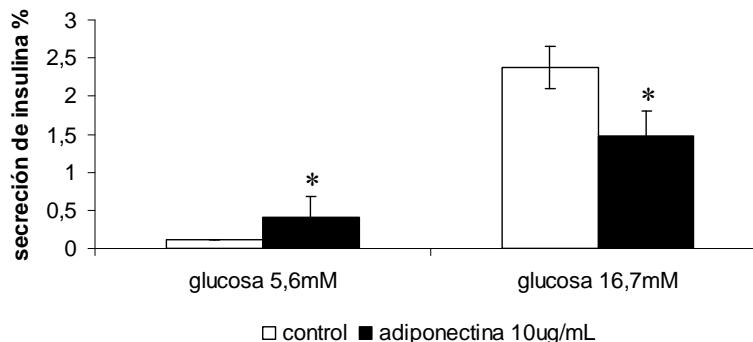

Figura 1. Efecto de la adiponectina sobre la secreción de insulina en los islotes pancreáticos. Los datos representan la media ± el error tipo de 3 experimentos independientes. En la prueba t de Student (p<0,05), * indica que es diferente del grupo control.

En presencia de adiponectina 10μg/mL, la actividad AMPK disminuía en condiciones de baja concentración de glucosa (Figura 2).
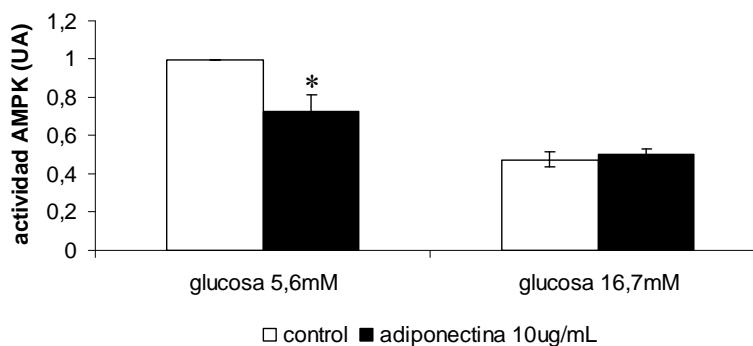

Figura 2. Efecto de la adiponectina sobre la actividad AMPK en los islotes pancreáticos. Los datos representan la media ± el error tipo de 2 experimentos independientes. En la prueba t de Student (p<0,05), * indica que es diferente del grupo control. UA, unidades arbitrarias.

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9. ANEXO 3.
PUBLICACIONES COMPLEMENTARIAS

Manuscrito V.

Gender related differences in paraoxonase 1 response to high-fat diet-induced oxidative stress.

Thomàs-Moyà E, Gómez-Pérez Y, Fiol M, Gianotti M, Lladó I, Proenza AM. Obesity (Silver Spring) 10:2232-8, 2008.

Gender Related Differences in Paraoxonase 1 Response to High-fat Diet–induced Oxidative Stress

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Objective: To evaluate the influence of the pro-oxidant and proinflammatory state related to dietary obesity on serum paraoxonase 1 (PON1) activity in male and female rats.

Methods and Procedures: Adult Wistar rats of both genders were fed on a high-fat diet to induce weight gain or standard diet for 14 weeks. Body weight was assessed weekly and food intake fortnightly throughout the dietary treatment. Biometrical parameters and serum lipid profile, glucose, insulin, and adipokine levels were measured. To assess the effect of dietary obesity on oxidative stress, levels of liver and serum thiobarbituric acid reactive substances, liver protein carbonyl groups, liver antioxidant enzymes activities, and serum PON1 activities were measured.

Results: High-fat diet feeding induced a significant body weight gain in both male and female rats, as well as a reduction of liver antioxidant protection. High-fat diet increased serum lipid peroxides in male rats and reduced serum PON1 activities and serum apolipoprotein A–I (apoA–I) levels in females, although did not alter serum PON1 or apolipoprotein J (apoJ) levels.

Discussion: Our results reveal a gender dimorphism in the high-fat diet–induced reduction of serum PON1 activity, which is likely to be related to the greater obese and proinflammatory state achieved in female rats. We suggest that the enhanced oxidative stress caused by dietary increased body weight, on leading to high-density lipoprotein (HDL), apoA–I or PON1 oxidation could entail the destabilization of the PON1 association to HDL or a direct inactivation of PON1 enzymatic activity, thus accounting for the decreased serum PON1 activities observed in female rats.

INTRODUCTION

Diet has been identified as one of the most important contributors in the etiology of obesity. Consumption of hypercaloric diets is associated with obesity development (1), and a wide variety of models of high-fat diets have been used to study their effects on body weight and metabolism. Among them, the cafeteria-diet, a palatable hypercaloric and hyperlipidic diet with similar characteristics to diets usually consumed by humans, has been shown to induce voluntary hyperphagia and fast body weight gain in rodents, thus it is a suitable model to study human obesity (2,3).

Obesity-related excessive adiposity is associated with increased systemic inflammation and cardiovascular risk (4) due to the secretion of a large number of adipokines that influence inflammation, coagulation, fibrinolysis, insulin resistance,

and atherosclerosis (4,5), thus contributing to the development of a systemic proinflammatory state. Tumor necrosis factor-α, interleukin 6, plasminogen activator inhibitor-1 (PAI-1), resistin, and leptin secretion is increased with obesity, whereas secretion of adiponectin is reduced (5).

A close association between adipokine profile and free-radical production has been described with obesity (6), and both increased oxidative stress and higher susceptibility to lipid peroxidation of low-density lipoproteins (LDLs) have been reported in some animal models of obesity (7,8). LDL oxidation, which represents one of the main events in atherosclerosis development (9), associates with impaired high-density lipoprotein (HDL) antioxidant defenses, such as decreased activity of the HDL-associated enzyme paraoxonase 1 (PON1) (10,11). PON1 enzyme is closely associated to circulating HDL

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containing both apolipoprotein A–I (apoA–I) and apolipoprotein J (apoJ) (12,13), and has been shown to prevent oxidation of LDL, thus protecting the endothelium from the pro-oxidant effect of oxidized LDL and from atherosclerosis development (14,15). Serum PON1 activity can be modulated by both dietary factors and oxidative stress (16), being decreased in obesity and inactivated by increased oxidative stress (10,17). Furthermore, in previous studies we have evidenced that gender influences the PON1 response to alterations in the oxidative environment in both dietary and physiological situations (18–20).

Considering that the obese state induced by cafeteria-diet feeding has been related to enhanced oxidative stress (21,22), and taking into account that we have previously reported gender-associated differences in response to cafeteria-diet feeding (3,23), the aim of this study was to evaluate whether the more pro-oxidant and proinflammatory state related to increased dietary adiposity could influence PON1 activity and the main factors involved in its expression, stability and function, in a gender-dependent manner.

METHODS AND PROCEDURES
Materials
Oligonucleotide primer sequences, Lightcycler-FastStart DNA Master SYBR Green I for real-time PCR, and Tripure isolation reagent were purchased from Roche Diagnostics (Basel, Switzerland). Reverse transcriptase PCR chemicals were purchased from Applied Biosystems (Foster City, CA). Rabbit polyclonal antibody to human PON1 was kindly provided by M. Mackness and B. Mackness (24). Rabbit polyclonal antibody to human apoA–I (Cat. No. 178422) was purchased from Calbiochem (San Diego, CA). Goat polyclonal antibodies to rat apoJ and PAI-1 (Cat. No. sc-13747 and Cat. No. sc-6644, respectively) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Chemiluminescence kit (ECL) for immunoblot development was purchased from Amersham (Little Chalfont, UK).

Kits for measurement of serum lipid profile—HDL-cholesterol direct (Cat. No. 1133505), LDL-cholesterol (Cat. No. 1142005), and Total cholesterol MR (Cat. No. 1118005)—were purchased from Linear Chemicals SL (Barcelona, Spain). Accutrend test strips and the Accutrend GCT-meter were used for the quantitative determination of glucose (cat. no. 11443054) and triglyceride levels (cat. no. 1538144). Nonesterified fatty acid assay kit (cat. no. 999-75406) was purchased from Wako Chemicals (Osaka, Japan). Enzyme immunoassays for measurement of rat adiponectin (cat. no. EK-ADI-02), rat leptin (cat. no. MOB00), and rat resistin (cat. no. K1014-1) were purchased from Phoenix Pharmaceuticals (Belmont, CA), R&D Sytems (Minneapolis, MN), and B-Bridge International (Mountain View, CA), respectively. The Oxyblot Protein Oxidation Detection Kit (cat. no. S7150) was purchased from Chemicon International (Temecula, CA). Substrates for the measurements of PON1 activities (diethyl p-nitrophenyl phosphate and phenylacetate) were from Sigma-Aldrich (St Louis, MO). Routine chemicals used were supplied by Pronadisa (Madrid, Spain), Panreac (Barcelona, Spain) and Sigma-Aldrich (St Louis, MO).

Animals, diets, and sample collection
Animal experiments were performed in accordance with general guidelines approved by our Institutional Ethics Committee and European Union (86/609/EEC) regulations. Fifteen-month-old Wistar rats (Charles River, Barcelona, Spain), 12 males and 12 females, were kept at 22 °C on a 12-h light–dark cycle. For each gender, rats having a similar mean body weight (556 ± 16 for male rats and 353 ± 7 for female rats) were divided into two dietary groups: a cafeteria diet–fed group and a control group. For 14 weeks, the cafeteria diet–fed group

had free access to a pelleted standard diet and to a variety of highly palatable food: cookies with pork liver paté, fresh bacon, fairy cakes, chocolate, carrots, and ensaïmada (a typical Majorcan pastry). In the cafeteria-diet, protein, carbohydrate, and lipids represent 8, 30, and 64% of the total energy content of the diet (18,894 kJ/kg), respectively. Fat content of the cafeteria-diet was 32% by weight (14% saturated fatty acids, 14.5% monounsaturated fatty acids, 3.4% polyunsaturated fatty acids and 0.06% cholesterol). The control group was fed a pelleted standard diet (Panlab, Barcelona, Spain) with protein, carbohydrate and fat representing 19, 73, and 8% of the total energy content of the diet (14,104 kJ/kg diet), respectively. Fat content of the standard diet was 2.9% by weight (0.8% saturated, 0.8% monounsaturated, 1.3% polyunsaturated and <0.01% cholesterol). Animals were allowed free access to water and food, freshly provided daily. Body weights of all animals were assessed once weekly and food intake fortnightly throughout the 14 weeks of dietary treatment. After a period of 12 h of fasting, rats were killed by decapitation. Liver and white adipose tissue depots were rapidly dissected, weighed, frozen in liquid N_2 and stored at −70 °C. Serum samples were also stored at −70 °C until analyzed.

Measurement of serum lipid profile, glucose, insulin, and adipokine levels
HDL-cholesterol levels were measured by using an enzymatic homogeneous assay. Total and LDL-cholesterol, as well as nonesterified free-fatty acids levels were measured by using spectrophotometric assay kits. Triglyceride and glucose levels were measured by using the Accutrend system. Serum levels of insulin, adiponectin, leptin, and resistin were measured by using enzyme immunoassay kits. To estimate insulin resistance, the homeostasis model assessment of insulin resistance index, which has been widely used in numerous human and animal studies (25–27), was calculated as (blood fasting glucose (mmol/l) × blood fasting insulin (μU/ml))/22.5.

Isolation of liver mitochondria and measurement of antioxidant enzymes
Pieces of ~2 g of liver were homogenized in sodium chloride–Tris–EDTA buffer (250 mmol/l sucrose, 5 mmol/l Tris–HCl, 2 mmol/l EGTA, pH 7.4). Homogenates were firstly centrifuged at 500 g to eliminate nucleus and homogenizing remains. Supernatants were subsequently centrifuged at 8,000 g to isolate the mitochondrial fraction, which was resuspended in sodium chloride–Tris–EDTA buffer.

Catalase activity was determined in liver homogenates (28) and activities of glutathione peroxidase, glutathione reductase, and Mn-superoxide dismutase were measured in liver mitochondria as previously described (29–31).

Measurement of TBARS levels, protein carbonyl groups, and serum PON1 activities
Levels of thiobarbituric acid reactive substance (TBARS), as an index of serum lipid peroxides, were measured in serum and liver homogenates as previously described (32). Protein carbonyl groups, as an index of protein oxidation, were measured in liver homogenates by immunoblot detection by using the OxyBlot Protein Oxidation Detection Kit according to the manufacturer's protocol. Serum PON1 activities were assayed in a microtiter plate spectrophotometer (Bio-Tek Instruments, Winooski, VT) by measuring the rate of hydrolysis of phenylacetate (arylesterase activity) and paraoxon (paraoxonase activity) as previously described (33).

Analysis of PON1 mRNA levels by real-time reverse transcriptase-PCR
Total cellular RNA was isolated from liver samples by using Tripure isolation reagent according to the manufacturer's protocol. One microgram of the total RNA was reverse transcribed to complementary DNA and subsequently diluted 1/10 and frozen at −70 °C until the PCRs were carried out. Real-time PCR was performed using SYBR Green detection

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technology in a Lightcycler Rapid Thermal Cycler (Roche Diagnostics, Basel, Switzerland), as previously described (18).

Real-time PCR efficiencies were calculated on average of efficiencies from each sample which were calculated by using the following formula: $e = (F/F_0)^{1/(n-n_0)}$, where F and F_0 mean fluorescence values belonging to the linear segment of each PCR quantification curve and n and n_0 represent their corresponding crossing points. PON1 and 18S ribosomal RNA real-time PCR efficiencies were 1.87 and 1.89, respectively.

Western blot analysis of serum PON1, apoA–I, apoJ, and PAI-1 protein levels

Equal amounts of serum protein (30 μg) were fractionated on 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and electrotransferred onto a nitrocellulose filter. Ponceau S staining was performed systematically to check the correct loading and electrophoretic transfer. Rabbit polyclonal antibodies against human apoA–I and rat PON1 and goat polyclonal antibodies against rat apoJ and PAI-1 were used as primary antibodies. Anti-rabbit and anti-goat immunoglobulin G–alkaline phosphatase antibodies were used as secondary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands in films were quantified by photodensitometric analysis (Kodak 1D Image Analysis Software). Autoradiograms of membrane proteins revealed an apparent molecular mass of 43, 50, 28, and 50 kd, for PON1, apoJ, apoA–I, and PAI-1, respectively.

Statistical analysis

All data are expressed as mean values ± s.e.m. of six animals per group. Statistical analysis were performed by using a statistical software package (SPSS 13.0 for Windows, Chicago, IL). Statistical differences between experimental groups were analyzed by two-way analysis of variance. Student's t-test, as *post hoc* comparison, was performed when an interactive effect of gender and diet was shown. A P-value of <0.05 was considered statistically significant.

The statistical PCR data analysis was performed by using the Relative Expression Software Tool (REST 2005 BETA V1.9.9) (34). Differences in mRNA levels between groups were analyzed by Pair Wise Fixed Reallocation Randomisation Test (35), a proper model to avoid the normal distribution of data.

RESULTS

Energy intake and body and tissue weights in male and female cafeteria diet–fed rats

The energy intake of cafeteria diet–fed rats was approximately three to four times higher than in control rats (**Table 1**).

Cafeteria-diet feeding induced a significant body weight gain in both male and female rats.

The weight of the liver was not affected by the cafeteria-diet feeding, whereas those of lumbar, inguinal, and gonadal white adipose tissue depots significantly increased. In the gonadal depot, this increase was statistically higher in female rats compared to males.

Serum lipid profile and serum glucose, insulin and adipokine levels in male and female cafeteria diet–fed rats

Cafeteria-diet feeding did not alter either serum lipid profile or serum glucose levels, whereas those of insulin significantly increased in both genders (**Table 2**). Therefore, the Homeostasis Model Assessment index was higher in the cafeteria diet–fed rats compared to the control ones. Adiponectin levels, which were threefold higher in female control rats compared to males, strongly decreased (43%) only in female rats with the cafeteria-diet consumption, thus reaching similar levels to cafeteria diet–fed males. In both genders, leptin levels of cafeteria diet–fed rats were twice their respective controls. Resistin levels were higher in male rats compared to females, and showed a tendency to increase with the cafeteria-diet feeding in female rats, although it did not reach statistical significance. PAI-1 levels did not show gender differences, but there was a tendency to decrease in male rats.

Serum lipid peroxide levels, PON1 activities and PON1, apoA–I and apoJ protein levels in male and female cafeteria diet–fed rats

In male cafeteria diet–fed rats, serum lipid peroxides were increased (63%) (**Table 3**). Both paraoxonase and arylesterase activities were higher in control female rats than in males and decreased with the consumption of the cafeteria diet, thus reaching the levels of males. In both genders, the TBARS/arylesterase activity and TBARS/paraoxonase activity ratios, indicative of the loss of antioxidant capacity of PON1, increased with cafeteria-diet feeding.

Table 1 Energy intake and body and tissue weights in male and female cafeteria diet–fed rats

	Male		Female		
	Control	Cafeteria-fed	Control	Cafeteria-fed	ANOVA
Food intake (g/kg day)	30.8 ± 1.1	112 ± 12	47.9 ± 3.0	130 ± 13	D
Energy intake (kJ/kg day)	335 ± 48	1,683 ± 279	596 ± 67	1,908 ± 304	D
Body weight gain (g)	12.8 ± 8.5	129 ± 17	19.7 ± 10.2	169 ± 18	D
Tissue weights (g/100g)					
Liver	2.26 ± 0.26	2.50 ± 0.11	2.43 ± 0.17	2.47 ± 0.19	NS
Lumbar	2.01 ± 0.21	2.77 ± 0.40	2.21 ± 0.22	3.43 ± 0.50	D
Inguinal	2.88 ± 0.31	3.53 ± 0.25	2.07 ± 0.25	3.08 ± 0.28	G,D
Epididymal	2.54 ± 0.12	3.23 ± 0.30	—	—	D
Periovarial	—	—	4.17 ± 0.35	6.11 ± 0.41	D

Values are expressed as the mean ± s.e.m. of six animals per group. ANOVA ($P < 0.05$): G, D, G × D and NS indicate gender effect, cafeteria-diet effect, interactive effect and nonsignificant effect, respectively.

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Table 2 Serum lipid profile and serum glucose, insulin and adipokine levels in male and female cafeteria diet–fed rats

	Male		Female		
	Control	Cafeteria-fed	Control	Cafeteria-fed	ANOVA
Triglyceride (mmol/l)	3.80 ± 0.60	3.09 ± 0.93	7.70 ± 0.31	7.91 ± 0.14	NS
Total cholesterol (mmol/l)	2.72 ± 0.30	3.03 ± 0.40	2.30 ± 0.26	2.26 ± 0.31	NS
HDL-cholesterol (mmol/l)	0.83 ± 0.08	0.90 ± 0.12	0.91 ± 0.04	0.70 ± 0.07	NS
LDL-cholesterol (mmol/l)	0.86 ± 0.20	0.78 ± 0.14	0.68 ± 0.01	0.56 ± 0.08	NS
LDL/HDL ratio	1.40 ± 0.35	1.00 ± 0.27	0.77 ± 0.03	0.73 ± 0.04	NS
Free-fatty acids (mmol/l)	0.94 ± 0.06	0.99 ± 0.14	3.00 ± 0.50	2.60 ± 0.41	NS
Glucose (mmol/l)	7.93 ± 0.21	7.79 ± 0.72	7.68 ± 0.31	7.91 ± 0.14	NS
Insulin (ng/ml)	2.89 ± 0.35	6.30 ± 0.93	1.33 ± 0.21	3.41 ± 0.59	G, D
HOMA-IR	24.4 ± 4.7	93.5 ± 5.9[a]	9.9 ± 1.9[b]	31.3 ± 4.8[a,b]	G, D, G × D
Adiponectin (ng/l)	7.9 ± 0.9	11.1 ± 1.9	24.4 ± 4.3[b]	13.9 ± 1.2[a]	G, G × D
Leptin (ng/ml)	9.1 ± 2.4	18.7 ± 0.9	6.8 ± 1.6	14.2 ± 1.6	D
PAI-1 (AU)	100 ± 16	73 ± 12	75 ± 12	72 ± 7	NS

Values are expressed as the mean ± s.e.m. of six animals per group. ANOVA ($P < 0.05$): G, D and NS indicate gender effect, cafeteria-diet effect and nonsignificant effect, respectively. Serum PAI-1 levels were measured by western blotting and control male rats value was set as 100%.
HDL, high-density lipoprotein; HOMA-IR, homeostasis model assessment of insulin resistance; LDL, low-density lipoprotein; PAI-1, plasminogen activator inhibitor-1.
t-Test ($P < 0.05$): [a]cafeteria-diet vs. control; [b]female vs. male.

Table 3 Serum lipid peroxide levels (TBARS), PON1 activities and PON1, apoA–I and apoJ levels in male and female cafeteria diet–fed rats

	Male		Female		
	Control	Cafeteria-fed	Control	Cafeteria-fed	ANOVA
TBARS (µmol/l)	23.7 ± 1.9	38.7 ± 7.8[a]	26.5 ± 2.2	23.9 ± 2.6	G × D
PON1 activities					
Paraoxonase activity (mIU/ml)	61.4 ± 5.1	60.9 ± 6.3	80.0 ± 4.4[b]	58.7 ± 4.7[a]	G × D
Arylesterase activity (IU/ml)	138 ± 8	146 ± 13	180 ± 13[b]	133 ± 7[a]	G × D
TBARS/paraoxonase activity (fmol/mIU)	177 ± 21	268 ± 50	151 ± 16	345 ± 30	D
TBARS/arylesterase activity (fmol/IU)	400 ± 40	650 ± 120	185 ± 27	461 ± 70	D
PON1 (%)	100 ± 26	58 ± 18	70 ± 21	63 ± 16	NS
ApoJ (%)	100 ± 7	122 ± 21	89 ± 14	72 ± 9	NS
ApoA–I (%)	100 ± 17	93 ± 19	176 ± 34[b]	59 ± 22[a]	G, G × D

Values are expressed as the mean ± s.e.m. of six animals per group. ANOVA ($P < 0.05$): G, D, G × D and NS indicate gender effect, cafeteria-diet effect, interactive effect and nonsignificant effect, respectively.Serum levels of PON1, apoA–I and apoJ were measured by western blotting and control male rats value was set as 100%.
Apo, apolipoprotein; PON1, paraoxonase 1; TBARS, thiobarbituric acid reactive substance.
t-Test ($P < 0.05$): [a]cafeteria-diet vs. control, [b]female vs. male.

Table 4 Liver PON1 mRNA levels in male and female cafeteria diet–fed rats

	Male		Female		
	Control	Cafeteria-fed	Control	Cafeteria-fed	REST
PON1					
Expression	1	1.248	1.955	1.209	NS
REST error		0.734–3.602	1.161–4.629	0.656–2.701	
18S					
Expression	1	0.694	0.630	0.800	NS
REST error		0.411–1.368	0.384–1.106	0.490–1.630	

Values are obtained from the mRNA expression of six animals per group and mRNA levels of control male rats were set as 1. 18s mRNA levels were used as a housekeeper. Statistical differences between experimental groups were assessed by Pair Wise Fixed Reallocation Randomisation Test ($P < 0.05$). Statistical comparisons were performed between cafeteria-fed vs. control rats and female vs. male rats.
18s, 18S ribosomal RNA; NS, nonsignificant; PON1, paraoxonase 1.

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Table 5 Liver lipid peroxide levels (TBARS), protein carbonyl groups and antioxidant enzymes activities in male and female cafeteria diet–fed rats

	Male		Female		
	Control	Cafeteria-fed	Control	Cafeteria-fed	ANOVA
TBARS (nmol/g tissue)	24.4 ± 1.3	33.9 ± 5.3	28.4 ± 1.2	56.0 ± 7.4	D
Protein carbonyl groups (%)	100 ± 25	186 ± 20[a]	92 ± 23	357 ± 46[a,b]	G, D, G × D
Liver antioxidant enzymes					
Catalase (µKat/mg protein)	13.4 ± 0.8	10.4 ± 0.7	11.1 ± 1.2	9.1 ± 0.6	G, D
Glutathione reductase (pKat/mg protein)	745 ± 82	556 ± 65	482 ± 13	459 ± 39	G
Glutathione peroxidase (nKat/mg protein)	17.3 ± 1.9	11.7 ± 0.8	25.3 ± 1.9	18.8 ± 0.4	G,D
Mn-superoxide dismutase (mIU/mg protein)	91.9 ± 22.2	62.4 ± 15.6	69.7 ± 22.5	49.0 ± 4.1	NS

Values are expressed as the mean ± s.e.m of six animals per group. ANOVA ($P < 0.05$): G, D, G × D and NS indicate gender effect, cafeteria-diet effect, interactive effect and nonsignificant effect, respectively. Protein carbonyl groups were assessed by immunoblot detection and control male rats value was set as 100%.
TBARS, thiobarbituric acid reactive substance.
t-Test ($P < 0.05$): [a]cafeteria-diet vs. control, [b]female vs. male.

The cafeteria diet did not alter serum PON1 or apoJ levels. Serum apoA–I levels were higher in female control rats compared to males and strongly dropped in the former with the cafeteria-diet feeding (66%).

Effect of cafeteria-diet feeding on liver PON1 mRNA levels in male and female cafeteria diet–fed rats
There were no gender or diet differences in liver PON1 mRNA levels (**Table 4**).

Liver lipid peroxide levels, protein carbonyl groups and antioxidant enzymes activities in male and female cafeteria diet–fed rats
Overfeeding induced 39 and 97% increase of liver lipid peroxide levels and 86 and 289% increase of liver protein content of carbonyl groups, in male and female rats, respectively (**Table 5**). Catalase and glutathione reductase activities were higher in male rats compared to females, but only catalase activity showed a diet effect. Glutathione peroxidase was higher in female rats compared to males, and decreased with the cafeteria-diet feeding. Mn-superoxide dismutase activity showed a tendency to decrease with cafeteria-diet feeding, although it did not reach statistical significance.

DISCUSSION
The cafeteria-diet feeding entailed a proinflammatory and pro-oxidant profile, represented by altered adipokine levels and by a loss of antioxidant protection in both serum and liver. Moreover, this study reveals a gender dimorphism in the response of rat serum PON1 activity to the cafeteria-diet feeding. Actually, the greater inflammatory state induced by the cafeteria-diet associated obesity in female rats could account for the changes observed in serum PON1, the activities of which were reduced only in this gender.

ApoA–I, the main protein component of HDL, is involved in the stability and function of PON1 (36,37), and thus, the higher serum apoA–I levels control female rats have compared to males would account for the higher serum PON1 activities also observed in this gender, which suggest a greater stability

of the PON1 association to HDL in control females. These gender differences in both PON1 activity and stability have been previously reported in younger rats (18). However, the inversion of gender differences in serum PON1 activity observed with aging (20) is not yet obvious at 18 months of age.

These greater serum PON1 activities in female control rats occur in parallel with a more beneficial adipokine profile—i.e., higher adiponectin and lower leptin and resistin levels—and lower serum insulin levels, thus contributing to confer on female rats a healthier state compared to males.

The cafeteria-diet feeding induced enhanced food and energy intake in both genders, thus resulting in increased adiposity and body weight, as previously described (3,38). However, female rats showed a more marked weight gain than males, which is in accordance with the reported greater resistance of males to diet-induced weight gain (3,23,39). In fact, female rats showed a 38.4% increase of body weight, whereas that of males was 16.2%. Interestingly, and dovetailing with this difference in weight gain, the cafeteria-diet feeding caused a reduction of serum PON1 activities only in female rats. In fact, a reduction of serum PON1 activity in response to obesity development has been previously reported (10,11).

Activities of the main antioxidant enzymes in the liver—catalase, glutathione peroxidase, and Mn-superoxide dismutase—were also reduced in response to the cafeteria-diet feeding in both male and female rats, thus accounting for the increased lipid and protein oxidation observed in this tissue and pointing to a greater oxidative damage in the liver. This impaired antioxidant status could be attributed to both the liver steatosis in cafeteria diet–fed rats (data not shown) and the higher content of monounsaturated fatty acids in the cafeteria diet compared to standard diet (40–42). Although both serum PON1 and the liver antioxidant enzymes activities decreased with the cafeteria-diet feeding, only serum PON1 response showed gender differences, thus suggesting a greater sensitivity of PON1 to the obese status. The inverse correlation between body weight gain and serum arylesterase activity found in female rats ($R = -0.658$, $P = 0.02$) further reinforce this idea. Nevertheless, the TBARS/paraoxonase activity and TBARS/arylesterase activity ratios reflect that

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in both male and female rats, the cafeteria-diet feeding impaired the antioxidant PON1 protection in the serum, thus being independent on the degree of overweight but rather more probably related to the higher energy intake, which was at the same level in both genders. As a whole, these results point to a greater oxidative status in the organism that, in part, would result from impaired antioxidant capacity. Although the antioxidant role of PON1 has recently been called into question (43), the present results and others previously described in different physiological situations characterized by changes in the oxidative status (18–20,44), suggest that this enzyme would be involved in the response to oxidative stress.

Alterations at the transcriptional level or as regards circulating levels of the HDL subpopulation in charge of PON1 transport in the blood would be ruled out as factors responsible for the reduction of serum PON1 activities in cafeteria diet–fed rats. In fact, neither liver PON1 mRNA levels nor serum PON1 nor apoJ levels—a marker of the HDL subpopulation to which PON1 associates (12)—were altered by the cafeteria-diet feeding. However, the reduced apoA–I levels observed in female cafeteria diet–fed rats indicate that a reduced stability of the PON1 association to HDL would rather be the factor responsible for the lower serum PON1 activity observed in this gender.

The appearance of a worsened proinflammatory profile and an hyperinsulinemic and insulin resistant state, which has been previously described with cafeteria-diet feeding (22,45), can be considered as a factor contributing to a more pro-oxidant environment. Decreased adiponectin and slightly increased resistin levels were observed in female cafeteria diet–fed rats, thus in agreement with the more proatherogenic adipokine profile reported in the obese status (4,46) and accounting, in part, for the increased insulin resistance observed with the cafeteria-diet feeding (5). Higher serum leptin levels were also observed in male and female rats fed the cafeteria-diet, as previously described (22). Both insulin resistance and leptin have been related to increased oxidative stress (47–50), thus in agreement with the enhanced pro-oxidant state and the impaired antioxidant capacity observed in both male and female cafeteria-fed rats. In fact, leptin, which stimulates *in vitro* and *in vivo* free-radical production (48,51,52) and induces oxidative stress in rats (49), has been inversely related to serum PON1 activity (49), in such a way that the cafeteria-diet associated higher leptin levels could account for the reduced serum PON1 activities observed in female rats. The strong negative correlation found between arylesterase activity and serum leptin levels in female rats further support this idea ($R = -0.684$, $P = 0.029$). The reduced serum PON1 activities in female rats are also in line with the reduction of adiponectin levels in this gender, showing a positive correlation ($R = 0.805$, $P = 0.02$) that has also been reported in humans (53), thus suggesting that variations in serum adiponectin levels could also account for the reduced serum activity of the enzyme. Concerning the greater oxidative environment related to the cafeteria-diet, it should be considered that both phospholipids and apoA–I present in HDL are susceptible to oxidation, which has been related to impaired serum PON1 activity and protection of LDL from

oxidation (54). Furthermore, and considering that PON1 protection of LDL against oxidation has been reported to result in a partial, time-dependent inactivation of the enzyme (17), we suggest that the reduced serum PON1 activities observed in female cafeteria diet–fed rats result from a direct inactivation of the enzyme. Although the cafeteria-diet is rich in monounsaturated fatty acids, the pro-oxidant state associated to body weight gain, especially in female rats, probably overrides the protective effects on PON1 attributed to monounsaturated fatty acids (55). In fact, in a previous study made by our group in which animals were fed a high-fat diet with the same amount and composition of fat as the cafeteria-diet (19), these animals did not show an increased inflammatory and oxidant state—due to the maintenance of body weight and to the high vitamin E content of the diet—a fact which would make the protection of PON1 less necessary. This decrease in PON1 activity was attributed to the decrease in both the PON1 mRNA levels and the PON1-HDL binding stability.

In conclusion, the response of serum PON1 activity to the consumption of the cafeteria-diet shows gender differences probably related to the greater obese and proinflammatory state achieved in female rats. We also suggest that the decreased PON1 activity in female cafeteria diet–fed rats is not due to reduced levels of serum PON1 or of the HDL subpopulation responsible for PON1 transport in the blood, but to the enhanced oxidative stress and damage caused by the obese status. This condition, on leading to apoA–I or PON1 oxidation could entail the destabilization of the PON1 association to HDL or a direct inactivation of the PON1 enzymatic activity.

DISCLOSURE
The authors declared no conflict of interest.

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Manuscrito VI.

The antioxidant effects of quercetin in a rat model of hyperoxaluria involve serum paraoxonase 1 activation

Emilia Amengual-Cladera, Antònia Nadal-Casellas, Yolanda Gómez-Pérez, Isabel Gomila, Rafael M. Prieto, Ana María Proenza, Isabel Lladó. Manuscript.

The antioxidant effects of quercetin in a rat model of hyperoxaluria involve serum paraoxonase 1 activation

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Short tittle: Serum PON1 in a rat model of hyperoxaluria

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ABSTRACT

Serum paraoxonase 1 (PON1) has been reported to be an important contributor to the antioxidant and anti-inflammatory activities of HDL, avoiding LDL oxidation. The activity of this enzyme is reduced in patients with renal insufficiency, caused by elevated oxidative stress and disturbances of apolipoprotein metabolism. Therapeutic utilization of antioxidants to control renal oxidative stress may be an effective therapy in renal protection. The aim was to investigate the protective effects of several antioxidant compounds against the oxidative stress associated to renal failure induced by ethylene glycol (EG), focusing on the possible role of serum PON1 activity. Fifty-four male Wistar rats were randomly assigned to six groups (n = 9): an untreated control (C) group, an EG treated group, a catechin (CAT) treated group, an epicatechin (EPI) treated group, a quercetin (QUE) treated group and a folk herbal extract (FHE) treated group. After 16 days of treatment, calcium oxalate lithiasis was induced in the rats using ethylene glycol. After 8 days (treatment + EG), the animals were sacrificed. EG treatment impaired kidney composition, increased oxidative damage and decreased serum paraoxonase and arylesterase activities. Catechin (CAT), quercetine (QUE) and the folk herbal extract *Fagolitos* (FHE) improved oxidative status by enhancing antioxidant defenses — superoxide dismutase and PON1 activities — and reducing oxidative damage generated by oxalate accumulation. Serum PON1 would play a role as a defense mechanism in kidney disease and could constitute a link between renal failure and the associated cardiovascular complications.

KEYWORDS

INTRODUCTION

Ethylene glycol (EG) administration to rats constitutes an useful model to evaluate renal papillary stone development linked to oxidative cell damage due to hyperoxaluria [11]. The oxalic acid that results from the detoxification of EG, precipitates as calcium oxalate monohydrate in numerous tissues, especially the kidney [23]. Oxalate exposure alters mitochondrial function, induces changes in gene expression, activates apoptosis and promotes free radical production and lipid peroxidation, which can result in renal failure [16, 23]. The oxidative damage appears primarily due to hyperoxaluria and is augmented by crystal deposition in the renal tubules [30].

Chronic renal failure is the end-stage of most chronic renal diseases and is associated with a tendency to atherosclerosis due to enhanced oxidative stress and insufficient antioxidant enzyme activities together with abnormalities in lipid parameters [1]. The incidence of cardiovascular complications in CRF is on average about 20 times higher than the normal population [29].

Serum paraoxonase 1 (PON1) is a calcium-dependent esterase synthesized in the liver and closely associated to HDL containing both apolipoprotein A-I and apolipoprotein J (apoA-I and apoJ) [22]. PON1 has been reported to be an important contributor to the antioxidant and anti-inflammatory activities of HDL, avoiding LDL oxidation. Thus, PON1 protects the endothelium from the pro-oxidant effect of oxidized LDL and from atherosclerosis development [28]. In addition, PON1 hydrolyses homocysteine thiolactone and prevents homocysteinemia, which is also involved in atherogenesis [15]. The activity of this enzyme is reduced in patients with enhanced atherosclerosis, in disorders of lipoprotein metabolism, in renal insufficiency and after renal transplantation, caused by elevated oxidative stress and disturbances of apolipoprotein metabolism [33]. Given that increased oxidative stress has been observed in patients with cardiovascular diseases, the use of nutritional antioxidants for the attenuation of atherosclerosis development has been suggested [8]. In fact, supplementation with antioxidant agents is reported to rescue renal epithelial cells from oxalate-induced generation of ROS, lipid peroxidation and other toxic effects [18]. Since ROS appears to be responsible for cellular injury, therapeutic utilization of antioxidants to control renal oxidative stress may prove an effective therapy in renal protection [20].

Plants remain the basis for a large proportion of the medications used today for the treatment of a variety of diseases [5], due to their content in polyphenolic compounds, such as quercetin and catechins, which have a high antioxidant activity. In fact, a traditional herbal preparation, very popular in Spain during the 1970s (known as *Fagolitos*), has been shown to have anti-lithiasic action probably due to its antioxidant properties [11].

This study was performed to investigate the potentially protective effects of the antioxidant flavonoids catechin, epicatechin and quercetin and of the traditional herbal preparation named *Fagolitos* against the oxidative stress associated with renal failure induced by EG. Since EG induces oxidative-stress related renal failure and subsequent atherogenesis, we focused on the possible involvement of serum PON1 activity in the antioxidant response and we analyzed the main factors involved in its stability and function.

MATERIALS AND METHODS

Materials

Catechin, epicatechin, quercetin and substrates for the measurement of PON1 activities (diethyl p-nitrophenyl phosphate and phenylacetate) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Folk herbal extract was prepared and supplied by Salva Trobat Chemist's (Palma de Mallorca, Spain). Protein Oxidation Detection kit was obtained from Invitrogen (Carlsbad, CA, USA). The kit for measurement of serum HDL-cholesterol (Ref. 1133505) was purchased from Linear Chemicals SL (Barcelona, Spain). Rabbit polyclonal antibody to human PON1 was kindly provided by M. Mackness and B. Mackness [21]. Rabbit polyclonal antibody to human apoA-I (Cat. No. 178422) was purchased from Calbiochem and goat polyclonal antibody to rat apoJ (Cat. No. sc-137147) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The enhanced chemiluminescence Western blotting analysis reagents were supplied by Bio-Rad (Hercules, CA, USA). Routine chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and Panreac (Barcelona, Spain).

Animals and treatments

Animal experiments were performed in accordance with general guidelines approved by our institutional ethics committee and EU regulations (86/609/CEE and 2003/65/CE). Fifty-four male Wistar rats, each weighing approximately 260 g, were acclimated for 7 days in cages prior to experimental treatment. Rats had ad libitum access to pelleted standard chow (A04 Panlab, Barcelona, Spain) and to tap water under a controlled 12-h light/dark cycle at $22 \pm 2°C$.

The animals were divided into six groups (nine animals per group): an untreated control (C) group, an EG treated group, a catechin (CAT) treated group, an epicatechin (EPI) treated group, a quercetin (QUE) treated group and a folk herbal extract (FHE) treated group. For 16 days, the control group and the EG group were supplied with drinking water without additives, the CAT, EPI and QUE groups were supplied with drinking water supplemented with 100 mg/L of catechin, epicatechin or quercetin, and the FHE-treated group was supplied with drinking water supplemented with 7 mL/L of the folk herbal extract. The dose of FHE administered to the rats was equivalent to the human dose and was calculated considering that a dose of 30 mL/day of herbal preparation was recommended for humans. FHE contains fluid extracts of Arctotaphylos uva-ursi L. (2.16%), Zea mays L. (2.16%) and Ricinus zanzibariensis L. (46.48%), tincture of Sabal serrulata L. (21.5%), mother tincture of Agathosma betulina L. (17.5%), glycerin (10%), and anis essence (0.2%). After 16 days, the drinking water that contained the corresponding additives was also supplemented for a further 8 days with 0.8% v/v EG plus 1% w/v NH_4Cl.

Sample preparation

Rats were sacrificed by decapitation. Blood was collected and serum was obtained and stored at -80ºC until analyzed. Kidneys were rapidly removed and weighed. Kidneys were minced and homogenized in an ice bath in 50mM potassium phosphate buffer (pH 7.4). The homogenate was divided into two aliquots. The first aliquot was centrifuged at 700xg for 20min and the supernatant was used to determine tissue composition and protein carbonyl levels. The second aliquot was centrifuged at 20000xg and the supernatant was used to determine Mn-superoxide dismutase (SOD) activity.

Serum PON1 activities and HDL-cholesterol levels

Serum PON1 activities were assayed in a microtitre plate spectrophotometer (Bio-Tek©️ Instruments, VT, USA) by measuring the rate of hydrolysis of phenylacetate (arylesterase activity, EC3.1.8.1) and paraoxon (paraoxonase activity, EC 3.1.1.2), as previously described [6]. HDL–cholesterol levels were measured using an enzymatic homogeneous assay kit.

Western blot analysis of serum PON1, apoA-I and apoJ protein levels

30 µg of serum proteins were fractionated on 15% (PON1) or 12.5% (apoA-I and apoJ) SDS-PAGE gels and electrotransferred onto a nitrocellulose filter. Rabbit polyclonal antibodies against human apoA-I and rat PON1 and goat polyclonal antibody against apoJ were used as primary antibodies. Development of immunoblots was performed using an enhanced chemiluminescence kit. Bands were quantified by ChemiDoc XRS (Bio-Rad, CA, USA). Autoradiograms revealed an apparent molecular mass of 43 kDa, 50 kDa and 28 kDa for PON1, apoJ and apoA-I, respectively.

Kidney composition, protein carbonyl groups and enzyme activities

Protein and total DNA levels were determined in the homogenate supernatants using the Bradford [3] and the diaminobenzoic acid [32] methods, respectively. Total lipid content was measured using the Folch method [7]. Protein carbonyl groups, as an index of protein oxidation, were measured in kidney homogenates by immunoblot detection using the OxyBlot™ Protein Oxidation Detection Kit according to the manufacturer's protocol with several modifications [12]. Citrate synthase (CS) [25], and SOD [26] activities were assayed by spectrophotometric methods.

Statistical analysis

All data are expressed as the mean values ± SEM of 9 animals per group. Statistical differences between groups were analyzed by Student's t-test and a p-value less than 0.05 was considered statistically significant. All statistical analyses were performed using a statistical software package (SPSS 17.0 for Windows, Inc., Chicago, IL, USA).

RESULTS

Kidney composition, protein oxidative damage and SOD and CS activities

In comparison to the C group, treatment with EG induced a decrease in kidney DNA content that was not prevented by QUE and FHE treatments (Table 1). QUE and FHE treatments decreased protein levels compared to both C and EPI groups. EG treatment induced an increase of kidney lipid content that was prevented by all the antioxidant compounds assayed. Carbonyl groups increased with EG treatment, but this increase was prevented by the treatments with QUE and CAT, but not by EPI or FHE. SOD and CS activities were not modified by EG treatment but SOD activity was increased by CAT, and CS activity was increased by QUE, CAT and FHE.

Serum HDL-cholesterol levels and paraoxonase activities

Serum PON1 activity (Table 2) decreased with EG treatment and values were completely restored by QUE treatment and partially restored by CAT, EPI and FHE, resulting in statistically significant higher activity values in the QUE group compared to the rest of the antioxidants tested. EG treatment decreased serum AE activity. QUE, EPI and, to a higher degree, FHE increased this activity. HDL cholesterol levels did not show significant differences between groups, except those of the CAT group that were higher compared to the C group.

Serum PON1, ApoA1, and ApoJ protein levels

FHE treatment increased PON1 protein levels compared to all groups except QUE (Table 3). In contrast, FHE treatment decreased ApoJ protein levels and differences were only significant compared to the CAT and EPI groups. No significant differences between groups were found in ApoA1 protein levels.

PON1 activity to ApoA1 and ApoJ protein level ratios

EG treatment induced a decrease of PON1 to APOA1 ratio that was prevented by QUE and FHE treatments (Fig 1 A). The same profile was observed for PON1 to ApoJ ratio (Fig 1 B).

DISCUSSION

Oral EG administration induces chronic hyperoxaluria and generates calcium oxalate deposition in kidneys [30]. Exposure to a high concentration of oxalate results in the production of free radicals, which induces oxidative stress and subsequent renal damage [35]. The results obtained in this study point to important changes in tissue composition induced by EG treatment. The kidney lipid content is increased but the effect of EG is counteracted by all the antioxidants tested, especially by QUE and FHE. The lipid accumulation in kidney associated with EG could be due to the mitochondrial dysfunction associated to hyperoxaluria [34], which impairs fatty acid oxidation and leads to fat accumulation in cells. In this sense, the effect of the antioxidants would occur through the improvement of mitochondrial function. In this sense, an enhancement of lipid catabolism by catechins in exercising muscle of high fat diet fed mice has been previously described [19, 24], and it is in accordance with the increased levels of citrate synthase activity observed in the CAT group.

The decrease in the kidney DNA content observed in EG-treated rats could be attributed to the cellular damage induced by oxalate accumulation [35]. Moreover, exposure to oxalate has been reported to generate bioactive lipids that activate caspases that may participate in apoptotic cell death [16]. All the antioxidants assayed but FHE alleviated the reduction of DNA content induced by EG, which, in the case of CAT, could be related to the decrease in apoptosis and the improvement of antioxidant defences that have been attributed to green tea catechins [14]. Interestingly, FHE is also associated with a decrease in kidney protein content, thus suggesting that this herbal extract does not protect the kidney from the detrimental effects of oxalate at least at a tissue composition level.

The increased oxidative damage shown by the kidney of EG-treated animals could be the result of both the increased ROS production associated with oxalate accumulation [35] and the lack of antioxidant response, as suggested by the maintenance of SOD activity. In fact, variations in SOD activity according to the EG dose and the duration of the treatment have been previously reported [4, 13]. Protein oxidative damage was prevented by all the antioxidants assayed, especially catechin whose effects were also extended to an increase of SOD activity. In fact, the inhibitory effect of green tea

(which contains approximately 13% catechins) on calcium oxalate urolithiasis is most likely due to its antioxidative effects, which includes the increase of SOD activity [14]. Paraoxonase activity has been suggested to play a role as an antioxidant defense [31]. In our study, the decreased serum PON1 activity induced by EG treatment could be related to the oxidative stress generated by oxalate accumulation [23]. Thus, PON1 protection against oxidation has been reported to result in a partial, time-dependent inactivation of the enzyme [2]. It is well known that PON1 loses its activity in an oxidative environment. Therefore, any factors that affect the status of oxidative stress will also affect PON1 activity status [10]. In fact, in a pro-oxidant state associated with body weight gain induced by high-fat diet feeding, we have found a decrease of PON1 activity [31]. The EG associated decrease in PON1 activity could be mainly attributed to alterations in the PON1 structure affecting its function, since no changes in the levels of PON1 protein or in the enzyme stability have been observed. ApoA–I, the main protein component of HDL, plays a key role in the stability and function of PON1 [9], being considered a PON1 stability marker, and their levels were unchanged in all the experimental groups. Our results suggest a role of PON1 in the defence against the oxidative stress associated with renal failure. This idea is reinforced by the decrease in PON1 activity reported in haemodialysis patients [17]. In this sense, a relationship between decreased PON1 activity and the higher incidence of cardiovascular disease in chronic renal failure patients [27] can not be ruled out.

All the antioxidants tested in this study induced an increase of serum PON1 activity, probably reflecting the prevention of the possible inactivation of PON1 induced by EG treatment. This protective action is more marked when rats are treated with QUE, a ubiquitous flavonoid present in fruits and vegetables, which is the only one among the assayed compounds that reverts the EG values to control values. Our results are in accordance with the previously reported capacity of quercetin to increase serum paraoxonase activity and with the idea of an antiatherogenic role for this dietary antioxidant [10]. Although the effects of the compounds tested on serum PON1 activity could be a consequence of their antioxidant properties, a direct effect on PON1 gene expression can not be ruled out, as has been reported for quercetin [10]. In fact, in the FHE supplemented rats the increase in serum PON1 activity is accompanied by an increase of serum PON1 levels suggesting an increased expression of PON1 in this

group. It is worth noticing that at least two of the plant extracts included in the FHE mixture contain quercetin, to which part of the observed effects of this herbal preparation could be attributed.

The decrease in Apo J levels, a marker of the HDL subpopulation to which PON1 associates [31], points to FHE supplemented rats having a lesser number of HDLs that carry PON1. The higher PON1 to ApoJ ratio shown by QUE and FHE supplemented rats compared to EG rats suggest that this HDL fraction is more enriched in the enzyme and further reinforces a role of quercetin enhancing PON1 antioxidant capacity.

In summary, EG treatment impairs kidney composition by increasing lipid accumulation and decreasing cell number. These effects could be attributed to an enhancement of cellular damage and to an alteration of mitochondrial oxidative capacity. Catechin, quercetin and the folk herbal extract *Fagolitos* are the antioxidants with a more marked protective effect on oxalate induced renal impairment. They would act by enhancing oxidative activity that results in a reduction of the renal accumulation of lipids. The oxidative damage protection exerted by these antioxidants involves an induction of SOD activity, in the case of catechin, and of PON1 activity, in the case of both quercetin and *Fagolitos*. Our results support a role of PON1 as a defense mechanism in kidney disease and a link between renal failure and associated cardiovascular complications.

Table 1. Kidney composition, protein oxidative damage and SOD and CS activities

	C	EG	QUE	CAT	EPI	FHE
DNA (mg/g tissue)	1.46 ± 0.09	1.14 ± 0.05^a	1.21 ± 0.08^a	1.35 ± 0.19	1.26 ± 0.09	$1.05 \pm 0.06^{a,e}$
Protein (mg/g tissue)	104 ± 2	98.5 ± 7.1	92.0 ± 5.0^a	93.1 ± 6.2	104 ± 2.9^c	$85.8 \pm 3.6^{a,e}$
Lipid (mg/g tissue)	31.7 ± 1.9	48.8 ± 3.4^a	31.4 ± 2.2^b	37.7 ± 2.8^b	$37.0 \pm 1.3^{a,b,c}$	$29.0 \pm 2.1^{b,d,e}$
Carbonyl groups (%)	100 ± 5	197 ± 40^a	116 ± 13^b	104 ± 15^b	129 ± 7^a	122 ± 19
SOD (I.U./g protein)	13.5 ± 0.4	13.6 ± 0.7	14.4 ± 0.6	$16.5 \pm 0.7^{a,b,c}$	13.8 ± 0.4^d	14.1 ± 0.5^d
CS (I.U./g protein)	69.0 ± 3.5	74.3 ± 2.9	80.4 ± 4.4^a	$83.1 \pm 3.0^{a,b}$	$67.9 \pm 2.7^{c,d}$	$76.8 \pm 2.4^{a,e}$

C, control; EG, ethylene glycol; QUE, quercetine; CAT, catechin; EPI, epicatechin; FHE, folk herbal extract; SOD, Mn-superoxide dismutase; CS, citrate synthase. Values are means $\pm$ SE of 9 animals per group. Student's t-test ($p<0.005$): **a** indicates EG, QUE, CAT, EPI and FHE *vs* C, **b** indicates QUE, CAT, EPI and FHE vs EG, **c** indicates CAT, EPI and FHE *vs* QUE, **d** indicates EPI and FHE vs CAT and **e** indicates FHE *vs* EPI.

Table 2. Serum HDL-cholesterol levels and paraoxonase activities

	C	EG	QUE	CAT	EPI	FHE
HDLc (mmol/L)	0.56 ± 0.10	0.83 ± 0.12	0.81 ± 0.13	0.86 ± 0.12^a	0.79 ± 0.14	0.86 ± 0.12
PON1 (mI.U./mL)	71.2 ± 5.8	26.0 ± 1.4^a	64.4 ± 3.1^b	31.9 ± 2.3^{a,b,c}	33.2 ± 1.6^{a,b,c}	43.2 ± 5.5^{a,b,c,d}
AE (I.U./mL)	22.3 ± 2.6	11.2 ± 1.0^a	15.4 ± 1.7^{a,b}	12.4 ± 1.3^a	14.3 ± 0.4^{a,b}	19.9 ± 1.6^{b,c,d,e}

C, control; EG, ethylene glycol; QUE, quercetine; CAT, catechin; EPI, epicatechin; FHE, folk herbal extract; HDLc, high density lipoprotein-cholesterol; PON1, paraoxonase 1; AE, arylesterase. Values are means ± SE of 9 animals per group. Student's t-test ($p<0.005$): **a** indicates EG, QUE, CAT, EPI and FHE *vs* C, **b** indicates QUE, CAT, EPI and FHE vs EG, **c** indicates CAT, EPI and FHE *vs* QUE, **d** indicates EPI and FHE vs CAT and **e** indicates FHE *vs* EPI.

Table 3. Serum PON1, ApoA1, and ApoJ protein levels

	C	EG	QUE	CAT	EPI	FHE
PON1 (%)	100 ± 20	93.9 ± 12.4	117 ± 12	100 ± 12	102 ± 17	180 ± 35[a,b,d,e]
ApoA1 (%)	100 ± 3	85.7 ± 6.3	82.9 ± 12.7	87.6 ± 7.4	103 ± 8	91.8 ± 6.4
ApoJ (%)	100 ± 5	98.2 ± 10.1	99.9 ± 6.7	111 ± 6	104 ± 8	83.0 ± 8.6[d,e]

C, control; EG, ethylene glycol; QUE, quercitine; CAT, catechin; EPI, epicatechin; FHE, folk herbal extract; PON1, paraoxonase 1; ApoA1, apolipoprotein A1; ApoJ, apolipoprotein J. Values are means ± SE of 9 animals per group. Student's t-test ($p<0.005$): **a** indicates EG, QUE, CAT, EPI and FHE *vs* C, **b** indicates QUE, CAT, EPI and FHE vs EG, **c** indicates CAT, EPI and FHE *vs* QUE, **d** indicates EPI and FHE vs CAT and **e** indicates FHE *vs* EPI.
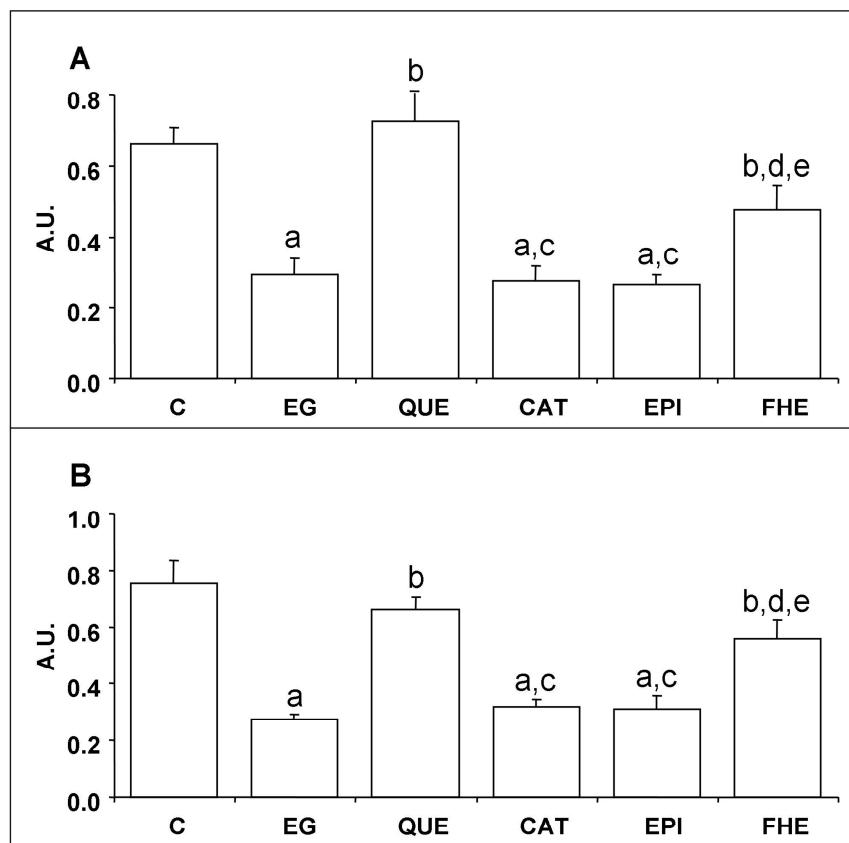
Figure 1. PON1 activity to ApoA1 ratio (A) and PON1 activity to ApoJ ratio (B). C, control; EG, ethylene glycol; QUE, quercitine; CAT, catechin; EPI, epicatechin; FHE, folk herbal extract; PON1, paraoxonase 1; ApoA1, apolipoprotein A1; ApoJ, apolipoprotein J. Values are means ± SE of 9 animals per group. Students's t-test (p<0.005): **a** indicates EG, QUE, CAT, EPI and FHE *vs* C, **b** indicates QUE, CAT, EPI and FHE vs EG, **c** indicates CAT, EPI and FHE *vs* QUE, **d** indicates EPI and FHE vs CAT and **e** indicates FHE *vs* EPI.

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