Modulación metabólica de la oleoil estrona en ratas con sobrepeso

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MODULACIÓN METABÓLICA DE LA OLEOIL ESTRONA EN RATAS CON SOBREPESO

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Resumen

Aunque se ha caracterizado extensamente la eficacia de la administración de la oleoil estrona sobre la pérdida de grasa corporal, todavía no se ha conseguido averiguar su mecanismo de acción. Esta tesis doctoral se ha centrado en el estudio de la acción de la oleoil estrona a nivel periférico, utilizando ratas Wistar macho con sobrepeso (previamente alimentadas con una dieta de cafetería), y en un modelo de restricción energética forzada equivalente (*pair fed*), con el fin de discernir los efectos específicos de la oleoil estrona sobre vías metabólicas, partición de la energía y regulación hormonal.

Los resultados obtenidos indican que la administración de oleoil estrona junto con una restricción energética adicional no potencia su efecto sobre la pérdida de grasa corporal, aunque se induce la movilización de proteína corporal.

Por otro lado, tanto la disminución de las reservas de grasa producidas por el tratamiento con oleoil estrona como el perfil de expresión génica relacionado con el metabolismo lipídico en el tejido adiposo, parecen ser consecuencia, en buena parte, de la disminución de la ingesta.

El hígado es, probablemente, el tejido que marca las diferencias en el metabolismo energético tras el tratamiento con oleoil estrona. La acción de la oleoil estrona en el hígado da lugar a cambios profundos y casi siempre en sentido contrario al observado en las ratas *pair fed*. Concretamente, la administración de oleoil estrona, a pesar de la diminución de la ingesta, disminuye la expresión hepática de los genes implicados en la gluconeogénesis, mantiene la de los relacionados con la glucolisis y la lipogénesis e incluso incrementa la expresión de los implicados en la síntesis de triacilgliceroles, de acuerdo con el incremento del factor de transcripción SREBP-1c, lo que diferencia claramente la acción de la oleoil estrona de la simple restricción energética. Estos resultados explican en parte el mantenimiento de la glucemia y el aumento del consumo de triacilgliceroles por parte de los órganos periféricos.

Además, el tratamiento con oleoil estrona incrementa en el hígado la expresión hepática de genes y niveles de proteínas que participan en el transporte reverso de colesterol y en su oxidación a ácidos biliares. Estos efectos están de acuerdo con la disminución del colesterol circulante que se observa tras el tratamiento con oleoil estrona.

El efecto adelgazante de la administración de la oleoil estrona se ve limitado por el efecto contraregulador de los glucocorticoides. Los resultados obtenidos indican que las ratas tratadas con oleoil estrona aumentan tanto la síntesis de glucocorticoides en las glándulas adrenales como su eliminación en el hígado, lo que modera el incremento de glucocorticoides circulantes, a diferencia del grupo *pair fed,* sometido a una restricción energética forzada.

La hidrólisis de la oleoil estrona libera estrona, un estrógeno débil pero fácilmente activable a estradiol. La sobrecarga de estrona producida tras la administración de la oleoil estrona inhibe su activación de estradiol, lo que limita los efectos estrogénicos, pero a la vez se inhibe la síntesis y la activación de testosterona, con lo que se produce una marcada disminución de la testosterona circulante.

En definitiva, el tratamiento con oleoil estrona disminuye la grasa corporal como consecuencia del incremento de la sensación de saciedad, pero a diferencia de una simple restricción energética mantiene la glucemia y la síntesis de lípidos hepáticos, a la vez que limita el incremento de glucocorticoides circulantes, lo que da lugar a la moderación del nivel de estrés de los animales.

Metabolic modulation of oleoyl estrone in overweight rats

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Abstract

This thesis has been focused on the study of the action of oleoyl estrone using overweight male Wistar rats, and a model of restricted energy intake (*pair fed*), in order to discern the specific effects on metabolic pathways of oleoyl estrone.

The decrease in fat reserves produced by treatment with oleoyl estrone as suggests by the expression profile of genes associated with lipid metabolism in adipose tissue seems to be due, largely, to the decrease in food intake.

The oleoyl estrone treatment results in profound changes in liver metabolism. In spite of decreased food intake, administration of oleoyl estrone decreases the hepatic expression of genes involved in gluconeogenesis, maintaining those of glycolysis and lipogenesis; there are, even, an increase in those of triacylglycerol synthesis. This is in agreement with SREBP-1c increase, a condition which clearly differentiates the action of oleoyl estrone from *pair fed* energy restriction. These results explain at least partly the maintenance of blood glucose and the increased consumption of triacylglycerols by peripheral organs.

Treatment with oleoyl estrone also increases the hepatic expression of genes involved in reverse cholesterol transport and its oxidation to bile acids. These effects are consistent with the marked decrease of circulating cholesterol observed under treatment with oleoyl estrone.

Rats treated with oleoyl estrone increase both glucocorticoid synthesis in the adrenal glands and their oxidative inactivation in the liver, thereby moderating the increase in circulating glucocorticoids, unlike the *pair fed* group, subjected to a non voluntary restriction in energy intake.

The estrone overload produced after the administration of the oleoyl estrone inhibits its own activation to estradiol, thereby limiting the overall estrogenic effects. However, this process also inhibits the synthesis and activation of testosterone, resulting in a decrease its circulating levels.

In conclusion, treatment with oleoyl estrone decreases body fat due to a higher level of satiety, but unlike pair fed energy restriction, blood glucose and hepatic lipid synthesis are maintained, and the increase of circulating glucocorticoids is limited, lowering the level of stress endured by the rats.

Publicaciones que han dado lugar a esta tesis:

Combined effect of oral oleoyl-estrone and limited food intake on body composition of young overweight male rats. Romero MM, Esteve M, Alemany M (2006) International Journal of Obesity 30: 1149-1156

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Oleoyl-estrone increases adrenal corticosteroid synthesis gene expression in overweight male rats Romero MM, Vilà R, Fernández-López JA, Esteve M, Alemany M (2010) Steroids 75: 20-26

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Índice

INTRODUCCIÓN

1 Introducción

1.1 La obesidad en el contexto del síndrome metabólico

El síndrome metabólico engloba una serie de patologías asociadas a la obesidad abdominal, entre las que destacan resistencia a la insulina, hiperlipemia, dislipoproteinemia, esteatosis hepática, hipertensión, una función alterada de las hormonas sexuales y de los glucocorticoides y un estado protrombótico e inflamatorio sostenido que predisponen a sufrir diabetes tipo 2, cardiopatías y accidentes vasculares, enfermedades del aparato motor como la artrosis, depresión y algunos tipos de cáncer como el de endometrio, mama y colon [1].

Se considera que el síndrome metabólico es principalmente una consecuencia de la inadaptación a las dietas actuales, a menudo muy ricas en energía, debido principalmente a que contienen una alta proporción de grasa, proteínas animales de alta calidad, azúcares y sal [2]. Tras su ingestión, el aumento de disponibilidad de ácidos grasos dificulta la oxidación de glucosa interfiriendo en la respuesta a la insulina [3]. Así, muchos investigadores consideran que la resistencia a la insulina es el principal factor común del síndrome metabólico, aunque en la actualidad empiezan a tomar mucha relevancia el papel de la respuesta inflamatoria del tejido adiposo [4], concretamente de la grasa abdominal [5] y la alteración de la función de las hormonas esteroideas [6].

La resistencia a la insulina disminuye la capacidad de controlar la concentración circulante de glucosa debido a la interferencia de los ácidos grasos. De hecho se considera que la resistencia a la insulina es un mecanismo de defensa, desarrollado a lo largo de la evolución, para proteger al organismo de la falta de glucosa en el ayuno, cuando la movilización de las reservas de triacilgliceroles procuran ácidos grasos para sostener las necesidades energéticas. Sin embargo, no estamos preparados para la situación opuesta: los efectos tóxicos de un exceso crónico de nutrientes [2]. El exceso de triacilgliceroles de la dieta, por tanto, limita la utilización de glucosa por el músculo y otros tejidos, aumentando sus niveles [7]. El incremento de concentración de glucosa plasmática debido a la resistencia a la insulina del músculo estimula la secreción de más insulina, y con ello, su utilización en la síntesis hepática de triacilgliceroles, lo que provoca, a su vez, el aumento de lipoproteínas circulantes y también que el contenido lipídico del hígado aumente [8]. Además la acumulación intracelular de triacilgliceroles y de algunos metabolitos derivados de los ácidos grasos (diacilgliceroles y ceramidas, entre otros) altera la señal de la insulina y disminuyen el transporte de glucosa [9]. Como consecuencia, el metabolismo glucídico del hígado se altera y ya no puede controlarse, a pesar del incremento de secreción y de niveles de insulina [10], mientras que el tejido adiposo tiene que hacer frente a un incremento de lípidos y glucosa circulantes cada vez mayor.

El exceso de energía al que el organismo tiene que hacer frente durante la obesidad inducido por la dieta se intenta compensar en parte incrementando el gasto energético,

pero, al final, la mayor parte de la energía se acaba acumulando en el tejido adiposo (y en parte en otros tejidos) en forma de triacilgliceroles [11]. El tejido adiposo incorpora los ácidos grasos de las lipoproteínas y también una parte del exceso de glucosa con la que sintetiza lípidos y/o produce lactato [12]. La creciente acumulación de triacilgliceroles en los adipocitos provoca su hipertrofia, hipoxia y acidosis. Todos estos factores dañan la función del tejido adiposo, que en respuesta a esta agresión, segrega citoquinas proinflamatorias [13]. También se alteran procesos metabólicos en los que están implicados el retículo endoplasmático y las mitocondrias [14]. Estos factores favorecen en conjunto la apoptosis de los adipocitos y provocan un incremento de la infiltración de células del sistema immune [15]. La infiltración de macrófagos y de otras células inflamatorias ayuda a frenar la expansión del tejido ya que limita el acúmulo de más energía aunque a costa de reducir la capacidad tamponadora de la disponibilidad de energía propia del tejido adiposo, y en detrimento de la función de otros órganos [16]. Así la sobrecarga de lípidos que ocurre en el tejido adiposo bajo condiciones de exceso crónico de disponibilidad de energía produce una activación defensiva (aunque ineficaz) del sistema immune y la sobreexpresión de genes que favorecen la inflamación y que contribuyen a una disfunción metabólica global.

En el síndrome metabólico el equilibrio funcional entre andrógenos, estrógenos y glucocorticoides se altera, con preponderancia de los glucocorticoides en detrimento de la acción las hormonas sexuales [17], lo que está de acuerdo con el aumento de la deposición de grasa abdominal en ambos sexos y de una mayor incidencia del síndrome metabólico en edades avanzadas, coincidiendo con la disminución de los niveles circulantes de andrógenos (hipoandrogenismo senil) o estrógenos (climaterio). Los glucocorticoides, que por un lado actúan contra la inflamación característica del síndrome metabólico, agravan la resistencia a la insulina y a la leptina [18], favoreciendo de este modo la síntesis de lípidos, la movilización proteica, el incremento de la producción de glucosa hepática y la desmineralización del hueso (osteoporosis).

1.1.1 Causas de la obesidad

La obesidad es una componente importante del síndrome metabólico. Es una enfermedad crónica multifactorial, consecuencia de la interacción entre el genotipo y el ambiente, que se caracteriza por un exceso de grasa corporal, es decir, la acumulación perjudicial de reservas de grasa muy por encima de las necesidades funcionales. Este exceso de grasa provoca una mayor masa corporal que sobrecarga el sistema respiratorio y el cardiovascular, limita la movilidad, impone un mayor esfuerzo al sistema locomotor y desestabiliza el equilibrio homeostático.

El contenido de grasa corporal está influenciado por factores genéticos, maternos (gestacionales, relacionados con la lactancia) y epigenéticos, pero el reciente incremento de obesidad mundial es debido principalmente a un cambio de dieta y de estilo de vida [19]. La causa fundamental del sobrepeso y de la obesidad es un desajuste en el equilibrio energético corporal, que se explica en buena parte por un aumento en la ingesta de

alimentos hipercalóricos que además son ricos en grasa, sal y azúcares de bajo peso molecular, y en un descenso de la actividad física como resultado de la naturaleza cada vez más sedentaria de muchas formas de trabajo, de los nuevos modos de desplazamiento y de una creciente urbanización. La Organización de Comida y Agricultura (FAO) ha registrado desde 1960 un incremento global de la ingesta calórica y un cambio en la composición de ésta, en la que aumenta la presencia de azúcares refinados y de alimentos con una alta densidad energética [20]. Dado que estos alimentos tienen una gran palatabilidad, provocan un incremento de su ingestión, alterando los mecanismos que modulan el apetito y la saciedad.

Se han descrito centenares de variantes alélicas que pueden predisponer a la obesidad, localizadas en distintos genes, contribuyendo cada una de ellas algo (habitualmente poco) al riesgo final de desarrollar la enfermedad. A pesar de la abundancia de estos estudios, la obesidad humana de base genética clara se limita a unos pocos casos de origen monogénico y alteraciones cromosómicas, en el resto de los casos la interacción con el ambiente es fundamental para su eventual manifestación [21].

A través del proyecto Genoma Humano, mediante el cual es posible analizar varios cientos de miles de variaciones (polimorfismos) en la secuencia del genoma, se han buscado variantes concretas que fueran más frecuentes en individuos obesos. Sin embargo sólo se ha identificado la asociación de una variante genética con el riesgo de desarrollar obesidad en el gen FTO (*fat mass and obesity-associated protein*) [22]. De hecho se ha constatado que, en general, una misma enfermedad poligénica puede estar causada por variantes genéticas distintas, complicándose de esta manera el estudio de las causas de la obesidad.

1.1.2 Origen de la obesidad

Se han planteado diversas hipótesis evolutivas para entender las raíces biológicas de la actual epidemia de obesidad y de sus comorbilidades. Por ejemplo, la teoría de Speakman, defendida como el origen del lipostato, señala que los genes se seleccionarían mediante la existencia de una doble presión evolutiva: un cierto acúmulo de grasa permitiría sobrevivir en situaciones de escasez, pero su exceso podría ser lesivo ya que limitaría la movilidad y la huida frente a los depredadores [23].

En paralelo, también se ha descrito que el aumento de proteínas en la dieta podría favorecer el incremento del tamaño del cerebro. En este contexto una cierta resistencia a la insulina protegería al cerebro, más evolucionado, desarrollado y con más requisitos energéticos del hombre carnívoro, frente a la disminución de glucosa en los periodos de escasez de comida [24]. Actualmente, en la sociedad industrializada que permite el fácil acceso a dietas de alto contenido calórico, la resistencia a la insulina deja de ser una ventaja evolutiva ya que predispone a padecer obesidad y diabetes tipo 2. Sin embargo, a pesar de su implicación en múltiples enfermedades metabólicas, tampoco se ha sometido a una presión evolutiva en su contra ya que hace muy poco tiempo que la Humanidad, en

general, dispone de acceso generalizado a estas dietas, y además las consecuencias nocivas de la resistencia a la insulina suelen aparecer en la edad post-reproductiva.

Desde un punto de vista evolutivo, la obesidad y la resistencia a la insulina pueden interpretarse también como una adaptación a la infección, ya que estas patologías están relacionadas con la activación de los marcadores del sistema inmunológico y de inflamación. Parece ser que la presión evolutiva sobre el control del almacenamiento de energía y la lucha contra patógenos coevolucionaron, lo que explicaría la relación de los preadipocitos con los macrófagos y el hecho de que ambos sean capaces de secretar citoquinas [4]. Empieza a extenderse la idea que el tejido adiposo es una parte importante del sistema de defensa del cuerpo, tanto por su íntima relación con el sistema inmunológico como por su capacidad de control y regeneración de tejidos y estructuras circundantes [25].

En la obesidad los adipocitos se hipertrofian porque no pueden hacer otra cosa con la sobrecarga energética que les llega que interiorizarla como reservas, y como respuesta a esta "agresión" se produce una inflamación de baja intensidad, pero crónica. Esta inflamación deteriora la señalización de la insulina al activarse quinasas a través de las citoquinas u otras señales de estrés [26]. Además, y a favor de este planteamiento, muchas de las moléculas o receptores asociados con la defensa frente a la infección también se han relacionado con el mantenimiento de la homeostasis energética [27].

1.1.3 Consecuencias de la obesidad

Disfunción del tejido adiposo en la obesidad

La remodelación del tejido adiposo es un proceso que se encuentra patológicamente acelerado en la obesidad. Muchos depósitos grasos en obesos presentan una vascularización reducida, una sobreproducción de matriz extracelular y una infiltración aumentada de células del sistema inmune [28]. El acumulo patológico de grasa puede, a su vez, disminuir la capacidad de acumulación de lípidos en el tejido adiposo facilitando de éste modo el flujo de lípidos hacia otros órganos y dando lugar a efectos lipotóxicos colaterales [16].

Así, el exceso de lípidos desencadena una respuesta inflamatoria crónica de baja intensidad (mediante el reclutamiento y activación de leucocitos, y el incremento de citoquinas inflamatorias) en el tejido adiposo pero también en el músculo, hígado, páncreas y sistema vascular, que es el nexo de unión entre el acumulo de grasa y las alteraciones metabólicas.

Concretamente la acumulación de reservas lipídicas en el tejido adiposo causa alteraciones funcionales en el retículo endoplasmático y en la mitocondria del adipocito [29]. En adipocitos hipertróficos, el retículo endoplasmático, que sintetiza proteínas pero también regula el almacenamiento de lípidos y los niveles de colesterol, se colapsa y altera el plegamiento de las proteínas, lo que provoca la acumulación de éstas en el citosol. La célula entonces responde inhibiendo la síntesis proteica y eliminando estos agregados para

evitar que interfieran con el resto de funciones celulares (*unfolded protein response* o respuesta de las proteínas desplegadas), proceso que puede acabar en muerte celular. Se cree que la muerte celular en el tejido adiposo juega un importante papel en la acumulación de macrófagos en la obesidad. Además el estado de estrés del retículo plasmático resulta a su vez en una liberación sistémica de ácidos grasos y de mediadores inflamatorios, generando un ciclo de empeoramiento de la resistencia a la insulina [30].

Como otros tejidos, el tejido adiposo tiene macrófagos residentes (fenotipo M2) que están "alternativamente" activados y producen citoquinas anti-inflamatorias como las interleucinas IL-4 y IL-13 que están implicadas en la remodelación y homeostasis del tejido. En la obesidad inducida por la dieta se observa un cambio del fenotipo M2 al M1, que da lugar a un incremento de producción de citoquinas proinflamatorias [31]. La infiltración de macrófagos de fenotipo M1 en el tejido adiposo aumenta el estado inflamatorio crónico que caracteriza a la obesidad ya que estas células infiltradas representan la principal fuente de las citoquinas pro-inflamatorias interleucina 6 (IL-6) y factor de necrosis tumoral (TNF), capaces de alterar la señal de la insulina en los adipocitos mediante la activación de la fosforilación del sustrato 1 del receptor de insulina (IRS-1)[32]. Las quinasas que participan en la alteración de la señal de la insulina son las quinasas c-jun N-terminal (JNK), que se activan durante la obesidad no sólo en respuesta a las adipoquinas sino también al aumento de ácidos grasos y al estrés oxidativo [32].

Se ha asociado la presencia de altas concentraciones de ácidos grasos libres y de triacilgliceroles circulantes con la acumulación de lípidos en hígado, músculo, corazón y páncreas [16]. Tanto en la obesidad como en los modelos animales lipodistróficos, la acumulación de lípidos intracelulares en estos tejidos está relacionada con esteatohepatitis, resistencia a la insulina en músculo y disfunción de las células beta del páncreas (fenómeno conocido como lipotoxicidad) [33]. Es por ello que la capacidad de expansión del tejido adiposo y su capacidad para tamponar de esta forma los lípidos circulantes protege en cierto modo al resto de órganos del incremento de lípidos circulantes y conlleva una mejora del estado de resistencia a la insulina [34]. El tratamiento de pacientes con hígado graso no alcohólico con tiazolidinedionas (TZD), que activan al receptor activado por proliferadores de peroxisomas gamma 2 (PPARg2) y aumentan la tasa de reclusión de adipocitos, permite la expansibilidad del tejido adiposo y conlleva una menor afectación del hígado.

La diversidad de localizaciones del tejido adiposo además refleja una gran heterogeneidad a nivel funcional, siendo el incremento de la parte visceral el más vinculado con el síndrome metabólico y otras enfermedades asociadas a la obesidad. El hígado recibe directamente los ácidos grasos y factores liberados por el tejido adiposo visceral, y más concretamente por el depósito mesentérico. En general en humanos, los adipocitos del depósito visceral son más pequeños y contienen una mayor proporción de macrófagos infiltrados productores de citoquinas proinflamatorias que los adipocitos localizados en la grasa subcutánea. A parte de la leptina, cuya fuente principal de producción es la grasa

subcutánea [35], muchas otras adipocitoquinas (TNF, proteína C reactiva o CRP, IL-6, inhibidor del activador del plasminógeno-1 o PAI-1) están secretadas principalmente por la grasa visceral [36]. Los adipocitos localizados en el depósito visceral son más resistentes al efecto antilipolítico de la insulina, están más vascularizados, son además metabólicamente más activos y tienen una mayor actividad lipolítica, siendo también más sensibles a la lipólisis inducida por catecolaminas [37]. La grasa visceral además presenta más receptores de glucocorticoides [38] que la grasa subcutánea.

Problemas asociados a la obesidad

Los problemas fisiológicos que conlleva la obesidad pueden dividirse en tres grupos, los derivados del incremento exagerado de la masa grasa, los derivados de la alteración neuroendocrina y metabólica producida por la acumulación de grasa y los derivados de la alteración del comportamiento. Dentro del primer grupo se incluyen los daños osteoarticulares, dermatológicos y los relacionados con la insuficiencia respiratoria y cardiovascular. Las alteraciones metabólicas tienen su origen principal en la resistencia a la insulina, que altera el metabolismo lipoproteico y la utilización de los triacilgliceroles, y la función del hígado y del páncreas, que finalmente contribuye al desarrollo de la diabetes. Los cambios en el comportamiento del obeso tienen un componente hormonal significativo, con predominio del sistema nervioso simpático y un aumento de la señal glucocorticoide. Además de problemas neurológicos definidos como la depresión, los pacientes obesos pueden presentar ansiedad y estrés relacionados con problemas de imagen, rechazo social y la incomprensión global de las causas de su enfermedad [39].

1.1.4 El ponderostato: la obesidad como alteración del mecanismo de control del peso corporal

El control del peso corporal es un sistema homeostático conceptualmente simple pero funcionalmente muy complejo, cuyo centro de control neural se ubica principalmente en el hipotalámo. El hipotálamo controla la ingesta mediante señales aferentes que provienen del intestino, niveles de metabolitos y hormonas circulantes y señales neurales procedentes de otros núcleos cerebrales y del sistema nervioso autónomo; también controla las señales eferentes que controlan la ingesta, las que actúan mediante el sistema nervioso simpático regulando la movilización de grasa y la termogénesis, así como el peristaltismo intestinal y el control global del proceso digestivo y absortivo.

La ingesta está regulada por un sistema complejo de señales centrales y periféricas que interactúan entre sí con la finalidad de modular la respuesta a la ingesta de nutrientes. Las señales de saciedad procedentes del tracto gastrointestinal durante la ingesta llegan al núcleo del tracto solitario del tallo caudal. Desde el núcleo del tracto solitario se proyectan fibras aferentes hacia el núcleo arcuado, donde las señales de saciedad se integran con las señales procedentes del tejido adiposo y con diversas entradas procedentes del hipotálamo, creándose una compleja red de circuitos neuronales que contribuyen a elaborar la respuesta

a la ingesta. En este proceso están implicados numerosos péptidos orexigénicos como el neuropéptido Y, las orexinas y péptidos anorexigénicos como las melanocortinas y la hormona liberadora de corticotropina (CRH) [40-42].

La disponibilidad energética viene establecida por la acción de metabolitos y hormonas, especialmente la insulina, que ante un incremento en la disponibilidad de sustratos activa la deposición de grasa, limita la oxidación de la glucosa, restringe la lipólisis y promueve la síntesis de ácidos grasos y la deposición de triacilgliceroles [43]. Los glucocorticoides pueden potenciar este efecto a largo plazo [44], mientras que las catecolaminas, las hormonas tiroideas y el glucagón tienden a contraregular estas acciones [45].

La termogénesis permite generar calor y también eliminar el exceso de energía disponible mediante la oxidación de sustratos sin obtener provecho energético, simplemente como fuente de calor. La estimulación adrenérgica a través de la vía simpática del tejido adiposo marrón induce la lipólisis y el desacoplamiento de la síntesis de ATP mediante la proteína UCP1 [46]. Existen otros mecanismos termogénicos no bien conocidos, probablemente regulados por las hormonas tiroideas, que disminuyen la eficiencia energética de órganos clave, como el músculo y el hígado [47,48].

En 1969 Hervey [49] avanzó la hipótesis que el peso corporal o el contenido de grasa corporal se mantienen constantes a lo largo de la vida porque se trata de una variable regulada homeostáticamente. Esta teoría es la alternativa a la que defendía que el peso corporal era el resultado de un simple flujo de energía, dependiente únicamente del balance entre la ingesta y el gasto energético.

Así la teoría del ponderostato de mantenimiento del peso corporal asume que el cerebro regula el peso corporal, mediante el control tanto del gasto energético como de la ingesta. El sistema ponderostato mantiene un nivel óptimo de reservas (de acuerdo con un patrón establecido durante el desarrollo) que permite hacer frente a cambios en la disponibilidad de energía y de gasto energético dentro de unos límites bastante amplios. La disminución de la ingesta se corrige disminuyendo la termogénesis y potenciando el aprovechamiento de la energía disponible, mientras que el exceso de ingesta se corrige aumentando la termogénesis y disminuyendo el apetito.

La obesidad sería, en este caso, la manifestación de una disfunción del sistema ponderostato que impide el ajuste de la masa de reservas a su tamaño óptimo [50,51]. El desajuste del ponderostato, es decir, la alteración de su nivel óptimo de reservas, puede ser debido a que las perturbaciones aplicadas al sistema sean muy intensas y/o prolongadas o porque se alteren los ajustes de referencia predeterminados por causas genéticas, epigenéticas, ambientales, neurales o metabólicas.

Un sistema ponderostato desajustado a un nivel más alto de su nivel óptimo defenderá la masa preestablecida (aunque el nivel de referencia esté desajustado y resulte lesivo) con todos los mecanismos de control disponibles. Según esta interpretación un obeso tendría

desajustado su ponderostato al alza, y su hipotálamo trataría de mantener el exceso de grasa contrarrestando las posibles acciones terapéuticas externas que se le apliquen para corregir la situación. Debido a que los mecanismos de control del peso corporal son múltiples, efectivos e interrelacionados, resulta muy difícil incidir en el peso corporal mediante acciones externas, y si se consigue es solo por un tiempo limitado, ya que si no se reajusta el ponderostato éste tiende a recuperar de nuevo la masa de reservas grasas que considera adecuado. Así el punto clave para el tratamiento efectivo y definitivo de la obesidad es llegar a modular el ajuste del ponderostato.

Se conoce muy poco acerca del mecanismo de control del propio ponderostato en su acción sobre el peso corporal. Está bien establecido que el sexo y la edad afectan a la cantidad de grasa corporal almacenada, y que algunos factores fisiológicos como la gestación y la lactancia también potencian la acumulación (en este caso temporal) de grasa. Se ha observado que el tipo de dieta, modulado por la herencia genética y ciertos mecanismos epigenéticos, influye en el desajuste de este sistema, pero aún no se ha establecido de modo definitivo cómo se produce. El problema es que una vez desajustado el ponderostato no se sabe cómo reajustarlo para que la obesidad desaparezca mediante los propios mecanismos de control de peso corporal.

1.1.5 El tratamiento de la obesidad

Las estrategias empleadas en el tratamiento de la obesidad se basan principalmente en la limitación de la ingesta y/o el incremento del gasto energético. Es decir, se actúa sobre la ecuación del balance energético alterando uno de los factores principales sin tener en cuenta la capacidad compensatoria del sistema ponderostato.

La limitación de la ingesta es la estrategia más utilizada para perder peso corporal, y se lleva a cabo principalmente mediante dietas hipocalóricas (menor aporte energético) o cirugía restrictiva o malabsortiva que limita la capacidad del estómago y/o la absorción intestinal (menor asimilación de nutrientes). También se ha intentado limitar la ingesta mediante fármacos que reducen el apetito, en general en combinación con dietas hipocalóricas.

El tratamiento clásico: dietas y ejercicio

Disminuir el peso corporal de un individuo obeso mediante dietas bajas en energía (se promueve la movilización de lípidos) y actividad física (se potencia la oxidación de sustratos, esperando que sean lípidos endógenos) conlleva a una normalización del perfil lipoproteico y de los niveles glucosa así como una disminución de la hiperinsulinemia [52]. Desafortunadamente se ha observado que la reducción de la ingesta incrementa el apetito, paralelamente al incremento de los niveles de cortisol (lo cual refleja una situación de estrés) [53] y también, de forma compensatoria, disminuye el gasto energético [54], lo que reduce la eficacia de la disminución de la ingesta energética [55].

Las dietas bajas en energía, ampliamente usadas a falta de otras soluciones, presentan una efectividad limitada que disminuye rápidamente con el tiempo, aunque pueden resultar útiles para corregir un exceso de peso moderado si se aplican correctamente. Estas dietas se clasifican por su contenido energético, en dietas bajas en energía (*Low Caloric Diets* o *LCD*) y las dietas muy bajas en energía (*Very Low Caloric Diets* o *VLCD*), que contienen entre el 40 y el 25% de la energía diaria recomendada. En la actualidad las dietas más bajas en energía contienen una cantidad de proteína de calidad suficiente para evitar al máximo las pérdidas de proteína corporal, pero aún así una buena parte de éstas se utiliza como fuente de energía resultando en un balance negativo de nitrógeno. Además de la dificultad de seguir este tipo de dietas debido al malestar y desasosiego que provoca el hambre, la exposición repetitiva con y sin control médico a este tipo de dietas resulta a menudo, paradójicamente, en una eficaz adaptación a periodos de baja disponibilidad energética y a una mayor protección de los depósitos grasos (dietas *yo-yo*).

Respecto al incremento del gasto energético, la eficacia del ejercicio como herramienta para disminuir el peso corporal en la obesidad es bastante limitada. El ejercicio incrementa el gasto energético pero también el apetito, y además el obeso se fatiga muy pronto y fuerza mucho más su sistema cardiovascular y respiratorio. Sin embargo, en algunos casos, el ejercicio moderado y prolongado junto con una dieta hipoenergética ayuda a prevenir la disminución de la tasa metabólica basal que ésta comporta, aumentando así su eficacia [56].

Fármacos

Los primeros fármacos anorécticos utilizados con este fin fueron las amfetaminas y otros agentes adrenérgicos, pero se descartaron al observarse que creaban dependencia, efectos secundarios como el aumento de la frecuencia cardíaca e hipertensión y además pérdida de sus efectos (ya limitados) sobre la ingesta al cabo de poco tiempo.

Posteriormente, en la década de los 90 se empezaron a comercializar la fenfluramina y dexfenfluramina (isómero *d* de la fenfluramina, más activo), que habían sido desarrollados como antidepresivos. Estos fármacos (igual que la fluoxetina) estimulan la saciedad inhibiendo la recaptación de serotonina [57]. La combinación de la fenfluramina con un estimulador adrenérgico, fentermina, resultaba más efectiva, pero poco después se confirmó la peligrosidad de la combinación y también de la fenfluramina al hallarse una clara relación entre su uso y la aparición de hipertensión pulmonar y otros trastornos cardiovasculares [58]. Cabe señalar que a pesar de su amplio consumo la efectividad de la fenfluramina fue muy limitada.

La sibutramina es otro inhibidor de la recaptación de serotonina que suprime el apetito y estimula la termogénesis mediante su efecto sobre la noradrenalina. La administración de sibutramina en combinación con una dieta hipocalórica induce una pérdida moderada de peso en obesos no mórbidos [59], pero se retiró del mercado el año 2010 porque también induce efectos deletéreos en el sistema cardiovascular [60].

El rimonabant es un antagonista selectivo del receptor CB1 de cannabinoides que provoca una pérdida de peso muy limitada en obesos no mórbidos mediante la reducción del apetito [61]. Sin embargo también se retiró del mercado en 2008 en relación a su asociación a la aparición de depresión y ansiedad.

La limitación de la extracción de energía de los alimentos se realiza mediante inhibidores de enzimas digestivos como es el caso del inhibidor de la lipasa pancreática Orlistat (tetrahidrolipstatina), que se debe acompañar de una dieta hipocalórica con limitación estricta de lípidos, ya que originan esteatorrea y otras molestias, con una eficacia global muy baja [62].

La cirugía bariátrica

La cirugía bariátrica es el tratamiento más común y efectivo en los pacientes obesos mórbidos, para los que otros tipos de tratamiento no son aplicables [63]. Hay, esencialmente, dos tipos de procedimientos, los restrictivos y los malabsortivos. En los procedimientos restrictivos sólo se limita la entrada de alimento, reduciendo el tamaño efectivo del estómago de forma (gastroplastia vertical anillada, por ejemplo, reversible, o la gastroplastia radical, que no lo es). Los procedimientos malabsortivos restringen la asimilación de la comida ingerida, acortando la longitud del intestino, de modo temporal o definitivo. En la actualidad, buena parte de los procedimientos combinan ambos enfoques (*Rou-en-Y*) con restricciones dietéticas importantes. Estas técnicas inducen pérdidas de peso mucho mayores que el resto de tratamientos, y se asocian con una menor intensidad de comorbilidades como la diabetes y la hipertensión, aunque no existen garantías de mantenimiento de la pérdida de peso a largo plazo. Además hay un elevado riesgo de complicaciones, e incluso mortalidad, durante el periodo postoperatorio; el paciente tiene que someterse a una larga adaptación, pero hay pocas alternativas terapéuticas a estos procedimientos.

1.2 La oleoil estrona

1.2.1 La oleoil estrona como posible señal de ponderostato

El mecanismo que regula el peso corporal, el ponderostato, como todo sistema homeostático, requiere la existencia de un sistema de medida de la variable controlada, en este caso la masa de tejido adiposo y los triacilgliceroles que almacena. En la teoría lipostática [64], previa pero coincidente con la del ponderostato, la variable a mantener es la reserva lipídica del organismo, de manera que cualquier desviación de su nivel establecido como óptimo debe dar lugar a un cambio compensatorio en sentido contrario para restablecer la situación inicial. En condiciones fisiológicas estos cambios se producen, aunque no siempre con total efectividad. Según este modelo existiría una señal que informara a nivel central de la cantidad de reserva grasa de que dispone el organismo para mantener su homeostasis.

Las hormonas esteroideas fueron consideradas como posibles candidatas a señal ponderostato porque su naturaleza lipófila y pequeño tamaño les permiten atravesar fácilmente la barrera hematoencefálica. El cerebro sintetiza y utiliza como señales internas un buen número de hormonas esteroideas, los neuroesteroides [65]. Además, las hormonas esteroideas actúan a nivel central y modulan, entre otras cosas, la distribución de grasa corporal, con patrones dependientes de la edad y el sexo [66]. La señal de ponderostato, según la teoría lipostática, tendría que sintetizarse o liberarse en proporción a la masa grasa, y de hecho el tejido adiposo tienen un papel importante en la síntesis y modificación de varias homonas esteroideas [67]. Además este tejido, junto con las lipoproteínas, contiene algunas hormonas esteroideas esterificadas con ácidos grasos, aumentando así su lipofilia [68].

La estrona, presente también en la leche materna, está asociada con la deposición de grasa y proteína en recién nacidos [69], y también está correlaciona con la masa de grasa corporal en mujeres tras la menopausia [70]. Se decidió estudiar la estrona y sus derivados como posibles señales de ponderostato porque se sintetiza ampliamente en el tejido adiposo que contiene la aromatasa [67] y se almacena en forma de acil estrona en proporción a la masa grasa, lo cual puede explicar los niveles elevados circulantes de estrona en personas obesas [71]. Por otro lado se planteó esterificarla con el ácido oleico por ser el mayoritario en la fracción lipídica de la rata [72], animal en el que se han desarrollado la mayoría de estudios sobre la oleoil estrona.

La oleato-3-estrona o oleoil estrona (OE) es un éster de ácido oleico y estrona que presenta una consistencia cerosa y una alta hidrofobicidad, por lo que en un principio se decidió administrarlo cargado en liposomas. Su administración intravenosa en ratas dio lugar a una disminución de la ingesta y a una pérdida de peso dosis-dependiente, acompañada del mantenimiento del gasto energético, mediante un incremento de la oxidación de lípidos y la preservación de la proteína corporal [73].

Los efectos metabólicos del OE están estrechamente relacionados con su estructura, ya que la modificación tanto del ácido graso como del núcleo esteroide provoca cambios en su acción [74].

El estudio de la OE como hormona adelgazante se basa en la hipótesis de que la OE es una señal que le indica al cerebro el estado de las reservas de triacilgliceroles del cuerpo. Durante más de diez años se han realizado numerosos estudios con animales de experimentación (ratas y ratones), entre ellos los que incluye esta tesis, para profundizar en el conocimiento de los efectos derivados de la administración de esta hormona y poder así comprender mejor su mecanismo de acción.

1.2.2 Efectos de la oleoil estrona

La oleoil estrona como agente adelgazante en modelos de roedor

Los primeros experimentos con OE se realizaron mediante infusión intravenosa continua de una suspensión de liposomas con OE. Las ratas experimentaron una pérdida de peso y de apetito dosis-dependiente, especialmente en los primeros días de tratamiento [73]. La cantidad de grasa corporal, y en concreto el tamaño de los adipocitos disminuyó [75], mientras que la glucemia [76] y el porcentaje de proteína corporal del animal se mantuvieron [76]. La disminución de la ingesta y del peso corporal fue más pronunciada en ratas genéticamente obesas que en animales con normopeso, y aunque las ratas obesas redujeron su gasto energético, la disminución de la ingesta fue tan marcada que produjo una gran movilización de sus depósitos grasos [76]. En el caso de las ratas con normopeso el mantenimiento de la pérdida de peso perduraba tras finalizar el tratamiento [77], lo que sugiere que la OE había podido modificar a la baja el nivel de peso a mantener, es decir, había alterado el ajuste ponderostato.

Un problema recurrente en la administración endovenosa de OE era la sobrecarga innecesaria de estrona directamente al torrente circulatorio, lo que producía efectos estrogénicos indeseados. La administración oral diaria de OE mediante sonda intragástrica evita en gran medida este problema [78]. La OE administrada oralmente se incorpora directamente a las lipoproteínas y la estrona procedente de la hidrólisis intestinal de la OE es mayoritariamente eliminada por el hígado [79]. El efecto dosis- dependiente se mantiene en el rango de administración de 1 a 20 nmol/g día, por lo que en la mayoría de los experimentos orales se administra una dosis de 10 nmol/g día, que da lugar a una pérdida de peso corporal del 10-15% en diez días de tratamiento [78]. La hidrólisis de la OE y el consiguiente aumento de estrona y de estradiol plasmáticos es menor que en el caso de la administración intravenosa, como se pudo comprobar por los bajos índices de sensibilidad a la estrogenización (tamaño del útero y de la glándula mamaria) sobretodo en comparación con la vía intravenosa [80].

La OE provoca una considerable pérdida de peso en modelos roedores con el sistema de la leptina alterado, como es el caso de las ratas genéticamente obesas *Lepr*$^{fa/fa}$ [76,81], así como en ratones *Lep*$^{ob/ob}$ y *Lepr*$^{db/db}$ [82]. Sin embargo, y a diferencia de las ratas, los ratones con normopeso no experimentan una disminución significativa de su peso corporal a las dosis estándar.

Las ratas a las que se administra estrona libre por vía intravenosa no pierden peso [74]. Sin embargo la administración oral de estrona a dosis elevadas, equivalentes a la dosis estandar de OE, y a diferencia de la vía intravenosa, produce una disminución de peso corporal que se ha atribuido a la esterificación secundaria de la estrona [83].

Efectos de la oleoil estrona sobre el apetito

La administración de OE no elimina la sensación de apetito, pero induce saciedad al cabo del poco tiempo de empezar a comer [84]. La inyección intracerebro-ventricular de OE provoca la pérdida de apetito y el mantenimiento del gasto energético en ratas, lo que demuestra su acción a nivel central [85]. La OE no altera los niveles hipotalámicos del neuropéptido orexigénico NPY [86] pero disminuye marcadamente la expresión del péptido orexigénico ghrelina en el estómago [87], que está implicado en la sensación de saciedad.

El tratamiento con OE mantiene la glucemia a pesar de disminuir la ingesta a través de mecanismos centrales, lo cual es también un factor importante para la disminución del apetito. El mantenimiento de la glucemia se consigue mediante una cierta resistencia periférica a la insulina, ya que disminuye la utilización periférica de glucosa a costa de la utilización de los lípidos movilizados desde las reservas grasas [88].

La combinación de señales centrales, señales de saciedad y el mantenimiento de la glucemia contribuyen (y se potencian mutuamente) a la disminución de la ingesta tras la administración de OE.

Efectos metabólicos de la oleoil estrona

La OE disminuye el tamaño de las reservas grasas, afectando principalmente el tamaño de los adipocitos, pero también disminuye el número de células mediante apoptosis [89]. Estos efectos son más marcados en el tejido adiposo mesentérico, que está directamente relacionado con el lecho esplácnico.

La leptina circulante, así como su expresión en el tejido adiposo, disminuye con el tratamiento con OE, aunque este efecto también se observa en ratas hembras sometidas a la misma restricción energética que muestran espontáneamente las ratas tratadas con OE, por lo que se podría tratar de un efecto colateral a la pérdida de masa grasa [90].

El tratamiento de ratas con normopeso con OE disminuye los niveles de insulina, pero mantiene los niveles de glucosa circulantes, lo que indica un incremento global de la sensibilidad a la insulina [78]. Este efecto, sin embargo, no es paralelo al incremento de captación de glucosa por los tejidos periféricos, ya que el músculo sigue utilizando como sustrato principal los lípidos liberados por el tejido adiposo [88].

El estado hiperglucémico de las ratas prediabéticas Zucker obesas mejora con la OE, ya que disminuye la insulinemia y se incrementa la respuesta de la insulina frente al aumento de glucosa [76,81] La administración combinada de OE y una tiazolidinadiona (rosiglitazona) no produce una mejora adicional de la glucemia o de la sensibilidad a la insulina [91]. Ambos compuestos aumentan la sensibilidad a la insulina por vías opuestas, ya que la OE disminuye la masa de tejido adiposo y la expresión de PPARg2, al contrario que la rosiglitazona [92].

El tratamiento con OE mantiene los niveles de glucosa circulantes en un rango de normalidad [78] o bien los normaliza cuando los animales presentan hiperglucemia, como es el caso de las ratas alimentadas con dieta de cafetería o las ratas Zucker obesas [76,81]. Las reservas de glucógeno hepático y muscular de las ratas hembra tratadas con OE se mantienen a pesar de la disminución de la ingesta [90].

La OE disminuye los niveles de triacilgliceroles circulantes tanto en ratas con sobrepeso como en obesas con hiperlipidemia, disminuyendo los triacilgliceroles en todos los tipos de lipoproteínas, especialmente en quilomicra y VLDL [93].

Además, la OE disminuye el colesterol circulante y este efecto se hace más evidente en el tratamiento oral, afectando por igual a ratas con normopeso [78] y obesas [81]. Esta disminución afecta principalmente al colesterol HDL [93]. Cabe señalar que en la rata la distribución del colesterol en lipoproteínas es diferente a la de los humanos, siendo mayoritaria la fracción HDL. También se ha observado que la OE el recambio de colesterol esterificado [94].

En el músculo, a pesar de que el transportador de glucosa GLUT4 no varía su expresión por el tratamiento con OE [95], el incremento de ácidos grasos circulantes que provienen del tejido adiposo podría facilitar su captación. La OE aumenta la actividad de la lipoproteína lipasa muscular en ratas obesas [93], por lo que también se incrementa la captación de ácidos grasos procedentes de las lipoproteínas circulantes.

La OE induce en las ratas una importante pérdida de energía pero preserva la masa de proteína corporal, sin afectar al balance nitrogenado [73,78]. Esto podría ser consecuencia, al menos en parte, del mantenimiento de la glucemia, ya que esta disponibilidad de glucosa evita la necesidad de activar la gluconeogénesis a partir de los esqueletos hidrocarbonados de los aminoácidos como ocurre en el ayuno.

1.2.3 Metabolismo de la oleoil estrona

La distribución y la hidrólisis del oleato de la OE, así como los efectos de la estrona resultante, dependen sobretodo del modo de administración. La infusión intravenosa de OE favorece su rápida hidrólisis, mientras que la administración oral favorece eliminar la estrona generada en el intestino, cargando la OE en las lipoproteínas, lo que aumenta la vida útil de la OE.

Estudios de trazabilidad con OE marcada, administrada por vía oral, demuestran que la estrona liberada en la hidrólisis de la OE en el tubo digestivo llega al hígado a través del sistema porta-hepático, mientras que la OE intacta es transportada por lipoproteínas en la linfa. Después de una carga oral con OE marcada, a las pocas horas se detecta su presencia en plasma, lo que está de acuerdo con que su absorción ocurre mayoritariamente en el estómago y en intestino delgado (yeyuno e íleon) [79].

La cantidad total de acil-estrona corporal se mantiene bastante constante y además positivamente relacionada con la masa de tejido adiposo [96]. La producción de acil-estrona en el tejido adiposo y sus niveles circulantes aumentan al incrementar el porcentaje de grasa corporal tanto en ratas como en humano. Pero a partir de una determinada proporción de grasa se pierde esta relación. Así se observa que las ratas Zucker obesas y los humanos con obesidad mórbida presentan niveles de acil-estrona más bajos que los que correspondería a su masa grasa [97,98]. Esta correlación entre los niveles de acil-estrona y la masa grasa apoya el posible rol de la OE como señal de ponderostato.

Tanto el intestino [99] como el hígado [79] presentan una gran actividad OE-esterasa, pudiendo convertir gran parte del OE administrado oralmente en estrona y a su vez transformarlo mediante la hidroxilación o la conjugación con grupos sulfato o glucoronatos. Estos metabolitos permiten la solubilización de la estrona al aumentar su hidrofilia, lo que facilita su excreción urinaria y fecal. Sin embargo la estrona sulfato constituye la principal forma química de estrona circulante [100], lo que la convierte en parte en el principal reservorio de estrógeno potencialmente utilizable.

1.2.4 Interacciones hormonales de la oleoil estrona

Oleoil estrona y glucocorticoides

El tratamiento intravenoso con OE en ratas hembra incrementa los niveles plasmáticos de corticotropina (ACTH) y de corticosterona a partir del sexto día de tratamiento, coincidiendo con el momento en el que la ingesta se empieza a recuperar y el peso corporal se estabiliza [101]. También se observa un incremento de los niveles hipotalámicos de hormona liberadora de corticotropina (CRH), pero éste es posterior al aumento de ACTH [102].

En ratas hembras la administración de OE, tanto intravenosa como oral, disminuye la capacidad de unión de la globulina fijadora de corticoesteroides (CBG) plasmática [103], por lo que incrementa la fracción de corticosterona libre y con ello la actividad glucocorticoide. La caída de la actividad CBG coincide con una disminución de la expresión de la CBG en el hígado [104]. En este mismo modelo de rata la administración oral de OE incrementa la expresión y la actividad hepática de la enzima 11-β-hidroxiesteroide deshidrogenasa de tipo 1 (11-β-HSD1) [105] que incrementa localmente la actividad glucocorticoide al potenciar la formación de la forma más activa (corticosterona a partir de 11-dehidrocorticosterona).

En ratas adrenalectomizadas (ADX), el tratamiento con OE intravenoso acentúa la disminución de la ingesta de un 30% (OE) a un 70% (ADX-OE) y la disminución del peso corporal del 3% (OE) al 16% (ADX-OE) en una semana [106]. Además estas ratas no sólo pierden grasa, sino que también movilizan proteína, agotan las reservas de glucógeno hepático y son hipoglucemicas. En el caso de las ratas adrenalectomizadas tratadas con OE por vía oral los efectos no son tan marcados sobre el peso, pero el análisis de la

composición corporal indica que la pérdida de lípidos se potencia [107]. Esto indica que los glucocorticoides juegan un importante papel modulador de los efectos de la OE, protegiendo las reservas energéticas de acuerdo con su papel amortiguador sobre los efectos de los agentes que tienden a modificar el balance energético [108].

La adrenalalectomía por si misma ya disminuye la ingesta, el peso y el lípido corporal de las ratas, y hay que tener en cuenta que esta técnica drástica no sólo impide la síntesis y secreción de los glucocorticoides sino que también afecta a los mineralocorticoides, la deshidroepiandrosterona y parte de las catecolaminas. Probablemente, es por esta razón que el tratamiento con OE a ratas adrenalectomizadas a las que se les administra niveles fisiológicos de corticosterona no disminuye el lípido corporal [107].

La administración de glucocorticoides en paralelo con el tratamiento con OE inhibe los efectos de la OE disminuyendo los lípidos del tejido adiposo, y también provoca la pérdida del efecto del OE sobre el apetito y la sensibilidad a la insulina [107]. El efecto antagónico de los glucocorticoides y la OE hace que la administración combinada de altas concentraciones de ambas hormonas de lugar a una paradójica acumulación neta de lípidos [107]. Así, un incremento de los niveles de glucocorticoides, incluso dentro del rango fisiológico, o inducido por el estrés, podrían bloquear los efectos adelgazantes de la OE.

Oleoil estrona y hormonas sexuales

Los estrógenos y los andrógenos juegan un importante (aunque en gran parte aún no bien conocido) papel en el control del metabolismo energético. Los andrógenos ejercen efectos anabólicos que protegen la proteína corporal, y favorecen la sensibilidad a la insulina [109]. Por su parte, los estrógenos afectan a la distribución de la grasa corporal [110] e inciden en el control de la homeostasis de la glucosa [111]. Las mujeres además, gracias a sus niveles de estrógenos, están más protegidas de las enfermedades cardiovasculares que los hombres, aunque sólo hasta la menopausia [112]. Los estrógenos ejercen, además, otros efectos beneficiosos para el sistema circulatorio como son sus efectos antiinflamatorios y antioxidantes [113].

Los estrógenos ejercen una amplia gran variedad de acciones en diversos tipos celulares, que se desarrollan mediante diferentes mecanismos de señalización. Una vez activados, los receptores clásicos de estrógenos ERα y ERβ, actúan como factores de transcripción mediante su unión al promotor de sus genes diana. Los estrógenos además se pueden unir a otros receptores de membrana tipo GRP30 o a canales de iones, iniciando cascadas de señales cuyos efectos son más rápidos y que en muchos casos también modulan la actividad transcripcional [114]. Estos receptores no clásicos de membrana están caracterizados por un perfil farmacológico diferente al de los receptores ERα y ERβ, no uniendo al antiestrógeno ICI182,780 [115].

El tejido adiposo es sexualmente dimórfico en humanos, con diferencias específicas ligadas al sexo en cuanto a la distribución de la grasa corporal, pero también en cuanto a la

funcionalidad y respuesta metabólica del tejido [116]. A partir de la adolescencia el porcentaje de grasa corporal empieza a ser más elevado en mujeres que, además, presentan una mayor proporción de grasa gluteo-femoral subcutánea y menos grasa visceral que los hombres. En las mujeres post-menopáusicas aumenta la grasa intra-abdominal y la resistencia a la insulina, efecto que se revierte en parte mediante terapia hormonal sustitutoria [117]. Es difícil determinar con precisión el papel real de los estrógenos sobre el peso corporal en condiciones de normalidad. Su déficit se asocia a menudo a un incremento de peso, pero el hecho de que las hembras tengan un porcentaje más alto de grasa podría sugerir que los estrógenos contribuyen a la acumulación de ésta.

Las ratas ovariectomizadas, los ratones *knout-out* para el receptor de estrógenos α (αERKO) y los ratones *knout-out* para la aromatasa (ArKO) presentan un fuerte incremento del peso corporal comparados con sus controles intactos [118-120]. Por otro lado, se ha descrito que la administración de estrógenos limita la deposición de grasa corporal, aunque el efecto de la administración de estrógenos exógenos en cuanto al peso y a la adiposidad corporal depende de varios factores, como la especie, el sexo y la edad, la forma química del estrógeno, la dosis administrada, la vía de administración y la duración del tratamiento. En todo caso, los efectos de la administración intravenosa de β-estradiol a ratas respecto la pérdida de peso corporal es menos marcada que la inducida por la OE [74].

En la mujer los estrógenos se sintetizan principalmente en los ovarios a partir del colesterol en respuesta a la estimulación por gonadotrofinas. Los estrógenos también se forman por aromatización de andrógenos en el tejido adiposo, la glándula mamaria, la piel, el hueso y otros tejidos [121]. Tras la menopausia, la síntesis ovárica de estrógenos disminuye, con lo que la mayor parte de los estrógenos se forman en el tejido adiposo por aromatización de la androstenediona sintetizada en las glándulas adrenales.

En humanos el β-estradiol y la testosterona (pero no otros estrógenos, como la estrona, y andrógenos) circulan en parte unidos a globulina transportadora de hormonas sexuales (SHBG), y con menor afinidad por otras proteínas plasmáticas [122], salvo una fracción libre (no unida a proteínas) que es considerada la concentración realmente activa de éstos. De esta manera los niveles de SHBG determinan la disponibilidad de las hormonas sexuales. En los roedores no hay SHBG, aunque en el testículo existe una proteína transportadora de testosterona [123], y las hormonas sexuales circulan en gran parte unidas a proteínas de baja afinidad, como la albúmina.

La estrona y el estradiol son interconvertibles mediante la acción de las 17β-HSD en sus distintas isoformas [124], pero su actividad biológica es muy diferente, por lo que el efecto biológico de los estrógenos depende en gran medida de su forma química, y ésta a su vez, de las posibles diferencias en cuanto su transformación metabólica.

La hidrólisis de la OE libera estrona, y es por ello por lo que su administración induce un incremento secundario de los niveles de estradiol circulantes, aunque su formación es

mucho más limitada que la de estrona-sulfato, el principal metabolito circulante de la OE tras su administración oral [100], y que probablemente constituye la principal forma en la que la estrona es eliminada.

1.2.5 Modos de administración de la oleoil estrona y sinergias con otros fármacos

En la administración prolongada en el tiempo con OE, es preferible el tratamiento discontinuo frente a continuo, ya que en los intervalos de descanso se normalizan los sistemas fisiológicos de control hormonal de la síntesis de estrógenos (y andrógenos), minimizándose de esta manera los posibles efectos nocivos del incremento de los estrógenos circulantes. Este planteamiento es terapéuticamente razonable por contribuir a evitar una caída de los andrógenos, hiperestrogenicidad y desajuste del eje hipotálamo-hipofisario-gonadal, pero también porque en el intervalo sin tratamiento no se produce una recuperación del peso corporal perdido.

La administración de OE junto con un agonista del receptor β_3-adrenérgico, como el CL316,243, produce un efecto combinado, con una masiva movilización de lípidos, ya que el agonista adrenérgico incrementa el gasto energético y acelera la lipólisis, mientras que la OE disminuye la ingesta [125]. El efecto estimulador de la termogénesis del agonista adrenérgico es más prolongado en combinación sinérgica con la OE que administrado solo, con lo que las ratas pueden llegar a perder hasta una cuarta parte de su peso corporal en sólo 10 días de tratamiento.

La administración de OE junto con los fármacos anorexigénicos sibutramina (actúa inhibiendo la recaptación postsináptica de serotonina y noradrenalina) [126] o rimonabant (antagonista selectivo del receptor cannabinoide CB1) [127] indican que la OE no actúa mediante las mismas vías que estas dos moléculas inhibidoras del apetito, ya que en ambos casos sus efectos son adicionales.

Estudios en humanos

El primer tratamiento con OE en un ser humano, y el único estudio publicado [128], consistió en un tratamiento diario de 1 nmol/día y g de masa magra (unos 100 mg) durante ciclos sucesivos de 3 semanas de una dosis diaria seguidas de 3 meses de descanso. La pérdida de peso total en 28 meses fue de 42 kg (peso inicial 172 kg), con la consiguiente mejora de los indicadores metabólicos. No se observaron efectos secundarios ni alteraciones metabólicas significativas.

Posteriormente se inició el desarrollo de la OE como fármaco. Los estudios de fase I corroboraron el efecto adelgazante de una única dosis alta. En los estudios de seguridad de fase II, se eligieron dosis de 5, 10 y 20 mg/día kg, demasiado bajas para obtener una pérdida de peso significativa, pero el aumento dosis-dependiente de estrona y estradiol y la disminución de la testosterona, a pesar de que se normalizaron sus niveles después de tratamiento, provocó formalmente la suspensión del estudio.

1.3 Metabolismo del colesterol

El colesterol tiene una función estructural en la membrana celular y además es el precursor de las hormonas esteroidales y de los ácidos biliares. Tanto el colesterol que proviene de la dieta como el que se sintetiza *de novo* circula en las lipoproteínas y se almacena en las células en forma de esteres de colesterol.

Del colesterol de la dieta, en el caso de que parte del alimento sea de origen animal, se absorbe un 50%, y dependiendo de la cantidad absorbida, se sintetiza más o menos colesterol. El hígado, además de sintetizar colesterol, capta eficientemente el colesterol procedente de la dieta y de los órganos periféricos. El organismo tiene la capacidad de degradar el colesterol, por lo que el excedente se destinada a la síntesis de ácidos biliares, que permite en parte su eliminación en las heces siendo ésta la única manera de excretar colesterol. La excreción de colesterol está regulada por el ciclo entero-hepático de los ácidos biliares, que tiene como finalidad preservar moléculas costosas de sintetizar.

1.3.1 Síntesis de colesterol

La síntesis *de novo* de colesterol es ubicua, siendo predominante en el hígado y en el intestino. La síntesis de colesterol se lleva a cabo en el citoplasma y en los microsomas a partir de acetil-CoA, que deriva de la oxidación mitocondrial de un ácido graso o del piruvato y que sale de la mitocondria en forma de citrato. Todas las reacciones de la reducción de la biosíntesis del colesterol utilizan NADPH como cofactor.

El acetil CoA se convierte en 3-hidroxi-3-metilglutaril-coenzima A (HMG-CoA) mediante la enzima HMG-CoA sintasa 1 (HMGS1) en el citoplasma. La HMG-CoA se convierte en mevalonato mediante la HMG-CoA reductasa (HMGR), que es una enzima unida al retículo endoplasmático y limitante en la síntesis del colesterol. La regulación a corto plazo de la HMGR tiene lugar mediante mecanismos post-transcripcionales, principalmente mediante su nivel de fosforilación a través de la quinasa dependiente de AMP (AMPK) [129] y de la fosfatasa 2A (PP2A) [130]. La insulina y la triyodotironina (T3) incrementan la síntesis de colesterol mediante la fosforilación de HMGR, mientras que el glucagón, la adrenalina y los glucocorticoides actúan en sentido contrario [131].

A nivel transcripcional, la concentración de colesterol intracelular regula la expresión de HMGR mediante el factor de transcripción SREBP-2 (*sterol response element binding protein 2*) [132]. La actividad de SREBP-2 está regulada por los niveles de colesterol de membrana, que son detectados mediante la proteína SCAP (*cleavage-activating protein*) [132]. Cuando el nivel de colesterol es alto, las proteínas INSIG (*insulin-induced gene*) se unen a SCAP reteniendo el complejo SCAP/SREBP-2 en el retículo endoplasmático. Al disminuir los niveles de colesterol, SCAP deja de interaccionar con INSIG y se transporta junto con SREBP-2 al aparato de Golgi formando parte de las vesículas COPII [133]. En el complejo de Golgi se encuentran las proteasas encargadas de la activación de SREBP, S1P

y S2P (*site-1 y site-2 proteases*), que actúan secuencialmente para liberar a la proteína SREBP madura de 68 kDa [134], que entra al núcleo dónde activa la transcripción de los genes que participan en la síntesis y metabolismo del colesterol, tales como la HMGR y el receptor de LDL (LDLR) [135].

1.3.2 Transporte de colesterol

El colesterol de la dieta es absorbido por los enterocitos dónde se esterifica mediante la acetil-CoA acetiltransferasa (ACAT) y es incorporado a los quilomicrones junto con los triglicéridos también procedentes de la dieta. Los quilomicrones pasan a la circulación sanguínea, y una vez se han vaciado de triacilglicéroles y se han enriquecido en colesterol y ApoE, son reconocidos por los receptores del hígado ApoE (también llamados receptores LRP). El hepatocito además sintetiza y libera VLDL que contiene tanto colesterol endógeno como procedente de la dieta, además de triglicéridos endógenos. Las partículas de VLDL una vez vaciadas de triacilgliceroles se convierten en partículas LDL, ricas en ApoB100 y en colesterol esterificado. En general, las células regulan la captación de colesterol exógeno mediante los receptores de LDL (LDLR).

Las HDL son partículas ricas en ApoA1 que captan colesterol desde las células periféricas, así como desde el resto de lipoproteínas. Las HDL capturan el exceso de colesterol periférico mediante la ApoA1 y en colaboración con la proteína ABCA1, presente en muchas células y que transfiere el colesterol a las partículas HDL desde los tejidos, y la lecitin colesterol acil transferasa (LCAT), también presente en las HDL, esterifica el colesterol adquirido [136]. La internalización hepática del colesterol contenido en las HDL se produce a través del vaciado selectivo de sus ésteres mediante el receptor SRBI, o mediante la captación de la partícula completa a través de receptores de ApoE o mediante un mecanismo indirecto a través de la proteína transferidora de ésteres de colesterol (CETP) que traspasa ésteres de colesterol hacia las LDL que finalmente son internalizadas por el hígado a través de LDLR [137].

Los roedores, que no tienen CETP, utilizan las HDL como principal transportador de colesterol, y son relativamente resistentes a la aterosclerosis de acuerdo con el potencial antiaterogénico de esta lipoproteína. Por el contrario, los conejos y los humanos, que expresan CETP, transportan el colesterol principalmente en las LDL, y ambas especies son susceptibles a padecer aterosclerosis bajo determinadas condiciones como puede ser una dieta rica en grasa [138]. De hecho los modelos de ratón a los que se les introduce el gen CETP humano disminuyen los niveles de HDL e incrementan de los niveles de LDL, y como consecuencia les aumenta la aparición de lesiones ateroscleróticas respecto a los ratones control [139].

En el hígado, los LDLR median la eliminación de LDL y de otras lipoproteínas remanentes de la circulación mediante su unión a la ApoB-100 y ApoE [140]. Las HDL, que contienen ApoE, también pueden ser reconocidas por los LDLR [141]. Los roedores presentan una

mayor proporción de proteína ApoE en las HDL que los humanos, por lo que una buena parte de sus HDL son reconocidas y captadas por los LDLR. La regulación de la expresión de LDLR está mediada por SREBP-2 y es clave en la acción de las estatinas, fármacos que disminuyen las LDL circulantes. Las estatinas se unen e inhiben a la HMGR, por lo que la célula, a través de SREBP-2, detecta una bajada de colesterol y aumenta la expresión de LDLR [142].

Los SRBI median la entrada selectiva de colesterol esterificado de las HDL principalmente en tejidos esteroidogénicos y en hígado, pero sobretodo contribuyen al flujo de colesterol libre desde las células periféricas a las HDL, favoreciendo el transporte reverso de colesterol [143]. Así, este transportador es capaz de captar selectivamente los diferentes componentes de las HDL sin internalizarlas ni degradarlas.

1.3.3 Regulación de los niveles de colesterol

El colesterol circulante está regulado principalmente por el nivel de colesterol intracelular. Cuando los niveles de colesterol aumentan la célula activa procesos encaminados a evitar la toxicidad que el colesterol libre provoca, mediante: (1) la disminución de la síntesis de colesterol endógeno, (2) la secreción de colesterol desde el hígado a la circulación mediante VLDL, (3) la disminución de los niveles de receptores LDL y el aumento del transporte reverso de colesterol desde las células periféricas, (4) el incremento de la síntesis de ácidos biliares así como el incremento del transporte de éstos y de colesterol desde el hepatocito hasta la bilis y (5) la disminución de la reabsorción de colesterol intestinal y el aumento de su excreción. Todas estas estrategias están reguladas por SREBP-2 y LXR. SREBP-2 se activa cuando los niveles de colesterol de la membrana disminuyen, y controla la expresión de los genes implicados en la síntesis de colesterol, así como el gen del receptor LDL [132]. LXR por su parte regula la síntesis de ácidos biliares (en roedores), el incremento del transporte de colesterol hacia la bilis, el transporte reverso de colesterol y la absorción/excreción intestinal de colesterol [144].

Los receptores nucleares LXRs del hígado detectan los niveles de colesterol celulares mediante su unión a oxiesteroles endógenos (derivados oxidados del colesterol) y regulan la expresión de los genes que protegen del exceso de colesterol. Existen dos isoformas, LXRα (NR1H3) y LXRβ (NR1H2). La expresión de LXR alfa predomina en los tejidos metabólicamente activos, tales como el hígado, el intestino delgado, el riñón, los macrófagos y el tejido adiposo, mientras que LXRβ se expresa más ubicuamente [144].

El transporte reverso de colesterol

Los niveles de HDL están inversamente correlacionados con la incidencia de enfermedades cardiovasculares ya que incrementan el transporte de colesterol reverso, vía por la cual el colesterol se transporta desde los tejidos periféricos hacia el hígado para su excreción.

Las HDL nacientes discoidales (ndHDL), que contienen ApoA-I, provocan la salida de colesterol desde la membrana celular de los tejidos periféricos mediante la proteína de membrana ABCA1. La ABCA1 además participa en la formación de HDL discoidales nacientes, proceso que tiene lugar principalmente en el hígado, lipidando con fosfolípidos a las proteínas ApoA-1. La sobrexpresión de ABCA1 en el hígado no sólo es fundamental en la biogénesis de las HDL sino que su sobrexpresión incrementa los niveles de colesterol HDL y de apoA-1 [145]. Una vez formadas, las partículas HDL se van enriqueciendo con ésteres de colesterol y aumentan su tamaño mediante la participación de la proteína transferidora de fosfolípidos (PLTP), que transfiere principalmente fosfolípidos desde lipoproteínas ricas en triacilgliceroles [144].

En las ratas, como no existe CETP y el principal transportador de colesterol son las HDL, existe una presencia prominente de la apoE asociada a las HDL. La ApoE es un ligando efectivo de los receptores de LDL, y las HDL en estas especies son internalizadas gracias a la interacción de la ApoE con los receptores LDL hepáticos. La ApoE además contribuye a la expansión del corazón de las HDL con ésteres de colesterol en colaboración con la LCAT.

La activación de LXRα estimula la expresión de ABCA1 [146], ABCG1 [147], ApoE [148] y PLTP [149], incrementando así la salida de colesterol de los tejidos periféricos. La acumulación de colesterol en los macrófagos localizados en la pared de los vasos sanguíneos se considera el acontecimiento inicial del desarrollo de la aterosclerosis y es por ello que la eliminación de colesterol de estas células previene las enfermedades cardiovasculares [150].

Síntesis ácidos biliares, circulación entero-hepática y eliminación de colesterol

La principal ruta de eliminación de colesterol es la síntesis y secreción hepática de ácidos biliares y colesterol en la bilis y su secreción fecal. El hígado metaboliza colesterol mediante su conversión en ácidos biliares que son secretados a través de los conductos biliares y de la vesícula biliar al lumen del intestino delgado dónde actúan como detergentes emulsionando los lípidos de la dieta. Los nutrientes emulsionados son absorbidos por los enterocitos proximales mientras que la mayor parte de los ácidos biliares son absorbidos en la parte más distal del intestino delgado.

La circulación enterohepática es el transporte de colesterol desde el intestino a la circulación portal, de la circulación portal al hepatocito, del hepatocito a la bilis y de la vesícula biliar al intestino. El 95% de los ácidos biliares siguen esta vía, eliminándose sólo el 5% a través de las heces. Debido a esta eficaz recirculación la cantidad de ácidos biliares sintetizados *de novo* es pequeña.

La síntesis de ácidos biliares se lleva a cabo a partir del colesterol, e implica la 7a-hidroxilación del esteroide precursor, modificaciones en su anillo esteroide, la oxidación de la cadena lateral y la conjugación de los ácidos biliares con taurina o glicina para aumentar su solubilidad. Existen dos rutas de síntesis, la vía neutra predominante y la vía

ácida alternativa que contribuye en menos de un 10% a la síntesis neta de ácidos biliares. La enzima limitante en la vía neutra, la colesterol 7α hidroxilasa (CYP7A1), cataliza la conversión de colesterol a ácidos biliares, y se regula principalmente a nivel transcripcional bajo el efecto del ritmo diurno, el estrés, los xenobioticos, el colesterol y los ácidos biliares[151]. Además en roedores la expresión de CYP7A1 incrementa en el estado de ayuno.

En roedores, el colesterol de la dieta activa LXRα, lo que estimula la transcripción del gen que codifica para CYP7A1, promoviendo de esta forma la conversión de colesterol a ácidos biliares, y su rápida adaptación a una dieta rica en colesterol [152]. El ratón deficiente en LXRα sometido a una dieta rica en colesterol sufre un incremento de colesterol hepático que no puede compensar al no sintetizar ácidos biliares [152]. Sin embargo en humanos el tratamiento con un agonista de LXRα disminuye la expresión de CYP7A1 [153].

LXRα disminuye la absorción intestinal de colesterol mediante el aumento de la expresión de los transportadores ABCG5/8, localizados en la membrana canalicular del hepatocito y en la membrana apical del intestino, y que favorecen la secreción de colesterol desde los hepatocitos hasta el conducto biliar y desde los enterocitos hasta el lumen intestinal. Además LXRα aumenta la glucoronización de los ácidos biliares, lo que facilitala excreción de éstos por la orina [154].

LXRα es pues es el principal factor de transcripción implicado en la detección de los niveles de colesterol mediante su interacción con oxiesteroles, que a su vez conduce a la eliminación de su exceso favoreciendo el transporte reverso de colesterol, disminuyendo la captación de colesterol de la dieta y la recaptación enterohepática de los ácidos biliares, y en el caso del roedor además incrementando la conversión de colesterol en ácidos biliares.

1.4 Metabolismo de las hormonas esteroideas

Los esteroides son moléculas lipofílicas y de bajo peso molecular derivadas del colesterol. Las hormonas esteroideas se sintetizan en las gónadas y en las glándulas adrenales, y actúan en los tejidos periféricos y en el sistema nervioso central. Las glándulas adrenales producen corticosteroides y andrógenos, los ovarios estrógenos y progestinas y los testículos principalmente andrógenos.

Las enzimas esteroidogénicas comprenden los citocromos P450 (CYPs), las hidroxiesteroides deshidrogenasas (HSDs) y las esteroides reductasas [155]. La esteroideogénesis requiere que la célula disponga de colesterol libre localizado en el interior de la membrana mitocondrial. A nivel de membrana plasmática el receptor de LDL (LDLR) (predomina en humanos) y el receptor *scavenger* clase B tipo I (SR-BI) (predomina en roedores) importan colesterol esterificado a partir de las lipoproteínas circulantes [156]. La lipasa sensible a hormonas (HSL) rompe las uniones éster liberando colesterol, que se transporta hacia el interior de la mitocondria mediante un complejo de

proteínas entre las que destaca la proteína reguladora de la esteroidogénesis *aguda* (StAR) [157]. Tanto la hormona adrenocorticotropa (ACTH) en el córtex adrenal como la hormona luteinizante (LH) en las gónadas aumentan la disponibilidad de colesterol libre intracelular y su entrada en la mitocondria.

1.4.1 Esteroidogénesis

El córtex adrenal es la zona esteroidogénica más importante en humanos, ya que aquí se encuentran todos los procesos esteroidogénicos divididos en zonas concéntricas. En la zona glomerulosa exterior del córtex adrenal se sintetizan los mineralocorticoides, en la zona *fasciculata* intermedia los glucocorticoides y en la zona *reticularis* los andrógenos precursores de las hormonas sexuales.

La síntesis *de novo* de todas las hormonas esteroidales (Figura 1) empieza con la conversión del colesterol a pregnonelona mediante la esteroide 20-22 liasa (CYP11A o P450scc) localizada en la mitocondria [158]. La pregnonelona puede dirigirse al retículo endoplasmático dónde se convierte en progesterona mediante la 3βHSD. La pregnonelona y la progesterona son las moléculas precursoras de todas las hormonas esteroideas.

La esteroide 21-hidroxilasa (CYP21 o P450c21) se expresa en el retículo endoplasmático liso y es responsable de la conversión de progesterona y de la 17α-hidroxiprogesterona en deoxicorticosterona y 11-deoxicortisol respectivamente [158]. Estos precursores se convierten en las hormonas activas corticosterona y cortisol mediante la esteroide 11 beta hidroxilasa (CYP11B1). La corticosterona, el glucocorticoide mayoritario en ratas y en otras especies que no producen cortisol, puede convertirse en aldosterona mediante la aldosterona sintasa (CYP11B2).

Los andrógenos precursores de las hormonas sexuales se forman mediante la esteroide 17-20 liasa (CYP17), enzima con actividad 17α-hidroxilasa y 17,20-liasa. La CYP17 se encuentra en el córtex adrenal y en las gónadas, siendo esta última su principal localización en el caso de los roedores. La CYP17 hidroxila la pregnonelona y la progesterona para formar los respectivos 17-hidroxiesteroides. La actividad 17,20-liasa de la CYP17 convierte los dos 17α-hidroxiesteroides en dehidroepiandrosterona (DHEA) y androstenediona respectivamente [158]. A partir de la pregnonelona existen dos vías posibles: la vía delta 5 (más común en humanos), en la que la pregnonelona pasa a DHEA y posteriomente a androstenediona y testosterona, y la vía delta 4 (dominante en roedor), en la que la pregnonelona pasa primero a progesterona y seguidamente a androstenediona y testosterona. La conversión de DHEA a androstenediol está catalizada por la 17bHSD 1 y 5, mientras que la reacción reversa mediante 17bHSD 2 y 4 [124]. DHEA y androstenediol se convierten en androstenediona y testosterona respectivamente mediante la acción unidireccional de 3bHSD.

La LH se une a su receptor en la células de Leydig de los testículos y estimula la síntesis de testosterona *de novo* a partir del colesterol [159]. La enzima más importante en la

producción de testosterona a partir de androstenediona es la 17bHSD3, que se expresa principalmente en testículo [124]. La oxidación de testosterona puede ser catalizada por la 17bHSD 2 y 8. El testículo también expresa la aromatasa, que convierte la testosterona en estradiol, necesaria para la espermatogenesis [160]. La dihidrotestosterona (DHT) se forma a partir de la testosterona mediante la 5a-reductasa 2, reacción que predomina en la próstata.

La aromatización de androstenediona y testosterona mediante la aromatasa (Cyp19) produce estrona y estradiol respectivamente. En el ovario la expresión de aromatasa se regula a través de FSH, mientras que en el tejido adiposo y en el hueso la expresión de aromatasa es dependendiente de glucocorticoides y citoquinas [161]. La reducción de estrona a estradiol está catalizada por 17bHSD1, 5 y 7. La oxidación del estradiol se lleva a cabo mediante 17bHSD 2, 4, 8 y 10. La 17bHSD 2 y 8 están más expresados en el epitelio de la glándula mamaria y del útero, mientras que 17bHSD 4 y 10 son ubicuos. La teca interna del ovario produce progesterona y andrógenos que actúan como precursores de la síntesis de estrógenos mediante la acción de la aromatasa y la 17bHSD1 y 7, que pasan la estrona a estradiol [124].

Se asume que los esteroides se liberan a la sangre una vez sintetizados, sin la mediación de transporte activo, siendo el grado de liberación directamente proporcional a la actividad biosintética de la glándula y a sus niveles plasmáticos.

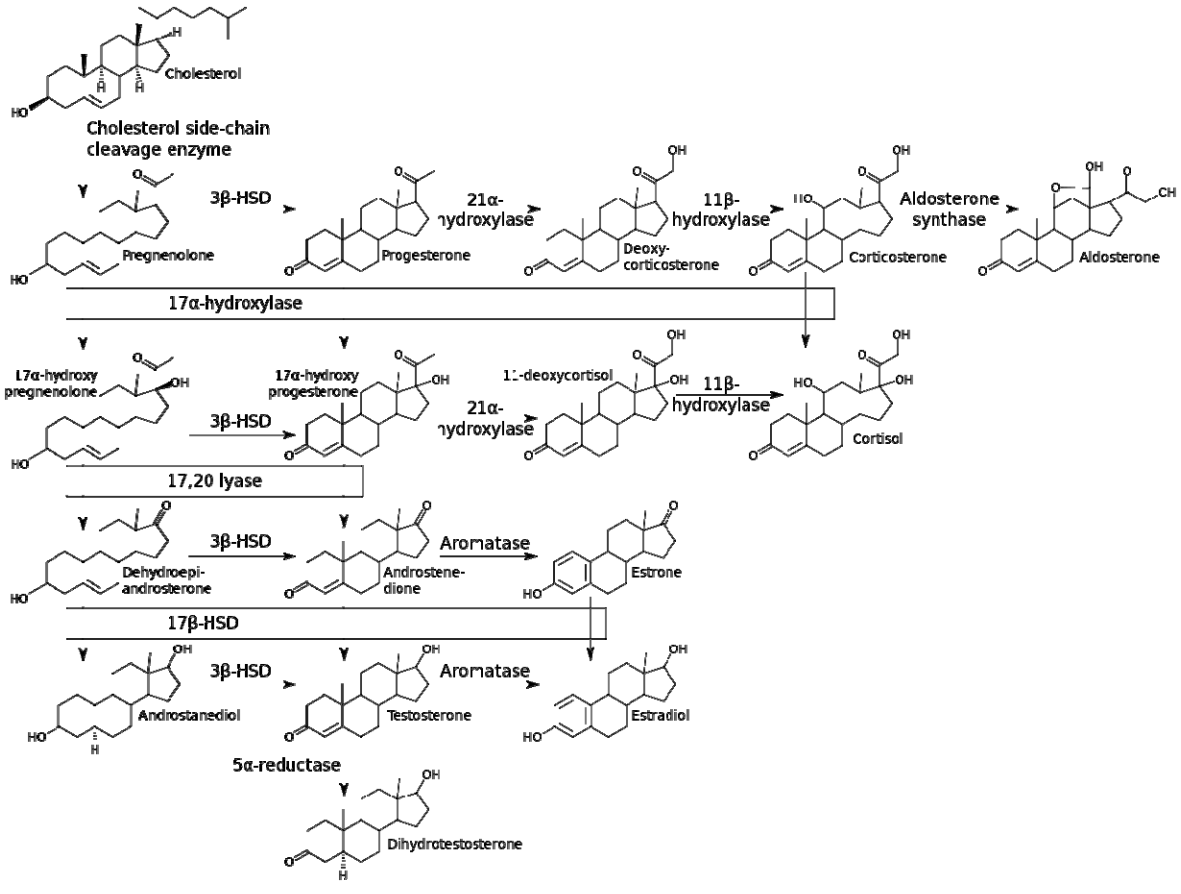

Figura 1. Esteroidogénesis

1.4.2 Modulación metabólica de las hormonas esteroideas

En los fluidos biológicos, y debido a su lipofilia, los esteroides se encuentran en forma conjugada unidos a una fracción hidrofílica (como sulfatos o glucoronatos) o unidos a proteínas mediante un enlace no covalente.

La mayor parte de la fracción unida circula junto con la globulina ligadora de corticosteroides (CBG) o a la globulina ligadora de hormonas sexuales (SHBG), pero una parte considerable de ésta fracción unida (de un 20 a un 50%) viaja junto a la albumina mediante enlaces no covalentes.

La fracción libre de las hormonas representa el 1-10% de la concentración total plasmática y se considera la fracción biologicamente activa, por lo que su unión con las proteínas transportadoras limita el acceso de las hormonas a los tejidos diana regulando de esta forma se disponibilidad. La CBG se sintetiza en el hígado desde dónde se secreta, pero también se expresa en algunos tejidos periféricos [162], en los que no se tiene clara su función.

Algunos esteroides en algunos tejidos diana deben convertirse en su forma activa antes de interactuar con sus receptores específicos. Este paso, además de un papel permisivo, cumple una función de modulación de la acción esteroidal. Los enzimas implicados en la activación metabólica suelen ser pasos irreversibles y, por lo tanto, limitantes de la acción esteroidal. Así, el metabolismo de las hormonas esteroidales en sus tejidos diana (activación, desactivación o transformación de las hormonas) puede ser más crítico en la especificidad y la magnitud de sus efectos que el número de receptores disponibles.

La 11β-HSD1, que se expresa principalmente en el hígado, en el tejido adiposo, cerebro y células del sistema inmune, activa los glucocorticoides en presencia de NADPH convirtiendo la 11-dehydrocorticorsterone/cortisona en corticosterona/cortisol y amplificando así la acción de los glucocorticoides en sus tejidos diana [163].

Numerosos tejidos expresan 3bHSD, 17bHSD y aromatasa, por lo que tienen la capacidad de sintetizar hormonas sexuales a partir de los precursores androgénicos adrenales DHEA y DHEA-S circulantes. En los primates una importante proporción de andrógenos y estrógenos se sintetizan localmente en los tejidos periféricos diana a partir de los precursores DHEA y DHEA-S producidos en el córtex adrenal. En la mujer postmenopausica la glándula adrenal es la fuente exclusiva de los andrógenos circulantes [164]. Estos andrógenos son a su vez los precursores de los estrógenos sintetizados a nivel periférico mediante la acción de la aromatasa, siendo el tejido adiposo una de las localizaciones más importantes. La intracrinología se refiere a la síntesis de esteroides activos en los tejidos diana periféricos, que pasa a depender de la expresión y actividad de los enzimas sintetizadores en cada uno de los tejidos [121,165].

Pero en roedores y mamíferos de pequeño, en general, la fuente exclusiva de andrógenos y estrógenos son los ovarios y el testículo. En estas especies las hormonas sexuales se sintetizan principalmente en las gónadas debido a que sus glándulas adrenales se dedican principalmente a la síntesis de glucocorticoides, de hecho en roedor los niveles circulantes de DHEA y la expresión de aromatasa en el tejido adiposo es menor que en humanos.

La inactivación de los esteroides ocurre principalmente en el hígado. Las hormonas inactivadas se eliminan en parte conjugadas por la orina, por lo que se requiere su transformación a compuestos hidrofílicos: glucurónidos (mediante glucuronil transferasas) y sulfatos (mediante sulfoquinasas). En el caso de los sulfatos, además de constituir productos de excreción, también se encuentran en los tejidos endocrinos o plasma como precursores de la síntesis hormonal, como en el caso de la DHEA-S. Las sulfatasas, que se encuentran en la fracción microsomal del hígado, las gónadas, las adrenales y la placenta, catalizan la hidrólisis de los esteroides sulfato. Así, los tejidos endocrinos con receptores esteroidales utilizan la sulfatación y la desulfatación para regular la unión de esteroides a su receptor [166]. El sistema sulfotransferasa/sulfatasa regula el nivel sistémico de hormonas así como sus niveles locales.

La esterificación con un ácido graso convierte a la hormona esteroidea en una molécula muy liposoluble, que pierde la afinidad por el receptor de la hormona, y cuya actividad resulta de la liberación de la hormona mediante esterasas [167]. Los ésteres de ácidos grasos circulan unidos a lipoproteínas, principalmente a las HDL, aunque pueden ser transferidas a las LDL [168]. Debido a su alta lipofilidad estos metabolitos endógenos se encuentran a bajas concentraciones en la sangre pero a relativamente altas concentraciones en tejidos acumuladores de lípidos [68]. La vida media de los ésteres de hormonas esteroideas es alta, por lo que podrían conformar una reserva hormonal que se transporta mediante lipoproteínas, o incluso ejercer alguna función independiente en el tejido adiposo dónde se acumulan.

JUSTIFICACIÓN Y OBJETIVOS

2 Justificación y objetivos

Esta tesis doctoral forma parte de un estudio mucho más amplio, el descubrimiento, caracterización y desarrollo de la oleoil-estrona (OE) como fármaco para el tratamiento de la obesidad. El grupo de investigación Nitrógeno-Obesidad, en el cual se ha realizado este trabajo, ha sido el responsable de la mayor parte de las publicaciones realizadas sobre el tema, y que han sido resumidas en dos publicaciones [169,170].

Aunque se ha caracterizado extensamente la eficacia de la administración de OE en cuanto a la pérdida de grasa corporal en diversos modelos experimentales (principalmente en rata), no se ha conseguido averiguar todavía mediante qué mecanismos actúa la OE. Los principales problemas que conllevan los estudios con OE son consecuencia de su naturaleza lipofílica (es insoluble en agua) y de la potente metabolización o transformación que parece experimentar tras su administración, lo que dificulta la determinación de sus niveles y su seguimiento *in vivo*. Además, el efecto central de la OE sobre la disminución de la ingesta hace difícil diferenciar si sus efectos globales son el simple resultado de la disminución de la ingesta o de si existen mecanismos de acción adicionales a éstos.

La acción de la OE está estrechamente relacionada con los glucocorticoides y las hormonas sexuales. Como bien se ha caracterizado en diversos estudios, el efecto adelgazante de la administración de OE se ve limitado por el efecto contraregulador de los glucocorticoides. Por otro lado la hidrólisis de OE libera estrona, estrógeno débil pero activable a estradiol, que puede alterar el metabolismo de las hormonas sexuales así como su funcionalidad y concentraciones plasmáticas.

El **objetivo** de este estudio es profundizar en el mecanismo de acción de la OE a nivel periférico, en paralelo a un modelo de restricción energética forzada equivalente, con el fin de analizar, comparar y discernir los efectos de la OE sobre diversas vías metabólicas, de partición de energía y de regulación hormonal que se ven modificadas tras su administración. Este objetivo general se ha desarrollado a lo largo de los siguientes objetivos parciales:

- Determinar si la combinación del tratamiento con OE con una restricción energética adicional potencia su efecto sobre la pérdida de peso y cómo afecta a la composición corporal, y a los balances energético y nitrogenado. Para este estudio, así como para los siguientes, se utiliza un modelo estándar con ratas Wistar macho alimentadas durante 5 semanas con una dieta de cafetería, con la finalidad de provocar un sobrepeso (un incremento del contenido de su grasa corporal) y poder analizar mejor sus variaciones frente al tratamiento con OE o a la restricción energética.

- Desarrollar una metodología adecuada basada en la utilización de la PCR a tiempo real que permita analizar y estimar, aunque de forma aproximada, el número total de tránscritos de la muestra para poder referir estos datos a las células o al tejido total del

que se parte, y poder comparar la expresión de un mismo gen entre diferentes órganos, localizaciones, grupos y tratamientos.

- Determinar cuál es el efecto de la OE sobre las vías que modulan la utilización de sustratos energéticos y el metabolismo del colesterol, principalmente mediante el estudio de la expresión génica y de algunas proteínas que participan en estas vías, realizando un análisis en paralelo de un grupo de animales *pair fed* para poder diferenciar la acción de la OE independientemente de la disminución de la ingesta.

- Analizar los cambios inducidos por la administración de OE en cuanto al metabolismo de los glucocorticoides y hormonas sexuales, mediante el estudio de sus niveles circulantes y de la expresión de los genes que codifican las enzimas que participan en su metabolismo.

RESULTADOS

3. Resultados

3.1 Efecto combinado de la oleoil estrona y la limitación de la ingesta

3.1.1 Efectos combinados de la oleoil estrona y la ingesta limitada de alimentos sobre la composición corporal de ratas macho con sobrepeso

En este estudio se analizan los efectos combinados del tratamiento con oleoil estrona (OE) y la limitación de la ingesta con el fin de determinar si las dietas bajas en energía potencian los efectos adelgazantes de la OE en ratas macho con sobrepeso. Se establecieron dos niveles de limitación de la ingesta, concretamente un 50 y un 25 % de la ingesta estándar, que equivale aproximadamente a las dietas humanas bajas en energía (LCD) y muy bajas en energía (VLCD).

Para ello se engordaron ratas Wistar macho con una dieta de "cafetería" durante 5 semanas. Después de un periodo de transición en el que las ratas fueron alimentadas con dieta estándar, se sometieron a dos niveles de restricción energética, 50 o 25 % con respecto a la ingesta del período de transición. A la mitad de los animales se les administró una dosis diaria de OE de 10 nmol/g durante 10 días, mientras que el resto recibió solamente el vehículo. Se determinaron el peso y los cambios en la composición corporal de las ratas (incremento de agua, lípidos, proteínas y energía total) comparadas con un grupo de ratas no tratadas. Se estimaron los balances de energía y nitrógeno. También se determinaron los niveles plasmáticos de metabolitos y hormonas.

La OE dio lugar a cambios en la composición corporal de los animales similares a los provocados mediante la reducción del 50 % de la ingesta, principalmente como consecuencia de una pérdida masiva de lípidos y energía. La combinación de OE con la restricción energética mejoró la homeostasis de los parámetros metabólicos estudiados y aumentó un poco más la pérdida del peso corporal, pero el análisis de la composición corporal nos indicó que mientras el tratamiento con OE afectaba principalmente a las reservas de grasa corporal, al combinarlo con una restricción energética también se movilizaba la proteína corporal.

En conclusión, los datos presentados indican que la combinación del tratamiento con OE y una dieta baja en energía, no conlleva beneficios adicionales, ya que los efectos no son aditivos. A pesar de que la OE disminuye la ingesta, hay otros mecanismos que participan en la movilización de la grasa corporal, ya que la ingesta por sí sola no puede explicar los cambios metabólicos y hormonales observados en las ratas tratadas con OE. La combinación de la OE con una restricción adicional de alimento puede dar lugar a un aumento no deseado de la movilización de proteína.

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ORIGINAL ARTICLE

Combined effects of oral oleoyl-estrone and limited food intake on body composition of young overweight male rats

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Objective: The combined effects of limited food intake and OE treatment have been analysed in order to determine whether hypocaloric diets enhance the slimming effects of OE on mature overweight male rats. Two levels of dietary limitation at 50 and 25% of a standard intake were established, roughly corresponding to the human LCDs and VLCDs.
Design: Wistar male rats (6 weeks old) were made overweight by a cafeteria diet. After transition to standard diet, they were subjected to food restriction: down to 50 or 25% with respect to the transition period. Half the animals were given daily oral gavages of 10 nmol/g oleoyl-estrone (OE), and the rest received only the vehicle during 10 days.
Measurements: Changes in weight and body composition: water, lipid, protein or gross energy were determined by comparing the final pool size with that of day 0, calculated from the initial body weight and the composition of untreated rats. Energy and nitrogen balances were estimated. Plasma levels of metabolites and hormones were also measured.
Results: OE induced changes in body composition similar to those elicited by a 50% reduction in food, with massive loss of lipid and energy. OE-treated rats ate less than the controls, but additional effects on body composition on reduced diet were minimal. OE improved metabolic homoeostasis: better maintained glycaemia, lower cholesterol and shallower hormonal changes, but at the expense of slightly increased protein mobilisation.
Conclusions: The data presented suggest that no advantages are accomplished by combining OE treatment and hypocaloric diets compared with OE alone, at least under the experimental conditions tested, since the effects were not additive. Despite OE affecting food intake, mechanisms other than that are deemed responsible for the mobilisation of body fat, since intake alone cannot explain the effects on body weight, nor the metabolic and hormonal changes in OE-treated rats. It is concluded that the combination of food restriction and OE may result in unwanted increased protein mobilisation with no synergy between both slimming treatments.
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Keywords: overweight; oleoyl-estrone; dietary restriction

Introduction

Food deprivation induces the rapid mobilisation of glycogen stores and the oxidation of protein as source of 3C fragments with which to sustain hepatic gluconeogenesis and thus help maintain glycaemia.[1] In a second phase, protein protection schemes take hold[2] preventing additional wasting and shifting the main energy source from glucose to fatty acids and ketone bodies.[3] This change is coupled to the progressive mobilisation of adipose tissue fat stores and the corresponding adaptation of muscle lipid and amino-acid metabolism, together with a marked decrease in energy expenditure[4] to save valuable (often irreplaceable) energy substrates.

Oleoyl-estrone (OE) is a fat-mobilising hormone[5] produced by adipose tissue, which is carried by the plasma lipoproteins.[6] The oral administration of OE to lean, genetically obese or dietary-obese rodents induces a marked loss of body fat,[7,8] primarily due to a decrease in voluntary food intake coupled with the maintenance of energy expenditure.[9] Glucose levels are maintained in spite of marked decreases in insulin and leptin,[10] a consequence of OE-elicited increase in insulin sensitivity/decrease in insulin resistance,[11] which results in the maintenance of liver glycogen stores regardless of the severe drainage of body

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energy.[10] Lipid mobilisation, however, does not massively increase circulating lipids, since lipid oxidation is also increased as shown by the lowering of the respiratory quotient,[5] and marked decrease in cholesterol levels[12] coupled with enhanced muscle lipoprotein lipase activity.[13]

Human obesity has been traditionally treated with hypocaloric diets, since it is assumed that a decrease in energy intake must compel the body to mobilise its fat stores to cope with the energy imbalance; however, dieting tends to decrease energy expenditure,[14] which makes much harder to significantly affect the mass of stored fat. The considerable difficulty in shedding off fat through dieting is compounded by the recovery of energy stores when food availability is restablished.[15]

Most antiobesity compounds are used in conjunction with hypocaloric diets, essentially to enhance their lipid-mobilising effects, to diminish the unwanted effects of limited food intake (i.e. hunger pangs, hypoglycaemia), to enhance energy expenditure to speed up the process of fat disposal, or to maintain the weight loss during and after treatment.[16] OE has been used, so far without association to limited food intake, both in rodents and humans,[17–19] since OE limits food intake by itself, and prevents the fall in energy expenditure[5,11] that accompanies food deprivation.[4] In the present study, we have explored the combined effects of severely limited food intake and OE treatment, in order to determine whether hypocaloric diets enhance the slimming effects of OE on mature overweight male rats. Since a typical hypocaloric diet provides 40–50% of a standard diet's energy, and a very low-calorie diet provides about half of that, we established two levels of dietary limitation, theoretically at 50 and 25% of a standard intake, roughly corresponding to the human low-calorie diets and very low-calorie diets.

Materials and methods

Male Wistar rats (6 weeks old) of Harlan-Interfauna (Sant Feliu de Codines, Spain) stock were used. They were kept in collective cages (two rats in each) in a light cycle, temperature, ventilation and humidity-controlled environment. All animal-handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, following the EU-, Spanish-, and Catalan Government-established norms and procedures.

A fattening protocol previously published[20] was used; the animals were fed for 5 weeks with a modified cafeteria diet[11,17] (fattening period). Afterwards, they were transferred to wire mesh-bottomed cages and were maintained for 5 days exclusively on a pellet ('maintenance' type, from Panlab, Barcelona, Spain) *ad libitum* diet (stabilisation period). Then, when the rat age was 12 weeks, they were randomly distributed in six experimental groups for the experimental phase of the study, which lasted 10 days. Initial body weights and food consumption during the different phases can be seen in Table 1. Half the animals were given a daily gavage of

Table 1 Food consumption and body weight change of male overweight rats subjected to limited energy intake and treatment with oral oleoyl-estrone for 10 days

Parameter	*Units*	*Period*	*Days*	*Diet*	*AL*		*HEA*		*QEA*		*P-value (ANOVA)*		
					Control	*OE*	*Control*	*OE*	*Control*	*OE*	*Diet*	*OE*	*Int*
Food available	%[a]	Study	10	Pellet/restricted	Unrestricted	Unrestricted	50.0	50.0	25.0	25.0			
	g/d	Stabilization	5	Pellet *ad libitum*			17.7±0.5						
Food consumption	g/d	Study	10	Pellet/restricted	20.6±1.6	13.5±0.9	8.9±0.9	7.6±0.8	4.5±0.3	4.4±0.3	<0.001	0.001	0.001
	%[a]				116.1±8.8	76.3±5.3	50.1±4.7	43.0±4.4	25.6±1.6	24.9±1.9	<0.001	0.001	0.001
Initial body weight	g	Study			380±15	360±12	378±6	358±12	356±17	360±8	NS	NS	NS
Final body weight	g	Study			387±13	320±9	329±5	298±8	294±15	289±7	<0.001	<0.001	0.018
Body weight change	g/d	Fattening	35	Cafeteria *ad libitum*			4.98±0.13						
	g/d	Stabilization	5	Pellet *ad libitum*			−1.63±0.23						
	g/d	Study	10	Pellet/restricted	0.67±0.62	−3.96±0.65	−4.88±0.18	−5.95±0.52	−6.28±0.15	−7.07±0.25	<0.001	<0.001	<0.001

Data are the mean±s.e.m. of six different animals per group. Statistical analysis of the differences (two-way ANOVA). NS = $P > 0.05$; int = interaction of diet and OE treatment. [a]Percentages refer to the mean food consumption of all animals during the stabilization period.

0.2 ml of sunflower oil by means of a gastric tube, the other half received the same gavage containing a dose of 10 nmol/g OE (OED, Barcelona, Spain). The animals were subdivided into three diet groups:

1. AL, fed *ad libitum*; the rats had unrestricted access to pellet food and water;
2. HEA, or half energy availability, that is, 50% dietary restriction, these animals had access to a fixed amount of pellet food, corresponding to half their mean food consumption during the stabilisation period, they had unrestricted access to water;
3. QEA, or quarter energy availability, that is, 75% dietary restriction, with access to only one-quarter of the mean food consumed during the stabilisation period.

Roughly, the HEA diet was considered akin to a 'human' LCD, and the QEA diet to a VLCD. An additional group of rats were killed just at the end of the stabilisation period, before the final gavage and diet-reduction experiment, in order to obtain the initial body composition data. Food consumption and body weight were measured daily in all the groups.

On day 10, the remaining rats were killed by decapitation. The blood was recovered and allowed to clot; the serum was stored at −80°C until processed. The stomach and intestinal contents were discarded; the rat remains were autoclaved, homogenised and used for the estimation of water, protein, lipid and energy content as previously described:[11] water was estimated by differential weighing before and after desiccation at 110°C; protein was estimated from the N content (Kjeldahl, using the 1007 Digestor and 1002 Distilling Unit, both from a Tecator Kjeltec System, Höganäs, Sweden) and conversion of N content into protein equivalence;[21] lipid by trichloromethane: methanol extraction;[22] and energy using a bomb calorimeter (C-7000 Ika, Heitersheim, Germany). The final body composition was determined from the percentages of body components measured experimentally and the estimated '*in vivo*' net body weight. For calculation of the body weight components' content of the group killed at the beginning of the study was used in conjunction with the initial body weight of each rat.

Metabolisable energy content of the pellet food was estimated from the standard caloric equivalence of its assimilable components (154 g/kg crude protein, 605 g/kg carbohydrate, 29 g/kg lipid) and the assumed efficiency of the digestive process, giving a yield of 13.9 kJ/g. The total energy content of the pellet was estimated with the bomb calorimeter (16.5 kJ/g), which represents that only about 84% of the total energy contained in the pellet (including that of fibre) was assumed to be taken up and used by the rat. Total nitrogen content of the pellet was also measured (Kjeldahl): 23.6 g/kg, and used for the estimation of N intake (Ni).

Energy expenditure was calculated as energy intake (energy correlate of the food ingested) minus the energy accretion calculated as the difference in the energy content of controls and experimental groups corrected by initial body weight. All data were expressed as rates, that is, accretion in g/day or, in the case of energy, in power units (watt = J/s).

Ni was estimated from the N content in the pellet, N accretion (Na) was determined from final N content and calculated initial N using the same reasoning described above for energy. Faecal N (Nf) was estimated by measuring (Kjeldahl) the N content of pooled droppings. It was assumed that the N excreted (Ne) was the difference:

$$Ni = Nf + Ne + Na$$

The nitrogen excreted included urinary nitrogen and other N losses, such as the 'nitrogen gap'.[23]

Serum samples were used for the estimation of glucose (Trinder glucose kit, Sigma), urea (kit B8035 from Menarini), triacylglycerols (kit 11528; Biosystems, Barcelona, Spain), total cholesterol (kit B7576; Menarini, Firenze, Italy), HDL-cholesterol (precipitating kit CH204 from Randox, Crumlin, UK; and kit B7576 from Menarini), non-esterified fatty acids (NEFA) (kit NEFA-C; Wako, Richmond, VA, USA), 3-hydroxybutyrate (kit 0907979; Boehringer-Mannheim, Mannheim, Germany), creatinine (kit 30982; Menarini), aspartate transaminase (kit 51-25 Infinity AST reagent, Sigma Diagnostics, St Louis, MO, USA), alanine transaminase (kit 51-25 Infinity ALT reagent, Sigma Diagnostics), insulin (rat insulin RIA kit; Linco, St Louis, MO, USA), adiponectin (mouse adiponectin RIA, Linco), leptin (rat leptin RIA, Linco). Homeostasis model assessment method (HOMA score) was used to assess insulin resistance.[24,25]

Statistical comparison between groups was carried out using a two-way ANOVA program from the Statgraphics Plus v.2.1 (Manugistics, Rockville, MD, USA) software.

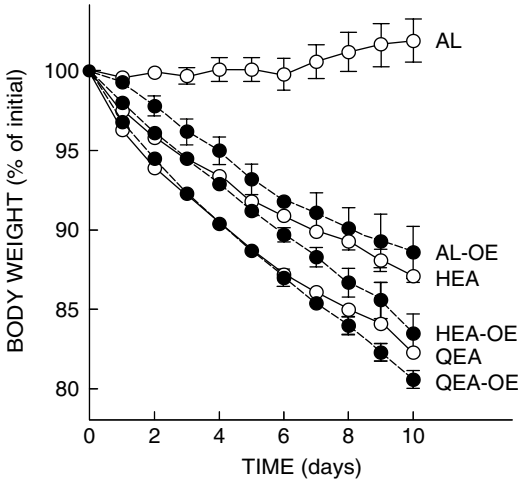

Figure 1 Body weight change of overweight male rats subjected to limited diet intake and oral oleoyl-estrone treatment. The data are presented as mean ± s.e.m. of six animals per group.

Results

The daily food consumption of all rats during the 5-day stabilisation period was 14% lower than that of AL controls during the following 10 days, probably because of diet-change stress (Table 1). The use of the food consumption data during this period to determine the overall reduction in food available for the restricted groups resulted, thus, in a more severe food allocation reduction than expected, but in any case the data are comparable between similarly treated groups, and inter-comparable with human dietary restriction treatments too, in spite of the experimental results showing a more intense or longer incidence of dietary change-related stress than expected.

Control rats killed at the end of the stabilisation period (i.e. day 0 of the food-restriction study) had a mean composition of 580±30 g/kg water, 177±3 g/kg protein, and 212±19 g/kg lipid; their energy content was 13.2±0.1 kJ/g.

Figure 1 shows the changes in body weight experienced by the six experimental groups during the 10-day study period. Table 1 also presents the data for the previous fattening and stabilisation periods, as well as the rates of body weight change and food consumption by the different groups. Control AL rats slightly increased their body weight (+1.8%) but had a higher food consumption than in the stabilisation period (+16%). OE decreased food consumption by 24%, and body weight by 11%; these data were fairly similar to those of the rats with HEA, which decreased body weight by 13%. The combination of HEA and OE further decreased food intake (−57%) and body weight (−17%), a figure similar to that of the rats with QEA, which decreased body weight by 17%. Finally, the maximal loss of weight (−20%) was observed in the rats subjected QEA and OE treatment.

In all the groups, the OE-treated rats ate less than their matched diet controls, the difference was maximal for AL: 66%, but with HEA, OE-treated rats did not consume all food available, only 85% of their matched controls; the figure for QEA was 98%.

The changes in body composition induced by dietary restriction and OE treatment are presented in Table 2. In the AL rats, the effects of OE were considerable, since in 10 days, differences of 7.5% in water pool, 4.7% in protein pool, 30.0% in lipid pool and 20.2% in total energy content were accumulated. However, the corresponding differences in the HEA rats were smaller: 5.7% in water pool, 1.4% in protein pool, −0.9% in lipid pool and 1.8% in total energy. Those obtained comparing the rats QEA groups were similar to the HEA data: 4.1% in water pool, 2.1% in protein pool, −1.7% in lipid pool and 2.0% in total energy.

Diet restriction resulted in significant effects on weight, water and protein content, those on lipid and energy were even more marked. OE induced further losses, especially of energy and lipid, but the differences between control and OE-treated rats in the HEA and QEA groups were relatively small, compared with AL. In fact, the similarities in body composition were higher when comparing the AL OE-treated rats with the HEA controls, and the HEA-OE rats with the

Table 2 Body composition of male overweight rats subjected to limited energy intake and treatment with oral oleoyl-estrone for 10 days

Parameter	*Units*	*AL*		*HEA*		*QEA*		P-*value (ANOVA)*		
		Control	*OE*	*Control*	*OE*	*Control*	*OE*	*Diet*	*OE*	*Int*
Water										
Content	g/kg	552±14	594±13	597±12	599±10	618±9	603±13	0.013	NS	NS
Pool	g	213±7	191±5	197±4	179±4	181±7	174±3	<0.001	0.001	NS
10 days accretion	g	7.3±6.3	−8.0±6.0	−8.9±4.0	−20.0±3.2	−16.5±2.1	−25.2±2.5	<0.001	0.002	NS
	% of initial	3.7±3.1	−3.8±3.0	−4.4±1.9	−10.1±1.6	−8.5±1.3	−12.6±1.1	<0.001	0.002	NS
Protein										
Content	g/kg	168.7±3.7	183.1±3.7	183.4±4.2	184.9±4.1	189.5±3.5	190.0±2.1	0.003	NS	NS
Pool	g	65.16±2.04	58.50±0.93	60.43±1.87	55.23±2.14	55.42±2.12	54.94±1.08	0.004	0.010	NS
10 days accretion	g	1.03±2.58	−1.71±2.25	−3.26±1.43	−4.64±2.06	−4.22±1.24	−5.33±0.54	0.046	NS	NS
	% of initial	+2.3±3.9	−2.4±3.7	−5.1±2.2	−7.6±3.4	−6.7±1.8	−8.8±0.7	0.025	NS	NS
Lipid										
Content	g/kg	180.1±18.7	130.7±17.8	118.9±18.4	121.7±12.8	108.1±9.1	122.7±11.7	0.026	NS	NS
Pool	g	70.34±9.16	40.20±6.50	39.23±6.20	36.86±4.54	32.23±4.00	35.83±4.25	0.002	NS	0.032
10 days accretion	g	−8.83±4.67	−29.63±2.99	−38.86±5.98	−34.45±3.06	−38.82±2.58	−36.04±2.89	<0.001	NS	0.005
	% of initial	−12.4±5.8	−42.4±6.0	−49.9±7.6	−49.0±5.1	−52.3±3.0	−50.6±4.4	<0.001	NS	0.004
Total energy										
Content	kJ/g	11.33±0.72	9.69±0.67	9.33±0.69	9.11±0.41	8.87±0.34	8.76±0.41	0.013	NS	NS
Pool	MJ	4.41±0.40	3.11±0.28	3.08±0.24	2.73±0.18	2.63±0.23	2.55±0.18	<0.001	0.012	NS
10 days accretion	kJ	−443±117	−1293±85	−1736±217	−1661±108	−1794±131	−1900±141	<0.001	0.017	0.007
	% of initial	−9.6±2.7	−29.8±2.8	−36.2±4.4	−38.0±2.6	−40.8±1.1	−42.8±2.8	<0.001	0.002	0.005

NS, not significant. Data are the mean ±s.e.m. of six different animals per group. Statistical analysis of the differences (two-way ANOVA). NS = $P > 0.05$; int = interaction of diet and OE treatment.

Table 3 Energy balance of male overweight rats subjected to limited energy intake and treatment with oral oleoyl-estrone for 10 days

Parameter	*Units*	*AL*		*HEA*		*QEA*		P-*value (ANOVA)*		
		Control	*OE*	*Control*	*OE*	*Control*	*OE*	*Diet*	*OE*	*Int*
Metabolisable energy intake	W	3.302±0.251	2.170±0.152	1.425±0.134	1.224±0.124	0.728±0.045	0.707±0.054	<0.001	<0.001	0.001
Energy accrual	W	+0.512±0.351	−1.496±0.099	−1.406±0.257	−1.923±0.125	−2.076±0.152	−1.884±0.370	<0.002	0.047	NS
Energy expenditure	W	3.815±0.349	3.666±0.222	2.831±0.370	3.147±0.066	2.804±0.191	2.597±0.386	0.004	NS	NS

NS, not significant. Data are the mean±s.e.m. of six different animals per group. Statistical analysis of the differences (two-way ANOVA). NS = $P>0.05$; int = interaction of diet and OE treatment.

Table 4 Nitrogen balance of male overweight rats subjected to limited energy intake and treatment with oral oleoyl-estrone for 10 days

Parameter	*Units*	*AL*		*HEA*		*QEA*		P-*value (ANOVA)*		
		Control	*OE*	*Control*	*OE*	*Control*	*OE*	*Diet*	*OE*	*Int*
N intake	mg/day	507±38	333±24	219±21	188±19	112±7	109±8	<0.001	<0.001	0.001
N accrual	mg/day	16.5±41.3	−27.4±36.0	−52.2±22.9	−74.2±32.9	−67.5±19.8	−85.3±8.6	0.046	NS	NS
Faecal N	mg/day	99.0±9.5	58.1±3.5	49.3±5.3	43.2±4.3	34.7±1.2	31.3±2.7	<0.001	<0.001	0.002
Total N excreted	mg/day	490±27	360±19	259±7	262±20	179±25	194±14	<0.001	0.005	0.004
Non-faecal N excreted	mg/day	391±26	302±19	210±11	219±20	144±24	163±12	<0.001	NS	0.020

NS, not significant. Data are the mean±s.e.m. of six different animals per group. The data for faecal N was obtained from pooled samples. Total N excreted and non-faecal excreted N were calculated as the difference between N intake, faecal N and accrued N. Statistical analysis of the differences (two-way ANOVA). NS = $P>0.05$; int = interaction of diet and OE treatment.

Table 5 Serum composition of male overweight rats subjected to limited energy intake and treatment with oral oleoyl-estrone for 10 days

Parameter	*Units*	*AL*		*HEA*		*QEA*		P-*value (ANOVA)*		
		Control	*OE*	*Control*	*OE*	*Control*	*OE*	*Diet*	*OE*	*Int*
Glucose	mM	7.35±0.08	7.71±0.08	6.64±0.18	6.47±0.15	5.63±0.09	6.32±0.10	<0.001	0.013	0.011
Urea	mM	7.12±0.33	6.73±0.47	5.39±0.22	7.47±0.11	4.22±0.18	6.91±0.27	0.001	<0.001	<0.001
Triacylglycerols	μM	1527±142	591±106	419±31	371±54	427±25	290±23	<0.001	<0.001	<0.001
Total cholesterol	μM	1525±129	487±73	1475±56	376±70	1334±100	350±25	NS	<0.001	NS
HDL-cholesterol	%	56.5±5.1	26.3±4.9	53.5±4.5	28.5±4.3	51.6±4.0	35.1±4.6	NS	<0.001	NS
3-hydroxybutyrate	μM	91±3	97±2	169±1	147±14	192±4	157±7	<0.001	0.034	NS
Non-esterified fatty acids	μM	463±27	487±23	771±29	615±35	716±29	607±36	<0.001	0.008	0.034
Creatinine	μM	46.0±1.7	55.5±4.6	64.4±4.1	57.2±3.4	50.1±4.4	61.9±5.2	NS	NS	NS
Aspartate transaminase	nkat/ml	1.86±0.08	1.69±0.12	1.84±0.12	1.88±0.05	1.88±0.12	1.79±0.14	NS	NS	NS
Alanine transaminase	pkat/ml	617±20	383±31	313±52	391±21	330±68	403±18	0.001	NS	<0.001
Insulin	pM	416±54	296±20	75±11	147±19	56±5	109±20	<0.001	NS	<0.001
Adiponectin	nM	89.4±10.2	71.0±13.7	67.5±6.6	51.6±2.6	94.3±12.1	62.8±8.0	NS	0.010	NS
Leptin	pM	729±42	205±34	108±33	127±22	100±3	133±28	<0.001	<0.001	<0.001
Urea/creatinine ratio	M/M	156±9	114±8	84±6	135±11	92±8	111±11	0.005	NS	<0.001
HOMA score		21.4±3.4	14.1±0.9	3.7±0.8	5.9±0.8	2.1±0.1	3.8±0.7	<0.001	NS	0.010

NS, not significant. Data are the mean±s.e.m. of six different animals per group. Statistical analysis of the differences (two-way ANOVA). NS = $P>0.05$; int = interaction of diet and OE treatment.

controls of the QEA. In any case, the overall effects of OE (ANOVA) over those of diet were significant for the combined energy content but not for protein or lipid.

The energy balance is shown in Table 3. Diet restriction, but not OE treatment, decreased energy expenditure, but to a lesser extent than changed energy intake, the difference being compensated by mobilisation of body energy substrates, mainly lipid, which accounted by 90–99% of energy accretion in all groups, the difference being the changes in protein stores. The maximal differences between energy intake, energy accretion and also calculated energy expenditure were found in the AL groups, there were no differences for energy balance figures between OE-treated and controls for the HEA and QEA groups.

The nitrogen balance is presented in Table 4. There were no significant effects of OE on Na. Decreased Ni in all groups was counteracted by decreased N excretion. However, OE elicited a shift in the form in which N was excreted, with

lower faecal losses, partly compensated by non-faecal excretion, suggesting a higher ability to extract dietary N in the OE-treated animals.

Table 5 presents the serum metabolites, enzymes and hormones of controls and OE-treated animals. Dietary restriction reduced the glucose levels, but the changes were smaller in the OE groups. Insulin levels decreased markedly with OE treatment in AL animals, but further diet restriction induced a more marked decrease in insulin levels in controls than in OE rats.

Urea levels decreased with diet restriction in controls, but not in OE-treated rats. Creatinine levels changed little, and thus the urea/creatinine (mol/mol) ratios were better maintained in all OE-treated rats (114, 135, 111, respectively, for AL, HEA and QEA groups) than in controls (156, 84, 92 for the corresponding groups), which suggests that under food restriction, the OE rats had a more active urea production than the untreated, in which it was reduced almost by half.

Dietary restriction markedly decreased serum triacylglycerols, and OE further decreased these levels; however, the increases on 3-hydroxybutyrate and NEFA were similar for matching OE/control food-restricted groups. Cholesterol levels were practically unchanged by dietary manipulation, but OE-induced marked, albeit similar, decreases irrespective of dietary status.

Aspartate transaminase did not show changes in any group; alanine transaminase levels were maximal in AL controls, with similar low levels in the other experimental groups.

Adiponectin levels showed no significant changes with dietary restriction, but the levels were lower in all OE-treated groups than in their matching controls. Leptin levels markedly decreased with either dietary restriction or OE treatment.

Discussion

The data presented suggest that no special advantages are gained by combining OE treatment and hypocaloric diets with respect to OE treatment alone, since the effects of both procedures are far from being additive. Notwithstanding, and in spite of OE deeply affecting food intake, mechanisms other than limited energy intake are responsible for the mobilisation of body fat, since food intake alone cannot explain the effects on body weight, nor, especially, the metabolite, cytokine and hormone changes observed in treated rats. In all cases, however, OE administration resulted in an improved metabolic homoeostasis: better maintained glycaemia, lower cholesterol and shallower hormonal changes, but at the expense of slightly increased protein metabolism.

OE treatment without the concourse of dietary restrictions protects body protein and carbohydrate, mobilising only fat.[11] This is accomplished essentially by facilitating the use of fatty acids by the muscle and other peripheral tissues,[13] decreasing the oxidation of glucose and increasing its storage as glycogen,[10] and, at the same time, decreasing insulin resistance.[11,26] Under OE, glucose levels are maintained in spite of a marked decrease of insulin.[10,11,17] In food deprivation or restriction, glycaemia is maintained at the expense of 3C fragments, essentially derived from amino acids through the glucose-alanine cycle.[27] However, under OE treatment there is no need for an enhanced liver glucose output, since peripheral utilisation of glucose is decreased,[28] which results in the sparing of protein.[10,11] However, when the rat is subjected to an additional and severe decrease in food available, the emergency mechanisms that maintain glycaemia are nevertheless activated. The higher sensitivity to insulin elicited by OE may result in enhanced hepatic gluconeogenesis, thus draining the 3C fragments available. As a consequence, more amino acids are mobilised in OE-treated rats than in food-deprived controls, since the former maintain better the glucose levels at the expense of higher amino-acid catabolism. The higher urea production (maintained urea/creatinine ratios) and the fractionally higher loss of body protein is not fully compensated by the also slightly higher nitrogen extraction from food.

Thus, paradoxically, the combination of a protein-sparing agent, OE,[9,11] with a process that actively mobilises body protein as is food deprivation,[29] results in small increases in body protein mobilisation. The low levels of insulin cannot be used to explain this situation (i.e. a decreased anabolic signal may facilitate catabolism), since the rats not receiving OE showed even lower levels of insulin than those treated with OE; in addition, OE increases insulin sensitivity,[26] insulin is largely responsible for muscle protein integrity, and muscle contains the largest body protein stores. Glucose availability was maintained in all groups, since the increases in 3-hydroxybutyrate were small and easily correlated with the increased levels of NEFA. The characteristic effect of OE decreasing circulating cholesterol,[10,12] also observed here, is largely independent of the mobilisation of lipids (controls' levels practically did not change with dietary restriction), but is directly correlated with an enhanced lipoprotein metabolism.[12,13]

The marked effects of OE on leptin[10,12] hint at deeply altered adipocyte signalling pathways, the uncharacteristic decreases of adiponectin elicited by OE attest that there is no direct or easy explanation of the mobilising effects of OE. Adiponectin and leptin levels usually show reversed patterns, leptin levels being high in the obese,[30] which usually show low adiponectin concentrations.[31] Adiponectin may be a harbinger of increased insulin sensitivity,[32] but in the food-deprived rats receiving OE, adiponectin, leptin and insulin levels were all decreased, under conditions of normoglycaemia, decreased appetite, maintained energy expenditure, and massive lipid mobilisation. This situation does not correspond with the usual paradigm of cytokine release by the adipocytes,[33] and may simply reflect increased OE-induced adipocyte apoptosis,[34] that eventually may result

in diminished overall secretion of adipocyte-derived proteins into the bloodstream.

The lack of changes in transaminases suggest that hepatic function is not compromised, independently of the higher protein mobilisation of semistarved OE-treated rats. This lack of hepatic damage agrees with previous findings in rats treated with OE.[35]

The similitudes in body composition parameters of *ad libitum*-fed OE-treated rats with the untreated HEA animals contrasts with the marked discordances in plasma composition found between these same groups. Conversely, the relative mild differential effects of OE on body composition parameters of matched (control and OE-treated) food-deprived groups sharply contrast with the marked OE-induced differences in plasma parameters, which suggests that, irrespective of the non-additive overall fat-mobilising effects of either diet restriction or OE treatment, the metabolic mechanisms through which these effects are elicited are basically different, as ascertained by the differences observed in serum composition patterns.

It is concluded that the combination of severe food restriction and OE treatment may result in unwanted increased protein mobilisation with no synergy between both procedures on weight loss.

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3.2 Efecto de la oleoil estrona sobre el metabolismo energético

3.2.1 Análisis semicuantitativo de la expresión génica en tejidos de rata mediante RT-PCR, incluyendo una corrección para las variaciones del tamaño y número de células

La metodología actual del análisis de la expresión génica suele representar los resultados en forma relativa en base a los porcentajes de cambio con respecto a un grupo control. Este planteamiento limita las posibilidades de comparación entre grupos y tejidos en los que el tamaño o el número de células varían como consecuencia del propio estudio (por ejemplo, la inanición). Un método que relacionara la abundancia relativa de copias de ARNm específicos podría permitir la comparación directa entre diferentes tratamientos y localizaciones y además permitiría referir las expresiones a unidades concretas en relación con el tejido, clarificando los cambios inducidos por el tratamiento.

Para la cuantificación del producto de la PCR a tiempo real se utilizó el marcador genérico fluorescente *SYBER Green* que detecta las cadenas dobles de ADN. Se prepararon una serie de estándares de ADNc de unos cuantos genes y se analizó la relación entre su número de bases y la fluorescencia registrada a un nivel fijo para crear una recta patrón. Se encontró una relación lineal entre la fluorescencia y la longitud del transcrito y partir de estos datos se dedujo una ecuación general que relaciona la fluorescencia de las muestras con el número inicial de copias de ARNm y la longitud del transcrito. La determinación del peso del tejido o del contenido de ADN por célula permite a su vez expresar los resultados de expresión génica como copias de ARNm por tejido total o por célula.

La aplicación de este procedimiento para seis genes diferentes, en el hígado y el tejido adiposo retroperitoneal de ratas control y ratas sometidas a una restricción energética, permitió obtener un resultado preciso de los cambios en su expresión génica, independientemente de la disminución de masa del tejido, el cambio del número de células o las modificaciones de su tamaño, factores que complican profundamente la comparación porcentual de los resultados cuando las condiciones experimentales inciden sobre estos parámetros. Los resultados porcentuales obtenidos con los presentes métodos fueron comparables a los obtenidos con el procedimiento de análisis semicuantitativo delta-delta y con el análisis cuantitativo basado en la curva estándar individual de ADNc.

En conclusión, el método presentado en este trabajo permite la comparación (como copias de ARNm por tejido o célula) entre diferentes grupos, tejidos o tratamientos, permitiendo a su vez comparar la abundancia de los diferentes transcritos.

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Methodology

Semiquantitative RT-PCR measurement of gene expression in rat tissues including a correction for varying cell size and number

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Abstract

Background: Current methodology of gene expression analysis limits the possibilities of comparison between cells/tissues of organs in which cell size and/or number changes as a consequence of the study (e.g. starvation). A method relating the abundance of specific mRNA copies per cell may allow direct comparison or different organs and/or changing physiological conditions.

Methods: With a number of selected genes, we analysed the relationship of the number of bases and the fluorescence recorded at a present level using cDNA standards. A lineal relationship was found between the final number of bases and the length of the transcript. The constants of this equation and those of the relationship between fluorescence and number of bases in cDNA were determined and a general equation linking the length of the transcript and the initial number of copies of mRNA was deduced for a given pre-established fluorescence setting. This allowed the calculation of the concentration of the corresponding mRNAs per g of tissue. The inclusion of tissue RNA and the DNA content per cell, allowed the calculation of the mRNA copies per cell.

Results: The application of this procedure to six genes: Arbp, cyclophilin, ChREBP, T4 deiodinase 2, acetyl-CoA carboxylase 1 and IRS-1, in liver and retroperitoneal adipose tissue of food-restricted rats allowed precise measures of their changes irrespective of the shrinking of the tissue, the loss of cells or changes in cell size, factors that deeply complicate the comparison between changing tissue conditions. The percentage results obtained with the present methods were essentially the same obtained with the delta-delta procedure and with individual cDNA standard curve quantitative RT-PCR estimation.

Conclusion: The method presented allows the comparison (i.e. as copies of mRNA per cell) between different genes and tissues, establishing the degree of abundance of the different molecular species tested.

Introduction

Real-time PCR analysis of gene expression is probably the method of choice for the establishment of hormone-, metabolite- or drug-induced modulation of the metabolic and hormonal milieu in most organs [1]. The results allow for comparison of the strength of replication (i.e. specific mRNAs abundance) of the genes/alleles under study between comparable groups of treated or experimental and control individuals. The comparisons are more often referred to "constitutive" genes, which are expected not to change under the experimental conditions because of their lack of reactivity, unrelatedness to the pathways studied or experimentally observed resilience to change. The results are usually presented as percentages of the controls or expressed in arbitrary units that allow for comparison, but seldom for quantitative analysis [2].

Quantitative PCR procedures are of limited application since they require considerable investments of time, resources and expertise [3,4].

The "comparative" approach is useful for a large number of experimental setups, and yields most of the data being currently published. A growing number of authors feel the need to include data on the number of complete PCR cycles necessary to achieve a given pre-established level of fluorescence in the analytical system used, but this is often additional information that just hints at the relative real abundance of experimental data. The "comparative" approach also requires that no additional changes in cellularity or tissue or organ mass occur, since then the comparisons with controls may be significantly skewed. The case of adipose tissue is paradigmatic of this situation: in the field of obesity many studies take this tissue as the subject of research, but its changes in size, cell count and cell size are seldom taken into account when comparing groups treated with powerful slimming agents and untreated (and unchanged) controls [5].

In this study we have developed a simple procedure for the quantification of specific mRNAs in relation to organ weight or cell numbers, so as to make fully comparable the data of controls and experimental subjects. Thus, we have studied the changes in the expression of a number of genes under the challenge of a 10-day period of limited food availability, a situation that is well within the physiological range and is akin to dietary energy restrictions in humans and induces significant changes in the expression of adipose tissue genes [6]. Decreased food energy reduces the mass of most adipose tissue locations in rats [7]. The study includes liver, i.e. an organ not expected to change too much under this limited dietary treatment and a location of white adipose tissue, which has been found to respond to decreased energy intake.

Methods

Animals and sample preparation

Adult male overweight Wistar rats [8], initially weighing 355 ± 5 g, and kept under standard conditions of housing and feeding were used. Two groups of 7 rats each were randomly selected: controls (C) and food-restricted (FR). The controls had free access to pellet food, and the FR were allowed only a 60% of the food consumed by C. FR rats completely ate the food allotted each day. On day 10, the rats were killed and the liver and retroperitoneal fat pads were excised, weighed, sampled, frozen and kept al -80°C.

The animals were handled and killed following the procedures approved by the University of Barcelona Animal Welfare and Ethics Committee in full compliment of the norms and procedures set forth by the European Union and the Governments of Spain and Catalonia.

Nucleic acids measurement and tissue cellularity

Tissue samples were used for the estimation of total DNA, using a standard fluorimetric method with 3,5-diaminobenzoic acid (Sigma, St Louis MO USA) and bovine DNA (Sigma) as standard [9]. Tissue DNA content allowed the calculation of the number of cells per g of tissue and in the whole liver, based on the assumption that the DNA content per cell is constant in mammals; here we used the genomic DNA size data [10] for somatic rat cells (5.60 pg/cell). Total liver/adipose tissue cell numbers included not only hepatocytes/adipocytes, but immune system, endothelial and other minority types of cells as well.

Mean cell volume was estimated from the number of cells and the volume of the organ, calculated using a liver density of 1.10 g/mL and 0.90 for adipose tissue [11].

Total tissue RNA was extracted using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and were quantified in a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) and oligo-dT primers.

Real-time PCR (RT-PCR) amplification was carried out using 10 μL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to OD 0.500 for all runs.

Outline of the semiquantitative method for the measurement of gene expression

The quantitative measurement of mRNAs concentration in a given tissue requires to know: a) the efficiency of RNA extraction from the tissue; b) the percent effectiveness of the mRNA to cDNA retro-transcriptase process; c) the quantitative efficiency of the RT-PCR amplification; and d) the quantitative estimation of the number of transcripts generated in the RT-PCR process. A fully quantitative analysis would require the specific measurement of each of these four parameters for each transcript analyzed. However, procedure a, i.e. the effectiveness of RNA extraction has been repeatedly studied and found to be practically quantitative. Likewise, the efficiency of the real time PCR amplification step (point c) is very high [12], and has been followed using cDNA probes (see below). The main problems arise from points b and d. The latter has been estimated using the parameters of the fluorescence analysis system as described below. However, the critical point of the efficiency of the retro-transcriptase step (point b) could not be easily circumvented and is, probably the link of the calculation chain with lower efficiency. Oligo dT primers were used to enhance the representativeness of cDNAs from mixed mRNA populations [13]. The efficiency of the retro-transcriptase step has been linked to the length of the transcripts and also to the "noise" and abundance of other transcripts [14]. Few studies have tackled the problem, and give indications that range from 20% to 6% efficiency for normal or poorly represented transcripts when using a system similar to ours [14].

In our approximation to more quantitatively comparable data we tried to estimate and apply all the corrections available to the calculations except for the critical point of retro-transcriptase efficiency. We applied a flat 20% efficiency (based on ref. 14 data) to all calculations, which results in the estimations being only approximate and not quantitative. For this reason we consider that our estimations are "semiquantitative" and treat them as such.

The system of calculation we applied requires the estimation of the number of transcripts resulting in the final lecture of fluorescence of the system. Since the PCR procedure implies a duplication of the cDNA chains at each cycle, we obtain the relationship:

$$Tf = Ti \cdot 2^Z \quad (1)$$

i.e. the number of final transcripts or copies Tf is dependent on the initial number of chains Ti and the number of duplication cycles Z. This equation is often [14] presented as:

$$Tf = Ti \cdot (1+R)^Z \quad (2)$$

where R is a factor that corrects for the eventual non-quantitative duplication of the initial transcripts because of possible alterations in the system. In the present study, in all measurements done, R was consistently equal to 1 – which means that the efficiency of the PCR step (point d) was quantitative and uniform for all samples-, thus we reverted to the simplified equation 1.

The system we used for real-time PCR established the number of cycles Z at which a given overall fluorescence is achieved. This set point is the same for all analyses and is preestablished in the instrument-based procedure. The linearity of the reaction is established by checking whether sequentially diluted cDNA samples result in proportionally increased numbers of cycles (in a log scale) [15]; we routinely included this check to ward off blank- or dilution-derived sources of error and to determine the sensitivity and efficiency of the amplification process.

The final (preset) fluorescence recorded by the system is proportional to the number of bases in the final reaction. We can thus rewrite equation 1:

$$Bf = Bi \cdot 2^Z \quad (3)$$

where Bf is the final number of bases in the cDNAs and Bi is the initial amount of bases present in the cDNA population obtained from tissue mRNAs. Obviously,

$$Bi = Ti \cdot L \quad (4)$$

where L is the length of the transcript, i.e. the number of bases between both extremes of the two probes for each gene. Similarly,

$$Bf = Tf \cdot L \quad (5)$$

Since we assume that the fluorescence is proportional to the number of bases, the estimation of Bf (or Tf) must be done using external calibration curves (as explained below). In an experimental setup, the determination of the number of cycles necessary to achieve the preset fluorescence threshold (i.e. using standard chains of cDNA for the gene including both up and down sequences), will allow us to apply this standardized and quantified Bf value to all other transcripts [15]:

$$Bf = (Ti \cdot L) \cdot 2^Z \quad (6)$$

which allows for the estimation of the (initial) number of cDNA copies for the given gene in the sample of cDNA used (Ti).

Since we can directly correlate the amount of cDNA used in a reaction vessel to a given weight of the tissue after

applying the corrections (measured, estimated or calculated) for the efficiency of the processes to obtain cDNAs, duplicate cycling of cDNA copies by the RT-PCR procedure, and estimation of the final number of copies of cDNA transcripts obtained, we can establish the number of copies of the mRNA per g of tissue or in the whole organ. These calculations allow us to present the concentration of each specific mRNA in molar units, or to express the value as the mean number of mRNA copies per cell simply by applying the Avogadro number (6.022×10^{23} molecules per mol). Since a critical step is only an approximate (not measured) value (i.e. the efficiency of the reverse transcriptase step) we present these data as simple approximations to the real figures (semiquantitative approach).

Establishment of the system basic parameters

The oligonucleotides used for the preparation of the external calibration curves were prepared from rat RNA. By using reverse transcriptase and oligo dT primers, cDNAs were obtained; they were amplified through the polymerase chain reaction, run on agarose gels and purified using the Hi-pure PCR Products Purification kit (Roche Applied Biosystems). Seven genes were used to establish the parameters of the system; the list, and the probes used can be seen in Table 1.

Thus, the final number of copies per cell will be the product of the number of initial cDNA transcripts (Bi/L), the tissue mRNA yield (corrected by the efficiency of extraction and cDNA copying) and the number of cells (i.e. DNA vs. weight) in the same tissue.

Comparison of the present method with standard procedures

The data obtained in the experiments described above were used to establish a direct standard comparison of expressions versus their corresponding controls using arbitrary units (delta-delta); the data were corrected by their relationships of mRNA abundance with respect to control constitutive genes (in this case cyclophilin and Arbp) [16].

A second -quantitative- approach was the comparison of the experimental data with standard calibration curves obtained using purified cDNAs at varying concentrations [13].

Results and Discussion

The set of calculations presented here facilitates the final estimation of the actual concentration or presence (in absolute numbers) of the mRNA corresponding to the genes studied through RT-PCR. This small advancement in the usefulness of that procedure improves its yield by allowing the comparison between the strength of the expression of different genes, correcting for changes in organ size and cell size. This is specially important for studies on WAT [17], but -as the results show- also for other organs such as liver.

Different initial amounts of these master genes were amplified through RT-PCR; Figure 1 shows the results obtained when plotting the number of bases found as a function of the number of cycles. In this case, the number of bases was derived from the known amount of cDNA standard for the specific gene used.

In Figure 1 (and the complementing Table 2), the equation for the regression line shows a slope close to -log 2 (deduced, but also found experimentally: -0.302 versus -0.293 ± 0.003, i.e. a mean 3% deviation over the theoretical figure). This formula coincides with a logarithmic expression of equation 3

Table 1: Genes for cDNA standards and sequences of the primers used for their estimation.

Gene name	Gene	Direction	Sequence	Length
60S acidic ribosomal protein P0	*Arbp*	3' > 5'	GAGCCAGCGAAGCCACACT	62
		5' > 3'	GATCAGCCCGAAGGAGAAGG	
Cyclophilin A	*Ppia*	3' > 5'	CTGAGCACTGGGGAGAAAGGA	87
		5' > 3'	GAAGTCACCACCCTGGCACA	
Carbohydrate-responsive element-binding protein	*Wbscr14*	3' > 5'	TACTGTTCCCTGCCTGCTCTCC	116
		5' > 3'	ACTGCCCTTGTGGCTTGCTC	
Type II iodothyronine deiodinase	*Dio2*	3' > 5'	CGGTGGCTGACTTCCTGTTG	123
		5' > 3'	CACATCGGTCCTCTTGGTTCC	
Acetyl-CoA carboxylase 1	*Acaca*	3' > 5'	AGGAAGATGGTGTCCGCTCTG	145
		5' > 3'	GGGGAGATGTGCTGGGTCAT	
60S acidic ribosomal protein P0	*Arbp*	3' > 5'	CCCTTCTCCTTCGGGCTGAT	165
		5' > 3'	TGAGGCAACAGTCGGGTAGC	
Insulin receptor substrate 1	*Irs1*	3' > 5'	AATGAGGGCAGCTCCCCAAG	198
		5' > 3'	GGTCCTGGTTGTGAATCGTGAA	

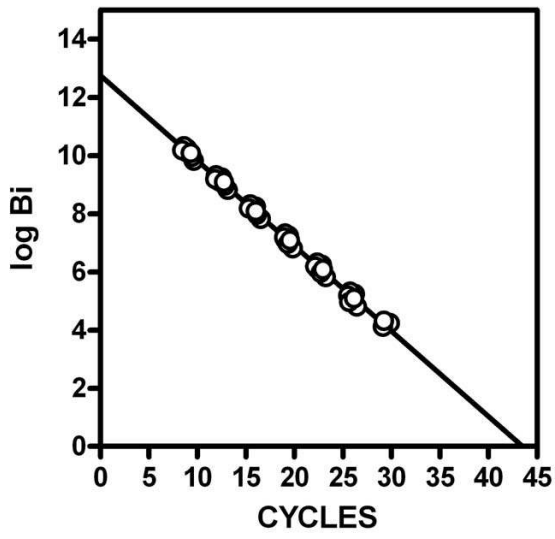

Figure 1
Relationship between the number of RT-PCR cycles and the initial copies used (expressed as the log of the initial number of bases) in seven cDNA standards. Parameters of the regression lines obtained for the individual (and combined) cDNAs are shown on Table 2. Each point of the line shown represents an individual measurement (N = 47). The most concentrated initial cDNA was 10^8, and was the same for all the transcripts tested. This stock solution was successively diluted 1/10 to obtain the rest of concentrations, down to 10^2 copies of cDNA per tube.

$$\log Bi = -\log 2 \cdot Z + \log Bf \quad (7)$$

We have found experimentally that the genes tested respond to equation 7 with a very high degree of correlation between expected and obtained values by using the cDNAs standards (Figure 2). The resulting line corresponds to the equation:

$$\log Ti_{real} = 1.01 \cdot \log Ti_{estimated} - 0.04 \quad (8)$$

that established a practical identity between both estimated and real Ti values ($r^2 = 0.999$), and confirms the applicability of the procedure to a number of different genes of different variability and abundance, and also using transcripts of different sizes.

Table 3 shows the weight and cellularity of the tissues used. As expected, the number of cells in liver was practically unchanged with limited feeding, but cell size shrunk by about 35% (in the same proportion as the liver weight). The differences in tissue weight and cell size can be directly traced to the loss of energy substrates, such as glycogen (and water) [18] and lipids [19].

In retroperitoneal WAT we observed a reduction in organ size (almost by half) due to both a decrease in the number of cells it contained (loss of about 22% of the cells) and decreased cell size (by about 32%). The decrease in mean cell size and cell numbers do not fully add to the tissue loss of WAT weight because with its mass shrinkage, largely due to the loss of fat [17] which affects its density. In addition their large mean size (in the range of ten-fold the mean size of liver cells) could not fully correspond to adipocytes, the prevailing cell type, because a variable number of other smaller cell types, such as preadipocytes, macrophages, and stem cells [20] coexist with adipocytes and their proportion is altered by obesity, stress and other conditions [21]. In any case, the data presented are a fairly valid approximation because of both the large predominance of hepatocytes in liver and adipocytes in WAT. In addition, the results, when presented per unit of cell-DNA (i.e. copies per cell of the mRNAs) fully correspond to the reality of the tissue irrespective of the type of cell that mostly contains the mRNA copies of the gene studied.

The loss of energy (mainly lipid) and loss of cells in WAT are in agreement with the supply of lipids to the liver under conditions of energy scarcity [22], resulting in the progressive mobilization of its triacylglycerol droplets and the corresponding diminution of its storage of circulating energy substrates.

Table 2: Parameters of the regression lines obtained for the individual (and combined) cDNAs shown on Figure 1

cDNA standards used	Slope	Log Bi (x = 0)	r^2
60S acidic ribosomal protein P0 (62 bp)	-0.298 ± 0.001	12.71 ± 0.04	9998
Cyclophilin A	-0.307 ± 0.003	12.98 ± 0.06	9996
Carbohydrate-responsive element-binding protein	-0.296 ± 0.002	12.85 ± 0.03	9999
Type II iodothyronine deiodinase	-0.296 ± 0.002	12.73 ± 0.01	10000
Acetyl-CoA carboxylase 1	-0.292 ± 0.002	12.65 ± 0.04	9998
60S acidic ribosomal protein P0 (165 bp)	-0.291 ± 0.001	12.88 ± 0.03	9999
Insulin receptor substrate 1	-0.291 ± 0.001	12.81 ± 0.03	9999
All data combined (line represented)	**-0.293 ± 0.003**	**12.75 ± 0.06**	**9960**

Table 3: Liver and adipose tissue cellularity

Parameter	Organ	Units	Control	Food-restricted
Tissue weight	liver	g	11.3 ± 0.4	7.4 ± 0.2 *
	WAT		9.07 ± 0.92	5.01 ± 0.67*
Number of cells	liver	× 10^9	4.44 ± 0.26	4.31 ± 0.18
	WAT		0.37 ± 0.02	0.29 ± 0.03*
Mean cell size	liver	pL	2.42 ± 0.11	1.55 ± 0.07 *
	WAT		25.7 ± 2.0	17.5 ± 2,6 *

The data are the mean ± sem of 7 different animals. Differences between groups: * = P < 0.05 versus controls.

Comparison of the data obtained using the present approach and those from the delta-delta or quantitative RT-PCR using standard cDNA standard curves can be seen in Table 4. The use of individual cDNA standards for the genes selected gave closely similar results to ours using a single generic common standard curve, and were also similar (when expressed as percentages) to those of the delta-delta procedure. The overall coincidence of results using genes of different abundance from different tissues supports the validity of our approach. The advantage gained by using the procedure we postulate here is, thus, that there is no need of individual specific cDNA calibration curves for each gene (the fluorescence-base pairs curve is sufficient) and, especially, that using our approach different tissues and different genes (i.e. of varying abundance) can be easily compared and conclusions as of their abundance can be drawn.

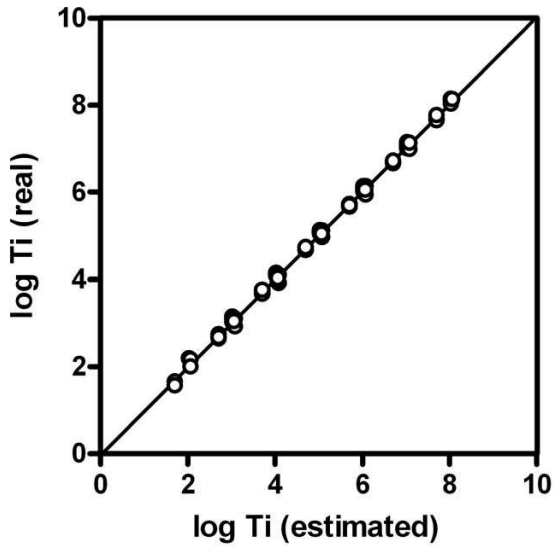

Figure 2
Relationship between the number of added cDNA standard transcripts and those experimentally found. The cDNA transcripts used were those listed in Figure 1. Each point represents an individual measurement (N = 87). Slope = 1.006 ± 0.004; Y intercept = -0.038 ± 0.021; r^2 = 0.9987.

In Table 5 we can see the application of the semiquantitative methodology proposed. Many of the genes tested gave very similar numbers of cycles in their RT-PCR estimation for both controls and food-restricted animals, in spite of small differences meaning large changes in specific mRNA abundance because of the logarithmic scale. If the only correction applied is the control of charge (comparison with constitutive gene expression), then the results obtained will miss any modification due to changes in cell size because of water, glycogen or fat content variation. In addition, the simple correction for RNA charge and constitutive gene expression does not take into account the overall effect of the selected gene expression for the whole organism because of changing size and cellularity of the organ studied.

When the additional corrections proposed are applied, even taking into account the relative individual imprecision of the effectiveness of the transcription process, we can give an estimate of the relative importance of the expression by relating the results to individual cells. This approach circumvents the problems posed by changes in tissue mass due to cell size changes, and can be further corrected by cell number changes for a better understanding of the physiological consequences of the gene expression analysis. The application of this copies-per-cell approach allows also for the relative comparison of the expression of different genes. Thus, we observe that the number of copies of the 60S acidic ribosomal protein is not altered in adipose tissue, but is down by a third in liver as a consequence of restricted feeding. A similar situation occurs with cyclophilin A; since both are commonly used "constitutive reference genes". In spite of the proven use of these genes for the control of RNA charge, the existence of differences in treated versus control groups when expressed as copies per cell cast doubts on their assumed unchanged physiological function.

The expression in liver of insulin receptor substrate 1 yields a small number of copies, but it is not changed by food restriction, whereas the more abundant acetyl-CoA

Table 4: Comparison of results obtained using a delta-delta (after correction using constitutive genes) method, quantitative RT-PCR approach (individual cDNA standard curves) and our postulated method (common cDNA bp-based standard curve) on liver and WAT from animals with restricted access to food and their controls.

ORGAN and gene	Group	Delta-delta	Individual RT-PCR	Present method
LIVER				
Carbohydrate-responsive element-binding protein	Control	100 ± 4	100 ± 5	100 ± 5
	Restricted	76 ± 4	79 ± 4	79 ± 4
Acetyl-CoA carboxylase 1	Control	100 ± 8	100 ± 3	100 ± 3
	Restricted	50 ± 12	47 ± 21	46 ± 1
Insulin receptor substrate 1	Control	100 ± 8	100 ± 9	100 ± 8
	Restricted	116 ± 10	115 ± 10	115 ± 10
ADIPOSE TISSUE				
Carbohydrate-responsive element-binding protein	Control	100 ± 12	100 ± 10	100 ± 9
	Restricted	24 ± 4	27 ± 5	27 ± 5
Type II iodothyronine deiodinase	Control	100 ± 6	100 ± 6	100 ± 5
	Restricted	45 ± 3	46 ± 2	46 ± 2
Acetyl-CoA carboxylase 1	Control	100 ± 8	100 ± 13	100 ± 11
	Restricted	55 ± 14	53 ± 14	53 ± 11
Insulin receptor substrate 1	Control	100 ± 13	100 ± 12	100 ± 11
	Restricted	28 ± 5	29 ± 5	28 ± 4

The data are the mean ± sem of 7 different animals and are expressed in all the cases as the percentage of the mean controls' values for easier comparison between the methods using homologous units. The base data used for comparisons in the case of the postulated method (and the individual quantitative RT-PCR method) was fmol/unit of total tissue RNA weight.

carboxylase gene expression is strongly reduced by food restriction, a logical change under the limited availability of lipogenic substrates. The copies-per-cell approach also allows for comparison between different tissues, thus we can observe that the number of copies per cell of the mRNAs for all the genes presented in Table 4 for WAT are lower than those of the much more active liver cells. In WAT, cyclophilin and 60S acidic ribosomal protein changes were small (not significant) in spite of a marked reduction in cell size (a behavior different from that of liver for these same genes). The expression of carbohydrate-responsive element-binding protein and insulin receptor substrate 1 decreased to about 20% in the food-restricted group, in agreement with the "wasting mode" adopted by the tissue under the ordeal of insufficient dietary fuels. A similar situation is observed in the T4-deiodinase, which main function is exacerbate thyroid hormone effects, akin to energy wasting [23]; under the strict conservation scheme of energy preservation, thyroid function is depressed [24] in part by decreasing peripheral T4 to T3

Table 5: Mean number of copies per cell of the mRNAs for the selected genes in liver and adipose tissue of overweight male rats subjected to food restriction

Gene name	Control		Restricted feeding	
	Cycles	cpc	Cycles	cpc
			Liver	
60S acidic ribosomal protein P0 (62 bp)	20.1 ± 0.1	1157 ± 50	20.1 ± 0.1	801 ± 38*
Cyclophilin A	19.4 ± 0.1	1261 ± 44	19.6 ± 0.1	855 ± 13*
Carbohydrate-responsive element-binding protein	22.8 ± 0.1	94 ± 6	23.3 ± 0.1*	54 ± 3*
Acetyl-CoA carboxylase 1	23.4 ± 0.2	50 ± 3	24.6 ± 0.1*	19 ± 1*
Insulin receptor substrate 1	25.0 ± 0.2	13 ± 1	24.9 ± 0.2	12 ± 1
			Retroperitoneal WAT	
60S acidic ribosomal protein P0 (62 bp)	19.7 ± 0.1	160 ± 18	19.7 ± 0.1	191 ± 29
Cyclophilin A	19.1 ± 0.1	193 ± 30	19.4 ± 0.1	149 ± 7
Carbohydrate-responsive element-binding protein	24.4 ± 0.3	4.3 ± 0.6	26.2 ± 0.4*	1.0 ± 0.2*
Type II iodothyronine deiodinase	27.0 ± 0.1	0.63 ± 0.10	28.1 ± 0.2*	0.29 ± 0.03*
Acetyl-CoA carboxylase 1	23.0 ± 0.2	7.6 ± 1.8	23.5 ± 0.6	6.8 ± 2.2
Insulin receptor substrate 1	26.6 ± 0.2	0.46 ± 0.05	27.9 ± 0.4*	0.15 ± 0.04*

The data are the mean ± sem of 7 different animals; cpc = copies of the corresponding mRNA per cell. Statistical significance of the differences between groups (Student's t test) * = $P < 0.05$.

conversion. WAT acetyl-CoA carboxylase expression was unchanged, suggesting a functionally active lipogenic pathway that may help to control the loss of energy endured by the tissue.

In the cases of a number of regulatory proteins of a tissue with limited metabolic activity as is the WAT, the copies per cell of their mRNAs may be very low, with values lower than a single copy per cell. This scarcity can only be interpreted as the combination of both a) the limited need for these particular proteins synthesis such as that related to hormone path signalling, and b) that not all the cells in the tissue contain the pathway or express the corresponding gene. The relative heterogeneity of WAT cellular populations [20] fits this interpretation: a few cells may have a sizeable number of copies of the mRNAs and most other have none; the postulated approach favours the identification of such cases, which may be easily passed over when the data are simply presented as a percentage of their controls.

In any case, the approach we postulate widens the possibility of physiological interpretation through comparison of the regulatory processes based on gene expression by extending the comparisons to other cells and genes and introducing a relative measure of quantitative importance of the specific gene expression studied.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

MMR did most of the experimental work; MMG and ME realized partial experiments and devised solutions to a number of experimental problems; JAFL established the numerical relationships and did all the computer work; MA designed the experiment and wrote the paper. All Authors participated in the design and final edition of the manuscript, as well as on the streamlining of the procedures and its direct application to experimental situations.

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3.2.2 Utilización de los lípidos en relación con su localización anatómica del tejido adiposo blanco bajo tratamiento con oleoil estrona en ratas macho con sobrepeso

La oleoil estrona (OE) disminuye la ingesta, mientras que mantiene la homeostasis de la glucosa y el gasto energético a expensas de la grasa corporal. Los diferentes depósitos de tejido adiposo blanco se comportan de forma diferente bajo condiciones de ayuno y alimentación.

El objetivo de este trabajo es comprender el mecanismo de la pérdida masiva de lípidos en el tejido adiposo blanco provocado por el tratamiento de OE.

Para ello se utilizaron ratas macho con sobrepeso. Las ratas recibieron una dosis oral de OE (10 nmol/g) durante 10 días y se compararon con un grupo control y un grupo *pair fed*. Se extrajeron las masas de tejido adiposo mesentérico, retroperitoneal, epidídimal y subcutáneo inguinal para determinar su peso y contenido de ADN, ARN, así como la expresión de los genes que codifican para enzimas lipogénicos, transportadores de ácidos grasos y proteínas lipasa.

Los pesos de las distintas localizaciones del tejido adiposo blanco disminuyeron en las ratas OE y ratas *pair fed*, en paralelo con una limitada pérdida de células. Los patrones de expresión de los genes en la mayoría de las localizaciones del tejido adiposo blanco fueron similares para los grupos OE y *pair fed*, lo que sugiere la existencia de un mecanismo común de movilización de la grasa, aunque en el tejido adiposo mesentérico el grupo *pair fed* aumentó la expresión de genes que codifican enzimas lipogénicas y transportadores de ácidos grasos. Por su parte el grupo OE inhibió las expresiones de los genes que codifican enzimas lipogénicas más profundamente que el grupo *pair fed.*

En conclusión, las diferentes localizaciones de tejido adiposo blanco muestran diferentes patrones de expresión, como respuesta a una especialización en el almacenamiento de la grasa, por lo que el análisis de una sola localización no puede ser extrapolado al conjunto del tejido adiposo blanco. Las diferencias entre el depósito mesentérico y el resto de localizaciones sugieren que el término "grasa visceral " debería reservarse sólo en la rata para este sitio, y no aplicarse a otros depósitos de grasa abdominal (epididimal, retroperitoneal).

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ORIGINAL CONTRIBUTION

Site-related white adipose tissue lipid-handling response to oleoyl-estrone treatment in overweight male rats

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Abstract
Background Oleoyl-estrone (OE) decreases energy intake while maintaining glucose homeostasis, and energy expenditure at the expense of body fat. White adipose tissue (WAT) depots behave differently under starvation, postprandial state and pharmacologically induced lipolysis.
Aim of the study To understand the mechanism of massive lipid loss from WAT elicited by OE treatment.
Methods We used overweight male rats. Rats receiving OE (10 nmol/g) gavages were compared with controls and a pair-fed group. Whole fat pads from the mesenteric, retroperitoneal, epididymal and inguinal subcutaneous sites were excised and analyzed for lipid, DNA, mRNA and the expression of lipogenic, fatty acid transporters and lipase genes.
Results In OE and pair-fed rats, WAT weights decreased, with the limited loss of cells. Patterns of gene expression in most WAT sites were similar for OE and PF, suggesting a shared mechanism of fat mobilization, but in mesenteric WAT, PF increased lipogenic and fatty acid transporter gene expressions. However, OE inhibited lipogenic expressions more deeply than PF.
Conclusions White adipose tissue sites showed different expression patterns, hinting at relatively specialized functions in fat storage; thus, single site analyses cannot be extrapolated to whole WAT. Differences between mesenteric and the other sites suggest that 'visceral fat' should be reserved for this site only, and not applied to other abdominal fat depots (epididymal, retroperitoneal).

Keywords White adipose tissue · Obesity · Oleoyl-estrone · Visceral fat · Lipogenesis

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Introduction

Obesity, a key constituent of the metabolic syndrome, is a direct correlate of the mass and distribution of white adipose tissue (WAT), and obesity treatments are focused on the elimination of these fat depots. WAT stores most of the body triacylglycerol reserves, and this storage function is yet considered to be its main function despite growing awareness of its many additional functions as stem cell repository [27], endocrine [36], paracrine [17], and immune/defense [22] site. WAT also influences, through paracrine secretion, the response of neighboring vessels and organs [15], and acts as insulating, filling, protecting or spacing material. Its site-dispersed nature and the multiple functions carried out by WAT agree with a logical specialization of different sites in functions additional to

energy storage, which translates into a variable structuring of cell types, sizes and expression patterns. The existence of metabolically differentiated 'visceral' and 'subcutaneous' fat depots has been repeatedly associated with different forms and intensities of pathological situations such as the metabolic syndrome [6] and, consequently, there is a higher cardiovascular risk for increased visceral versus subcutaneous fat carriers [25]. However, this gross distinction is often insufficient, since the term 'visceral' is imprecise and comprises a variety of fat pads with different and distinct anatomical locations inside the visceral cavity, with different irrigation schemes that largely determine their different physiological function.

There is a number of studies on the composition and metabolic orientation of the WAT at different sites of the mammal [5, 21], especially oriented to their different ability to express or release cytokines [30, 44], other hormones [12] or their potential response to metabolic regulation agents [31, 45]. The direct analysis of their handling of fat has been sparsely analyzed in relation to site distribution.

Oleoyl-estrone is a powerful slimming agent [41] that induces an energy imbalance by decreasing food intake and maintaining energy expenditure [40]. The action of OE results in the maintenance of body protein [13, 41] and glycemia as well as liver glycogen [38]. However, muscle utilization of lipid in obese rats is enhanced in parallel to the normalization of circulating lipids and the wasting of overall body fat [4]. The administration of OE induces the fast loss of body fat with little change on other metabolic parameters (e.g. protein turnover/nitrogen balance, glycemia) [13, 40, 41].

A key objective of this study was to determine how OE exerts this marked reduction in adipose tissue lipid. However, in order to differentiate the effects of OE from those elicited by reduced food intake, a group of animals pair-fed with respect to those receiving OE was included. This study allowed us to establish basal and OE-induced differences in the main agents regulating triacylglycerol metabolism through gene expression.

An additional objective of the study was to determine whether OE affected differently the main WAT sites and the eventual attribution of these effects to its hormonal signaling [1] other than those simply derived from limited access to food.

The overweight (and otherwise normal) rat model [10] allows for a fair anatomical distinction of a number of WAT sites, yielding sufficient material for analysis and adequate 'slimming times' for investigative consistency. Thus, we used medium-sized overweight male rats to ascertain whether the loss of fat caused similar decreases in the most commonly studied WAT sites, and, especially, whether the abdominal (i.e. 'visceral') adipose tissues behaved in a similar way as to made any of them representative of WAT metabolic changes as assumed in many studies that distinguish only 'visceral' and 'subcutaneous' WAT.

Methods

Animals and sample preparation

Adult male Wistar rats were made overweight by a limited period of cafeteria diet feeding, as previously described [10]. The rats, initially weighing 355 ± 5 g, were kept under standard conditions of housing and feeding [10]. Three groups of eight rats each were randomly selected: controls, OE and 'pair-fed' (PF). All animals received every day an oral gavage of 0.2 mL of sunflower oil (7 kJ), which was supplemented in the OE group with 10 nmol/g oleoyl-estrone (OED, Barcelona, Spain). The controls and OE group had free access to pellet food (maintenance chow, Panlab, Barcelona, Spain), and the PF rats were allowed to eat only every day the mean food consumption on the matching day of the OE group; all rats had water available ad libitum. On day 10, the rats were killed by decapitation. The following WAT sites were isolated and completely dissected: intestine-related mesenteric WAT [MAT], perigonadal (epididymal) [EAT], retroperitoneal cordons [RPAT] and the subcutaneous inguinal [SCAT] fat pads. The samples were blotted and carefully cleaned of extraneous material (epididymis, pancreas, dermis), weighed, frozen in liquid nitrogen and kept at −80° C.

The animals were kept, handled and killed following the specific procedures approved by the University of Barcelona Animal Welfare and Ethics Committee, in full conformity with the norms and proceedings set forth by the European Union and the Governments of Spain and Catalonia.

Nucleic acid analyses, cellularity

Tissue samples were used for the estimation of total DNA, using a standard fluorimetric method with 3,5-diaminobenzoic acid (Sigma, St Louis MO, USA) and bovine DNA (Sigma) as standard [46]. Tissue DNA content allowed the calculation of the number of cells per gram of tissue and in the whole tissues sampled, based on the assumption that the DNA content per cell is constant in mammals; here, we used the genomic DNA size data [26] for somatic rat cells (5.6 pg/cell). Mean cell mass was estimated from the number of cells and the weight of the organ.

Total tissue RNA was extracted using the Tripure reagent (Roche Applied Science, Indianapolis, IN, USA), and were quantified in a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Tissue total mRNA was determined using the poly-(A) mRNA detection system kit (Promega).

Semiquantitative RT-PCR analysis of protein expression

RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) and oligo-dT primers.

Real-time PCR (RT-PCR) amplification was carried out using 0.010 mL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to OD 0.500 for all runs.

We have grouped the studied genes in three groups: (1) lipogenic (citrate: ATP lyase, acetyl-CoA carboxylase, fatty acid synthase and glycerol-3P acyl-transferase; (2) proteins binding/translocating or transporting fatty acids (fatty acid transporter protein 1, fatty acid translocase, carnitine-palmitoleoyl transferase, and fatty acid-binding protein 4); and (3) lipases (lipoprotein lipase, hormone-sensitive lipase, adiponutrin, and adipose triacylglycerol lipase). The list of primers used is given in Supplemental Table 1.

A semiquantitative approach for the ultimate estimation of the number of copies of each expressed gene mRNAs per tissue weight was used as previously described [34]. In any case, cyclophilin was used as charge control gene in all samples.

Statistical comparison between the groups was established using the unpaired Student's t test and one-way ANOVA tests, with a limit of significance of $P < 0.05$.

Results

Body weight and food intake

During the 10-day period studied, control rats gained 9.1 ± 1.4 g (i.e. 2.6% of initial body weight), while OE rats lost 34.9 ± 2.3 g (9.9%), and the pair-fed animals lost 29.8 ± 2.4 g (8.5%), the differences versus controls being significant for both OE and PF rats. Mean daily food consumption was 18.0 ± 0.2 g (i.e. 257 kJ) for controls, and 10.4 ± 0.3 g (187 kJ) for both OE and PF rats (i.e. a mean 58% of controls), the differences being statistically significant.

Nucleic acid content and cellularity

Table 1 shows the weight and cellularity of the four WAT sites studied; in the PF group, the loss of weight for the WAT sites was: RPAT 45%, EAT 41%, MAT 37% and SCAT 33%; OE rats lost slightly less: RPAT 45%, MAT 36%, and EAT or SCAT 30%. In all cases, the differences were significant versus controls but not between OE and PF groups.

The mean cell weight was higher for RPAT, followed by EAT and SCAT; MAT showing the lowest mean size, less than half the RPAT mean cell weight. However, and despite the loss of weight, the number of cells contained in WAT pads changed little, with only significant losses for PF EAT, and PF and OE RPAT. As a consequence, the mean cell size decreased significantly in all sites (PF and OE alike) versus controls, except for RPAT of OE-treated rats.

The proportion of mRNA with respect to total RNA showed little overall differences in controls (2.8–3.5%); the range for PF was 2.7–4.0, and 2.7–4.6% for OE rats. However, the ratio of total RNA to DNA increased in all sites in OE and PF rats, with the exception of unchanged ratio in the OE EAT and decreased ratios in SCAT samples.

Gene expression analysis

Table 2 presents the lipid-related gene expressions in the four WAT sites of control rats. Arbitrarily using SCAT as reference, values for lipogenic enzyme expressions tended to be higher in RPAT (and, to a lesser extent, MAT) when expressed per unit of tissue weight, while EAT tended to be lower. The pattern for fatty acid transporters (i.e. fatty acid transport protein 1, the translocase and carnitine-palmitoleoyl transferase genes) were similar in SCAT, RPAT and MAT, but lower in EAT. Fatty acid-binding protein gene showed the highest abundance of specific mRNA in RPAT and lowest in EAT. The four lipase genes studied showed different patterns, with closely similar expressions for adiponutrin gene in all sites, lowest lipoprotein lipase and hormone-sensitive lipase gene expressions in EAT, and highest adipose triacylglycerol lipase gene in RPAT.

The effects of OE or pair-feeding are shown in Fig. 1. The data are presented as percent values, versus controls, of the fmoles of each gene-specific mRNA found in the whole fat pad, irrespective of its actual size. Because both OE and PF treatments resulted in the shrinking of WAT masses, the relative size of the resulting fat pads is also presented as a percentage of controls' weight for comparisons.

The patterns of gene expression for OE and PF were fairly similar in EAT, and, to a lower extent in SCAT, the differences being more marked in RPAT and, especially, in MAT.

Oleoyl-estrone tended to decrease the tissue lipogenic enzyme gene expressions in MAT with respect to controls, but those in the whole tissue were somewhat higher than

Table 1 WAT sites weight and cellularity of overweight male rats treated for 10 days with oral OE or pair-fed compared with controls

Parameter (units)	Group	Mesenteric	Subcutaneous	Epididymal	Retroperitoneal
Tissue weight (g)	C	2.47 ± 0.16	2.34 ± 0.24	7.54 ± 0.65	9.07 ± 0.92
	PF	1.56 ± 0.22*	1.58 ± 0.05*	4.43 ± 0.47*	5.01 ± 0.67*
	OE	1.57 ± 0.14*	1.64 ± 0.12*	5.25 ± 0.19*	4.98 ± 0.51*
DNA content (μg/g)	C	618 ± 50^{A}	335 ± 33^{BC}	334 ± 12^{B}	255 ± 20^{C}
	PF	960 ± 85*^{A}	555 ± 35*^{B}	381 ± 4*^{C}	390 ± 49*^{D}
	OE	902 ± 50*^{A}	484 ± 42*^{B}	374 ± 22^{C}	288 ± 24^{D}
Total RNA content (μg/g)	C	137 ± 9^{A}	195 ± 8^{B}	52 ± 4^{C}	80 ± 8^{D}
	PF	319 ± 15^{A}	239 ± 5*^{B}	75 ± 4*°^{C}	138 ± 19*^{D}
	OE	274 ± 22^{A}	208 ± 15^{B}	61 ± 3^{C}	158 ± 15*^{D}
mRNA content (fg/cell)	C	41 ± 5^{AB}	93 ± 12^{C}	29 ± 3^{B}	63 ± 9^{AC}
	PF	77 ± 9*^{A}	65 ± 8^{A}	28 ± 2^{B}	58 ± 9^{A}
	OE	76 ± 6*^{A}	65 ± 4^{A}	26 ± 2^{B}	110 ± 10^{C}
Cell number in tissue ($\times 10^6$)	C	274 ± 10	150 ± 10	410 ± 29	371 ± 17
	PF	253 ± 16	157 ± 14	300 ± 30*	291 ± 25*
	OE	263 ± 10	139 ± 14	351 ± 26	247 ± 24*
Mean cell weight (ng)	C	8.1 ± 0.6^{A}	16.0 ± 1.0^{B}	15.3 ± 0.6^{B}	20.8 ± 1.6^{C}
	PF	5.5 ± 0.5*^{A}	9.2 ± 0.5*^{B}	13.2 ± 0.1*^{C}	14.2 ± 2.1*^{C}
	OE	5.5 ± 0.4*^{A}	10.3 ± 0.7*^{B}	13.1 ± 0.7*^{C}	16.9 ± 1.1^{D}
RNA/DNA ratio (×1,000)	C	210 ± 22^{A}	610 ± 49^{B}	158 ± 12^{A}	322 ± 27^{C}
	PF	289 ± 19*^{A}	442 ± 30*^{B}	196 ± 10*°^{C}	420 ± 34*^{B}
	OE	341 ± 20*^{A}	439 ± 29*^{B}	160 ± 5^{C}	563 ± 51*^{B}

Data with different superscript letters in the same row are significantly different ($P < 0.05$; one-way ANOVA and post hoc Bonferroni test)

The values are the mean ± SEM of eight different animals

C controls, *OE* OE-treated-group; *PF* pair-fed group

Statistical significance of the differences between groups: *$P < 0.05$ versus controls; °$P < 0.05$ of OE versus PF

those expected from the reduced fat pad mass. Fatty acid transport protein 1 gene expression increased versus controls. All other transporter genes showed similar expression values to those of controls, which means that their total tissue expression was higher than that expected from a smaller fat pad size. The hormone-sensitive lipase gene showed the same pattern, but lipoprotein lipase, with lower gene expression than controls, showed little change when the mass of adipose tissue was taken into account. OE markedly decreased adiponutrin gene expression but increased that of adipose triacylglycerol lipase. The pattern elicited by pair-feeding reversed that of OE for lipogenic enzyme genes, and showed a trend towards higher expression versus controls for fatty acid transporter, lipoprotein lipase and adipose triacyl-glycerol lipase gene expression.

In contrast with the apparently enhanced metabolic activity of MAT, SCAT showed a pattern fairly in line with its reduced tissue mass, with grossly decreased lipogenic gene expressions in OE rats, and the same pattern of MAT (but at a lower setting) for fatty acid transport and binding proteins, carnitine-palmitoleoyl transferase, and lipases' gene expressions; the main difference being the lack of changes versus controls of adipose triacylglycerol lipase gene expression. PF resulted in a closely similar pattern to that described for OE, with only marked differences for acetyl-CoA carboxylase gene, which increased its expression in PF versus OE. The pattern of EAT was practically coincident with that of SCAT for OE rats; the PF animals showed no differences with respect to OE in total specific mRNA organ contents (except for higher acetyl-CoA carboxylase gene expression), despite a more marked loss of tissue weight.

The RPAT again repeated a similar pattern for OE-driven changes in gene expression, with lower than control lipogenic, enhanced fatty acid transporting and lowered lipase gene expressions, except for the adipose triacylglycerol lipase gene. The main difference with respect to the other WAT sites may be the much increased expression of the carnitine-palmitoleoyl transferase gene. In PF rats, gene expression was lower than in OE in fatty acid binders/transporter genes.

Table 3 shows the expression of the SREBP1c gene in the four WAT sites. No changes were observed with respect to controls in MAT, but in all the other WAT sites, both OE and PF resulted in significantly decreased

Table 2 Gene expression of lipid-handling proteins (fmol of the corresponding mRNAs) in the four WAT locations studied of control rats

Gene	Symbol	Units	Mesenteric	Subcutaneous	Epididymal	Retroperitoneal
ATP citrate lyase	Acly	fmol/g	0.58 ± 0.13^{AB}	0.47 ± 0.06^{AB}	0.39 ± 0.02^{A}	0.76 ± 0.16^{B}
		fmol	*1.42 ± 0.34*	*1.01 ± 0.14*	*3.47 ± 0.44*	*8.00 ± 2.63*
Acetyl-coa carboxylase α	Acaca	fmol/g	0.33 ± 0.05^{A}	0.35 ± 0.04^{A}	0.29 ± 0.02^{A}	0.43 ± 0.08^{A}
		fmol	*0.74 ± 0.09*	*0.89 ± 0.13*	*2.44 ± 0.42*	*3.79 ± 0.55*
Fatty acid synthase	Fasn	fmol/g	7.4 ± 1.3^{A}	4.3 ± 0.7^{B}	2.6 ± 0.4^{B}	7.3 ± 1.2^{A}
		fmol	*15.8 ± 2.8*	*10.6 ± 2.0*	*20.2 ± 3.5*	*63.3 ± 7.9*
Glycerol-3-phosphate acyltransferase, mit.	Gpam	fmol/g	0.40 ± 0.05^{A}	0.25 ± 0.04^{B}	0.30 ± 0.02^{AB}	0.64 ± 0.08^{C}
		fmol	*0.94 ± 0.12*	*0.54 ± 0.09*	*2.52 ± 0.32*	*6.51 ± 1.16*
Fatty acid transport protein 1	Fatp1	fmol/g	0.40 ± 0.05^{A}	0.37 ± 0.03^{A}	0.19 ± 0.03^{B}	0.35 ± 0.04^{A}
		fmol	*0.95 ± 0.13*	*0.82 ± 0.09*	*1.55 ± 0.30*	*3.57 ± 0.73*
Fatty acid translocase	Cd36	fmol/g	7.3 ± 0.9^{A}	13.0 ± 1.5^{B}	5.4 ± 0.6^{A}	11.9 ± 1.6^{B}
		fmol	*17.1 ± 2.2*	*32.8 ± 5.7*	*42.2 ± 6.6*	*105 ± 11*
Fatty acid-binding protein 4	Fabp4	fmol/g	118 ± 21^{AB}	93 ± 9^{A}	80 ± 5^{A}	180 ± 24^{B}
		fmol	*270 ± 45*	*222 ± 30*	*632 ± 73*	*1,686 ± 210*
Carnitine-palmitoleoyl transferase 1β	Cpt1b	fmol/g	0.35 ± 0.06^{AB}	0.37 ± 0.04^{A}	0.20 ± 0.01^{B}	0.40 ± 0.07^{A}
		fmol	*0.81 ± 0.14*	*0.86 ± 0.14*	*1.70 ± 0.27*	*3.43 ± 0.33*
Lipoprotein lipase	Lpl	fmol/g	16.9 ± 1.7^{A}	21.8 ± 2.7^{A}	11.3 ± 1.7^{B}	22.2 ± 3.4^{A}
		fmol	*35.9 ± 5.2*	*52.8 ± 8.0*	*84.8 ± 11.3*	*232 ± 26*
Lipase, hormone-sensitive	Lipe	fmol/g	6.1 ± 1.1^{AB}	6.2 ± 0.9^{AB}	4.6 ± 0.5^{A}	6.9 ± 0.9^{B}
		fmol	*13.4 ± 1.7*	*15.6 ± 2.5*	*36.4 ± 6.2*	*68.3 ± 10.9*
Adiponutrin	Adpn	fmol/g	1.20 ± 0.29^{A}	0.70 ± 0.11^{A}	0.56 ± 0.08^{A}	0.93 ± 0.18^{A}
		fmol	*2.73 ± 0.41*	*1.65 ± 0.25*	*4.50 ± 0.88*	*8.20 ± 1.19*
Adipose triacylglycerol lipase	ATGL	fmol/g	12.6 ± 1.5^{AB}	10.5 ± 1.3^{A}	12.6 ± 1.5^{AB}	16.7 ± 2.5^{B}
		fmol	*29.6 ± 4.1*	*23.9 ± 3.6*	*95.7 ± 13.5*	*157 ± 24*

The values are the mean ± SEM of eight different animals

Data with different superscript letters in the same row are significantly different ($P < 0.05$; one-way anova and post hoc Bonferroni test)

expression of the gene when referred to the whole fat pad mass.

Discussion

The present data show that different WAT sites actually show different metabolic patterns even for a function that is assumed constant and primary for this tissue: the management of fat reserves. The decreased cell size but unchanged number of cells (except in RPAT), suggest a limited role of apoptosis after 10 days of OE treatment, in contrast with our previous findings of active loss of cells in the short term in young female rats [39]. This was reinforced by the analysis of the ratios of Bcl2/Bax expression in a number of WAT sites, which yielded similar values in all sites; nevertheless, they were lower (not statistically significant differences) in OE-treated rats [unpublished results].

Loss of body weight in PF and OE were similar, which agrees with our previous findings that lower food intake is largely responsible for the loss of weight [38]. However, the different regulation of metabolic pathways [35] strongly suggests that OE-induced changes are largely independent of food intake [34, 38]. The powerful slimming effects of OE have been attested in rats [13] and humans [3], always inducing a marked loss of appetite and body fat, with no additional secondary or lasting effects [1].

The marked differences in mean cell size can be attributed in part to actually different adipocyte sizes [9, 16], but also to a different percentage of smaller non-lipid carrier cells (stem, endothelial, macrophages, etc.) [2], which reduce the mean cell weight when computed from tissue DNA content. In any case, the ample differences in mean size suggest that probably adipocyte size is also different [23], with larger cells showing slower metabolic responses [16, 43] and thus a higher functional inertia to change. Thus, it can be expected that EAT and RPAT will show lower protein expression per unit of weight than small-cell WATs such as MAT and SCAT; since in these sites mRNA content per gram of tissue was, respectively, 4.6 and 6.0 versus 1.6 and 2.6 μg/g of EAT and RPAT. These differences contrast with the fairly uniform values of

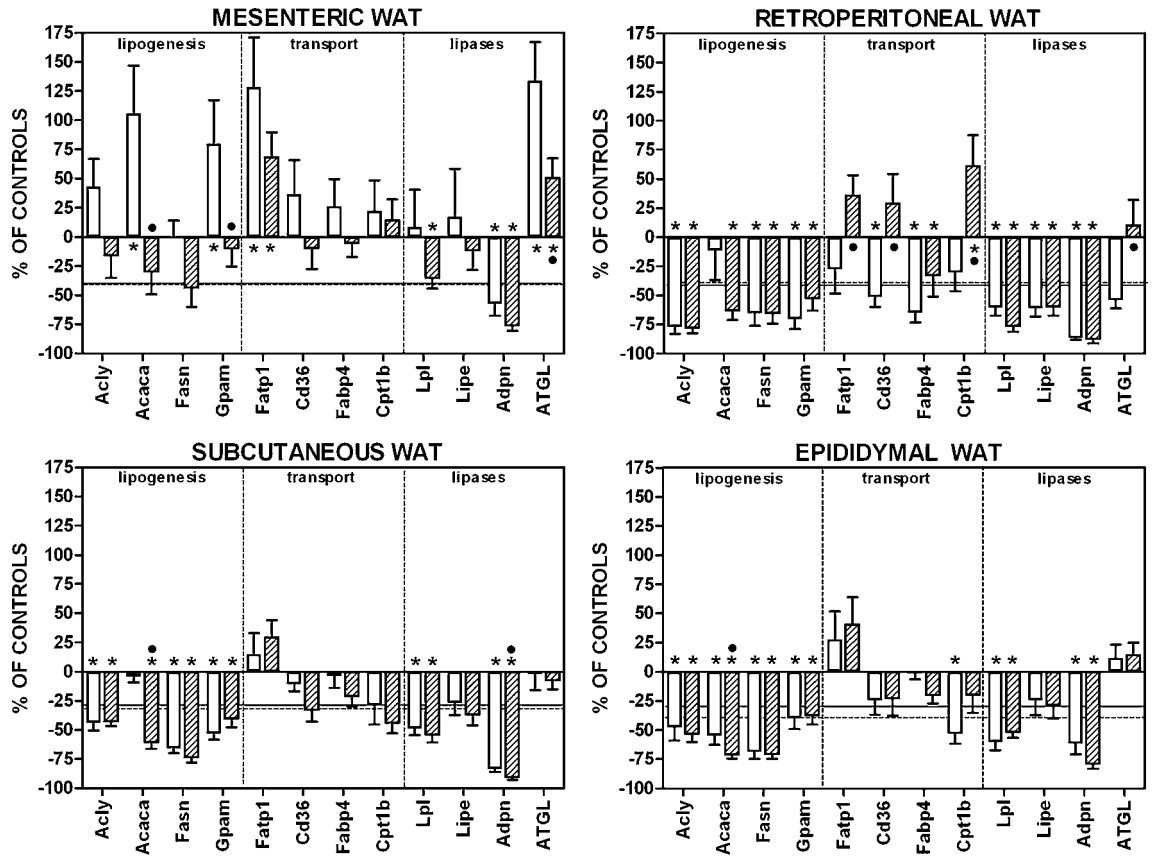

Fig. 1 Patterns of gene expression of lipid-handling proteins in four WAT sites of OE- and pair-fed-treated rats. The values are the mean ± SEM of eight different animals, and are expressed as percentages, versus the control values of the total amount (fmol) of the given transcript in the whole fat pad. *White columns* pair-fed; *dashed columns*: OE. The *horizontal line* at 0 represents the 100% value of controls' transcript content. The *fine horizontal dashed line* corresponds to the mean percentage of the specific site weight (g) of PF rats versus the corresponding controls; the *continuous line* corresponds to the percentage of OE vs. controls' weight. An *asterisk* indicates a significant ($P < 0.05$) difference of the data with respect to controls, and a *black dot* represents a significant ($P < 0.05$) difference between matching OE and PF groups (one-way anova and post hoc Bonferroni test)

Table 3 Gene expression (fmol of the corresponding mRNAs) of SREBP1c in the four WAT locations studied of control rats

Gene	Symbol	Group	Mesenteric	Subcutaneous	Epididymal	Retroperitoneal
Sterol regulatory element binding factor 1	Srebf1	C	2.22 ± 0.33	3.55 ± 0.68	6.83 ± 1.22	9.86 ± 1.76
		PF	3.23 ± 0.68	1.92 ± 0.27*	3.11 ± 0.54*	2.38 ± 0.76*
		OE	3.00 ± 0.38	1.86 ± 0.28*	3.44 ± 0.46*	4.57 ± 0.79*

The values are the mean ± SEM of eight different animals, and are expressed as fmol of SREBP1c mRNA per gram of tissue

Statistical significance of the differences between groups: *$P < 0.05$ versus controls (Student's t test)

There were no statistical differences between PF and OE groups

C controls, *OE* OE-treated-group, *PF* pair-fed group

the gene expressions per unit of tissue weight in the four sites, which hints at a special preservation of the lipid metabolism functions studied under severe energy drainage: the 'share' of mRNA resulting from these genes' expression constituted a larger share of total mRNA than in cells under normal conditions and with higher total mRNA.

The fat mobilizing effect of OE has been found not to depend solely on decreased food intake [32]. By design, pair-feeding equals the decreases in food intake of the OE-treated animals, but OE also induces a severe drain in body reserves [29] imposed by the maintenance of energy expenditure [40, 41]. Despite similar effect on WAT lipid mobilization found here, OE effects on circulating glucose, fatty acids and other energy parameters are much milder than those elicited by food deprivation, including pair-feeding [38], which attests to a different handling of the

reserves by other tissues and a lower degree of metabolic stress by OE under identical energy availability decreases [38].

Oleoyl-estrone induced a marked decrease in lipogenesis in all WAT sites with the exception of MAT; PF resulted in a similar pattern, but a key lipogenic enzyme gene expression (acetyl-CoA carboxylase) did not change, in agreement with previous reports [20]; thus, consistently showing higher values than in OE, hinting at different mechanisms (or intensity) of inhibition of lipogenic gene expression in either group.

SREBP1c is a powerful lipogenic enhancer [20, 43], activated by energy availability, insulin [11], and a number of lipogenic signals [42]. We have found recently that SREBP1c upregulates liver lipogenesis under OE treatment [33], a probable consequence of the maintenance of glycemia and glucose availability in OE rats [8, 13] in contrast with semistarved or pair-fed animals [38]. The fall in SREBP1c expression, observed here for SCAT, EAT and RPAT, fully agrees with their markedly decreased lipogenic enzyme gene expressions. In MAT, the lack of overall OE-induced changes in lipogenic gene expressions was paralleled by a similar lack of changes in the expression of the SREBP1c gene, but the actual increase of some lipogenic enzyme gene expressions induced by PF was not correlated with changes in the expression of SREBP1c. This difference suggests that factors additional to SREBP1c may influence the observed effects of PF on lipogenic gene expression; however, the more consistent effects of OE were closely correlated with this regulator, which further supports its probable implication on the mechanism of action of OE [33].

Lipoprotein lipase gene expression was diminished in OE rats, in agreement with the OE-elicited decrease in its activity observed in Zucker obese rats [4], and is justified by the direction of fatty acid flow from WAT to other tissues, as in starvation [24]. The marked decrease in the expression of adiponutrin in OE and PF was again correlated with the assumed decrease in lipogenesis, because of the association of this lipase with the lipogenic process [19].

Hormone-sensitive lipase plays a key role in the mobilization of the WAT fat stores [37]. The expression of the hormone-sensitive lipase gene was lower than in controls in all sites in OE and PF rats, but the values were similar to those of controls when expressed per gram of tissue. Thus, this gene was not affected by OE, which agrees with its regulation via kinases and not through transcription [14]; this stability helps us to assess by contrast the deep changes experienced in the expression of other genes. Its lack of expression changes with respect to tissue weight, together with the relative increases observed in all sites in the expression of the adipose triacylglycerol lipase gene, may help explain that, overall, lipolytic gene expressions relatively surpassed the lipogenic ones (when compared with controls) in OE and PF, which is in agreement with the wasting of WAT, the predominance of lipolysis and the constant flow of fatty acids from WAT in rats under OE treatment. This is compounded by the eventual intervention of the adrenergic-driven cAMP cascade regulation of hormone-sensitive lipase (expression unchanged), since OE increases cAMP availability [7]. Increased expression of the carnitine-palmitoleoyl transferase gene in the RPAT of OE rats also suggests that at least part of the lipid lost may be used for energy maintenance in the tissue itself.

A large part of the process of reduction of fat stores elicited by OE is comparable to that induced by pair-feeding, suggesting that the main factor driving the loss of WAT fat is the response to low energy availability, probably mediated by changes in catecholamine and leptin signaling. This similarity of mechanisms hints at a common regulation of lipolysis, that is not coincident with that of lipogenesis, and this difference may mark the dissimilitude between the exhaustion of WAT energy under normoglycemia characteristic of OE [13] with the mobilization parallel to hypoglycemia typical of limited feeding [38].

The MAT was, again, peculiar in its pattern of expression changes induced by OE and PF. Despite an overall shrinking of body WAT (and that of its triacylglycerol stores) [11, 28], with highly increased lipase expressions and even higher fatty acid handling protein genes', lipogenesis was not inhibited by OE, in contrast with the other WAT sites. The picture was the same for PF (but with higher gene expression values throughout), suggesting a common origin and regulation. This difference may stem from the privileged position of MAT, the only truly anatomically visceral WAT, receiving substrates from the intestine and voiding its metabolic proceeds through the portal vein directly to the liver. Probably the assumed high-lipogenic activity and active handling and transport of lipids may be related to the synthesis of nascent lipoproteins and the integration of fatty acids derived from intestinal absorption into these lipoproteins [18]. The relatively low-lipid content of MAT [28], small mean cell size (i.e. suggesting the presence of a large number of non-adipocyte cells, and smaller, and thus more active, adipocytes), and a marked enhancement in the expression of intracellular fatty acid-binding and compartment shifting protein genes, suggests a rapid turnover of lipids that contrasts with the more or less long-term storage and paucity of metabolism of the other WAT sites studied.

In conclusion, the data presented suggest that OE effects on WAT may be more a consequence of inhibited lipogenesis than enhanced lipolysis, an effect fully comparable to that produced by pair-feeding. There is a considerable coincidence on the patterns of expression of lipid-handling

protein genes of WAT in different sites for OE or PF rats, supporting largely shared mechanisms of fat mobilization. The four sites tested show different lipid-handling gene expression patterns, hinting at different functions / regulation sensitivity that apply also to the tissue-defining fat storage role, in addition to their known diversity in other signaling and hormonal functions [2, 17, 36]. Thus, the generalization of conclusions from the analysis of WAT samples from a single site cannot be extended to the whole body WAT because of the differences are present even in such a common and basic function of WAT as is lipid energy storage. Our results are in line with the previous studies on WAT site-related differences in gene expression and the response to pharmacological or physiological challenges [28, 30, 31, 40].

A fair conclusion derived from this study is the marked metabolic differences between MAT and the other WAT sites studied. Truly, MAT is the best representative example of 'visceral' fat depot, different from all others because of its direct relationship with the intestinal circulation and portal venous drainage. However, the differences between SCAT and RPAT or EAT were considerably smaller, and could not sustain the common practice of considering 'visceral' all intra-abdominal depots (such as RPAT or EAT) as counterposed to peripheral depots such as SCAT. The data presented here should be taken as a further contribution to the strict differentiation of WAT function depending on anatomy, blood supply, and drainage and not only on general visceral or subcutaneous location.

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3.2.3 Modulación de la expresión génica del metabolismo energético hepático tras la administración de oleoil-estrona a ratas con sobrepeso

En este trabajo se intentó caracterizar la movilización masiva de lípidos inducida por la oleoil-estrona (OE), y diferenciar sus efectos independientes de la disminución de la ingesta de alimentos.

Para ello se utilizaron ratas macho tratadas con OE (10 nmol/g por día) por vía oral durante 10 días en comparación con un grupo control y un grupo *pair fed*. Se determinaron los parámetros plasmáticos y los lípidos totales de hígado, así como el ADN y ARN total. Se analizó la expresión de genes que codifican enzimas y factores de regulación que participan en el metabolismo energético del hígado mediante PCR en tiempo real.

La mayoría de las expresiones génicas fueron similares entre el grupo OE y el grupo control, mientras que se observaron marcadas diferencias entre los grupos OE y *pair fed*, de acuerdo con el postulado de que los efectos de la OE no son solamente consecuencia de una menor ingesta. El hígado de las ratas tratadas con OE, a pesar de la disminución de la ingesta, mantuvo en gran medida su capacidad de sintetizar lípidos a partir de la glucosa, lo que parece haberse logrado en parte mediante una mayor disponibilidad de lípidos plasmáticos procedentes de la movilización de las reservas grasas.

En conclusión, los resultados sugieren que el efecto de la OE sobre el metabolismo energético del hígado puedo ser, al menos en parte, mediada a través del incremento de la sensibilidad a la insulina y del aumento de la expresión del factor regulador de la lipogénesis SREBP-1c, de acuerdo con el mantenimiento de la lipogénesis y de la disponibilidad de la glucosa.

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Gene expression modulation of liver energy metabolism by oleoyl-estrone in overweight rats

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Synopsis

We intended to determine how the liver copes with the massive handling of lipids induced by OE (oleoyl-estrone), as well as to characterize and differentiate the actual OE effects from those that may be only the consequence of decreased food intake. Thus we used male rats treated with oral OE (10 nmol/g per day) compared with a vehicle only PF (pair-fed) group and controls fed *ad libitum* (vehicle only). Plasma parameters, and total liver lipids, glycogen, DNA and total mRNA were measured. RNA was extracted and used for real-time PCR analysis of the gene expression of enzymes and regulatory factors of liver energy metabolism. Most hepatic proteins showed similar gene expressions in OE and controls, but the differences widened between OE and PF rats, showing that OE effects could not be merely attributed to a lower energy intake. The liver of OE-treated rats largely maintained its ability to mobilize glucose for the synthesis of fats; this was achieved in part by a peculiar combination of regulative modifications that facilitate both fatty acid disposal and restrained glucose utilization under conditions of limited food supply but ample availability of internal energy stores. In conclusion, the results presented suggest that the effect of OE on liver metabolism may be (at least in part) mediated through an insulin-sensitivity-dependent modulation of the expression of SREBP-1c (sterol-regulatory-element-binding protein-1c), resulting in the unique combined effect of mildly increased (or maintained) glucose disposal but also limited enhancement of lipogenesis.

Key words: energy metabolism, lipogenesis, liver, oleoyl-estrone, pair-feeding, sterol-regulatory-element-binding protein-1c (SREBP-1c)

INTRODUCTION

OE (oleoyl-estrone) is a signal of adipose tissue carried in the plasma by lipoproteins [1] that elicits marked (and dose-dependent) decreases in body fat [2,3], in part through actions on WAT (white adipose tissue), such as the massive loss of lipid, decreased cell size and increased apoptosis [4,5]. Other actions are central, such as the decrease in food intake [2] and the maintenance of energy expenditure [6] in spite of massive energy losses [3,7], nevertheless sparing body protein [3].

Under conditions of low energy intake (resulting in decreased glucose availability and enhanced lipolysis), OE maintains glycaemia, and liver glycogen stores [8,9], but lowers insulinaemia, and overall glucose utilization [10]. These combined effects suggest that no additional liver glucose output is needed, since the flow of food-derived glucose is enough to maintain glucose homoeostasis.

The fatty acids freed by WAT lipolysis are largely used by peripheral tissues as the main energy substrate [3,4,7]. The OE-induced shift in the distribution of tissue lipoprotein lipase activity favours the use of fats by the muscle, and decreases its liver and WAT storage in obese rats [11]. This situation contrasts sharply with the metabolic environment caused by a simple decrease in food intake, when lowered glucose levels are largely maintained by increased liver glucose output [12]. Low glucose also results in increased lipolysis and synthesis of ketone bodies [13]. These effects are the consequence energy saving [14], resulting in decreased thermogenesis [15] and overall energy expenditure [16].

The regulation of hepatic metabolism plays a key role in the overall energy balance, since the liver is the major site of carbohydrate metabolism and lipogenesis. The synthesis of most glycolytic and lipogenic enzymes is eventually regulated by dietary status and nutrient (e.g. glucose) levels [17]. The latter response takes place largely at the transcriptional level, modulating the

Abbreviations used: ChREBP, carbohydrate-responsive element-binding protein; LXR, liver X receptor; OE, oleoyl-estrone; PEPCK, phosphoenolpyruvate carboxykinase; PPARα, peroxisome-proliferator-activated receptor α; WAT, white adipose tissue.

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expression of genes encoding glycolytic and lipogenic enzymes [18].

Since the liver is a key organ for body energy management, we centred our study on the analysis of the gene expressions of key enzymes and regulatory factors in response to the challenges of OE treatment or relative food deprivation (pair-feeding). We intended to better understand the easiness with which OE facilitates the use of internal energy stores and to obtain information on the regulatory mechanisms modulated by OE in liver.

MATERIALS AND METHODS

Animals and sample preparation

Adult male Wistar rats, kept under standard conditions of housing and feeding, were made overweight by a limited period of cafeteria diet feeding, as previously described [19]. The rats weighed 355 ± 5 g when the experiment began; three groups, of eight rats each, were randomly selected: controls, OE and PF (pair-fed). All animals received every day an oral gavage of 0.2 ml of sunflower oil (7 kJ), which was supplemented in the OE group with 10 nmol/g OE (OED, Barcelona, Spain). Controls and OE had free access to pellet food (maintenance chow; Panlab, Barcelona, Spain); and the PF were allowed to eat every day only the amount of food consumed by the OE group; all rats had water available *ad libitum*. PF rats completely ate the food allotted each day. On day 10, the rats were killed by decapitation and blood was recovered in plastic beakers; serum was separated and frozen for later analysis. The liver was rapidly excised, weighed, sampled, frozen and kept at –80°C until processed.

The animals were kept, handled and killed following the procedures approved by the University of Barcelona Animal Welfare and Ethics Committee, in full agreement with the norms and procedures set forth by the European Union and the Governments of Catalonia and Spain.

Analytical procedures

Serum was used to measure glucose (Trinder glucose kit; Sigma, St. Louis, MO, U.S.A.), triacylglycerols (kit 11528; Biosystems, Barcelona, Spain), non-esterified fatty acids (kit NEFA-C; Wako, Richmond, VA, U.S.A.) and insulin (rat insulin RIA kit; Linco, St. Louis, MO, U.S.A.). Liver samples were used for glycogen determination as glycosyl residues [20]. Lipid content of liver samples were extracted with trichloromethane/methanol (2:1), dried and weighed [21].

Total DNA liver was measured using a standard fluorimetric method with 3,5-diaminobenzoic acid (Sigma) and bovine DNA (Sigma) as the standard [22]. Tissue DNA content allowed the calculation of the number of cells per g of tissue and in the whole liver, based on the assumption that the DNA content per cell is constant in mammals; here we used the genomic DNA size data [23] for somatic rat cells (5.60 pg per cell). Mean cell volume was estimated from the number of cells and the volume of the organ, calculated using a liver density of 1.10 g/ml [24].

Real-time PCR expression analysis

Total tissue RNA was extracted using the Tripure reagent (Roche Applied Science, Indianapolis, IN, U.S.A.) and quantified with an ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A.). Tissue total mRNA was determined by using the poly-(A) mRNA detection system kit (Promega, Madison, WI, U.S.A.).

RNA samples were reverse transcribed using the MMLV (Moloney-murine-leukaemia virus) reverse transcriptase (Promega) and oligo-dT primers. Real-time PCR amplification was carried out using 10 μl amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems). The list of genes and primes used is given in Supplementary Table S1 (http://www.bioscirep.org/bsr/029/bsr0290000add.htm). A semi-quantitative approach for the ultimate estimation of the number of copies of each expressed gene mRNAs was used as previously described [25]. Cyclophilin was used as charge control gene in all samples.

Preparation of crude membrane fraction, nuclear extracts and Western-blot analysis

Liver samples were homogenized in a buffer containing 10 mM Tris/HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4 (sodium orthovanadate) and Complete™ protease inhibitor cocktail (Sigma). Nuclei were pelleted with a 10 min centrifugation (at 500 ***g***) at 4°C and washed once with the same buffer. The nuclear pellet was resuspended and kept for 30 min at 4°C in a hypertonic buffer: 10 mM Hepes (pH 7.4) containing 0.42 M NaCl, 1.5 mM $MgCl_2$, 2% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 1 mM sodium orthovanadate and Complete™ protease inhibitor cocktail (Sigma). A clear nuclear extract was obtained by 30 min centrifugation (100 000 ***g***) at 4°C. The supernatant from initial low-speed centrifugation was further spun at 100 000 ***g*** for 30 min at 4°C to obtain a pellet of the crude membrane fraction. The membrane pellet was resuspended in 10 mM Tris/HCl (pH 6.8) containing 10 mM NaCl, 1% SDS, 1 mM EDTA and 1 mM EGTA.

Proteins from the membrane fraction and nuclear extracts (50 μg) were separated by SDS/PAGE in a 7.5% gel and electrotransferred on to a PVDF membrane (Millipore, Billerica, MA, U.S.A.). Membranes were incubated with a mouse monoclonal antibody for SREBP-1c (sterol-regulatory-element-binding protein-1c) (2A4), lamin B1(2L-5) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and tubulin (DM1A) (Abcam, Cambridge, U.K.). Anti-mouse secondary antibody (IgG1–horseradish peroxidase human adsorbed) was also obtained from Santa Cruz Biotechnology. ECL® detection

Table 1 Plasma parameters of overweight rats treated for 10 days with oral OE compared with controls and a PF group
The results are the means ± S.E.M. for eight different animals. Differences between groups: *$P < 0.05$ compared with controls; †$P < 0.05$ between OE and PF groups.

Parameter	Units	Control	PF	OE-treated
Glucose	mM	8.01 ± 0.09	6.02 ± 0.13*	7.19 ± 0.15*†
Insulin	pM	574 ± 79	71 ± 10*	333 ± 72*†
Triacylglycerols	mM	1.58 ± 0.13	0.62 ± 0.05*	0.47 ± 0.06*
Non-esterified fatty acid	mM	0.32 ± 0.02	0.56 ± 0.03*	0.23 ± 0.02*†

Table 2 Liver nucleic acid content and cellularity of overweight rats treated for 10 days with oral OE compared with controls and a PF group
The results are the means ± S.E.M. for eight different animals. Differences between groups: *$P < 0.05$ compared with controls; †$P < 0.05$ between OE and PF groups.

Parameter	Units	Control	PF	OE-treated
Liver weight	g	11.3 ± 0.4	7.4 ± 0.2*	9.9 ± 0.5†
DNA content	mg/g	2.13 ± 0.07	3.31 ± 0.15*	2.21 ± 0.13†
	mg	24.8 ± 1.5	24.2 ± 1.0	21.5 ± 1.1
Number of cells	$\times 10^9$	4.44 ± 0.26	4.31 ± 0.18	3.84 ± 0.19
Mean cell size	pL	2.42 ± 0.11	1.55 ± 0.07*	2.37 ± 0.14†
RNA/DNA ratio		2.84 ± 0.19	2.01 ± 0.09*	3.00 ± 0.20†
RNA content	mg/g	5.59 ± 0.24	6.26 ± 0.15	6.49 ± 0.30*
	mg	63.6 ± 4.4	46.7 ± 0.5*	64.0 ± 3.9†
mRNA content	μg/g	253 ± 31	84 ± 12*	159 ± 12*†
	% of RNA	4.70 ± 0.63	1.31 ± 0.20*	2.51 ± 0.25*
	fg/cell	712 ± 97	156 ± 22*	411 ± 38*†

reagent (GE Healthcare, Amersham) analysis was performed and the resulting signal was scanned and quantified using the Total-Lab v2003.3 program (Non-Linear Dynamics, Newcastle, U.K.).

Statistical analysis

Statistical analysis was carried out using the Prism 4 program (GraphPad Software, San Diego, CA, U.S.A.). Differences between groups were established by using a one-way ANOVA. A Tukey's multiple comparison test was performed for the evaluation of significant differences between groups. Differences were assumed to be significant when $P < 0.05$.

RESULTS

Body weight and plasma parameters

After 10 days of treatment, the body weight of control rats increased by 2.6 ± 0.4 %; PF lost 8.5 ± 0.8 % and OE rats lost 9.9 ± 0.8 %. Mean food intake of controls was 18.0 ± 0.2 g/day and that of OE (and thus PF) was 10.4 ± 0.3 g, representing a mean 58 ± 2 % of the food (energy) ingested by controls.

Plasma parameters are shown in Table 1. In OE rats, plasma glucose and insulin were lower than in controls, but in the PF rats the levels were even lower. The decrease in triacylglycerols and the increase in non-esterified fatty acids observed in PF versus controls were consistent with food restriction, but the OE group showed a significant decrease in plasma non-esterified fatty acids with respect to controls and PF groups.

Liver cellularity and composition

Table 2 shows the liver weight, DNA and RNA contents of control, PF and OE-treated rats. Pair-feeding resulted in the loss of liver weight (down by approx. 35 %), but not of liver cells. Neither liver weight nor its cellularity was significantly affected by OE. As a consequence, the liver cells of PF rats were smaller than those of controls or OE. Total RNA content was, again, lower in PF compared with controls; with OE being not different from controls. However, OE-treated rats contained approx. 53 % of the mRNA controls; the figure for PF was even lower, in the range of 28 %. When correcting these values by the number of cells, we found that cells from OE-treated rats contained 58 % of the mRNA of controls, and PF animals only 22 %.

Table 3 shows the exhaustion of glycogen reserves in PF but its remaining constant in OE-treated rats. Total liver lipids tended to be lower in PF rats and remained constant in OE.

Gene expression of energy metabolism enzymes and regulating factors

Figure 1 shows the gene expression of enzymes involved in carbohydrate metabolism in the liver. Supplementary Table S2

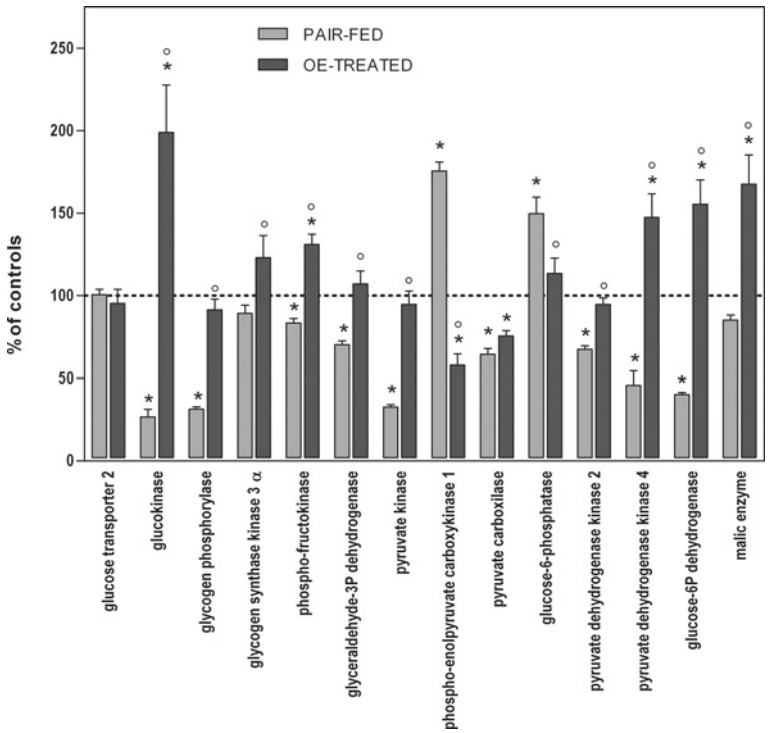

Figure 1 Liver gene expression of enzymes involved in carbohydrate metabolism of male overweight rats treated with oral OE and PF

Data are the means ± S.E.M. for eight different animals, and are expressed as percentages of control values of the total amount (fmol) of the given transcript in whole liver. Grey columns: PF; black columns: OE. The horizontal dashed line represents the 100% value of controls' transcript content. An asterisk indicates a significant ($P < 0.05$) difference with respect to controls, and an open circle represents a significant ($P < 0.05$) difference between OE and PF groups.

Table 3 Liver lipids and glycogen of overweight rats treated for 10 days with oral OE compared with controls and a PF group

The results are the means ± S.E.M. for eight different animals. Differences between groups: *$P < 0.05$ compared with controls; †$P < 0.05$ between OE and PF groups.

Parameter	Units	Control	PF	OE-treated
Total lipids	mg	508 ± 27	431 ± 16	514 ± 34
Glycogen	mg	467.5 ± 24.7	0.98 ± 0.29*	244.5 ± 22.6*†

(http://www.bioscirep.org/bsr/029/bsr0290000add.htm) shows, in absolute terms, the expressions of the genes presented in Figure 1.

Pair-feeding resulted in a generalized decrease in the gene expression of most enzymes: glucokinase, glycogen phosphorylase, phospho-fructokinase, glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, pyruvate carboxylase, pyruvate dehydrogenase kinases 2 and 4 and glucose-6-phosphate dehydrogenase. The only increases in gene expression were those of PEPCK (phosphoenolpyruvate carboxykinase) and glucose-6-phosphatase. The pattern shown by OE rats was much closer to controls and markedly different from PF. The only genes decreasing their expressions were PEPCK and pyruvate carboxylase. All other gene expressions were similar to those of controls except for actual increases in glucokinase, phospho-fructokinase, pyruvate dehydrogenase kinase 4, glucose-6-phosphate dehydrogenase and the malic enzyme.

The expressions of genes codifying enzymes of lipid metabolism are presented in Figure 2; Supplementary Table S3 (http://www.bioscirep.org/bsr/029/bsr0290000add.htm) shows these expressions in absolute values. Again, the PF group showed a generalized decrease in gene expression, affecting hepatic lipase, adiponutrin, citrate:ATP lyase, acetyl-CoA carboxylases 1 and 2, fatty acid synthase, steroyl-CoA desaturase 1 and glycerol-3-phosphate acyl-transferase. There were increases only in fatty acid transport protein 2, hormone-sensitive lipase and carnitine palmitoleoyl-transferase expressions. The pattern for OE rats was very different, with a marked increase in gene expression of fatty acid translocase, and a less marked (albeit significant) increase in fatty acid transport protein 2, hormone-sensitive lipase, carnitine palmitoleoyl-transferase, acyl-CoA dehydrogenase (long chain), steroyl-CoA desaturase, glycerol-3-phosphate acyl-transferase and long-chain acyl-CoA synthetase compared with controls. Q4

Figure 3 shows the expression of the genes for a number of energy metabolism regulating factors (the absolute values are given in Supplementary Table S4 at http://www.bioscirep.org/bsr/029/bsr0290000add.htm). Pair-feeding decreased the gene expression of most of the factors studied, in particular

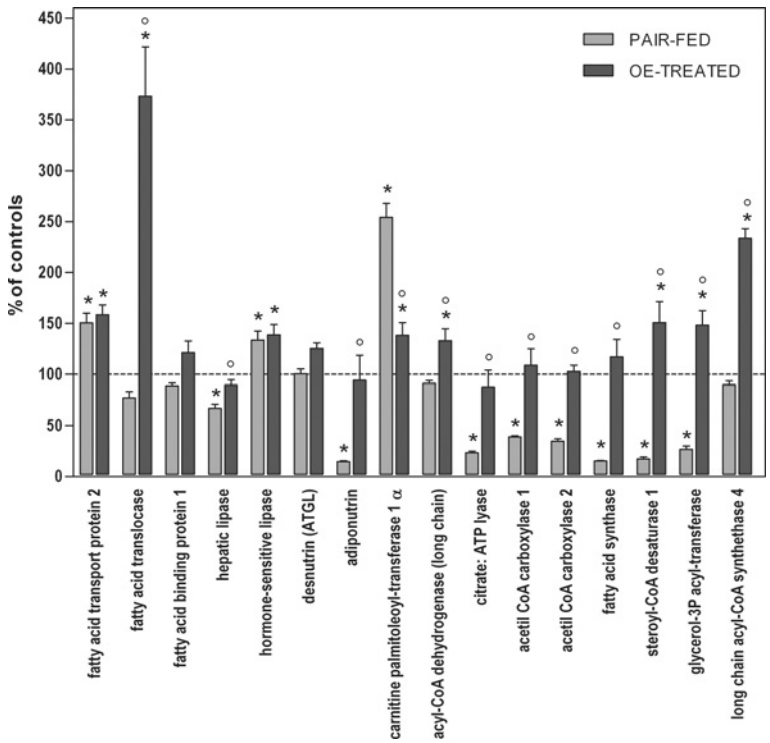

Figure 2 Liver gene expression of enzymes involved in lipid metabolism of male overweight rats treated with oral OE and PF

Results are the means ± S.E.M. for eight different animals, and are expressed as percentages of control values of the total amount (fmol) of the given transcript in whole liver. Grey columns: PF; black columns: OE. The horizontal dashed line represents the 100% value of controls' transcript content. An asterisk indicates a significant ($P < 0.05$) difference with respect to controls, and an open circle represents a significant ($P < 0.05$) difference between OE and PF groups.

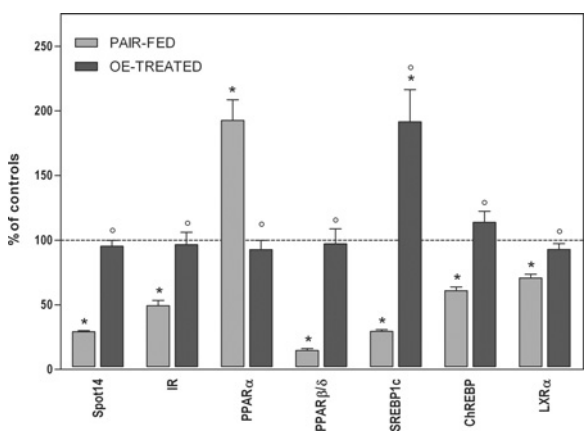

Figure 3 Liver gene expression of metabolic regulation indicators of male overweight rats treated with oral OE or PF

Results are the means ± S.E.M. for eight different animals, and are expressed as percentages of control values of the total amount (fmol) of the given transcript in whole liver. Grey columns: PF; black columns: OE. The horizontal dashed line represents the 100% value of controls' transcript content. An asterisk indicates a significant ($P < 0.05$) difference with respect to controls, and an open circle represents a significant ($P < 0.05$) difference between OE and PF groups.

glucose-related [ChREBP (carbohydrate-responsive element-binding protein) and LXR (liver X receptor)] and insulin-related (insulin receptor and SREBP-1c) regulatory genes. This pattern, along with an increase in PPARα (peroxisome-proliferator-activated receptor α) and a marked decrease in PPARβ/δ gene expression, was indicative of an adaptation to low levels of intake. OE treatment induced fewer changes with respect to controls, the only difference being a marked increase in the expression of SREBP-1c.

SREBP-1c protein levels

Figure 4 shows the protein levels of both precursor and nuclear SREBP-1c in membrane and nuclear extracts of liver. In PF rats, both forms decreased when compared with controls; however, in the OE group precursor and nuclear protein levels were increased compared with controls, in parallel with SREBP-1c gene expression.

DISCUSSION

Simple food restriction induces the loss of liver energy: i.e. glycogen, lipid and protein content [26], which result in smaller PF

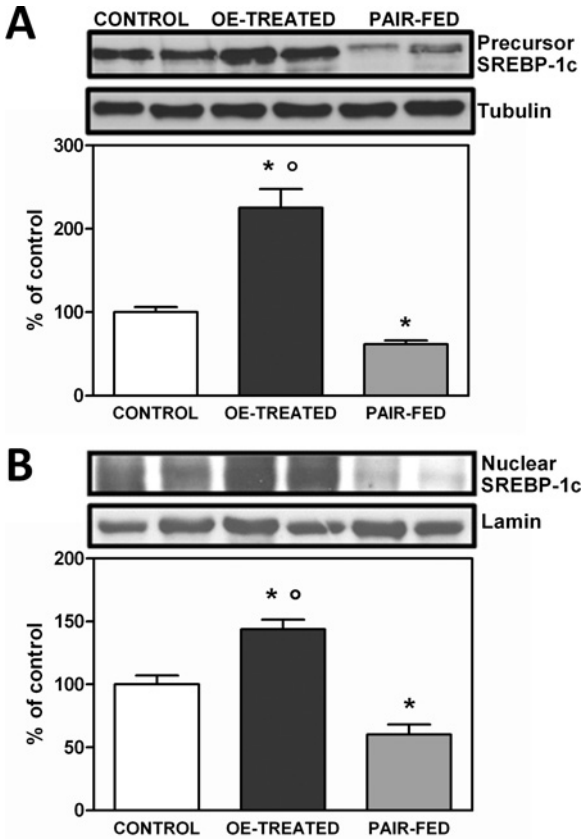

Figure 4 Characterization, by Western-blot analysis, of SREBP-1c forms in liver as consequence of food restriction and OE treatment

(A) Abundance of precursor SREBP-1c. (B) Abundance of nuclear SREBP-1c. An asterisk indicates a significant ($P < 0.05$) difference with respect to controls, and an open circle represents a significant ($P < 0.05$) difference between OE and PF groups.

cell size but not in the loss of cells. PF rat cells lost a large share of their total RNA, with mRNA being only a fraction of its proportion in controls. OE treatment also reduced liver mRNA levels, which suggests a generalized decrease in liver protein synthesis. This is an important point, since the reduction of total mRNAs to approximately half the mRNA of controls represents an overall trend in the same line but less marked in OE than that observed in PF rats. The uniformity in the measurement of expression between controls and OE represents a relative increase in the given protein expression with respect to the whole mRNA pool. As a consequence, the apparent lack of change in most of the pathways investigated suggests that there is a metabolic effort to maintain these pathways close to controls' activity at the expense of the synthesis of other proteins.

Figure 5 presents a comparative scheme of the main liver energy metabolism pathways. The genes for the main paths controlling enzymes have been represented in parallel for PF and OE as a way to compare the differential effects on liver metabolism of dietary restriction and OE.

In PF rats, there was a generalized decrease in the gene expression of most enzymes, but there was a fair relationship between the gene expression data and the metabolic changes observed. Thus lipogenesis enzyme gene expressions were decreased in PF rats. The only gene-expression increases in lipid metabolism were related to enhanced lipid utilization: increased uptake of non-esterified fatty acids (fatty acid transport protein), which agrees with higher plasma levels, hydrolysis of triacylglycerols (hormone-sensitive lipase), and transference of fatty acids into the mitochondria (carnitine palmitoleoyl-transferase), all consistent with a lower liver lipid content. Glycolytic enzyme gene expressions were also decreased, but those related to gluconeogenesis (PEPCK and glucose-6-phosphatase) were increased. This profile is coherent with the exhausted liver glycogen stores. The decrease in pyruvate dehydrogenase kinases' gene expressions suggests that pyruvate dehydrogenase activity may also be increased, thus providing extra acetyl-CoA for the tricarboxylic acid cycle operation under conditions of limited energy supply.

In contrast, OE showed only limited differences compared with controls. Liver glucose utilization was probably increased by OE treatment, since both pentose-phosphate and glycolytic pathway enzyme gene expressions were increased. The parallel inhibition of the gluconeogenic enzyme PEPCK seems to indicate that glycolysis may be favoured over gluconeogenesis. In OE-treated rats, glucose utilization in peripheral tissues is decreased [10], and there is plenty of glucose available the liver, since glycogen stores are essentially maintained with respect to controls [8,9]. Consequently, it may be inferred that high glucose availability may facilitate that part of this glucose be derived to synthetic processes. In addition, the parallel increases in glucose-6-phosphate dehydrogenase and malic enzyme gene expressions hint at an increased availability of NADPH for biosynthesis. The uptake of non-esterified fatty acids may be enhanced, as suggested by the increased expression of fatty acid transport protein 2, and especially that of fatty acid translocase. There were no differences between OE and controls in the expression of acetyl-CoA carboxylase and fatty acid synthase, which suggest that *de novo* synthesis from glucose was unchanged. These results suggest that at least part of the fatty acids taken up from the bloodstream may be further unsaturated and re-esterified (increased steroyl-CoA desaturase and glycerol-3-phosphate acyl-transferase gene expressions) to render triacylglycerols, exported in lipoproteins. It may be further speculated that these processes are helped by the abundance of NADPH used to generate glycerol phosphate or sustain fatty acid insaturation [27]. However, the increased expression of carnitine palmitoleoyl-transferase points to a higher availability of fatty acids for mitochondrial oxidation, as suggested by the increased long-chain acyl-CoA dehydrogenase gene expression.

PPARs are important effectors regulating lipid metabolism [28]; PPARα is expressed mainly in the liver where it up-regulates genes involved in lipid oxidation in the fasted state [29]. This is in agreement with the increased expression of PPARα in PF rats. In the liver, PPARβ/δ-specific agonists suppress hepatic glucose output, promote glucose flux through the pentose phosphate shunt

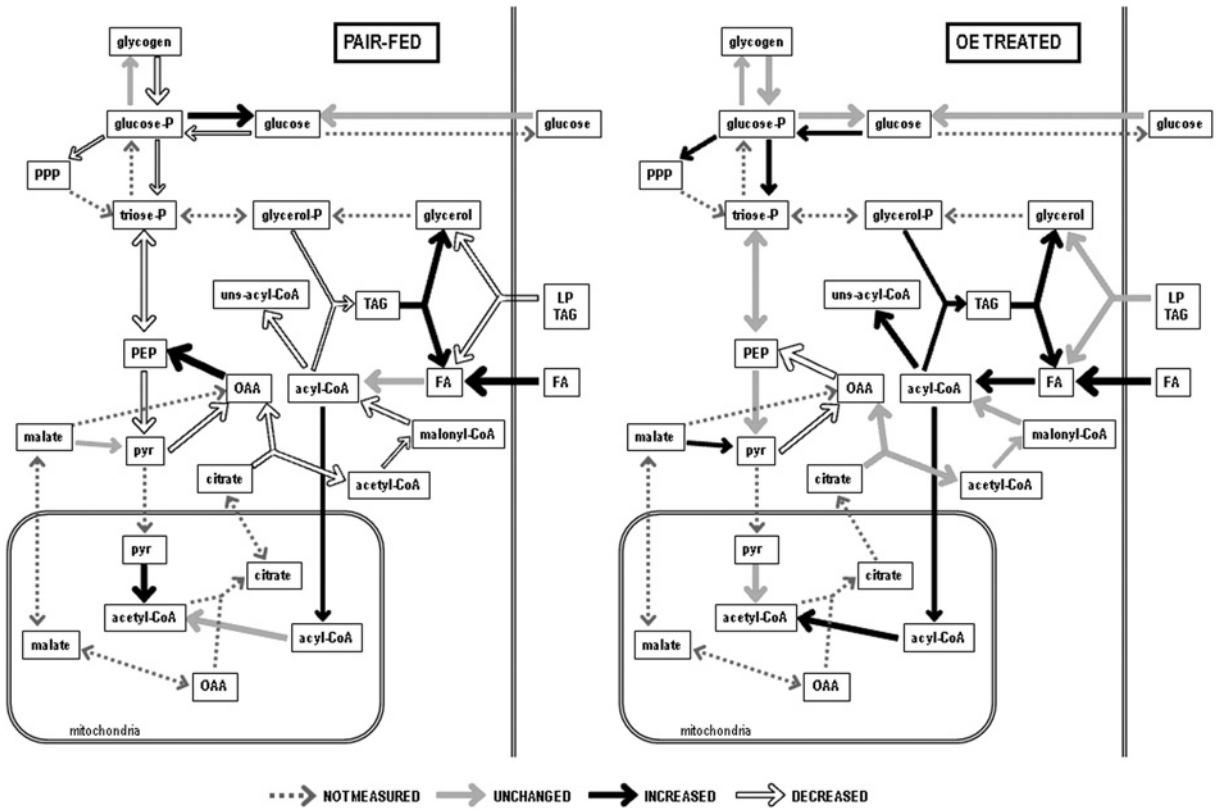

Figure 5 Scheme of the main traits of liver energy metabolism showing the differential effects of OE treatment and pair-feeding on the gene expression of enzymes

Left-hand panel: PF animals. Right-hand panel: OE-treated rats. The results presented summarize those presented in Figures 1 and 2. In both graphs, the width of the arrows corresponds to the relative abundance of the transcripts in control animals. PPP, pentose-phosphate pathway; uns-acyl-CoA, unsaturated acyl-CoA; LP TAG, triacylglycerols in lipoproteins; FA, fatty acids; PEP, phosphoenolpyruvate; OAA, oxaloacetate; pyr, pyruvate.

and stimulate fatty acid synthesis [30]. The marked decrease in PPARβ/δ expression in the PF group is, again, consistent with a situation of limited glucose availability. In contrast, in OE rats, the expression of PPARβ/δ was not inhibited, which can be related to higher activity of the pentose phosphate shunt, and the preservation of glycogen stores. In addition, PPARβ/δ increases the expression of liver fatty acid translocase [31], as found in the OE group.

The decreased insulin pathway signalling activity in PF is consistent with low plasma levels of insulin and glucose, and the increased liver glucose output typical of a low energy availability situation. However, OE treatment did not change (compared with controls) the insulin receptor gene expression. OE-treated rats maintain quite normal blood glucose, with a marked decrease in insulin levels [3,8]. Insulin-mediated repression of gluconeogenesis involves the inhibition of PEPCK transcription [32], as found in OE rats in spite of lower insulin levels, which points to an increase in liver insulin-sensitivity.

SREBP-1c activity is primarily regulated by insulin, which activates its expression and proteolytic cleavage, increasing the nuclear active form [33,34]. SREBP-1c enhances the transcription of genes favouring the catabolism of glucose and lipogenesis [27,33]. ChREBP is primarily regulated by pentose phosphate pathway products, generated under conditions of high glucose availability [35]. In PF animals, as expected, gene expressions of both SREBP-1c and ChREBP were decreased, as were those of the enzymes they regulated, resulting in lower glucose utilization and lipogenesis. The situation contrasts with that of OE rats where the expression of SREBP-1c was increased, but that of ChREBP remained unchanged. As a consequence, the gene expressions of the enzymes controlled more markedly by ChREBP (pyruvate kinase, acetyl-CoA carboxylase and fatty acid synthase) [27,35] remained unchanged. However, all those regulated more specifically by SREBP-1c (acyl-CoA desaturase, glycerol-3-phosphate-acyl-transferase, glucose-6-phosphate dehydrogenase and the malic enzyme) [18] increased their gene expressions. Since OE rats showed low insulinaemia, normoglycaemia and a postulated higher liver insulin-sensitivity, the control of lipogenesis and glycolysis became partially distorted: the lack of changes in glucose resulted in unchanged ChREBP, whereas

increased insulin-sensitivity resulted in an enhanced SREBP-1c response.

LXR transcription factors are also involved in the regulation of liver energy and cholesterol metabolism [27]. LXR increases the mRNA levels of SREBP-1c but has little effect inducing its activation to the nuclear form [34]. Recent studies have shown that glucose also regulates LXR [36]. OE maintained unchanged LXR gene expression, in contrast with PF rats' decrease; thus it may be related to sustained glucose availability. The increase in the active nuclear form of SREBP-1c of OE rats gives support to the critical role of insulin in its activation [34] and helps explain ours results.

In conclusion, the results presented suggest that the effect of OE on liver metabolism may be (at least in part) mediated through an insulin-sensitivity-dependent modulation of the expression of SREBP-1c, resulting in the unique combined effect of mildly increased (or maintained) glucose disposal but also limited enhancement of lipogenesis. Other transcription factors such as PPARβ/δ, LXR and ChREBP may play complementary functions in the regulation of liver energy metabolism under OE treatment. In addition we showed that the slimming effect of OE treatment could not be explained exclusively on the basis of the simple decrease in food intake.

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online data

SUPPLEMENTARY ONLINE DATA

Gene expression modulation of liver energy metabolism by oleoyl-estrone in overweight rats

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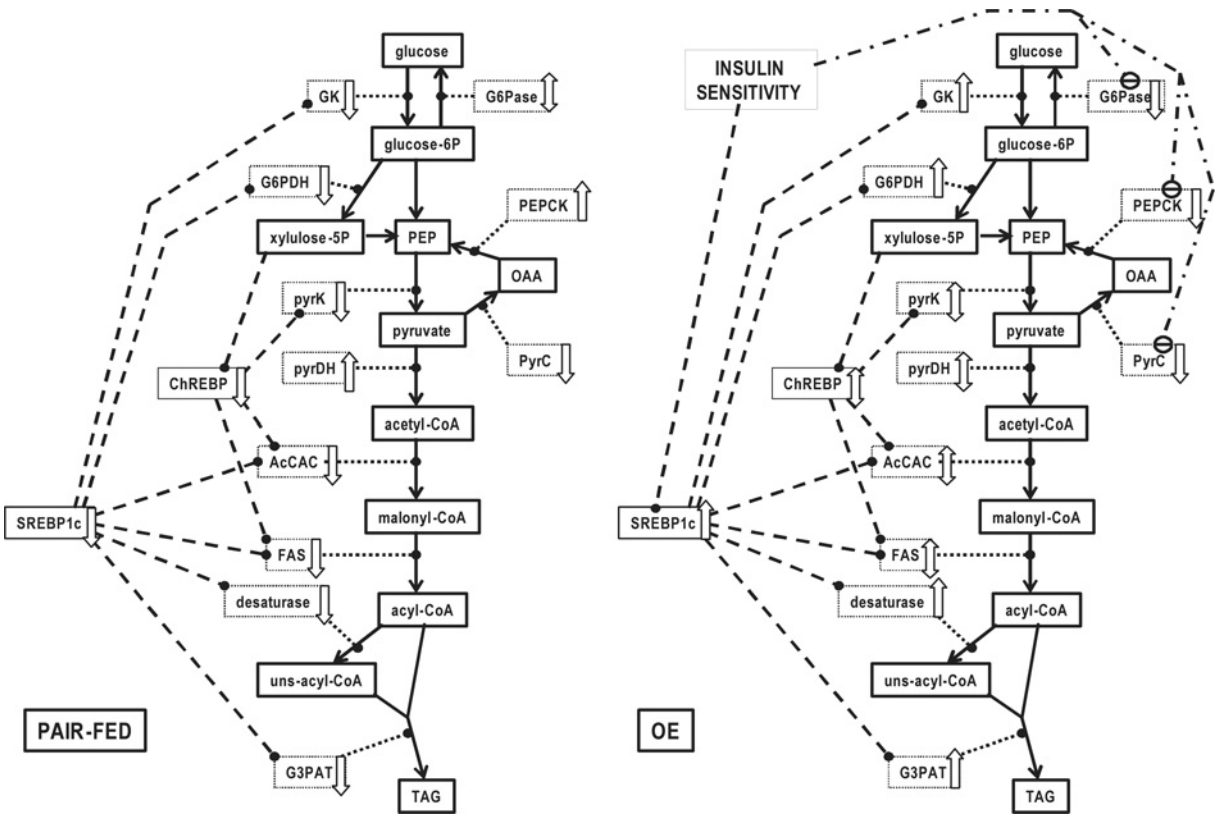

Figure S1 Scheme of the postulated implication of SREBP-1c and ChREBP in the control of lipid synthesis under OE treatment and pair-feeding

Left-hand panel: PF animals. Right-hand panel: OE-treated rats. The data presented summarize part of the data in Figures 1–4 in the main paper. Continuous lines correspond to metabolic paths controlled by enzymes (dotted squares and dotted lines). Dashed lines correspond to activation relationships. Dashed-and-dotted lines correspond to inhibitions. The downward arrow indicates significantly decreased expression compared with controls; double-headed arrow indicates absence of significantly different expression compared with controls; upward arrow indicates significantly increased expression compared with controls. GK, glucokinase; G6PDH ,glucose-6-dehydrogenase; PEP, phosphoenolpyruvate; pyrK, pyruvate kinase; pyrDH, pyruvate dehydrogenase; AcCAC, acetyl-CoA carboxylase; FAS, fatty acid synthase; desaturase, palmitoleoyl-CoA desaturase; uns-acyl-CoA, unsaturated acyl-CoA; G3PAT, glycerol-3-phosphate acyl-transferase; G6Pase, glucose 6-phosphatase.

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Table S1 Genes and sequences of the primers used for estimation

Protein name	Gene	Forward 5′–3′	Reverse 5′–3′	Length
Glucose transporter type 2, liver	*Slc2a2/Glut2*	CATCATCGCCCTCTGCTTCC	ATCAGGACCACCCCAGCAAA	81
Glucokinase	*Gck*	GCTTTTGAGACCCGTTTCGT	GCCCCAGAGTGCTTAGGATGT	85
Glycogen phosphorylase, liver	*Pygl*	ATGTGGCCGCTCTGGACAAG	GAGGTCTGGCTGATTGGGAGAA	122
Glycogen synthase kinase-3α	*Gsk3a*	TCCTCAAGGCTCTCCCCACT	AGACGGTTGGATGGAAAGTTCA	141
Phosphofructokinase, liver	*Pfkl*	CCAGCCACCATCAGCAACA	CAATGAACACACGCCGCTTT	133
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	CAAATGGGGTGATGCTGGTG	GAAGGGGCGGAGATGATGAC	118
Pyruvate kinase, liver	*Pklr*	GTTCCAGACAAGGGGCGATG	TTCCTGCTGCCCAAGATACCA	180
Phosphoenolpyruvate carboxykinase 1	*Pck1*	CCCCCTTGTCTACGAAGCTC	ACCTTGCCCTTATGCTCTGC	99
Pyruvate carboxylase	*Pc*	GCCAGAGGCAGGTGTTCTTTG	TTTGGCCCTTCACATCCTTCA	120
Glucose-6-phosphatase	*G6pc*	TCAGGTGGTGGCTGGAGTCTT	CTGGAGGCTGGCATTGTAGATG	85
Pyruvate dehydrogenase kinase 2	*Pdk2*	ATGGAGGGCTTTGGGACAG	CGCCAGGCAGACTTGTTGTAG	95
Pyruvate dehydrogenase kinase 4	*Pdk4*	GTCAGGCTATGGGACAGATGC	TTGGGATACACCAGTCATCAGC	142
Glucose-6-phosphate dehydrogenase X-linked	*G6pdx*	GACTGTGGGCAAGCTCCTCAA	GCTAGTGTGGCTATGGGCAGGT	77
Malic enzyme 1	*Me1*	GGAGTTGCTCTTGGGGTAGTGG	CGGATGGTGTTCAAAGGAGGA	143
Fatty acid transport protein 2	***Slc27a2****/Fatp2*	GCATCAAAGTCCCCAAAGGT	TTTCCTCCAGCATAGCCAAA	85
Fatty acid translocase	*Cd36*	TTGGATGTGGAACCCATAACTG	TGGTCCCAGTCTCATTTAGCC	154
Fatty acid binding protein 1, liver	*Fabp1*	CGGCAAGTACCAAGTGCAGAG	TCCTTCCCTTTCTGGATGAGGT	90
Hepatic lipase	*Lipc*	GGTGGCTGCTCTTCTCCTATGG	GCCTGCGAATCCTGAAACG	106
Hormone-sensitive, lipase	*Lipe*	CAAGCCCCATAAGACCCCATT	CCGTAAGTCGCCCAGAATCC	68
Desnutrin	*Pnpla2/Atgl*	CGGTGGATGAAGGAGCAGACA	TGGCACAGACGGCAGAGACT	138
Adiponutrin	*Pnpla3/Adpn*	GTGTGCCCGAATGACCATGT	GCCTTGGGGTTTGTGGAGAG	11
Carnitine palmitoleoyltransferase 1a	*Cpt1a*	CCGCTCATGGTCAACAGCA	CAGCAGTATGGCGTGGATGG	105
Acetyl-CoA dehydrogenase, long chain	*Acadl*	ATGTGGGAATACCCGATTGC	GGATTTCAGGATGTAGGCAGATG	141
ATP citrate-lyase	*Acly*	GACCAGAAGGGCGTGACCAT	GTTGTCCAGCATCCCACCAGT	96
Acetyl-CoA carboxylase 1	*Acaca*	AGGAAGATGGTGTCCGCTCTG	GGGGAGATGTGCTGGGTCAT	145
Acetyl-CoA carboxylase 2	*Acacb*	CTGGAACAGCACTGGTCAGC	GGCTCAGGTATGCCACACAG	151
Fatty acid synthase	*Fasn*	CTTGGGTGCCGATTACAACC	GCCCTCCCGTACACTCACTC	163
Stearoyl-CoA desaturase 1	*Scd1*	AGAAGGGCGGAAAGCTGGAC	AGTGTGGGCAGGATGAAGCA	112
Glycerol-3-phosphate acyltransferase	*Gpam*	GGTGAGGAGCAGCGTGATTG	GGACAAAGATGGCAGCAGAGC	127
Long-chain acyl-CoA synthetase 4	*Acsl4*	CGCTCCTCTTATTTGCTGTGA	CCTCTGGGGTTTGGCTTATC	84
Thyroid hormone responsive element spot14	*Thrsp*	GCACTTCTCCAGCCTCCATCA	GACCTGCCCCGTCATTTCCT	91
Insulin receptor	*Insr*	TTGCTGAGGTGGGAGCCCTA	GCCCGTCAAACTCTGTCACG	106
Peroxisome-proliferator-activated receptor α	*Pparαa*	TTCAATGCCCTCGAACTGGA	GCACAATCCCCTCCTGCAAC	124
Peroxisome-proliferator-activated receptor δ	*Pparbδ*	CCAGCCATAACGCACCCTTC	TTCCACACCAGGCCCTTCTC	73
Sterol-regulatory-element-binding factor 1 isoform c	*Srebf1*	AAAACCAGCCTCCCCAGAGC	CCAGTCCCCATCCACGAAGA	153
Carbohydrate-responsive element-binding protein	*Wbscr14/ChREBP*	TACTGTTCCCTGCCTGCTCTCC	ACTGCCCTTGTGGCTTGCTC	116
Liver X receptor α	*Nr1h3/LXRα*	CCCACGACCGACTGATGTTC	TGCTCTGAATGGACGCTGCT	79

Table S2 Liver gene expression of enzymes involved in carbohydrate metabolism of male overweight rats treated with oral OE or PF

The results are the means ± S.E.M. for eight different animals. Differences between groups: *$P < 0.05$ compared with controls; †$P < 0.05$ between OE and PF groups.

		Whole liver mRNAs content (fmol)		
Protein	**Gene**	**Control**	**PF**	**OE**
Glucose transporter 2	*Glut2*	1843 ± 82	1853 ± 58	1757 ± 154
Glucokinase	*Gck*	256 ± 44	68 ± 12*	509 ± 74*†
Glycogen phosphorylase	*Pygl*	2452 ± 211	772 ± 33*	2239 ± 158†
Glycogen synthase kinase 3α	*Gsk3a*	170 ± 12	151 ± 9	209 ± 23†
Phospho-fructokinase	*Pfkl*	174 ± 15	145 ± 5	228 ± 11*†
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	4183 ± 319	2936 ± 98*	4476 ± 331†
Pyruvate kinase	*Pklr*	795 ± 84	259 ± 13*	751 ± 64†
Phosphoenolpyruvate carboxykinase 1	*Pck1*	8115 ± 659	14242 ± 438*	4692 ± 557*†
Glucose-6-phosphatase	*G6pc*	379 ± 41	568 ± 37*	430 ± 36†
Pyruvate carboxylase	*Pc*	2479 ± 200	1590 ± 88*	1871 ± 80*
Pyruvate dehydrogenase kinase 2	*Pdk2*	2435 ± 224	1641 ± 55*	2304 ± 96†
Pyruvate dehydrogenase kinase 4	*Pdk4*	128 ± 13	59 ± 11*	189 ± 18*†
Glucose-6-phosphate dehydrogenase	*G6pdx*	154 ± 15	62 ± 2*	224 ± 26*†
Malic enzyme	*Me1*	142 ± 10	121 ± 5	261 ± 26*†

Table S3 Liver expression of lipid metabolism indicators of male overweight rats treated with oral OE or PF

The results are the means ± S.E.M. for eight different animals. Differences between groups: *$P < 0.05$ compared with controls; †$P < 0.05$ between OE and PF groups.

		Whole liver mRNA content (fmol)		
Protein	**Gene**	**Control**	**PF**	**OE**
Long-chain fatty acid transporting protein 2	*Fatp2*	87 ± 7	131 ± 8*	138 ± 8*
Fatty acid-binding protein 1	*Fabp1*	23412 ± 1649	20870 ± 610	28450 ± 2610†
Fatty acid translocase	*Cd36*	59 ± 5	46 ± 4	221 ± 28*†
Hepatic lipase	*Lipc*	1752 ± 73	1170 ± 70*	1570 ± 90†
Hormone-sensitive lipase	*Lipe*	71 ± 5	89 ± 8	98 ± 7*
Adiponutrin (lipase)	*Adpn*	75 ± 17	11 ± 1*	70 ± 18†
Desnutrin (lipase)	*Atgl*	414 ± 40	417 ± 20	519 ± 23*
Carnitine palmitoyltransferase 1a	*Cpt1a*	161 ± 18	409 ± 22*	223 ± 20†
Acyl-CoA dehydrogenase (long chain)	*Acadl*	2512 ± 129	2290 ± 80	3340 ± 297*†
Citrate:ATP lyase	*Acly*	807 ± 116	189 ± 11*	708 ± 134†
Acetyl-CoA carboxylase a	*Acaca*	340 ± 33	133 ± 4*	371 ± 55†
Acetyl-CoA carboxylase b	*Acacb*	202 ± 12	70 ± 5*	208 ± 12†
Fatty acid synthase	*Fasn*	1196 ± 135	185 ± 5*	1400 ± 200†
Steroyl-CoA desaturase 1	*Scd1*	3878 ± 449	678 ± 72*	5847 ± 801*†
Glycerol-3-phosphate acyl-transferase	*Gpam*	380 ± 39	102 ± 12*	564 ± 50*†
Long-chain acyl-CoA synthetase	*Acsl4*	137 ± 14	123 ± 5	320 ± 13*†

Table S4 Liver gene expression of metabolic regulation indicators of male overweight rats treated with oral OE or PF
The results are the means ± S.E.M. for eight different animals. Differences between groups: *$P < 0.05$ compared with controls; †$P < 0.05$ between OE and PF groups.

		Whole liver mRNA content (fmol)		
Protein	**Gene**	**Control**	**PF**	**OE**
Thyroid hormone responsive element (Spot14)	*Thrsp*	4905 ± 178	1434 ± 32*	4677 ± 220†
Insulin receptor	*Insr*	118 ± 12	58 ± 5*	114 ± 11†
PPARα	*Ppara*	201 ± 20	388 ± 32*	187 ± 14†
PPARβ/δ	*Pparbd*	248 ± 23	37 ± 4*	241 ± 29†
SREBP-1c	*Srebf1*	1340 ± 152	394 ± 17*	2567 ± 335*†
ChREBP	*Chrebp*	639 ± 43	389 ± 19*	727 ± 55†
LXRα	*LxRa*	2626 ± 290	1859 ± 72*	2436 ± 119

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3.2.4 La oleoil estrona inhibe la expresión de genes lipogénicos pero mantiene la termogénesis en el tejido adiposo marrón de ratas con sobrepeso

En este estudio se determinó cómo el tejido adiposo marrón interescapular mantiene la termogénesis durante el tratamiento con oleoil estrona (OE), una potente hormona que disminuye el contenido corporal de lípidos sin disminuir la tasa metabólica basal.

Se administró una dosis diaria de 10 nmol/g de OE a ratas macho con sobrepeso durante 10 días y se compararon con un grupo control y un grupo *pair fed*. Se determinó la masa del tejido adiposo marrón interescapular, su contenido lípidico y de ácidos nucleicos, así como la expresión de genes que codifican proteínas implicadas en el metabolismo lipídico y energético.

La masa del tejido adiposo marrón y su contenido lipídico disminuyó en los grupos OE y *pair fed*, aunque de modo más marcado en este último, que mostró además una reducción del contenido total de ARNm total. El mantenimiento de la expresión génica del gen que codifica la perilipina en los grupos OE y *pair fed,* a pesar de la pérdida de lípidos, podría indicar que se conservó la superficie interactiva vacuolar, un factor crítico para la respuesta termogénica. El grupo OE y, en menor medida el grupo *pair fed*, mantuvieron la expresión de genes que controlan la lipólisis y la oxidación de ácidos grasos, pero disminuyeron marcadamente la expresión de los genes implicados en la síntesis de ácidos grasos y triacilgliceroles. La OE no afectó la expresión de los genes que codifican la UCP1, el receptor adrenérgico β_3 y la lipasa sensible a las hormonas, lo que indica que la OE mantuvo funcional en buena parte el sistema termogénico.

En conclusión, el grupo OE mantuvo al tejido adiposo marrón menos estresado energéticamente que el grupo *pair fed*, probablemente a través de la utilización de la glucosa circulante. Por el contrario, el grupo *pair fed* disminuyó más marcadamente la expresión del gen que codifica la UCP1 y su contenido lipídico.

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Original Paper

Oleoyl-oestrone inhibits lipogenic, but maintains thermogenic, gene expression of brown adipose tissue in overweight rats

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Synopsis

In the present study we intended to determine how BAT (brown adipose tissue) maintained thermogenesis under treatment with OE (oleoyl-oestrone), a powerful slimming hormone that sheds off body lipid but maintains the metabolic rate. Overweight male rats were subjected to daily gavages of 10 nmol/g of OE or vehicle (control) for 10 days. A PF (pair-fed) vehicle-receiving group was used to discount the effects attributable to energy availability limitation. Interscapular BAT mass, lipid, DNA, mRNA and the RT-PCR (real-time PCR) expression of lipid and energy metabolism genes for enzymes and regulatory proteins were measured. BAT mass and lipid were decreased in OE and PF, with the latter showing a marked reduction in tissue mRNA. Maintenance of perilipin gene expression in PF and OE rats despite the loss of lipid suggests the preservation of the vacuolar interactive surface, a critical factor for thermogenic responsiveness. OE and, to a lesser extent, PF maintained the expression of genes controlling lipolysis and fatty acid oxidation, but markedly decreased the expression of those genes involved in lipogenic and acyl-glycerol synthesis. OE did not affect UCP1 (uncoupling protein 1) (decreased in PF), β_3 adrenergic receptors or hormone-sensitive lipase gene mRNAs, which may translate in maintaining a full thermogenic system potential. OE rats were able to maintain a less energetically stressed BAT (probably through glucose utilization) than PF rats. These changes were not paralleled in PF rats, in which lower thermogenesis and glucose preservation resulted in a heavier toll on internal fat stores. Thus the mechanism of action of OE is more complex and tissue-specific than previously assumed.

Key words: brown adipose tissue (BAT), energy metabolism, lipogenesis, oleoyl-oestrone (OE), thermogenesis, UCP1 (uncoupling protein 1)

INTRODUCTION

OE (oleoyl-oestrone) is a powerful slimming signal from adipose tissue that elicits both central and peripheral energy wasting effects [1,2], and has been postulated as a ponderostat signal [3]. OE decreases food intake without significant changes in energy expenditure [1], and the resulting energy gap is fulfilled essentially by lipid mobilization [1,3] in WAT (white adipose tissue). This lipid is mainly used by muscle, at least in obese rats [4], decreasing hyperlipidaemia and inducing a marked decrease in circulating cholesterol [5]. Under OE treatment, glycaemia is maintained in the range of normalcy, but with lower insulin and leptin levels [5].

The marked WAT energy wasting induced by OE cannot be justified by increased catecholamine sensitivity [6], or increased stimulation of expression of key regulators of lipolytic pathways [4], but essentially occurs by powerful inhibition of lipogenesis [7], which results in an unbalanced lipid equilibrium favouring the massive release of fatty acids and glycerol. This selective voiding of fat reserves is complemented by a lowered peripheral utilization of glucose [8], which reinforces the decreased food intake, partly induced by the inhibition of ghrelin expression in the stomach, and central effects on the brain [2] not mediated by neuropeptide Y. The relative availability of glucose can be attested by the accumulation of liver glycogen [1] in spite of the partially maintained hepatic conversion of glucose into triacylglycerols which, together with those from WAT, increase the liver

Abbreviations used: BAT, brown adipose tissue; ChREBP, carbohydrate-responsive element-binding protein; OE, oleoyl-oestrone; PF, pair-fed; PPAR, peroxisome-proliferator-activated receptor; SREBP1c, sterol-regulatory-element-binding protein 1c; UCP, uncoupling protein; WAT, white adipose tissue.

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lipoprotein output in order to fuel muscle and other peripheral tissues (M. M. Romero, J. A. Fernández-López, M. Alemany and M. Esteve, unpublished work).

BAT (brown adipose tissue) is a particular type of adipose tissue that contains all of the basic metabolic machinery of WAT, complemented by a powerful oxidative mitochondrial apparatus and the unique presence of a functional UCP1 (uncoupling protein 1) [9], as well as a tighter control of both blood flow and direct stimulation of cells by sympathetic nerves [10]. The apparent 'quietness' of BAT during the energy turmoil elicited by OE may be the consequence of unaltered thermogenic activity, which corresponds with energy wasting elsewhere. This peculiarity has been tested in the present study compared with a situation in which the limited energy intake (pair-feeding) theoretically exerts the same metabolic pressure on the rat body energy budget management, independently of OE treatment. The effects of OE are quite different in liver and WAT, but BAT could not be readily compared with either model because of the unique function of this tissue in thermogenesis, at least in rodents.

MATERIALS AND METHODS

Adult male Wistar rats were made overweight by cafeteria diet feeding for a limited period, as previously described [11]. The rats, initially weighing 355 ± 5 g, were kept under standard conditions for housing and feeding. Three groups of eight rats each were randomly selected: control (C), OE and PF (pair-fed). Each day, all of the animals received an oral gavage of 0.2 ml of sunflower oil (7 kJ), which was supplemented in the OE group with 10 nmol/g of OE (OED, Barcelona, Spain). The control and OE groups had free access to pellet food (maintenance chow, Panlab, Barcelona, Spain), and the PF group were allowed only the amount of food consumed by the OE group each day; all rats had water available *ad libitum*. PF rats completely ate the food allotted each day. On day 10, the rats were killed and the interscapular BAT was rapidly excised, weighed, sampled, frozen and stored at -80°C until processed.

The animals were housed, handled and killed following the procedures approved by the University of Barcelona Animal Welfare and Ethics Committee, in full complement of the procedures set forth by the European Union and the Governments of Spain and Catalonia.

Tissue samples were used for the estimation of total lipid by extraction with trichloromethane/methanol [12]. DNA was measured using a standard fluorimetric method with 3,5-diaminobenzoic acid (Sigma, St Louis, MO, U.S.A.) and bovine DNA (Sigma) as a standard [13]. Tissue DNA content allowed the calculation of the approximate number of cells per gram of tissue and in the whole interscapular BAT pad, based on the assumption that, in mammals, the cell DNA content is constant; we used the genomic DNA size data [14] for somatic rat cells (5.6 pg/cell). Mean cell mass was estimated from the number of cells and the organ mass.

Total tissue RNA was extracted using Tripure reagent (Roche Applied Science, Indianapolis, IN, U.S.A.), and was quantified in a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, U.S.A.). RNA samples were reverse transcribed using the MMLV (Moloney-murine-leukaemia virus) reverse transcriptase (Promega, Madison, WI, U.S.A.) and oligo-dT primers. Total tissue mRNA was determined by using the poly-(A)mRNA detection system kit (Promega).

RT-PCR (real-time PCR) amplification was carried out using reaction mixtures (10 μl final volume) containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, U.S.A.), the equivalent of 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were performed on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to an absorbance (A) of 0.500 for all runs. The primers used for the estimation of gene expression in interscapular BAT are shown in Supplementary Table S1 (at http://www.bioscirep.org/bsr/029/bsr0290237add.htm).

A semi-quantitative approach for the estimation of the concentration of specific gene mRNAs per unit of tissue mass was performed as previously described [15]. In any case, cyclophilin was used as the control gene in all samples. The data are presented as the amount of copies (fmoles) present in the whole interscapular BAT mass, as an indication of the potential for synthesis of these specific proteins in an organ which size changes with treatment. This may allow for comparison between groups in a more direct way than a simple comparison of relative expressions.

Statistical comparison between groups was established by using the unpaired Student's t test with a limit of significance of $P < 0.05$ using the GraphPad Prism5 program (GraphPad, La Jolla, CA, U.S.A.).

RESULTS

At the end of the study, and in relation to their initial masses, control rats gained $2.6 \pm 0.4\%$ (not significant compared with initial values), PF rats lost $8.5 \pm 0.7\%$ ($P < 0.05$ compared with initial values and the gain of the control group) and OE rats lost $9.9 \pm 0.7\%$ ($P < 0.05$ compared with initial values and the gain of the control group). Table 1 presents the nucleic acid and cell contents of the interscapular BAT of overweight male rats treated with OE compared with control and PF rats. OE treatment resulted in the loss of approx. 40% of whole BAT lipids compared with control rats, whereas PF rats lost 57%; the differences being significant compared with control rats, but not between OE and PF groups. Both OE and PF treatments resulted in the loss of approx. one-third of the tissue mass, but with no parallel loss of cells. In any case, the RNA/DNA ratio decreased, which suggests a diminished cell function overall. However, the cells of the OE group maintained the same amount of mRNA per cell as control cells, and PF cell mRNA levels were approximately half of those of control and OE cells. The loss of tissue mass and the maintenance of cell number resulted in smaller mean cell

Table 1 Nucleic acid content and cellularity in interscapular BAT of OE-treated and PF male overweight rats
The parameters represent the absolute content in the total interscapular BAT. The data are the means ± S.E.M. for eight different animals. *$P < 0.05$ compared with controls; †$P < 0.05$ between PF and OE-treated rats.

Parameter	Control group	PF group	OE group
Tissue mass (mg)	551 ± 30	363 ± 29*	379 ± 21*
Lipid content (mg per g of tissue)	569 ± 34	374 ± 64*	497 ± 19
Lipid content (mg per BAT site)	314 ± 18	136 ± 20*	188 ± 16*
DNA content (μg per g of tissue)	1.57 ± 0.12	2.68 ± 0.12*	2.54 ± 0.12
DNA content (mg per BAT site)	0.87 ± 0.06	0.94 ± 0.06	0.95 ± 0.06
Total RNA content (mg per g of tissue)	1.05 ± 0.02	1.29 ± 0.04*†	1.17 ± 0.03*
Total RNA content (mg per BAT site)	0.58 ± 0.03	0.46 ± 0.03*	0.44 ± 0.02*
RNA/DNA ratio	0.70 ± 0.06	0.49 ± 0.01*	0.47 ± 0.02*
Number of cells ($\times 10^6$ cells per BAT site)	155 ± 13	168 ± 9	170 ± 11
Mean cell mass (ng)	3.72 ± 0.30	2.15 ± 0.10*	2.25 ± 0.11*
Total mRNA content (μg per g of tissue)	20.7 ± 2.3	14.5 ± 1.9*†	28.3 ± 2.6*
Total mRNA content (fg/cell)	70.7 ± 8.4	32.3 ± 3.3*†	62.5 ± 6.7

sizes (the results include both adipocytes and non-adipocyte cells) for OE and PF rats compared with control rats.

Figure 1 shows the changes induced either by pair-feeding or OE treatment on the expression of a number of energy handling and control-related genes, presented as a percentage of the corresponding control values. OE treatment and pair-feeding induced a similar marked decrease in the expression of GLUT4 and hexokinase, and OE decreased the expression of the NADPH-producing malic enzyme. PF raised the expression of phosphoenolpyruvate carboxykinase and pyruvate dehydrogenase kinase 4 compared with both control and OE rats. Supplementary Tables S2 and S3 (at http://www.bioscirep.org/bsr/029/bsr0290237add.htm) show in absolute terms, i.e. fmoles in the whole interscapular BAT mass, expression of the genes presented in Figure 1 in order to allow for more quantitative comparisons.

OE induced a marked decrease in the expression of lipogenic enzyme genes, a decrease that was less extensive in PF rats (which even showed an increased expression of acetyl-CoA carboxylase 2 compared with control rats). OE induced a dramatic decrease in the expression of stearoyl-CoA desaturase, which was unaffected in PF rats. Glycerol-phosphate acyl-transferase gene expression was decreased in both groups compared with control rats. Fatty acid transport, translocation and binding enzyme gene expressions were not altered by either treatment, the exceptions being a decrease in expression of fatty acid transport protein in OE rats (unaffected in PF rats) and a reduction in expression of fatty acid-binding protein 3 in PF rats, which was not altered by OE. Gene expression of the β-oxidation enzyme acyl-CoA dehydrogenase isoenzyme was unaffected by OE and PF, except for decreased medium-chain dehydrogenase expression in PF rats.

With the exception of a considerable decrease in the expression of the adiponutrin gene in both OE and PF rats, gene expression of lipases was unaffected by OE, and only lipoprotein lipase expression was enhanced by pair-feeding. Perilipin expression was unchanged in both OE and PF rats compared with control rats.

The expression of the UCP1 gene was decreased in PF rats compared with control rats, but this did not occur in those treated with OE. The expression of the β_1 adrenergic receptor gene was decreased in both OE and PF rats, but that of the β_3 adrenergic receptor was unchanged, and the expression of the β_2 adrenergic receptor was considerably higher in PF rats compared with both control and OE rats. No differences were observed in the expression of phosphodiesterase in comparison with control rats. The oestrogen receptor α gene more than doubled its expression in PF rats, but OE and control rats showed similar values. Neither treatment changed the expression of the thyroid hormone receptor gene, but both PF and OE treatment decreased the expression of thyroxine deiodinase.

OE decreased the gene expression of PPAR (peroxisome-proliferator-activated receptor) γ_1 and its coactivator 1α, as well as the expression of phosphatase and tensin homologues, nuclear respiratory factor 1, ChREBP (carbohydrate-responsive element-binding protein) and the Spot 14/thyroid hormone-responsive protein. PF rats only displayed a reduction in the gene expression of ChREBP and the Spot 14/thyroid hormone-responsive protein, and displayed an increase in expression of the PPARα gene.

DISCUSSION

Under normal conditions, perilipin levels (and gene expression) are assumed to be related to the vacuole size (surface) [16] and BAT contains multiple vacuoles per cell that facilitate the rapid mobilization of fat. The lack of change in perilipin expression despite a decrease in cell size due to the observed loss of fat (and maintenance of cell numbers) resulted in the following values for perilipin mRNA (in fmol/g of tissue lipid): 148 ± 20 (control rats), 309 ± 22 (PF) and 207 ± 17 (OE), the differences in PF compared with control and OE rats being significant. Expression of perilipin in PF rats was actually increased when compared with total tissue mRNA, resulting in remarkably similar expression in all of the three groups studied, irrespective of the much lower lipid content and smaller mean cell size of PF and OE. The data

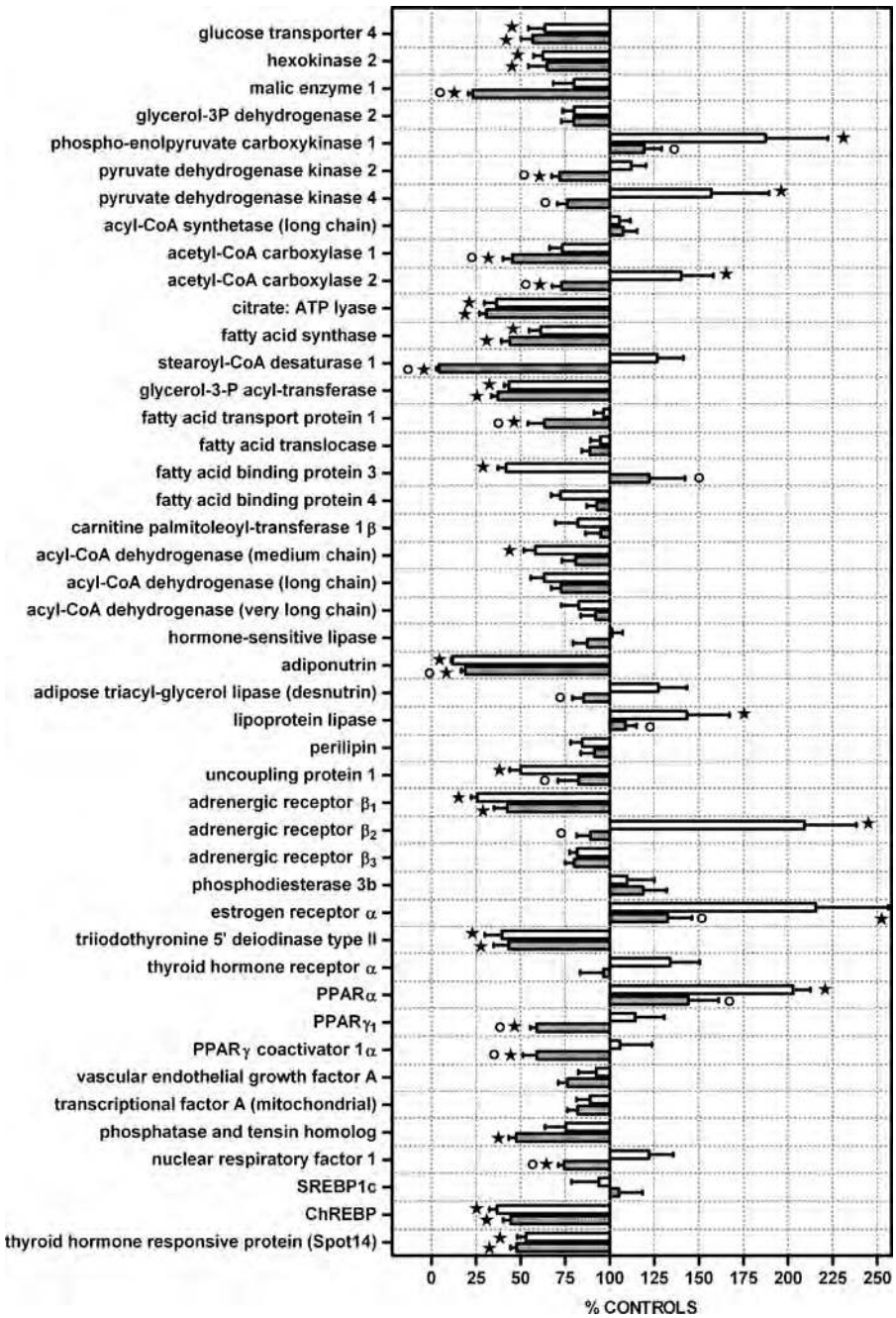

Figure 1 Percentage of mRNA for enzyme and regulatory protein expression in the interscapular BAT mass of overweight male Wistar rats treated with OE or PF compared with control rats

The values represent the content of specific gene mRNA in the whole interscapular BAT mass, and are expressed as a percentage of the expression in the control rats (see Supplementary Tables S2 and S3 at http://www.biosciep.org/bsr/029/bsr0290237add.htm), and are the means ± S.E.M. for eight different animals. PF, white bars; OE, grey bars. Statistical significance of the differences between groups: stars indicate $P < 0.05$ compared with the control group (100%), and open circles indicate $P < 0.05$ between OE and PF groups.

on fat and cell number indicate that the mean lipid vacuole size must be smaller in OE and PF rats than in control rats, despite the other differences in metabolism. However, unchanged perilipin expression is probably correlated to maintenance of perilipin levels, and to unchanged vacuolar lining surface area, roughly proportional to perilipin content [16]. Consequently, the total

surface area of vacuoles could not be maintained following vacuolar shrinking, which occurs as a result of the loss of cell fat. This means that the total number of vacuoles must be increased to pack a lower volume of fat with a closely similar estimated surface area in PF and OE rats than in control rats, thus preserving the cytoplasm/fat droplet interface area and consequently the responsiveness to hormonal stimulation. The unchanged expression of the hormone-sensitive lipase gene agrees with this interpretation. The data presented indirectly hint at the maintenance of the overall interactive surface of the BAT vacuole as being an important factor in the thermogenic-response capability of the tissue.

The loss of fat (and thus postulated droplet fractionation) was more marked in PF than in OE rats, which somehow were able to maintain a much less energetically stressed tissue (probably through glucose utilization) than PF animals. This buffered effect of energy loss in OE rats is again exemplified by the marked difference in total tissue mRNA content between control rats and OE rats on one side and PF on the other, which in BAT agrees with our previous findings that the effects of OE on energy balance are not mediated simply by a decrease in dietary energy availability [17]. However, this lower mRNA content in PF rats is in full agreement with the decreased thermogenesis and BAT function [18] of food-deprived animals. Lowered mRNA levels are an index of decreased protein synthesis, in itself a normal mechanism of decreasing energy expenditure through slower protein turnover [19]. The loss of lipids in PF rats tends to be higher than in OE rats, the latter maintaining UCP levels and the full complement of mRNA (i.e. protein synthesis), which means that the flow of energy substrates to and from BAT should be different for PF and OE rats. This is supported by the differences in lipoprotein lipase gene expression, but not by the gene expression of other lipases and most fatty acid transportation/translocation enzyme genes. It should be noted, however, that the presence of the same amount of a given specific mRNA in BAT from PF rats represents a much higher 'selective effort' of translation than in OE or control animals, which hints at the relative importance for the tissue of the maintenance of the corresponding pathway with respect to all other pathways. This way we can observe that lipase and β-oxidation operation, as well as 3-carbon fragment preservation through activation of pyruvate dehydrogenase kinases are important pathways that seem to receive this special treatment in food restriction.

The effects of limited food availability and OE treatment on the cellularity, cell size and mass of lipid reserves of BAT were apparently similar. Analysis of the expression of the main genes controlling lipid and energy metabolism seems to confirm this initial assumption, but the effects induced by OE were deeper than those of simple food restriction (pair-feeding), since lipogenesis gene expression was more markedly inhibited in OE than in PF rats.

The postulated reduction of NADPH synthesis in OE rats (malic enzyme) suggest a lower synthetic drive [20], this is further stressed by a much decreased expression of stearoyl desaturase which is not observed in PF rats, and the differentially more marked decrease in the expression of lipogenic enzyme genes in OE rats compared with both control and PF rats. The marked decrease in adiponutrin expression in OE and PF supports a probable inhibition of lipogenesis, given the nature of adiponutrin as a marker of lipid biosynthesis [21] in addition to reflecting decreased food intake.

Glucose uptake and phosphorylation seem to be inhibited in both OE and PF animals because of the decreased expression of GLUT4 and hexokinase 2. However, 3-carbon fragment utilization through the pyruvate dehydrogenase pathway was probably increased in OE rats and decreased in PF rats (lower kinase 2 gene expression in OE rats and higher kinase 4 gene expression in PF rats).

The potential to dispose of internal fat stores remained unchanged in all three groups, as was β-oxidation (only a decrease in medium-chain acyl-CoA dehydrogenase gene expression in PF rats was shown). The possible incorporation of lipid from lipoproteins was unchanged in OE rats, since the decreased activity found in WAT [5] was not paralleled in BAT by the expression (unchanged) of lipoprotein lipase. In PF rats, lipoprotein lipase expression actually increased, which suggests that circulating lipids may continue to be taken up for energy supply under food restriction conditions [22]. The differential expression of fatty acid-binding protein 3 [23] and fatty acid-transport protein 1 genes [24] in PF and OE rats may be directly related to this different potential use of circulating lipoprotein lipid, since the latter is closely related to fatty acid transport into the cell [25].

The raised expression of the PPARα gene in PF rats compared with control rats and its lack of difference in OE rats agree with a maintainance of lipolytic activity in OE rats. The more lipogenic PPARγ [26] expression was again strongly inhibited by OE, since PPARγ activation increases both lipid and adipose tissue masses [27].

Lipid synthesis is partially controlled in adipose tissue by the insulin signalling cascade [28] through modulation of SREBP1c (sterol-regulatory-element-binding protein 1c) [29] and ChREBP [30]. Both factors have been found to play a key role in OE-stimulated liver lipogenesis [7], which is in part fuelled by glucose in spite of the generalized body lipid mobilization and decreased food intake. In the present study, however, SREBP1c remained unchanged in both PF and OE rats, even when the Spot 14/thyroid hormone-responsive protein, closely related to SREBP1c action [31] and also a marker of lipogenesis, was inhibited, as was that of ChREBP. These results suggest that OE modulation of lipid metabolism did not proceed along the same pathways in liver and BAT.

However, the possibly most far-reaching 'metabolic' difference between PF and OE rats was the relative maintenance of the expression of UCP1 in OE rats compared with its significant fall in PF rats. This finding is in agreement with the known maintenance of energy expenditure under OE treatment [1], in contrast with its decrease under conditions of starvation or food restriction.

The maintenance of an unchanged adrenergic pathway (β_3 adrenergic receptors, phosphodiesterase, hormone-sensitive lipase and maintained vacuolar surface area) and UCP1 ensures the full operativity of the thermogenic response to eventual adrenergic

stimuli in OE rats. These arrangements are less complete or probably less efficient in PF animals. Decreased adrenergic receptor expression is often a consequence of saving strategies under conditions of limited energy availability; the differential expression of UCP1 in OE and PF rats contrasts with the lack of change in β_3 adrenergic receptors that control UCP1 expression [32]. A reduction in expression of UCP1 in PF rats may be related to the effects of oestrogen signalling, which reduces the sensitivity to catecholamine stimulation in BAT [33] as a way to decrease thermogenic responsiveness. However, the less marked increase in oestrogen receptor expression in OE rats may be either a consequence of increased circulating oestrogen in OE rats [34] that may help down-regulate the expression of the receptor, or another way of maintaining the ability of the BAT thermogenic system to respond to hormonal signals.

The data presented on gene expression suggest that, in PF rats, the BAT energy needs are low, and thus limited glucose availability (very low 3-carbon fragment conversion into acetyl-CoA) was translated into decreased fat stores despite external lipid utilization. However, in OE rats, this same overall energy scarcity was compounded by the need to fuel thermogenesis, which poses an additional burden for BAT, and can explain why glucose utilization (in spite of assumedly limited entry) may be increased, and why lipid synthesis is strongly limited in contrast with β-oxidation, which is only slightly decreased. The success of this OE-driven strategy is proven by both the minor change in metabolic orientation, maintenance of thermogenesis and the lower overall loss of lipid. Since the main difference between PF and OE rats seems to be in the regulation of pyruvate dehydrogenase (and a stronger inhibition of lipid synthesis in OE) through differential gene expression, it can be speculated that the continuous availability of glucose in OE rats [5,8] may be a key factor in the preservation of part of the BAT lipid and the full operativity of thermogenesis.

Pair-feeding changed the expression of growth and differentiation factors little compared with control rats, in contrast with OE-treated rats. The expression of the phosphatase and tensin homologue, which is implicated in cell survival [35], as well as the PPARγ coactivator 1α [36] and the nuclear respiratory factor 1 genes [37] related to mitochondriogenesis, were inhibited by OE, but not by pair-feeding. However, the gene expression of neither the vascular endothelial growth factor A, an angiogenic factor [38], nor the mitochondriogenic transcriptional factor A [39] were altered by OE (or by PF), suggesting few changes in tissue architecture in parallel with the loss of tissue mass and fat (but not cells). In any case, the effects of OE were more marked than those of pair-feeding. The better maintenance of expression of cell development factors by PF rats disagrees with the decrease in UCP1 expression, which is presumably an index of thermogenic capability. The cell changes cannot be attributed directly to a change in thyroid receptor regulation, in spite of the important control of BAT development and function by the thyroid. The decrease in thyroxine deiodinase expression may be a direct consequence of energy scarcity [40].

In spite of some differences in the control of tissue factors, the limits set for the synthesis of fat, management of its stores, the use of glucose and the maintenance of thermogenic potential conform with the bulk of the changes in gene expression induced by OE on BAT. These changes were not paralleled in PF rats, which used a lower thermogenesis and glucose-preservation strategy which takes a heavier toll on internal fat stores. The effects of OE on BAT energy metabolism are, thus, fairly different from those observed in the liver, which suggests that the mechanism of action of OE is more complex and tissue-specific than previously assumed.

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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

Oleoyl-oestrone inhibits lipogenic, but maintains thermogenic, gene expression of brown adipose tissue in overweight rats

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Table S1 Genes and sequences of the primers used in the present study

Gene	Gene symbol	Forward primer (5′→3′)	Reverse primer (5′→3′)	Size of amplified gene (bp)
Glucose transporter type 4	Slc2a4	CTTGATGACGGTGGCTCTGC	CACAATGAACCAGGGGATGG	127
Hexokinase 2	Hk2	GCCTCAAGACAAGGGGAATCT	CCTCCTTCACGATGATGCTGT	121
Malic enzyme 1	Me1	GGAGTTGCTCTTGGGGTAGTGG	CGGATGGTGTTCAAAGGAGGA	143
Glycerol 3-phosphate dehydrogenase 2	Gpd2	TGGGCTTGAGAGTGAGGTTGC	GCCACCGTTTTCCAGTCACA	98
Phosphoenolpyruvate carboxykinase 1	Pck1	CCCCCTTGTCTACGAAGCTC	ACCTTGCCCTTATGCTCTGC	99
Pyruvate dehydrogenase kinase 2	Pdk2	ATGGAGGGCTTTGGGACAG	CGCCAGGCAGACTTGTTGTAG	95
Pyruvate dehydrogenase kinase 4	Pdk4	GTCAGGCTATGGGACAGATGC	TTGGGATACACCAGTCATCAGC	142
Acetyl-CoA carboxylase 1	Acaca	AGGAAGATGGTGTCCGCTCTG	GGGGAGATGTGCTGGGTCAT	145
Acetyl-CoA carboxylase 2	Acacb	CTGGAACAGCACTGGTCAGC	GGCTCAGGTATGCCACACAG	151
Citrate: ATP lyase	Acly	GACCAGAAGGGCGTGACCAT	GTTGTCCAGCATCCCACCAGT	96
Fatty acid synthase	Fasn	CTTGGGTGCCGATTACAACC	GCCCTCCCGTACACTCACTC	163
Stearoyl-CoA desaturase 1	Scd1	AGAAGGGCGGAAAGCTGGAC	AGTGTGGGCAGGATGAAGCA	112
Glycerol 3-phosphate acyl-transferase	Gpam	GGTGAGGAGCAGCGTGATTG	GGACAAAGATGGCAGCAGAGC	127
Fatty acid-transport protein 1	Slc27a1	GTGCGACAGATTGGCGAGTT	TGCGTGAGGATACGGCTGTT	107
Fatty acid translocase	Cd36	TTGGATGTGGAACCCATAACTG	TGGTCCCAGTCTCATTTAGCC	154
Fatty acid-binding protein 3	Fabp3	GACCAAGCCGACCACAATCA	TTTGCCTCCGTCCAGTGTCA	166
Fatty acid-binding protein 4	Fabp4	CCTTTGTGGGGACCTGGAAA	TGACCGGATGACGACCAAGT	152
Carnitine palmitoleoyl transferase 1β	Cpt1a	CCGCTCATGGTCAACAGCA	CAGCAGTATGGCGTGGATGG	105
Acyl-CoA dehydrogenase, medium chain	Acadm	TCGGAGGCTATGGATTCAACACTG	AGCGTAGTTACATGAGGGTGAAGC	185
Acyl-CoA dehydrogenase, long chain	Acadl	ATGTGGGAATACCCGATTGC	GGATTTCAGGATGTAGGCAGATG	141
Acyl-CoA dehydrogenase, very long chain	Acadvl	GGAGCCATTGACCTCTACGC	TTTCTCATGCTGTGCTGTCG	90
Hormone-sensitive lipase	Lipe	CAAGCCCCATAAGACCCCATT	CCGTAAGTCGCCCAGAATCC	68
Adiponutrin	Pnpla3/Adpn	GTGTGCCCGAATGACCATGT	GCCTTGGGGTTTGTGGAGAG	11
Adipose triacylglycerol lipase (desnutrin)	Pnpla2/Atgl	CGGTGGATGAAGGAGCAGACA	TGGCACAGACGGCAGAGACT	138
Lipoprotein lipase	Lpl	GAAGGGGCTTGGAGATGTGG	TGCCTTGCTGGGGTTTTCTT	103
Perilipin	Plin	GAGGGGCTGATCTGGCTTTG	GCATCTTTTGCCGTCCTGAA	102
UCP1	Ucp1	CCTCTCCGGTGGATGTGGTAAA	CGCAGAAAAGAAGGCGCAAA	142
β_1 adrenergic receptor	Adrb1	TTCAACTGGCTGGGCTACGC	CAAAGCAGGCGCTGGAAA	92
β_2 adrenergic receptor	Adrb2	GGACAGACTACACAGGGGAGCA	CCAGGGGCTTCCTCACAAA	82
β_3 adrenergic receptor	Adrb3	GGCTATGAAGGTGAGCGTCCA	CTGAGCAGAAATCAAGGGCTG	149
Phosphodiesterase 3b	Pde3b	GCCAGGTGTGCATCAAATTAGC	CCAGGGTTGCTTCTTCATCTCC	123
5′-Triiodothyronine deiodinase type II	Dio1	GACGACTTTGCCTCCACAGC	TCCACCACCACAGGACACTG	176
Oestrogen receptor α	Esr1	GCCGAAATGAAATGGGCACT	ACAGGGCGGGGCTATTCTTC	99
Thyroid hormone receptor α	Thrb	GTGACGGACCTGCGGATGAT	TCCAAGAAGAGAGGCGGGAAG	98
PPARα	Ppara	TTCAATGCCCTCGAACTGGA	GCACAATCCCCTCCTGCAAC	124
PPARγ1	Pparg1	CACTTTCTGACCGGACTGTGTG	AAGTTGGTGGGCCAGA	144
Vascular endothelial growth factor	Vegfa	CGTCCTGTGTGCCCCTAATG	TGTGCTGGCTTTGGTGAGGT	126
Transcriptional factor A (mitochondrial)	Tfam	TGATCTCATCCGTCGCAGTGT	GTGCCCAATCCCAATGACAAC	89
Phosphatase and tensin homologue	Pten	TGGGAAAGGACGGACTGGTG	CATAGCGCCTCTGACTGGGAAT	146
Nuclear respiratory factor 1	Nrf1	TGCTGTGGCTGATGGAGAGG	GCTTGCGTCGTCTGGATGGT	78
SREB1c	Srebf1	AAAACCAGCCTCCCCAGAGC	CCAGTCCCCATCCACGAAGA	153
ChREBP	ChREBP	TACTGTTCCCTGCCTGCTCTCC	ACTGCCCTTGTGGCTTGCTC	116
Thyroid hormone-responsive protein (Spot 14)	Thrsp	GCACTTCTCCAGCCTCCATCA	GACCTGCCCCGTCATTTCCT	91

Table S2 Nucleic acid content and cellularity in interscapular BAT of OE-treated and PF male overweight rats
The data are expressed as fmol of the corresponding mRNA in the whole interscapular BAT and are the means ± S.E.M. for eight different animals. *$P < 0.05$ compared with controls; †$P < 0.05$ between PF and OE-treated rats.

mRNA	Control group (fmol)	PF group (fmol)	OE group (fmol)
GLUT4	19.9 ± 2.1	12.7 ± 1.9*	11.3 ± 1.3*
Hexokinase 2	2.08 ± 0.27	1.30 ± 0.11*	1.35 ± 0.12*
Malic enzyme 1	120 ± 13	95.9 ± 14	27.9 ± 3.1*†
Glycerol 3-phosphate dehydrogenase 2	33.0 ± 3.1	26.4 ± 2.1	26.3 ± 2.3
Phosphoenolpyruvate carboxykinase 1	134 ± 18	251 ± 47*	160 ± 13†
Pyruvate dehydrogenase kinase 2	15.7 ± 1.0	17.6 ± 1.3	11.3 ± 0.7*†
Pyruvate dehydrogenase kinase 4	22.8 ± 3.0	35.8 ± 7.4	17.4 ± 1.3†
Acyl-CoA synthetase (long chain)	61.5 ± 6.3	64.8 ± 3.8	66.2 ± 4.8
Acetyl-CoA carboxylase 1	38.0 ± 4.4	27.9 ± 2.7	17.2 ± 2.0*†
Acetyl-CoA carboxylase 2	16.7 ± 1.5	23.4 ± 3.0*	12.2 ± 0.9*†
Citrate: ATP lyase	59.8 ± 4.4	21.9 ± 4.1*	18.4 ± 2.3*
Fatty acid synthase	433 ± 55	266 ± 28*	191 ± 21*
Stearoyl-CoA desaturase 1	184 ± 37	233 ± 27	8.0 ± 3.1*†
Glycerol 3-phosphate acyl-transferase	11.3 ± 1.4	4.9 ± 0.3*	4.2 ± 0.4*
Fatty acid-transport protein 1	17.0 ± 1.5	16.4 ± 0.9	10.8 ± 1.6*†
Fatty acid translocase	99.0 ± 8.1	93.9 ± 5.5	87.9 ± 4.4
Fatty acid-binding protein 3	39.6 ± 3.6	16.6 ± 1.8*	48.4 ± 7.9†
Fatty acid-binding protein 4	823 ± 92	611 ± 44	764 ± 46
Carnitine palmitoleoyl transferase 1β	50.9 ± 7.0	41.8 ± 6.4	48.3 ± 3.3
Acyl-CoA dehydrogenase (medium chain)	153 ± 17	89 ± 10*	124 ± 12
Acyl-CoA dehydrogenase (long chain)	297 ± 37	188 ± 23	216 ± 37
Acyl-CoA dehydrogenase (very long chain)	36.9 ± 4.5	30.5 ± 3.6	34.0 ± 3.1
Hormone-sensitive lipase	59.3 ± 7.6	60.0 ± 3.7	51.8 ± 4.7
Adiponutrin	24.6 ± 4.0	2.9 ± 0.2*	4.7 ± 0.6*†
Adipose triacyl-glycerol lipase (desnutrin)	226 ± 21	288 ± 36	193 ± 14†
Lipoprotein lipase	67.0 ± 6.1	95.8 ± 16*	73.4 ± 4.3†
Perilipin	45.9 ± 6.0	39.1 ± 3.0	43.3 ± 3.6
UCP1	487 ± 57	243 ± 29*	402 ± 56†

Table S3 Gene expression of the metabolic control-related, hormone-signalling pathway, and adipose tissue differentiation and proteins in BAT of male overweight rats

The data are expressed as fmol of the corresponding mRNA in the whole interscapular BAT mass and are the means ± S.E.M. of eight different animals. *$P < 0.05$ compared with controls; †$P < 0.05$ between PF and OE-treated rats.

mRNA	Control group (fmol)	PF group (fmol)	OE group (fmol)
β_1 adrenergic receptor	2.33 ± 0.38	0.60 ± 0.08*	0.99 ± 0.08*
β_2 adrenergic receptor	0.65 ± 0.07	1.36 ± 0.10*	0.58 ± 0.05†
β_3 adrenergic receptor	11.6 ± 1.2	9.5 ± 0.5	9.3 ± 0.6
Phosphodiesterase 3b	1.44 ± 0.29	1.58 ± 0.22	1.71 ± 0.19
Oestrogen receptor α	0.089 ± 0.014	0.192 ± 0.036*	0.118 ± 0.012†
Triiodothyronine 5′-deiodinase type II	2.17 ± 0.24	0.86 ± 0.21*	0.94 ± 0.18*
thyroid hormone receptor α	1.15 ± 0.15	1.54 ± 0.10	1.11 ± 0.15
PPARα	3.56 ± 0.59	7.22 ± 0.25*	5.13 ± 0.61†
PPARγ1	0.98 ± 0.09	1.12 ± 0.16	0.88 ± 0.03*†
PPARγ coactivator 1α	1.52 ± 0.22	1.61 ± 0.27	0.90 ± 0.12*†
Vascular endothelial growth factor A	8.65 ± 1.09	7.98 ± 0.86	6.60 ± 0.44
Transcriptional factor A (mitochondrial)	4.95 ± 0.48	4.41 ± 0.38	4.06 ± 0.28
Phosphatase and tensin homologue	5.84 ± 0.43	4.40 ± 0.67	2.78 ± 0.29*
Nuclear respiratory factor 1	0.59 ± 0.06	0.72 ± 0.08	0.44 ± 0.02*†
SREBP1c	9.81 ± 2.00	9.2 ± 1.5	10.3 ± 1.3
ChREBP	11.4 ± 1.1	4.2 ± 0.5*	5.1 ± 0.5*
Thyroid hormone-responsive protein (Spot 14)	993 ± 87	527 ± 51*	474 ± 34*

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3.2.5 Modulación de la expresión génica del metabolismo del colesterol en el hígado de ratas por la oleoil estrona

La oleoil estrona (OE) disminuye los niveles de colesterol circulante en rata.

En este trabajo se analizaron las expresiones de genes relacionados con el metabolismo del colesterol en el hígado tras el tratamiento con OE. Se compararon ratas macho con sobrepeso tratadas con una dosis oral diaria de OE (10 nmol/g) con un grupo control y un grupo *pair fed*. Se determinaron algunos parámetros lipídicos en el suero e hígado y su contenido de colesterol. El ARN total del hígado se utilizó para el análisis de la expresión de los genes que codifican las enzimas y factores de regulación implicados en el metabolismo del colesterol en el hígado mediante la PCR en tiempo real. Se estimaron los niveles del transportador ABCA1 y colesterol 7α-hidroxilasa mediante *Western blot*.

Los grupos OE y *pair fed* disminuyeron la expresión de la 3-hidroxi-3-metil-glutaril-CoA sintasa. El grupo OE aumentó la expresión del receptor de LDL. La eliminación de colesterol, a través de la síntesis de ácidos biliares, se incrementó en el grupo *pair fed,* pero más notablemente en el grupo OE. La expresión génica del transportador ABCA1 y de las apolipoproteínas A1 y E se incrementó en el grupo OE. La expresión del receptor X del hígado fue menor en el grupo *pair fed* que en los grupos OE y control.

En conclusión, la rápida desaparición de colesterol circulante provocada por la administración de OE es consecuencia de: (1) la disminución de la actividad de la vía del mevalonato, (2) una mayor expresión del receptor de LDL, y (3) la activación de la oxidación de colesterol para formar ácidos biliares como consecuencia del incremento de colesterol en el hígado, aunque esta última vía también parece incrementar debido a la disminución de la disponibilidad energética.

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ORIGINAL ARTICLE

Gene expression modulation of rat liver cholesterol metabolism by oleoyl-estrone

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Summary
Objective: Since oleoyl-estrone (OE) decreases circulating cholesterol in the rat, we analyzed the response to OE treatment of hepatic gene expressions related with cholesterol metabolism.
Methods: Male overweight rats treated with oral OE (10 nmol/g daily) were compared with a pair-fed (PF) group and controls fed *ad libitum*. Serum parameters and liver lipid and cholesterol contents were measured. Total tissue RNA was used for real-time PCR analysis of the gene expression of enzymes and regulatory factors of liver cholesterol metabolism. Cholesterol-7α-hydroxylase and ABC transporter A1 protein levels were estimated by Western blot.
Results: Pair-feeding and OE treatment reduced the expression of 3-hydroxy-3-methyl-glutaryl-CoA synthase. OE increased the expression of the LDL receptor. Cholesterol disposal, through bile acids synthesis, was increased in PF and more markedly in OE rats. Gene expressions of the ABC transporter A1 and apolipoproteins A1 and E were increased in OE rats. The expression of liver X receptor was lower in PF than in OE and controls.
Conclusion: The rapid disappearance of circulating cholesterol elicited by OE is consequence of: (1) decreased mevalonate pathway activity, (2) a higher expression of the LDL-receptor, and (3) the activation of the oxidation of cholesterol to form bile acids as a consequence of the higher cholesterol concentrations found in liver, also affected by energy availability.

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Introduction

Oleoyl-estrone (OE) is a powerful slimming hormone ester synthesized in adipose tissue and related to plasma lipoproteins [1,2]. Its administration to rats decreases food intake [1], and maintains energy expenditure [3], sparing protein [4,5]. This is mainly accomplished through white adipose tissue (WAT) lipid wasting, decreased cell size and increased apoptosis [6,7]. The fatty acids freed by WAT lipolysis are largely used by peripheral tissues as main energy substrate; the OE-induced shift in the distribution of tissue lipoprotein lipase activity favors the use of fat by the muscle, decreasing its liver and WAT storage in obese rats [8]. OE treatment maintains glycemia and lowers insulinemia; with decreased overall glucose utilization and maintenance of liver glycogen stores [9]; thus, no additional liver glucose output is needed, since food-derived glucose is enough to maintain glucose homeostasis. This situation contrasts sharply with the metabolic adaptation elicited by a marked reduction of food intake: glycemia decreases, and is ultimately maintained at the expense of increased liver glucose output [10]. Low glucose elicits the activation of WAT lipolysis and the liver synthesis of ketone bodies [11] in order to save energy and spare glucose and amino acids [12]. This energy-saving strategy results in a decreased overall energy expenditure [13] largely because of diminished thermogenesis [14].

In the rat, OE decreases circulating cholesterol, especially in the HDL fraction [8,15], but whole body cholesterol pool is not altered to a significant extent [15]. The effects of OE on cholesterol levels are fast and primarily affect the cholesteroyl-esters fraction [15]. The effects are more marked on hyperlipidemic obese rats, in which plasma lipids are rapidly normalized [8].

In the process of cholesterol transport from the periphery to the liver, HDL, which contain apoAI and apoAII proteins, act as cholesterol acceptors and are efficiently cleared from the bloodstream by the liver. Cholesterol export from cells requires both the expression of specific transporters of the ATP binding cassette (ABC) superfamily and the presence in the extracellular space of apolipoproteins (i.e. A1 and E) that act as free cholesterol acceptors [16]. These proteins are major components of the reverse cholesterol pathway, and are critical for the disposal of excess cell cholesterol. An excess of cholesterol activates its oxidation by the liver to bile acids [17], which results in increased fecal elimination irrespective of the enterohepatic recycling circuit. In fact, this is the only significant cholesterol excretion pathway.

Changes in plasma cholesterol dynamics may reflect changes in the rates of synthesis or excretion of cholesterol. Since the liver is the main controller of cholesterol metabolism, in this study we analyzed the response of the liver to the challenge of OE-induced massive lipid mobilization on the gene expression of the key enzymes and control factors regulating hepatic cholesterol metabolism.

Since hypercholesterolemia is a first-order risk for cardiovascular disease [18], we decided to investigate further how OE lowers circulating cholesterol as the basis for the pharmacological design of better hypocholesterolemic drugs for humans.

Materials and methods

Animals

Adult male Wistar rats were made overweight by a limited period of cafeteria diet feeding, as previously described [19], reaching a weight of 355 ± 5 g at the beginning of the experiment. Tree groups of 8 rats each were randomly selected: controls, oleoyl-estrone (OE) and pair fed (PF); all animals received, daily for ten days, an oral gavage of 0.2 mL of sunflower oil (7 kJ), supplemented in the OE group with 10 nmol/g oleoyl-estrone (OED, Barcelona, Spain). The control and OE groups had free access to pellet food (maintenance chow, Panlab, Barcelona, Spain); and the PF rats were allowed every day only the amount of food consumed by the OE group; all rats had water available *ad libitum*. PF rats completely ate the food allotted each day.

The animals were kept under standard conditions of housing and feeding, and were handled and killed following protocols approved by the University of Barcelona Animal Welfare and Ethics Committee, in full compliment of the norms and procedures established by the European Union and the Governments of Spain and Catalonia.

Analytical procedures

On day 10, the rats were killed by decapitation, and the blood was allowed to clot to obtain serum, that was kept at $-80\,^{\circ}$C until processed. The liver was also rapidly excised, blotted, weighed, sampled, frozen and stored at $-80\,^{\circ}$C.

Blood serum was used for the measurement of glucose by an enzymatic-colorimetric method (Trinder kit, Sigma, St. Louis, MO, USA), non-esterified fatty acids by an enzymatic-colorimetric method (NEFA kit, Wako Chemicals, Neuss, Ger-

Table 1 Liver parameters in overweight rats after 10-days of treatment with oral OE or pair-fed.

Parameter	Units	Control	Pair-fed	OE-treated
Weight	g	11.3 ± 0.4	7.2 ± 0.2*	9.9 ± 0.5*°
Total lipids	mg/g	41.6 ± 1.9	44.8 ± 1.7	48.3 ± 2.7
	mg	459 ± 26	395 ± 6	451 ± 26
Cholesterol	mg/g	1.89 ± 0.20	2.68 ± 0.35	3.23 ± 0.21*
	mg	21.4 ± 2.3	19.3 ± 2.5	32.0 ± 2.1*°

The data are the mean ± SEM of 8 different animals. Differences between groups: *$P < 0.05$ versus controls; °$P < 0.05$ between OE and pair-fed groups.

many), total triacylglycerols by a lipase-glycerol kinase spectrophotometric method (kit 11528, Biosystems, Barcelona, Spain), and total cholesterol (both in serum and liver homogenates) by an enzymatic-colorimetric method (Cholesterol reagent easy, Menarini, Firenze, Italy). Liver lipids were extracted with trichloromethane—methanol (2:1), dried and weighed [20].

Total tissue RNA was extracted using the Tripure reagent (Roche Applied Science, Indianapolis, IN, USA), and was quantified in an ND-100 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA samples were reverse-transcribed using the MMLV reverse transcriptase (Promega, Madison, WI, USA) and oligo-dT primers.

Real-time PCR (RT-PCR) amplification was carried out using 0.010 mL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems). The primers used for the estimation of gene expression in liver are presented in Table S1 of the *Supplemental online material*.

A semiquantitative approach for the ultimate estimation of the number of copies of each expressed gene mRNAs per tissue weight was used as previously described [21]. In any case, cyclophilin was used as charge control gene in all samples.

Liver samples were homogenized in a buffer containing 1× Tris buffered saline, 1% Igepal, 0.5% sodium deoxycholate, 0.1% sodium lauryl-sulfate, 1 mM phenylmethylsulphonyl fluoride, 1 mM sodium orthovanadate and protease inhibitor mix complete (Sigma, St. Louis MO, USA). Aliquots of 0.05 mg of supernatant liver proteins were separated by SDS—PAGE in a 7.5% gel, and electrotransferred onto a PVDF-membrane (Millipore, Billerica, MA, USA). Western blot analysis of cholesterol-7α-hydroxylase and ABC transporter A1 was performed using goat polyclonal antibodies for cholesterol-7α-hydroxylase (N-17) and ABC transporter A1 (T-15) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal antibodies for tubulin (DM1A) (Abcam, Cambridge, UK), antigoat and antimouse secondary antibodies (IgG-HRP human absorbed; Santa Cruz Biotechnology), and the fluorescence detection system (ECL™ Detection Reagents; GE Healthcare, Amersham, Buckinghamshire, UK) performed with a Total-Lab v2003.3 program (Non-Linear Dynamics Ltd., Newcastle, UK).

Statistical analysis

Statistical comparison between groups was established by using the unpaired Student's *t* test with a limit of significance of $P < 0.05$.

Table 2 Serum metabolites in overweight rats after 10-days of treatment with oral OE or pair-fed.

Parameter	Control	Pair-fed	OE-treated
Glucose	8.01 ± 0.09	6.02 ± 0.15*	7.19 ± 0.13*°
Triacylglycerols	1.58 ± 0.13	0.62 ± 0.05*	0.47 ± 0.06*
Non-esterified fatty acids	0.35 ± 0.03	0.50 ± 0.07*	0.28 ± 0.05°
Total cholesterol	1.36 ± 0.03	1.46 ± 0.11	0.53 ± 0.11*°

The data are the mean ± SEM of 8 different animals. All units are expressed in mM. Differences between groups: *$P < 0.05$ versus controls; °$P < 0.05$ between OE and pair-fed groups.

Table 3 Liver expression of cholesterol and lipoprotein metabolism indicators of male overweight rats treated with oral OE or pair-fed.

Protein	Gene	Units	Whole liver mRNAs content		
			Control	Pair-fed	OE-treated
3-Hydroxy-3-methyl-glutaryl-CoA synthase 1	*Hmgcs1*	fmol	1009 ± 105	624 ± 46*	634 ± 77*
3-Hydroxy-3-methyl-glutaryl-CoA reductase	*Hmgcr*	fmol	212 ± 26	163 ± 28	173 ± 17
Lecithin-cholesterol acyl-transferase	*Lcat*	pmol	2.25 ± 0.07	1.84 ± 0.10	2.34 ± 0.13°
Cholesterol-7α-hydroxylase	*CyP7a1*	fmol	181 ± 42	398 ± 56*	740 ± 140°*
ABC transporter A1	*Abca1*	fmol	111 ± 10	111 ± 11	139 ± 8°*
Scavenger receptor class B1	*Scarb1*	fmol	211 ± 11	213 ± 15	201 ± 11
LDL-receptor	*Ldlr*	fmol	245 ± 21	127 ± 6*	350 ± 25*°
Apolipoprotein A1	*Apoa1*	pmol	8.77 ± 0.51	7.28 ± 0.62	13.1 ± 1.1*°
Apolipoprotein A2	*Apoa2*	pmol	30.7 ± 2.0	28.4 ± 1.3	28.3 ± 2.5
Apolipoprotein E	*Apoe*	pmol	96.2 ± 7.0	89.5 ± 7.1	128.2 ± 9.5*°
SREBP2	*Srebf2*	fmol	201 ± 17	171 ± 18	209 ± 12
Liver X receptor α	*LxRa*	pmol	2.63 ± 0.29	1.86 ± 0.07*	2.33 ± 0.15°
Liver X receptor β	*LxRb*	fmol	109 ± 6	88 ± 7*	128 ± 12°

The data are the mean ± SEM of 8 different animals. Differences between groups: *$P < 0.05$ versus controls; °$P < 0.05$ between OE and pair-fed groups.

Results

Body weight and liver content

Control rats increased their weight by 2.6 ± 0.4% in the 10-day experiment; PF lost 8.5 ± 0.8% and OE rats lost 9.9 ± 0.8%. Mean food intake of controls was 18.0 ± 0.2 g/d, and that of OE (and, thus, PF) was 10.4 ± 0.3 g, representing a mean 58 ± 2% of the food (energy) ingested by controls.

Table 1 shows the liver weights and total lipid and cholesterol content in the liver of control, pair-fed and OE-treated rats. Animals with decreased energy intake (i.e. PF and OE) had lower liver weights that controls; in PF animals the liver weight was significantly smaller than in the OE group. There were no significant differences in total liver lipids between groups, but OE-treated rats showed higher liver cholesterol values, both when expressed per unit of tissue weight or as whole liver content.

Serum parameters

Table 2 shows serum glucose and lipid values after ten days of treatment. Despite identical energy intake of PF and OE, only triacylglycerols showed a similar decline in both groups of animals compared to controls. In all other parameters, different effects were found between PF and OE. In the animals treated with OE there was a relative maintenance of serum glucose (in the range of 90% of controls), not observed in the pair-fed group (75% of the values of controls). PF animals, but not the OE group, also showed higher concentrations of non-esterified fatty acids. The animals treated with OE also showed lower cholesterol levels, in the range of 40% of those of control and PF animals.

Gene expression and protein levels

Table 3 shows the gene expressions of key liver cholesterol metabolism enzymes and control factors for all experimental groups, expressed as total liver content of the corresponding mRNAs. It is interesting to note that mRNAs for apolipoproteins, synthesized for their exportation with lipid in lipoproteins, were found in higher concentrations (i.e. one or two orders of magnitude higher) than other transcripts corresponding to enzymes, transporters or receptors.

Pair-feeding and OE treatment reduced the expression of cytosolic 3-hydroxy-3-methyl-glutaril-CoA synthase, the first enzyme of cholesterol synthesis (mevalonate pathway). However PF also diminished the expression of the LDL receptor, whereas OE increased it. The expression of the lecithin: cholesterol acyl-transferase was maintained at the same level for all three groups. Disposal of cholesterol toward the synthesis of bile acids, as shown by the expression of the cholesterol-7α-hydroxylase gene, was increased in PF and OE rats; the mean values for the latter, however, were fourfold those of the controls, suggesting an important activation of the disposal pathway. In addition, the gene expressions of the ABC transporter A1, a marker of reverse choles-

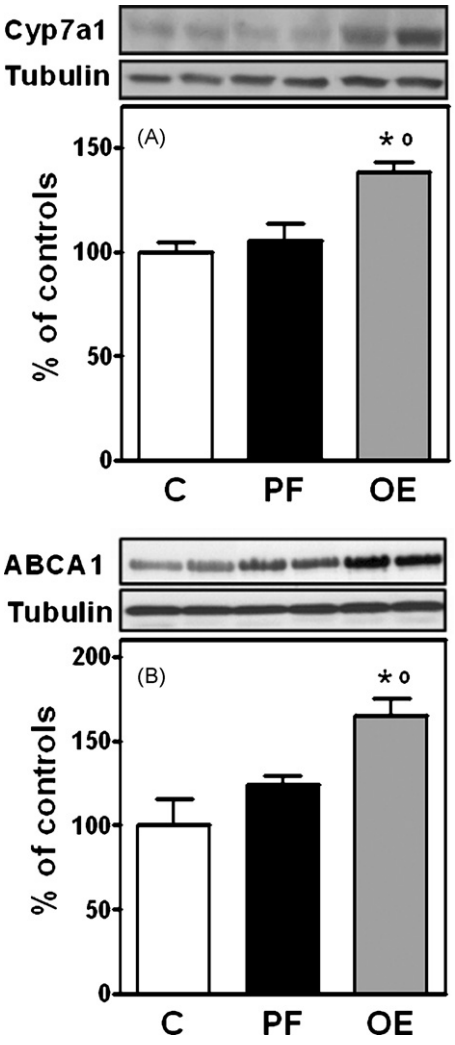

Figure 1 Quantification by Western blot analysis, of cholesterol-7α-hydroxylase (panel A), and ABC transporter A1 (panel B) proteins in liver. Data are expressed as arbitrary units per gram of liver protein. *Significant ($P < 0.05$) difference respect to controls, and ◦ significant ($P < 0.05$) difference between OE and PF groups.

terol transport, and apolipoproteins A1 and E were increased in OE rats compared with controls and PF.

Pair-feeding significantly decreased the expression of the liver X receptor α and β, but not that of SREBP2 when compared with both the control and OE groups. In spite of different enzyme-related gene expression changes, there were no differences between OE and controls for the expression of any of the transcription factor genes studied.

Fig. 1 shows the cholesterol-7α-hydroxylase and ABC transporter A1 protein levels in liver, which were increased in the OE group compared with both, controls and PF animals.

Discussion

As previously described, the loss of body weight of OE-treated rats is largely a consequence of decreased energy intake [9]. However, liver weight and serum parameters were widely different between OE and PF rats, in spite of their identical energy input. Low energy availability induces lower serum glycemia and compensatory glycogenolysis and gluconeogenesis, decreasing liver glycogen levels [9], and, consequently, liver weight. This effect, classically found in PF, was not observed in OE-treated rats, which maintained their hepatic energy content unchanged. The PF rats show a lipid metabolism pattern typical of energy restriction, i.e. decreased serum triacylglycerols and increased non-esterified fatty acids, a telltale of the mobilization of peripheral (mainly WAT) fat stores.

Although OE-treated rats, similarly to the PF animals, showed a severe shrinkage of fat reserves [6], serum non-esterified fatty acid levels did not increase in the OE group, with values similar to those of controls, suggesting the existence of a high uptake of circulating fatty acids by the liver, where they were esterified to form triacylglycerols — as suggested by the high expression of the hepatic genes involved in its synthesis [22] — and released as triacylglycerol-rich lipoproteins, accompanied by its higher peripheral utilization, which may explain the decreased serum triacylglycerols of OE rats. This interpretation agrees with the increase in muscle drainage of plasma lipids through enhanced lipoprotein lipase activity, as observed in obese rats [8].

The lack of differences, versus controls, of the cholesterol/triacylgycerol ratio of OE rats suggests that not only there is a higher turnover of triacylglycerol-rich lipoproteins in OE rats, but also an increased turnover of cholesterol-rich lipoproteins. In liver, this same ratio, twice in OE than in control rats, suggest that in spite of a parallel increase in serum triacylglycerol and cholesterol turnovers, these changes can be attributed to different mechanisms.

In OE-treated rats, the high liver cholesterol, coupled with the lack of significant changes in total body cholesterol pool [15], suggests that liver accumulation is the primary factor responsible for the drop in serum cholesterol. This is in agreement with a higher expression of liver LDL receptor gene, a main regulatory step for adjusting the import of cholesterol [23].

High liver cell cholesterol strongly inhibits cholesterol hepatic synthesis; this agrees with the observed inhibition of the expression of 3-hydroxy-3-methyl-glutaryl-CoA synthase. The expression of

the LDL receptor is largely downregulated by SREBP2 [24], which contrasts with our data showing that SREBP2 expression is not affected by OE in spite of a marked increase in the LDL receptor. In addition, since SREBP2 posttranscriptional activation is mainly up-regulated by cholesterol levels, we expected a LDL receptor expression change (under OE treatment, i.e. high liver cholesterol) in the opposite direction to that found.

We have previously described [22] that the effect of OE on liver metabolism may be — at least in part — mediated through an insulin sensitivity-dependent modulation of the expression of SREBP1c. Since both, SREBP1c and SREBP2, activate the expression of the LDL receptor in liver [25] we can hypothesize that the higher expression of LDL-receptors in OE-treated animals may be a consequence of the OE-induced increase in hepatic insulin sensitivity. Although oral administration of OE to rats has no significant estrogenic effects, and estradiol levels increase only to a very limited extent after oral OE administration [26], we cannot overlook that OE effects on the LDL receptor could be at least in part mediated by OE-derived estrogen, since a large share of the protective influence of estrogens in cardiovascular disease is believed to be mediated by effects on cholesterol metabolism: estradiol increases the number of liver LDL receptors at pharmacological doses [27].

The transient accumulation of cholesterol from plasma in the liver of OE-treated animals may help explain how OE affected cholesterol metabolism in a way parallel to that described for lipid and lipoprotein release. There was a statistically significant correlation between the expression of cholesterol-7α-hydroxylase and liver cholesterol levels ($r = 0.6756$; $p = 0.0006$) for all experimental groups taken together, reinforcing the idea that liver cholesterol levels are probably, the main factor responsible for the increase in cholesterol disposal as bile acids, increasing proportionally the expression of the main enzyme regulating cholesterol catabolism, cholesterol-7α-hydroxylase [28]. However, the liver levels of this enzyme protein were not correlated with liver cholesterol ($r = 0.245$; $p = 0.342$). There is not, then, a direct relationship between mRNA and protein levels for this key liver enzyme, despite transcriptional regulation being considered the main mechanism controlling 7α-hydroxylase activity [29]. However, changes in mRNA do not always correlate with changes in enzyme activity [30,31]. In our experimental setup, the cholesterol-7α-hydroxylase protein/mRNA ratio was a similar fraction of that of controls, both for OE and pair-fed animals: $24 \pm 8\%$ and $32 \pm 8\%$, respectively ($P < 0.05$). The lower relative protein abundance versus expression of pair-fed and OE rats, compensated in the latter for a more marked level of expression, hint at energy restriction hampering the actual synthesis of protein.

The ABC transporter A1, which plays a major role in regulating plasma HDL levels and the cholesterol reverse transport, mediating and rate-limiting HDL biogenesis, is also up-regulated by increased cell cholesterol [32].

LXR is the major transcription factor that acts as a sensor of cholesterol levels via its interaction with oxysterols [33]. It was initially characterized by its role in the positive regulation of the gene encoding cholesterol-7α-hydroxylase [34], but it also up regulates the expression of the ABC transporter A1 [32] and ApoE [35]. Since OE rats showed higher expression and protein levels of cholesterol-7α-hydroxylase, and ABC transporter A1 and higher ApoE expressions, compared with PF animals, probably these effects on cholesterol metabolism may be regulated, at least in part, by LRX, although other additional factors, as the redox potential or cell energy availability, could be involved (helping to explain the differences between OE-treated and control animals).

In spite of the unexpected — and so far unexplained — lack of inhibition of LDL receptor expression, this may help the liver of OE rats to take up cholesterol for ulterior processing and (mainly) excretion in a globally fairly well orchestrated process.

The rapid disappearance of circulating cholesterol elicited by OE is the consequence of three main hepatic factors: (1) decreased mevalonate pathway activity, (2) a higher expression of the LDL-receptor that explains the higher cholesterol uptake by liver and faster body cholesterol turnover, and (3) the activation of the oxidation of cholesterol to form bile acids as a consequence of the higher cholesterol concentrations found in liver, also affected by energy availability.

These combined factors allow for an assumedly steady transfer of liver cholesterol to lipoproteins for export. The sum of reverse transport, diminished synthesis and enhanced disposal to bile acids accounts for the overall removal of circulating cholesterol and increased liver cholesterol metabolism.

In summary, OE is effective in enhancing cholesterol disposal through its combined action facilitating liver uptake, blocking further synthesis, and increasing disposal in addition to increasing its plasma turnover through enhanced overall lipoprotein handling. The possibilities of this multifaceted effect of OE on this target may open the way for the

development of new hypocholesterolemic drugs for human use.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.orcp.2009.09.001.

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3.3 Relaciones de la oleoil estrona con las hormonas esteroideas

3.3.1 La oleoil estrona incrementa la expresión génica de la síntesis adrenal de corticosteroides en ratas macho con sobrepeso

La oleoil estrona (OE) induce una marcada pérdida de grasa corporal en ratas a través del mantenimiento del gasto energético, de la proteína corporal y de la glucosa sanguínea a pesar de la disminución de la ingesta de alimentos. La OE también promueve un incremento de los glucocorticoides que contrarresta la movilización de lípidos.

Se estudió si la OE inducía un efecto directo sobre la función de las glándulas adrenales como parte de esta regulación por retroalimentación. Para ello se utilizaron ratas macho con sobrepeso a las que se les administró diariamente una dosis oral de 10 nmol OE/ g peso corporal durante diez días. Se comparo con un grupo de animales control y un grupo de animales *pair fed*, ambos grupos recibieron la dosis oral sólo del vehículo (aceite de girasol).

Los animales del grupo OE perdieron un poco más de peso corporal que el grupo *pair fed*, pero sus glándulas adrenales aumentaron de tamaño. En las adrenales, los niveles de corticosterona y las expresiones de genes que codifican las enzimas de la vía de síntesis de los glucocorticoides se incrementaron en el grupo OE frente al grupo control y *pair fed*, indicando que el crecimiento suprarrenal afectaba esencialmente la zona cortical, ya que además, la OE redujo la expresión de los genes que codifican para las enzimas de síntesis de catecolaminas (de localización medular). Los niveles de corticosterona en suero fueron mayores en el grupo *pair fed* que en el grupo OE y control, pero la expresión hepática del enzima 5α-reductasa 1, la principal responsable del catabolismo de los corticosteroides, fue tres veces mayor en los animales tratados con OE que en los *pair fed* y control. Los niveles de glucocorticoides circulantes mostraron pocos cambios por efecto de la OE a pesar de su mayor contenido, tanto en las glándulas adrenales como en el hígado, indicando la importancia de la regulación del recambio de los glucocorticoides en la para modulación de su actividad.

En conclusión, en la rata, la OE promueve un considerable aumento de la expresión de los genes que controlan la síntesis de glucocorticoides a partir del colesterol, incrementando en consecuencia el contenido de corticosterona en la glándula adrenal, al igual que el tamaño de su corteza y su celularidad. Pero a la vez, la OE incrementa la eliminación hepática de los corticosteroides, lo que sugiere que la OE aumenta la síntesis y degradación de estas hormonas (es decir, su recambio en plasma), un proceso que no se rige por la limitación de la energía disponible, sino que está directamente relacionado con la OE.

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Oleoyl-estrone increases adrenal corticosteroid synthesis gene expression in overweight male rats

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ABSTRACT

Oleoyl-estrone (OE) induces a marked loss of body fat in rats by maintaining energy expenditure, body protein and blood glucose despite decreasing food intake. OE increases glucocorticoids, but they arrest OE lipid-mobilization. We studied here whether OE induces a direct effect on adrenal glands function as part of this feedback regulation. Dietary overweight male rats were given oral 10 nmol/g OE gavages for ten days. A group (PF) of pair-fed to OE rats, and controls received vehicle-only gavages. OE rats lost slightly more body than PF, but had larger adrenal glands. Tissue corticosterone levels, and gene expressions for glucocorticoid-synthesizing enzymes were increased in OE versus controls and PF; thus, we assumed that adrenal growth affected essentially its cortex since OE also lowered the expression of the medullar catecholamine synthesis enzyme genes. Serum corticosterone was higher in PF than in OE and controls, but liver expression of corticosteroid-disposing steroid 5α-reductase was 3× larger in OE than PF and controls. Circulating glucocorticoids changed little under OE, in spite of higher adrenal gland and liver content, hinting at modulation of glucocorticoid turnover as instrumental in their purported increased activity. In conclusion, we have observed that OE considerable enhanced the expression of the genes controlling the synthesis of glucocorticoids from cholesterol in the rat and increasing the adrenal glands' corticosterone, size and cellularity, but also the liver disposal of corticosteroids, suggesting that OE increases corticosterone synthesis and degradation (i.e. serum turnover), a process not driven by limited energy availability but directly related to the administration of OE.

1. Introduction

Glucocorticoids play a key role in the body response to aggression, largely controlling the hystic response to extraneous or potentially dangerous compounds and restoring the alterations induced by the body responses to insult, exercise or harm [1]. The soothing effect of glucocorticoids, i.e. after a catecholamine-driven muscular exercise, largely consists in: controlling the immune system responses, restoring the energy metabolism parameters and activate the reparation of damages [2]. Stress, continuing dietary aggression and other prolonged situations eliciting a glucocorticoid response may result in overstretching their corrective function, depressing a number of paths below normalcy: altered circadian rhythms [3], appetite [4] and sleep [5], depression [6], deregulation of inflammatory [7] and immune [8] responses and a derangement of the energy homeostasis, starting with insulin resistance [9] and continuing with severe accumulation of fat in the blood, liver, muscle and adipose tissue [10], which starts the cascade of counteractions that generate the multiple facets of the metabolic syndrome [11].

The metabolic syndrome, and obesity as one of its main pathological manifestations is directly related to inflammation [12]. It is widely accepted that the alteration of adipose tissue endothelial function and signalling may be the consequence of long-term or sustained excess nutrient availability [13]; this results in a continued saturation of the adipose tissue capability to store fat that elicits a cytokine-driven response [14,15] that, in the long-term, results in additional alteration of energy metabolism and homeostasis [16]. The basis of the tissue insult lies on the endothelium, which inflammation (involving active radicals of oxygen and nitrogen) [12,17] starts a response cascade in which the counteractive actions of adipokines, cytokines and other hormonal signals, both peptidic and steroidal acquire a protagonist role [17,18]. The removal of the

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adrenal gland prevents or even reverses obesity in several animal models [19,20] enhancing the slimming effects of leptin [21] and oleoyl-estrone [22].

Oleoyl-estrone (OE) is a powerful slimming agent [23] that induces an energy imbalance [24] by decreasing food intake and maintaining energy expenditure [25]. The action of OE results in the maintenance of body protein [23,24] and glycemia as well as liver glycogen [26]. But muscle utilization of lipid in obese rats is enhanced in parallel to the normalization of circulating lipids and the wasting of overall body fat [27]. Oral OE administration does not produce estrogenic effects [28], in spite of the considerable release of estrone by tissue OE hydrolysis [29], because conversion of estrone into estradiol is limited by OE itself via inhibition of 17β-hydroxysteroid dehydrogenase [30]. The administration of estrone alone does not induce the slimming effects of OE either [31]; the effects of OE, thus, could not be explained by estrogenic effects, irrespective of the limited increase in circulating estrogen induced by OE treatment [32]: OE is a ponderostat signal [33] rather than a simple estrogen-carrier molecule.

OE increases tissue glucocorticoid availability in female rats by (a) increasing hypothalamic CRH, plasma corticotropin and circulating corticosterone [34,35], (b) decreasing plasma binding, thus enlarging the free fraction of corticosterone [36], and (c) increasing the expression and activity of 11β-hydroxysteroid dehydrogenase in the liver of adrenalectomized rats, even after compensatory corticosterone administration [37].

Corticosterone administration inhibits the lipid-mobilizing action of OE in adrenalectomized rats [38]. The overall effects of OE on energy metabolism (including insulin resistance) are fairly opposed to those of glucocorticoids, which may suggest that the rise in corticosteroids may be a reaction to the energy-wasting counteractive action of OE.

In the present study we intended to check whether the effects of OE on the circulating glucocorticoids of overweight (but otherwise normal) rats are a consequence of altered adrenal function. We included a pair-fed group in the study as a way to discern whether the effects observed were due to OE action or to the decreased energy intake consequence of this treatment.

2. Experimental

2.1. Animals and sample preparation

Adult male Wistar rats were made overweight by a limited period of cafeteria diet feeding, as previously described [39]. The rats, initially weighing 355 ± 5 g, were kept under standard conditions of housing and feeding [39]. Three groups of 8 rats each were randomly selected: controls, OE and 'pair-fed' (PF). All animals received every day an oral gavage of 0.2 mL of sunflower oil (7 kJ), which was supplemented in the OE group with 10 nmol/g oleoyl-estrone (OED, Barcelona, Spain). The controls and OE group had free access to pellet food (maintenance chow, Panlab, Barcelona, Spain), and the PF rats were allowed to eat only the mean food consumption on the matching day of the OE group; all rats had water available *ad libitum*. On day 10, the rats were killed by decapitation and the adrenal glands, and liver were dissected, weighed, frozen in liquid nitrogen and kept at −80 °C. Blood samples were allowed to clot and serum was also stored at −80 °C until processed.

The animals were kept, handled and killed following the specific procedures approved by the University of Barcelona Animal Welfare and Ethics Committee, in full conformity with the norms and proceedings set forth by the European Union and the Governments of Spain and Catalonia.

2.2. Serum steroid hormone analysis

Aliquots (0.400 mL) of rat serum were mixed with 0.025 mL of a working stock mixture of d8-corticosterone (1×10^{-5} M), added as an internal standard, and the tubes were incubated on ice for 20 min. Acetonitrile (1.5 mL) was then added and the tubes were vortexed and centrifuged to remove proteins. Supernatants were transferred into glass tubes and evaporated to dryness under nitrogen; dried residues were redissolved in 0.120 mL of the mobile phase.

Sample analyses were performed by LC–MS/MS (Varian, Palo Alto, CA, USA) consisting of a 210 pump with an online degassifier, a 410 autosampler, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operated in positive ion mode. The chromatographic separation was performed in a 150 × 3.0 (i.d.) mm Intersil 3 ODS-3 column (Varian, Palo Alto, CA, USA). The mobile phase consisted of two eluents, solvent A (5 mM ammonium acetate, 0.1% formic acid) and solvent B (acetonitrile), at a flow rate of 0.2 mL/min. Samples of 0.050 mL were injected into the LC–MS/MS, after which the injector was washed five times with mobile phase. The total run time was 55 min. A linear gradient from 30% to 55% B was programmed for the first 28 min, followed by a second linear gradient to 90% B for 4 min, and isocratic mode for the next 16 min. Then, the system was returned to the initial proportion of 30% solvent B over the following 2 min and maintained for the final 5 min of each run.

Deuterated steroids were purchased from CDN Isotopes Inc (Pointe Claire, Canada). All other steroid standards were from Sigma (St Louis, MO, USA). Calibration curves were prepared in the range 1–4000 nM. The ratio between the peak area of each endogenous steroid and the deuterated steroid (d8-corticosterone) was correlated with the concentration using linear regression analysis to quantify serum steroid levels. Detection limits of the method were 2 nM for dehydrocorticosterone and 10 nM for corticosterone.

Serum corticosterone binding was carried out as previously described [40] using ^{3}H-corticosterone (NET399, PerkinElmer, Boston, MA, USA).

2.3. Tissue corticosterone analysis

Frozen adrenal glands and liver samples were homogenized, in about 10 volumes of chilled 0.3 N perchloric acid containing 9 g/L NaCl [41], using a Polytron (Kinematica, Luzern, Switzerland) homogenizer. After centrifugation, clear supernantants were diluted (1/3 liver, 1/400 adrenals) and used for a corticosterone radioimmunoanalysis (MP Biomedical, Oranburg, NY, USA) [42]. The data were expressed per unit of tissue weight.

2.4. Nucleic acid analyses, cellularity

Tissue samples were used for the estimation of total DNA, using a standard fluorimetric method with 3,5-diaminobenzoic acid (Sigma, St Louis, MO, USA) and bovine DNA (Sigma) as standard [43]. Tissue DNA content allowed the calculation of the number of cells per gram of tissue and in the whole tissues sampled, based on the assumption that the DNA content per cell is constant in mammals; here we used the genomic DNA size data [44] for somatic rat cells (5.6 pg/cell). Mean cell mass was estimated from the number of cells and the weight of the organ.

Total tissue RNA was extracted using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and were quantified in a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington DE USA).

Table 1
Effects of pair-feeding and OE on body weight, adrenal glands weight and cellularity and circulating serum glucocorticoids of male overweight rats.

Parameter	Units	Control	Pair-fed	OE-treated
Initial body weight	g	355 ± 12	350 ± 3	358 ± 7
Body weight change	g	+9.1 ± 1.4	−29.3 ± 3.0*	−34.9 ± 2.3*
Adrenal glands weight	mg	57 ± 5	55 ± 1	85 ± 6*◦
Adrenal glands DNA	μg	360 ± 9	411 ± 43	457 ± 25*
Adrenal glands cell number	$\times 10^6$	60.0 ± 1.5	68.5 ± 7.2	76.1 ± 4.1*

All data are the mean ± sem of 8 different animals. Statistical significance of the differences between groups: *$P < 0.05$ vs. controls; ◦$P < 0.05$ between OE and PF.

2.5. Semiquantitative RT-PCR analysis of protein expression

RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) and oligo-dT primers. Real-time PCR (RT-PCR) amplification was carried out using 0.010 mL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to OD 0.500 for all runs.

The expression of the genes listed in Tables 2 and 3 was estimated using specific primers (the list is given in the online-only Supplemental Table 1) by using a semiquantitative approach for the ultimate estimation of the number of copies of each expressed gene mRNAs per tissue weight was used as previously described [45]. In any case, cyclophilin was used as charge control gene in all samples.

Statistical comparison between groups was established by using the unpaired Student's *t* and one-way ANOVA tests, with a limit of significance of $P < 0.05$.

3. Results

Table 1 shows the weights of the animals and their adrenal glands, as well as the calculated cellularity of adrenals. Pair-feeding and OE treatment resulted in a significant loss of body weight in only 10 days. However, the size of adrenal glands was increased in OE rats, with no significant changes in the PF group compared with controls. This increase was in part due to an increase in DNA content, which represents a marked increase in adrenal cell numbers induced by OE.

Table 2 shows the tissue and serum levels of corticosteroids at the moment of sacrifice. Serum levels of dehydrocorticosterone were not different from controls in PF and OE rats; corticosterone levels were slightly raised (NS) in OE-treated animals, but there was a marked increase in pair-fed rats. Serum corticosterone binding showed no differences between groups, which represents that OE and, especially PF rats had a higher proportion of free corticosterone than controls, since the corticosterone levels to binding ratio were, respectively 0.53, 2.31 and 0.85 for controls, PF and OE rats.

Corticosterone content in the adrenals was highest in OE rats in absolute values, with no differences between controls and PF animals. The adrenals/serum concentration ratio showed the lowest values for PF, and the highest for OE, a five-fold (significant) difference range. In liver, OE rats showed a similar pattern, with higher corticosterone concentrations than controls and PF. The tissue/serum ratios showed a similar pattern than in the adrenals, but the ratios were much lower; PF showed the lowest ratios, followed by controls and OE, again a five-fold difference range.

Table 3 presents the comparative gene expression of the main enzymes controlling the synthesis of glucocorticoids in the rat adrenal glands. The data are presented as total amount (fmol) of the corresponding mRNAs in the whole adrenal organ (both glands) as a way to compare the overall adrenal capability to express the corresponding proteins and thus establish comparisons between the three experimental groups.

The steroid-modifying complexes 20,22-hydroxylase 30-22-lyase, 3β-dehydrogenase 4–5 isomerase and the 21-hydroxylase gene expressions behaved in the same way, increasing their expressions under OE treatment, but remained unchanged in PF animals. The expression of 11β-hydroxylase gene, rendering corticosterone, was higher in both PF and OE than in controls. Finally, the gene for 11β-hydroxysteroid dehydrogenase type 1, acting essentially in the direction of corticosterone synthesis [46], was underexpressed in OE and unchanged in PF rats versus controls. Consequently, OE treatment resulted in significant increases in the expression of the enzyme complex genes of corticosterone synthesis, a result not observed in PF.

The corticotropin receptor gene expression was unchanged in OE but was lower in PF versus controls, exactly the reverse situation (i.e. increased by OE) found in the expression of the scavenger receptor for cholesterol, main responsible for the incorporation of cholesterol as substrate for steroidogenesis in the adrenal gland.

Table 3 also shows the expressions of the genes for enzymes participating in the catecholamine synthesis cascade: (a) phenylalanine hydroxylase, which expression was markedly depressed by OE; (b) tyrosine oxidase, unchanged in PF but depressed in OE; (c) dopa-decarboxylase, decreased in PF and unchanged in OE; and (d) dopamine oxidase, unchanged by OE and pair-feeding.

Fig. 1 shows the expressions in liver, of the steroid 5α-reductase 1 and 11β-hydroxysteroid dehydrogenase 1 genes. OE treatment induced a considerable increase in the expression of the reduc-

Table 2
Effects of pair-feeding and OE on circulating serum and tissue glucocorticoids of male overweight rats.

Parameter	Units	Control	Pair-fed	OE-treated
Serum corticosterone	nM	196 ± 30	782 ± 166*	385 ± 139
Serum dehydrocorticosterone	nM	20.1 ± 5.0	40.0 ± 13.3	33.9 ± 11.7
Serum corticosterone binding	nM	369 ± 31	339 ± 63	452 ± 43
Adrenal gland	nmol/g	11.7 ± 1.1	17.9 ± 4.5	40.4 ± 14.5
corticosterone	nmol	0.67 ± 0.06	0.99 ± 0.33	3.43 ± 1.04*◦
Adrenal gland/serum corticosterone concentrations ratio	nmol/kg/nM	55.6 ± 2.3	22.5 ± 3.5*	130.0 ± 29.1*◦
Liver corticosterone	pmol/g	18.6 ± 2.3	37.7 ± 4.7*	120.3 ± 34.1*◦
	nmol	0.21 ± 0.03	0.28 ± 0.07	1.19 ± 0.44*
Liver/serum corticosterone concentrations ratio	nmol/kg/nM	0.088 ± 0.004	0.051 ± 0.004*	0.265 ± 0.029*◦

All data are the mean ± sem of 8 different animals. Statistical significance of the differences between groups: *$P < 0.05$ vs. controls; ◦$P < 0.05$ between OE and PF.

Table 3
Effects of pair-feeding and OE on the gene expression of the enzymes and enzyme complexes involved in the adrenal synthesis of glucocorticoids and catecholamines of male overweight rats.

Gene expressing the protein:	Gene name	Control	Pair-fed	OE-treated
20,22-Hydroxylase 30-22-lyase	Cyp11a1	3072 ± 223	2756 ± 177	5434 ± 442*◦
3β-Dehydrogenase 4–5 Isomerase	3bhsd1	2008 ± 143	1751 ± 177	2630 ± 211*◦
21-Hydroxylase	Cyp21	4032 ± 231	3856 ± 335	6279 ± 526*◦
11β-Hydroxylase	Cyp11b1	6.8 ± 0.9	17.7 ± 5.5*	21.3 ± 3.5*
11β-Hydroxysteroid dehydrogenase 1	11bhsd1	14.8 ± 2.2	15.8 ± 2.8	9.0 ± 1.6*◦
Scavenger receptor for cholesterol	Scarb	254 ± 31	330 ± 62	644 ± 85*◦
Corticotropin receptor	Mc2r	102 ± 7	69 ± 7*	121 ± 13◦
Phenylalanine hydroxylase	Pah	3.37 ± 0.48	2.22 ± 0.39	0.31 ± 0.08*◦
Tyrosine oxidase	Th	1.40 ± 0.14	1.28 ± 0.11	0.9 ± 0.06*◦
Dopa-decarboxylase	Ddc	23 ± 2	17 ± 1*	24 ± 4
Dopamine oxidase	Dbh	57 ± 5	58 ± 3	57 ± 4

All data are expressed as fmoles of the corresponding mRNAs present in both adrenal glands, and are the mean ± sem of 8 different animals. Statistical significance of the differences between groups: *$P < 0.05$ vs. controls; ◦$P < 0.05$ between OE and PF.

tase, with unchanged levels of expression in PF rats compared with controls. The expression of the dehydrogenase, however, showed a fairly different picture, with only a limited decrease in the expression in PF rats.

4. Discussion

Since adrenal glands contain an important catecholaminergic core, the changes in its weight may reflect not only changes in their steroidogenic activity, but also a change in the ability to respond with catecholamine secretion to a number of challenges. We found here that the path for the synthesis of noradrenaline from either phenylalanine or tyrosine was depressed by OE, and remained unchanged by PF. In fact, potential conversion of Phe to Tyr was slightly depressed by pair-feeding, since the essential amino acid metabolism (and specifically that of tyrosine, at least in liver) is decreased by limited food availability [34]. Nevertheless, the difference between PF and OE is too extensive to assume that this is the sole reason for a lower expression. The whole path from Phe (or Tyr) to noradrenaline is depressed by OE. This may translate probably in a lower extension of the adrenergic responses originated in the medullary portion of the adrenal glands in rats treated with OE, which agrees with the unchanged expression of most adrenergic-driven paths in OE rats [47].

These data, however, clearly hint at the cortical zone as the more than probable responsible for adrenal increase in weight and cellularity, which in turn raise the question of why this increase occurred? The higher steroidogenic (glucocorticoid) synthetic capability of OE rats results in an actually depressed overall glucocorticoid response *in vivo,* with limited increases in their circulating levels, compared with a – more stressful, indeed [48] – situation such as pair-feeding, in which adrenal steroidogenesis is not enhanced, but circulating levels are increased and overall glucocorticoid activity is higher.Fig. 2 shows a scheme of the cholesterol to glucocorticoid synthetic pathway in rat adrenals. This is fairly different from that of humans because of the rat's lack of functional 17α-hydroxylase [49], that cuts the way to the synthesis of cortisol, but also to DHEA and from that to androgens and estrogens. The rat adrenal cortex is thus essentially limited to the production of corticosteroids. The marked and consistent increases induced by OE in the expression of the genes controlling the enzyme complexes of this path hint at a marked stimulation of glucocorticoid synthesis by OE. This is further strengthened by an increase in the expression of *Scarb* [50] induced by OE, which probably results in enhanced availability of cholesterol for steroidogenesis in the adrenal glands of OE rats (but not in those of PF animals). The increase in serum corticosterone agrees with this interpretation, but it is fairly apparent that adrenal synthesis is probably not the main path controlling circulating glucocorticoids, since pair-feeding increased more markedly circulating corticosterone (with unchanged 11-dehydrocorticosterone) than OE rats, in spite of lower expressions of the genes controlling its adrenal synthesis. The high content of corticosteron in the adrenal glands and the steep tissue to serum concentration differences suggest an increased adrenal production of corticosterone in OE rats, and a restrained synthesis in PF rats, despite them showing the highest circulating corticosterone.

Previous studies have shown that OE treatment elicits a restrained glucocorticoid response [37], probably a consequence of the profound metabolic (energy handling) changes induced by

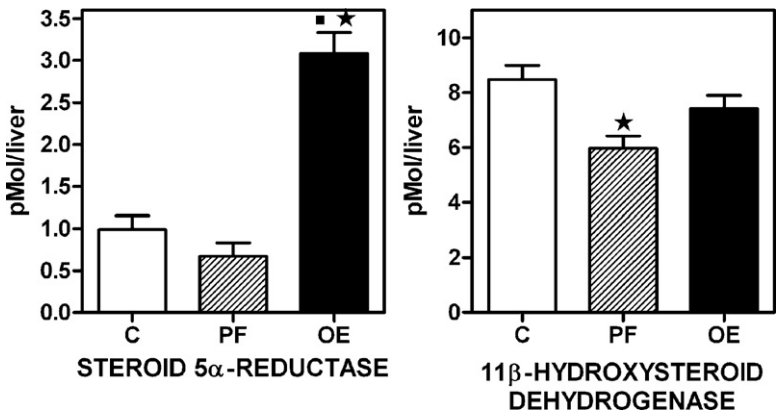

Fig. 1. Effects of pair-feeding and OE on the gene expression of steroid 5α-reductase 1 and 11β-hydroxysteroid dehydrogenase 1 in the liver of male overweight rats. The values represent the mean ± sem of 8 different animals per group, and are expressed as picomols of the corresponding mRNAs in the whole liver. Statistical significance of the differences between groups: (⋆) $P < 0.05$ versus the control group; (■) $P < 0.05$ between PF and OE groups.

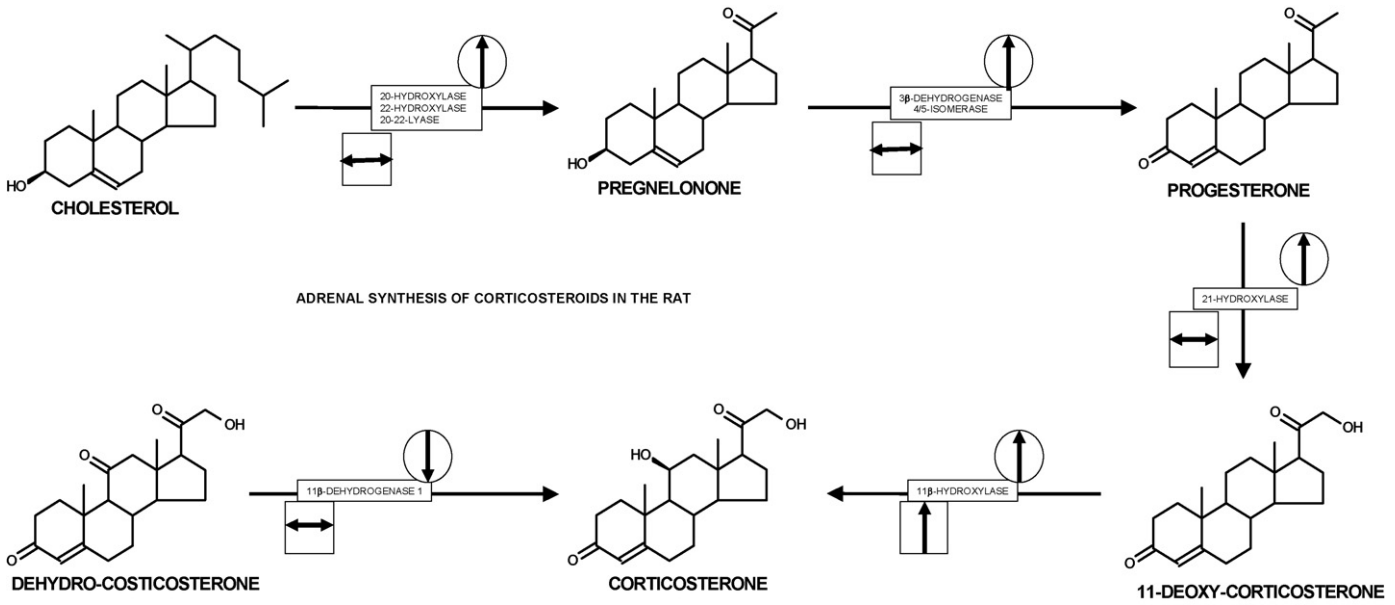

Fig. 2. Glucocorticoid synthesis pathway of the rat adrenal gland. The enzymes/enzymatic complexes are indicated as boxes on the arrows. The effects of PF [squares] or OE [circles] are also presented. An upward arrow indicates a significant ($P < 0.05$) increase in the expression of the enzyme or complex versus controls; a downward arrow indicates a significant decrease, and a horizontal two-headed arrow indicates a non-significant difference ($P > 0.05$) versus controls. Specific numeric data for gene expression can be found in Table 2.

the steroid ester [23]. In female rats, the main effect observed was a relative increase in glucocorticoid availability due to an increase in circulating free corticosterone fraction and [36] the active reconversion of 11-dehydrocorticosterone to the more active corticosterone in liver, via increased activity and gene expression of 11β-hydroxysteroid dehydrogenase [37]. The lack of increase in this enzyme expression observed on our – male – model, and the lack of differences versus controls in the serum availability of free corticosterone suggests a marked sexual difference on OE modulation of glucocorticoid response, which is in agreement with the widely different overall lipid wasting response to OE treatment [51].

The data obtained in the present study using overweight males do not match these effects found in female rats, since OE induced no significant changes in the expression of liver 11β-hydroxysteroid dehydrogenase or serum corticosterone binding. However, the powerful boost of corticosterone synthesis in the adrenals elicited by OE and the limited changes in serum corticosterone (in a situation of unchanged serum glucocorticoid binding) attest to a necessarily increased corticosterone turnover. The lack of change in liver dehydrogenase 1, one of the key elements in the recycling of dehydrocorticosterone to corticosterone [52,53], the decreased expression of this same enzyme in other peripheral tissues (data not shown), and the maintenance of the serum ratio of corticosterone to dehydrocorticosterone concentrations, suggest that 11β oxidation is not the critical path in the removal of active glucocorticoid from rat serum under the conditions tested.

The liver actively takes up most steroid hormones [54], in a two-step process of inactivation: uptake and chemical transformation by oxidation, conjugation or other mechanisms. The tissue/serum concentration ratios show that in controls, tissue hormone was about 1/10th of that of serum, in PF, the ratio was even lower, but the amount of hormone in the liver was similar to that of controls. However in OE rats, the ratio was much higher, about 1/4th. This is probably a consequence of increased liver corticosterone uptake, which in turn elicits the activation of the hormone chemical inactivation. It has been reported that steroid 5α-reductase is the main outlet for the permanent elimination of glucocorticoids [55], thus, the massive increase in steroid 5α-reductase expression in the liver of the rats treated with OE may respond to this accumulation. Taken together, the hormone accumulation in liver and the increased 5α-reductase expression suggest that, probably, liver is the main site for definitive corticosteroid destruction, and can help explain the maintenance of relatively unchanged serum levels of both oxidized and reduced glucocorticoids studied in spite of increased adrenal synthesis.The obvious conclusion to these data is, thus, that OE induces a higher rate of circulating corticosteroids' turnover: there is an increased adrenal synthesis, but there is also an increase in liver disposal, the question is to discriminate which of both is the primary factor and which is the homeostatic compensatory response.

The data observed for PF rats show a completely different situation from OE and help us discard dietary energy availability as the main driving factor for the changes observed. PF values for all these parameters are close to those of controls, but circulating corticosterone levels were higher, and there was an increase in the proportion of free (unbound) hormone, which hints at a higher level of stress than in controls and OE; this can be expected because PF rats are chronically hungry (and thus stressed) [56]. This marks a clear difference versus the OE group (but also controls) in which weight loss was slightly higher, but adrenal glands weighed more than in PF. Weight loss was a direct consequence of limited energy availability, common to PF and OE, which in the latter was compounded by a maintained energy expenditure [25]. Thus, the observed adrenal gland differences of OE rats could not be attributed to energy availability, being genuine effects induced by the ester. These data agree with the circulating and tissue levels of glucocorticoids presented here: the alteration induced by insufficient feeding (akin to dieting) of PF rats was more marked than that induced by OE in spite of a more profound wasting of body energy [57].

The limited increase of the HPA axis induced by OE [34] could not explain by itself the increase in glucocorticoid synthesis pathway enzyme gene expressions, but the difference versus PF observed in the expression of the corticotropin receptor fits in this interpretation. The role of white adipose tissue interconversion of the active / inactive glucocorticoid forms probably is critical to understand the glucocorticoid dynamics in obesity [48,58], since its large

size (at least compared with the adrenals) may result in massive changes affecting not only its own response to glucocorticoids, but also deeply modulating the whole body response to them [10,59].

In obesity, glucocorticoid activity is apparently increased, but the circulating levels of active glucocorticoids do not match this enhanced function [60], suggesting that the adrenals are only part (a critical part, however) in the control of overall glucocorticoid response. The results presented here add to the picture the possible relevance of increased glucocorticoid turnover, observed in a situation in which the metabolic derangements induced by obesity tend to be corrected by OE treatment.

In conclusion, we have observed that OE considerable enhanced the expression of the genes controlling the synthesis of glucocorticoids from cholesterol in the rat and increasing the adrenal glands' corticosterone content, size and cellularity, but also the liver disposal of corticosteroids, suggesting that OE increases corticosterone synthesis and degradation (i.e. serum turnover), a process not driven by the limitation of energy availability but directly related to the administration of OE.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.steroids.2009.09.009.

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3.3.2 Efectos del tratamiento con oleoil estrona en los niveles circulantes de testosterona. Papel de las enzimas 17β-hidroxiesteroide deshidrogenasa

Se administró por vía oral una dosis diaria de oleoil estrona (OE) a ratas macho con sobrepeso durante 10 días, y se compararon con animales controles que recibían una dosis oral del vehículo (aceite de girasol). Mediante un método semicuantitativo de PCR a tiempo real se analizó la expresión génica de las isoenzimas de la 17β-hidroxiesteroide deshidrogenasa (17βHSDH) y de otras proteínas relacionadas con el metabolismo de las hormonas sexuales en testículo, hígado, glándulas suprarrenales y dos localizaciones de tejido adiposo blanco (subcutáneo inguinal y epididimal). Los niveles de androstenediona, testosterona, estradiol y estrona se midieron mediante HPLC-MS/MS. Las expresiones de las isoenzimas fueron agrupadas de acuerdo con su función fisiológica principal (oxidante o reductora) conocida y su principal sustrato (andrógenos o estrógenos).

Como era de esperar, el testículo fue el tejido cuantitativamente más importante para la síntesis de testosterona y estradiol, y el hígado el principal órgano encargado de oxidarlos a androstenediona y estrona, respectivamente. La capacidad total oxidativa fue aproximadamente 6,5 veces mayor que la capacidad reductora. La síntesis y el potencial oxidativo para el estradiol fue mayor que para la testosterona. La OE disminuyó los niveles de andrógenos séricos e incrementó los de estrona, pero no los de estradiol. Ello fue debido a que disminuyó la capacidad el testículo para producir testosterona como consecuencia de la disminución de su tamaño, la caída de la expresión génica de la 17βHSD3 y también a una menor disponibilidad de moléculas precursoras.

La alta disponibilidad de estrona exógena (generada a partir de la hidrolisis de la OE) no se traduce en un aumento paralelo de estradiol debido a la disminución en el testículo de la expresión génica de la 17βHSD3 (reductora) y de la aromatasa. En consecuencia, podemos suponer que los efectos de la OE sobre los andrógenos y el eje hipotálamo-hipófisis-gonadal se limita esencialmente a los testículos.

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INFLUENCE OF OLEOYL-ESTRONE TREATMENT ON CIRCULATING TESTOSTERONE. ROLE OF 17β-HYDROXYSTEROID DEHYDROGENASE ISOENZYMES

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Overweight male rats received oral oleoyl-estrone (OE) for 10 days, and were compared with controls. The expression of 17β-hydroxysteroid dehydrogenase (17βHSDH) isoenzymes, and other proteins related to sex hormone metabolism, were analyzed in testicle, liver, adrenals and two white adipose sites: subcutaneous inguinal and epididymal pads using a semiquantitative RT-PCR method. Androstenedione, testosterone, estrone and estradiol levels were measured by HPLC-MS/MS. Isoenzyme expressions were grouped according to their main physiological function (oxidative or reductive) and preferred substrate (androgen or estrogen). As expected, testicle was the main site for synthesis of testosterone and estradiol, and the liver the main organ oxidizing them to androstenedione and estrone. Overall oxidative capacity was 6.5-fold higher than the reductive, and estradiol synthesis and oxidation potential were higher than for testosterone. OE decreased serum androgens, and increased estrone, but not estradiol. This was due to decreased testicle ability to produce testosterone, because of smaller size and decreased 17βHSDH3 expression, but also to lower availability of precursors. High estrone availability (from OE hydrolysis) does not translate into higher estradiol because of decreased testicle reductive 17βHSDH expression and decreased aromatase. In consequence, we can assume that OE effects on androgens, and the hypothalamic-pituitary-gonadal axis are limited to testicles.

Keywords: *oleoyl-estrone; androgen; estrogen; testosterone; 17βHSDH*

INTRODUCTION

Oleoyl-estrone (OE) induces a dose-dependent loss of body fat (1) by decreasing food intake and maintaining energy expenditure (2) in rats. These effects essentially induce a resetting of the ponderostat (3) and are independent of the diet, since there are no additive effects with energy intake restriction (4), and its induction of body fat loss is unaffected by high-energy lipid-laden diets (5). Loss of body energy affects essentially the adipose tissue (6), sparing protein (2), and maintaining glucose and overall circulating energy homeostasis (7) thanks to an increased usage of lipid by muscle and decreased incorporation of lipids to adipose tissue (8).

When given i.v. in liposomes, OE is rapidly hydrolyzed by tissue esterases (9). Oral OE is in part hydrolyzed to estrone and oleate by pancreatic and enteric esterases, including cholesterol-ester esterases (10). From the experimental data gathered, it seems that only intact OE, absorbed in the upper part of the intestine, may have significant pharmacological effects (10), since most of the intestine-released estrone is absorbed and converted to hydrophillic conjugates by the liver (11). In any case, the ultimate fate of OE is a final hydrolysis to estrone, which may be used by adipocytes to

regenerate OE (12). Circulating levels of estrone (and to a lower extent, estradiol) rapidly increase during i.v. OE administration (9), but oral OE administration results in limited increases of both, being estrone sulphate the main circulating catabolite (13), with no significant estrogenic effects observed both in rats (14) and a human (15).

17β-hydroxysteroid dehydrogenases (17βHSDH) are an important family of enzymes which catalyze the interconversion of inactive (or less active) 17-keto and active 17β-hydroxy forms of sex steroids, regulating the intracellular levels of biologically active androgens and estrogens (17). The types of 17βHSDH are largely grouped into *in vivo* oxidative enzymes (types 2, 4, 6, 8, 9, 11, and 14) and reductive enzymes (types 1, 3, 5, 7, and 12). These enzymes differ in tissue and subcellular distribution, catalytic preferences, substrate specificity and regulation mechanisms (18). The peculiarities of rat sex hormones metabolism, such as limited adipose tissue aromatase activity, and decreased role of adrenal glands supplying precursors, may justify the sex hormone metabolism differences between rodents and humans.

The relatively low increase in estradiol elicited by a significant load of estrone resulting from OE treatment was speculated may be a consequence of the inhibition of 17βHSDH activity in the direction of reducing estrone, thus minimizing the potential estrogenic effects of the estrone load because of its lower estrogenic effects (19). However, this same pathway also reduces androstenedione to testosterone; thus, the protection against estrogenicity may result in a loss of androgenic potential as an unwanted side-effect. This study was devised to analyze how a standard oral OE treatment of overweight male rats affects the circulating levels of these hormones as well as the potential of key tissues in male sexual hormone metabolism: testicle, adrenal glands, liver and white adipose tissue (WAT) to affect this transformation.

MATERIALS AND METHODS

Animals and sample preparation

Adult male Wistar rats were made overweight by a limited period of cafeteria diet feeding, as previously described (20). The rats, initially weighing 355 ± 5 g, were kept under standard conditions of housing and feeding (20). Two groups of 8 rats each were randomly selected: controls and OE. All animals received every day an oral gavage of 0.2 mL of sunflower oil (7 kJ), which was supplemented in the OE group with 10 nmol/g oleoyl-estrone (OED, Barcelona, Spain). The controls and OE group had free access to pellet food (maintenance chow, Panlab, Barcelona, Spain); all rats had water available *ad libitum*. On day 10, the rats were killed by decapitation; testicles, adrenal glands, liver and two well-defined WAT sites: the perigonadal (epididymal) and subcutaneous inguinal fat pads were excised, weighed and samples were frozen and kept at -80°C. Blood was recovered and allowed to clot; the serum was stored at -80°C until processed.

The animals were kept, handled and killed following the procedures approved by the University of Barcelona Animal Welfare and Ethics Committee, in full compliment of the norms and procedures set forth by the European Union and the Governments of Spain and Catalonia.

Tissue samples were used for the estimation of total DNA, using a standard fluorimetric method with 3,5-diaminobenzoic acid (Sigma, St Louis MO USA) and bovine DNA (Sigma) as standard (21). Tissue DNA content allowed the calculation of the number of cells per g of tissue and in the whole tissues sampled, based on the assumption that the DNA content per cell is constant in mammals; here we used the genomic DNA size data (22) for somatic rat cells (5.60 pg/cell). Mean cell volume was estimated from the number of cells and the volume of the organ, calculated using known tissue density data (23).

Semiquantitative RT-PCR analysis of protein expression

Total tissue RNA was extracted using the Tripure reagent (Roche Applied Science, Indianapolis IN USA), and were quantified in a ND-100 spectrophotometer (Nanodrop Technologies, Wilmington DE USA). RNA samples were reverse transcribed using the MMLV reverse transcriptase (Promega, Madison, WI USA) and oligo-dT primers.

Real-time PCR (RT-PCR) amplification was carried out using 0.010 mL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems) using a fluorescent threshold manually set to OD 0.500 for all runs. The list of genes and primers used is given in Supplemental Table 1.

A semiquantitative approach for the ultimate estimation of the number of copies of each expressed gene mRNAs per cell or tissue weight was used as previously described (24). In any case, cyclophilin was used as charge control gene in all samples.

Table 1: Organ weights and cellularity of overweight male rats subjected to a 10-day treatment with oleoyl-estrone

parameter	units	C/OE	testicle	liver	WAT SC	WAT PG	adrenals
organ weight	g	C	3.27 ± 0.07	11.34 ± 0.42	2.58 ± 0.34	8.06 ± 0.83	0.057 ± 0.05
		OE	2.65 ± 0.03*	9.90 ± 0.53	1.83 ± 0.22	6.08 ± 0.80	0.085 ± 0.006*
organ number of cells	$x10^7$	C	124 ± 4	387 ± 34	16.8 ± 3.0	37.9 ± 3.0	6.04 ± 0.15
		OE	126 ± 6	371 ± 12	14.4 ± 3.0	29.0 ± 2.2*	7.61 ± 0.41*
mean cell size	pL	C	2.93 ± 0.12	3.14 ± 0.15	15.9 ± 2.0	15.5 ± 0.3	1.082 ± 0.018
		OE	2.31 ± 0.08*	3.07 ± 0.18	13.9 ± 3.1	14.1 ± 0.7	0.976 ± 0.017*

Weights were directly estimated, cell data were calculated from measured DNA and referenced density data. The values shown are the mean ± sem of 8 different animals; * = $p < 0.05$ vs. controls.

HPLC-MS/MS hormone measurements

Two sets of serum samples (0.400 mL each, in duplicate) were used. The first was duped with 0.010 mL of 50 nM d4-estrone and 25 nM d4-estradiol as internal standards. Samples were extracted with 2 mL dichloromethane. The organic phase was dried under a gentle nitrogen stream; dry residues were resuspended in 0.075 mL of 100 mM bicarbonate buffer, pH 10.5, and 0.075 mL of 1g/L dansyl-chloride (Sigma) in acetone (25) were added, then vortexed and incubated 5 min at 60 °C. This suspension was directly injected to the HPLC-MS/MS system.

The second set of samples was loaded with 0.025 mL of 1 µM d7-androstenedione and 1 µM d2-testosterone as internal standards. Samples were extracted with 1.5 mL acetonitrile; the organic phase was dried with nitrogen and resuspended in 0.120 mL of 5 mM ammonium acetate buffer containing 1g/L formic acid, pH 4.0. This suspension was directly injected to the HPLC-MS/MS system.

Hormone standards were obtained from Steraloids (Newport, RI USA) and the deuterated internal standards from CDN Isotopes (CIL Cluzeau, Sainte Foy La Grande, France). Chromatographic-grade solvents were from Riedel de Haën (Seelze, Germany).

Chromatographic separation was carried out using an Atlantis column 50 x 2.1 mm 3 µm (Waters, Milford, USA) at 0.3 mL/min for estrogens and an Inertsil ODS-3 column 150 x 3.0 mm 3 µm (Varian, Walnut Creek, USA) at 0.2 mL/min for androgens. The MS configuration consisted of two 210 HPLC pumps, a 410 automatic injector and a 1200L tripleQ (Varian) apparatus, with an electrospray interface operating in positive ionization mode.

Gradient for estrogens was: a) 95% water-5% acetonitrile (with 0.1% formic acid), b) 95% acetonitrile-5% water (with 0.1% formic acid). The gradient (for b) was: min 0: 70 %, min 0.24-2: 85%, min 2-3.3: 85›95%, min 3.3-4: 95›70%, min 4-7: 70 %. The limit of sensitivity for both estrogens was established at 25 pM.

Gradient for androgens was: a) 5 mM ammonium acetate (with 0.1% formic acid), b) acetonitrile. The gradient (for b) was: min 0-28: 30›55 %, min 28-32: 55›90 %, min 32-48: 90 %, min 48-50: 90›30 %, min 50-55: 30 %. The limit of sensitivity for both androgens was set at 0.6 nM. Interassay precision for hormone analyses within the present range of measurement were: 20% estrone, 21 % estradiol, 16% androstenedione and 17% testosterone.

WAT calculations and statistical comparisons

Testicle, liver and adrenal gland weights are directly available, but the mass of WAT is not readily available. The sampling of two representative and major sites: perigonadal and subcutaneous cordons –cells of different size and metabolic activity (26), allows for a more balanced estimation of their gene expression data. In order to obtain an approximate estimation of the whole-body WAT expression of a given gene, we used the data for lipid content in this same rat model of controls and OE rats (6): 180 g/kg for controls and 131 g/kg for OE rats. The final weights for control rats were 364 ± 12 g and 323 ± 8 g for OE rats. Using the percentage of lipids in WAT sites of control and OE rats previously published (6), we derived an estimation of percent body fat in controls at 21 %, and 15 % in OE rats. Then, a weighed mean of perigonadal and subcutaneous sites' data was calculated; and the results were extrapolated to whole body WAT in order to obtain an estimate of overall WAT 17βHSDH expression. Evidently, the results obtained are only an indication of the actual contribution of WAT to whole body 17βHSDH activity, but allow for a rough comparison of the probable contribution of WAT to sexual

hormone metabolism in comparison with liver, testicle and adrenal glands.

Statistical comparison between groups was established by using the unpaired Student's *t* test with a limit of significance of $P < 0.05$.

RESULTS

Table 1 presents the weights and cellularity of the organs studied. OE decreased the weight of testicles but not its number of cells (in fact its DNA content). No significant differences were attributable to OE treatment in liver or subcutaneous WAT in spite of a noticeable trend to smaller weights and cell size in both tissues; in perigonadal WAT, the decrease in weight was not significant either, but the decrease in cell numbers became significant (i.e. there was a loss of 23 % of cells vs. only 14 % lost in subcutaneous WAT). Adrenals increased their weight and cell count with OE treatment, but the size of the cells was smaller than in controls.

Table 2 shows the serum levels of the four steroid hormones studied. OE treatment markedly decreased the levels of both androstenedione and testosterone, and increased those of estrone, but not those of estradiol. The estrone / estradiol concentrations ratio changed from 3.3 in controls to 6.5 in OE rats, hinting at a limited ability to reduce estrone to estradiol. Since the values for androstenedione in the OE group fell below the safe limit of detection of the system the androstenedione / testosterone ratio (0.20 in controls) could not be compared.

Table 2: Serum levels of androgens and estrogens of overweight male rats subjected to a 10-day treatment with oleoyl-estrone

hormone	units	control	OE
testosterone	nM	11.2 ± 5.4	1.23 ± 0.64 *
androstendione	nM	2.20 ± 0.23	<0.6 *
estradiol	pM	41.6 ± 11.8	91.8 ± 32.7
estrone	pM	137 ± 14	595 ± 137 *

Values are the mean ± sem of 6 different animals; * = $p < 0.05$ *vs*. controls.

Table 3: Predominance of the reductive/oxidative direction and substrate preferences for the isoenzymes studied

direction	reaction	isoenzymes	references
reducing	A ➜ T	1,3	(25-29)
oxidizing	T ➜ A	2,8	(29-31)
reducing	E_1 ➜ E_2	1,7	(25-27,32,33)
oxidizing	E_2 ➜ E_1	2,4,8,11	(30,31,34-36)

A = androstenedione; T = testosterone; E_1 = estrone; E_2 = estradiol

The pattern of distribution of the different isoenzymes, and the relative abundance of their mRNAs showed considerable differences between the organs studied. *Table 3* shows the available data on the preferred substrate and oxidative or reductive direction, which translates in the purported physiological function of the isoenzymes studied.

Table 4 depicts the expression of 17βHSDH isoenzymes as the number of the corresponding mRNAs copies per cell in the five organs studied. In testicle, there was a significant decrease in the expression of all isoenzyme expressions, but the decrease was more marked for isoenzyme 3 (reducing). In liver only a slight decrease in isoenzyme 7 (reducing) and a marked increase in isoenzyme 2 (oxidizing) were observed. Perigonadal adipose tissue showed no changes in isoenzyme expression, but in the subcutaneous site, decreases in isoenzymes 1, 7 (reducing) and 4 (oxidizing) were observed. In contrast with humans, we did not detect the expression of isoenzymes 2 and 3 in either WAT site. In adrenal glands, there was only a significant change, an increase in the expression of isoenzyme 4 (oxidizing).

When the data of *Table 3* are translated to the total number of copies for each isoenzyme mRNA found in the tissue mass (combining the WAT data to obtain an extrapolated figure for whole-body WAT) we observe that the ability to synthesize testosterone under basal conditions is concentrated in the testicle (90.9 %), with small contributions of liver (5.5 %), WAT (3.0 %) and adrenals (0.6 %); testosterone oxidation, however is mainly carried out in the liver (79.2 %), followed by testicle (11.9 %), adrenals (7.5 %) and WAT (1.4 %). The corresponding data for estradiol were: synthesis in testicles (66.9 %), liver (28.6 %), WAT (2.6 %), and adrenals (1.9 %); estradiol oxidation: liver (90.9 %), testicle (4.8 %), adrenals (2.5 %) and WAT (1.8 %). *Fig. 1* presents a comparative histogram of these data, showing that in addition to the differences in oxidation / reduction, the ability to synthesize estradiol was also higher than that of testosterone (1.4-fold); the difference being mainly due to the selectiveness of the liver for estrone rather than androstenedione reduction. On the oxidative side, the differences between androgen and estrogen handling are more pronounced (6.5-fold), and again the role of liver is overwhelming.

The distribution of isoenzyme expressions does not reflect the unequal distribution of oxidizing versus reducing isoenzymes, heavily decanted towards oxidation (i.e. inactivation of both the most effective hormones, testosterone and estradiol).

Table 4: Tissue 17βHSDH isoenzyme gene expression in organs of overweight male rats subjected to a 10-day treatment with oleoyl-estrone

isoenzyme	C/OE	testicle (cpc)	liver (cpc)	WAT-SC (cpc)	WAT-PG (cpc)	adrenals cpc
17βHSDH1	C	3.74±0.09	1.30±0.12	1.11±0.11	0.413±0.060	8.86±0.31
	OE	2.59±0.12*	1.11±0.05	0.73±0.06*	0.331±0.013	9.63±0.55
17βHSDH2	C	7.11±0.51	56±4	nd	nd	nd
	OE	4.56±0.23*	142±31*	nd	nd	nd
17βHSDH3	C	2.63±0.38	nd	nd	nd	0.104±0.002
	OE	0.16±0.03*	nd	nd	nd	0.071±0.021
17βHSDH4	C	53.2±3.2	281±15	17.3±1.2	7.9±0.5	465±31
	OE	37.2±2.8*	322±22	10.4±1.1*	8.8±0.8	560±23*
17βHSDH7	C	7.06±0.29	8.86±0.33	0.212±0.013	0.145±0.009	29.6±3.0
	OE	5.55±0.15*	6.97±0.58*	0.131±0.013*	0.163±0.018	29.5±3.1
17βHSDH8	C	40.4±0.7	44.0±2.8	2.43±0.27	1.26±0.10	602±28
	OE	34.3±1.8*	45.7±1.6	1.79±0.18	1.38±0.08	618±32
17βHSDH11	C	25.1±1.4	359±22	5.07±0.36	2.00±0.22	224±11
	OE	17.9±0.7*	347±18	4.02±0.48	2.34±0.20	231±14

Values are the mean ± sem of 8 different animals; * = $p < 0.05$ *vs*. controls; cpc = copies of the specific mRNA per cell: nd = not detected (*i.e.* not observed at more than 30 cycles)

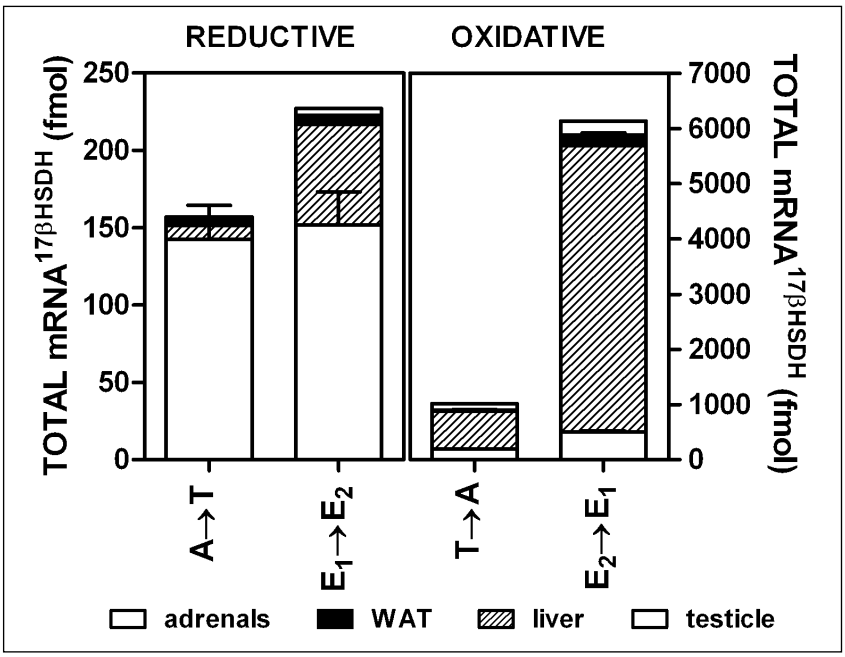

Fig. 1: Sum of combined oxidative or reductive 17βHSDH isoenzyme expressions in the organs of overweight male rats under basal conditions.
The data are the mean ± sem of 8 different animals. statistical significance of the differences vs. controls: * = $p < 0.05$. The hormone symbols used are the same as in *Table 3*.

Fig. 2 shows the marked decrease in reductive synthesis of both testosterone and estradiol induced by OE treatment; the increase in testosterone oxidation, however, is not paralleled by a similar decrease in estradiol oxidation.

The expression of other important enzymes involved in the metabolism of steroidal hormones are shown in *Table 5*. The ability to process cholesterol into steroid hormones in its first steps (20,22-hydroxylase - 20-22 lyase) is notoriously a role for the adrenal glands, with testicle in a far second place. However, once the lateral chain of cholesterol was pruned to pregnelonone, the conversion of this precursor to

Table 5: Tissue gene expression of sexual hormone metabolism enzymes in organs of overweight male rats subjected to a 10-day treatment with oleoyl-estrone

gene	C/OE	testicle (cpc)	liver (cpc)	WAT-SC (cpc)	WAT-PG (cpc)	adrenals (cpc)
20,22 hydroxylase +20-22 lyase	C	64.5±9.0	nd	nm	nm	28000±1700
	OE	9.9±2.3*	nd	nm	nm	37300±2000*
17-hydroxylase +17-20 lyase	C	94.7±8.8	4.00±0.59	0.186±0.029	0.116±0.026	0.69±0.14
	OE	19.4±5.2*	2.70±0.31	0.119±0.018	0.136±0.018	0.54±0.14
3β-hydroxysteroid dehydrogenase	C	63.4±8.8	176±12	1.58±0.26	0.73±0.10	18300±1100
	OE	39.0±5.1*	133±16	0.90±0.09*	0.94±0.11	18600±950
aromatase	C	0.36±0.05	nd	nd	nd	nd
	OE	0.16±0.01*	nd	nd	nd	nd
steroid sulfatase	C	1.12±0.17	14.3±1.6	1.01±0.09	0.570±0.063	64.5±3.9
	OE	0.64±0.04*	12.6±0.4	0.71±0.09*	0.680±0.041	55.5±1.3
estrogen sulfotransferase	C	0.339±0.075	3200±230	0.431±0.103	0.609±0.219	nd
	OE	0.049±0.010*	354±128*	0.026±0.005*	0.037±0.012*	nd
UDP-glycosyl-transferase 2-3	C	nm	3100±510	nd	nd	nd
	OE	nm	3000±360	nd	nd	nd

Values are the mean ± sem of 8 different animals; * = $p < 0.05$ *vs*. controls; cpc = copies of the specific mRNA per cell: nd = not detected (*i.e.* not observed at more than 30 cycles); nm = not measured

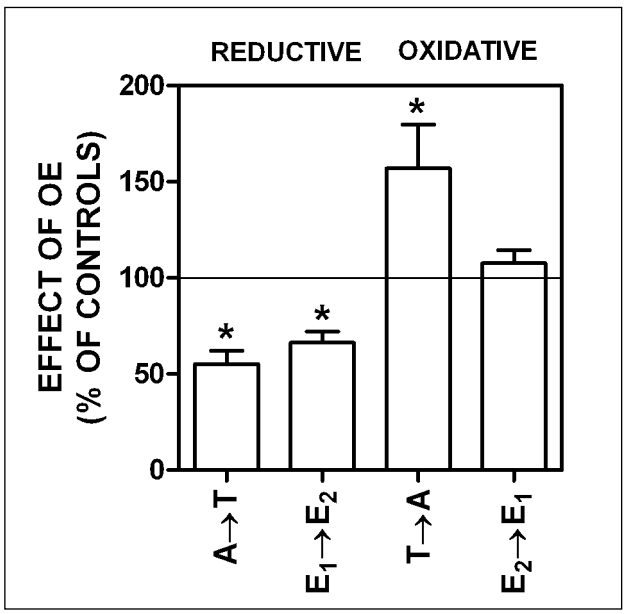

Fig. 2: Effects of 10-day OE treatment on the combined oxidative or reductive 17βHSDH isoenzyme expressions in the organs of overweight male rats.
The data are expressed as percentage of the controls' values, and are the mean ± sem of 8 different animals; statistical significance of the differences vs. controls: * = $p < 0.05$. The hormone symbols used are the same as in *Table 3.*

dehydroepiandrosterone (17α-hydroxylase, 17-20-lyase) was principally expressed in testicle (but also, to a lower extent, in all the other tissues studied). Conversion of dehydroepiandrosterone to androstenedione (3βHSDH) is again highly concentrated in the adrenals, but its small size and the fair levels found in testicle and liver suggest that this part may be also actively pursued in these organs. The effects of OE on this part of the synthesis pathway show a consistent decrease in testicular expression of all three enzyme complexes, in part compensated by a higher expression of the first enzyme of the series acting on cholesterol in the adrenals. The ability to take up sulfated steroid hormones (steroid sulfatase), i.e. estrone or dehydroepiandrosterone decreased in testicle and WAT, but was unchanged in liver (and adrenals), where it is more abundant.

Aromatase activity was also decreased in the testicle by OE, and was not found in sufficient levels in all other tissues studied, even after using a number of different probes, to show whether this trend was overall maintained.

Steroid glucuronization was unchanged by OE in liver, which contained a very high level of

expression, but estrogen sulfotransferase expression decreased sharply in liver (very high number of copies per cell) and also in testicle and WAT sites, suggesting a decreased ability to yield estrone sulfate.

DISCUSSION

The extrapolation of expression data to putative enzyme activities is not a direct relationship. However, the whole organ expression of a given gene for an enzyme reflects, in most cases (specially when no post-translational modulation has been described), the overall potency of the organ to produce the enzyme and, consequently, to justify its metabolic function.

The semiquantitative approach used here allows for such an approximation because data from different organs can be compared in terms of absolute number of copies (i.e. absolute number of enzyme molecules and their activity correlates) (24). Evidently, things never are as simple under physiological conditions, since blood flow, availability of substrates and coenzymes, as well as other regulatory elements –with substrate availability being a critical factor– may play a significantly modifying role. However, in the very limited domain of a single key enzyme, 17βHSDH, which expression has been often used as a sign of enzyme potential activity, we have been able to compare the relative weight of this activity in different organs. Thus, we analyzed the main organs that contain this enzyme, acting on two critical pairs of substrates: androgens and estrogens, in which the reduced form is the most active, and the oxidized is the less active. These comparisons, albeit incomplete, may provide us with insight as to how the system is regulated, eventually resulting in changes in serum hormones.

In the male overweight (but otherwise normal) rat, almost all testosterone is synthesized in the testicle, but the capacity to oxidize testosterone essentially belongs to the liver. This is in agreement with previous studies showing the liver as main site for testosterone processing (27) and the unique function of the testicle as testosterone provider (27-30), with only a limited (under normal conditions) synthesis in the adrenal glands (31). The oxidizing/reducing capability ratio (O/R) for the testicle is 0.73, versus 81 for the liver, and 66 for adrenals. In WAT, in spite of a significant presence of 17βHSDH, the distribution of isoenzymes does not favour the conversion of androstenedione to testosterone (O/R = 2.6), but it is far away from the oxidative preference and overall capability of the liver.

The case for estradiol (in a male rat) is fairly different, most of the reductive capacity to generate estradiol from estrone can be found again in the testicle, with liver, which is also the main site for oxidative destruction of the hormone, following suit (32). Their low estrogen levels, thus, attest to both a limited overall synthetic ability (notwithstanding several fold higher in the testicle than that of testosterone) of estradiol and the existence of powerful sulfation/glucuronization enzyme expressions that can eventually dispose of any excess of these hormones. This theoretically higher ability to produce estradiol in comparison with testosterone was probably compensated by the limited synthesis of estrogen from androgen precursors, consequence of a low aromatase expression. In parallel, the absence of significant aromatase expression in WAT attests to the limited effectivity of extragonadal estrogen synthesis in rodents (33), that contrast with the sizeable levels of aromatase found in human WAT (31, 34). Consequently, the limiting step in the formation of estradiol in male rat testicles is probably aromatization, in agreement with the data obtained in other tissues and conditions (35).

The other enzyme expressions studied agree with a powerful ability of the adrenals to convert cholesterol to steroidal hormones, including corticosteroids, but also including androgens (especially dehydroepiandrosterone in humans (36)); however, their small size relativizes their eventual contributions to sexual hormones' levels, compounded in rodents by their deficit in 17α-hydroxylase (31). The main proof that the handling of the pairs: testosterone-androstenedione and estradiol-estrone is not carried out in a similar way is the 5.1 vs. 0.3 concentrations' ratios in serum levels.

Treatment with oleoyl-estrone deeply alters these equilibria, since WAT mass is reduced in parallel to food intake (1, 5), and lipid metabolism is enhanced (37). In addition, there is a large exogenous source of estrone, that eventually coming from oleoyl-estrone hydrolysis. Unregulated, the system would markedly increase the levels of estradiol, but the lack of estrogenic effects (19), medium-term unchanged estradiol levels (13) and the non-significant change observed here attest that in some way, the serum estrone/estradiol ratio, – in the end the responsibility of whole-body 17βHSDH activity – is doubled, from 3.3 to 6.5, probably part of a conterregulatory mechanism preventing feminization because of excess estrone. In extreme human obesity, estrone levels are raised (38), but feminization is limited, probably because of this high preponderance of built-in oxidative potential.

The effect of OE decreasing testosterone synthesis is compounded by the increase in its oxidizing capability, a drive that does not affect the ability to oxidize estradiol, but nevertheless, estradiol levels in plasma do not increase in parallel to those of estrone. The O/R for estrogens changed from 25 to 41, whilst that for androgens increased more sharply, from 5.6 to 16. This may help explain the marked fall in plasma testosterone compared with that of estradiol. The increased oxidative capacity for testosterone is not parallelled by that of estradiol, but the overall capacity to oxidize estrogen is 6.5-fold higher than that of oxidizing androgens, slightly decreasing to 4.5-fold under OE treatment.

High estradiol levels may affect plasma transport of testosterone (39), but also elicit a hypothalamic response through GnRH (40), that results in an overall decrease in the synthesis of estrogens and, concurrently of their precursors, androgens (41). This may explain the general decrease in hormone-related genes of the testicle (compared with other organs) and the loss of testicular mass. The pincer effect of blocked reducing 17βHSDH isoenzymes' expression and decreased arrival of precursors of androstenedione (including its synthesis from dehydroepiandrosterone) could not compensate the decrease in aromatase to prevent enhancement of estradiol availability. The consequence is a drop in testosterone levels.

It seems that the gonadotropin path may ultimately explain most of the inhibition of testosterone synthesis and the drop in its serum levels, but the OE-elicited estradiol levels do not seem high enough to trigger an hypothalamic response of the HPG axis. However, OE induces deep changes in the hormonal function of WAT; one of its earlier and most marked effects is the inhibition of the *lep* gene (42), that is rapidly correlated with markedly decreased circulating leptin (2). This is a fairly rapid effect that may be related to the control of appetite (43), but which also affects the HPG axis, since leptin is a key hormone controlling the release of GnRH (44-45), and, consequently, of gonadotropins (46). Decreased leptin stimulation of the hypothalamus results in decreased signaling in the HPG axis (47, 48). In this same animal model and OE administration protocol, we have previously observed a marked decrease of serum leptin under OE treatment (20). The low leptin levels probably act as a powerful inhibiting mechanism limiting the release of gonadotropins (49), a decrease which in turn may result in the dramatic loss of androgenic function in spite of normalized energy homeostasis and fairly contained prevention of estrogenic responses (14). However, factors other than leptin may affect the purported decrease in testicular size and testosterone levels (probably *via* gonadotropin release), since we have observed in Zucker fa/fa rats ($lepr^{ob}/lepr^{ob}$) that prolonged treatment with OE also decreases testicle size and circulating testosterone (unpublished results).

The data presented also confirm 17βHSDH3 is the key testosterone-synthesizing isoenzyme in the testicle (29, 50) and hints at a special and marked control of its expression by gonadotropic - or direct leptin - stimulation (30, 51).

In conclusion, OE, especially in continuous treatment, induces a marked hypoandrogenic effect, mainly because of the alteration of the expression of testicular 17βHSDH3. Since in discontinuous studies no negative effects have been observed (15,16), we can assume that OE effects on androgen synthesis, and thus on the hypothalamic-pituitary-gonadal axis are limited to the testicle. This allows for a rapid recovery when using discontinuous OE administration.

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DISCUSIÓN GENERAL

4 Discusión general

Efecto combinado de la OE y la limitación de la ingesta

La administración de OE produce una pérdida significativa de peso en los animales de experimentación, principalmente mediante la reducción de la ingesta y el mantenimiento del gasto energético [78].

La mayoría de los tratamientos (dieta, ejercicio, cirugía bariátrica, incluso los farmacológicos supresores del apetito o activadores del gasto energético) utilizados en la actualidad para combatir la obesidad requieren la combinación continuada con dietas hipo-energéticas para mejorar su limitada efectividad.

En la primera parte de este trabajo se planteó la cuestión de si el tratamiento con OE también aumentaría su efectividad en combinación con una dieta hipo-energética. Se utilizaron ratas macho previamente sometidas a un periodo de 30 días de dieta de cafetería para aumentar sus reservas lipídicas [171]. La administración de esta dieta rica en grasa y azúcares incrementó moderadamente el peso del animal y la masa de tejido adiposo respecto a los controles alimentados con pienso estándar, lo que permitió estudiar mejor la modificación de las reservas corporales por el tratamiento, ya que se partía de una masa de reservas significativa.

En concreto se estudiaron los efectos combinados de la OE y la restricción adicional de la ingesta energética, para analizar si esta combinación potenciaba la disminución de peso que produce la OE. Este estudio permitió también comparar los efectos de la restricción calórica con los del tratamiento con OE.

La administración oral de OE durante 10 días disminuyó la ingesta voluntaria de alimento en un 30-40% con respecto las ratas control, mientras que las restricciones energéticas que se aplicaron en paralelo y en combinación con la administración de OE fueron un 50% y un 75% de la ingesta *ad libitum* de las ratas control. Estas dos dietas restringidas, bajas en energía, pretendían ser equivalentes a las dietas bajas en energía (LCD) y muy bajas en energía (VLCD) utilizadas en el tratamiento de la obesidad en humanos, aunque su diseño fue más simple, ya que se aplicó únicamente una restricción energética, sin modificar las proporciones de proteína ni de microcomponentes.

La pérdida de peso observada al combinar la administración de OE con una dieta LCD o VLCD dio lugar a una pérdida de peso significativa con respecto al grupo tratado con OE sin restricciones, es decir, con libre acceso a la comida. La pérdida de peso corporal del grupo OE combinado con las dietas afectó poco la pérdida de lípido corporal con respecto al grupo OE, y no hubo diferencias entre la dieta más restrictiva con respecto a la menos restrictiva; es posible que se hubiera alcanzado el límite de las reservas lipídicas más fácilmente disponibles. Sin embargo, la combinación de OE con estas dietas dio lugar, en

los dos casos, a la pérdida de proteína corporal, que fue proporcional al grado de restricción energética. Así, la diferencia de peso entre el grupo OE combinado con las dietas restrictivas y el grupo OE se debió, en parte, a la pérdida neta de proteína corporal, y no sólo a la movilización de grasa.

Las restricciones energéticas se compensan en parte por la disminución del gasto energético [172]. Tanto las dietas LCD y VLCD como su combinación con OE disminuyeron el gasto energético. Se observó que la disminución del gasto energético compensatorio a la disminución de la ingesta también tiene un límite, ya que el gasto energético fue el mismo para ambas dietas restrictivas. El tratamiento con OE mantiene el gasto energético [78], y este mantenimiento refleja una respuesta diferente frente a la limitación de la ingesta energética, que parecía disminuir parcialmente al combinar la OE con la restricción del acceso a la comida.

Un aspecto característico del tratamiento con OE es que mantiene la glucemia [90] a pesar de la disminución de la ingesta, mientras que la restricción energética (sin OE) disminuye la glucemia y la insulinemia e incrementa los niveles circulantes de ácidos grasos libres y cuerpos cetónicos. La combinación de OE con las dietas LCD y VLCD dio lugar en general a una contención del perfil plasmático característico de una restricción energética, ya que no disminuyó tanto los niveles de glucosa y de insulina, ni aumentó tanto los de ácidos grasos libres y cuerpos cetónicos como en la ausencia de OE.

A la vista de estos resultados no parece derivarse ninguna ventaja adicional de combinar el tratamiento con OE con una restricción energética adicional, ya que los efectos de ambas estrategias no son aditivos. Además, el análisis de la composición corporal nos indica que mientras que el tratamiento con OE afecta principalmente a las reservas de grasa corporal, al combinarlo con una restricción energética adicional, se moviliza también la proteína corporal, incluso en mayor grado que en los grupos sólo sometidos a restricción calórica, por lo que la combinación de OE con una restricción energética adicional podría comprometer negativamente el balance nitrogenado.

Por tanto, y a diferencia del resto de tratamientos anti-obesidad, la administración de OE no requiere, y ni tan sólo es aconsejable, limitar adicionalmente la ingesta de energía, evitándose así no sólo la sensación de hambre sino también posibles daños derivados de la alteración del metabolismo proteico.

Efectos de la OE sobre el metabolismo energético

A pesar de que uno de los efectos más destacables de la administración de OE es la disminución de la ingesta, este primer trabajo indicó la existencia de otros mecanismos metabólicos adicionales como el mantenimiento del gasto energético [82] y de la homeostasis glucídica (glucemia y glucógeno hepático) [90], así como la disminución de los niveles de colesterol circulantes [94].

En la segunda parte de esta tesis se estudiaron los efectos de la administración de OE sobre el tejido adiposo y el hígado, utilizando ratas sometidas previamente a una dieta de cafetería, con el fin de profundizar en el análisis de su mecanismo de acción. Paralelamente, se comparó la administración de OE con un grupo sometido a una restricción energética equivalente (grupo *pair fed*) a la ingesta real del grupo tratado con OE para poder distinguir los efectos metabólicos de la OE de la simple disminución del apetito, y valorar de este modo hasta qué punto los efectos de la administración de OE son consecuencia de la disminución de la energía ingerida.

La principal dificultad que plantea el modelo *pair fed* es que se trata de una restricción no voluntaria de la ingesta, a diferencia de la administración de OE, que disminuye el apetito. En estos dos modelos, la disminución de la ingesta por saciedad (OE) y la restricción de la ingesta forzada (*pair fed*), son diferentes, ya que en la restricción de la ingesta participan mecanismos relacionados con el ahorro y el estrés, por lo que no se puede considerar que el modelo *pair fed* constituya un simple control de la ingesta respecto al tratamiento con OE. Además, aunque la cantidad diaria de comida ingerida fue la misma en los dos grupos, el perfil de ingesta fue muy diferente, ya que mientras que el grupo OE tuvo libre acceso a la comida, el grupo *pair fed* consumía rápidamente la comida que se le ofrecía una vez al día pasando el resto del día en situación de ayuno.

Buena parte de este estudio se centró en la valoración de las expresiones génicas de enzimas que participan en las principales vías metabólicas implicadas en el metabolismo energético y del colesterol. El análisis de expresiones génicas en tejidos con una alta variabilidad respecto su celularidad o extensión hizo necesario adaptar la metodología a valoraciones no basadas en porcentajes de cambio con respecto a los controles (cuantificación relativa), con poco sentido ya que grandes variaciones de tamaño celular pueden dar resultados muy dispares en la comparación. Por ello se desarrolló un enfoque sencillo a partir de la metodología disponible para el estudio de la expresión génica mediante PCR a tiempo real que nos permitió estimar de manera aproximada el número de tránscritos de cada ARN mensajero por célula o por tejido total. Esta aproximación cuantitativa, a diferencia de la cuantificación relativa que expresa los resultados como unidades arbitrarias dependientes de cada ensayo, permitió comparar los resultados entre diferentes grupos experimentales y tejidos, referir el número de transcritos al tejido total y además hacerse una idea de la abundancia relativa de los transcritos al permitir comparaciones entre diferentes genes.

En las ratas tratadas con OE y en las ratas *pair fed*, la disminución de peso corporal (del orden de un 10 % con respecto al peso inicial) durante los 10 días de tratamiento estándar fue similar. Esto nos indica que la pérdida de peso producida por el tratamiento con OE sí que es consecuencia, al menos en parte, de la disminución de la ingesta. Sin embargo, muchos de los parámetros estudiados en estos dos grupos de ratas, de acuerdo con los resultados previos, mostraron importantes divergencias en cuanto a su estrategia

metabólica, destacando las diferencias en los niveles circulantes de hormonas y metabolitos.

La administración de OE normalizó la glucemia de las ratas previamente sometidas a una dieta de cafetería, que presentaban niveles de glucosa algo elevados; pero además, la OE mantuvo su glucemia (normoglucemia) por encima de los niveles del grupo *pair fed* (hipoglucémicas). Precisamente este mantenimiento de la glucemia es una de las razones que podría explicar la disminución del hambre que provoca la administración de la OE y que incide con tanta eficacia en el control de la ingesta.

La disminución de los niveles circulantes de insulina y el efecto sensibilizador a la insulina es una característica ya descrita desde los primeros experimentos con OE intravenosa [101]. Sin embargo, en comparación con los bajos niveles de insulina que presentó el grupo *pair fed*, se podría decir que la OE lo que hizo en realidad fue moderar la disminución de los niveles de insulina frente a una misma cantidad de alimento ingerido. Un efecto comparable se observó con la leptina circulante, que disminuyó en ambos grupos, pero más marcadamente en el grupo *pair fed*.

El tejido adiposo se ha considerado desde los inicios del estudio de la OE uno de sus principales tejidos diana, ya que tras su administración se observa una gran pérdida de masa grasa [73]. En este trabajo se compararon cuatro localizaciones distintas de tejido adiposo, con funciones, morfología y disposición anatómica diferentes: el tejido adiposo subcutáneo inguinal y tres localizaciones independientes de tejido adiposo intraabdominal: el retroperitoneal, el epididimal y el mesentérico, este último comparable al tejido adiposo visceral de los humanos.

Tanto en el grupo OE como en el grupo *pair fed* se observó una disminución del peso de las cuatro localizaciones de tejido adiposo estudiadas, del 30 al 45 % respecto las ratas control. En los cuatro depósitos adiposos disminuyó también el tamaño celular, mientras que el número de células no varió en el caso de los depósitos mesentérico y subcutáneo, aunque sí que lo hizo el epididimal en el grupo OE y el retroperitoneal en ambos grupos. De hecho, la OE incrementa la expresión de factores apoptóticos a las 48 horas de tratamiento en la localización perigonadal [89]. A su vez se ha descrito que en la rata la restricción calórica disminuye tanto la masa global de tejido adiposo como el tamaño medio de los adipocitos [173]. Cabe señalar que la reducción del tamaño de los adipocitos está asociada a un incremento de la sensibilidad a la insulina [174].

En ambos grupos se observó una disminución de la expresión de los genes que codifican enzimas lipogénicas (aunque de un modo más marcado en el grupo OE) en todas las localizaciones del tejido adiposo estudiadas, excepto en el mesentérico, que a pesar de disminuir su tamaño respecto al grupo control, no solo no disminuyó la expresión de los genes relacionados con la síntesis de lípidos (excepto la expresión de la triacilglicerol lipasa (*Atgl*) de tejido adiposo) sino que incluso, en el grupo *pair fed*, la expresión de éstos

aumentó. Un perfil de expresión similar se observó en el caso del gen que codifica para la lipoproteína lipasa (*Lpl*), que disminuyó en general en todas las localizaciones, de acuerdo con una menor posibilidad de captación de lípidos de lipoproteínas circulantes, excepto en el tejido adiposo mesentérico, en contacto directo con el intestino. La expresión del gen que codifica la ATGL aumentó también en el tejido adiposo mesentérico, con un perfil similar al del transportador de ácidos grasos FAPT1.

La insulina juega un papel importante en el ajuste de la función metabólica específica del tejido adiposo en el período postprandrial y el ayuno. La disminución de los niveles circulantes de glucosa e insulina inducidas por la restricción energética está asociada a la menor captación de ácidos grasos, y de la lipogénesis, en el tejido adiposo. El ayuno y la restricción energética sensibilizan al adipocito frente a la acción de la insulina para potenciar su capacidad lipogénica y de almacenamiento de energía cuando los niveles de substratos energéticos vuelvan a aumentar. Esto se consigue en parte incrementando la respuesta de la LPL y, en paralelo, de la lipogénesis tras la ingesta de comida [175]. Así, en ratas sometidas a una restricción energética del 30% a las que se le suministra pienso una vez al día (y que consumen toda la comida en un corto espacio de tiempo, como en el grupo *pair fed* de nuestro experimento), se observa un marcado y breve período diario de síntesis y captación de ácidos grasos seguido de otro mucho más prolongado en el que predomina el catabolismo de lípidos [176]. En este estudio, las ratas *pair fed* se sacrificaron por la mañana tras de toda una noche sin acceso al alimento, por lo que los datos obtenidos podrían estar enmascarando los picos de lipogénesis y captación de ácidos grasos que caracterizan a este modelo de restricción. Sin embargo, en el grupo *pair fed* sí que se observó, a diferencia del grupo OE, que el depósito mesentérico, descrito como el más dinámico en cuanto a la movilización de lípidos a corto plazo [177], mantuvo incrementada la expresión de los genes que codifican enzimas lipogénicas clave.

El aumento de sensibilidad a la insulina en el tejido adiposo durante la restricción calórica facilita la captación y almacenamiento de los nutrientes en forma de triacilgliceroles, con la ventaja de permitir la salida regulada de ácidos grasos (constituyen una alternativa a la oxidación de glucosa) y de glicerol (substrato de la gluconeogénesis hepática) cuando descienden los niveles de insulina. Además, la exposición aguda a los glucocorticoides durante los períodos de privación de alimento (estrés por hambre) incrementa la actividad de la vía lipolítica [178]. La extrema sensibilidad a los cambios de suministro de nutrientes del adipocito bajo restricción calórica está relacionada con el "efecto rebote" de las dietas bajas en energía, y con el incremento de peso corporal que se suele producir tras de períodos de escasez de alimentos. En el caso de las ratas tratadas con OE, el mantenimiento o descenso limitado de los niveles de glucosa y de insulina, junto con un menor nivel de estrés debido al hambre (la disminución de la ingesta era voluntaria), dio lugar a una pérdida de grasa equivalente a la de una simple restricción, pero bajo condiciones mucho más tolerables y de menor estrés, que probablemente contribuyen a

evitar la recuperación del peso perdido al finalizar el tratamiento [77], independientemente de sus posibles efectos sobre el ajuste del ponderostato [85].

En los seres humanos se suele diferenciar el tejido adiposo visceral del subcutáneo, en cuanto al tamaño más pequeño de sus adipocitos, a una menor capacidad proliferativa y a una mayor capacidad de recambio lipídico, entre otras características [177]. Sin embargo, en ratas, el tejido adiposo visceral es muy limitado (tejido adiposo mesentérico), y comparte espacio (cavidad visceral) con otras masas de tejido adiposo que representan en conjunto una elevada proporción respecto del tejido adiposo total [179]. Esta ubicación hace que muy a menudo se confunda la grasa intraabdominal con la grasa visceral. En los humanos, la masa grasa visceral (mesentérica, omental) tiene el retorno venoso a través de la vena porta hepática, con conexiones importantes al sistema linfático (gran conducto torácico) y una importante integración anatómica con el intestino. En los roedores, esta función la desempeña el tejido adiposo mesentérico, mientras que las otras masas intraabdominales, sobre todo el tejido adiposo retroperitoneal y el perigonadal (periovárico/epididimal), tienen una importante función de reserva y su retorno venoso se realiza por la cava inferior, es decir, están fuera del circuito enterohepático.

De acuerdo con esta distinta funcionalidad, en los experimentos descritos en la presente tesis, el tejido adiposo mesentérico mostró un comportamiento muy diferente al del resto de las localizaciones citadas: no disminuyó la expresión de los genes lipogénicos, sino que incluso aumentó en el caso del grupo *pair fed* y a su vez incrementó la expresión del gen que codifica la triacilglicerol lipasa, lo que indica una mayor actividad, tanto lipogénica como lipolítica. Nuestros resultados analíticos, por tanto, están plenamente de acuerdo con los paralelismos anatómicos indicados más arriba que establecen la homología de esta localización con el tejido adiposo visceral humano.

El hígado es un órgano determinante en la homeostasis de la glucosa. Una de las principales características del tratamiento con la OE es el mantenimiento de los niveles de glucosa circulante así como de las reservas de glucógeno hepático y muscular, a pesar de la disminución de la ingesta [90]. En el presente estudio, la administración de OE disminuyó a la mitad la cantidad de glucógeno hepático con respecto al grupo control, mientras que prácticamente desapareció en las ratas *pair fed*.

Bajo condiciones de restricción energética, el hígado activa la gluconeogénesis frente a la glucolisis para mantener la homeostasis glucídica. Así, el grupo *pair fed* disminuyó la expresión de los genes que codifican la glucoquinasa (*Gk*) y las enzimas glucolíticas fosfofructoquinasa 1 (*Pfkl*), gliceraldehído-3-fosfato deshidrogenasa (*Gapdh*) y piruvato quinasa (*Pk*), mientras que aumentó la expresión de los genes que codifican las enzimas gluconeogenicas fofoenolpiruvato-carboxiquinasa (*Pck1*) y glucosa-6 fosfatasa (*G6pc*). La marcada disminución de los niveles circulantes de insulina en el grupo *pair fed* podría favorecer la proteólisis muscular y lipólisis del tejido adiposo; de esta forma, la liberación

periférica de alanina, glutamina, y glicerol podría aumentar el suministro de sustratos gluconeogénicos para el hígado.

En cuanto a la síntesis de lípidos en el hígado, en el grupo *pair fed* disminuyó la expresión de los genes que codifican las enzimas lipogénicas citrato: ATP liasa (*Acly*), acetil-coenzima A carboxilasa 1 (*Acca*), ácido graso sintetasa (*Fasn*) y adiponutrina (*Adpn*), en paralelo con la disminución de la expresión de los genes que codifican las enzimas glucolíticas.

En el grupo *pair fed*, a diferencia del grupo OE, aumentaron los niveles de ácidos grasos libres circulantes, así como la expresión hepática del gen que codifica la carnitina-palmitoeloil-transferasa 1a (*Cpt1a*), mientras que disminuyó la de la acetil-coenzima A carboxilasa 2 (*Accb*). La CPT1a es la enzima limitante en el transporte de ácidos grasos a través de la membrana mitocondrial interna para su oxidación (mediante la ß-oxidación), y el malonil-CoA, producto de la ACC2, controla la oxidación de éstos por su efecto inhibidor de la carnitina-palmitoleoil-transferasa. El incremento de la oxidación de ácidos grasos que se produce durante la restricción energética favorece la producción de cuerpos cetónicos, al bajar la disponibilidad de insulina y aumentar la de acetil-CoA, como se observó en el grupo *pair fed*. El 3-hidroxibutirato y el acetoacetato pueden ser utilizados por muchos tejidos como substrato energético, contribuyendo con ello al ahorro de glucosa, y con ello de proteína.

De acuerdo con este perfil ahorrador, en el hígado del grupo *pair fed* disminuyó la expresión del gen que codifica el receptor de insulina, así como de los genes que codifican los factores de transcripción implicados en la síntesis de lípidos SREBP-1c (*Srebf1*), ChREBP (*Wbscr14*) y LXRa (*Nr1h3*) mientras que incrementó la expresión del gen que codifica el PPARα (*Ppara*). El incremento de PPARα, activador de la gluconeogénesis y de la β-oxidación, está de acuerdo con el incremento de la expresión de los genes *Pck1* y *Cpt1a*, respectivamente. El grupo *pair fed* también disminuyó la expresión del gen de la proteína Spot 14 (*Thrsp*), que es un factor relacionado con la lipogénesis y cuya expresión depende de los niveles de T_3, insulina y glucosa. Además, en el grupo *pair fed* disminuyó la cantidad de proteína SREBP-1c, tanto de la forma precursora residente en el retículo endoplasmático como de la forma madura localizada en el núcleo celular.

En contraste con el grupo *pair fed*, el grupo OE incrementó la expresión del gen que codifica la glucoquinasa (*Gk*), mantuvo la de los genes que codifican las enzimas glucolíticas sin cambios con respecto a los controles y disminuyó la expresión de la *Pck1*. Además, en el grupo OE se incrementó la expresión de los genes que codifican las proteínas que participan en la síntesis de NADPH como la glucosa 6 fosfato deshidrogenasa (*G6pdx*) y el enzima málico (*Me1*), que permiten aportar el NADPH necesario para los procesos de síntesis [180].

A pesar de la disminución de la ingesta la administración de OE mantuvo inalterada la expresión de los genes que codifican las enzimas lipogénicas, e incluso se observó un incremento en la expresión de genes que codifican enzimas implicadas en las síntesis de triacilgliceroles estearoil-Coa desaturasa 1 (*Scd1*) y glicerol-3P acil-transferasa (*Gpam*), sin que se observara un aumento del contenido de lipídos del hígado. La probable utilización de la glucosa en el hígado por parte del grupo OE, aunque limitada, pudo verse favorecida por el mantenimiento de la glucemia y la disminución de la captación de glucosa a nivel de los tejidos periféricos [88]. También mantuvo la expresión del gen que codifica ChREBP (*Wbscr14*), factor de transcripción que estimula la lipogenesis mediante el aumento de la disponibilidad de glucosa. El aumento de la expresión génica y proteica del factor SREBP-1c, mediador de los efectos de la insulina en la vía lipogénica y de la síntesis de triacilgliceroles, y de acuerdo con la mayor la expresión de enzimas de síntesis de tricilgliceroles y de generación de NADPH descritos más arriba, parece confirmar el mantenimiento de una cierta capacidad de síntesis de triacilgliceroles por el hígado de las ratas sometidas a tratamiento con OE.

La lipogenesis hepática y la activación hepática de SREBP-1c se modulan, en parte, en función de la cantidad de alimento ingerido; la relación entre glúcidos y grasa disponibles y la concentración de insulina circulante [181]. La insulina activa la expresión y la maduración de SREBP-1c; de hecho se ha relaciona la activación de SREBP-1c en el hígado con el aumento de la disponibilidad de aminoácidos mediante mTORC1, un complejo dependiente de insulina que favorece el metabolismo anabólico [182]. El aumento de la expresión de SREBP-1c se asocia además en muchos estudios a modelos de obesidad con esteatosis hepática, en los que el exceso de nutrientes estimula la formación de triacilgliceroles hepáticos [183] a los que no puede dar salida. En el grupo OE, el hígado se comportó, con respecto al metabolismo energético, como si el organismo en conjunto no estuviera sometido a un balance de energía negativo, manteniendo la lipogénesis e incluso incrementando la síntesis de triacilgliceroles. Tanto los ácidos grasos sintetizados *de novo* como los procedentes del tejido adiposo podrían formar parte de las lipoproteínas que serían utilizadas como sustrato energético por el resto de órganos, manteniendo de esta forma los niveles de glucosa dentro de la normalidad, con la única excepción del "vaciado" energético del tejido adiposo. La rata mantendría sus niveles de gasto energético con una ingesta disminuida y el aporte masivo de lípidos del tejido adiposo, manteniendo la normalidad homeostática en el resto de tejidos y órganos.

El incremento de SREBP-1c en las ratas a OE podría ser la consecuencia de un incremento de la sensibilización hepática a la insulina, pero también de la activación del receptor nuclear LXR que une oxiesteroles y elimina colesterol hepático, ya que se ha descrito que también activa la lipogénesis mediante el aumento de la expresión de SREBP-1c [184].

El tratamiento con OE mantiene el gasto energético a pesar de la disminución de la ingesta, ya que no disminuye el consumo de oxígeno ni la expresión (ni niveles) de la proteína desacopladora UCP1 del tejido adiposo marrón interescapular (TAMi) [73,185]. Por contra, se

ha descrito que el balance energético negativo que comporta la restricción calórica involuntaria da lugar al incremento del apetito, la disminución de la tasa metabólica y una disminución de la actividad simpática [172]. El incremento de eficiencia metabólica en roedores producido por la escasez de energía está relacionado con la disminución de la capacidad termogénica del TAM y ayuda a proteger en parte las reservas (glucógeno, triacilgliceroles), pero sobre todo la proteína corporal, factor clave para la supervivencia durante los periodos de ayuno. La reducción calórica da lugar a una disminución global de leptina, que a su vez disminuye la expresión de TRH, TSH y con ellas de las hormonas tiroideas, sobre todo T_4, lo que también conduce a una disminución de la termogenesis [48].

De hecho en la situación contraria, que sería la hiperfagia/sobrealimentación, como es el caso de las ratas sometidas a una dieta de cafetería, se produce un aumento de la actividad del sistema nervioso simpático, así como de la concentración intracelular de T_3 a través de la activación de la desyodasa 2 [186]. Este incremento de actividad del sistema nervioso simpático y de la formación de T_3 contribuyen a aumentar de forma sinérgica la tasa metabólica basal y hasta cierto punto limita o retrasa la aparición de la obesidad.

En nuestro estudio, la administración de OE no disminuyó la expresión génica de la *Ucp1*, a diferencia del grupo *pair fed* en el que sí disminuyó. De acuerdo con esto, la concentración de leptina circulante disminuyó en el grupo OE, aunque no tan marcadamente como en el caso del grupo *pair fed*. Sin embargo, la expresión del gen que codifica la desyodasa 2 (*Dio2*) (que convierte la tiroxina T_4 en triodotironina T_3), disminuyó en ambos grupos.

Tanto el grupo OE como el grupo *pair fed* experimentaron una pérdida significativa de la masa del TAMi respecto al grupo control, aunque no varió el número de células del tejido, por lo que se deduce que el TAMi de los grupos OE y *pair fed* contienen células más pequeñas debido, principalmente, a la pérdida de lípido, que fue más pronunciada en el grupo *pair fed*. El mantenimiento de una reserva adecuada de triacilgliceroles es esencial para la termogénesis, ya que los ácidos grasos que se derivan de su hidrólisis representan, al menos inicialmente, el principal sustrato para la producción de calor y además activan a la UCP1 [187].

La captación de glucosa por el TAM está incrementada por la insulina y la activación del sistema nervioso simpático [46]. Las expresiones de los genes que codifican el transportador de glucosa GLUT4 (*Slc2a4*), de la hexoquinasa 2 (*Hk2*) y diversas enzimas implicadas en la glucolisis y la lipogénesis (*Acly*, *Acca*, *Fasn*, *Gpam*, *Adpn*, *Spot14* y *Wbscr14*) disminuyeron tanto en el grupo OE como en el grupo *pair fed*, aunque de manera más marcada en el grupo OE (*Acca*, *Fasn*, *Scd1* y *Me1*), a pesar de los niveles de glucosa y de insulina circulantes mantener más elevados que el grupo *pair fed.*

Los inductores reconocidos del reclutamiento del TAM son la estimulación crónica de los terminales nerviosos simpáticos y el PPARγ. Mientras que el reclutamiento en el caso del

sistema simpático se traduce en una actividad termogénica, el reclutamiento mediante PPARγ está asociado a una reducción de la actividad simpática y a un incremento de la reserva lipídica en los adipocitos marrones. En el grupo OE la expresión de *Pparγ* disminuyó, lo que podría explicar la reducción, más marcada, de la expresión de *Acc1*, *Fas*, *Me1* y *Scd1* [188].

Por otro lado el grupo OE disminuyó la expresión de los genes que codifican los factores PGC1a (*Ppargc1a*) y NRF1 (*Nrf1*). La expresión del gen que codifica para el cofactor PGC1a incrementa paralelamente con la diferenciación del adipocito marrón e induce la expresión del gen que codifica el factor respiratorio nuclear NRF1, con el que interacciona activando la transcripción de genes de la cadena respiratoria mitocondrial [189].

La capacidad termogénica del TAMi en las ratas OE al parecer se mantuvo por encima de la correspondiente a la simple restricción energética mediante el mantenimiento de la expresión de *Ucp1* y de una menor disminución de las reservas lipídicas del tejido, probablemente gracias al mantenimiento de los niveles de glucosa circulante, a pesar de que este tejido mostró una inhibición más profunda, bajo tratamiento con OE, de la expresión de los genes que codifican enzimas lipogénicas y factores de transcripción implicados en los procesos oxidativos mitocondriales.

Cabe señalar que el estudio se ha centrado en el estudio del TAM interescapular, que en roedores representa el depósito de TAM de mayor tamaño. Sin embargo, el mantenimiento del gasto metabólico observado durante la administración de la OE podría tener lugar, en parte, en depósitos de tejido adiposo blanco, ya que se reconoce cada vez más la importancia de la coexistencia de adipocitos marrones, "beige" y blancos en los diferentes depósitos grasos [190], así como la gran plasticidad de los adipocitos para transdiferenciarse o diferenciarse de nuevo bajo determinados estímulos [191]. De hecho, en un estudio posterior, se observó que la administración de OE incrementa la expresión de la UCP-1 en algunas localizaciones del tejido adiposo blanco [192]. Además, existen otras vías metabólicas relevantes (que en parte aún nos son desconocidas) en otros tejidos, como el músculo y el hígado, que participan en el gasto energético global, mediante la modulación hormonal o metabólica de la eficiencia de algunos procesos metabólicos [47,48] y que también podrían participar en el mantenimiento de la tasa metabólica observado en el tratamiento con OE.

La administración de OE en rata disminuye los niveles circulantes de colesterol total en ratas de ambos sexos; este efecto se observa incluso cuando los animales se someten a una dieta hiperlipídica [84]. Como la mayor parte del colesterol circulante en rata se encuentra en forma de HDL, el tratamiento con OE disminuye la proporción de estas lipoproteínas con respecto al colesterol total [93]. Los roedores, que no tienen CETP, utilizan las HDL como principal transportador plasmático de colesterol, y son relativamente resistentes a la aterosclerosis, lo que está de acuerdo con el potencial antiaterogénico de la HDL.

Estudios previos ya indicaron que el tratamiento oral con OE disminuye, en cuestión de horas, los niveles circulantes de colesterol total, incrementando también el recambio de colesterol, como parece indicar la marcada disminución de la fracción de colesterol esterificado [94]. Los cambios en la dinámica del colesterol circulante pueden ser el resultado de cambios en su síntesis o en su excreción (catabolismo a ácidos biliares, excreción biliar), por lo que en este trabajo se estudió el efecto de la OE sobre la expresión de los genes que codifican las principales proteínas hepáticas implicadas en el metabolismo del colesterol.

La administración de OE a ratas macho previamente alimentadas con una dieta de cafetería hizo disminuir cerca del 60% los niveles de colesterol circulante con respecto a los grupos control y *pair fed*, lo que indicó, como previamente se había observado [90], que la disminución del colesterol plasmático debida al tratamiento con OE es un proceso independiente de la disminución de la ingesta.

Por otro lado, el grupo de ratas tratadas con OE mostró un incremento del colesterol hepático total. El aumento de la expresión del gen que codifica el receptor de LDL (*Ldlr*), que se observó en el hígado del grupo OE, está de acuerdo con el incremento de la captación hepática de colesterol, lo que podría contribuir a explicar su acumulación en el hígado. El receptor de LDL permite incorporar el colesterol a las células mediante endocitosis de las LDL y de otras lipoproteínas ricas en ApoE, como es el caso de las HDL de rata [193].

Por lo que respecta a la síntesis de colesterol, nuestros resultados indicaron que la expresión hepática del gen que codifica la enzima hidroximetil-glutaril-coenzima A reductasa (*Hmgcr*) no presentó variaciones por efecto de la OE, aunque cabe señalar que la regulación de esta reductasa en ratas se produce principalmente a nivel post-transcripcional [194]. Tampoco se observaron cambios en la expresión hepática del gen que codifica el factor de transcripción SREBP-2 (*Srebf2*), y que se activa mediante la disminución de los niveles de colesterol celular, y que, a su vez, activa la expresión del gen *Hmgcr* [195]. El aumento de los niveles de colesterol en el hígado observado en el grupo tratado con OE no parece que sea la consecuencia de un incremento de la síntesis hepática de colesterol, ya que, además, la expresión del gen que codifica la enzima hidroximetil-glutaril-coenzima A sintetasa 1 (*Hmgs1*), limitante en la síntesis de colesterol (vía del mevalonato), también disminuyó. Es más, parece probable que la HMGR se encuentre inhibida a nivel post-transcripcional en el grupo OE simplemente por los elevados niveles de colesterol presentes en el hígado, ya que el colesterol hepático disminuye la actividad de esta enzima mediante la inhibición de la maduración de SREBP-2 , así como aumentando la degradación de la HMGR [196].

El hecho de que la expresión hepática del gen que codifica el receptor de LDL (*Ldlr*), gen diana de la SREBP2, aumente a pesar del incremento del colesterol hepático, podría ser, en parte, la consecuencia de la activación de otras vías de regulación en las que podrían estar

implicados los estrógenos liberados en la hidrólisis de la OE. Se ha comprobado que los estrógenos a dosis farmacológicas disminuyen el colesterol circulante mediante el incremento de la expresión del receptor de LDL en el hígado [197].

El receptor nuclear LXRα se activa al aumentar los niveles de colesterol celular mediante su unión a los oxiesteroles producidos en el metabolismo del colesterol [198]. La activación del receptor LXRα induce la expresión de los genesque codifican las apolipoproteínas ApoA1 (*Apoa1*), ApoE (*Apoe*) y el transportador ABCA1 (*Abca1*) [199]; estas proteínas participan en el transporte reverso de colesterol, que recoge el colesterol desde los tejidos periféricos y ayudan a conducirlo hacia el hígado para su catabolismo oxidativo, excreción o reciclado. En los roedores, la activación del LXRα también incrementa la expresión del gen *Cyp7a1* [198] que codifica la colesterol 7α-hidroxilasa, que es la enzima limitante del catabolismo del colesterol y paralelamente de la síntesis de ácidos biliares. La administración de OE produjo un incremento en la expresión de todos estos genes implicados en la remoción del exceso de colesterol y activados por LXRα, aunque no se observó ningún incremento en la propia expresión del gen que codifica LXRα (*Nr1h3*). La activación de los genes diana de LXRα está de acuerdo con el aumento de los niveles de colesterol hepático observado en el grupo OE. Además, el aumento en el grupo OE de los niveles de proteína del transportador ABCA1 y de la colesterol 7α-hidroxilasa confirmaron el aumento de la captación periférica de colesterol, su transporte al hígado y la eliminación del exceso de colesterol por este órgano.

La hipótesis de que la OE activa la función del factor de transcripción hepático LXRα viene reforzada por el incremento de la síntesis hepática de triacilgliceroles descrita más arriba. El receptor LXRα, además de su papel como sensor de los niveles de colesterol hepáticos, estimula la lipogénesis activando la transcripción de SREBP-1c y de la vía lipogénica [184]. Tanto el uso de dietas ricas en colesterol como de agonistas del LXRα aumentan la expresión de SREBP-1c. Se ha postulado que la inducción de la expresión de SREBP-1c por parte del LXRα tiene como finalidad generar ácidos grasos suficientes para la permitir la formación de los ésteres de colesterol, que a su vez ayudan a modular los niveles de colesterol libre [200]. Esta regulación dual mediada por la insulina y por los oxiesteroles parece ser un factor clave para la regulación de la homeostasis energética a través de SREBP-1c.

Relaciones de la OE con las hormonas esteroideas

Una de las principales limitaciones del tratamiento con OE es la interferencia con los glucocorticoides, que contraregulan su mecanismo de acción [106,107]. Durante la administración intravenosa de OE a ratas hembra con normopeso se produce un incremento de los niveles de ACTH y corticosterona plasmática a partir del sexto día de tratamiento, coincidiendo con el momento en el que la caída del peso corporal empieza a estabilizarse [101]. Además, la administración de OE a ratas hembra disminuye la expresión y la capacidad de unión de glucocorticoides a la globulina transportadora de glucocorticoides (CBG), lo

que implica el aumento de la fracción de corticosterona libre. De esta manera los glucocorticoides pueden limitar muchos de los efectos de la administración de OE al cumplir su función de proteger al organismo frente a la pérdida de sus reservas energéticas [108].

En el modelo experimental utilizado en este estudio (ratas macho alimentadas previamente con una dieta de cafetería para incrementar su contenido corporal de grasa) se observó que la administración de OE dio lugar a un incremento del peso de las glándulas adrenales, aumentando también su número de células. Estos datos indujeron el estudio de cómo la OE podía afectar el funcionamiento de esta glándula, responsable tanto de la síntesis de catecolaminas (zona medular) como de glucocorticoides (zona cortical). En roedores, a diferencia de los humanos, en la zona cortical de la glándula adrenal se sintetizan solamente mineralocorticoides y glucocorticoides, al no expresar la enzima 17α hidroxilasa/17,20-liasa (CYP17α1) [201], paso limitante en la síntesis de deshidroepiandrosterona (DHEA) y de los demás andrógenos.

El análisis de los niveles circulantes de corticosterona indicó que, a pesar de que en el grupo OE se produjo un incremento de esta hormona, las diferencias con el grupo control no fueron significativas. Sin embargo, los niveles de corticosterona sí que aumentaron significativamente en el grupo *pair fed*, sometido a una restricción energética forzada, con respecto tanto al grupo control como al tratado con OE. La restricción energética crónica promueve el aumento de la corticosterona plasmática en rata [202] en respuesta a la hipoglucemia. En humanos bajo restricción energética, la hipercortisolemia es una respuesta adaptativa frente al estrés nutricional, que estimula el apetito y promueve la lipolisis, a la vez que reduce el gasto energético.

Los niveles de CBG circulantes y la actividad de la 11β-hidroxiesteroide deshidrogenasa1 (11βHSD1) en los tejidos determinan la disponibilidad de los glucocorticoides. La mayor parte de la corticosterona circula unida a la CBG, que a su vez controla la fracción de hormona libre activa [203]. En ninguno de los tres grupos experimentales se apreciaron variaciones en la capacidad de unión de la corticosterona circulante a la CBG, lo que indica que el grupo *pair fed* tenía los niveles de corticosterona activa más elevados que los controles o las ratas tratadas con OE, de acuerdo con su restricción de ingesta y el estrés generado por no poder comer en cantidad suficiente. Por otro lado, la 11βHSD1, que activa la corticosterona *in vivo*, se expresa abundantemente en hígado y en el tejido adiposo, donde regula el acceso de los glucocorticoides a su receptor [204]. La expresión hepática del gen que codifica esta enzima (*11βHSD1*) disminuyó en el grupo *pair fed*, lo que probablemente tiene una finalidad compensatoria frente al incremento de corticosterona circulante.

La administración de OE no sólo incrementó el tamaño de las glándulas adrenales, sino que también aumentó su contenido de corticosterona. Respecto a la expresión génica de esta glándula, el grupo OE incrementó la expresión de los genes que codifican las enzimas

que participan en la síntesis de corticosterona colesterol desmolasa (*Cyp11a1*), 3ß-hidroxiesteroide-deshidrogenasa 1 (*3βHsd1*), 21-hidroxilasa (*Cyp21*) y 11β-hidroxilasa (*Cyp11b1*), mientras que disminuyó la expresión de las que sintetizan catecolaminas fenilalanina hidroxilasa (*Pah*) y tirosina hidroxilasa (*Th*). Además, se observó un incremento de la expresión del gen que codifica para el transportador de colesterol SCARB1 (*Scarb1*) y que media la captación de éste como substrato para la esteroidogénesis [205]. En conjunto, los datos obtenidos parecen indicar que el tratamiento con OE estimula la síntesis de corticosterona a nivel de la corteza adrenal y, en cambio, disminuye la capacidad de generar catecolaminas, un punto que merece un estudio posterior más detallado.

En el grupo OE también aumentó el contenido de corticosterona en el hígado, con un marcado incremento de la expresión hepática del gen que codifica la enzima 5α reductasa 1 (*Srd5a1*), principal responsable del catabolismo (inactivación) de la corticosterona [206], incremento que podría ser una respuesta homeostática a la acumulación hepática de la hormona. El aumento de la expresión de la 5α reductasa 1 en hígado podría explicar la falta de cambios en los niveles de corticosterona circulante en las ratas OE a pesar de la activación de su síntesis adrenal.

En el grupo *pair fed* no se observó, en general, ningún incremento de la expresión de las enzimas de la síntesis de glucocorticoides en la glándula adrenal. Sí disminuyó, en cambio, la expresión del gen que codifica el receptor de la ACTH (*Mc2r*) en la glándula adrenal en este grupo, probablemente como consecuencia de la retroalimentación negativa causada por los elevados niveles de corticosterona circulantes. Tampoco se observó un aumento del contenido de corticosterona en la glándula adrenal en el grupo *pair fed*, dato que refuerza la idea de que la síntesis de glucocorticoides en este grupo no incrementó a pesar del marcado aumento de sus niveles circulantes. Por otro lado, el grupo *pair fed* disminuyó la expresión hepática de la 5α reductasa 1 (*Srd5a1*), aunque no significativamente. Durante la restricción energética se produce una disminución de la actividad 5α reductasa 1 [202], que podría explicar en parte el incremento plasmático de corticosterona bajo restricción energética.

En este trabajo se observó una marcada diferencia sexual en cuanto a la modulación del metabolismo de los glucocorticoides frente al tratamiento con OE. La administración de OE a ratas macho incrementó el recambio de corticosterona mediante el aumento paralelo de su síntesis adrenal y de su catabolismo hepático, de modo que los niveles circulantes no aumentaron significativamente, a diferencia de lo observado hasta el momento en ratas hembra [105]. Esta diferencia sexual está de acuerdo con la pérdida de peso corporal más marcada en machos que en hembras bajo el tratamiento con OE [81]. Estos cambios en el metabolismo de los glucocorticoides inducidos por la OE tampoco parecen tener relación con la disminución de la ingesta.

El principal problema en cuanto a la utilización de la OE como fármaco anti-obesidad es la liberación de estrona por hidrólisis del enlace éster por diversas esterasas de localización ubicua [99]. El principal metabolito de la degradación de OE tras su administración oral a ratas hembra es el sulfato de estrona; también aumentan los niveles circulantes de estrona y 17β-estradiol, pero más moderadamente [100]. Aunque la estrona es un estrógeno débil, puede convertirse (reversiblemente) en estradiol mediante la acción de las 17ß-hidroxiesteroide deshidrogenasas [165]. El aumento de estrogenicidad aparejado a la síntesis de 17β-estradiol da lugar a efectos indeseables, ya que la exposición prolongada en el tiempo de altas concentraciones de estrógenos puede favorecer la aparición de ciertos tipos de cáncer, alterar el control del eje hipotálamo-hipófisis gónadas y producir feminización en machos.

La administración de OE a ratas macho produjo una fuerte disminución de los niveles de testosterona, en paralelo a incrementos en los niveles de estrona y 17β-estradiol, aunque la relación estrona/estradiol fue el doble que en las ratas control. Estos cambios en los niveles de hormonas sexuales son indicativos de importantes modificaciones en su metabolismo, por lo que se estudió la expresión génica de las principales enzimas que participan en la síntesis de hormonas sexuales y en la reducción/oxidación de dichas hormonas en las glándulas adrenales, los testículos, el hígado y el tejido adiposo.

En el hombre, una elevada proporción de las hormonas sexuales se sintetiza localmente en los tejidos periféricos a partir de los abundantes precursores adrenales circulantes DHEA y DHEA-sulfato [121]. En el caso de los roedores, las hormonas sexuales se sintetizan principalmente en las gónadas, donde además se encuentra la mayor parte de la enzima aromatasa, responsable de la síntesis de estrógenos a partir de andrógenos precursores. Sin embargo, los tejidos periféricos de los roedores presentan, al igual que ocurre en humanos, la capacidad de regular la actividad de las hormonas sexuales mediante las diversas isoenzimas de la 17β-hidroxiesteroide deshidrogenasa [165] y las enzimas que cambian la solubilidad de estas hormonas (sulfo-transferasas y sulfatasas) [166].

La familia de las 17β-hidroxiesteroide deshidrogenasas modula la potencia de estrógenos y andrógenos antes de su unión al receptor mediante la conversión de la posición 17 de la molécula, siendo las formas ceto (DHEA, androstenodiona y estrona) inactivas y las formas hidroxi (androstenodiol, testosterona, 17β-estradiol) activas y más accesibles al receptor [165]. Esta interconversión entre moléculas es unidireccional según la isoenzima implicada y las concentraciones de substratos, y permite regular los niveles intracelulares de esteroides sexuales activos a nivel local, aunque también influyen en los niveles globales circulantes.

En ratas macho, según el resultado del análisis de la expresión génica extrapolada al peso de los diferentes tejidos, el principal lugar de síntesis de andrógenos y de activación de éstos a testosterona resultó ser el testículo, como era de esperar. Dos tercios de las enzimas activadoras de estrógenos en estas mismas ratas se localizaron principalmente en el

testículo, y un tercio en hígado. La ubicación de la mayoría de las enzimas oxidativas se encontraron esencialmente en el hígado.

El aumento de estrógenos disminuye el nivel de testosterona circulante mediante la inhibición de la secreción de gonadotropinas por el hipotálamo. La disminución de LH inhibe la espermatogénesis y la producción de testosterona en los testículos. Por esta misma vía, los estrógenos pueden reducir la síntesis y liberación de testosterona [207]. La administración de OE disminuyó el peso de los testículos, la expresión génica de las enzimas implicadas en la síntesis de hormonas sexuales y la expresión de la isoenzima testicular 17β-hidroxiesteroide deshidrogenasa 3, clave en la activación de los andrógenos y en el mantenimiento de los niveles de testosterona circulantes. Por otro lado en el grupo OE aumentó la expresión génica de las enzimas oxidantes (inactivadoras) sobre todo en el hígado, principal centro de catabolismo de las hormonas esteroideas, como es el caso de la 17β-hidroxiesteroide deshidrogenasa 2, que actúa sobre el 17β-estradiol, pero también sobre la androstenodiona y la testosterona [165]. Estas respuestas seguramente tienen como finalidad contrarestar los efectos de los estrógenos exógenos, con niveles elevados tras la administración de la OE, mediante la disminución compensatoria de la síntesis endógena de hormonas sexuales.

Los tejidos endocrinos con receptores esteroideos utilizan la conjugación con sulfato y la acción de sulfatasas para regular la unión de esteroides a su receptor [166] además de modular su solubilidad, y con ello la posibilidad de excreción urinaria. La conjugación de la estrona con sulfato mediante la estrógeno sufotransferasa (EST) anula la capacidad de unión del estrógeno a su receptor, mientras que la esteroide sulfatasa (STS) estimula la actividad estrogénica.

En el testículo de rata el tratamiento con OE disminuyó la expresión del gen que codifica la sulfatasa STS, y que se traduce en una menor disponibilidad de estrógenos, por otro lado esenciales para el funcionamiento del testículo [208]. A su vez el tratamiento con OE disminuyó marcadamente la expresión del gen que codifica la sulfotransferasa EST en todos los tejidos estudiados (testículo, hígado y tejido adiposo). La sulfotransferasa EST, que inactiva los estrógenos por conjugación con sulfato, protege en condiciones fisiológicas al aparato reproductivo masculino y a la placenta del exceso de estrógenos [209]. La EST se expresa dimórficamente en machos y hembras bajo estimulación con testosterona, lo que explica que disminuyera bajo el tratamiento con OE.

Por otro lado, la administración de OE produce un importante incremento de estrona sulfato [100], seguramente como resultado del marcado incremento de expresión que experimenta el gen que codifica la DHEA sulfotransferasa (*SMP2*) en el hígado tras la administración de OE (resultados posteriores a la presente tesis). La DHEA sulfotransferasa o SMP2 opera a concentraciones más elevadas de estrógenos que la esteroide sulfotransferasa o EST, y su expresión, además, es mayor en las hembras, ya que

se inhibe por los andrógenos [210,] lo que explica el incremento observado en las ratas tratadas con OE.

El incremento de los niveles circulantes de estrona como consecuencia del tratamiento oral con OE, aunque mucho menos marcado que tras su inyección endovenosa continuada [78], podría ser consecuencia, en parte, de la inhibición de la actividad reductora de la 17ß-hidroxiesteroide deshidrogenasa, de acuerdo con la disminución observada de su expresión, limitando de esta manera la conversión de estrona a estradiol y por tanto la acción estrogénica global. Esta protección frente a la acción de la sobrecarga de estrógenos dio lugar, a su vez, a una disminución de la síntesis y activación de andrógenos, probablemente por ser precursores de los estrógenos. De este modo, el hipoandrogenismo observado en la acción de la OE podría ser parte del mecanismo que protege al cuerpo del exceso de estrógenos, disminuyendo la disponibilidad de substratos para la aromatasa y el mecanismo de activación (reducción en C17) que comparten andrógenos y estrógenos (estrona a 17β-estradiol y androstenodiona a testosterona).

El hígado fue el órgano que más diferencias reveló en cuanto a los efectos de la OE y, sobre todo, en comparación con el grupo *pair fed*. Una de las razones por las que la OE tiene una acción más marcada en machos que en hembras podría ser precisamente el importante dimorfismo sexual del hígado. Las hormonas sexuales controlan las diferencias sexuales en el hígado, y la mayoría de sus efectos están mediados indirectamente por el patrón diferencial de secreción de GH entre machos y hembras [211]. Los andrógenos y los estrógenos determinan el perfil de liberación de GH desde el lóbulo hipofisario anterior. Las ratas macho secretan GH de un modo pulsátil con picos de 200 ng/ml cada 3 horas y media seguido de intervalos de unas dos horas sin GH, mientras que las hembras liberan GH de un modo más continuo, a niveles de 30-60 ng/ml. La disminución de andrógenos circulantes en ratas macho tratadas con OE podría favorecer un perfil hepático más "femenino", de acuerdo con algunas de los resultados que se han descrito en la presente tesis, como el hecho de que el hígado bajo el tratamiento con la OE promueva la síntesis de lípidos, la activación del transporte reverso del colesterol e incluso la protección frente a la elevación de los niveles de estrógenos mediante el incremento de la inactivación de hormonas sexuales. El perfil de secreción de GH tiene un vasto impacto sobre el metabolismo de esteroides y fármacos [212], pero también sobre las proteínas del plasma, enzimas y receptores relacionados con el metabolismo de los lípidos y glúcidos [213].

CONCLUSIONES

5 Conclusiones

1. No parece existir ninguna ventaja adicional en cuanto a la disminución del peso corporal al combinar la administración de OE con una restricción energética forzada adicional, ya que mientras que el tratamiento con OE afecta principalmente a las reservas de grasa corporal, al incluir la restricción energética se produce también una movilización de la masa proteica, lo que implica un claro riesgo para el mantenimiento de la proteína corporal sin beneficios adicionales.

2. La disminución de la grasa corporal que conlleva el tratamiento con OE, así como el perfil de expresión génica relacionado con el metabolismo lipídico en el tejido adiposo, parecen ser debidos en parte a la disminución de la ingesta, pero la OE produce efectos adicionales positivos sobre el metabolismo energético, principalmente en el mantenimiento de la homeostasis de la glucosa.

3. El tratamiento con OE, a pesar de la diminución de la ingesta, disminuye la expresión hepática de los genes implicados en la gluconeogénesis, mantiene la de los genes relacionados con la glucolisis y la lipogénesis e incluso incrementa la expresión de los implicados en la síntesis de triacilgliceroles, de acuerdo con el incremento de la proteína SREBP-1c, lo que diferencia claramente la acción de la OE de la simple restricción energética.

4. El tratamiento con OE disminuye los niveles de colesterol circulante independientemente de la disminución de la ingesta. La OE incrementa la expresión hepática de genes y proteínas que participan en el transporte reverso de colesterol y en su conversión a ácidos biliares. El tratamiento con OE podría activar al factor de transcripción LXRα, que favorece el transporte de colesterol hacia el hígado y su eliminación, de acuerdo con el incremento del colesterol hepático y la activación de la lipogénesis a través de SREBP-1c.

5. El tratamiento con OE incrementa el recambio de corticosterona mediante el aumento de su síntesis adrenal y su inactivación hepática, por lo que el aumento de sus niveles circulantes es más moderado que en el caso de una simple restricción energética, en la que hay que contar con el factor adicional de estrés por hambre.

6. El exceso de estrona resultante de la hidrólisis de la OE tras su administración a ratas macho da lugar a una disminución de la síntesis y activación de la testosterona en el testículo y a un incremento de la oxidación de testosterona y estradiol en el hígado. El incremento de estrona exógena da lugar a un efecto estrogénico limitado a expensas de una marcada disminución de la testosterona.

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Apéndice

Otras publicaciones realizadas durante la ejecución de la tesis doctoral

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