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Nuevas interacciones nutrientes-genes en conexión con el problema de la obesidad y la función adipocitaria

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La interesada

Núria Granados Borbolla

A mis padres,

a mis hijos,

y

a Loren

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Lista de abreviaturas más utilizadas

- **AGs:** ácidos grasos
- **AGTs:** ácidos grasos *trans*
- **AGTs-PI:** ácidos grasos *trans* producidos industrialmente
- **AR***:* ácido retinoico *(retinoic acid)*
- **ARNm:** ácido ribonucleico mensajero
- **ATP:** adenosín trifosfato *(Adenosine Tri-Phosphate)*
- **ATRA:** ácido retinoico todo-*trans (All-Trans Retinoic Acid)*
- **BMSCs:** células mesenquimales de médula ósea *(Bone Marrow Mesenchimal Stem cells)*
- **BMP2:** proteína morfogénica 2 *(Bone Morphogenic Protein 2)*
- **C/EBP:** proteína de unión al estimulador CCAAT(*CCAAT-Enhancer-Binding Protein)*
- **Col2a1:** Colágeno tipo 2 alfa 1
- **DAG:** diacilglicerol
- **DT2:** diabetes tipo 2
- **ECV**: enfermedad cardiovascular
- **FAS:** ácido graso sintasa *(Fatty Acid Synthase)*
- **GLUT4:** trasportador 4 de glucosa *(GLUcose Transporter 4)*
- **HPLC:** cromatografía líquida de alta resolución *(High Pressure Liquid Chromatography)*
- **ICAM-1:** molécula de adhesión intercelular 1 *(Inter-Cellular Adhesion Molecule 1)*
- **IL:** Interleuquina
- **IRS:** sustrato del receptor de la insulina *(insulin receptor substrate)*
- **IKK:** quinasa inhibidora del factor nuclear kappa B (*Inhibitor of nuclear factor kappa-B kinase)*
- **JAK:** quinasa de Janus *(JAnusKinase)*
- **JNK:** quinasa terminal de c-JUN *(c-JuNKinase)*
- **LDL:** lipoproteínas de baja densidad (Low Density Lipoproteins)
- **LPL:** lipoproteína lipasa *(Lipo Protein Lipase)*
- **MCP-1:** proteína quimioatrayente de monocitos 1 *(monocyte chemoattractant protein-1*)
- **MEFs:** fibroblastos embrionarios de ratón *(Mouse Embrionic Fibroblasts)*
- **NAD:** dinucleótido de nicotinamida *(Nicotinamide Adenine Dinucleotide*
- **Nampt:** nicotinamida fosforibosil transferasa *(Nicotin Amide Phosphoribosil transferasa)*
- **NFκB***:* factor nuclear kappa B *(Nucear Factor Kappa B)*
- **ON:** óxido nítrico
- **PGC1-α:** coactivador 1α del *PPARγ (Peroxisome Proliferator-Activated Receptor Coactivator 1α)*
- **PPAR:** receptor activado por proliferadores peroxisomales *(Peroxisome Proliferator-Activated Receptor)*
- **Pref-1**: factor de preadipocitos-1 *(Pre adipocyte Factor -1)*
- **RBP:** proteína de unión al retinol *(Retinol Binding Protein)*
- **ROS:** especies reactivas del oxígeno (Reactive Oxigen Species)
- **PCR:** reacción en cadena de la polimerasa (Polimerase Chain Reaction)
- **Sox-9:** factor de transcripción SOX-9 (*SRY –related high mobility group – Box gene 9*)

- **TAB:** tejido adiposo blanco
- **TAM:** tejido adiposo marrón
- **TNFα:** factor de necrosis tumoral alfa *(Tumor Necrosis Factor alpha)*
- **TGs**: triacilgliceroles
- **UCP:** proteína desacopladora *(UnCoupling Protein)*
- **VCAM-1:** molécula de adhesión vascular *(Vascular Cell Adhesion Molecule-1)*

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Resumen

Inflamación sub-crónica y resistencia a la insulina constituyen el eje de toda una serie de complicaciones médicas asociadas a la obesidad a las que se les atribuye la tercera parte de todas las muertes y discapacidades a nivel mundial. Más allá del exceso, los nutrientes impactan específicamente sobre marcadores de pérdida de la homeostasia metabólica. Los objetivos de esta tesis se centran en el estudio de interacciones entre nutrientes y genes de potencial relevancia en el contexto de la obesidad y sus co-morbilidades. En particular se ha estudiado: a) la regulación de proteínas bioactivas de secreción de adipocitos y miocitos por obesidad, vitamina A y ácidos grasos *trans*; b) la regulación de la adipogénesis por nutrientes con efectos saludables sobre la función articular; y c) los efectos a largo plazo de una suplementación con vitamina A durante la lactancia sobre la susceptibilidad a la obesidad.

Las alteraciones de la proteína de unión a retinol (RBP) y la Nampt/visfatina de origen adipocitario en el estado obeso y su posible papel en las complicaciones metabólicas asociadas al mismo son aspectos controvertidos. Nuestros resultados sobre los niveles circulantes y de expresión en tejido adiposo blanco de RBP y Nampt/visfatina en dos modelos de obesidad en rata, uno dietético (ratas Wistar alimentadas con dieta de cafetería) y uno genético (ratas obesas Zucker *fa/fa),* indican que, en estos modelos, el desarrollo de resistencia a la insulina es independiente de incrementos en estas dos proteínas.

Además de por la obesidad, la función secretora del tejido adiposo puede verse afectada por nutrientes específicos, como la vitamina A y ciertos ácidos grasos. Nuestros resultados muestran que el ácido retinoico (forma carboxílica de la vitamina A) reprime la expresión de RBP en modelos de adipocitos en cultivo y selectivamente en el tejido adiposo blanco, pero no en el hígado, *in vivo*. La RBP de origen adipocitario se ha postulado como un factor de resistencia a la insulina y, de acuerdo con este rol, su reducción en respuesta al tratamiento agudo *in vivo* con ácido retinoico se acompañó de un aumento de la sensibilidad a la insulina en los animales. Todo ello refuerza el concepto de que vitámeros de la vitamina A pueden afectar la sensibilidad a la insulina vía efectos sobre adipoquinas.

La ingesta de ácidos grasos *trans* de origen industrial se asocia con efectos deletéreos sobre la salud cardiovascular y metabólica. Nuestros resultados muestran efectos directos diferenciales del ácido oleico y de su isómero *trans*, el ácido elaídico, el más abundante de los ácidos grasos *trans* de origen industrial, sobre la función secretora de adipocitos y células musculares en cultivo. En conjunto los resultados muestran que la exposición a ácido oleico, pero no elaídico, induce la expresión de proteínas que mejoran la sensibilidad a la insulina, como la interleuquina-6 en células musculares y la adiponectina en células adiposas, al tiempo que reduce la expresión adipocitaria de resistina, proteína relacionada con la resistencia a la insulina y de potencial pro-inflamatorio. Por el contrario, la exposición a ácido elaídico, pero no oleico, induce en células musculares la expresión de un factor de resistencia a la insulina, el factor de

necrosis tumoral α, al tiempo que disminuye la expresión de interleuquina-15, un factor anabólico muscular de efectos anti-obesogénicos. Estos resultados contribuyen a establecer un vínculo molecular entre los efectos adversos del ácido elaídico y beneficiosos del ácido oleico sobre la sensibilidad a la insulina y el riesgo cardiovascular y la función secretora de adipocitos y miocitos.

La relación entre obesidad y osteoartritis tiene bases biomecánicas, metabólicas, y posiblemente también celulares. La diferenciación de células multipotentes en adipocitos o condrocitos está competitivamente equilibrada. Nuestros resultados muestran que nutrientes con conocidos efectos sobre la condrogénesis y la salud articular inhiben la adipogénesis en fibroblastos embrionarios de ratón, al tiempo que disminuyen en estas células la expresión de proteínas consideradas pro-inflamatorias y/o de efectos negativos sobre la sensibilidad a la insulina, como la resistina y la RBP, e incrementan la expresión de proteínas de efecto anti-adipogénico, como la leptina. Se sugiere que los nutrientes ensayados poseen una cierta capacidad de regular el balance de diferenciación de estas células multipotentes hacia unos u otros tipos celulares, lo que podría ser de interés en estrategias para el control simultáneo de la adiposidad corporal y la salud articular.

Las interacciones nutrientes-genes pueden tener consecuencias diferentes dependiendo de la etapa del desarrollo. Así, mientras resultados previos indican un efecto anti-obesogénico de la vitamina A en animales adultos, los resultados de esta tesis muestran que ratas que recibieron una suplementación moderada de vitamina A (como retinil palmitato) durante la lactancia presentan un mayor potencial de proliferación celular en el tejido adiposo blanco en el momento del destete, y acumulan subsiguientemente más grasa corporal que las controles en respuesta a una dieta hiperlipídica. Los resultados sugieren que la vitamina A en etapas tempranas de la vida puede condicionar efectos a largo plazo sobre la adiposidad corporal vía efectos sobre el desarrollo y la celularidad del tejido adiposo.

En definitiva, el conjunto de resultados presentados nos aportan nueva información acerca de los efectos de varios nutrientes sobre la secreción de proteínas bioactivas del tejido adiposo y muscular, la adipogénesis y también, administrados en etapas tempranas del desarrollo, la futura susceptibilidad a la obesidad. El conocimiento de las interacciones nutrientes-genes en procesos que regulan la adiposidad corporal y la sensibilidad a la insulina en etapas tempranas y en la edad adulta puede ayudar al diseño de nuevas estrategias para prevenir/tratar la obesidad y complicaciones médicas asociadas.

Listado de artículos originales

Esta tesis se basa en los siguientes manuscritos originales:

I. Mercader J, **Granados N**, Caimari A, Oliver P, Bonet ML, Palou A. Retinol-binding Protein 4 and Nicotinamide Phosphoribosyltransferase/Visfatin in Rat Obesity Models. Horm Metab Res, 40:462-72, 2008.

II. Mercader J, **Granados N**, Bonet ML, Palou A. All-trans retinoic acid decreases murine adipose retinol binding protein 4 production. Cell Physiol Biochem, 22:362-72, 2008.

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V. Bonet ML, **Granados N**, Palou A. Molecular players at the intersection of obesity and osteoarthritis. Current Drugs Targets, en prensa.

VI. **Granados N**, Amengual J, Ribot J, Musinovic H, Ceresi E, Von Lintig J, Palou A, Bonet ML. Moderate vitamin A supplementation in early life affects later adiposity in rats. Manuscrito en vías de publicación.

1. INTRODUCCIÓN

La obesidad es un estado en el que la salud se ve comprometida por el exceso de grasa acumulada. De una forma simplificada, su aparición se explica como el resultado de un desequilibrio sostenido del balance energético en el que la ingesta calórica supera el gasto. Sin embargo, los mecanismos fisiológicos y procesos moleculares encargados de mantener la homeostasia energética constituyen una red muy compleja dónde interactúan toda una serie de señales producidas por el sistema nervioso central y los tejidos periféricos.

El conocimiento de la biología de los tejidos periféricos y de los procesos bioquímicos que determinan el balance energético y la deposición de grasas puede ser decisivo para combatir la obesidad y sus complicaciones médicas asociadas de una forma racional y eficaz. Algunos nutrientes intervienen en la regulación de los procesos bioquímicos implicados y la presente Tesis se enmarca dentro de esta línea de investigación, que es una de las principales líneas en las que trabaja nuestro grupo de investigación, el *Laboratorio de Biología Molecular, Nutrición y Biotecnología-Nutrigenómica* de la UIB.

1.1. LA OBESIDAD Y COMPLICACIONES MÉDICAS ASOCIADAS

Según datos de la Organización Mundial de la Salud (OMS), 1,5 billones de adultos en el año 2008 y 43 millones de niños menores de 5 años en el 2010 padecían sobrepeso u obesidad (índice de masa corporal (IMC) ≥ 25) (WHO, 2011). La gran disponibilidad de alimentos energéticamente muy densos y la reducción de la actividad física, lo que se ha dado en llamar "ambiente obesogénico", en el contexto de una evolución génica que ha favorecido la predisposición a almacenar energía, explicaría esta epidemia global de obesidad. La prevalencia de la obesidad es mucho mayor de lo que se había estimado previamente, en gran parte debido a su actual incremento en regiones donde tradicionalmente la malnutrición había sido el problema dominante, como Latinoamérica, el Caribe, China, Australasia, Vietnam e Islas del Pacífico. La OMS prevé que en el 2015 se alcance la cifra de 2,3 billones de adultos con sobrepeso (IMC ≥ 25) y más de 700 millones de obesos (IMC ≥30). En España, el porcentaje de obesidad en la población adulta se ha duplicado desde el año 1987, y se estima que hoy en día el 62% de los adultos tiene sobrepeso u obesidad (Rodriguez-Artalejo et al., 2011).

La obesidad infantil preocupa especialmente. A nivel mundial, se ha duplicado en tan sólo cinco años, afectando actualmente a un 10% de la población pediátrica de todo el mundo. Los niños con sobrepeso tienen muchas probabilidades de convertirse en adultos obesos y, en comparación con los niños sin sobrepeso, tienen más probabilidades de sufrir a edades más tempranas diabetes y enfermedades

cardiovasculares, que a su vez se asocian a un aumento de la probabilidad de muerte prematura y discapacidad. En España, uno de cada tres niños padece sobrepeso u obesidad y según datos recientes de la Sociedad Gallega de Endocrinología, Nutrición y Metabolismo, uno de cada cuatro casos nuevos de diabetes que se dan en Galicia es un menor de diez años (Sociedad Gallega de Endocrinología, 2011).

Hasta principio de los años 80 no empezó a considerarse la obesidad como un problema médico que inducía o amplificaba toda una serie enfermedades tales como la enfermedad cardiovascular, hipertensión o diabetes (Black, 1983). Varios grupos de trabajo en Escandinavia, Inglaterra, Francia y Estados Unidos estaban ya investigando intensamente sobre el control del apetito y las consecuencias metabólicas del aumento de peso. Asimismo, se comenzaba a estudiar la influencia de la nutrición en etapas tempranas del desarrollo sobre la susceptibilidad a padecer obesidad y desórdenes metabólicos en la edad adulta (McCance and Widdowson, 1974). Sin embargo, no fue hasta finales de los 90 cuando se documentó con gran precisión que toda una gama de condiciones médicas y factores de riesgo cardiovascular son mucho más frecuentes a medida que el IMC incrementa a partir de 20 (Shaper et al., 1997; Willett et al., 1999). Según datos de la OMS, la obesidad se considera actualmente el tercer factor de riesgo en sociedades desarrolladas, tras el consumo de alcohol y tabaco, para todas las discapacidades y muertes prematuras (WHO, 2009).

Por todo ello es necesaria una mayor comprensión de los procesos asociados a la obesidad, así como la búsqueda de nuevas estrategias dirigidas a prevenirla ya desde etapas tempranas del desarrollo y a favorecer y mantener la pérdida de peso en sujetos obesos.

1.1.1. Obesidad e inflamación

El sistema metabólico (vías de detección de nutrientes) y el sistema inmune (vías de detección de patógenos) están estrechamente relacionados (Hotamisligil, 2006). El equilibrio entre las respuestas inmune y metabólica puede verse amenazado tanto por un déficit crónico de nutrientes como por una ingesta calórica excesiva continuada. Bajo condiciones de exceso de energía, el potencial inflamatorio de tejidos y órganos puede verse reactivado, y de hecho la obesidad está asociada a un estado de inflamación crónica de bajo grado, si bien hay que señalar que no se trata de la clásica respuesta inflamatoria, sino más bien debería considerarse como una forma aberrante de inmunidad desencadenada por nutrientes, u otras señales intrínsecas, a la que se ha denominado meta-inflamación o para-inflamación (Medzhitov, 2008). La cronificación de este estado de inflamación incrementa el riesgo de dañar múltiples sistemas, incluidos,

entre otros, aquellos involucrados en la homeostasis de la glucosa (Gregor and Hotamisligil, 2011; Wellen and Hotamisligil, 2005).

Durante la última década, han sido intensamente estudiadas las principales vías de señalización involucradas en la respuesta inflamatoria (ver descripción a continuación) y los principales mediadores inflamatorios que activan dichas vías y que resultan estar incrementados en el estado obeso, entre los que se incluyen proteínas, lípidos y especies reactivas del oxígeno (véase el apartado 1.2.5) (Figura 1).

Las principales vías de señalización pro-inflamatorias asociadas a la obesidad son la vía del factor nuclear kappa B (NFκB), la vía de la C-JUN N-terminal quinasa (JNK) y la vía Janus quinasa/transductor de la señal y activador de la transcripción (vía JAK/STAT). Estas vías se introducen brevemente a continuación.

a) Vía IKKβ/NFκB

Es la vía de señalización dominante en la inflamación. Se relacionó por primera vez con la obesidad en el año 2001, cuando se observó que ratones transgénicos IKKβ +/- no desarrollaban resistencia a la insulina en el estado obeso (Yuan et al., 2001). Desde entonces, la activación de esta vía se ha observado en adipocitos, hepatocitos y neuronas en condiciones de obesidad.

IKKβ (quinasa inhibidora del inhibidor kappa B) es una proteína quinasa que cataliza la fosforilación del inhibidor kappa B (IκBα), marcándolo de ese modo para su posterior ubiquitinización y degradación en el proteosoma (Baeuerle and Henkel, 1994); la degradación de IκBα permite la liberación del factor de transcripción NFκB, que puede entonces viajar al núcleo y ejercer su actividad transcripcional (Karin and Ben-Neriah, 2000).

La actividad de NFκB inhibe la expresión y actividad del PPARγ (*peroxisome proliferator-activated receptor* gamma, principal factor de transcripción adipogénico) (Ye, 2008), e incrementa la síntesis de citoquinas pro-inflamatorias – factor de necrosis tumoral α (TNFα), interleuquina-1 (IL-1), interleuquina-6 (IL-6), factor quimiotáctico de macrófagos (MCP-1) – moléculas de adhesión intercelular (ICAM-1) y moléculas de adhesión vascular (VCAM-1). Además, IKKβ tiene la capacidad de fosforilar directamente al sustrato 1 del receptor de insulina (IRS-1) en residuos de serina, inhibiendo así la señalización de la insulina (Gao et al., 2002).

En el estado obeso la vía IKKβ/NFκB puede ser activada por señales extracelulares – e.g. TNFα (Hotamisligil, 1999; Peraldi and Spiegelman, 1998), IL-1, ácidos grasos (Itani et al., 2002; Kim et al., 2001; Yuan et al., 2001) – y por señales intracelulares – e. g.

especies reactivas del oxígeno (Mercurio et al., 1997; Piette et al., 1997), señales de estrés del retículo endoplásmico, diacilglicerol y ceramidas (de Luca and Olefsky, 2008). Entre las quinasas corriente arriba activadoras de IKKβ está la proteína quinasa C (PKC), activada por diacilglicerol (Tojima et al., 2000).

b) Vía de la JNK

JNK es una proteína serina quinasa ubicua. Se la empezó a relacionar con la obesidad/metabolismo cuando se descubrió que, en respuesta a TNFα, JNK inhibía la respuesta a la insulina mediante fosforilación de un residuo de serina de IRS-1 (Aguirre et al., 2000; Rui et al., 2001). Además, JNK activa el factor de transcripción activador de la proteína 1 (AP1), que a su vez regula la transcripción génica de factores inflamatorios. De las tres isoformas conocidas, JNK1 ha sido la más estudiada en el campo de la obesidad. JNK1 es activada en el estado obeso por TNFα, ácidos grasos, insulina, activadores de la PKC (diacilglicerol), ceramidas y señales de estrés del retículo endoplásmico (Aguirre et al., 2002; Gao et al., 2004; Gao et al., 2003; Jiang et al., 2003; Lee et al., 2003b; Ozcan et al., 2004; Rui et al., 2001). JNK1 promueve la resistencia a la insulina sistémica a través de la inflamación del hígado (Nakatani et al., 2004) y el tejido adiposo (Sabio et al., 2008). En páncreas, la activación de JNK por citoquinas media la inflamación y la apoptosis (Abdelli et al., 2004; Ammendrup et al., 2000) y disfunción de las células β (Kaneto et al., 2004; Kaneto et al., 2002). La inhibición de JNK mejora la sensibilidad a la insulina en animales obesos con resistencia a la insulina (Bennett et al., 2003).

c) Vía JAK/STAT

Muchas citoquinas y factores de crecimiento ponen en marcha esta vía en sus células diana. En respuesta a la unión de la citoquina a su correspondiente receptor de membrana se activan tirosina proteína quinasas intracelulares (de la familia de las Janus quinasas, JAKs) que fosforilan e inducen la dimerización y el transporte al núcleo de factores de transcripción de la familia STAT. La actividad de las STATs se asocia a respuestas metabólicas e inflamatorias. La vía JAK/STAT es regulada negativamente por los “supresores de la señalización por citoquinas” (SOCS), que son importantes reguladores fisiológicos de la inflamación y la inmunidad (Tamiya et al., 2011). Las SOCS (e.g. SOCS3) también están implicadas en la atenuación de la señalización por insulina y leptina.

STAT3 ha sido estrechamente relacionado con la obesidad ya que es activado por leptina, IL-6 e IL-10. Esta vía de regulación metabólica actúa tanto a nivel central como periférico. En el sistema nervioso central inhibe la ingesta y promueve el gasto

energético (Inoue et al., 2006). A nivel periférico, la activación de STAT3 induce lipólisis en el tejido adiposo; inhibe la secreción de insulina en células β pancreáticas (Gorogawa et al., 2004; Handschin et al., 2007); induce la expresión del factor de crecimiento del endotelio vascular (VEGF) (Niu et al., 2002; Wei et al., 2003); promueve la proliferación de hepatocitos (Taub, 2003); inhibe la producción de glucosa en hígado (Inoue et al., 2006; Moh et al., 2008); y promueve la oxidación de lípidos y la captación de glucosa en músculo esquelético (Pedersen, 2007).

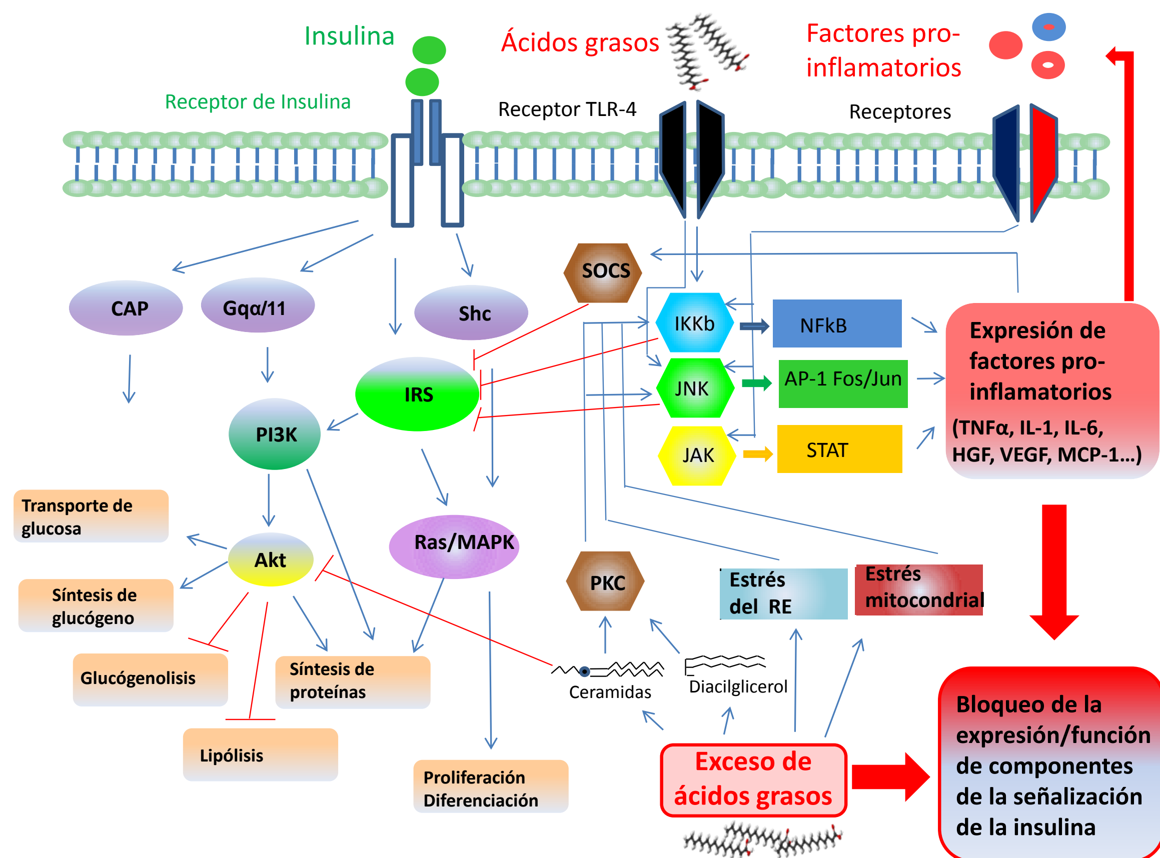

Figura 1. *Interacción entre la señalización de la insulina y las vías inflamatorias. Ácidos grasos y factores pro-inflamatorios bloquean la función/expresión de componentes de la señalización de la insulina. Adaptado de (Serrano Rios et al., 2011).*

1.1.2. Resistencia a la insulina y diabetes tipo 2

En la patogenia de la diabetes tipo 2 (DT2) el equilibrio entre la sensibilidad y la secreción de insulina se ve comprometido por factores ambientales y/o genéticos (Bell and Polonsky, 2001; Kahn et al., 2006; Weir and Bonner-Weir, 2004). La obesidad es el principal factor de riesgo de DT2 y de otros grados de la pérdida de la homeostasia de la

glucosa, hasta el punto que se ha acuñado el término *diabesity* para referirse a esta pandemia de enfermedades gemelas (Astrup and Finer, 2000).

Los parámetros de diagnóstico de la DT2 incluyen resistencia a la insulina, hiperinsulinemia e hiperglucemia. En general, se acepta que la hiperinsulinemia es una respuesta compensatoria a la resistencia a la insulina, resultado de una hipertrofia de las células β pancreáticas y un deficiente mecanismo de captación de insulina por parte del hígado. La hiperglucemia, que define esta enfermedad, tiene lugar cuando los mecanismos de compensación fallan.

La insulina es la principal hormona anabólica en mamíferos y es esencial para la homeostasis metabólica. Sintetizada y secretada por las células β de los islotes pancreáticos de Langerhans, juega un papel crucial en la homeostasia de la glucosa mediante efectos coordinados sobre la captación de glucosa, el metabolismo y el almacenamiento de energía en tejidos clave como músculo esquelético, células β, tejido adiposo e hígado. En particular, la insulina es fundamental en la regulación de todos los aspectos de la biología de los adipocitos: promueve la adipogénesis, la síntesis de triacilgliceroles, induce la lipogénesis, e inhibe la lipólisis.

La acción de la insulina empieza con su unión a receptores de membrana en tejidos diana, desencadenando una cascada de señalizaciones intracelulares que se inicia con la activación de la actividad tirosina quinasa del propio receptor de insulina, seguida del reclutamiento y la fosforilación en residuos de tirosina de los “sustratos intracelulares del receptor de insulina” (IRSs, particularmente IRS-1 e RS-2), que a su vez activarán a la fosfatidil inositol 3 quinasa (PI3Ks) y a la proteína quinasa B (PKB/Akt), críticas en la mediación de los efectos metabólicos de la insulina, a través de una compleja red de señales corriente abajo (Figura 1). De igual relevancia es la activación de la vía Ras-proteína quinasa activada por mitógenos (MAPK), crucial en los efectos mitogénicos de la insulina.

La resistencia a la insulina es una constante en la fisiopatología de la obesidad y la DT2, constituyendo el mayor enlace entre ambas condiciones. La inflamación crónica de bajo grado asociada a la obesidad es un mecanismo etiológico importante en la disminución de la señalización de la insulina.

Distintos mecanismos, no mutuamente excluyentes, han sido propuestos para explicar el desarrollo de la resistencia a la insulina en la obesidad. En síntesis, todos los individuos tenemos una capacidad máxima de expansión del tejido adiposo, determinada por factores genéticos y ambientales. Cuando se alcanza este máximo, numerosos mecanismos (hipertrofia del adipocito, necrosis, hipoxia y señales de estrés

oxidativo y del retículo endoplásmico) activan vías inflamatorias que interfieren con la señalización de la insulina, a la vez que contribuyen a la infiltración y activación de macrófagos que a su vez se convertirán en una fuente importante de señales inflamatorias (véase el apartado 1.2.5). Así pues los propios adipocitos, “sordos” a la señalización de la insulina, inician un metabolismo lipolítico con la consecuente liberación de ácidos grasos, además de adipoquinas y mediadores inflamatorios que junto a los producidos por los macrófagos tendrán un efecto sistémico sobre la sensibilidad a la insulina. Mucha evidencia científica respalda el concepto de que las células adiposa obesas liberan ácidos grasos a la circulación, que podrán ser captados por tejidos periféricos que, en condiciones fisiológicas, también poseen cierta capacidad de almacenar grasa, como son el músculo esquelético, el hígado, las células β pancreáticas, y probablemente otros (Smith and Ravussin, 2002).

Los ácidos grasos inducen inflamación y resistencia a la insulina por varios mecanismos. Por una parte, son capaces de activar la vía IKK/NFκB en adipocitos y macrófagos mediante su unión al receptor de membrana TLR4 (*Toll Like Receptor* 4) (Gao et al., 2004; Lee et al., 2003a; Shi et al., 2006) (Figura 1). Un segundo mecanismo ocurre a nivel intracelular. Cuando el flujo de ácidos grasos procedentes del adipocito obeso es excesivo, la capacidad de síntesis de triacilgliceroles en las células que los captan puede llegar a saturarse, y se acumulan productos intermedios como ácido lisofosfatídico, ácido fosfatídico y diaciglicerol, que a su vez estimulan la síntesis de ceramidas. Todos estos compuestos interfieren con las vías de señalización de la insulina, produciendo lipotoxicidad (de Luca and Olefsky, 2008; Schenk et al., 2008). Por ejemplo, el diacilglicerol y las ceramidas son activadores alostéricos de ciertas isoformas de PKC inactivadoras de IRS-1 e inducen las vías inflamatorias IKKB/ NFκB y JNK (Ballou et al., 1996; Brose and Rosenmund, 2002) (Figura 1). Las ceramidas poseen también la capacidad de inactivar la PKB/Akt en hígado, en células β y en músculo (Corcoran et al., 2007; de Luca and Olefsky, 2008; Donnelly et al., 2005; Haber et al., 2003; Schenk et al., 2008; Zierath, 2007). Por último, el exceso de ácidos grasos en adipocitos y tejidos con capacidad de almacenar lípidos provoca estrés en la mitocondria y el retículo endoplásmico que se traduce en la producción de señales activadoras de las vías inflamatorias (de Luca and Olefsky, 2008; Gambert and Ricquier, 2007; Hotamisligil, 2010; Schenk et al., 2008).

En resumen, en el estado obeso, el incremento de los niveles de ácidos grasos circulantes procedentes de la lipólisis en el tejido adiposo y señales inflamatorias procedentes del tejido adiposo y de otros tejidos serán los principales desencadenantes de la resistencia a la insulina sistémica y en tejidos clave (véase la Figura 2). En

humanos, por ejemplo, se ha demostrado que un exceso de ácidos grasos circulantes puede disminuir en un 50% la captación y metabolismo de la glucosa en músculo esquelético (Lois and Kumar, 2009; Roden, 2004; Shulman, 2000).

A pesar de la extensa evidencia epidemiológica de la asociación entre obesidad y DT2, existe aproximadamente un 23-24% de obesos que no desarrollan la enfermedad. Esto ha sugerido que la disfunción de las células β del páncreas podría ser decisiva en el desarrollo de la hiperglucemia que define la DT2 (Ahren and Pacini, 2005; Kahn et al., 2006; Muoio and Newgard, 2008; Weir and Bonner-Weir, 2004). De hecho, recientes estudios de asociación del genoma completo con la DT2 muestran que la mayoría de los loci génicos identificados están relacionados con la alteración de la función de células β. Estos hallazgos, aunque no descartan la importancia de la resistencia a la insulina, ponen de relieve la contribución genética de la disfunción de las células β en la patogénesis de la DT2 (Florez, 2008).

1.1.3. Enfermedad cardiovascular

La obesidad es uno de los principales factores de riesgo de enfermedad cardiovascular (ECV). La relación entre obesidad y riesgo cardiovascular es compleja y está mediada por varios mecanismos biológicos. Aunque el término ECV incluya todas las enfermedades que afectan al sistema cardiovascular (hipertensión, disfunción endotelial, enfermedad coronaria, etc.), usualmente se utiliza para referirse a aquellas que afectan a las arterias, y en concreto a la aterosclerosis (del griego *athero* (pasta) y *skleros* (duro, piedra)).

La aterosclerosis es un proceso inflamatorio crónico en la pared arterial por el depósito e infiltración de sustancias lipídicas en las paredes de mediano y grueso calibre (Ross, 1999). Provoca una reacción inflamatoria y la multiplicación y migración de las células musculares lisas de la pared que van produciendo estrechamientos de la luz arterial. Los engrosamientos concretos se denominan placas de ateroma. En casos avanzados se observa un proceso de calcificación de las placas de ateroma que aumenta el riesgo de ruptura, ulceración o erosión de las mismas, además de su exposición a agentes trombogénicos. Si los trombos se desprenden y viajan por el torrente sanguíneo pueden ocluir el flujo en arterias coronarias, cerebrales o pulmonares causando ataques al corazón, accidentes cerebrovasculares o embolias pulmonares que junto con aneurismas (debilitamiento de la pared arterial causado principalmente por las placas de ateroma) constituye la principal causa de muerte en los países occidentales.

Inflamación y resistencia a la insulina en el endotelio, dislipidemia, y acumulación patológica de lípidos en el corazón y la médula ósea son condiciones asociadas al

estado obeso que impactan negativamente en todos los elementos de la patogénesis de la enfermedad cardiovascular (véase la Figura 2):

a) Obesidad y disfunción endotelial

En la obesidad, los elevados niveles circulantes de ácidos grasos, citoquinas pro-inflamatorias y especies reactivas del oxígeno interfieren con importantes vías de señalización intracelular en células endoteliales; las consecuencias más destacables son el desarrollo de resistencia a la insulina y la producción de mediadores inflamatorios (TNFα, MCP-1, IL-8, ICAM-1 y VCAM-1) que promueven la infiltración de monocitos circulantes y su activación a macrófagos en el espacio sub-endotelial (Boden, 2008; Curat et al., 2004; Gruen et al., 2007; Pilz and Marz, 2008; Steinberg et al., 2000).

Una de las manifestaciones de la resistencia a la insulina en el endotelio es la inhibición de la óxido nítrico sintasa endotelial (eNOS), y con ello una menor producción de óxido nítrico (ON) (Cersosimo and DeFronzo, 2006). El ON secretado por las células endoteliales es un vasodilatador y un potente inhibidor de la agregación plaquetaria y la adhesión a la pared vascular (Caballero, 2003). Además, este gas reduce la permeabilidad vascular y la tasa de oxidación de las LDL, e inhibe la proliferación de células musculares lisas (Kim et al., 2006). La insulina normalmente estimula la producción de ON endotelial, en lo que parece ser un mecanismo destinado a incrementar la disponibilidad de glucosa para los tejidos; de hecho, se ha estimado que entre el 25-40% del incremento de captación de glucosa inducida por insulina en el músculo se debe al incremento del flujo sanguíneo mediado por ON (Kim et al., 2006).

En respuesta a la insulina, la eNOS es normalmente activada por fosforilación catalizada por la PKB/Akt. La hiperglucemia inhibe *per se* la producción de ON por favorecer la glicosilación de la eNOS en el residuo de serina diana de la PKB/Akt (Du et al., 2001). Esto es parte de la relación subyacente entre diabetes y aterosclerosis (Caballero, 2003).

b) Obesidad y retención sub-endotelial de las LDL

La retención de las LDL en el sub-endotelio es un evento temprano en la aterosclerosis que queda potenciado en condiciones de obesidad. En primer lugar, porque sobrepeso y obesidad se asocian a niveles circulantes aumentados de LDL. Y en segundo lugar, porque en la obesidad se favorecen determinadas modificaciones de las LDL que suponen un aumento del potencial aterogénico de estas lipoproteínas. La interacción entre las LDL y los proteoglicanos de la pared arterial depende de enlaces electrostáticos entre grupos amino (+) de la apolipoproteína B100 (apoB100) y grupos

cargados negativamente de los proteoglicanos. Modificaciones post- traduccionales de la apoB100 pueden modificar la afinidad de esta proteína por los proteoglicanos y en consecuencia modificar el potencial aterogénico de las LDL. Es bien conocido el incremento del potencial aterogénico de las LDL tras ser oxidadas (Steinberg et al., 1989). Más recientemente, se ha descrito que la glicosilación de la apoB100 cuadruplica su potencial aterogénico (Rabbani et al., 2011). Este hallazgo contribuye a comprender a nivel molecular la relación entre DT2 y enfermedad cardiovascular.

c) Obesidad y respuesta inflamatoria originada por el material retenido

Los macrófagos infiltrados en el espacio sub-endotelial ingieren lípidos y colesterol de las LDL y se convierten en las llamadas células espumosas, que contribuyen al crecimiento de la placa de ateroma. Los ácidos grasos, cuyos niveles circulantes están aumentados en la obesidad, son ligandos activadores de los receptores TLR4 de la superficie de macrófagos, y la activación de estos receptores desemboca en el reclutamiento y activación de más monocitos circulantes, y la retención de más lípidos y proteínas aterogénicas. En definitiva, se inicia un proceso que se retroalimenta positivamente y finaliza con la formación de la placa de ateroma.

d) Obesidad y lipotoxicidad cardiaca

En el estado obeso, el exceso de lípidos puede acumularse tanto dentro del corazón, en adipocitos inter-miocitos, como en la capa superficial de tejido adiposo que envuelve al corazón. Esta capa de tejido adiposo tiene una gran capacidad de secretar ácidos grasos que sirven de sustrato metabólico para el miocardio, pero también puede convertirse en una fuente de proteínas pro-aterogénicas y pro- inflamatorias como ocurre en pacientes con enfermedad coronaria (Baker et al., 2009). Además, en el estado obeso incrementa el número de adipocitos intercalados entre los miocitos, que pueden provenir de células madre mesenquimales (destinadas normalmente a reemplazar miocitos) (Gesta et al., 2007; Hill et al., 2009) o de la transdiferenciacion de miocitos maduros (Aguiari et al., 2008). Este incremento de masa adiposa (interna y externa) no contráctil contribuye al fallo cardiaco y a la ateroesclerosis de las coronarias.

e) Obesidad y recambio/reparación del endotelio

Dentro de la población de células multipotentes de la médula ósea, las células progenitoras endoteliales (EPCs, *endothelial progenitor cells*) son aquellas que han adquirido el compromiso para diferenciarse en células endoteliales y tienen la capacidad de migrar desde la medula ósea para reparar el endotelio dañado o para crear nuevos

vasos sanguíneos en respuesta a ON y varias citoquinas, incluyendo VEGF (*vascular endothelial growth factor*) y SCF (*stem cell factor*) (Aicher et al., 2003). Las EPCs juegan un papel crucial en la biología cardiovascular, hasta tal punto que el número de EPCs circulantes se considera un reflejo de la salud cardiovascular (Fadini et al., 2006). Todos los factores de riesgo aterogénico (diabetes, hipercolesterolemia, hipertensión y tabaquismo) se han asociado con una menor cantidad o disfunción de las EPCs (Fadini et al., 2005; Kondo et al., 2004; Levy, 2005; Urbich and Dimmeler, 2005).

La obesidad abdominal y el síndrome metabólico se asocian a una menor cantidad de EPCs circulantes (Westerweel et al., 2008), hecho que se ha relacionado con un incremento de la adipogénesis en la médula ósea en detrimento de la hematopoyesis (Calvo et al., 1976; Naveiras et al., 2009). Recíprocamente, la pérdida de peso se asocia con un incremento de las EPCs circulantes (Muller-Ehmsen et al., 2008). La adiponectina, una adipoquina con actividad anti-diabética y anti-aterosclerótica cuyos niveles circulantes están reducidos en la obesidad (ver apartado 1.2.4), es un quimioatrayente y estimulador de la diferenciación de las EPCs (Kondo et al., 2009; Shibata et al., 2008).

1.1.4. Osteoartritis

La osteoartritis o enfermedad degenerativa articular es una patología multifactorial caracterizada por la pérdida de cartílago articular. La visión actual es que se trata de un desorden sistémico músculo-esquelético que afecta a la articulación en su conjunto, incluido el hueso subcondral y la cápsula sinovial (membrana y tejido periarticular) (Goldring and Goldring, 2007; Samuels et al., 2008). También se ha reconsiderado el papel de la inflamación en el desarrollo de la osteoartritis. Así, aunque la osteoartritis no haya sido tradicionalmente considerada una enfermedad inflamatoria (ya que no se da un incremento importante del número de leucocitos en el líquido sinovial), creciente evidencia científica demuestra la existencia de inflamación del sinovio en etapas tempranas de la enfermedad. Los sinoviocitos inflamados secretan citoquinas pro-inflamatorias que favorecen la infiltración de células del sistema inmune, y finalmente tanto el hueso subcondral como el propio cartílago activan la destrucción del cartílago mediante la producción de enzimas que degradan la matriz extracelular (Aspden, 2008; Iannone and Lapadula, 2010).

La obesidad es un factor de riesgo de desarrollo de osteoartritis. Tradicionalmente, la relación entre obesidad y osteoartritis se explicaba únicamente por razones biomecánicas. Sin embargo, actualmente se considera que esta relación tiene una base metabólica, como lo indican el hecho de que la obesidad es un factor de riesgo de

osteoartritis no sólo en las articulaciones que deben soportar un exceso de peso (rodillas, caderas) sino también en las que no soportan peso, como las de las manos, y que la pérdida de masa grasa mejora los síntomas de la osteoartritis más que la pérdida de peso corporal *per se* (Aspden et al., 2001; Griffin and Guilak, 2008; Iannone and Lapadula, 2010; Pottie et al., 2006).

Existen varios niveles de conexión entre obesidad y osteoartritis (véase la Figura 2). Por una parte, a nivel sistémico, la producción y secreción alterada de adipoquinas y mediadores inflamatorios en el tejido adiposo, la hiperlipidemia y el estrés oxidativo son condiciones frecuentemente asociadas a la obesidad que favorecen la degeneración de las articulaciones. Por otra parte, existen depósitos de grasa en las articulaciones (adipocitos en grasa infrapatelar de la rodilla y en la médula ósea) cuya secreción de adipoquinas y factores inflamatorios también puede afectar a la funcionalidad de los tejidos de la articulación. Además, en la obesidad podría verse comprometida la capacidad del organismo de mantener y regenerar las articulaciones (véase a continuación).

Complejas vías de señalización y factores de crecimiento regulan la formación de las articulaciones durante el desarrollo embrionario, y estas mismas vías, aunque menos activas, son responsables de mantener la homeostasia articular en la vida adulta (De Bari et al., 2010). La pérdida de esa homeostasia, como ocurre con la edad y probablemente en el estado obeso, conduce a la aparición de osteoartritis en múltiples articulaciones (De Bari et al., 2010). Los mecanismos subyacentes a esta pérdida de homeostasia podrían en parte tener su origen, tal y como hemos visto en el apartado anterior, en la degeneración grasa de la médula ósea. Aquí hay células madre mesenquimales (BMSCs, bone marrow stem cells) capaces de diferenciarse en diferentes tipos celulares incluidos adipocitos, condro-/osteo-blastos y miocitos. La diferenciación de las BMSCs en los diferentes linajes está competitivamente equilibrada: factores de transcripción que promueven la diferenciación en un determinado linaje inhiben activamente la diferenciación en los otros linajes. Por ejemplo, el PPARγ, factor clave para la adipogénesis, inhibe la diferenciación terminal de osteoblastos al inhibir la expresión de Runx2 (Takada et al., 2009b); en relación con ello, es bien conocido el efecto de las tiazolidinedionas – ligandos sintéticos del PPARγ utilizados en clínica como anti-diabéticos – sobre la reducción de masa ósea (Lecka-Czernik, 2010). Recíprocamente, vías de señalización que estimulan la osteoblastogénesis de BMSCs, como las iniciadas por Wnt-5a, TNFα e IL-1, promueven la represión del PPARγ (Takada et al., 2009a). También hay un control recíproco de la adipogénesis y la

condrogénesis de BMSCs: Sox9, factor clave de la condrogénesis, inhibe la adipogénesis al inhibir la expresión de los factores de transcripción adipogénicos tempranos CEBPβ y CEBPδ, y Pref-1, un marcador de preadipocitos e inhibidor de la adipogénesis, induce la expresión de Sox9 (Wang and Sul, 2009).

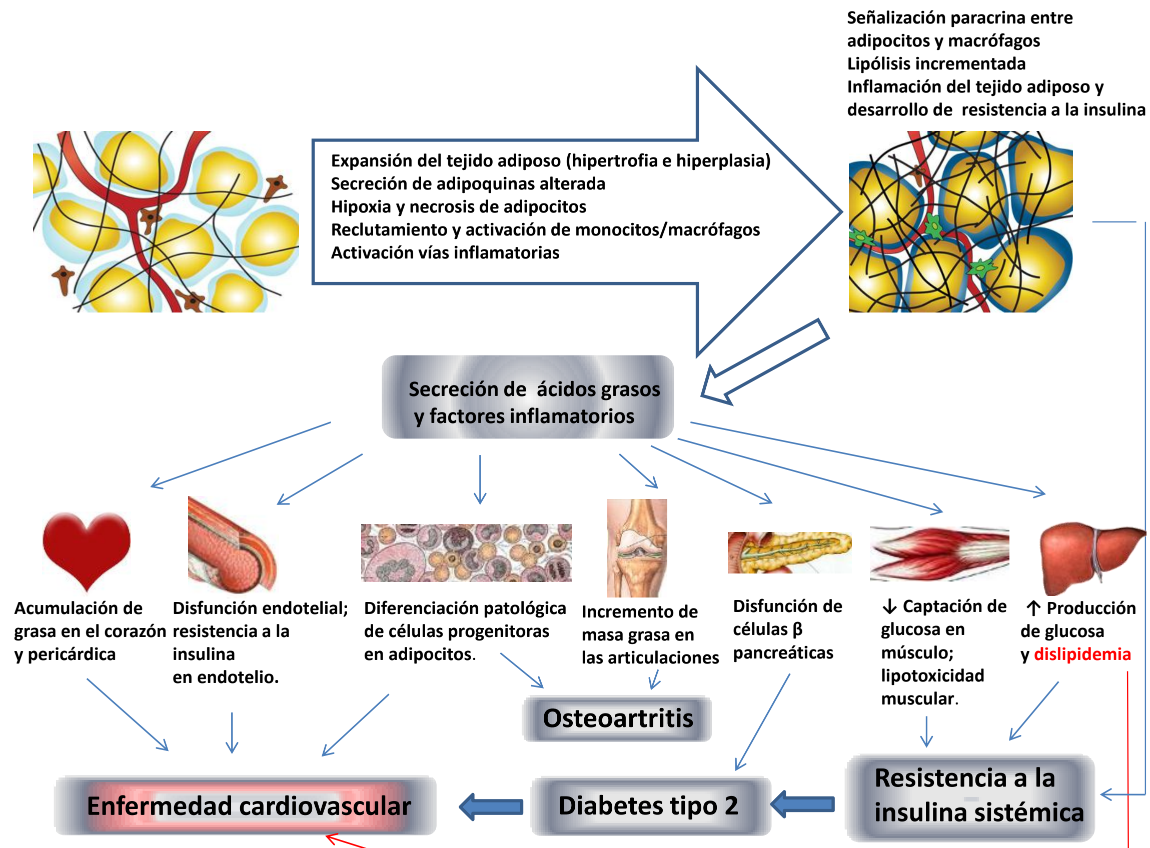

Figura 2. *Impacto de la lipotoxicidad e inflamación sistémica en las comorbilidades de la obesidad.*

1.2. EL TEJIDO ADIPOSO EN EL CONTEXTO DE LA OBESIDAD Y SUS COMPLICACIONES

El tejido adiposo es un tejido conjuntivo de origen mesenquimal formado mayoritariamente por células especializadas en la acumulación de lípidos en su citoplasma: los adipocitos. Además de éstos, se encuentran en el tejido adiposo otros tipos celulares tales células mesenquimales multipotentes, células del sistema inmune (macrófagos, células dendríticas, mastocitos, linfocitos y granulocitos), células endoteliales y fibroblastos, que en conjunto constituyen la llamada fracción estromo-vascular del tejido.

Desde un enfoque energético-funcional, existen dos tipos básicos de adipocitos: blancos y marrones. Mientras los adipocitos blancos están especializados en almacenar energía, los adipocitos marrones disipan energía en forma de calor. La cantidad de adipocitos blancos y marrones en un determinado depósito graso depende de la edad, el género así como de las condiciones ambientales y nutricionales (Cinti, 2005; Cinti, 2006). Recientemente, se ha postulado la existencia de un tercer tipo de adipocitos, similares a los adipocitos marrones en su función de disipar energía pero cuyo origen sería distinto: los "adipocitos marrones en tejido adiposo blanco" o adipocitos BRITE (de *BRrown In whiTE*) (Petrovic et al., 2010). Los adipocitos BRITE parecen compartir un progenitor mesenquimal común con los adipocitos blancos, mientras que los adipocitos marrones "genuinos" parecen compartir un progenitor común con las células musculares (Petrovic et al., 2010; Timmons et al., 2007) (Figura 3).

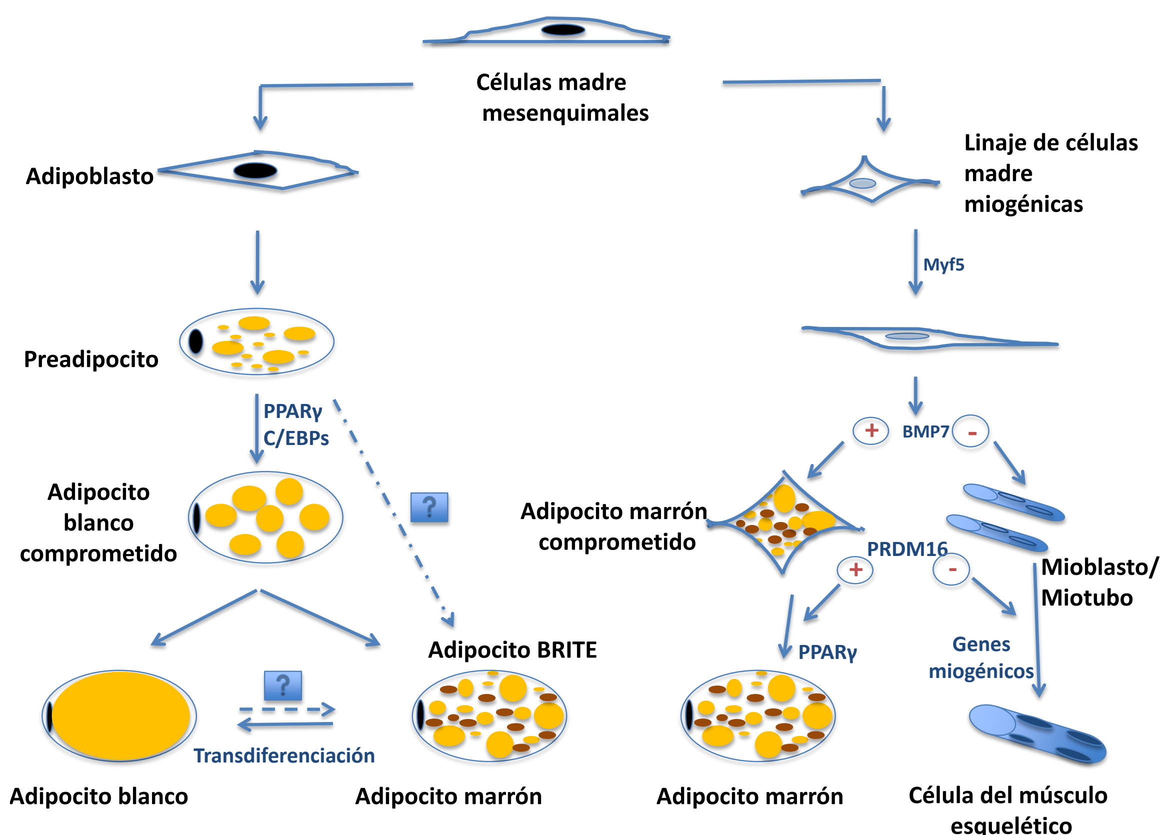

Figura 3. *Vías de diferenciación de adipocitos blancos, marrones y BRITE. Adaptado de (Serrano Rios et al., 2011).*

1.2.1. El tejido adiposo marrón

El tejido adiposo marrón (TAM) está formado principalmente por adipocitos marrones que se caracterizan por almacenar los triacilgliceroles en múltiples vacuolas y, metabólicamente, por presentar una gran capacidad oxidativa y un gran número de mitocondrias especializadas, que confieren el color característico del tejido. Se trata además de un tejido muy vascularizado y con una rica inervación simpática. Su principal función es la de disipar energía en forma de calor (termogénesis adaptativa), proceso que puede ser activado en respuesta al frío y a un exceso de ingesta (Rothwell and Stock, 1979) o en procesos febriles.

La base molecular de la termogénesis en el TAM es la actividad de la proteína desacopladora 1 (UCP1), presente en la membrana interna mitocondrial, que disipa en forma de calor el gradiente de protones generado por la actividad de la cadena respiratoria, desacoplando de este modo la oxidación de combustibles de la síntesis de ATP (Cannon and Nedergaard, 2004). La importancia de la termogénesis adaptativa en el TAM para la protección frente a la obesidad está demostrada en modelos animales (Cummings et al., 1996; Lowell et al., 1993). No obstante, la disminución de la UCP1 no implica necesariamente el desarrollo de obesidad (Enerback et al., 1997).

El interés por el TAM se ha acrecentado a raíz de investigaciones recientes que demuestran que, contrariamente a lo que venía asumiendo, este tejido no desaparece en humanos adultos, sino que permanece activo en localizaciones específicas (Nedergaard et al., 2007), y que individuos con poco TAM funcional son más propensos a la obesidad y desordenes metabólicos asociados (Cypess et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). Se estima que tan sólo 50 g de TAM activo pueden incrementar el gasto energético diario en un 20% (Virtanen and Nuutila, 2011). Además, investigaciones en animales indican que la actividad del TAM puede reducir la hipertrigliceridemia (Bartelt et al., 2011) y tener efectos beneficiosos sobre el metabolismo de la glucosa y la sensibilidad a la insulina, vía producción de señales endocrinas como el factor de crecimiento de fibroblastos 21 (Hondares et al., 2011).

Junto con la activación de los depósitos endógenos de TAM, la estimulación de la formación de adipocitos BRITE en el tejido adiposo blanco y la adquisición de características propias de los adipocitos marrones en adipocitos blancos son estrategias de interés potencial en la prevención y tratamiento de la obesidad y sus comorbilidades.

1.2.2. El tejido adiposo blanco

El tejido adiposo blanco (TAB) se encarga de almacenar el exceso de energía en forma de triacilgliceroles y de proporcionarla cuando el organismo lo requiere. En una situación de demanda energética, los triacilgliceroles almacenados en el TAB son hidrolizados por lipasas, en un proceso conocido como lipólisis, de forma que se liberan ácidos grasos no esterificados y glicerol a la circulación, los cuales serán utilizados como sustratos oxidables para satisfacer los requerimientos energéticos de otros tejidos, principalmente músculo, hígado y riñón.

Dos enzimas juegan un papel clave en el proceso lipolítico: la triacilglicerol lipasa adiposa (ATGL, *adipose triglyceride lipase*) y la lipasa sensible a las hormonas (HSL, *hormone sensitive lipase)*. Se acepta hoy día que la ATGL inicia la movilización de los triacilgliceroles produciendo diacilgliceroles que son subsecuentemente hidrolizados por la HSL. El proceso lipolítico en el TAB mediado por estas enzimas se encuentra finamente controlado por ciertas hormonas tales como la insulina, las catecolaminas y los glucocorticoides, cuya secreción, a su vez, está regulada por el estatus nutricional del organismo (Jaworski et al., 2007).

En situaciones de abundancia energética, la insulina inhibe normalmente la lipólisis (inhibición de HSL y ATGL) a la vez que estimula la lipogénesis en el TAB, al estimular la captación por los adipocitos de ácidos grasos de los triacilgliceroles contenidos en lipoproteínas circulantes (vía activación de la lipoproteína lipasa, LPL) y de glucosa circulante (vía inducción de la síntesis y translocación a la membrana plasmática del transportador de glucosa 4, GLUT4). Dentro del adipocito, ácidos grasos y glucosa sirven como precursores para la síntesis de triacilgliceroles.

El TAB no es un tejido uniforme, sino que se encuentra distribuido en forma de depósitos en distintos lugares anatómicos. De forma convencional, se pueden dividir en subcutáneos y viscerales y tienen función biológica y actividad metabólica distintas (Einstein et al., 2005; Giorgino et al., 2005; Klaus and Keijer, 2004). Los desórdenes metabólicos asociados al exceso de grasa se relacionan específicamente con la acumulación de grasa visceral en la región abdominal, probablemente por poseer esta grasa mayor actividad metabólica que la grasa subcutánea. El TAB visceral capta más glucosa en respuesta a la insulina (Virtanen et al., 2002) y también posee mayor actividad lipolítica (Giorgino et al., 2005). Los ácidos grasos liberados por el TAB visceral alcanzan rápidamente el hígado vía circulación portal, disminuyendo en ese tejido la acción de la insulina e incrementando la salida de glucosa y VLDL (Boden, 1998; Giorgino et al., 2005).

1.2.3. La adipogénesis

La adipogénesis es el proceso que implica la determinación y la diferenciación de una célula precursora en un adipocito. Durante este proceso un gran número de genes son regulados de forma selectiva, secuencial y coordinada, lo que se refleja en cambios sustanciales de la morfología celular, la sensibilidad a hormonas y las capacidades metabólicas celulares, particularmente para la lipogénesis y la lipólisis, y en el caso de los adipocitos marrones también para la termogénesis.

Durante el desarrollo la formación de células adiposas ocurre a partir del mesodermo. Los depósitos de TAM, necesarios para el control térmico en el nacimiento, aparecen durante la fase final de la gestación, mientras que el TAB crece fundamentalmente en la etapa post-natal temprana, como resultado del incremento del tamaño de adipocitos existentes y la proliferación de células precursoras (Feve, 2005).

La regulación de la adipogénesis es muy compleja. Depende de estímulos hormonales y nutricionales que controlan la expresión y acción de una serie de factores de transcripción pro- y anti- adipogénicos (Feve, 2005). Entre los pro- adipogénicos destacan el PPARγ y los de la familia de las C/EBPs *(CCAAT/enhancer-binding proteins)* que incluye C/EBPα, C/EBPβ y C/EBPδ. En respuesta a estímulos pro-adipogénicos se produce una inducción transitoria de C/EBPβ y C/EBPδ, que a su vez inducen la expresión de PPARγ. PPARγ induce la expresión de C/EBPα y éste, de forma recíproca, induce la de PPARγ. PPARγ y C/EBPα presentan un comportamiento sinérgico en la diferenciación de adipocitos, ya que en el promotor de muchos genes marcadores adipocitarios co-existen elementos de respuesta para ambos factores de transcripción (Rosen et al., 2000). Se considera al PPARγ el regulador clave de la adipogénesis, siendo su actividad necesaria y suficiente para la adipogénesis, y al C/EBPα necesario para mantener elevados los niveles de PPARγ y para la adquisición de la sensibilidad a la insulina (Rosen, 2005). Modificaciones post-traduccionales, interacción con coactivadores/correpresores y unión de ligandos, constituyen toda una gama de mecanismos que integran todas las señales presentes y modulan finamente la actividad de estos factores de transcripción.

Mientras que el proceso de diferenciación de preadipocitos en adipocitos ha sido muy estudiado, especialmente en líneas celulares inmortalizadas comprometidas a diferenciarse en adipocitos blancos (como las células 3T3-L1), se sabe relativamente poco del origen de los preadipocitos. Las células madre mesenquimales residen principalmente en la médula ósea (BMSCs) pero también se las encuentra en la fracción estromo-vascular del tejido adiposo, así como en músculo, sangre periférica, hígado y

tejidos embrionarios. Algunos estudios han sugerido que el precursor del adipocito es una célula circulante derivada de la médula ósea, pero esto es controvertido (Kahn, 2008). Otros estudios indican que las células progenitoras de los adipocitos blancos son pericitos residentes en la pared de los vasos sanguíneos del tejido adiposo (Tang et al., 2008) [los pericitos son células parecidas a células musculares lisas, que recubren el endotelio capilar].

En todo caso, la capacidad de diferenciación de adipocitos a partir de células precursoras se mantiene toda la vida. De hecho, estudios recientes indican que la población de adipocitos es más dinámica de lo que se creía. Así, en humanos, aunque el número de adipocitos blancos queda establecido en la adolescencia, un porcentaje considerable (10%) es renovado anualmente durante toda la vida adulta, mediante la coordinación de adipogénesis *de novo* y muerte de adipocitos preexistentes (Spalding et al., 2008). Muchos adultos obesos presentan un exceso de celularidad (hiperplasia) en el TAB, y estos individuos pueden ser especialmente refractarios a la pérdida de peso a largo plazo y propensos al conocido efecto yo-yo. El control de la adipogénesis emerge en este contexto como una nueva diana terapéutica en el control de la obesidad, que, complementando a la clásica intervención de dieta y ejercicio, favorecería el mantenimiento de la reducción de las reservas de grasa.

Mención aparte merece la adipogénesis en células mesenquimales de la médula ósea (BMSCs). Éstas constituyen una población heterogénea de células madre con capacidad de diferenciarse en linajes celulares tanto mesodérmicos como no mesodérmicos, incluyendo adipocitos, osteocitos, condrocitos, miocitos, cardiomiocitos, fibroblastos, células epiteliales y neuronas. El control de la adipogénesis de las células mesenquimales de la médula ósea favorecería la homeostasia de múltiples órganos y tejidos cuya funcionalidad, como hemos ido viendo en apartados anteriores, está comprometida en el estado obeso (e.g. corazón, vasos sanguíneos, articulaciones).

1.2.4. El tejido adiposo como órgano secretor

El tejido adiposo, particularmente el blanco, lejos de ser una reserva inerte de grasa es un importante órgano endocrino y secretor (Trayhurn and Beattie, 2001). Produce y secreta una gran variedad de compuestos, incluidos ácidos grasos y una variedad de proteínas con función reguladora denominadas colectivamente adipoquinas. Funcionando como señales autocrinas, paracrinas y endocrinas, las adipoquinas modulan el crecimiento y metabolismo del propio tejido adiposo y de otros órganos, y juegan un papel clave en la regulación del equilibrio energético y otros procesos

fisiológicos y patológicos, como el mantenimiento de la homeostasia de la glucosa y las respuestas inflamatoria y aterogénica.

A continuación se introducen las principales adipoquinas que han sido objeto de estudio en esta tesis:

Nicotinamida fosforibosil transferasa (Nampt)/visfatina

Nampt/visfatina fue originalmente identificada como una citoquina que estimula la maduración de precursores de linfocitos B (Samal et al., 1994) y después como una enzima citosólica que cataliza el primer paso en la síntesis de nicotinamin adenin dinucleótido (NAD), la condensación de nicotinamida (vitamina B3) con 5-fosfo ribosil-1-pirofosfato para rendir el mononucleótido de nicotinamida (NMN) (Rongvaux et al., 2002). Más recientemente, esta proteína fue descrita como una adipoquina (visfatina) secretada principalmente por la grasa visceral en el estado obeso y con efectos análogos a la insulina (Fukuhara et al., 2005). Sin embargo, ante la irreproducibildad de estos últimos resultados, los autores se vieron obligados a retirar la publicación (Fukuhara et al., 2007).

La importancia de la Nampt/visfatina en la respuesta inmune, metabólica y frente a situaciones de estrés radica en sus funciones extra-celulares (de citoquina/enzima) e intra-celulares (de enzima). Como citoquina sus funciones son básicamente pro-inflamatorias, ya que induce la producción de citoquinas pro-inflamatorias como el TNFα, IL-1β o IL-6 en monocitos CD14+ (Luk et al., 2008; Moschen et al., 2007; Revollo et al., 2007a; Tilg and Moschen, 2008). Sin embargo, existen resultados contradictorios respecto del efecto de citoquinas pro-inflamatorias sobre la producción adipocitaria de visfatina: mientras algunos estudios en adipocitos 3T3-L1 muestran un efecto inhibitorio de TNFα e IL-6 (Kralisch et al., 2005a; Kralisch et al., 2005b), otros estudios realizados en adipocitos humanos muestran una activación de la producción de visfatina en estas células por TNFα (Hector et al., 2007).

La actividad enzimática de la Nampt/visfatina en plasma parece jugar un papel importante en la provisión de NMN a tejidos/órganos donde la actividad de Nampt/visfatina es insuficiente para cubrir las necesidades celulares de NAD. En este contexto, la actividad sistémica de la Nampt/visfatina es importante para la secreción de insulina por las células pancreáticas β, que depende del suministro de NMN a estas células (Imai, 2009b; Revollo et al., 2007b).

La actividad intracelular de la Nampt/visfatina interviene en el control de enzimas dependientes de NAD, como por ejemplo las sirtuínas, afectando de ese modo a

múltiples procesos relacionados con la restricción calórica (lipólisis en tejido adiposo, oxidación de ácidos grasos en músculo, gluconeogénesis en hígado, etc.) y la supervivencia celular (Imai, 2009a).

A diferencia de adipoquinas prototipo como leptina y adiponectina expresadas preferencialmente por adipocitos blancos, la Nampt/visfatina se expresa en una variedad de tipos celulares y abundantemente en adipocitos marrones (Revollo et al., 2007b) y monocitos/granulocitos (Friebe et al., 2011).

Proteína 4 de unión al retinol (RBP4, *retinol binding protein* 4)

La RBP4 (o simplemente RBP) es una proteína de 21 KD de la familia de las lipocalinas que se expresa principalmente en hígado (Blaner, 1989; Soprano et al., 1986), seguido del TAB (Tsutsumi et al., 1992). La función más conocida de la RBP es la de proteína transportadora de retinol, desde los tejidos que almacenan vitamina A (principalmente el hígado, y en menor medida TAB) hasta los tejidos que la requieren. En el TAB la expresión de RBP es prácticamente exclusiva de adipocitos, y la expresión de RBP aumenta durante la diferenciación de los adipocitos (Tsutsumi et al., 1992).

La RBP derivada específicamente del TAB fue propuesta como un factor circulante que inducía resistencia a la insulina a partir de estudios en ratones deficientes en GLUT4 en el TAB (Yang et al., 2005). Los mismos autores mostraron altos niveles circulantes de RBP en otros modelos animales de resistencia a la insulina y en humanos insulino-resistentes, y que, en roedores, manipulaciones genéticas y farmacológicas que suponen un incremento de los niveles séricos de RBP provocaban resistencia a la insulina, mientras que la reducción de la RBP sérica mejoraba la sensibilidad a la insulina (Yang et al., 2005).

Con posterioridad, diversos estudios en humanos han encontrado una correlación directa entre los niveles séricos de RBP y el grado de resistencia a la insulina en individuos obesos con baja tolerancia a la glucosa o DT2 (Cho et al., 2006; Graham et al., 2006; Lee et al., 2007; Ribel-Madsen et al., 2009; Yang et al., 2005). En concordancia, niveles séricos bajos de RBP se correlacionan con una mayor sensibilidad a la insulina en individuos sanos (Aeberli et al., 2007; Cho et al., 2006; Stefan et al., 2007). Asimismo, se han descrito polimorfismos del gen de la *rbp* que se han asociado con una mayor susceptibilidad a padecer DT2 en humanos (Craig et al., 2007; Kovacs et al., 2007; Munkhtulga et al., 2007). No obstante, también existen estudios que no encuentran ninguna correlación entre niveles séricos de RBP y

sensibilidad a la insulina (Broch et al., 2007; Lewis et al., 2007; Yao-Borengasser et al., 2007).

A pesar de las discrepancias, se han demostrado efectos de la RBP contrarrestando la acción de la insulina en diferentes sistemas. Así, se ha descrito que la RBP atenúa la señalización de la insulina en cultivos primarios de adipocitos humanos (Ost et al., 2007) y en músculo esquelético de ratones (Yang et al., 2005), incrementa la producción hepática de glucosa en ratones (Yang et al., 2005), y reduce la secreción de insulina en células β pancreáticas humanas (Broch et al., 2007). Es más, recientemente se ha descrito que la holo-RBP (RBP con retinol unido) es funcionalmente una citoquina capaz de inducir la expresión de SOCS3, un conocido supresor de la señalización por insulina, en tejidos diana como el músculo esquelético y el TAB (Berry et al., 2011). En particular, se ha descrito que la proteína de membrana que media el transporte del retinol desde la holo-RBP circulante al interior celular, STRA6, funciona como un receptor de citoquinas que, tras ser activado por holo-RBP, media la expresión de SOCS3 y la atenuación de la señalización por insulina (Berry et al., 2011).

Además de con la resistencia a la insulina, se han descrito correlaciones directas de los niveles séricos de RBP sérica con marcadores de inflamación (von Eynatten et al., 2007; Yao-Borengasser et al., 2007) y con la cantidad de grasa hepática (Ribel-Madsen et al., 2009; Seo et al., 2008; Stefan et al., 2007).

Leptina

La leptina es secretada principalmente por adipocitos blancos, pero también por otros tipos celulares: estómago (Bado et al., 1998); músculo (Wang et al., 1998); sinoviocitos y osteocitos (Presle et al., 2006); placenta y epitelio mamario (Casabiell et al., 1997). Sus niveles circulantes correlacionan con el tamaño de las reservas grasas y el estatus nutricional. Tiene un papel importante en la regulación del balance energético, inhibiendo la ingesta e incrementando el gasto energético, fundamentalmente a través de efectos a nivel central (hipotalámico) (Gautron and Elmquist, 2011; Zhang et al., 1994). Además, la leptina inhibe la adipogénesis e incrementa la lipólisis en el músculo y TAB y la oxidación de ácidos grasos en músculo e hígado (Muoio y Lynis Dohm, 2002). En humanos obesos lo habitual es la hiperleptinemia y la resistencia a la leptina, que puede tener diversos orígenes.

Además de sus efectos metabólicos, la leptina está involucrada en la respuesta inmune e inflamatoria actuando como una señal pro-inflamatoria (Lago et al., 2008; Stofkova, 2009), pudiendo actuar, por ejemplo, como quimioatrayente de monocitos (Curat et al., 2004; Gruen et al., 2007). Se la ha relacionado con diferentes patologías inflamatorias.

Específicamente, en relación a la patofisiología de la osteoartritis, se han descrito tanto acciones anabólicas (Dumond et al., 2003; Figenschau et al., 2001) como catabólicas (Bao et al.; Griffin et al., 2009; Simopoulou et al., 2007; Vuolteenaho et al., 2009) de la leptina. En células mesenquimales de la médula ósea la leptina inhibe la adipogénesis y estimula la osteo-condrogénesis (Hamrick and Ferrari, 2008).

La leptina se encuentra en la leche materna, y estudios de nuestro grupo han mostrado que la suplementación oral con leptina a dosis fisiológicas durante el periodo de lactancia previene la obesidad, mejora la resistencia a la insulina y disminuye la preferencia por dietas ricas en grasa en la edad adulta (Pico et al., 2007; Priego et al., 2010; Sanchez et al., 2008).

Resistina

La resistina fue descubierta como una proteína secretada por adipocitos murinos cuya producción disminuía en respuesta a drogas anti-diabéticas y que causaba resistencia a la insulina cuando se inyectaba a ratones (Steppan et al., 2001). Sin embargo, actualmente existe una gran controversia en torno a este rol, principalmente en humanos.

Estudios recientes han demostrado la expresión de resistina en muchos otros tipos celulares además del adipocito, notablemente en humanos en células del sistema inmunitario (monocitos y macrófagos) (Patel et al., 2003; Savage et al., 2001), y han relacionado a la resistina con la respuesta inflamatoria (Pang and Le, 2006). Se ha descrito que la activación de vías inflamatorias induce la expresión de resistina en monocitos humanos y que, a su vez, la resistina induce la expresión de citoquinas pro-inflamatorias en monocitos y macrófagos humanos (Anderson et al., 2007; Bokarewa et al., 2005; Kaser et al., 2003; Lehrke et al., 2004). Además, los niveles plasmáticos de resistina correlacionan directamente con marcadores de inflamación en varias condiciones patofisiológicas en humanos (Pang and Le, 2006). Se ha sugerido que la resistina podría constituir un enlace entre inflamación y resistencia a la insulina (McTernan et al., 2006).

Adiponectina

La adiponectina es una hormona proteica secretada en gran cantidad por el TAB y específicamente por los adipocitos que circula en plasma en diferentes isoformas producidas por modificaciones post-traduccionales. Desempeña un papel en la prevención de la resistencia a la insulina y de la aterosclerosis, teniendo propiedades anti-diabéticas y propiedades anti-inflamatorias en el endotelio vascular (Akiyama et al.,

2005; Kadowaki et al., 2006; Wellen and Hotamisligil, 2005). En humanos y roedores, los niveles circulantes de adiponectina están reducidos en la obesidad, estados de resistencia a la insulina y DT2, así como en la enfermedad cardiovascular y la hipertensión (Kadowaki et al., 2006).

La adiponectina inhibe la gluconeogénesis hepática, aumenta la captación y utilización de la glucosa por el músculo esquelético, y favorece la reducción del contenido en triacilgliceroles en hígado y músculo, ya que estimula la oxidación de ácidos grasos y suprime la lipogénesis (Kadowaki et al., 2006). Estos efectos están mediados por la activación de la proteína quinasa activada por AMP (AMPK) y de la isoforma alfa del PPAR (PPARα). La AMPK es una enzima reguladora que se activa en condiciones de depleción de energía intracelular (aumento de la ratio AMP/ATP) y por fosforilación, cuya actividad inhibe rutas metabólicas consumidoras de energía y activa rutas metabólicas productoras de energía. El PPARα es una isoforma de PPAR que controla al alza la transcripción de genes relacionados con la oxidación de ácidos grasos y el metabolismo oxidativo en general.

En células endoteliales, la adiponectina inhibe la vía inflamatoria IKK/NFκB (Ouchi et al., 2000) a la vez que es un quimioatrayente y estimulador de la diferenciación de las células progenitoras endoteliales (EPCs), procesos necesarios para mantener y reparar el tejido vascular (Kondo et al., 2009; Shibata et al., 2008) .

1.2.5. Alteraciones en el tejido adiposo en la obesidad

El tejido adiposo en el estado obeso se caracteriza por una lipólisis basal incrementada, y por una expresión alterada de adipoquinas y factores pro-inflamatorios a la que contribuyen los adipocitos hipertrofiados (Halberg et al., 2008; Kershaw and Flier, 2004; Skurk et al., 2007) y en especial los macrófagos, cuya presencia en el tejido adiposo obeso se encuentra aumentada (Weisberg et al., 2003; Xu et al., 2003) (Tabla 1).

En condiciones de balance energético positivo, el exceso de energía se almacena primero en los adipocitos maduros, causando un incremento de su tamaño (hipertrofia), a lo que sigue un incremento de la adipogénesis de células mesenquimales del tejido adiposo, con aumento del número de adipocitos (hiperplasia) (Bjorntorp et al., 1982; de Ferranti and Mozaffarian, 2008). Ambos procesos permitirían mantener la funcionalidad del tejido adiposo. Cuando se alcanza el límite de expansión del tejido adiposo (determinado por factores genéticos y ambientales), se inicia una cascada inflamatoria con efectos locales y sistémicos que conducen al desarrollo de resistencia a la insulina y sus consecuencias metabólicas, muchas de las cuales han sido descritas en apartados anteriores (Medina-Gomez et al., 2007; Virtue and Vidal-Puig, 2010).

Varios mecanismos relacionados entre sí han sido propuestos para explicar la inflamación en el tejido adiposo en la obesidad, y se comentan a continuación.

Los adipocitos hipertrofiados incrementan la producción de señales, como la MCP1 (*monocyte chemoattractant protein 1*) o la propia leptina que actúan como quimioatrayentes de monocitos, promoviendo la infiltración de células inmunitarias en el tejido adiposo (Curat et al., 2004; Gruen et al., 2007).

Citoquina	Adipocitos	Macrófagos	Efecto sobre la inflamación
Adiponectina	↓	-	**Inhibición**
Apelina	↑	↑	
Leptina	↑	-	Activación
Resistina	↑	↑	Activación
RBP	↓	-	Activación
Nampt/visfatina	↑	↑	Activación
Proteína amiloide sérica A3	↑	↑	Activación
Factor de necrosis tumoral α (TNFα)	↑	↑	Activación
IL-1β	↑	↑	Activación
IL-4	↑	↓	**Inhibición**
IL-6	↑	↑	Activación
IL-7	↑	-	Activación
IL-8	↑	↑	Activación
IL-10	↑	↑	**Inhibición**
IL-12	↑	↑	Activación
IL-18	↑	↑	Activación
Proteína inflamatoria de macrófagos-1 (MIP-1)	-	↑	Activación
Proteína quimiotáctica de macrófagos-1 (MCP-1)	↑	↑	Activación
Inhibidor del activador del plasminógeno-1 (PAI-1)	↑	↑	Activación
Proteína C-reactiva (CRP)	↑	↑	Activación
Factor estimulador de monocitos-1 (CSF-1)	↑	↑	Activación
Factor de crecimiento del endotelio vascular (VEGF)	↑	↑	Activación
Factor de crecimiento de plaquetas (PDGF)	-	↑	Activación
Factor del endotelio pigmentado (PEDF)	↑	-	**Inhibición**
Complemento C1, C3	↑	↑	Activación
Complemento C2, C4, C7	↑	-	Activación
Factor de crecimiento de fibroblastos 2 (FGF2)	↑	↑	Activación
Factor de crecimiento transformante β (TGFβ)	↑	↑	Activación
Prostaglandina E2	↑	↑	Activación
Trombospondina-1,2	↑	↑	Activación
Osteopontina	↑	↑	Activación
Interferón- α,γ IFN-α,γ	-	↑	Activación
Oxido nítrico sintasa inducible (iNOS)	-	↑	Activación
Factor de crecimiento de hepatocitos (HGF)	↑	↑	Activación
Factor tisular	↑	↑	Activación
Angiopoyetina1	↑	↑	Activación
Angiopoyetina2	↑	↑	Activación
Angiotensinógeno	↑	↑	Activación
Metaloproteinasas de la matriz extracelular (MMP)	↑	↑	Activación

Tabla 1*. La producción de citoquinas pro-inflamatorias está incrementada en la obesidad. Adipocitos y macrófagos infiltrados en el tejido adiposo son la principal fuente de factores inflamatorios circulantes en la obesidad. Adaptado de (Serrano Rios et al., 2011).*

El retículo endoplásmico (RE) juega un papel muy importante como sensor de nutrientes (colesterol, otros lípidos y proteínas) en el ambiente citoplasmático. Un exceso de

nutrientes como en el estado obeso causa estrés en el RE, y la célula en respuesta pone en marcha una serie de funciones adaptativas (defensivas) colectivamente llamadas *unfolded protein response* (UPR), que incluyen la activación de la vía inflamatoria de JNK y la inducción de la expresión del factor pro-inflamatorio TNFα (de Ferranti and Mozaffarian, 2008; Hotamisligil, 2010).

Además, por mecanismos que no son del todo comprendidos, el exceso de ácidos grasos, puede favorecer la generación de especies reactivas del oxígeno (ROS) en las mitocondrias y con ello el estrés oxidativo mitocondrial (de Ferranti and Mozaffarian, 2008). ROS y TNFα secretados por los adipocitos activan la respuesta inflamatoria en macrófagos, que a su vez secretan más factores inflamatorios que atraen a más monocitos circulantes (Surmi and Hasty, 2008).

Una menor vascularización debida a la rápida expansión del tejido puede conducir a una situación de hipoxia y, en consecuencia, producirse un incremento de la expresión de múltiples genes inflamatorios y el desencadenamiento de procesos de muerte celular (necrosis de adipocitos) que a su vez, atraen la llegada de células fagocitarias (macrófagos y neutrófilos) (Surmi and Hasty, 2008). También se ha propuesto que los adipocitos que alcanzan un tamaño máximo entran en necrosis espontáneamente, lo que favorece la infiltración de macrófagos en torno a ellos (Cinti et al., 2005).

La comunicación paracrina que se establece entre adipocitos y macrófagos estaría promoviendo el inicio y la perpetuación del estado de inflamación que caracteriza a la obesidad (Permana et al., 2006; Suganami et al., 2005; Suganami et al., 2007). Los factores inflamatorios producidos por macrófagos se unen a sus respectivos receptores en la membrana de los adipocitos, desencadenando la activación de diferentes MAPKs, así como del NFκβ, a través de los cuales se produce una mayor activación de la lipólisis y de la expresión de genes inflamatorios por parte de los adipocitos (Capel et al., 2009). A su vez, los ácidos grasos saturados liberados por los adipocitos se unen al receptor TLR4 de macrófagos, cuya activación conduce a la producción de TNFα vía NFκB (de Ferranti and Mozaffarian, 2008). Este circuito se considera que juega un importante papel en el desarrollo de la inflamación en el tejido adiposo obeso (Suganami et al., 2007).

1.3. EL MÚSCULO ESQUELÉTICO EN EL CONTEXTO DE LA OBESIDAD Y SUS COMPLICACIONES

El músculo, al igual que el TAB, es un tejido de origen mesenquimal, en este caso formado por células contráctiles llamadas miocitos. Existen varios tipos de tejido

muscular en el organismo, clasificados según su organización estructural en músculo liso, músculo cardíaco y músculo esquelético. El músculo esquelético está implicado en el movimiento, el mantenimiento de la postura, la estabilidad, la producción de calor, la tolerancia al frío y la comunicación inter-órganos.

1.3.1. Metabolismo en el músculo esquelético

El músculo esquelético representa aproximadamente el 40% del peso corporal y su actividad aproximadamente el 50% del gasto energético diario, y juega un papel clave la homeostasia de la glucosa y el metabolismo glucídico y lipídico. Es responsable del 80% de la captación de glucosa estimulada por insulina y un importante órgano catabolizador de ácidos grasos (AGs). Alteraciones del metabolismo en el músculo esquelético están implicadas en la resistencia a la insulina, la DT2 y el síndrome metabólico (Parish and Petersen, 2005; Perseghin, 2005; Sell et al., 2006).

Clásicamente, se ha considerado al músculo esquelético como un tejido oxidativo en el que los AGs suponen su mayor fuente de energía. Los AGs son transportados al interior de la célula muscular a través de la ácido graso translocasa (FAT/CD36) y otras proteínas transportadoras de AGs (FATPs, principalmente FATP1) (Sebastian et al., 2009). En la célula muscular, los AGs pueden ser oxidados en la mitocondria mediante la β-oxidación o almacenados como triacilgliceroles intramiocelulares. Las mismas proteínas que facilitan el transporte de AGs a través de la membrana plasmática, como la FAT/CD36 y la FATP1, colaboran con la carnitina palmitoil transferasa 1 (CPT1) en el transporte de ácidos grasos activados a la matriz mitocondrial

La tasa de captación de AGs en el músculo está determinada por la concentración de lípidos plasmáticos, especialmente en condiciones de hipersinsulinemia (Brechtel et al., 2001). En pacientes obesos y con DT2, la concentración de AGs libres circulantes está incrementada, situándose típicamente en rangos de 600-800 μmol/L comparado con 300-400 μmol/L en individuos sanos normopeso. Esto, unido a la reducida capacidad de oxidación lipídica que caracteriza al músculo obeso, conduce a una acumulación de lípidos intramiocelulares, especialmente metabolitos intermedios como diacilgicerol y ceramida, que interfieren con la acción de la insulina (Corcoran et al., 2007; Sabin et al., 2007) (véase también el apartado 1.1.2 y la Figura 1). Ello se traduce en un bloqueo de la translocación de GLUT4 a la membrana, una menor captación de glucosa circulante y una menor síntesis de glucógeno en las células musculares que puede alterar la homeostasis de la glucosa a nivel sistémico (Turcotte and Fisher, 2008).

La acumulación de triacilgliceroles intramiocelulares no es *per se* un indicador de resistencia a la insulina, como lo demuestra el hecho de que los atletas bien entrenados son muy sensibles a la insulina y poseen un elevado contenido de triacilgliceroles intramiocelulares, rasgo que constituye una de las adaptaciones al entrenamiento. En respuesta a una mayor demanda de energía, el músculo esquelético incrementa la captación, transporte, utilización y oxidación de AGs, y en este sentido otro efecto bien conocido del entrenamiento es el incremento de la biogénesis de mitocondrias y de la tasa de oxidación mitocondrial de AGs (Corcoran et al., 2007; Menshikova et al., 2006). El problema en la obesidad y la DT2 es que, al estar reducida la capacidad de oxidación de AGs (Morino et al., 2005; Parish and Petersen, 2005), la tasa de recambio de los lípidos intramiocelulares es baja, lo que favorece la peroxidación lipídica y la acumulación de metabolitos intermedios inhibidores de la señalización por insulina, del tipo del diacilglicerol y la ceramida (Corcoran et al., 2007). De hecho, los efectos beneficiosos del ejercicio físico en relación a la sensibilidad a la insulina se relacionan con una mayor tasa de recambio de los lípidos musculares, que a su vez implica la eliminación de estos metabolitos intermedios (Moro et al., 2008).

1.3.2. El músculo esquelético cómo órgano secretor

El músculo esquelético ha sido recientemente descrito como un órgano endocrino que, especialmente en respuesta al ejercicio, produce y secreta, citoquinas y otros péptidos que se han denominado colectivamente mioquinas (Pedersen and Febbraio, 2008). Las mioquinas ejercen sus efectos a nivel local y/o sistémico: sus efectos auto y paracrinos modulan el metabolismo del músculo, y sus efectos endocrinos, el metabolismo de otros órganos como el tejido adiposo. El impacto de las mioquinas sobre el metabolismo es actualmente un importante tema de investigación. Al igual que en el caso de las adipoquinas, a las mioquinas se les atribuye un papel importante en la regulación de la sensibilidad a la insulina y en la protección o mejora inducida por el ejercicio físico sobre muchas enfermedades crónicas. (Pedersen, 2010; Pedersen, 2011; Pedersen et al., 2007)

A continuación se introducen las mioquinas descritas hasta el momento, con especial énfasis en las que han sido objeto de estudio en esta tesis:

Interleuquina-6 (IL-6)

Fue la primera mioquina descrita y ha sido la más estudiada. Una gran cantidad de estudios demuestran que los niveles plasmáticos de IL-6 aumentan exponencialmente en respuesta al ejercicio y que este aumento, contrariamente a lo que se pensaba hasta hace pocos años, no está relacionado con el daño muscular. El incremento, que puede

alcanzar hasta 100 veces los niveles previos a la actividad física, refleja la duración e intensidad del ejercicio, la masa muscular involucrada y la capacidad de resistencia. Tras el ejercicio, la concentración sérica de IL-6 desciende hasta niveles iniciales. Se ha demostrado que son las fibras musculares activas las que producen y secretan IL-6 en respuesta al ejercicio, mientras que en reposo la principal fuente de IL-6 circulante es el tejido adiposo (Pedersen, 2007; Pedersen and Febbraio, 2008).

En el músculo esquelético, la IL-6 estimula la captación de glucosa basal y estimulada por insulina y la oxidación de lípidos intramiocelulares, probablemente mediante la activación de la AMPK (Carey et al., 2006). A su vez, la AMPK regularía positivamente la secreción de IL-6 por el músculo (Pedersen and Febbraio, 2008). La producción y secreción de IL-6 en el músculo activo tiene lugar en condiciones en que la disponibilidad de carbohidratos está reducida, y la IL-6 tiene efectos extra-musculares estimulando la lipólisis en adipocitos. Esto sugiere que la IL-6 actuaría como un sensor energético que activaría la lipólisis en tejido adiposo con el fin de proveer sustratos energéticos al músculo (Pedersen and Febbraio, 2008).

La IL-6 se detecta y expresa, junto con sus receptores (muy similares a los de la leptina), en conjuntos de neuronas hipotalámicas implicadas en el control de la homeostasis energética, y tiene efectos anti-obesogénicos que dependerían, al menos en parte, de acciones al nivel del sistema nervioso central. Posiblemente la prueba más convincente de los efectos anti-obesogénicos de la IL-6 es la observación de que los ratones deficientes (*knockout*) en IL-6 desarrollan obesidad e intolerancia a la glucosa (Wallenius et al., 2002), y que ratones transgénicos que sobreexpresan IL-6 humana predominantemente en cerebro y pulmón son resistentes a la obesidad dietética, y más sensibles a la insulina y a las acciones centrales de la leptina (Sadagurski et al., 2010; Wallenius et al., 2002).

Aunque la IL-6 se considera clásicamente una citoquina pro-inflamatoria, también existen evidencias de una acción anti-inflamatoria, basados en la capacidad de la IL-6 de estimular la producción de citoquinas anti-inflamatorias como la IL-1ra y la IL-10 (Steensberg et al., 2003).

El concepto de la IL-6 como una mioquina de efectos anti-obesogénicos y que aumenta la sensibilidad a la insulina en el músculo choca con la visión tradicional de la IL-6 como una citoquina pro-inflamatoria, aumentada en la obesidad y promotora de resistencia a la insulina. Ciertamente, estudios en hepatocitos aislados e *in vivo* en ratones han mostrado que la IL-6 reduce la sensibilidad hepática al efecto supresor de la insulina sobre la gluconeogénesis, al incrementar la expresión de SOCS3 en hepatocitos (Bonet

et al., 2009). Además, la elevación crónica de IL-6 se ha asociado con reducción de peso, hiperinsulinemia, deficiente captación de glucosa estimulada por insulina en músculo e inflamación hepática (Franckhauser et al., 2008).

La controversia suscitada por las diferentes observaciones podría ser explicada, entre otras cosas, por diferencias entre un efecto agudo (producción asociada a la contracción muscular, que se traduce en un incremento de hasta 100 veces los niveles circulantes previos al ejercicio) *vs* crónico (producción en el tejido adiposo obeso/inflamado, que se traduce en unos niveles circulantes de 3 a 5 veces superiores a los observados en sujetos normopeso). Se ha sugerido que, en este último contexto, podría existir un fenómeno de “resistencia a la IL-6” similar a lo que ocurre con la insulina y leptina cuando sus niveles permanecen elevados de forma crónica (Pedersen and Febbraio, 2008).

Interleuquina-15

La IL-15 se expresa a alto nivel en el músculo esquelético, donde su expresión es inducida por el ejercicio intenso (Nielsen et al., 2007). La IL-15 tiene importantes efectos en el músculo: estimula la diferenciación de las células musculares y la acumulación de miosina en miocitos diferenciados, antagoniza la proteólisis muscular, y promueve la oxidación de ácidos grasos y la captación y utilización de glucosa. (Busquets et al., 2005; Furmanczyk and Quinn, 2003; Quinn et al., 1997). Además de estos efectos anti-diabetogénicos y anabólicos en músculo, la IL-15 parece tener un efecto anti-adiposidad, reduciendo el tejido adiposo visceral. (Nielsen et al., 2008). Los adipocitos no expresan IL-15, pero sí su receptor, lo que reforzaría la hipótesis de que una acción endocrina de la IL-15, desde el músculo al tejido adiposo (Quinn et al., 2005).

Otras mioquinas

Otras mioquinas recientemente descubiertas de efectos locales y cuyos niveles incrementan con el ejercicio son la IL-8, que estimula la angiogénesis; *leukaemia inhibitory factor* (LIF), que estimula la proliferación de células musculares satélites (Alvarez et al., 2002; Pedersen et al., 2007; Quinn et al., 2009; Quinn et al., 2005); y *brain derived neurotrofic factor (BDNF)*, cuyo efecto auto-paracrino estimula la oxidación de ácidos grasos en las células musculares, vía activación de la AMPK (Broholm and Pedersen, 2010).

1.4. INTERACCIONES NUTRIENTES–GENES EN EL CONTROL DEL BALANCE ENERGÉTICO Y LAS COMPLICACIONES METABÓLICAS DE LA OBESIDAD

El desequilibrio energético crónico que conduce al estado obeso es fruto de alteraciones o deficiencias genéticas o adquiridas en procesos bioquímicos que afectan al control de la ingesta, la eficiencia energética, la adipogénesis y/o la distribución de los nutrientes entre tejidos y vías metabólicas (Palou et al., 2004). Del mismo modo que la susceptibilidad genética individual (e.g., polimorfismos en genes específicos) puede influir sobre estos procesos, se ha demostrado que los nutrientes pueden afectar todos estos procesos a distintos niveles:

- Como sustratos de las vías metabólicas. La disponibilidad de ciertos nutrientes regula la velocidad de las reacciones enzimáticas de las cuales son sustrato, y por tanto el flujo de las vías metabólicas en las que participan.
- Como reguladores de la secreción hormonal y de diferentes eslabones en las vías de señalización hormonal que impactan sobre el metabolismo.
- Como reguladores de la actividad de enzimas y otras proteínas reguladoras, bien por unión directa o promoviendo modificaciones post-tradicionales (fosforilación /desfosforilación, glucosilación, proteólisis parcial, etc.).
- Como reguladores de la concentración de enzimas y otras proteínas reguladoras, actuando como modificadores específicos de la expresión génica.
- Como factores que condicionan la impronta metabólica causada por efectos epigenéticos a nivel de ADN, histonas y otras biomoléculas.

A continuación se introducen los nutrientes o condiciones nutricionales que han sido objeto de estudio en esta tesis.

1.4.1. Papel de la grasa dietética y de los ácidos grasos *trans*

La calidad de la grasa de la dieta impacta sobre procesos implicados en el control de la adiposidad corporal (e.g. adipogénesis, lipogénesis, oxidación de ácidos grasos, expresión de proteínas desacoplantes) y en parámetros relacionados como la lipidemia y la sensibilidad a la insulina. En general, los efectos de la composición grasa de la dieta se relacionan con: a) cambios en la composición lipídica de las membranas celulares, que afectan a su fluidez y a funciones de señalización, incluyendo la sensibilidad a señales extracelulares que interaccionan con receptores de membrana; b) cambios en la cantidad y el tipo de segundos mensajeros lipídicos que se puedan generar (e.g.,

eicosanoides, diacilglicerol, ceramidas); y c) los efectos de ácidos grasos particulares y derivados estimulando determinadas vías y factores de transcripción (e.g., PPARs).

Numerosos estudios correlacionan la ingesta de grasa saturada, en especial ácidos grasos saturados de cadena larga (16-20 C), con la síntesis de diacilglicerol y ceramidas y el desarrollo de resistencia a la insulina en el músculo (Corcoran et al., 2007). Además, los ácidos grasos saturados favorecen el aumento de la colesterolemia, principal factor de riesgo de ECV, y tienen efectos pro-inflamatorios en el endotelio y otros tejidos vía activación del TLR4 (Boden, 2008; Pilz and Marz, 2008). Por el contrario, abundante evidencia científica avala los efectos beneficiosos de la grasa insaturada, especialmente de los ácidos grasos poliinsaturados n-3 (PUFA n-3) de cadena larga, como el ácido eicosapentaenoico (20:5 cis-$\Delta^{5,8,11,14,17}$) y el docosahexaenoico (22:6 cis-$\Delta^{4,7,10,13,16,19}$). En comparación con la grasa saturada y los PUFA n-6, los PUFA n-3 tienen efectos anti-inflamatorios, y pueden favorecer la reducción de la colesterolemia, la trigliceredemia y la grasa visceral, y preservar la sensibilidad a la insulina (Corcoran et al., 2007; Oliver et al., 2010).

La ingesta de ácidos grasos *trans* (AGTs) también se ha relacionado con muchos de los efectos deletéreos de la grasa saturada. Los AGTs son ácidos grasos insaturados con al menos un doble enlace en configuración *trans*, en lugar de la configuración *cis* habitual en la naturaleza. Son por lo tanto isómeros geométricos cuya estructura molecular resulta más rígida y les confiere propiedades físicas distintivas, fundamentalmente un punto de fusión más elevado y una mayor estabilidad termodinámica.

Los AGTs aparecen de forma natural en productos lácteos y otras grasas animales, pero la gran mayoría de AGTs que se consumen se producen industrialmente (AGTs de producción industrial, AGTs-PI) durante la hidrogenación catalítica de aceites vegetales (*hardening*). Esta técnica, desarrollada ya en los años 30, consiste en la introducción de gas hidrógeno en aceite vegetal a temperaturas de más de 400ºC a elevadas presiones y en presencia de diferentes catalizadores, con el objetivo de conseguir ácidos grasos saturados. Sin embargo, dependiendo de las condiciones, la hidrogenación no llega a ser completa y permanecen algunos dobles enlaces ahora en configuración *trans*. Esta grasa parcialmente hidrogenada, sólida a temperatura ambiente, se utiliza en una amplia variedad de productos: margarinas, bollería industrial, frituras, aperitivos y *fast food* De los productos del mercado español con más porcentaje de AGTs en relación con el total de ácidos grasos destacan las palomitas de maíz para preparar en microondas (36%), las patatas fritas (20,9%), sopas deshidratadas (15,4%), bollería industrial (hasta un 10%), hamburguesas (4%) y pizzas (3-15%; aquí también pueden provenir del queso) (Fernández-San Juan, 2009). Las margarinas comercializadas en

España han reducido su porcentaje de trans en los últimos años mediante el proceso de interesterificación documentándose actualmente una media del 3% de trans respecto al total de ácidos grasos (Fernández-San Juan, 2009). El ácido elaídico (trans-9-18:1), el isómero trans del ácido oleico, es el AGT de producción industrial predominante (Mensink, 2005; Stender et al., 2008).

Mientras la evidencia científica avala los efectos favorables de las dietas enriquecidas en ácido oleico, y del ácido oleico en sí mismo, sobre el riesgo de padecer enfermedad cardiovascular, el mantenimiento del peso corporal y la sensibilidad a la insulina (Bondia-Pons et al., 2007; Fito et al., 2008; Fito et al., 2007; Lopez-Miranda et al., 2010; Riserus et al., 2009; Schroeder et al., 2008), las dietas ricas en AGTs-PI se asocian generalmente con efectos perjudiciales sobre la salud (Stender et al., 2008). Los efectos adversos de los AGTs-PI sobre múltiples factores de riesgo cardiovascular están bien establecidos, entre ellos la promoción de un perfil lipídico/lipoproteínico en suero pro-aterogénico, la disfunción endotelial y la producción de citoquinas pro-inflamatorias (Dorfman et al., 2009; Lemaitre et al., 2006; Micha and Mozaffarian, 2008; Micha and Mozaffarian, 2009; Mozaffarian et al., 2007; Salmeron et al., 2001). Los efectos de los AGTs-PI sobre la resistencia a la insulina y el riesgo de DT2 son más controvertidos, ya que se han descrito efectos perjudiciales en este sentido en algunos, pero no todos, los estudios observacionales y experimentos cortos de intervención dietética realizados en humanos (Lovejoy et al., 2002; Odegaard and Pereira, 2006; Riserus et al., 2009; Tardy et al., 2008). Análogamente, en ratas alimentadas con dietas ricas en AGTs-PI, se ha descrito el desarrollo de resistencia a la insulina en algunos estudios (Dorfman et al., 2009; Ibrahim et al., 2005; Natarajan and Ibrahim, 2005), pero no en otros (Tardy et al., 2008). En un estudio a largo plazo (5 años) en monos en el que se compararon los efectos de una dieta rica en AGTs-PI y otra rica en ácido oleico se encontró que los animales sometidos a la dieta rica en AGTs-PI desarrollaron resistencia a la insulina, alteraciones en el metabolismo de la glucosa y más obesidad abdominal (Kavanagh et al., 2007).

Los mecanismos que subyacen a los efectos adversos de los AGTs-PI sobre la salud metabólica son múltiples y se conocen sólo parcialmente (Riserus et al., 2009), y podrían incluir efectos sobre la producción de adipoquinas y mioquinas relacionadas con la inflamación y la resistencia a la insulina, siendo este último un aspecto poco estudiado que se ha abordado específicamente en la presente tesis.

1.4.2. Papel de la vitamina A

La vitamina A es un nutriente esencial liposoluble que se encuentra en el organismo en varias formas que difieren en su estado de oxidación: retinal, retinol, y ácido retinoico (AR). Estos tres vitámeros, junto con sus metabolitos derivados y ciertos análogos sintéticos, reciben colectivamente el nombre de retinoides. Los retinoides juegan un papel crítico en muchos procesos esenciales para la vida como la visión, reproducción, función inmunitaria, desarrollo embrionario y modulación de la proliferación, diferenciación y muerte celular de un gran número de tipos celulares, incluidos los adipocitos (Blomhoff and Blomhoff, 2006).

La vitamina A está presente en la dieta de los animales en dos formas: como vitamina A preformada, principalmente ésteres de retinol con ácido graso (retinil ésteres) contenidos en grasa e hígado de otros animales, y como carotenoides precursores contenidos en alimentos de origen vegetal. De los más de 700 carotenoides identificados en la naturaleza, unos 50 pueden dar lugar a la formación de vitamina A. El más importante desde el punto de vista nutricional es el β-caroteno (40 C), cuya escisión por la enzima β,β-caroteno-15,15´-oxigenasa (Bcmo1) rinde dos moléculas de retinal (20 C) (von Lintig and Vogt, 2004). El metabolismo del β-caroteno difiere en función del organismo: en roedores, prácticamente todo el β-caroteno absorbido es transformado en vitamina A en los enterocitos, mientras que en humanos tan sólo un 60-70% lo es, y el resto pasa intacto empaquetado en los quilomicrones. Recientemente, se ha descrito un factor de transcripción, ISX (*Intestin specific homeobox*), cuya expresión en el epitelio intestinal resulta inducida por AR y cuya actividad limita la captación del β-caroteno de la dieta y su conversión en vitamina A mediante efectos sobre la expresión génica del receptor de β-caroteno y la Bcmo1 (Seino et al., 2008).

Los retinil ésteres y los carotenoides de la dieta son transformados en el lumen del intestino y en la mucosa intestinal principalmente en retinol, el cual se esterifica a ácido graso (e.g., palmítico) formando nuevamente retinil ésteres que pasan a la circulación asociados a los quilomicrones (Harrison and Hussain, 2001). Una parte minoritaria del retinol dietético se convierte dentro de los enterocitos en AR, que entra por el sistema portal unido a albúmina sérica (Harrison and Hussain, 2001). El hígado capta la mayor parte del retinol dietético, que le llega a través de los quilomicrones remanentes, y constituye el principal órgano de reserva de vitamina A. No obstante, otros tejidos incluyendo el tejido adiposo pueden derivar retinol y carotenoides a partir de los quilomicrones (Blaner et al., 1994; Harrison and Hussain, 2001; Paik et al., 2004). El retinol almacenado en el hígado y tejido adiposo puede volver a la circulación unido a su proteína de transporte específica, la RBP (ver apartado 1.2.4). El retinol unido a RBP es

captado por diferentes tejidos que expresan un receptor de membrana específico, STRA6 (Kawaguchi et al., 2007). Una vez en el interior celular, el retinol puede ser almacenado como retinil éster o metabolizado a retinal y, por oxidación de éste, a la forma más activa de AR. Este último tiene una vida media breve, ya que es metabolizado por diversas enzimas de la familia de la citocromo P450 (CYP26A1, CYP26B1, CYP26C1) que rinden una gran variedad de productos más hidrosolubles, con aún cierta actividad biológica (Idres et al., 2001), que serán excretados por la orina y/o la bilis. Todo el metabolismo intracelular de los retinoides ocurre con éstos unidos a proteínas intracelulares de unión específicas.

A nivel celular, los distintos tipos de retinoides pueden actuar provocando cambios en la función de las biomembranas y modificaciones post-traduccionales de ciertas proteínas, como retinilización (unión covalente de retinilo) o fosforilación (hay proteína quinasas cuya actividad resulta modulada por retinoides). No obstante, la mayoría de los efectos de los retinoides se dan a nivel de la regulación transcripcional de la expresión génica principalmente (aunque no sólo) a través de su interacción con los receptores de retinoides, que son factores de transcripción dependientes de ligando de la superfamilia de receptores nucleares (Bonet et al., 2011; Ziouzenkova and Plutzky, 2008). El AR se considera la forma más activa de la vitamina A en la regulación de la expresión génica (Bastien and Rochette-Egly, 2004). Existen dos familias de receptores de retinoides: los RAR (*retinoic acid receptors*), que responden a AR todo *trans* y AR 9-*cis*, y los RXR (*retinoid X receptors*), que responden específicamente al isómero 9-*cis* AR y también a otros compuestos como los ácidos grasos insaturados (Wolf, 2006). Se han descrito tres subtipos diferentes para cada familia, α, β y γ, codificados por genes individuales y cuyo patrón de expresión es específico de cada tejido y etapa del desarrollo (Mangelsdorf and Evans, 1995).

Los RAR se unen en forma de heterodímeros con el RXR a elementos de respuesta al AR (RARE, *retinoic acid response element*) situados en la región reguladora (promotor génico) de genes sensibles a retinoides y, en general, incrementan la tasa de inicio de la transcripción de estos genes en función de la unión del ligando (AR todo *trans* o 9 *cis*) a la mitad RAR, aunque también hay genes reprimidos activamente por RAR de manera dependiente de ligando (Aranda and Pascual, 2001; Bastien and Rochette-Egly, 2004). Los RXR también pueden formar homodímeros, que se unen a otro tipo de RAREs, y heterodímeros con otros miembros de la superfamilia de receptores nucleares como el receptor de hormona tiroidea (TR), el receptor de vitamina D (VDR) o los PPARs, entre otros. Algunos heterodímeros de RXR con receptores nucleares, incluyendo el PPAR:RXR, se pueden activar por ligandos de los dos componentes, resultando la

activación máxima por unión simultánea de ambos ligandos (Aranda and Pascual, 2001). Numerosos factores adicionales pueden modular los efectos sobre la transcripción génica mediados por receptores de retinoides, entre ellos la interacción con proteínas reguladoras (co-activadores y co-represores) cuya actividad, así como la de los propios receptores de retinoides, puede ser controlada por modificaciones post-traduccionales (fosforilación, ubiquitinización, etc.) (Bour et al., 2007).

El espectro de mecanismos a través de los cuales los retinoides pueden impactar la expresión génica es todavía más amplio. Los receptores de retinoides activados por ligando pueden interferir con la actividad de otros factores de transcripción como AP1, NF-κB o C/EBPs (Blomhoff and Blomhoff, 2006). Además, los retinoides pueden afectar la expresión génica por mecanismos independientes de su interacción con RAR o RXR. Se ha descrito, por ejemplo, que el AR todo-*trans*, además de a los RAR, puede activar al PPARβ/δ (una isoforma de PPAR abundante en el músculo, que controla genes implicados en la oxidación de ácidos grasos) (Shaw et al., 2003). Asimismo, se han descrito efectos de la RBP con retinol unido (holo-RBP) modulando la expresión génica desde el exterior celular, vía activación del receptor de membrana STRA6 (Berry et al., 2011).

La actividad del AR como modulador de la adipogénesis se conoce desde hace tiempo. En estudios *in vitro* se han demostrado efectos que son dependientes de la dosis y el estado de la diferenciación. A dosis relativamente altas (0.1-10μM) y en una etapa temprana de la diferenciación, el AR todo-*trans* es un potente inhibidor de la adipogénesis (Kuri-Harcuch, 1982; Murray and Russell, 1980), porque los RAR activados interfieren con la actividad del C/EBPβ, que es un factor de transcripción adipogénico temprano (Schwarz et al., 1997). Otros metabolitos en la vía del β-caroteno/vitamina A, en particular el retinal y el β-apo-14'-carotenal (un derivado de la rotura asimétrica del β-caroteno), también reprimen la adipogénesis *in vitro* por un mecanismo distinto, que implicaría su unión física a PPARγ y RXR, y con ello la supresión de la actividad del heterodímeros PPARγ:RXR (Ziouzenkova and Plutzky, 2008). Por el contrario, a dosis relativamente bajas (1pM-10nM) y en un estado terminal de la diferenciación, el AR todo-*trans* actúa como una hormona pro-adipogénica (Safonova et al., 1994a), e incluso se han descrito efectos sinérgicos sobre la adipogénesis del AR todo-*trans* junto a los ácidos grasos (Safonova et al., 1994b). Además, el compromiso de células madre embrionarias hacia la línea adipocitaria es dependiente de la presencia de AR todo-trans en el medio (Bost et al., 2002; Dani et al., 1997) y se ha descrito que el AR favorece la proliferación de preadipocitos *in vitro.*(Gupta et al., 2007).

Más allá de los efectos sobre la adipogénesis, en los últimos años se ha evidenciado un papel regulador de la vitamina A y sus derivados en la biología del adipocito y la adiposidad corporal (Bonet et al., 2003; Bonet et al., 2011; Villarroya et al., 2004). La manipulación genética (deleción o sobreexpresión) de varias enzimas y proteínas de transporte implicadas en el metabolismo de los retinoides se ha demostrado que resulta en alteraciones de la adiposidad en roedores (Amengual et al., 2011; Hessel et al., 2007; Schupp et al., 2009; Zhang et al., 2007; Ziouzenkova et al., 2007; Zizola et al., 2010; Zizola et al., 2008). Asimismo, se ha demostrado que, tanto en ratones normopeso como obesos, el tratamiento con AR reduce el peso corporal y la masa de los tejidos adiposos independientemente de cambios en la ingesta (Berry and Noy, 2009; Bonet et al., 2000; Felipe et al., 2005; Mercader et al., 2006; Puigserver et al., 1996), y mejora la tolerancia a la glucosa y la sensibilidad a la insulina (Berry and Noy, 2009; Felipe et al., 2004; Mercader et al., 2008). El tratamiento con retinal, otro vitámero de la vitamina A, también reduce la masa grasa e incrementa la sensibilidad a la insulina en ratones genéticamente obesos (Ziouzenkova et al., 2007).

La pérdida de masa grasa inducida por el AR se correlaciona con una activación del tejido adiposo marrón (Bonet et al., 2000; Puigserver et al., 1996), una reducción de la capacidad adipogénica/lipogénica en tejido adiposo blanco (Ribot et al., 2001) e hígado (Amengual et al., 2010), y una capacidad incrementada para el metabolismo energético y la termogénesis en tejido adiposo blanco (Berry and Noy, 2009; Mercader et al., 2007; Mercader et al., 2006), músculo esquelético (Berry and Noy, 2009; Felipe et al., 2003) e hígado (Amengual et al., 2010). Al incremento de la sensibilidad a la insulina tras el tratamiento agudo con AR en ratones podrían contribuir asimismo efectos sobre la expresión de determinadas adipoquinas (Felipe et al., 2004), siendo éste un aspecto que se ha abordado específicamente como parte de esta tesis (Mercader et al., 2008).

En concordancia con lo anterior, hay evidencia que el estatus en vitamina A puede tener un impacto sobre la adiposidad corporal. Estudios en animales adultos indican que un estatus pobre en vitamina A favorece la formación de tejido adiposo (Kawada et al., 1996; Ribot et al., 2001). Además, existen resultados en humanos que relacionan los niveles de carotenoides/retinoides circulantes con la adiposidad corporal, siendo éstos inferiores en niños y adolescentes con sobrepeso que en normopesos (de Souza Valente da Silva et al., 2007). Unos niveles de carotenoides/retinoides inadecuadamente bajos se asocian también con ciertos desórdenes como la resistencia a la insulina y el desarrollo de hígado graso en humanos obesos (Chaves et al., 2007; Pereira et al., 2009; Villaca Chaves et al., 2008). Recíprocamente, se ha reportado que, en ratas y ratones adultos, la suplementación con vitamina A (como retinil palmitato) reduce el

contenido en masa grasa e impide parcialmente el desarrollo de obesidad (Jeyakumar et al., 2005; Kumar et al., 1999) e incrementa el potencial termogénico del tejido adiposo marrón y el músculo esquelético (Felipe et al., 2005; Jeyakumar et al., 2005; Kumar et al., 1999). No obstante, en un estudio realizado en ratas jóvenes (50-70 g, 21 días de edad) la vitamina A dietética sinergizó los efectos de una dieta de cafetería en la promoción de la expansión del tejido adiposo (Redonnet et al., 2008).

En resumen, el posible papel de la vitamina A en el control de la adiposidad corporal está suscitando un interés científico creciente. Principalmente en forma de AR, la vitamina A afecta diferentes procesos implicados en el control de la adiposidad corporal incluyendo: la proliferación de preadipocitos; la diferenciación de preadipocitos en adipocitos maduros o adipogénesis; la expresión de proteínas termogénicas desacoplantes; el metabolismo lipídico en tejidos adiposos blanco y marrón, hígado y músculo esquelético; y la producción de adipoquinas que participan en el control del balance energético y la sensibilidad a la insulina. No obstante, la mayor parte de los estudios que relacionan la vitamina A con el control de la adiposidad corporal se han realizado en modelos de células en cultivo (estudios *in vitro)* y/o en animales adultos, y no han cubierto las etapas tempranas de la vida postnatal.

1.4.3. Papel de la nutrición perinatal

Estudios epidemiológicos y en modelos animales revelan que los orígenes de la obesidad y desórdenes metabólicos asociados que aparecen en la edad adulta se encuentran no sólo en la dotación genética individual y en los clásicos factores de riesgo como el sedentarismo y la incorrecta alimentación, sino que también se adquieren, o están ligados, a factores del entorno que actúan mucho antes, durante el periodo perinatal (Taylor and Poston, 2007). Alteraciones hormonales, metabólicas o nutricionales en etapas críticas del desarrollo pueden determinar la propensión a padecer obesidad y complicaciones metabólicas asociadas en la edad adulta (Breier et al., 2001; Gluckman and Hanson, 2004a; Godfrey and Barker, 2001). Frecuentemente la obesidad es resistente a la terapia (Gunnell et al., 1998) por lo que su prevención es de crucial importancia, y cada vez hay mayor conciencia de que dicha prevención, para ser eficaz, pasa por la implementación de estrategias integradas ya desde la infancia, y aún antes.

En mamíferos la nutrición de la madre es un factor clave que determina la alimentación de su descendencia, primero vía placentaria y después mediante la leche materna. Numerosos estudios relacionan una alimentación inadecuada durante etapas críticas del desarrollo con la aparición de la obesidad y enfermedades asociadas, en la edad adulta

(ECV, DT2, etc.). Parece ser que el desarrollo del sistema cardiovascular, del sistema de control del balance energético y de las vías metabólicas relacionadas con la homeostasia de la glucosa y de los lípidos es particularmente propenso a las perturbaciones a nivel nutricional (Taylor and Poston, 2007). Cambios en la alimentación de la madre durante la gestación y la lactancia producen cambios en el ambiente nutricional de la descendencia que podrían traducirse en alteraciones en la programación de los mecanismos de regulación del balance energético de los individuos. En este contexto, se ha planteado que la nutrición en etapas tempranas del desarrollo puede condicionar adaptaciones perdurables del organismo con el fin de optimizar su supervivencia que, en otros contextos futuros, pueden propiciar la obesidad y el síndrome metabólico (Cripps et al., 2005; Fowden et al., 2005; Gluckman and Hanson, 2004b; Martorell et al., 2001).

Los mecanismos de la programación metabólica incluirían efectos de nutrientes/dietas sobre el control epigenético de la expresión génica (e.g. cambios en el estado de metilación del DNA o de modificaciones covalentes de histonas) (Godfrey et al., 2011; Palou et al., 2011) y sobre el desarrollo estructural de órganos importantes en el control del balance energético y la adiposidad corporal como los circuitos neuronales hipotalámicos involucrados en este control o el propio tejido adiposo (Delahaye et al., 2008; Garcia et al., 2010; Garcia et al., 2011). En particular, la programación del número de adipocitos presentes en los depósitos grasos puede ser muy importante, ya que hay evidencia de que un número incrementado de adipocitos puede favorecer el desarrollo de obesidad, y de que dicho número queda fijado en etapas tempranas de la vida, permaneciendo constante durante la edad adulta, aun cuando aproximadamente un 10% de los adipocitos se renueven anualmente en la especie humana (Arner and Spalding, 2010; Spalding et al., 2008).

La sobrealimentación, la infralimentación y la composición en macronutrientes de la dieta son factores nutricionales relevantes en la programación metabólica conocidos desde hace tiempo. También han sido implicados algunos nutrientes específicos. Se ha propuesto, por ejemplo, la implicación de la leptina (Palou and Pico, 2009), que se sabe está presente en la leche materna y es absorbida intacta por los neonatos, y de ciertas vitaminas y otros factores dietéticos que intervienen en reacciones bioquímicas de metilación relacionadas con cambios epigenéticos, como el folato y la vitamina B12 (Rosenberg, 2008). Asimismo, diversos estudios han mostrado una correlación directa entre los niveles de ácidos grasos *trans* de la leche materna y de la dieta de la madre (Mojska et al., 2003; Ross et al., 1985), así como el impacto negativo que la grasa *trans* puede tener sobre el metabolismo a largo plazo (Albuquerque et al., 2006; Pisani et al.,

2008). No obstante, la identificación de factores nutricionales concretos implicados en los fenómenos de programación metabólica de la obesidad y el conocimiento de sus mecanismos de acción es un campo todavía en sus inicios, importante por cuanto puede permitir recomendaciones concretas y menos generalistas.

En particular, se sabe relativamente poco del impacto de la ingesta de vitamina A en etapas tempranas de la vida sobre el desarrollo del tejido adiposo y la susceptibilidad futura a la obesidad. Y ello a pesar de que es sabido que la vitamina A juega un papel crucial en el desarrollo; que la actividad de los retinoides modula diferentes procesos relacionados con el control de la adiposidad corporal, incluyendo la adipogénesis y la proliferación de preadipocitos (véase el apartado 1.4.2.); y que el contenido en vitamina A de la leche materna depende del contenido en vitamina A y β-caroteno de la dieta, tanto en humanos (Rice et al., 1999; Villard and Bates, 1987) como en roedores (Akohoue et al., 2006; Davila et al., 1985; Green et al., 2001).

2. OBJETIVOS

Los objetivos de esta tesis se enmarcan dentro del principal campo de investigación de nuestro grupo: la identificación y caracterización de interacciones nutrientes-genes de potencial relevancia en el contexto de la obesidad y sus complicaciones clínicas asociadas. Los objetivos de la tesis reflejan la evolución de este campo en los últimos años, así como la implicación creciente del grupo en proyectos de investigación aplicada en colaboración con la industria. En particular, se ha investigado en las siguientes líneas:

a) Modulación de la función secretora del tejido adiposo por estatus nutricional y nutrientes específicos

El inicio de esta tesis coincidió con el redescubrimiento de RBP y Nampt/visfatina, como posibles adipoquinas potencialmente relacionadas con la sensibilidad/acción de la insulina, cuyos cambios en el estado obeso eran poco conocidos. La controversia en torno a ello, junto con la escasez de estudios en modelos animales, nos motivó a estudiar los cambios de expresión de RBP y Nampt/visfatina en dos modelos de obesidad en ratas, uno genético (Zucker fa/fa) y uno inducido por la dieta (ratas Wistar alimentadas con dieta de cafetería). Los resultados de este estudio condujeron a la publicación del **manuscrito I.**

Trabajos previos de nuestro grupo indicaban que, en animales adultos, el tratamiento con ácido retinoico mejora la sensibilidad a la insulina y afecta la producción de adipoquinas relacionadas con la sensibilidad a la insulina, como la resistina. Esto nos condujo a plantearnos la hipótesis que el ácido retinoico podría afectar la producción adipocitaria de RBP, otra adipoquina que, como la resistina, puede contribuir a la resistencia a la insulina. Además, la RBP es clave en el transporte de la vitamina A (retinol), y su regulación por vitamina A había sido muy estudiada en el hígado, principal lugar de almacenamiento de retinol y síntesis de RBP, pero no en el tejido adiposo. Así pues, un objetivo particular fue estudiar la modulación de la expresión de RBP por ácido retinoico en modelos celulares de adipocitos, e *in vivo* comparativamente en hígado y tejido adiposo. Los resultados de este estudio condujeron a la publicación del **manuscrito II**.

La ingesta de ácidos grasos *trans*, especialmente de origen industrial, ha sido relacionada con complicaciones clínicas frecuentes en la obesidad como la ECV y la resistencia a la insulina. Los mecanismos subyacentes a la relación de los ácidos grasos *trans* con la salud metabólica son variados y podrían incluir efectos sobre la producción de adipoquinas y también mioquinas, a la luz de la evidencia científica que ha ido consolidando al músculo como un importante órgano de secreción de proteínas reguladoras del metabolismo. Al inicio de esta tesis, no había apenas estudios

comparando los efectos directos de especies concretas de ácidos grasos monoinsaturados *cis* y *trans* sobre la producción de adipoquinas y mioquinas. Por ello, nos planteamos como objetivo comparar los efectos del ácido elaídico, principal ácido graso *trans* de origen industrial, y su isómero *cis*, el ácido oleico, sobre la expresión, en modelos celulares, de adipoquinas y mioquinas relacionadas con la inflamación y la modulación de la sensibilidad a la insulina. Los resultados de este estudio condujeron a la publicación del **manuscrito III.**

b) Impacto de compuestos de interés sobre la adipogénesis

El número de adipocitos es un determinante importante de la adiposidad corporal. En muchos casos, el fenotipo obeso no sólo se caracteriza por un mayor volumen sino también por un mayor número de adipocitos. La inhibición de la adipogénesis se valora como una posible diana terapéutica coadyuvante en estrategias de control del peso. Obesidad y osteoartritis están relacionadas por factores que van más allá de los biomecánicos y podrían incluir interrelaciones entre adipogénesis y condrogénesis. Así las cosas, en el contexto de un contrato del grupo dentro del programa CENIT, nos planteamos evaluar el posible impacto de compuestos relacionados con la salud articular sobre el proceso adipogénico. Los resultados de este estudio se recogen en el **manuscrito IV** (Dossier). Paralelamente, se planteó como objetivo hacer una revisión bibliográfica de los factores moleculares en la intersección entre obesidad y disfunción articular **(manuscrito V)**.

c) Nutrición en etapas tempranas de la vida y susceptibilidad futura a la obesidad

La nutrición en la etapa perinatal tiene consecuencias a largo plazo sobre la susceptibilidad a la obesidad y el síndrome metabólico. La identificación de factores nutricionales concretos implicados en estos fenómenos de programación temprana y el conocimiento de sus mecanismos de acción es de gran interés en el contexto de la prevención de la obesidad, y un campo emergente en el que nuestro grupo ha hecho importantes aportaciones. La vitamina A juega un papel crucial en el desarrollo y – fundamentalmente como ácido retinoico – afecta la adipogénesis, la función adipocitaria y la adiposidad corporal en animales adultos, siendo esta otra línea de trabajo importante en nuestro grupo. No obstante, pocos estudios han evaluado el impacto de la vitamina A en etapas tempranas de la vida sobre la adiposidad futura. Por todo ello, nos planteamos estudiar los efectos a largo plazo de una suplementación con vitamina A durante el período de lactancia sobre el desarrollo del tejido adiposo y la susceptibilidad a la obesidad dietética en ratas Wistar. Los resultados de este estudio se recogen en el **manuscrito VI**.

3. PLANTEAMIENTO EXPERIMENTAL

Para la consecución de los objetivos de la presente tesis se han utilizado distintos modelos experimentales que se describen brevemente en este apartado. Los detalles de las técnicas individuales utilizadas se encuentran expuestos en los correspondientes manuscritos y en el apéndice I. Toda la experimentación con animales llevada a cabo se ha realizado de acuerdo con estándares de cuidado aceptados y ha sido aprobada por el comité de bioética de la Universidad de las Islas Baleares.

Modelos *in vivo*

1) Modelos de obesidad genética y dietética

Estos modelos fueron empleados para estudiar la regulación de la RBP y la Nampt/visfatina en la obesidad **(manuscrito I)**.

Como modelo de obesidad genética se utilizaron ratas obesas macho de tres meses de edad de la cepa Zucker que presentaban una mutación del receptor de la leptina en homocigosis (ratas Zucker fa/fa); como grupo control se emplearon ratas de las mismas características pero normopeso y con al menos un alelo salvaje del gen para el receptor de la leptina (ratas Zucker -/?). Como modelo de obesidad inducida por la dieta se emplearon ratas macho de la cepa Wistar alimentadas desde los dos hasta los seis meses de edad con una dieta de cafetería consistente en: galletas con paté de hígado y sobrasada, caramelos, galletas dulces, chocolate, queso, cacahuetes salados, ensaimada, tocino y leche con un 20% de azúcar; como grupo control se emplearon ratas Wistar alimentadas con una dieta estándar.

En ambos modelos se determinaron: los niveles circulantes de glucosa e insulina tras un periodo de ayuno y, a partir de ellos, el índice HOMA-IR como medida de la resistencia a la insulina; el peso y la adiposidad corporal; el peso de los depósitos adiposos de TAB epididimal, retroperitoneal, mesentérico e inguinal; los niveles circulantes de leptina, insulina, glucosa, Nampt/visfatina y RBP; y la expresión de Nampt/visfatina y RBP a nivel de ARNm en los distintos depósitos adiposos de TAB.

2) *Modelo de tratamiento agudo con ácido retinoico*

Este modelo ha sido utilizado repetidamente en nuestro laboratorio y aquí se empleó para estudiar específicamente los efectos de la vitamina A como AR sobre la expresión de RBP en tejidos adiposos e hígado **(manuscrito II)**.

Se emplearon ratones macho NMRI de 12 semanas de edad, alimentados con una dieta estándar. Se trataron con una dosis diaria de AR todo *trans* (10, 50 o 100 mg/kg de animal) durante los 4 días previos al sacrificio. El AR todo *trans* se administró por

inyección subcutánea disuelto en aceite de oliva, y como control del tratamiento se inyectó aceite de oliva a animales de las mismas características.

Se determinó el efecto del tratamiento sobre la producción de RBP en depósitos de TAB y en hígado a nivel de ARNm y proteína, y sobre los niveles circulantes de RBP, medidos por immunoblotting tras electroforesis desnaturalizante del suero. Los complejos de RBP en suero también fueron analizados por immunoblotting tras una electroforesis no desnaturalizante, que puede permitir distinguir los complejos con retinol-RBP, AR-RBP y apo-RBP (RBP sin retinoides unido); en este análisis, suero delipidado de animales control sirvió como marcador de los complejos conteniendo apo-RBP. Asimismo, se determinó el índice HOMA-IR en una cohorte independiente de animales control y tratados con AR a una dosis de 50 mg/kg de animal durante los 4 días previos a la medida. (Los efectos del tratamiento con AR sobre la ingesta energética y parámetros biométricos y sanguíneos habían sido previamente publicados en otros trabajos del grupo).

3*) Modelo de intervención nutricional temprana*

Este modelo se empleó para el estudio del efecto de la suplementación con vitamina A durante el periodo de lactancia sobre la ulterior susceptibilidad a la obesidad (**manuscrito VI)**.

Se utilizaron crías de ratas de la cepa Wistar recién nacidas a las que se suministró diariamente durante todo el periodo de lactancia (desde el día 0 al 20 de vida) una dosis suplementaria de vitamina A en forma de retinil palmitato, disuelta en aceite de oliva, equivalente aproximadamente a tres veces la ingerida a través de la leche materna. Los animales del grupo control recibieron la misma cantidad del vehículo (aceite de oliva). Una parte de las crías se sacrificaron al final de la lactancia y el resto de ratas macho de cada grupo (control y tratado con vitamina A) se subdividieron en dos grupos en función de la dieta suministrada desde el destete (en el día 21 de vida) hasta el sacrificio (en el día 135 de vida): animales alimentados con una dieta rica en grasa (60% de las calorías en forma de grasa) y animales alimentados con una dieta estándar (10% de calorías en forma de grasa), generándose un total de cuatro grupos experimentales.

En todos los animales se determinaron: parámetros biométricos (peso, adiposidad corporal y peso de depósitos adiposos e hígado); niveles circulantes de glucosa, leptina, resistina, y retinoides; niveles de retinoides en TAB inguinal e hígado; análisis morfométrico del TAB inguinal; expresión de ISX a nivel de ARNm en intestino; expresión de UCP1 en TAM; niveles de ARNm en TAB inguinal de genes que codifican

para proteínas relacionadas con la adipogénesis y la lipogénesis (PPARγ, CEBPα, LPL, FAS, GLUT4).

En las crías sacrificadas en el momento del destete, adicionalmente se determinó: en TAB inguinal, la expresión de PCNA a nivel de ARNm y proteína y niveles de ARNm de p21, PPARγ y CEBPα; y en el hígado, los niveles de ARNm de Cyp26a1.

En las ratas adultas, adicionalmente se determinó: ingesta; índice HOMA-IR; tolerancia a la glucosa; niveles circulantes de insulina, triacilgliceroles, ácidos grasos libres y resistina; análisis morfológico y composición tisular de los distintos depósitos adiposos (contenido total de ADN, proteína y lípidos); análisis morfológico del hígado; contenido total de lípidos y triacilgliceroles hepáticos; temperatura rectal; niveles de ARNm de genes para proteínas relacionadas con la lipogénesis (SREBP1-c, FAS, SCD1) y con la oxidación de ácidos grasos (PPARα) en hígado; y niveles de ARNm de genes para proteínas relacionadas con la oxidación de ácidos grasos y proteínas desacoplantes de la cadena respiratoria en TAM (PGC-1α, UCP1) y músculo esquelético (PPARβ/δ, UCP3).

Modelos celulares

1) Modelo de miocitos C2C12

Este modelo se utilizó para comparar los efectos del ácido oleico y su isómero *trans*, el ácido elaídico, sobre la expresión de mioquinas y la captación de glucosa basal y estimulada por insulina **(manuscrito III)**.

Cultivos de mioblastos C2C12 fueron diferenciados a miocitos utilizando suero de caballo y se incubaron durante 24 horas con los ácidos grasos de interés (oleico y elaídico), disueltos en DMSO, a tres concentraciones finales diferentes: 20, 100 y 500 µM. Como control se emplearon miocitos C2C12 tratados con vehículo (DMSO).

En este modelo se determinó la expresión a nivel de ARNm y la secreción de proteína al medio de cultivo de IL-6 y TNFα, así como los niveles de ARNm de IL-15. También se determinó la captación de glucosa basal y estimulada por insulina, previa incubación con los ácidos grasos mencionados, o con DMSO como control, durante 24 horas.

2) Modelo de adipocitos blancos 3T3-L1

Este modelo se empleó para estudiar el efecto del AR todo *trans* sobre la producción adipocitaria de RBP **(manuscrito II)** y para comparar los efectos del ácido oleico y su isómero *trans,* el ácido elaídico, sobre la expresión de adipoquinas y la captación de glucosa basal y estimulada por insulina en adipocitos **(manuscrito III)**.

En el primer caso, cultivos de fibroblastos 3T3-L1 fueron diferenciados a adipocitos blancos utilizando un coctel hormonal estándar (consistente en dexametasona, 3-isobutil -1- metilxantina (IBMX) e insulina) administrado (en el que se considera el día 0 de cultivo) dos días después de alcanzada la confluencia. A día 8-10 de cultivo, las células, ya diferenciadas en adipocitos, fueron tratadas con distintas dosis de AR todo *trans* (0,1, 1 y 10 μM) durante 48 horas, y se determinó la expresión de RBP a nivel de ARNm. Como control se emplearon adipocitos 3T3-L1 tratados con vehículo (DMSO).

En el segundo caso, los adipocitos 3T3-L1, una vez diferenciados como en el caso anterior, fueron incubados durante 24 horas con los ácidos grasos de interés (oleico y elaídico), disueltos en DMSO, a una concentración final de 100 μM. Como control se emplearon adipocitos 3T3-L1 tratados con el vehículo (DMSO). En este sistema se determinó la expresión a nivel de ARNm de resistina y adiponectina. También se determinó la captación de glucosa basal y estimulada por insulina tras 24 horas de incubación con los ácidos grasos citados o DMSO como control.

3) Modelo de adipocitos blancos derivados de MEFs (Mouse Embryonic Fibroblasts)

Este modelo, se empleó para dos propósitos. En primer lugar, para comparar los efectos de AR todo *trans*, retinal y agonistas de RAR y RXR sobre la producción de RBP en adipocitos **(manuscrito II)**. En segundo lugar, para estudiar los efectos de compuestos empleados en salud articular sobre la adipogénesis **(manuscrito IV)**.

En el primer caso, cultivos post-confluentes de fibroblastos de embrión de ratón C57BL/6J de 13 días de edad embrionaria fueron diferenciados a adipocitos utilizando un coctel hormonal estándar (conteniendo dexametasona, IBMX, insulina y rosiglitazona) (día 0). A día 8, se trataron durante 48 horas con distintas concentraciones de AR todo *trans*, retinal o agonistas de los receptores de retinoides RAR y RXR. Se analizó la expresión de RBP a nivel de ARNm y la acumulación de RBP proteína en el medio de cultivo condicionado por las células.

En el segundo caso, cultivos post-confluentes de fibroblastos de embrión de ratón C57BL/6J de 13 días de edad embrionaria fueron estimulados a diferenciarse en adipocitos como en el caso anterior, pero al tiempo que se añadían los compuestos de interés desde el día 0 hasta el día 8. Como control se emplearon fibroblastos estimulados a adipocitos de la manera estándar. Se analizó el contenido intracelular de triacilgliceroles y los niveles de ARNm de: marcadores adipocitarios (PPARγ, CEBPα, FAS); proteínas de secreción (leptina, RBP, adiponectina, resistina); proteínas inhibidoras de la adipogénesis y/o relacionadas con la condrogénesis (Sox-9, PGC1α,

leptina, TNFα); y componentes de la matriz extracelular (proteína del agrecan, colágeno tipo 2a1).

4) Modelo de MEFs no estimulados con coctel adipogénico

Este modelo se empleó para estudiar el impacto de compuestos empleados en salud articular sobre el devenir “espontáneo” de los MEFs y compararlo con el de una proteína con conocida actividad pro-condrogénica en estas células, la proteína 2 morfogénica del hueso (BMP2) **(manuscrito IV)**. Se analizaron los efectos en dos tipos de cultivo, cultivo en monocapa y cultivo de alta densidad celular o también llamado cultivo en tres dimensiones.

Desde el día 0 (equivalente a 48 horas tras la confluencia en cultivos en monocapa y al día de la siembra en cultivos en tres dimensiones) hasta el día 13 se añadieron los compuestos de interés a una dosis de 100 μM, o BMP2 a una dosis de 100 nM. Como control se emplearon cultivos a los que no se les añadió ningún tratamiento. Las células fueron cosechadas a diferentes días de cultivo (día 0, día 3, día 8 y día 13). Se midieron los niveles de ARNm de genes que codifican para proteínas involucradas en la diferenciación de condrocitos (Pref-1, Sox-9) y para componentes estructurales (proteína del agrecan, colágeno tipo 2a1) y enzimas remodeladoras (agrecanasa) de la matriz extracelular de condrocitos. La diferenciación espontánea en adipocitos se evaluó por la presencia de acumulación lipídica intracelular al microscopio de contraste de fase y a partir de los niveles de ARNm de marcadores adipocitarios (PPARγ, FAS), a día 8 de cultivo.

4. RESULTADOS Y DISCUSIÓN

MANUSCRITO I

Retinol-binding Protein 4 and Nicotinamide Phosphoribosyltransferase/Visfatin in Rat Obesity Models.

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Retinol-binding Protein 4 and Nicotinamide Phosphoribosyltransferase/Visfatin in Rat Obesity Models

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- insulin sensitivity
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Abstract

Retinol-binding protein 4 (RBP4) and nicotinamide phosphoribosyltransferase/visfatin (Nampt/visfatin) are adipocyte-secreted proteins (adipokines) whose relevance to the metabolic syndrome and regulation in obesity remain controversial. Here, we tested the hypothesis that adipose tissue expression and circulating levels of these two adipokines are elevated in obesity by analyzing their changes in both a genetic and a diet-induced model of obesity in the rat (obese *fa/fa* Zucker rats and Wistar rats fed a cafeteria diet, respectively). Compared with lean controls, obese *fa/fa* rats were hyperleptinemic, hyperinsulinemic, and insulin resistant and had reduced RBP4 serum levels and mRNA levels in adipose depots, unchanged Nampt/visfatin serum levels, and reduced Nampt/visfatin mRNA levels selectively in the inguinal adipose depot. Cafeteria diet-induced obesity resulted in increased fed blood glucose levels, a variable degree of insulin resistance, unchanged serum Nampt/visfatin and RBP4 levels, and reduced mRNA levels of both adipokines in several adipose depots. Hence, increases in RBP4 or Nampt/visfatin do not accompany obesity and insulin resistance in the models examined.

Introduction

Adipokines are proteins secreted by adipose tissue that affect whole-body energy metabolism. Their dysregulated production in obesity has implicated them as potential mediators in the pathogenesis of obesity-related risk factors (reviewed in [1]). Retinol-binding protein 4 (RBP4) and visfatin—the latter previously known as pre-B cell colony-enhancing factor and nicotinamide phosphoribosyltransferase (Nampt) and referred to throughout the rest of this paper as Nampt/visfatin—are two proteins that have been recently rediscovered as adipokines. Evidence was presented of effects of adipose tissue-derived RBP4 promoting systemic insulin resistance [2] and of insulin-mimetic effects of Nampt/visfatin [3]. Though the insulin-mimetic function of Nampt/visfatin has not consistently been reproduced and therefore remains unproved, it is accepted that differentiated adipocytes are likely to be a major source of circulating Nampt/visfatin and that Nampt/visfatin, acting as a systemic NAD biosynthetic enzyme, affects insulin secretion in pancreatic β-cells [4]. Therefore, both RBP4 and Nampt/visfatin might have a role in the modulation of insulin biology and glucose homeostasis.

Following the re-identification of RBP4 and Nampt/visfatin as adipokines, attention focused mainly on possible correlations between the levels and genetic variation of these proteins with parameters of obesity, insulin resistance, and other components of the metabolic syndrome in humans, giving rise to controversial results. However, the number of reports dealing with the expression of these novel adipokines in animal models of obesity/insulin resistance is surprisingly small [2, 3, 5]. This prompted us to examine RBP4 and Nampt/visfatin serum levels and relative adipose tissue gene expression in two well-established rat models of obesity, namely, Zucker *fa/fa* rats, which display genetic obesity due to deficiencies in the leptin receptors, and cafeteria diet–fed Wistar rats, which develop diet-induced hyperphagia and obesity [6]. Expression of RBP4 and Nampt/visfatin in these models had not been previously addressed.

Materials and Methods

Animals

Three-month-old male lean (–/?) and obese (*fa/fa*) Zucker rats fed regular chow (n = 10 animals per group, distributed in two independent experiments) and six-month-old male Wistar rats fed from the age of two months either regular chow or a cafeteria diet (n = 5 animals per group) were used (from Charles River Laboratories España SA, Barcelona, Spain). The cafeteria diet included cookies with liver pâté and sobrassada (a typical Majorcan sausage), candies, fresh bacon, biscuits, chocolate, salted peanuts, cheese, ensaïmada (a typical Majorcan pastry), and milk containing 20 % (w/v) sucrose.

Animals were housed at 22 °C with a period of light/dark of 12 hours and sacrificed by decapitation at the start of the light period. White adipose tissue (WAT) depots (epididymal, retroperitoneal, mesenteric, and inguinal; eWAT, rWAT, mWAT, and iWAT, respectively) were excised in their entirety, weighed, frozen in liquid nitrogen, and stored at −80 °C until analysis. Troncular blood was collected from the neck, stored at room temperature for 1 hour, and centrifuged at 1000 × g for 10 minutes to collect serum.

Adiposity index

The adiposity index was computed as the sum of the mass of all WAT depots taken expressed as percentage of total body weight.

HOMA-IR analysis

Insulin resistance was assessed by the homeostatic model assessment for insulin resistance (HOMA-IR) in parallel groups of lean and obese Zucker rats and control and cafeteria diet–fed Wistar rats that had been submitted to overnight (14 h) fasting (n = 5 for all groups). The HOMA-IR score was calculated from fasting insulin and glucose concentrations using the formula of Matthews et al. [7]: HOMA-IR = fasting glucose (mmol/l) × fasting insulin (mU/l)/22.5.

Western blotting analysis

A total of 3 μl of serum was mixed with 10 μl phosphate-buffered saline and solubilized and boiled for three minutes in Laemmli sample buffer containing 20 % 2-mercaptoethanol. Total protein was fractionated by SDS-polyacrylamide gel electrophoresis (13 % polyacrylamide) and electrotransferred onto a PVDF membrane (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, membranes were incubated with primary mouse monoclonal antibodies (anti-RBP4 from Abcam, Cambridge, UK or anti-visfatin from Axxora LLC, San Diego, CA) and then with secondary horseradish peroxidase-linked anti-mouse IgG antibody (Amersham Biosciences, Barcelona, Spain). Immunocomplexes were revealed using an enhanced chemiluminescence detection system (ECL, Biosciences, Barcelona, Spain). Membranes were exposed to Hyperfilm ECL (Amersham Biosciences, Barcelona, Spain). Bands in films were analyzed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, MA).

Tissue RNA extraction and real-time PCR analysis

Total RNA was extracted using Trizol (Invitrogen, Carlsbad CA, USA) according to the supplier's instructions. cDNA synthesis was performed from 1 μg total RNA using leukemia virus reverse transcriptase (Perkin-Elmer, Madrid, Spain) in the presence of 50 pmol of random primers, in a Perkin-Elmer 2400 Thermal Cycler. After diluting the cDNA samples 1:10, real-time PCR analyses were conducted in the LightCycler System with SYBR Green I sequence nonspecific detection (Roche Diagnostic GmbH, Mannheim, Germany). LDL receptor-related protein 10 (LRP10) was used as a reference gene, after verifying that its mRNA expression levels varied very little between the different adipose depots and between lean and obese animals (data not shown), in agreement with a previous report [8]. Sense and antisense primers used in the PCR reactions were designed with specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA) to hybridize to different exons and to avoid amplification of any residual genomic DNA, and their specificity was analyzed by the ENTREZ and BLAST databases utilities (National Center for Biotechnology Information, Bethesda, MD). The following primers were used. For RBP4 (Genbank accession number NM_013162), 5′-ACTGGGGTGTAGCCTCCTTT-3′ and 5′-GGTGTCGTAGTCCGTGTCG-3′; for Nampt/visfatin (Genbank accession number NM_177298), 5′-CATAGGGGCATCTGCTCATT-3′ and 5′-GCTATCGCTGACCACAGACA-3′; and for LRP10 (Genbank accession number NM_001037777), 5′-TCCCCTTTCTTCTCCTCCTC-3′ and 5′-TTACCGTCTGTTCCTTGCTG-3′. Each PCR was performed in a total volume of 8 μl, made from 2 μl diluted cDNA template, 0.375 μl forward and reverse primers, 0.6 μl $MgCl_2$, 3.9 μl PCR-grade water, and 0.75 μl SYBR Green I master mix. After an initial Taq activation at 95 °C for 10 minutes, LightCycler PCR was performed using 40 cycles with the following cycling conditions: denaturation at 95 °C for two seconds, annealing at 61 °C (for RBP4) or 60 °C (for Nampt/visfatin and LRP10) for six seconds, and extension at 72 °C for four seconds (for RBP4) or nine seconds (for Nampt/visfatin and LRP10). To verify the purity of the PCR products, a melting curve was produced after each run and these products were separated in a 2 % agarose gel (Agarose D-1 Low EEO; Pronadisa, Madrid, Spain) in 0.5 × Tris-borate-EDTA buffer and stained with ethidium bromide. Threshold cycle values were determined using the LightCycler software. Relative gene expression numbers were calculated using the $2^{-\Delta\Delta Ct}$ method [9].

Quantification of circulating insulin, leptin and glucose

Serum insulin and leptin were measured using ELISA kits (from DRG Instruments, Marburg, Germany, and R&D Systems, Minneapolis, MN, respectively), and blood glucose was measured using an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain).

Statistical analysis

Data are presented as mean ± SEM. Statistical significance of differences between lean and obese rats was assessed by two-tailed Student's *t*-test. Statistical significance of differences in relative gene expression among fat depots was assessed by one-way ANOVA followed by least-significant difference (LSD) *post hoc* comparison. Results were considered statistically significant when $p < 0.05$.

Results

Compared to lean (–/?) controls, Zucker *fa/fa* rats displayed a 24 % increase in body weight, a 3.2-fold increase in adiposity index, and a marked increase in circulating leptin levels and

were euglycemic but hyperinsulinemic in the fed state (**Table 1**). HOMA-IR determined in parallel groups of fasted animals confirmed systemic insulin resistance in the *fa/fa* rats, as expected (HOMA-IR: lean Zucker rats, 1.80 ± 0.20; obese *fa/fa* Zucker rats, 21.18 ± 3.26; p = 0.000). RBP4 mRNA levels (determined per microgram of tissue RNA relative to LRP10 as a reference gene) were lower in WAT depots of obese Zucker *fa/fa* rats than in their lean controls: the reduction reached statistical significance in the epididymal (p = 0.013) and retroperitoneal (p = 0.002) depots and showed a clear trend in the mesenteric depot (p = 0.056) (**Fig. 1A**). In line with the mRNA profile, serum RBP4 levels were significantly lower in obese Zucker *fa/fa* rats than in their lean controls (p = 0.01) (**Fig. 1B**). Obesity in Zucker *fa/fa* rats did not bring about noticeable changes in serum Nampt/visfatin or relative Nampt/visfatin gene expression in visceral WAT depots, but it was accompanied by a significant reduction in Nampt/visfatin gene expression in the inguinal depot (p = 0.023) (**Fig. 1C,D**).

Compared with age-matched controls fed a standard chow diet, Wistar rats fed a cafeteria diet for 16 weeks from the age of two months exhibited a 37 % increase in body weight, a 2.2-fold increase in body adiposity, and a 3.5-fold increase in leptinemia (**Table 2**). Cafeteria diet-induced obesity did not significantly alter circulating insulin levels but resulted in increased blood glucose levels in the fed state (**Table 2**). HOMA-IR analysis performed in parallel groups of fasted cafeteria and control Wistar rats indicated reduced insulin sensitivity in the former, although the difference did not reach statistical significance owing to interindividual variability in the cafeteria group (HOMA-IR score: control Wistar rats, 5.25 ± 1.52; cafeteria-fed Wistar rats, 10.96 ± 4.91). Cafeteria diet-induced obesity did not affect serum RBP4 or Nampt/visfatin levels (**Fig. 2B,D**) and was accompanied by a significant decrease in RBP4 gene expression in the inguinal adipose depot (p = 0.036) (**Fig. 2A**) and in Nampt/visfatin gene expression in the mesenteric (p = 0.019) and inguinal (p = 0.000) adipose depots (**Fig. 2C**).

We compared RBP4 and Nampt/visfatin gene expression levels in subcutaneous (inguinal) and visceral depots of Zucker and Wistar lean rats (**Table 3**). In lean Zucker rats, RBP4 mRNA levels were similar in all fat depots analyzed, and no increased Nampt/visfatin expression in visceral depots over that found in the inguinal depot was detected. In lean Wistar rats, RBP4 mRNA levels were lower in the inguinal depot than in visceral depots (epididymal and mesenteric), and Nampt/visfatin mRNA levels were maximal in the mesenteric depot, followed by the inguinal depot.

Table 1 Biometric parameters, glycemia, insulinemia, and leptinemia in lean and obese Zucker rats

Parameter	(–/?) rats	*fa/fa* rats	*t*-test*
body weight (g)	321 ± 6	397 ± 8	p = 0.000
eWAT mass (g)	1.87 ± 0.13	5.50 ± 0.52	p = 0.000
rWAT mass (g)	1.71 ± 0.16	5.04 ± 0.38	p = 0.000
mWAT mass (g)	1.40 ± 0.11	4.04 ± 0.43	p = 0.000
iWAT mass (g)	4.09 ± 0.27	21.6 ± 0.5	p = 0.000
adiposity index (%)	2.82 ± 0.17	9.13 ± 0.21	p = 0.000
circulating glucose (mM)	6.29 ± 0.30	6.49 ± 0.49	
circulating insulin (μg/l)	0.72 ± 0.13	15.4 ± 3.7	p = 0.003
circulating leptin (μg/l)	3.47 ± 0.24	41.1 ± 2.9	p = 0.000

Three-month-old male lean (–/?) and obese (fa/fa) Zucker rats fed regular chow were used. Biometric data are the mean ± SEM of 10 animals per group, distributed in two independent experiments. Blood parameters are the mean ± SEM of 5 animals per group in a single experiment

*Student's t-test significance is given

Table 2 Biometric parameters, glycemia, insulinemia, and leptinemia in Wistar rats made obese by cafeteria diet feeding and their controls

Parameter	Control group	Cafeteria group	*t*-test*
body weight (g)	475 ± 22	652 ± 4	p = 0.000
eWAT mass (g)	11.7 ± 1.5	34.5 ± 2.5	p = 0.000
rWAT mass (g)	12.1 ± 2.2	33.9 ± 2.6	p = 0.000
mWAT mass (g)	6.40 ± 1.62	18.6 ± 0.6	p = 0.001
iWAT mass (g)	8.06 ± 1.02	27.0 ± 2.7	p = 0.001
adiposity index (%)	7.95 ± 0.86	17.5 ± 0.9	p = 0.000
circulating glucose (mM)	6.43 ± 0.31	8.11 ± 0.35	p = 0.012
circulating insulin (μg/l)	3.25 ± 0.47	3.41 ± 0.98	
circulating leptin (μg/l)	10.7 ± 1.8	38.0 ± 4.9	p = 0.001

Six-month-old male Wistar rats fed from the age of 2 months with either regular chow (control group) or a cafeteria diet (cafeteria group) were used. Data are the mean ± SEM of 5 animals per group

*Student's t-test significance is given

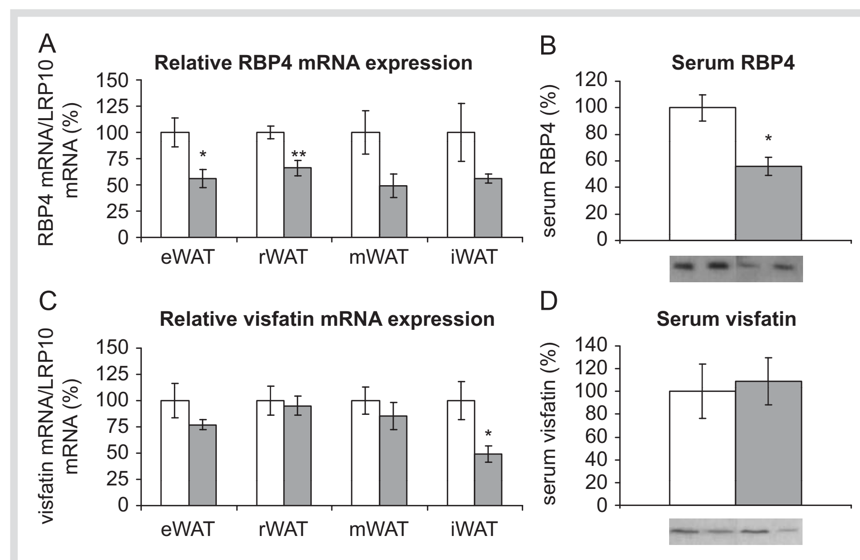

Fig. 1 Adipose tissue expression and serum levels of RBP4 (**A**, **B**) and visfatin (**C**, **D**) in lean and obese Zucker rats. Three-month-old male lean (–/?) and obese (*fa/fa*) Zucker rats fed regular chow were used (white bars and gray bars, respectively). Gene expression data in mWAT and circulating RBP4 and visfatin data are the mean ± SEM of 5 animals per group in a single experiment. Gene expression data in eWAT, rWAT, and iWAT are the mean ± SEM of 10 animals per group, distributed in two independent experiments. Data are expressed relative to the mean value of the lean group, which was set as 100 %. Representative immunoblots of RBP4 and visfatin in 3 μl of serum are shown at the bottom of panels **B** and **D**, respectively. Student's *t*-test significance: *p < 0.05; **p < 0.01.

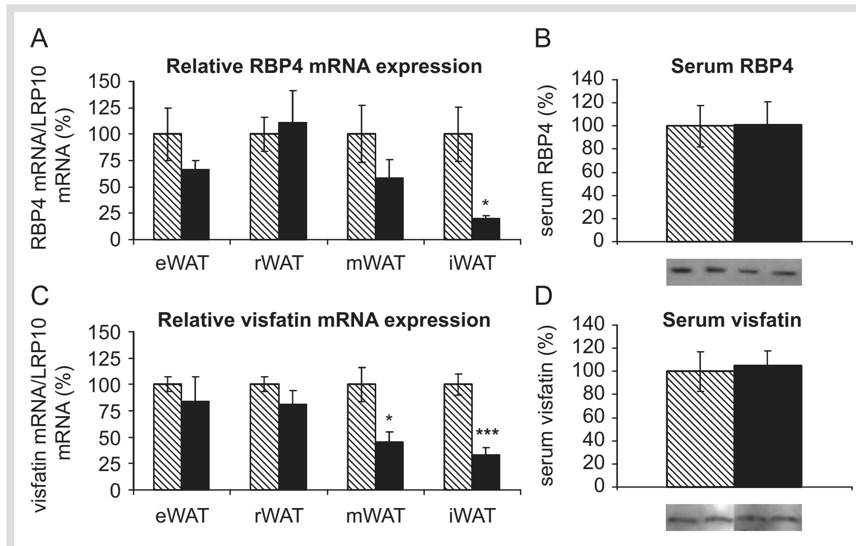

Fig. 2 Adipose tissue expression and serum levels of RBP4 (**A**, **B**) and visfatin (**C**, **D**) in Wistar rats made obese by cafeteria diet feeding and their controls. Six-month-old male Wistar rats fed from the age of 2 months either with regular chow (control group) or a cafeteria diet were used (dashed bars and black bars, respectively). Data are the mean ± SEM of 5 animals per group and are expressed relative to the mean value of the control group, which was set as 100 %. Representative immunoblots of RBP4 and visfatin in 3 μl of serum are shown at the bottom of panels **B** and **D**, respectively. Student's *t*-test significance: *$p<0.05$; ***$p<0.001$.

Table 3 Expression of RBP4 and Nampt/visfatin mRNA in different adipose tissue depots of three-month-old male lean (–/?) Zucker rats and six-month-old male Wistar rats fed regular chow

		iWAT	eWAT	rWAT	mWAT	ANOVA
RBP4 mRNA	Zucker lean	0.96 ± 0.50	1.48 ± 0.21	1.87 ± 0.33	1.11 ± 0.10	
	Wistar lean	1.29 ± 0.12^{a}	3.44 ± 0.83^{b}	1.14 ± 0.16^{a}	4.44 ± 0.33^{b}	D
Nampt/visfatin mRNA	Zucker lean	1.18 ± 0.33^{a}	1.17 ± 0.14^{a}	0.56 ± 0.10^{b}	0.99 ± 0.15^{a,b}	D
	Wistar lean	1.23 ± 0.15^{a}	0.88 ± 0.13^{b}	0.79 ± 0.07^{b}	2.01 ± 0.08^{c}	D

Ratios of gene expression to LRP10 as a reference gene are given as mean ± SEM of n = 5 animals per group
D = one-way ANOVA significance, $p<0.05$, effect of depot
In each row, values not sharing a common letter are statistically different by LSD post hoc comparison ($p<0.05$)

Discussion and Conclusions

Serum RBP4 levels have been reported to be increased in insulin-resistant mouse models, some involving obesity (such as mice on a high-fat diet and *ob/ob* mice) and others not (such as adipose-specific GLUT4-deficient mice) [2]. Biochemical, genetic, and pharmacological studies have suggested that in mice, adipose tissue–derived RBP4 can cause insulin resistance by mechanisms that might include impairment of insulin signaling in muscle and enhancement of hepatic glucose output [2]. However, the relationship between RBP4 and obesity and insulin resistance remains unclear. Some authors reported a positive association between serum RBP4 levels and general measures of obesity such as BMI or percentage body fat in humans (e.g. [2, 10, 11],); other authors did not detect such correlations (e.g. [12–15],) but found a correlation with liver fat [14] or percentage trunk fat [15]. An association between high serum RBP4 levels and impaired insulin sensitivity, even independent of obesity, was found in some human studies (e.g. [10, 11, 14–16],) but not in others (e.g. [12, 13, 17],). Our results do not sustain a role for RBP4 in the development of systemic insulin resistance in the rat models examined, as serum RBP4 levels—measured by "gold standard" Western blotting [18]—were clearly reduced in insulin-resistant Zucker *fa/fa* rats and unchanged in cafeteria diet–fed rats relative to lean controls.

A reduced RBP4 level in the serum of *fa/fa* rats was an unexpected finding. It could have been expected that deficiencies in leptin and leptin receptors lead to a similar RBP4 phenotype. In fact, in both leptin-deficient *ob/ob* mice [2] and obese Zucker *fa/fa* rats (this work), specific RBP4 gene expression in adipose tissues is reduced by 40–50 % compared with lean controls, suggesting that leptin signaling normally upregulates RBP4 gene transcription in adipocytes. This transcriptional response could contribute to local effects of leptin antagonizing insulin signaling in adipose tissues, as effects of RBP4 attenuating insulin signaling in adipocytes have been described [19]. However, serum RBP4 levels are reduced in hyperleptinemic *fa/fa* Zucker rats (this work) and elevated by a factor of 13 (remarkably much more than in the other mouse models of insulin resistance analyzed) in *ob/ob* mice lacking leptin [2]. This difference may suggest that high leptin normally has side effects independent of leptin receptors that favor reduction of RBP4 levels in serum (e.g., increased clearance and/or reduced secretion). This scenario, with hyperleptinemia having opposite effects on adipose RBP4 gene expression and RBP4 serum levels, may partly explain inconsistencies in the literature regarding RBP4 in obesity, especially when it is considered that hyperleptinemia and leptin resistance coexist at various degrees in many forms of obesity. It is also possible that species differences exist between rats and mice concerning RBP4 regulation.

The biology of Nampt/visfatin and its connections with pathophysiological conditions such as obesity and type 2 diabetes remain poorly understood. Acting as a systemic NAD biosynthetic enzyme, Nampt/visfatin has been shown to positively modulate insulin secretion in pancreatic -cells, suggesting a role for this protein in the regulation of glucose homeostasis [4]. Nampt/visfatin may also represent a survival signal for adipocytes and other cell types as a consequence of its intracellular role in the provision of NAD for cell survival reactions linked to the sirtuin family of protein deacetylases [20]. In fact, Nampt/

visfatin is upregulated by hypoxia in cell types including adipocytes [21,22] and reportedly stimulates angiogenesis [23], inhibits apoptosis [24], and extends the cell's lifespan [20]. Hence, Nampt/visfatin may act as an intracellular and/or paracrine signal for sustained WAT development/maintenance and as a systemic positive modulator of insulin secretion.

Changes in Nampt/visfatin levels in obesity are controversial. Nampt/visfatin serum levels and expression in visceral fat were reported to increase in KKAy mice (a polygenic model of diabesity) at the onset of obesity and to be increased in mice on a high-fat diet [3]. In humans, plasma levels of Nampt/visfatin have been reported to correlate with measures of obesity (BMI, percentage body fat) [3,25–27], to be increased in proportion to visceral fat accumulation [3,27], and to be reduced after weight loss [28]. However, others found reduced plasma Nampt/visfatin levels in obese compared with normal-weight subjects [29,30] or lack of association between plasma Nampt/visfatin and BMI [31] or whole-body adiposity [32]. Changes in Nampt/visfatin levels in insulin resistance and type 2 diabetes are also controversial. Increased serum concentrations of Nampt/visfatin in type 2 diabetic humans independent of their BMI have been reported [33], as has increased expression of Nampt/visfatin mRNA in peripheral blood mononuclear cells of type 2 diabetic women independent of their BMI [34]. However, other authors found no association between serum visfatin and parameters of insulin sensitivity in humans [25,29]. Our results do not sustain the concept that Nampt/visfatin is characteristically upregulated in obesity or insulin resistance states and are in line with previous findings in obese Wistar Ottawa Karlsburg rats with the MHC RT1u haplotype (WOKW rats), an animal model for polygenically inherited metabolic syndrome [5].

We found a preferential expression of both Nampt/visfatin and RBP4 in the mesenteric vs. the subcutaneous inguinal depot in lean Wistar rats, but not in lean Zucker rats. Our results are compatible, however, with a preferential expression of Nampt/visfatin in the visceral depots in the obese state because, in both models, obesity was accompanied by a downregulation of Nampt/visfatin gene expression in the inguinal depot that was absent or less marked in the visceral depots analyzed.

In conclusion, our results in both a genetic and a diet-induced rat model of obesity do not support a major role for increased RBP4 or Nampt/visfatin in the development of insulin resistance in these models, or an effect of increased adiposity *per se* inducing the expression of these two adipokines in WAT depots.

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MANUSCRITO II

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All-Trans Retinoic Acid Decreases Murine Adipose Retinol Binding Protein 4 Production

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Key Words

Vitamin A • Retinoic acid • Retinaldehyde • Adipokines • Insulin resistance • Adipocyte

Abstract

Background/Aims: Adipose-derived retinol binding protein 4 (RBP4) might contribute to the development of insulin resistance, and therefore further knowledge of factors regulating it is of interest. Retinoic acid, the acid form of vitamin A, affects the expression of several adipokines related to insulin sensitivity in mice. Here, we sought to investigate its impact on adipose RBP4 production. Methods: Changes in RBP4 expression were analyzed in adipose tissues and liver of mice treated *in vivo* with all-trans retinoic acid (ATRA), and in 3T3-L1 adipocytes and adipocytes derived from mouse embryonic fibroblasts exposed to ATRA. Results: ATRA treatment in mice increased insulin sensitivity as assessed by the homeostatic model assessment for insulin resistance, and led to a reduction of RBP4 mRNA and protein levels in adipose tissues, a reduction of RBP4 protein but not RBP4 mRNA levels in the liver, and a marked increase in circulating RBP4 protein levels. In adipocyte cell models, ATRA down-regulated RBP4 mRNA levels in a dose-dependent manner: this effect was reproduced by retinaldehyde and retinoid receptors agonists, and correlated with a reduced accumulation of RBP4 protein in the culture medium. Conclusion: These results reveal a selective effect of ATRA inhibiting RBP4 expression specifically in adipocytes, and reinforce the concept that vitamin A vitamers may affect insulin sensitivity through effects on adipokine production.

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Introduction

Retinol binding protein 4 (RBP4) is a 21-kDa single polypeptide chain protein best known for being the principal physiological carrier of retinol, the parent vitamin A molecule, in blood. RBP4 helps vertebrates adapt to fluctuations in dietary vitamin A intake by allowing the delivery of retinol from tissue storage sites to target tissues, which express a specific membrane receptor for RBP4 (STRA6) that mediates retinol uptake from vitamin A-loaded RBP4 (holo-RBP4) complexes [1]. Both apo- and holo-RBP4 circulate in blood complexed to plasma transthyretin in a 1:1 molar ratio. Liver and, to a lesser extent, white adipose tissue (WAT) are two main sites of

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vitamin A storage and RBP4 synthesis. RBP4 is abundantly expressed in WAT depots [2-4], almost exclusively in the adipocytes and only weakly in the stromal-vascular cells [3], and adipocytes express RBP4 in a differentiation-dependent manner [5].

RBP4 was recently identified by microarray profiling as a gene overexpressed in WAT (but not liver) of adipose-specific GLUT4 knockout mice, which are insulin resistant, and underexpressed in WAT of transgenic mice overexpressing GLUT4 in adipose tissues, which have enhanced insulin sensitivity, suggesting adipocyte-derived RBP4 may act as an insulin resistance factor [6]. In keeping with this role, serum RBP4 levels are elevated in insulin-resistant mouse models; the anti-diabetic drug rosiglitazone reduces serum RBP4 levels and RBP4 mRNA expression in WAT (but not liver) of adipose-specific GLUT4 knockout mice; and genetic and pharmacological interventions that elevate serum RBP4 impair, while those reducing serum RBP4 enhance, insulin sensitivity in mice [6]. In humans, several studies have found a correlation between serum RBP4 levels and the magnitude of insulin resistance in subjects with obesity, impaired glucose tolerance or type 2 diabetes [6-10]. Some authors even reported a negative correlation between serum RBP4 and insulin sensitivity in healthy human subjects [9, 11, 12]. The association between serum RBP4 levels and insulin resistance remains, however, controversial, as several studies failed to find it [13-19], although in some of these reports an association of RBP4 with inflammatory markers and other components of the metabolic syndrome was noticed [17, 18]. Despite these discrepancies, there is evidence of effects of RBP4 counteracting insulin action, as RBP4 has been reported to attenuate insulin signaling in primary human adipocytes [20] and in skeletal muscle of mice [6], to increase hepatic glucose output in mice [6], and to negatively affect insulin secretion by pancreatic beta-cells in humans [16]. In addition, single nucleotide polymorphisms in the RBP4 gene have been found to be associated with susceptibility to type 2 diabetes and insulin resistance in humans [21-23].

RBP4 regulation by vitamin A has been most studied in the liver, but not in the adipose tissue, and this becomes of special interest in view of the potential role of adipose-derived RBP4 in the modulation of insulin action. It is unclear whether the link between RBP4 and insulin resistance occurs through vitamin A-dependent and/or vitamin A-independent mechanisms [6]. Retinoic acid, the carboxylic acid form of vitamin A, is a well known regulator of gene expression in mammals, mainly though not solely through binding to and activation of specific retinoid receptors that behave as ligand-modulated transcription factors. Retinoic acid has many remarkable effects on adipocyte biology (reviewed in [24]), including effects on the production of several adipocyte-secreted bioactive proteins (adipokines), such as resistin and leptin [25-27]. The aim of this study was to evaluate RBP4 regulation by retinoic acid in murine adipose tissues and cultured white adipocytes.

Materials and Methods

Animal studies

Twelve-week-old NMRI male mice (CRIFFA, Barcelona, Spain) fed *ad libitum* regular chow (Panlab, Barcelona, Spain) and kept at 22°C with a 12-h light/dark cycle (lights on at 08:00) received one daily subcutaneous injection of all-trans retinoic acid (ATRA, Sigma, St. Louis, MO) at a dose of 10, 50, or 100 mg/kg body weight during the 4 days before they were killed (5-6 animals/group). Controls were injected the vehicle (olive oil). The animals were killed with CO_2 and decapitated at the start of the light cycle. Blood was collected from the neck and serum prepared and frozen at –20ºC. Interscapular brown adipose tissue (BAT), inguinal WAT, epididymal WAT, retroperitoneal WAT and liver were excised in their entirety, weighed, frozen in liquid nitrogen and stored at –70ºC until analysis. Biometric and blood parameters of animals used in the present study have already been published [28]. Insulin resistance was assessed by the homeostatic model assessment for insulin resistance (HOMA-IR) in additional twelve-week-old NMRI male mice treated as described above with vehicle (n=6) or 50 mg ATRA/kg body weight (n=6) and submitted to a 6-h fast (from 24:00 to 06:00) after the last vehicle/ATRA injection. The HOMA-IR score was calculated from fasting insulin and glucose concentrations using the formula of Matthews et al. [29]: HOMA-IR = fasting glucose (mmol/L) x fasting insulin (mU/L)/22.5. All animal experimentation was conducted according to accepted standards of humane care and use of laboratory animals and the studies were approved by our institutional bioethical committee.

Cell culture and differentiation

3T3-L1 cells (American Type Culture Collection, LGC Deselaers SL, Barcelona, Spain) were grown to confluence in basal medium – Dulbecco's modified Eagle's medium (DMEM; Sigma) with 50 IU/ml penicillin (Sigma), 50 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma) – supplemented with 10% new born calf serum (Linus, Madrid, Spain). Two days after the cells reached confluence (referred as day 0) they were induced to differentiate in basal medium containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), 1 µM dexamethasone (DEX; Sigma), 0.5 mM methylisobutylxanthine (MIX; Sigma) and 1 µg/ml insulin (Sigma) for 2 days, followed by 2 days in basal medium containing 10% FBS and 1 µg/ml insulin. The cells were subsequently refed every other day fresh

basal medium supplemented with 10% FBS (without insulin). Primary mouse embryonic fibroblasts (MEFs) were prepared and cultured essentially as described previously [30]. In brief, MEFs on passage 3-4 were grown to confluence in AmnioMAX™-C100 basal medium (Invitrogen) supplemented with 7.5% AmnioMAX™-C100 supplement (Invitrogen), 7.5% FBS, 50 IU/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine. For differentiation, 2-day postconfluent cells were treated (day 0) with growth medium containing 1 µM DEX, 0.5 mM MIX, 5 µg/ml insulin and 0.5 µM rosiglitazone (BioVision, Mountain View, CA) for 2 days; the cells were subsequently refed every other day fresh medium containing 5 µg/ml insulin and 0.5 µM rosiglitazone, and were deprived of rosiglitazone from day 6 on. In both cell models, adipogenesis was monitored by examination of the cells for lipid accumulation using phase-contrast microscopy. ATRA, all-trans retinaldehyde or the retinoid receptors agonists isopropyl- (E,E)-(R,S)-11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate (methoprene) and p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid (TTNPB) (all from Sigma) were added together with fresh medium when more than 90% of the cells had acquired the adipose phenotype, at the doses and times indicated in the figure legends; control cells received an equal volume of vehicle (DMSO). Cytotoxicity of ATRA and the retinoid receptors agonists at the working concentrations used over that of DMSO was measured by the lactate dehydrogenase method [31], using a commercial kit (Roche Diagnostics GmbH, Mannheim, Germany).

Real-time PCR

Total RNA was extracted from cultured cells and tissues using Trizol reagent (Invitrogen) according to the supplier's instructions. For cDNA synthesis, 1 µg total RNA was denatured at 65ºC for 10 min prior to being reverse transcribed using murine leukemia virus reverse transcriptase (Perkin-Elmer, Madrid, Spain) in the presence of 50 pmol of random primers, in a Perkin-Elmer 2400 Thermal Cycler. After diluting the cDNA samples, real time PCR analyses were conducted using the LightCycler System with SYBR Green I sequence nonspecific detection (Roche Diagnostic Gmbh, Mannheim, Germany) and LRP10 [32] and β-actin as reference genes. Sense and antisense primers used were designed with specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA), and their specificity analyzed by the ENTREZ and BLAST databases utilities (National Center for Biotechnology Information, Bethesda, MD). The primers used were: for RBP4, 5'-ACT GGG GTG TAG CCT CCT TT-3' and 5'-GGT GTC GTA GTC CGT GTC G-3'; for LRP10, 5'-TCC CCT TTC TTC TCC TCC TC-3' and 5'-TTA CCG TCT GTT CCT TGC TG-3'; and for β-actin, 5'-TAC AGC TTC ACC ACC ACA GC-3' and 5'-TCT CCA GGG AGG AAG AGG A T-3'.

Immunoblotting analysis

Adipose tissue and liver samples were homogenized in phosphate-buffered saline supplemented with protease inhibitors using a Teflon/glass homogenizer. After centrigugation at 500 g for 10 min at 4°C, the supernatant was used for total protein quantification with the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein in supernatants (20-60 µg) and in 3 µl of serum was solubilized and boiled in Laemmli sample buffer containing 20% 2-mercaptoethanol, fractionated in 13% SDS-PAGE gels, and electrotransferred onto PVDF membranes (Bio-Rad, Madrid, Spain). Black amide B10 staining provided visual evidence for correct loading and blotting of proteins. After blocking, membranes were incubated with polyclonal anti-mouse RBP4 antibody raised in rabbits (Axxora, LLC, San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as secondary antibody (from Amersham Biosciences, UK). The immunocomplexes were revealed using an enhanced chemiluminiscence detection system (Amersham Biosciences, Barcelona, Spain). Membranes were exposed to Hyperfilm ECL (Amersham Biosciences), and bands in films were analyzed by scanner photodensitometry and quantified using the BioImage program (Millipore, Bedford, MA). Serum RBP4 was also assessed by immunoblotting after fractionation of whole serum by nondenaturing PAGE (7% acrylamide), which has been reported to allow the separation of retinol bound-, retinoic acid bound- and apo-human RBP4 complexes [33-35]. Delipidized serum from control mice was prepared as previously described to serve as a marker for the apo-RBP4:transthyretin complex in nondenaturing PAGE [35].

Adipose depot	RBP4 mRNA levels (% of liver level)
epidiymal WAT	6.20 ± 0.77
retroperitoneal WAT	23.25 ± 7.56
inguinal WAT	6.31 ± 2.10
interscapular BAT	10.27 ± 1.17

Table 1. RBP4 mRNA levels in adipose depots of NMRI mice: Relative RBP4 mRNA levels were determined by real time PCR in the indicated adipose depots and liver of twelve-week-old NMRI male mice (n=6) using β-actin as reference gene. Results are expressed as a percentage of the relative RBP4 mRNA levels in liver, which was set as 100%.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance of effects was assessed by Student's *t*-test or one-way ANOVA followed by least-significance difference (LSD) post-hoc comparison. Results were considered statistically significant when $P < 0.05$.

Results

RBP4 mRNA expression pattern in mouse fat depots

The anatomic pattern of RBP4 mRNA expression in the different fat depots has been reported in the rat [3] but not, to our knowledge, in the mouse. We compared

	Control	ATRA treatment			ANOVA
		10 mg/kg	50 mg/kg	100 mg/kg	
epididymal WAT	100 ± 15[a]	78 ± 12[ab]	47 ± 8[bc]	69 ± 7[ac]	P<0.05
inguinal WAT	100 ± 20[a]	24 ± 7[b]	20 ± 5[b]	7 ± 3[b]	P<0.001
retroperitoneal WAT	100 ± 13[a]	60 ± 17[b]	62 ± 5[b]	58 ± 16[b]	P<0.05
interscapular BAT	100 ± 11	100 ± 15	86 ± 17	54 ± 7	P=0.056
liver	100 ± 6	74 ± 5	87 ± 7	93 ± 24	

Table 2. Effect of ATRA treatment on RBP4 mRNA levels in adipose depots and liver of NMRI mice: RBP4 mRNA levels were determined in the indicated tissues of twelve-week-old NMRI male mice treated with vehicle (olive oil) or ATRA (one daily subcutaneous injection, at the indicated dose, during the 4 days before the animals were killed). Data are the mean ± SEM of 8-12 animals per group distributed in two independent experiments and are expressed relative to the mean value of the control group, which was set as 100%. Relative levels of RBP4 mRNA were analyzed by real time PCR using β-actin as the reference gene. Values within a row not sharing a common letter are statistically different by LSD post-hoc comparison (P <0.05).

the expression levels of RBP4 mRNA in visceral WAT depots (retroperitoneal and epididymal), subcutaneous (inguinal) WAT and interscapular BAT of NMRI mice relative to the liver levels by real time PCR (Table 1). Levels of RBP4 mRNA in the retroperitoneal, epididymal, inguinal and interscapular mouse adipose depots averaged, respectively, 23.3%, 6.2%, 6.3% and 10.3% the levels in mouse liver. This expression pattern is similar to the rat pattern except for the epididymal depot, for which RBP4 expression was proportionally higher in the rat [3]. In humans, a higher RBP4 gene expression in visceral *versus* subcutaneous fat and correlation of RBP4 serum levels with intra-abdominal fat mass have been reported [36, 37].

ATRA treatment in NMRI mice led to reduced RBP4 mRNA and protein levels in fat depots and triggered an increase in serum RBP4 protein levels.

We first examined RBP4 regulation by ATRA in a model of acute ATRA treatment *in vivo* that has previously been shown to improve glucose tolerance and insulin sensitivity, to decrease body weight and adiposity independent of changes in food intake, and to suppress adipose expression and circulating levels of resistin and leptin in the treated mice [25, 27, 28, 38-40]. This treatment also increases thermogenic capacity in BAT [38, 39] and thermogenic and fat oxidation capacity in skeletal muscle [41], and favors the acquisition of BAT-like properties in WAT depots [28], without increasing aminotransferase activity in serum [27] – a marker of hepatic damage, which can arise from vitamin A excess. Further confirming an effect of this treatment in increasing insulin sensitivity, we report here that the HOMA-IR score was lower in mice treated with 50 mg ATRA/kg body weight than in vehicle-treated mice (ATRA-treated group, 1.69 ± 0.35; vehicle-treated group, 4.22 ± 0.91; P< 0.05, Student's t test).

ATRA treatment triggered a significant down-regulation of RBP4 mRNA levels in all WAT depots examined, but not in the liver (Table 2). Down-regulation was especially marked in the inguinal WAT depot, where RBP4 mRNA levels were already reduced by 76% at the lowest ATRA dose tested (10 mg per kg body weight) and almost completely suppressed at the highest (100 mg ATRA per kg body weight). In the retroperitoneal WAT depot, RBP4 mRNA levels were reduced by 40% following treatment with 10 mg ATRA per kg body weight, and treatment with higher doses of ATRA resulted in similar reductions. In the epididymal WAT depot, down-regulation was maximal (53% reduction) in the animals treated with 50 mg ATRA per kg of body weight. A down-regulatory effect of ATRA treatment on RBP4 mRNA expression in the interscapular BAT depot did not reach statistical significance (P=0.056) but became evident (46% decrease) at the highest ATRA dose used.

Immunoblotting following SDS-PAGE was used to estimate total RBP4 protein levels in tissues and serum. In keeping with the down-regulation seen at the mRNA level, RBP4 protein levels were significantly reduced in the epididymal WAT depot (Figure 1a) and had a tendency (that just failed to reach statistical significance, P=0.056) to be reduced in the inguinal WAT depot of ATRA-treated mice as compared with vehicle-treated controls (Figure

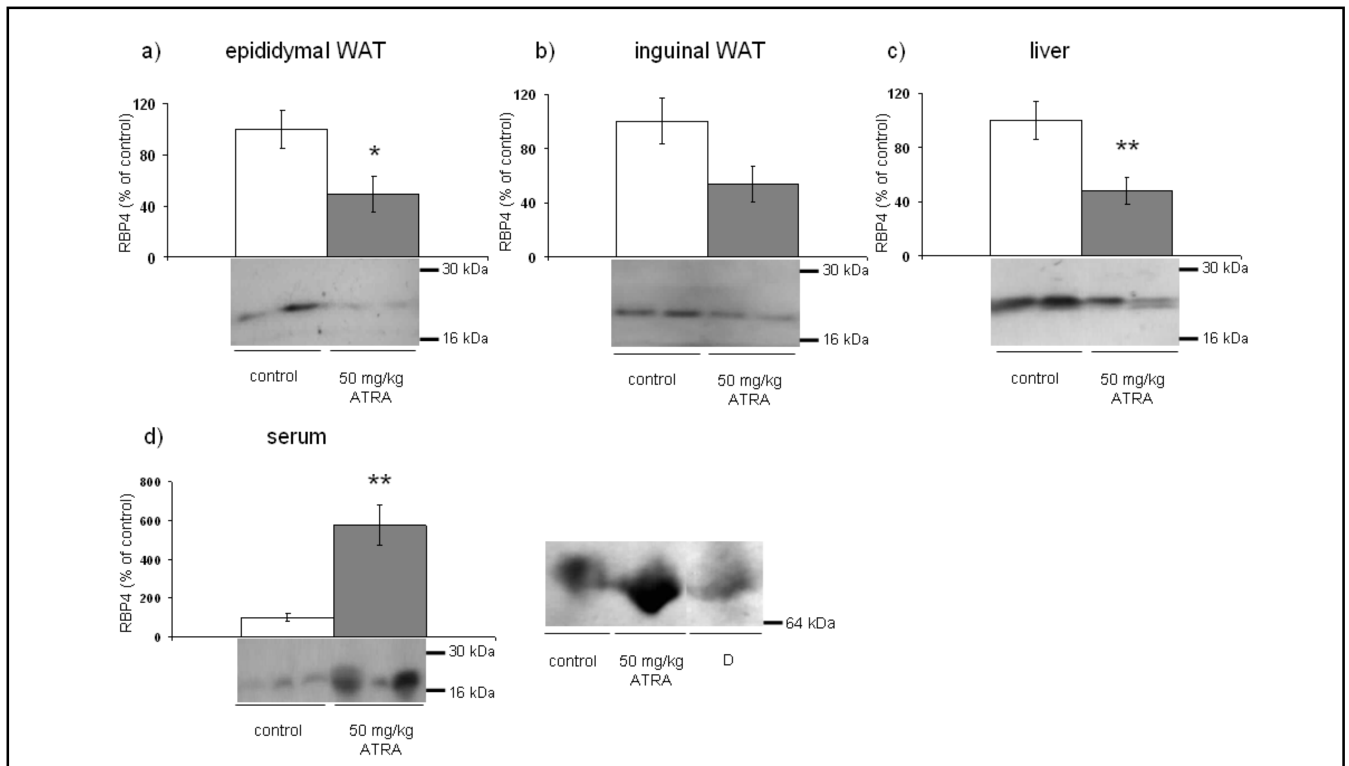


Fig. 1. Effect of ATRA treatment on tissue and serum RBP4 protein levels in NMRI mice. RBP4 protein levels were analyzed by immunoblotting after SDS-PAGE in epididymal WAT (a), inguinal WAT (b), liver (c), and serum (d, right) of twelve-week-old NMRI male mice treated with vehicle (control group) or 50 mg ATRA/kg body weight (one daily subcutaneous injection during the 4 days before the animals were killed). The blot to the left of panel d shows immunodetection of serum RBP4 complexes after nondenaturing PAGE (7% acrylamide) in control mice and ATRA-treated mice; delipidized serum (D) was run to serve as a marker for the apo-RBP4:transthyretin complex . Data in the bar diagrams are the mean ± SEM of 7-10 animals per group distributed in two independent experiments, and are expressed relative to the mean value of the control group, which was set as 100%; at the bottom of each bar diagram representative Western blots are shown (60 µg of total WAT protein, 20 µg total liver protein and 3 µl of serum per lane). Student's *t* test significance: *, *P* <0.05; ** *P*<0.01.

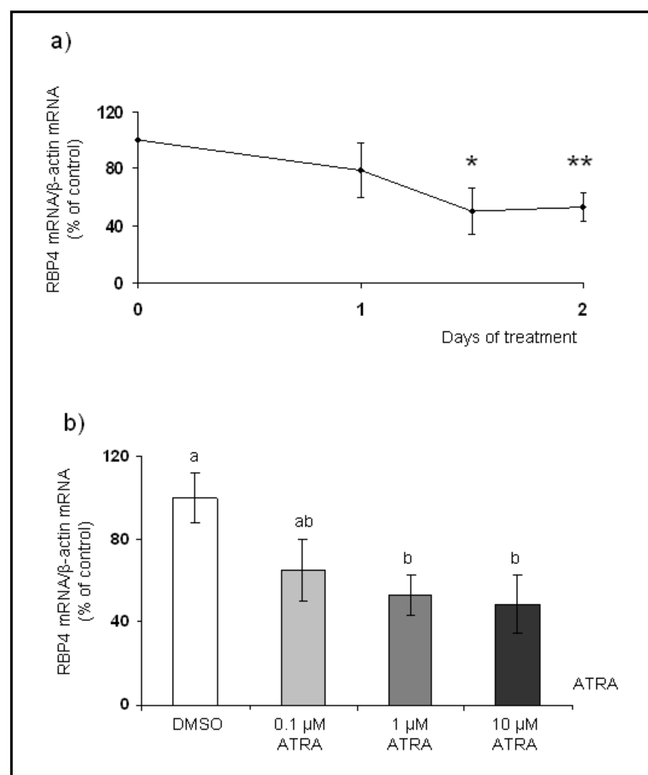
1 a and b, respectively). RBP4 protein content was also significantly decreased after ATRA treatment in the liver (Figure 1c), despite no treatment effect on liver RBP4 mRNA levels (Table 2). Serum total RBP4 levels were approx. 6-fold higher in the ATRA-treated mice than in the vehicle-treated mice (Figure 1d left). We also performed immunodetection of RBP4 following nondenaturing PAGE of whole serum. Results of this analysis indicated the presence in the serum of ATRA-treated mice of RBP4 complexes of higher mobility than those present in the serum of control mice (Figure 1 d, right). Delipidized mouse serum, which was used to locate the apo-RBP4:transthyretin complex, gave a band with mobility in between that of the RBP4 complexes present in serum of control and ATRA-treated mice. In the light of previous literature [33-35], this pattern of electrophoretic mobilities is consistent with serum complexes consisting mainly of retinol-RBP4:transthyretin in the control mice, and of retinoic acid-RBP4: transthyretin in the ATRA-treated mice.

ATRA reduced RBP4 mRNA expression and the amount of secreted RBP4 in cultured white adipocytes.

We next examined RBP4 regulation by ATRA in adipocyte cell model systems. Exposure of mature 3T3-L1 adipocytes to 1 µM ATRA reduced RBP4 mRNA expression levels by approx. 50% after 36 h and 48 h treatment (Figure 2a). ATRA-induced down-regulation of RBP4 gene expression in 3T3-L1 adipocytes was dose-dependent, reaching a plateau at 1 µM (Figure 2b). Exposure to 1 µM rosiglitazone for 48 h also resulted in a significant (*P*<0.05), 40% decrease of RBP4 mRNA levels in 3T3-L1 adipocytes, in keeping with previous results *in vivo* [6].

Since RBP4 was expressed at very low levels in

Fig. 2. ATRA down-regulates RBP4 mRNA levels in 3T3-L1 mature adipocytes. Differentiated 3T3-L1 adipocytes (on day 8-10) were treated with vehicle (DMSO) and 1 μM ATRA for the indicated time-intervals (a) or the indicated ATRA concentrations for 48 h (b) before harvesting and total RNA extraction. RBP4 mRNA levels were analyzed by real time PCR and normalized to the expression of β-actin. Data are expressed relative to the mean value of the vehicle-treated cells, which was set as 100%, and are the mean ± SEM of n= 5-12 plates (a) or n= 7-10 plates (b) per treatment condition, distributed in at least two independent experiments. *, P <0.05, Student's t test; **, P<0.01, Student's t test; ATRA, effect of ATRA treatment, one-way-ANOVA. In panel b, bar values not sharing a common letter are statistically different by LSD post-hoc comparison (P <0.05).



3T3-L1 adipocytes, making reproducibility difficult, we sought to contrast the above results in an alternate adipocyte cell model system, namely primary MEFs-derived adipocytes, which we found express RBP4 mRNA at much higher levels (approx. 100-fold higher). Exposure of primary MEFs-derived adipocytes for 48 h to 0.1 μM, 1 μM and 10 μM ATRA reduced RBP4 mRNA levels by 46%, 56% and 69%, respectively (Figure 3a). Exposure to ATRA also reduced the amount of RBP4 protein accumulated in the culture medium (Figure 3b), indicating that the effect on RBP4 gene transcription translated into reduced secreted RBP4 protein. Retinaldehyde – which is primarily considered to be a precursor for retinoic acid formation but may also exert distinct, retinoic acid-independent effects in adipose cells [42] – also down-regulated RBP4 mRNA levels in MEFs-derived adipocytes, although with a lower potency than ATRA (Figure 3c). We also assayed whether synthetic compounds that selectively bind to and activate the retinoid receptors were able to reproduce the inhibitory effect of ATRA on RBP4 gene expression (Figure 3d). Similar to exposure to 1 μM ATRA, exposure for 48 h to 1 μM TTNPB – a pan-agonist of all retinoic acid receptors (RARs) – reduced RBP4 mRNA levels by 60% in primary MEFs-derived adipocytes. Methoprene – a pan-agonist of the rexinoid receptors (RXRs) – also reduced RBP4 mRNA levels in these cells in a dose-dependent manner, although with a lower potency than ATRA and TTNPB. Similar effects of TTNPB and methoprene on RBP4 gene expression were found in 3T3-L1 adipocytes (data not shown). No cytotoxicity of ATRA or the retinoid receptors agonists at the working concentrations used over that of vehicle (DMSO) was detected in the cell models used by the lactate dehydrogenase method.

Discussion

In this work, we provide novel evidence of a down-regulatory effect of retinoic acid on adipose RBP4 gene expression. This down-regulation was apparent both in adipose depots of ATRA-treated mice and, after exposure to ATRA, in two adipocyte cell model systems with different endogenous levels of RBP4 expression, namely 3T3-L1 adipocytes and primary MEFs-derived adipocytes. Down-regulation of adipose RBP4 mRNA levels by ATRA correlated with reduced RBP4 protein content in adipose tissues of ATRA-treated mice and, in the cultured adipocytes, was accompanied by a reduction in the amount of RBP4 secreted in the culture medium and reproduced by retinaldehyde and by both RAR and RXR agonists.

Previous literature on the regulation of adipose RBP4 expression by retinoids is scarce. RBP4 expression has

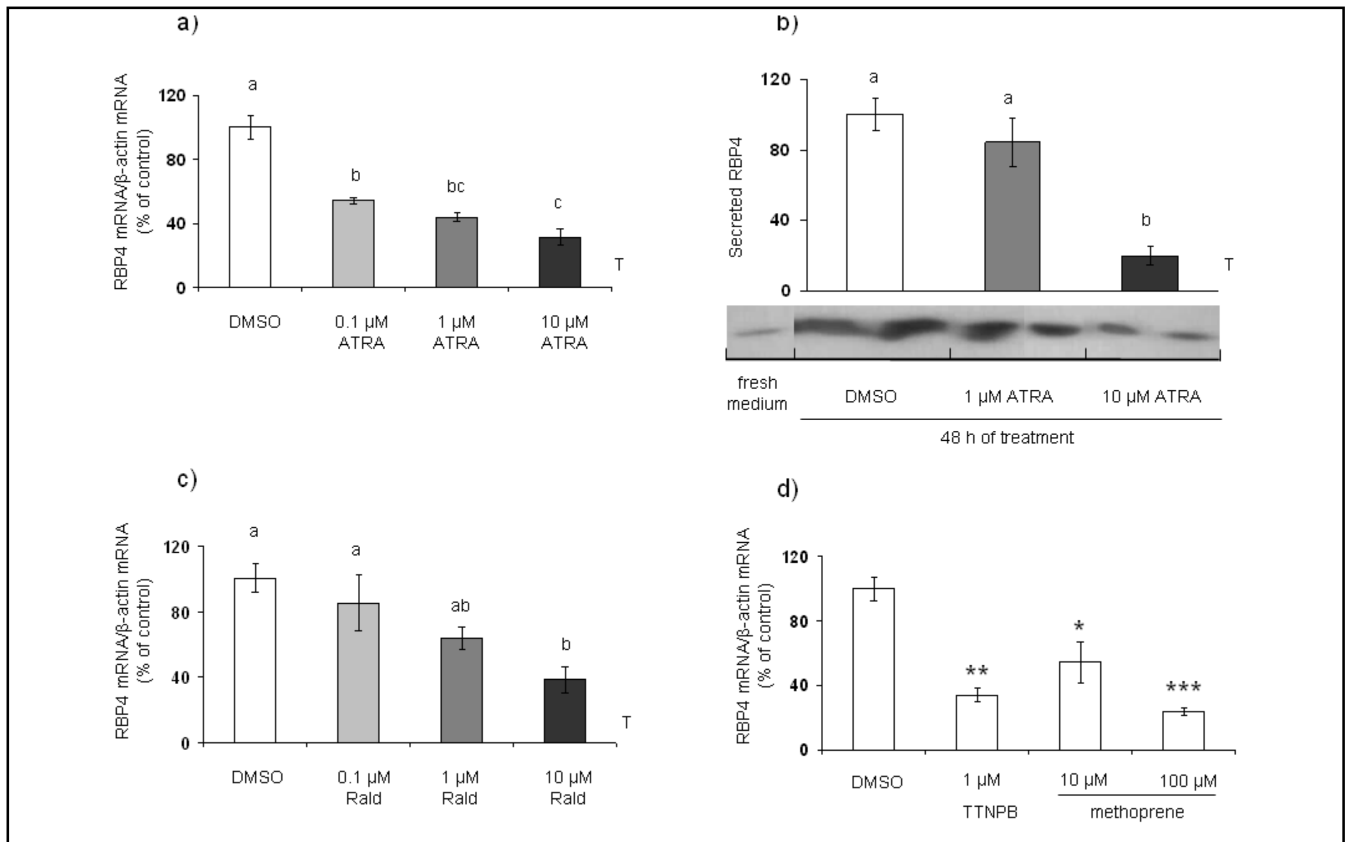


Fig. 3. Retinoids down-regulate RBP4 expression in primary MEFs-derived adipocytes. MEFs-derived adipocytes that had been deprived of rosiglitazone from day 6 were treated on day 8 with vehicle (DMSO) or problem compounds for 48 h, followed by total cellular RNA extraction and medium collection. a) Effect of ATRA on RBP4 mRNA levels. b) Effect of ATRA on RBP4 protein accumulation in the culture medium. Representative Western blots for RBP4 in fresh medium and in medium after 48 h incubation with cells and the indicated additions are shown at the bottom (25 µl of medium per lane). c) Effect of retinaldehyde (Rald) on RBP4 mRNA levels. d) Effect of retinoid receptors agonists on RBP4 mRNA levels. Data in the bar diagrams are expressed relative to the mean value of the vehicle-treated cells, which was set as 100%, and are the mean ± SEM of n= 6-7 plates per treatment condition distributed in two independent experiments (a-c) or n= 3 plates per treatment condition (d). T: one-way-ANOVA, effect of treatment; bar values not sharing a common letter are statistically different by LSD post-hoc comparison ($P < 0.05$). *, **, ***: different from vehicle, Student's t ($P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively).

been reported to be unaffected either by retinol in differentiating BFC-1β cells (a preadipocyte cell line derived from mouse BAT) [5] or by 24 h ATRA treatment in primary cultures of rat adipocytes [43]. Though species-specific differences cannot be discarded, differences between the latter report and our work may be related to differences in the duration of the treatment, because we found the down-regulatory effect of ATRA on adipocyte RBP4 mRNA levels following a relatively lengthy time-course. This suggests that ATRA may be inducing the expression in adipose cells of a protein or proteins that repress RBP4 gene transcription. Attempts to contrast this hypothesis by using an inhibitor of translation (cycloheximide) were hindered by the fact that the inhibitor itself markedly suppressed RBP4 mRNA levels in MEFs-derived adipocytes, suggesting that relatively short-lived transcription factors are involved in basal RBP4 gene transcription in these cells. In any case, it is possible that repression of RBP4 expression is secondary to ATRA-induced metabolic/morphological changes in mature adipocytes, in which ATRA has been shown to favor remodeling toward increased oxidative metabolism and decreased triacylglycerol content [28, 44].

In contrast with the down-regulation seen in adipose depots, ATRA treatment had no effect on RBP4 mRNA levels in liver. Previous studies on the effect of retinoids

on liver RBP4 expression reveal a complex picture. On the one hand, animal studies have shown that liver RBP4 mRNA levels do not change in response to changes in the vitamin A status (reviewed in [45]). On the other hand, it has been reported that retinol and retinoic acid induce RBP4 mRNA expression in human and mouse hepatoma cell lines [4, 46] and that, in mice, a single oral dose of retinoic acid (either ATRA or the 9-cis isomer) up-regulates liver RBP4 mRNA levels [4]. Moreover, a retinoic acid response element in the promoter region of the RBP4 gene has been described that allows transactivation of transcription by liganded RAR homodimers, RXR homodimers and, especially, RAR:RXR heterodimers in cells of hepatic origin [47]. We were unable to detect an increase in liver RBP4 mRNA levels following subcutaneous ATRA injection for 4 consecutive days in mice. It is possible that both the regimen of ATRA treatment (single *versus* repeated doses) and the way of administration (oral *versus* subcutaneous) are factors that influence effects on liver RBP4 gene transcription. In any case, our results reveal a differential regulation of liver and adipose RBP4 gene expression by ATRA that deserves further investigation and may be related to differences in the battery of retinoid receptors and transcriptional cofactors expressed in each tissue.

Treatment of adult, vitamin A-sufficient mice with ATRA led to a marked increase of RBP4 levels in serum. Adipose tissue is unlikely to be a source for this increase, because ATRA reduced RBP4 gene expression both in WAT depots *in vivo* and in cultured adipocytes and, in the cell model, this was accompanied by a reduction in RBP4 protein accumulation in the culture medium. The liver is a likely source, not only because it is the main site of RBP4 production but also because in this tissue ATRA treatment depleted RBP4 protein levels without affecting RBP4 mRNA levels, suggesting that reduction in liver RBP4 protein content reflects enhanced release into the circulation. Importantly, in support of this interpretation, previous reports have shown that retinoic acid can bind to RBP4 [48, 49] and stimulate secretion of RBP4 by cultured liver cells [50, 51], similar to retinol, whose positive effect on hepatic RBP4 secretion (in the form of holo-protein) is well established [45]. Thus, a plausible explanation for the increased serum RBP4 levels in ATRA-treated mice is that excess ATRA in liver cells is favoring the export of the retinoid bound to RBP4, potentially minimizing toxicity. This is in line with our nondenaturing PAGE results, which are consistent with a preponderance of the retinoic acid-RBP4:transthyretin complex in serum of the ATRA-treated mice.

Since glucose tolerance and insulin sensitivity were improved after ATRA treatment ([25] and this work), our results would indicate that increased serum levels of holo-RBP4 of non-adipose origin do not cause *per se* insulin resistance in mice. Although the idea that insulin resistance is related to adipose-derived rather than liver-derived RBP4 was implicit in the seminal work by Yang et al. [6], the molecular basis for this difference remains unclear (whether or not there may be differences in the glycoprotein moiety, for instance). It is also unclear whether apo-RBP4, holo-RBP4 or both are the active species for the effect [6]. In fact, an involvement of apo-RBP4 seems more plausible, as some of the manipulations that were reported [6] to result in increased insulin resistance (such as overexpression of RBP4 in transgenic mice and injection of RBP4 in normal mice) are not to be expected to translate into differences in serum holo-RBP4 levels in the absence of changes in the vitamin A status of the animals. Moreover, a recent report showed that, in children, the serum RBP4 to serum retinol ratio more strongly correlates with components of the metabolic syndrome than serum RBP4, suggesting a specific involvement of apo-RBP4 [10].

Results in this work are compatible with adipose-derived RBP4 being a contributor factor to systemic insulin resistance in mice, as ATRA treatment down-regulated RBP4 expression specifically in adipocytes (but not in the liver) while it enhanced systemic glucose tolerance and insulin sensitivity of the animals ([25] and this work). Remarkably, ATRA treatment also suppresses the adipose expression of another insulin resistance factor of adipose origin in mice, namely resistin [25], as well as the adipose expression of leptin [25-27]. RBP4 [20], resistin [52-54] and leptin [55] are known to exert paracrine effects in WAT antagonizing insulin signaling, which in WAT is pro-adipogenic and pro-lipogenic, and ATRA is an agent that promotes body fat loss in mice *in vivo* and a metabolic remodeling of adipocytes (both *in vivo* and in cell models) toward increased oxidative metabolism [24, 28, 44]. Given the essential role of adipose cells in fat storage, from a teleological point of view the ATRA-induced down-regulation of adipose RBP4, resistin and leptin expression may be viewed as part of a security mechanism to avoid fat depletion (by increasing local insulin sensitivity in the adipocytes, and also through the CNS-mediated consequences of reduced leptinemia) in the context of an overall fat-mobilizing effect of ATRA. Agents displaying fat-mobilizing effects coupled to down-regulatory effects on the expression of insulin resistance

factors of adipose origin might be ideally suited to serve as insulin sensitizers, provided that safety issues can be guaranteed.

In summary, this work demonstrates a selective effect of ATRA inhibiting RBP4 expression specifically in adipocytes, which correlated with increased systemic insulin sensitivity in the ATRA-treated mice. These results are compatible with an involvement of adipose-derived RBP4 in the development of insulin resistance, and reinforce the concept that vitamin A vitamers may affect insulin sensitivity through effects on adipokine production.

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MANUSCRITO III

Distinct effect of oleic acid and its trans isomer elaidic acid on the expression of miokines and adipokines in cell models.

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Distinct effects of oleic acid and its *trans*-isomer elaidic acid on the expression of myokines and adipokines in cell models

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Abstract

Trans-fatty acids (TFA) and *cis*-monounsaturated fat appear to exert detrimental and beneficial effects, respectively, on glucose metabolism and insulin sensitivity. Adipose tissue and skeletal muscle are a source of signalling proteins (adipokines and myokines), some of which have been related to the control of insulin sensitivity. Here, we investigated the possible differential effects of elaidic acid (EA; *trans*-9-18 : 1) – the major component in industrially produced TFA – and oleic acid (OA; *cis*-9-18 : 1) – its *cis*-isomer naturally present in food – on cellular glucose uptake and the expression of selected myokines and adipokines using cell models. Differentiated C2C12 myotubes and 3T3-L1 adipocytes were pretreated with the vehicle (control cells) or fatty acids for 24 h, after which basal and insulin-stimulated 2-deoxyglucose uptake and the expression of selected signalling proteins were measured. In C2C12 myotubes, pretreatment with OA, but not with EA, led to increased insulin-stimulated 2-deoxyglucose uptake and IL-6 expression levels, while pretreatment with EA, but not with OA, led to reduced IL-15 mRNA levels and increased TNF-α expression levels. In 3T3-L1 adipocytes, exposure to OA, but not to EA, resulted in reduced resistin gene expression and increased adiponectin gene expression. The results show evidence of distinct, direct effects of OA and EA on muscle glucose uptake and the expression of target myokines and adipokines, thus suggesting novel mechanisms by which *cis*- and *trans*-monounsaturated fat may differentially affect systemic functions.

Key words: Adipokines and myokines: Glucose uptake: *Trans*-fatty acids: Oleic acid: Cell models

Trans-fatty acids (TFA) are unsaturated fatty acids with at least one double bond in the *trans*, rather than the typical *cis*, configuration. A major source of TFA in food is industrially produced *trans*-fatty acids (IP-TFA) generated during partial hydrogenation ('hardening') of vegetable fat. Industrial hydrogenation involves heating the raw oils to about 400°C under high pressure and with the addition of different catalysts, and in this process, *trans*-double bonds are undesirably formed along with fatty acid molecules from position six and higher, at the same time as SFA are formed. Partially hydrogenated fat is used in a variety of food products, especially spreads, baked goods and fast food. Elaidic acid (EA; *trans*-9-18 : 1), the *trans*-isomer of oleic acid (OA), is the predominant IP-TFA[1,2].

While there is evidence pointing to favourable effects of OA-enriched diets and of OA itself on CVD risk, weight maintenance and insulin sensitivity[3–5], dietary TFA is generally associated with detrimental effects on health[2,6,7]. Importantly, deleterious effects are well documented for IP-TFA, but not for TFA of ruminant origin, in the low amounts usually consumed[2,6]. In fact, health benefits have been suggested for conjugated linoleic acid and vaccenic acid (*trans*-11-18 : 1), the major TFA naturally present in beef and dairy products[8,9].

Adverse effects of IP-TFA consumption on multiple cardiovascular risk factors are well established, including promotion of a pro-atherogenic serum lipid/lipoprotein profile, endothelial dysfunction and an increased production of pro-inflammatory cytokines[6,7,10]. The impact of IP-TFA consumption on insulin resistance and the risk of type 2 diabetes is more controversial, since detrimental effects have been reported in some, but not all, human observational studies and short-term intervention trials on the topic[11–14]. Likewise, in rats, the effects of TFA-enriched diets (fed for 8–12 weeks) increasing insulin resistance and impairing systemic and tissue-specific glucose disposal have been reported in some studies[15–17], but not in others[18]. Nevertheless, in

Abbreviations: 2-DOG, 2-deoxy-D-glucose; EA, elaidic acid; FBS, fetal bovine serum; IP-TFA, industrially produced *trans*-fatty acid; OA, oleic acid; TFA, *trans*-fatty acids.

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a long-term, 5-year controlled study in monkeys, TFA feeding (*v.* OA) caused insulin resistance and impaired glucose metabolism along with abdominal obesity[19]. Two long-term prospective observational studies in humans have also suggested that TFA consumption promotes weight gain, and particularly accumulation of abdominal fat[20,21]. Abdominal obesity, insulin resistance and CVD risk are intimately linked[22].

Mechanisms underlying the relationship between dietary fatty acids and metabolic health are incompletely understood and seem to include the effects on cell-membrane fatty acid composition and function, as well as on gene expression and enzyme activity in key tissues[5]. Both adipose tissue and skeletal muscle produce and secrete signalling proteins (named adipokines and myokines, respectively), which may affect metabolic health, and particularly glucose metabolism and insulin sensitivity locally and systemically. Studies examining the possible differential, direct effects of concrete species of *cis*- and *trans*-MUFA on the cellular production of such signals are lacking, yet of potential interest to support the biological plausibility of observations derived from epidemiological studies and studies using complex mixtures. Therefore, the aim of the present study was to compare the direct effects of EA, the major IP-TFA, and its *cis*-isomer, OA, naturally present in foods on the expression of myokines and adipokines possibly involved in the control of insulin sensitivity, along with their effects on basal and insulin-stimulated glucose uptake, in muscle and adipocyte cell models.

Experimental methods

Cell culture and fatty acid treatment

All reagents used for cell culture and treatment including insulin from bovine pancreas (product no. I4011), OA (product no. O1008) and EA (product no. E4637) were from Sigma (St Louis, MO, USA), unless otherwise indicated. The purity of the fatty acids was at least 99 %. 3T3-L1 mouse pre-adipocytes (ATTC CL-173™; LGC Deselaers SL, Barcelona, Spain) were grown to confluence in basal medium – Dulbecco's modified Eagle's medium with penicillin (50 U/ml), streptomycin (50 μg/ml) and 4 mM-L-glutamine – supplemented with 10 % newborn calf serum (Linus, Madrid, Spain) under 8 % CO_2 and 92 % air at 37°C in six- or twelve-well culture plates. At 2 d after the cells reached confluence (referred to as day 0), they were induced to differentiate in basal medium containing 10 % fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), dexamethasone (1 μM), methylisobutylxanthine (0·5 mM) and insulin (1 μg/ml) for 48 h, followed by 48 h in basal medium containing 10 % FBS and insulin (1 μg/ml). The cells were subsequently refed every other day with fresh basal medium supplemented with 10 % FBS (without insulin). Adipogenesis was monitored by morphological examination of the cells for lipid accumulation using phase contrast microscopy. The cells were treated with fatty acids along with fresh medium on days 8–10 of culture, when more than 90 % had acquired the adipose phenotype. For treatment, stock solutions of fatty acids in dimethyl sulphoxide as the vehicle were prepared at 500× the desired final working concentration and diluted 1:500 with basal medium containing 10 % FBS. This mixture was pre-warmed for 45 min at 37°C with vortex every 15 min before being added to the differentiated cells[23]. Control cells received the same medium incubated with the corresponding volume of the vehicle (0·2 % dimethyl sulphoxide). Experimental parameters were measured following 24 h treatment with the fatty acids or the vehicle.

C2C12 mouse myoblasts (ATTC CRL-1772™; LGC Promochem SL, Barcelona, Spain) were cultured at 37°C with

Table 1. Primers used in the quantitative PCR

Genes	NCBI no.	Primers	Amplicon (bp)
IL-6	NM_031168.1	F: 5′-TGGGAAATCGTGGAAATGAG-3′ R: 5′-GAAGGACTCTGGCTTTGTCTT-3′	250
IL-15	NM_008357.1	F: 5′-TGTCTTCATTTTGGGCTGTG-3′ R: 5′-TGCAACTGGGATGAAAGTCA-3′	155
TNF-α	NM_013693.2	F: 5′-CGTCGTAGCAAACCACCAA-3′ R: 5′-GAGAACCTGGGAGTAGACAAGG-3′	147
Resistin	NM_022984.3	F: 5′-TTCCTTTTCTTCCTTGTCCCTG-3′ R: 5′-CTTTTTCTTCACGAATGTCCC-3′	247
Adiponectin	NM_009605.4	F: 5′-GCTCAGGATGCTACTGTTG-3′ R: 5′-TCTCACCCTTAGGACCAAG-3′	256
Retinol-binding protein 4	NM_001159487.1	F: 5′-ACTGGGGTGTAGCCTCCTTT-3′ R: 5′-GGTGTCGTAGTCCGTGTCG-3′	71
GLUT1	NM_011400.3	F: 5′-GCCCCCGCTTCCTGCTCATC-3′ R: 5′-CCCGCATCATCTGCCGACCC-3′	132
GLUT4	NM_009204.2	F: 5′-GGCATGCGTTTCCAGTATGT-3′ R: 5′-TGCCCCTCAGTCATTCTCATC-3′	234
β-Actin	NM_007393.3	F: 5′-TACAGCTTCACCACCACAGC-3′ R: 5′-TCTCCAGGGAGGAAGAGGAT-3′	120

F, forward; R, reverse.

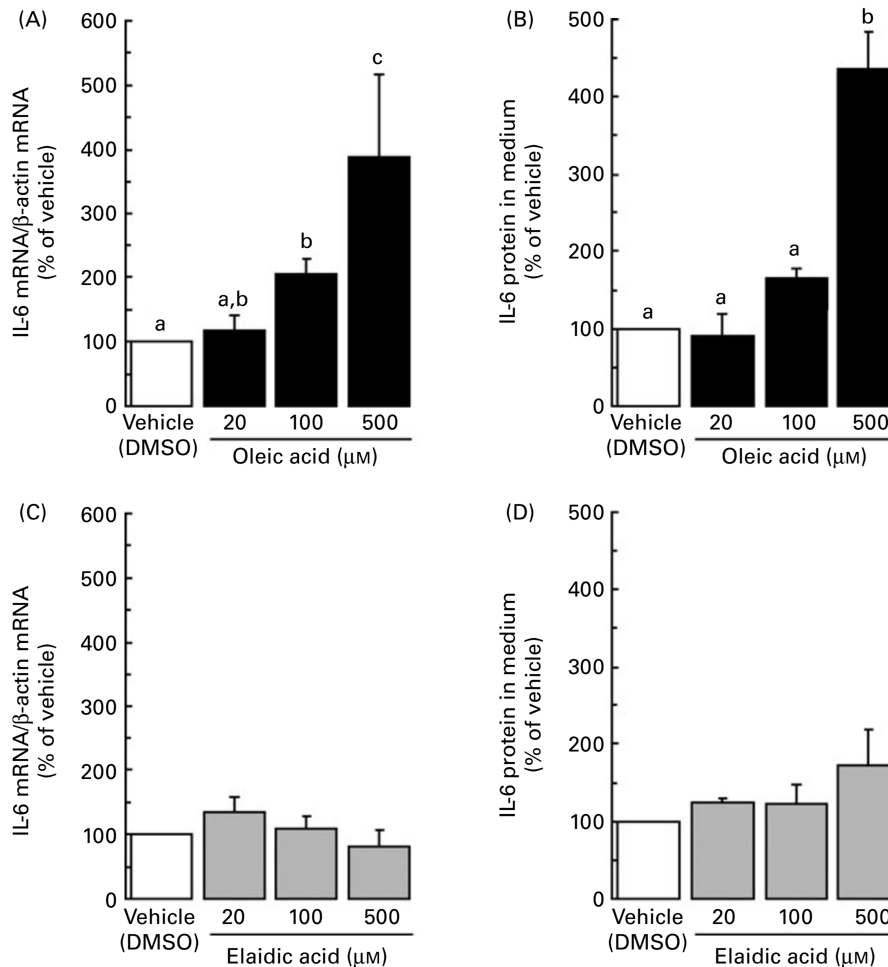

Fig. 1. Effects of 24 h exposure to varying doses of oleic acid or elaidic acid on IL-6 gene expression and secreted levels in C2C12 myotubes. Gene expression of IL-6 ((A) and (B)) was measured by real-time quantitative PCR and normalised to β-actin. IL-6 protein in the medium ((C) and (D)) was measured by ELISA. Values are means of at least three experiments (*n* 3–7) each run in triplicate, with standard errors represented by vertical bars, and are expressed relative to the mean value in the vehicle dimethyl sulphoxide (DMSO)-treated control cells, which was set at 100 %. The absolute mean value of IL-6 in the medium of the vehicle-treated cells was 429 (SE 62) pg/mg of cell protein. Data were analysed by one-way ANOVA followed by the least significant difference *post hoc* test. [a,b,c] Mean values with unlike letters were significantly different ($P < 0{\cdot}05$).

humidified 95 % air and 5 % CO_2 in Dulbecco's modified Eagle's medium supplemented with 10 % FBS, penicillin (50 U/ml), streptomycin (50 μg/ml) and 3 mM-L-glutamine (growth medium) in twelve-well culture plates. To induce differentiation, C2C12 myoblasts were allowed to reach 80 % confluence in growth medium and then changed to Dulbecco's modified Eagle's medium containing 2 % horse serum (differentiation medium). After ten additional days, the cells had differentiated and fused into myotubes, as assessed by microscopic examination. Differentiated myotubes were treated with the fatty acids or the vehicle for 24 h using the same procedure described above for adipocytes, before RNA isolation or the measurement of glucose uptake.

RNA isolation and analysis

Total RNA was extracted from 3T3-L1 adipocytes and C2C12 myotubes using Tripure reagent (Roche, Barcelona, Spain). Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), and its integrity was confirmed using agarose gel electrophoresis. A260:A280 ratios were between 1·9 and 2 for all samples, indicating good RNA purity. Retrotranscription followed by real-time quantitative PCR was used to measure the mRNA levels of resistin, adiponectin, retinol-binding protein 4, IL-6, IL-15, TNF-α, GLUT1, GLUT4 and β-actin as an internal control. In brief, 0·25 μg of total RNA (in a final volume of 5 μl) was denatured at 65°C for 10 min and then reverse transcribed to complementary DNA using murine leukaemia virus RT (Applied Biosystems, Madrid, Spain) in the presence of 50 pmol of random primers in an Applied Biosystems 2720 Thermal Cycler. Sense and antisense primers used in the PCR were designed with specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA), and their specificity was analysed by the ENTREZ and BLAST

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database utilities (National Center for Biotechnology Information, Bethesda, MD, USA). The primers were produced by Sigma (Madrid, Spain), and their sequences are shown in Table 1. Each PCR was performed from diluted (1:20) complementary DNA template, forward and reverse primers (1 μM each), and Power SYBR Green PCR Master Mix (Applied Biosystems, Madrid, Spain) on a StepOnePlusTM Real-Time PCR System (Applied Biosystems). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The relative expression of each mRNA was calculated according to Pfaffl[24], using β-actin as a reference gene.

Quantification of cytokines

IL-6 and TNF-α concentrations in the cell's conditioned culture medium were measured using ELISA kits (both from Pierce, Rockford, IL, USA), following the manufacturer's instructions.

2-Deoxy-D-glucose uptake

Analysis of 2-deoxy-D-glucose (2-DOG) uptake was performed immediately after completion of the 24 h incubation with fatty acids, essentially as described previously[25]. After washing with Krebs–Ringer phosphate buffer (10 mM-KH_2PO_4, pH 7·4, containing 136 mM-NaCl, 4·7 mM-KCl, 1·25 mM-$CaCl_2$, 1·25 mM-$MgSO_4$ and 0·05 % bovine serum albumin), cells were incubated with Krebs–Ringer phosphate buffer without glucose in the absence of insulin or in the presence of 100 nM-insulin for 15 min at 37°C. The transport assay was then initiated by the addition of ^{3}H-2-DOG (3700 Bq/ml in 1 μM unlabelled 2-DOG), and the cells were further incubated

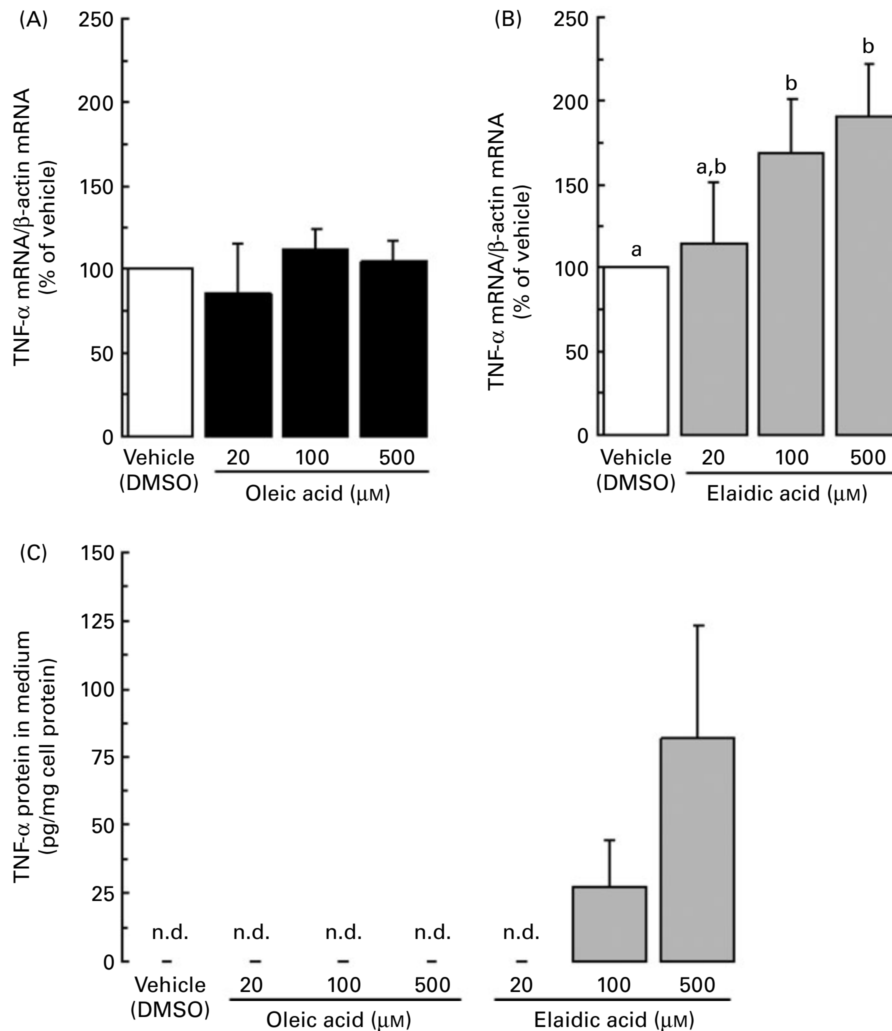

Fig. 2. Effects of 24 h exposure to varying doses of oleic acid or elaidic acid on TNF-α gene expression and secreted levels in C2C12 myotubes. Gene expression of TNF-α ((A) and (B)) was measured by real-time quantitative PCR and normalised to β-actin, and is expressed relative to the mean value in the vehicle dimethyl sulphoxide (DMSO)-treated control cells, which was set at 100 %. TNF-α protein in the medium (C) was measured by ELISA and is expressed as absolute values (n.d., not detected). Values are means of at least three experiments (*n* 3–7) each run in triplicate, with standard errors represented by vertical bars. Gene expression data were analysed by one-way ANOVA followed by the least significant difference *post hoc* test. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

for 10 min with or without 100 nM-insulin at 37°C. Cells were then washed three times with ice-cold PBS and lysed with 0·1 M-NaOH. Radioactivity in cell lysates was counted in Hi-safe 3 scintillant (Perkin Elmer, Shelton, CT, USA) using a Beckman Coulter LS 6500 multi-purpose liquid scintillation counter (Beckman Coulter, Brea, CA, USA). Counting efficiency was about 47 %. Protein concentration in cell lysates was evaluated via the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Statistical analysis

Data are expressed as means with their standard errors of at least three individual experiments, each run in triplicate or quadruplicate. Differences between treatments were assessed by one-way ANOVA, followed by a least significant differences *post hoc* comparison. Student's *t* test was used to assess the effect of insulin on glucose uptake. The threshold of significance was set at $P<0·05$.

Results

Distinct effects of oleic acid and elaidic acid on myokine expression and insulin-stimulated 2-deoxy-D-glucose uptake in C2C12 myotubes

Differentiated C2C12 myotubes were pretreated for 24 h with 20–500 μM-OA, 20–500 μM-EA or the vehicle (0·2 % dimethyl sulphoxide), and the changes elicited by fatty acid treatment on IL-6, TNF-α and IL-15 expression were analysed. Treatment with OA caused a dose-dependent increase in cellular IL-6 mRNA levels (Fig. 1(A)) and in IL-6 protein levels in the cell's conditioned culture medium (Fig. 1(B)), whereas treatment with EA had no effect on IL-6 expression either at the mRNA or at the secreted protein level (Fig. 1(C) and (D)). TNF-α mRNA levels were unaffected by OA treatment (Fig. 2(A)) and were dose dependently increased following treatment with EA by 69 % at the 100 μM dose and by 90 % at the 500 μM dose (Fig. 2(B)). In good concordance with the mRNA results, TNF-α protein was undetectable in the conditioned culture medium of the vehicle- and OA-treated cells, yet it could be consistently detected, though at very low levels, in the medium of cells treated with the highest EA doses (Fig. 2(C)). IL-15 mRNA levels were unaffected by OA treatment (Fig. 3(A)) and were significantly reduced by about 25 % following treatment with 100 or 500 μM-EA (Fig. 3(B)).

Basal and insulin-stimulated 2-DOG uptake was measured in differentiated C2C12 myotubes following 24 h exposure to 100 μM-OA, 100 μM-EA or the vehicle (Fig. 4(A)). Basal 2-DOG uptake was slightly but significantly lower in the cells that had been treated with either fatty acid compared with the vehicle-treated cells. Stimulation of the cells with 100 nM-insulin produced, as expected, a significant increase in 2-DOG uptake. Insulin-stimulated 2-DOG uptake was approximately 35 % higher ($P<0·05$) in the OA-treated cells compared with the vehicle- or EA-treated cells. Reduced basal 2-DOG uptake in fatty acid-treated myotubes was not related to reduced GLUT1 mRNA expression levels, which did not show differences between treatments (data not shown). Likewise, increased insulin-stimulated 2-DOG uptake in the OA-treated cells was not related to the differences in GLUT4 mRNA levels in these cells compared with the vehicle- or EA-treated cells (data not shown).

Distinct effects of oleic acid and elaidic acid on adipokine expression in 3T3-L1 adipocytes

Adipokine mRNA levels and 2-DOG uptake were measured in differentiated 3T3-L1 adipocytes following 24 h exposure to 100 μM-OA, 100 μM-EA or the vehicle

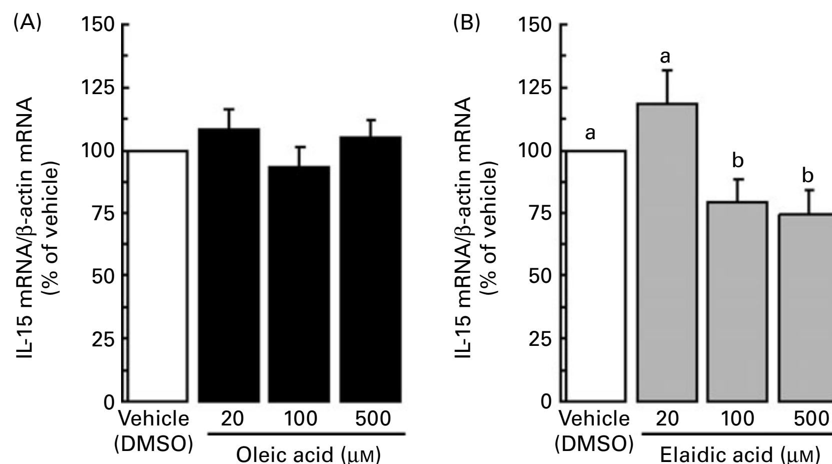

Fig. 3. Effects of 24 h exposure to varying doses of (A) oleic acid or (B) elaidic acid on IL-15 gene expression in C2C12 myotubes. Values are means of at least three experiments (*n* 3–7) each run in triplicate, with standard errors represented by vertical bars, and are expressed relative to the mean value in the vehicle dimethyl sulphoxide (DMSO)-treated control cells, which was set at 100 %. Gene expression of IL-15 was measured by real-time quantitative PCR and normalised to β-actin. Data were analysed by one-way ANOVA followed by the least significant difference *post hoc* test. [a,b] Mean values with unlike letters were significantly different ($P<0·05$).

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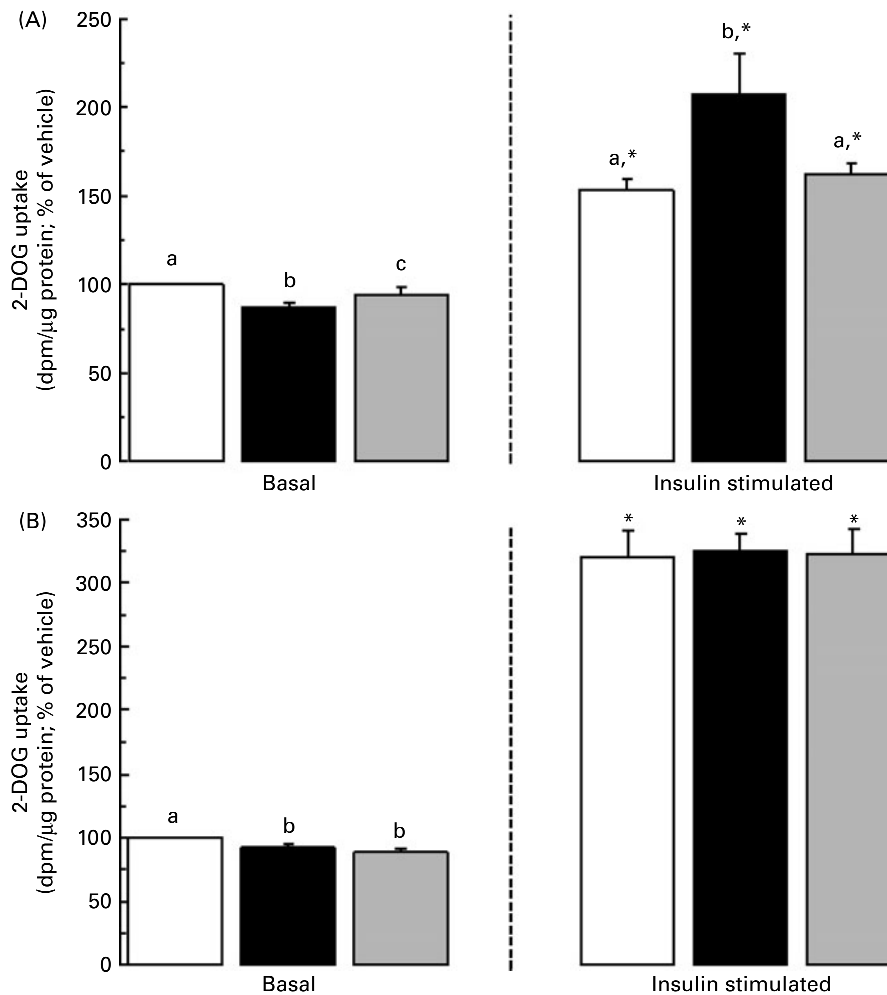

Fig. 4. Effects of 24 h exposure to 100 µM-oleic or -elaidic acid on basal and insulin-stimulated 2-deoxy-D-glucose (2-DOG) uptake in (A) C2C12 myotubes and (B) 3T3-L1 adipocytes. 2-DOG uptake was measured in the absence of insulin (basal) and in the presence of 100 nM-insulin as described in the Experimental methods. Values are means of at least three experiments (*n* 3–8) each run in quadruplicate, with standard errors represented by vertical bars, and are expressed relative to the mean value in the vehicle dimethyl sulphoxide (DMSO)-treated control cells, which was set at 100 %. One-way ANOVA followed by the least significant difference *post hoc* test was used to analyse the significance of differences among treatments under the basal and insulin-stimulated conditions; [a,b,c] for each condition, mean values with unlike letters were statistically different ($P<0·05$). * Statistically significant difference ($P<0·05$) *v.* the basal condition assessed by Student's *t* test. □, Vehicle (DMSO); ■, 100 µM-oleic acid; ▨, 100 µM-elaidic acid; dpm, disintegrations per min.

(0·2 % dimethyl sulphoxide), and the changes elicited by fatty acid treatment relative to the vehicle were analysed. Treatment with EA did not affect resistin or adiponectin mRNA levels, whereas treatment with OA resulted in a significant 28 % decrease in resistin mRNA levels and a significant 25 % increase in adiponectin mRNA levels (Fig. 5). Similar results were obtained in a separate experiment in which the fatty acids were assayed at 500 µM concentration (data not shown). The mRNA levels of the other cytokines/adipokines assayed, namely IL-6, IL-15, TNF-α and retinol-binding protein 4, were very low in 3T3-L1 adipocytes, both under the control condition and after 24 h treatment with OA or EA up to 500 µM (crossing points ≥30 in the real-time quantitative PCR). Low basal levels of expression of retinol-binding protein 4 and IL-15 mRNA in 3T3-L1 adipocytes are in agreement with previous reports[26,27]. No differential effects of OA and EA on 2-DOG uptake in 3T3-L1 adipocytes were found: pretreatment with either fatty acid similarly slightly lowered basal 2-DOG uptake relative to the vehicle, and insulin-stimulated 2-DOG uptake was equal in adipocytes pre-exposed to OA, EA or the vehicle (Fig. 4(B)).

Discussion

Skeletal muscle plays a major role in insulin-stimulated glucose disposal[28], and is nowadays recognised as a secretory organ that produces and releases bioactive proteins exerting autocrine, paracrine and endocrine effects, including local effects on muscle glucose uptake[29]. Among these is IL-6, a cytokine produced by many cell types and by muscle fibres in response to contraction[29,30]. IL-6 enhances glucose uptake by human and rat skeletal muscle cells[31–33], and secretion of IL-6 is reduced in human skeletal muscle cells that are made insulin-resistant by incubation with

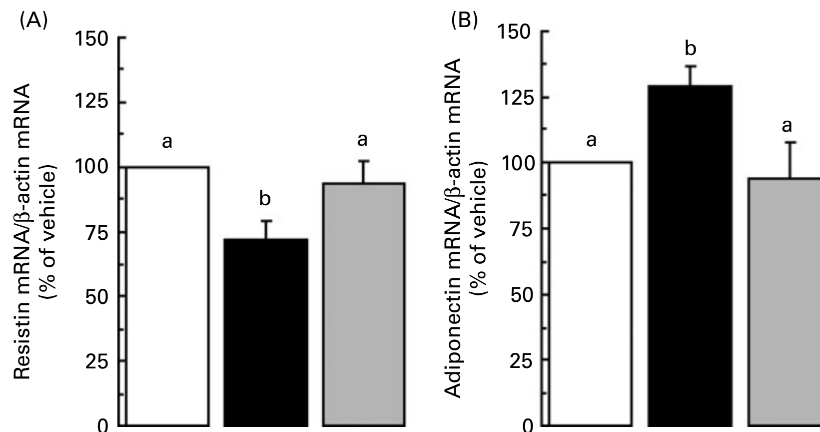

Fig. 5. Effects of 24 h exposure to 100 μM-oleic or -elaidic acid on the gene expression of (A) resistin and (B) adiponectin in 3T3-L1 adipocytes. Values are means of four experiments (*n* 4) each run in triplicate, with standard errors represented by vertical bars, and are expressed relative to the mean value in the vehicle dimethyl sulphoxide (DMSO)-treated control cells, which was set at 100 %. Gene expression was measured by real-time quantitative PCR and normalised to β-actin. Data were analysed by one-way ANOVA followed by the least significant difference *post hoc* test. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$). □, Vehicle (DMSO); ■, 100 μM-oleic acid; ▨, 100 μM-elaidic acid.

adipocyte-conditioned medium[34]. These and other findings, including reports of central and peripheral anti-obesity effects of IL-6, have challenged the classical view of IL-6 as a pro-inflammatory cytokine associated with obesity and reduced insulin sensitivity[29,30]. Our finding that incubation with OA increased both IL-6 expression and insulin-stimulated 2-DOG uptake in C2C12 myotubes supports this novel scenario for muscle-derived IL-6. The present results are in concordance with reported stimulatory effects of IL-6 on skeletal muscle glucose uptake[31–33] and with reported beneficial effects of OA on insulin sensitivity, systemically and at the skeletal muscle level[4,5,35]. In particular, OA has been shown to protect against palmitate-induced insulin resistance at the level of glucose uptake in rat L6 myotubes[35]. In that study[35], however, OA alone did not increase insulin-stimulated glucose uptake as in ours; the reasons for this difference remain unclear and may relate to differences in the cell model and treatment conditions used. Importantly, we found that the stimulatory effects of OA on insulin-stimulated 2-DOG uptake and IL-6 expression at both the mRNA and secreted protein levels in skeletal muscle cells were not reproduced by its *trans*-isomer, EA.

Treatment with either OA or EA caused in our hands a small, but consistent, reduction in cellular basal 2-DOG glucose. The present results indicate that the observed effects of fatty acids on 2-DOG uptake in muscle cells were unrelated to changes in GLUT1 or GLUT4 mRNA levels. Muscle has a high potential to adjust fuel selection depending on energy supply and demand. Fatty acids have been shown to induce the expression in muscle cells (including C2C12 myotubes) of key genes involved in their catabolism, which may facilitate the transition from a more glycolytic to a more lipid-oxidative metabolism during episodes of heavy influx of fatty acids to skeletal muscle, such as in fasting[36]. It is possible, therefore, that reduced basal glucose uptake may, by default, result as a consequence of increased cellular fatty acid catabolism. In any case, the significance and mechanistic basis of fatty acid effects on glucose uptake observed in the present study requires further investigation.

IL-15 is another myokine possibly involved in the control of muscle glucose disposal. IL-15 is highly expressed in skeletal muscle compared with other tissues and plays important regulatory roles in skeletal muscle itself, where it promotes protein synthesis, fatty acid oxidation, and glucose uptake and utilisation[37,38]. In addition to anti-diabetogenic effects, anti-adiposity effects have been proposed for muscle-derived IL-15 acting on multiple target tissues including adipose tissue[38,39]. We report here, for the first time to our knowledge, a down-regulatory effect of EA on IL-15 gene expression in skeletal muscle cells, which could have implications for the effects of TFA promoting insulin resistance and obesity evidenced in previous studies[12,13,15–17,19–21].

Adipose tissue plays a relatively modest role in insulin-stimulated glucose disposal compared with skeletal muscle, yet it contributes to the control of systemic insulin sensitivity through the secretion of NEFA and adipokines that have an impact on skeletal muscle and liver metabolism, among other mechanisms. Two important adipokines in this context are resistin and adiponectin, which exert opposite effects as an insulin resistance factor[40,41] and an insulin-sensitising factor[42], respectively. Resistin and adiponectin might also oppositely relate to inflammation, as there is evidence of pro-inflammatory properties of resistin[41] and anti-inflammatory properties of adiponectin especially with regard to atherosclerosis[43]. In our hands, exposure to OA significantly reduced gene expression of resistin and increased that of adiponectin in mature 3T3-L1 adipocytes, and these putatively beneficial effects were not reproduced by EA, which did not affect adiponectin or resistin gene

expression. We also investigated the effects on retinol-binding protein 4 – which, similar to resistin, might function as an insulin resistance factor secreted by adipocytes[44] – and found no effect of OA or EA treatment on its (very low) gene expression in 3T3-L1 adipocytes. Repression of adipocyte resistin expression by OA has been previously reported[45], and a stimulatory effect of OA on adiponectin expression might be in line with a recent report showing a positive association between serum adiponectin levels and OA content in white adipose tissue in rats[46]. In a previous study in 3T3-L1 adipocytes, however, 48 h incubation with 250 μM-OA had no effect on adiponectin gene expression[47]. Studies examining the effects of TFA on adipokine expression in adipocyte cell models are lacking, to our knowledge. There are, however, reports indicating that, in rats, consumption of TFA-enriched diets is associated with increased resistin expression[48,49] and reduced adiponectin expression in white adipose tissue[49,50]. Additionally, also in rats, a negative correlation between adipose adiponectin gene expression and TFA levels in the serum has been described[46]. Taken together, these *in vivo* studies and our cell studies suggest that the effects of TFA-enriched diets on adipokine expression observed *in vivo* are likely to be indirect.

TNF-α is a cytokine linked to inflammation and obesity-associated insulin resistance[51]. TFA intake has been reported to be associated with increased TNF system activity in observational studies in humans[10] and with increased TNF-α expression in white adipose tissue in experimental studies in rats[49,50]. In our hands, gene expression of TNF-α was negligible in 3T3-L1 adipocytes yet readily detectable in C2C12 myotubes, in which both TNF-α mRNA levels and secreted TNF-α protein increased following exposure to EA. TNF-α activity favours insulin resistance and protein wasting in skeletal muscle[52], effects that are the opposite to those exerted in muscle by IL-15[38], and, remarkably, we found here that EA oppositely affected gene expression of these two cytokines in muscle cells.

In summary, the present study revealed the distinct, direct effects of OA and EA on the expression of target myokines and adipokines. OA, but not EA, dose dependently induced IL-6 expression in skeletal muscle cells, and repressed resistin and induced adiponectin gene expression in cultured adipocytes. EA, but not OA, repressed IL-15 gene expression and induced TNF-α expression in cultured skeletal muscle cells. Overall, these findings suggest novel mechanisms by which *cis*- and *trans*-monounsaturated fat may differentially affect systemic functions.

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MANUSCRITO IV

Effects of selected compounds related to joint health on the differentiation of mouse embryo fibroblast cells: Cross-talk with the adipogenic and the chondrogenic programs.

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Dossier

DOSSIER

Effects of selected compounds related to joint health on the differentiation of mouse embryo fibroblast cells: Cross-talk with the adipogenic and the chondrogenic programs

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SUMMARY

Obesity and degenerative joint disease (osteoarthritis) are two multifactorial pathologies that are becoming major medical issues with the aging of the world population and which often appear together. The relationship of osteoarthritis with obesity is complex, underlying both biomechanical and metabolic/developmental links. Various transcription-related factors and regulatory signals have been implicated in the differentiation and/or function of both adipocytes and joint cells. Mouse embryo fibroblast (MEFs) are multipotent cells which among other lineages give rise to adipocytes and chondrocytes through mechanisms competitively balanced, and are considered a powerful tool for cartilage regeneration studies. Here, we investigated in these cells the effects of specific compounds best known for their possible usefulness in promoting joint health on some regulatory factors implicated in the development, maintenance and function of adipocytes and chondrocytes. We show that these compounds can block the adipogenic differentiation of MEFs while promoting osteo-/chondrogenic gene expression and regulating the production of adipokines in the cells. Overall, these constitute promising data suggesting that some of the compounds tested could be useful in the simultaneous management of obesity and osteoarthritis.

INTRODUCTION

Obesity is a condition where excess body fat accumulates to such an extent that health may be affected. The expanded body fat mass characteristic of the obese phenotype is associated with increased morbidity and mortality and causes an enormous disease burden to the society. Degenerative joint disease (osteoarthritis) is another pathology that, like obesity, is becoming a major public issue. The hallmarks of osteoarthritis are the irreversible destruction of cartilage, subchondral bone alterations (thickening, osteophyte formation) and synovitis (inflammation of the synovial membrane). Obesity and osteoarthritis are interrelated, and often appear together. This relationship includes biomechanical and metabolic and developmental links. Dysregulated production of adipokines and inflammatory mediators, hyperlipidemia, and increased systemic oxidative stress are, for instance, conditions frequently associated with obesity that may favor joint degeneration (1-4).

Obesity is ultimately caused by a chronic energy imbalance whereby energy intake exceeds energy expenditure. However, alterations in metabolic pathways and in processes affecting adipocyte number may also contribute to the development of obesity as well as to its treatment. Traditional weight loss regimes, such as diet and exercise, are successful in decreasing adipocyte size, but fail to reduce adipocyte number. Since many obese and almost all severely obese individuals have more than the average number of adipocytes, control of adipogenesis (the process of adipocyte differentiation from precursor cells) might contribute, along with diet and exercise, to reduce and maintain the fat mass to lean levels. In this context, a dynamic and highly regulated turnover of adipocytes has been demonstrated in adults humans− so that the total number of adipocytes remains remarkably constant but about 10% of them are renewed every year −, which establishes a new therapeutic target whereby pharmacological or nutritional intervention may potentially tip the adipocyte birth-death balance in favor of reducing the number of fat cells (5).

Mesenchymal stem cells (MSCs), or multipotent mesenchymal stromal cells as they are also known, have been identified in bone marrow as well as in other tissues of the joint, including adipose, synovium, periosteum, perichondrium, and cartilage. These cells are characterized by their ability to differentiate into chondrocytes, osteoblasts and adipocytes. The potential of stem cells to repair compromised cartilage is a promising tool for cell therapy, either through direct contribution to the repair of bone, tendon and cartilage or as an adjunct therapy through protein production and immune mediation (6).

The differentiation of MSCs into chondrocytes, osteoblasts or adipocytes occurs through the crosstalk between complex signaling pathways - including those derived from bone morphogenic proteins, wingless- type MMTV integration site proteins, hedgehogs, delta/jagged proteins, fibroblast growth factors, insulin, insulin-like growth factors and cytokines – which in concert with cell-matrix and cell-cell interactions control the expression and activity of key lineage-specific transcription factors. The differentiation of MSCs into these three lineages is competitively balanced, in that mechanisms that promote one cell fate often actively suppress mechanisms that induce alternative lineages. In this context, many common regulatory factors have been implicated not only in the development but also in the maintenance of the cell terminal differentiated state and healthy function of adipose tissue, cartilage and other articular joint tissues.

The aim of this work was to elucidate the effects of selected compounds mainly used for joint health on the expression of key molecular players at the intersection of adipose tissue and joint cell's biology. As a model system, we used primary mouse embryo fibroblasts (MEFs), which have the ability to differentiate to adipocytes and chondrocytes among other lineages. We show in these cells dual effects of tested compounds preventing adipose conversion and promoting the expression of some markers of chondrocyte differentiation, while modulating the expression of secreted regulatory protein factors of pathophysiological significance. The work has been developed mainly as part of a contract between Bioibérica S.A. (Spain) and the University of the Balearic Islands within the CENIT PRONAOS project, and it is an example of translational academy-industry research.

MATERIALS AND METHODS

MEFs isolation and culture

MEFs were isolated from mouse embryos derived from mothers at day 13.5 of gestation. Briefly head, liver and other organs were removed and the remaining carcass was minced and incubated at 37 ºC for one hour with collagenase After that, fragment embryos were passed over a syringe (20G) in order to obtain single cells. Collagenase was inactivated by addition of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. The outgrowing primary cell population was passaged by trypsinization at a ratio of 1:3 to 1:4 upon confluency and continuously cultured in AmnioMAX-C100 basal medium supplemented with 7.5% AmnioMAX-C100 supplement, 7.5% FCS, 50 IU/mL penicillin, 50 µg/ml streptomycin,

and 2 mmol/L l-glutamine (growth medium). Differentiation experiments were carried out using cells between passage 3 and 5.

Adipogenic induction

For adipocyte differentiation, 2-day post confluent MEFs were treated (day 0) with growth medium containing 1µmol/L dexamethasone, 0.5 mmol/L methylisobutylxanthine, 5 µg/mL insulin and 0.5 µmol/l rosiglitazone (adipogenic cocktail) for 48 h. The cells were subsequently refed every other day with fresh medium containing 5 µg/mL insulin and 0.5 µmol/L rosiglitazone (adipogenic medium). From day 6 onwards, cells were deprived of insulin and rosiglitazone. Adipogenesis was routinely monitored by morphological examination of the cells for lipid accumulation using phase-contrast microscopy. Compounds tested − C1 and C2, and their derivates obtained by enzymatic modification, named C1' and C2', respectively − were added to the cells from day 0 to day 8, at the doses indicated in the figure legends; control cells received an equal volume of vehicle (TRIS 50 mM pH 8). Cells were harvested at day 8. (The actual names of the compounds under investigation are subjected to confidentiality issues).

Chondrogenic induction

MEFs were plated onto 12-well-plates in monolayer culture as in the prior section (1.2 x 10^5 cells/well) or in high-density micromass culture (see below), and were treated with vehicle, compound C1, compound C2, or human recombinant bone morphogenic protein 2 (BMP2, Sigma Catalog number B3555, used at 100 ng/mL) in the absence of adipogenic stimuli. For micromass culture, 1.2 x 10^5 cells in a 10 µL droplet were plated in the center of each well of a 24-well plate and left to adhere for four hours at 37 ºC in a humidified atmosphere. Once the cells had adhered, medium was added and cells were cultured as usual. BMP2 has previously been shown to have the potential to induce chondrogenic differentiation of MEFs in ex vivo cultures (7), and it plays an important role in the induction of chondrogenesis of condensed mesenchyme in vivo (8). The treatments started (day 0) after attachment of the cells, in the case of the micromass cultures, and 2 days after the cells had reached confluence, in the case of the monolayer cultures. In both systems, the cells received fresh medium and treatment every two to three days, and were harvested at days 0, 3, 8 and 13 of culture.

RNA isolation and analysis

Total RNA was extracted from monolayer cultures using Trizol reagent (Invitrogen) and from micromass cultures using E.Z.N.A columns, according to the supplier's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc Wilmington, DE, USA) and its integrity confirmed using

agarose gel electrophoresis. A260/A280 ratios were between 1.9 and 2 for all samples, indicating good purity. Retrotranscription followed by real-time polymerase chain reaction (RT-qPCR) was used to measure the mRNA expression levels of: peroxisome proliferator-activated receptor gamma (PPARγ); CCAAT/enhancer binding protein alpha (C/EBPα); fatty acid synthase (FAS); Sry-related high-mobility-group box 9 (Sox9); collagen type II, alpha 1 (Col2a1); PPARγ coactivator 1 alpha (PGC1α); cytochrome-oxidase subunit II (COX II); leptin; adiponectin; resistin; retinol binding protein 4 (RBP4); preadipocyte factor 1 (Pref-1); disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif 5 (ADAMTS5, also known as aggrecanase 2); and β-actin, the latter as internal control. In brief, 0.25 µg of total RNA (in a final volume of 5 µL) was denatured at 65 °C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20ºC for 15 min, 42 °C for 30 min, with a final step of 5 min at 95 °C in an Applied Biosystem 2720 Thermal Cycler. Sense and antisense primers used in the PCR reactions were designed with specific primer analysis software Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA), and their specificity analyzed by the ENTREZ and BLAST databases utilities (National Center for Biotechnology Information, Bethesda, MD, USA). The primers were produced by Sigma (Madrid, Spain), and their sequences are shown in Table 1. Each PCR was performed from diluted (1/20) cDNA template, forward and reverse primers (1μM each), and Power SYBER Green PCR Master Mix (Applied Biosystems). Real time PCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95 ºC, followed by a total of 40 two- temperature cycles (15 s at 95 ºC and 1 min at 60 ºC). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated according to Pfaffl (9), using β-actin as a reference gene.

Oil Red O Staining

Cells were fixed in 4% paraformaldehyde for 30 min at room temperature, washed with PBS, rinsed with isopropanol and stained with 0.2% Oil red O (Sigma, St. Louis, Mo., USA) in 60% isopropanol for 1 hour. The intracytoplasmatic dye extracted with isopropanol was quantified as described previously (10).

Statistical analysis

Data are expressed as means ± s.e.m. The statistical differences in mean values were assessed by Student's *t* test. All experiments were repeated at least four times (in triplicates or quadruplicates per each tested condition) except time-course experiments for chondrogenic induction that were performed once (in triplicates). Threshold of significance was set at $P<0.05$.

RESULTS

Effects of the tested compounds on terminal adipocyte differentiation

MEFs were induced to differentiate into adipocytes using a standard adipogenic mixture in the presence of vehicle or individual problem compounds at two different doses (20 and 200 μg/mL). After 8 days of differentiation, the cells were stained for neutral lipids with Oil Red O. As expected, most cells exposed to the vehicle had by then differentiated into adipocytes and were filled with lipids. In contrast, there were few lipid-laden cells present in the cultures treated with compounds C1' and C2' (Figure 1A). Semi-quantification of the accumulated lipids by spectrophotometric analysis of the amount of extracted dye (10) revealed a dose–response effect more pronounced for the modified compounds C1' and C2' (data not shown).

To characterize the pattern of gene expression in these cells, total RNA was isolated at day 8 of culture and subjected to RT-qPCR. We first examined the expression of key players of the transcriptional cascade that controls adipocyte differentiation. It has been well established that transcription factors of the C/EBP family, in particular C/EBPβ and C/EBPδ are induced very early in adipogenesis and play a crucial role in initiating the differentiation of adipocytes by promoting cell cycle arrest and clonal expansion (case of C/EBPβ) and activating the expression of PPARγ (case of C/EBPδ). PPARγ, upon agonist ligand binding, activates the expression of C/EBPα (11). PPARγ and C/EBPα control each other expression and function together to activate the entire program of morphological and biochemical adipocyte terminal differentiation (12). We measured PPARγ and C/EBPα mRNA levels in our cells at day 8 of culture (Figure 1B). We did not find significant changes in PPARγ mRNA expression levels, except for a decrease in the cells treated with C1' at the low dose. However, gene expression of C/EBPα was strongly decreased by compounds C1', C2 and C2', in a manner that was dose-dependent for C1' and C2'. We also measured the gene expression of fatty acid synthase (FAS), an end product of adipogenesis. Changes in FAS mRNA levels largely paralleled those of CEBPα, being strongly decreased by C1', C2 and C2' (Figure 1B).

A key aspect of this experimental design is the use of a mixture of stimulus to induce adipogenesis. The adipogenic stimuli include isobutylmethylxanthine, dexamethasone, insulin, and rosiglitazone. The individual components of this mixture facilitate different aspects of adipogenesis. Isobutylmethylxanthine, a phosphodiesterase inhibitor, elevates cellular cAMP levels leading to activation of protein kinase A (PKA). One of the PKA substrates in adipocytes is the transcription factor cAMP-response element-binding protein (CREB). Activated CREB positively regulates the expression of C/EBPβ. Dexamethasone activates the glucocorticoid receptor, whose targets include C/EBPδ, a transcription factor that induces the expression of the PPARγ gene (13). Rosiglitazone serves as an activating ligand of PPARγ, which as previously stated regulates target gene mRNA expression in a ligand-dependent manner. Importantly, modifications in the PPARγ molecule or associations with intracellular signal transducers or transcriptional factors play a pivotal role in the modulation of PPARγ physiological action (14). This opens the possibility that, even if PPARγ expression levels are unaffected, C1', C2 and C2' act by inhibiting the ligand-dependent transactivation function of PPARγ on target genes, such as C/EBPα. The observed repression of FAS expression would, in turn, be consistent with previous studies demonstrating an important role for C/EBPα in lipogenesis per se and specifically in FAS expression (11).

Overall, these results indicate that enzimatically modified compound 1 (C1') and compound 2, either intact (C2) or enzymatically modified (C2'), inhibit adipocyte terminal differentiation by suppressing C/EBPα expression and, possibly, the transactivation function of PPARγ, through as yet unknown mechanisms.

Effects of the tested compounds on adipokine expression

Dysregulated production of adipokines is frequently associated with obesity and several adipokines, such as leptin, adiponectin and resistin, have been implicated specifically in the pathophysiology of joint disease. Adipokines are supposed to be released specifically by adipocytes, however most of them are in fact produced in a variety of cell types. In particular, joint tissues including synovium, osteophytes, cartilage and bone have been demonstrated to produce adipokines such as leptin and adiponectin (15). The joint cavity is a special space where each adipokine undergoes specific regulatory pathways, reinforcing the idea that adipokines may have local effects in the joint (15). In view of the connections between adipokines and joint health and of effects on adipogenesis presented in the prior section, we decided to analyze adipokine

expression levels in MEFs following 8 days of culture under adipogenic conditions in the presence or absence of the problem compounds.

It has been described that leptin inhibits adipogenic differentiation and stimulates osteo-/chondrogenic differentiation in bone marrow mesenchymal stem cells (16). Moreover, previous studies showed that chondrocytes treated with leptin exhibited increased proliferation, proteoglycan and collagen synthesis and expression of leptin itself (17, 18), although other studies found evidence of catabolic effects of leptin in cartilage (19, 20). Our results show a trend of leptin expression to be increased in MEFs following treatment with all the problem compounds, particularly at the low dose (Figure 2a). This trend reached statistical significance for compound C2 at the low dose, whereas variability among samples precluded the attainment of statistical significance for the other treatments assayed.

There is some controversy whether increased adiponectin in the diseased joint serves to counteract local inflammation or, on the opposite, contributes to cartilage destruction. The mRNA levels of adiponectin were not significantly changed by any of the treatments (Figure 2b).

Resistin and RBP4 adipose expression and circulating levels positively correlate with adiposity (21, 22), and these two protein signals have been implicated in the development of insulin resistance in some studies in both rodents and humans (23, 24). Resistin has also been implicated as a pro-inflammatory and pro-catabolic factor in the diseased joint (25, 26). The mRNA expression levels of resistin (Figure 2c) and RBP4 (Figure 2d) were reduced by all treatments. Down-regulation of resistin expression reached statistical significance for C1' and C2', and down-regulation of RBP4 expression for C1', C2 and C2'.

Effects of the tested compounds on osteo-/chondrogenic markers

a) Studies in MEFS subjected to adipogenic stimulation

MEFs have the potential to differentiate into different lineages, including adipocytes, chondrocytes and osteoblasts. To investigate a possible effect of treatments promoting chondrogenic induction, we first measured Sox9 mRNA levels in MEFs following 8 days of culture under adipogenic conditions, in the presence or absence of the problem compounds (Figure 3a). Sox9 is a transcription factor considered the canonical "master regulator" of chondrogenesis, since it is required for mesenchymal condensation and for initiation of early chondrogenesis to generate immature chondrocytes that express cartilaginous matrix proteins, such as type II and IX collagens and aggrecan (27). Thus,

Sox9 is expressed in all chondro-osteoprogenitor cells and in mesenchymal condensations, and it promotes chondrogenic commitment. Lineage-specific transcription factors can actively inhibit differentiation of other lineages and, interestingly, Sox9 has been shown to directly bind to the promoter region of the C/EBPβ and C/EBPδ encoding genes to suppress their activity, thus preventing adipocyte differentiation (28). There was a trend of Sox9 mRNA levels to be increased in cells treated with C2 and, especially, the modified compounds C1' and C2' (Figure 3a). Remarkably, the gene expression of a Sox9 target gene, collagen type II alpha 1 (Col2a1), although highly variable, also exhibited a clear trend to be increased in the cells exposed to the treatments, particularly to compound 2 intact or modified (Figure 3b).

Sox9 requires molecular partners to control chondrogenesis and chondrogenic gene expression. PGC-1α in particular was identified as a coactivator for Sox9 during chondrogenesis (29), and it is also an important transcriptional coactivator for mitochondrial biogenesis and brown versus white adipogenesis (30). Interestingly, expression levels of PGC-1α were consistently increased in MEFs exposed to C2 and C2', an effect that reached statistical significance for compound C2' (Fig 3c). Considering the well known role of PGC-1α on mitochondrial biogenesis, we next measured the expression of cytochrome C oxidase subunit II (COX II), a component of the mitochondrial respiratory chain. Exposure to C2, and to C2' at the low dose, significantly resulted in increased COXII mRNA levels in cells (Figure 3d).

Cytokines such as TNFα and IL-1 appear to alter the fate of pluripotent mesenchymal stem cells directing cellular differentiation towards osteoblasts rather than adipocytes through activation of NF-κB (31). In its active form, NF-κB blocks the ligand-induced transactivation function of PPARγ by forming a complex with PPARγ (31). In two of the four independent experiments performed, TNFα expression was barely detected in control cells and significantly induced by C1', C2 and C2' treatments in a dose-dependent manner, whereas in the other two experiments TNFα expression was very low irrespective of treatment (data not shown).

b) Studies in MEFS not subjected to adipogenic stimulation

In an attempt to get further insight into the capacity of the compounds tested to influence chondrogenic differentiation, we assayed their effects in MEFs not submitted to adipogenic induction (i.e., cultured in growth medium not supplemented with the adipogenic stimuli). In particular, the time-course of the effects of compounds C1 and C2

(both at 100 μg/mL) on the expression of selected chondrogenesis/chondrocyte-related genes were analyzed and compared to the effects of BMP2 (100 ng/mL), which has been shown to induce chondrogenic differentiation, both in cultured precursor cells and in vivo (7, 8). We measured the expression levels over time of Sox9, Col2a1,Pref-1, aggrecan protein and aggrecanase 2, first in monolayer cultures (Figure 4). Pref-1 is a well-known pre-adipocyte marker that has recently been shown to inhibit adipocyte differentiation by up-regulating Sox9 (28). Aggrecan protein is the core protein of the cartilage-specific proteoglycan aggrecan, which together with type II collagen are the main components of the extracellular matrix surrounding chondrocytes in cartilage. Aggrecanase 2 (also known as ADAMTS-5) is a proteinase that appears to be the primary enzyme responsible for aggrecan degradation in mice, and it plays a role in terminal differentiation of chondrocytes (32). Pref-1 was transiently induced at day 3 and then significantly decreased in cells treated with C2 and BMP2 (Figure 4a), but only in BMP2-treated cells were Sox9 mRNA levels increased relative to vehicle-treated cells (Figure 4b). Aggrecan protein expression was powerfully increased from day 3 on in cells exposed to C2 or BMP2, reaching an 8-fold and a 5-fold increase by day 13 of culture, respectively (Figure 4c). Aggrecanase 2 levels increased along the culture history irrespective of treatments (Figure 4d). Col2a1 expression was not increased upon BMP2, or C1 or C2 treatment (Figure 4e); this was not unexpected, since a previous study found that BMP2 treatment induced Sox9 expression but failed to induce Col2a1 expression in MEFs in monolayer culture (7).

In contrast to conventional monolayer cell cultures in which cells grow in only two dimensions on the flat surface of a plastic dish, high-density micromass culture provides cell-cell contacts in 3 dimensions (3D) similar to what occurs in vivo in condensing mesenchyme prior to induction of chondrogenesis, and is preferred for chondrogenesis induction. For instance, whereas (as stated above) BMP2 treatment failed to induce Col2a1expresion in MEFs in monolayer culture, it has been reported that BMP2 synergizes with 3D cell contacts resulting in a robust Col2a1 (and Sox9) induction in MEFs in micromass culture (7). This background prompted us to repeat the experiment described in the previous paragraph using MEFs in micromass culture (Figure 5). As in monolayer culture, in micromass culture aggrecan protein mRNA levels were robustly induced by C2 and BMP2, but not C1 (Figure 5c), and aggrecanase 2 mRNA levels showed a sustained increase along the culture history with all treatments (Figure 5d). Additionally, C2 and BMP2 induced similar time-dependent changes in Pref-1 mRNA levels and Col2a1 mRNA levels as compared to vehicle or C1 (Figures 5a and 5e). Nevertheless, neither C2 nor BMP2 induced Sox9 or Col2a1 mRNA levels in MEFs in

micromass culture over the levels in vehicle-treated time control cells; on the contrary, down-regulation of Col2a1 levels was apparent (Figures 5b and 5e).

Both in monolayer (Figure 6A) and micromass (not shown) cultures, morphological examination of the cells by phase-contrast microscopy revealed little islands of fully differentiated adipocytes in vehicle- and C1-treated cultures, but not in C2- or BMP2-treated cultures. Some studies have reported that serum may induce spontaneous adipogenesis while inhibiting chondrogenesis in bovine synovial explants (33) and human bone-marrow derived stem cells (34). To confirm that C2 and BMP2 were inhibiting the spontaneous adipogenic conversion of MEFs, we measured the gene expression of PPARγ and FAS in cells at day 8 in monolayer culture (Figure 6B). As expected from the microscopic examination of the cells, the mRNA levels of PPARγ and FAS were powerfully decreased after C2 and BMP2 treatment, while C1 treatment had no effect.

DISCUSSION

The results of this study indicate that compound 2 − either intact (C2) or enzymatically modified (C2') − and modified compound 1 (C1') have the potential to inhibit terminal adipocyte differentiation of MEFs even in the presence of adipogenic stimuli. Thus, lipid content and expression of lipogenic genes such as CEBPα and FAS was decreased in MEFs undergoing adipogenic differentiation in the presence of these chemical species. Nevertheless, the expression levels of the master regulator of adipogenesis, PPARγ, which are induced by the presence of glucocorticoid (dexamethasone) in the adipogenic cocktail, remained unchanged, pointing to direct or indirect effects of the compounds on PPARγ transcriptional activity. When C1 and C2 were tested in MEFs not exposed to the adipogenic stimuli, compound C2 but not compound C1 powerfully inhibited spontaneous adipogenesis and the cellular expression of PPARγ and FAS.

Mounting evidence indicates that crosstalk exists among potential differentiation programs of pluripotent cells, such as bone marrow mesenchymal stem cells or adipose-derived stem cells. MEFs have the ability to differentiate, among other lineages, to adipocytes and chondrocytes. Our results show that, when these cells were cultured in adipogenic medium, tested compounds that inhibited adipogenesis (C1', C2 and C2') also tended to increase chondrogenic markers (Sox9, Col2a1). Furthermore, the expression of PCG-1α (a transcriptional coactivator of Sox9) and TNFα (an inhibitor of

adipogenesis and inductor of osteoblastogenesis) was also increased, reinforcing the idea that these compounds may act to favor the chondrogenic, over the adipogenic, program. Interestingly, in cells cultured without adipogenic stimuli (both in monolayer and in 3D cultures) compound C2 had effects that were similar to those exerted by the well-known chondrogenic inducer, BMP2. Thus, C2 and BMP2, but not C1, inhibited spontaneous adipogenesis in MEFs, and the expression patterns over time of genes related to the chondrogenic program were similarly affected by C2 and BMP2, but not C1, treatment. In particular, both C2 and BMP2 led to a robust induction of aggrecan protein over time, which is consistent with the profile of immature chondrocytes and the subsequent formation of cartilage tissue (28, 35). Nevertheless, the decrease of Col2a1 mRNA levels that we found under both C2 and BMP2 treatments is not consistent with a chondrogenic effect and conflicts with previous results regarding BMP2 effects on MEFs (7). Further analysis − such as Col2a1 and aggrecan protein levels (by immunoblotting or immunostaining) and production of other chondrocyte markers − are required to further test the hypothesis that compounds tested, particularly C2, may positively affect chondrogenesis of pluripotent MEF cells.

Taken together, we conclude that compound 2 either intact or in modified form and compound 1 only in modified form (C1') have the potential to inhibit adipocyte differentiation and promote to some extent the expression of chondrogenesis-related genes in pluripotent MEFs cells. These compounds also had effects on the adipokine profile of potential interest in the context of the management of obesity and osteoarthritis, as they induced leptin and repressed resistin and RBP4.

Joint morphogenesis involves signaling pathways and growth factors that recur in the adult life with less redundancy to safeguard joint homeostasis (36). Loss of such homeostasis due to abnormal signaling networks, as might occur in obesity, can lead to osteoarthritis. It is foreseen that compounds with effects both on the underlying mechanisms involved in obesity and osteoarthritis and on the stem cell niches and related remodeling signals, may emerge as useful interventions to support effective joint tissue regeneration, to restore joint homeostasis, and to prevent osteoarthritis, while possibly serving simultaneously as coadjuvants in strategies to control and manage obesity.

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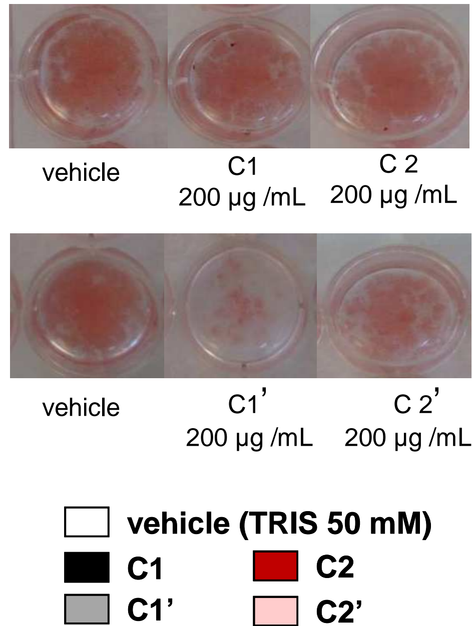
Table 1. List of primers

gene	NCBI number	Primer forward(5’-3’)	Primer reverse (5’-3’)	Product size pb
PPARγ	NM_001127330.1	AGACCACTCGCATTCCTTTG	TCGCACTTTGGTATTCTTGG	154
C/EBPα	NM_007678.3	AGGTGCTGGAGTTGACCAGT	CAGCCTAGAGATCCAGCGAC	233
FAS	NM_007988.3	TTCGGTGTATCCTGCTGTCC	TGGGCTTGTCCTGCTCTAAC	167
Col2a1	NM_031163.3	TGCAGAATGGGCAGAGGTAT	GGACAGCACTCTCCGAAGG	243
PGC1α	NM_008904	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA	70
COX II	NC_012310.1	AAGACGCCACATCCCCTATT	CTTCAGTATCATTGGTGCCCT	291
Leptin	NM_008493.3	AGGAAAATGTGCTGGAGACC	ATACCGACTGCGTGTGTGAA	160
Adiponectin	NM_009605.4	TCTCACCCTTAGGACCAAG	GCTCAGGATGCTACTGTTG	247
Resistin	NM_022984.3	TTCCTTTTCTTCCTTGTCCCTG	CTTTTTCTTCACGAATGTCCC	247
RBP4	NM_001159487.1	ACTGGGGTGTAGCCTCCTTT	GGTGTCGTAGTCCGTGTCG	71
Pref 1	NM_001190705.1	TTCTGCGAAATAGACGTTCGG	TCGTTGTCGCATGGGTTAGG	202
Sox 9	NM_011448.4	GAGGCCACGGAACAGACTCA	CAGCGCCTTGAAGATAGCATT	51
aggrecan	NM_007424.2	GGCGTGCGCCCATCATCAGA	CCACTGACACACCTCGGAAGCA	364
aggrecanase-2	NM_011782.2	GCCTCCATCGCCAACAGGCT	AGATGCCCAATTTCATGAGCCACA	362
β actin	NM_007393.3	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT	120

A



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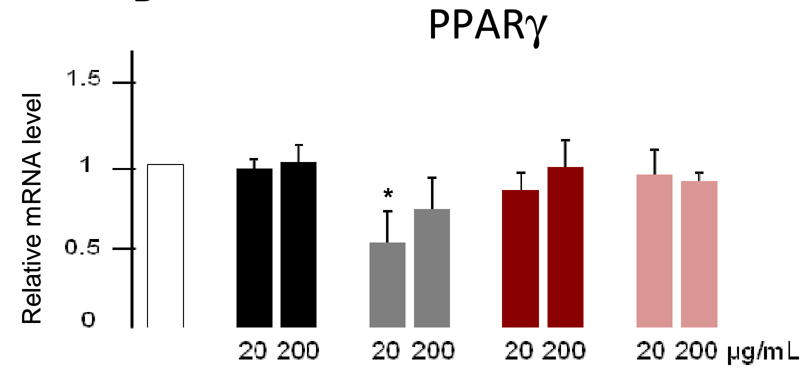

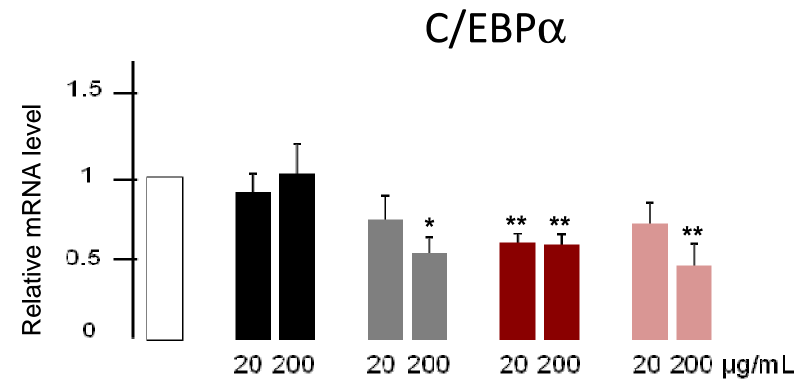

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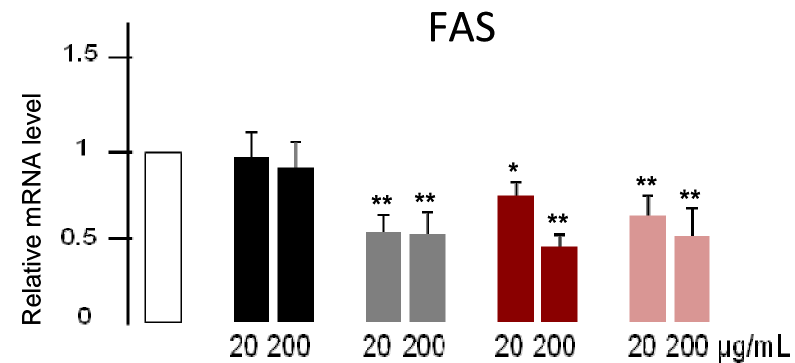


Figure 1.- Effects of compounds C1, C1', C2 and C2' on adipogenesis in MEFs subjected to adipogenic differentiation. MEFs were subjected to adipogenic differentiation in monolayer culture in the presence of vehicle (TRIS 50 mM) or C1, C2, C1' and C2' at 20 µg/mL and 200 µg/mL from day 0 of culture (two-day post-confluent cells), as described in the Materials and Method section. Oil red O staining (A) and RT-qPCR of selected genes (B) was performed in cells at day 8 of culture. Expression levels of PPARγ, CEBPα and FAS were normalized to β-actin. Values are the means of at least four experiments (carried out in quadruplicates) with standard error represented by vertical bars, and are expressed relative to the mean value in the vehicle-treated control cells. *, $p < 0.05$ and **, $p < 0.01$ in Student's *t* test, treatment *versus* vehicle.

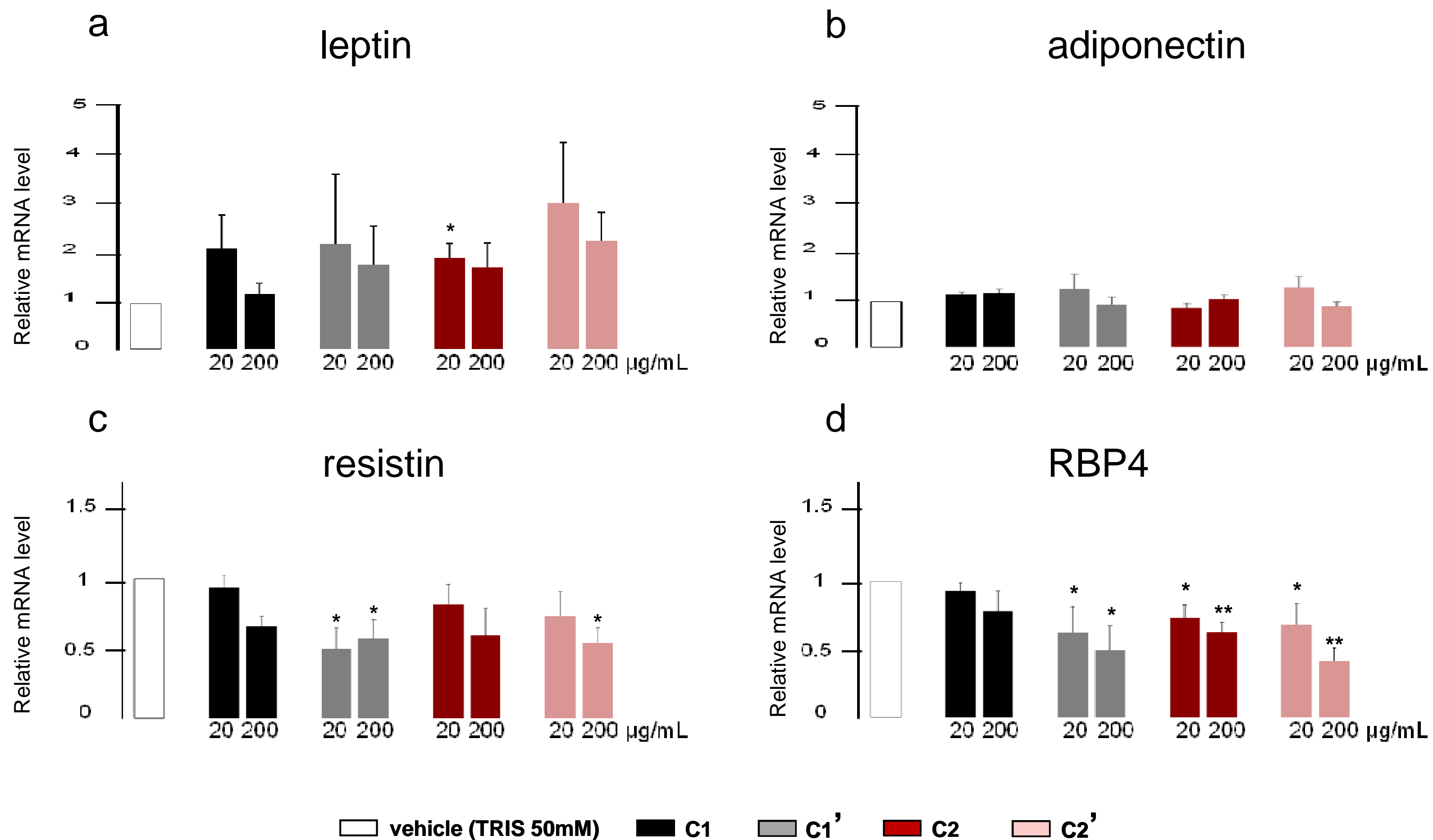


Figure 2.- Effects of compounds C1, C1', C2 and C2' on adipokine expression in MEFs subjected to adipogenic differentiation. MEFs were subjected to adipogenic differentiation in monolayer culture in the presence of vehicle (TRIS 50 mM) or C1, C2, C1' and C2' at 20 µg/mL and 200 µg/mL from day 0 (two-day post-confluent cells), as described in the Materials and Method section. Expression levels of leptin (a), adiponectin (b), resistin (c) and RBP4 (d) in cells at day 8 of culture were measured by RT-qPCR and normalized to β-actin. Values are the means of at least three experiments (carried out in quadruplicates) with standard error represented by vertical bars, and are expressed relative to the mean value in the vehicle-treated control cells (which was set as 1). *, p<0.05 and **, p<0.01 in Student's *t* test, treatment *versus* vehicle.

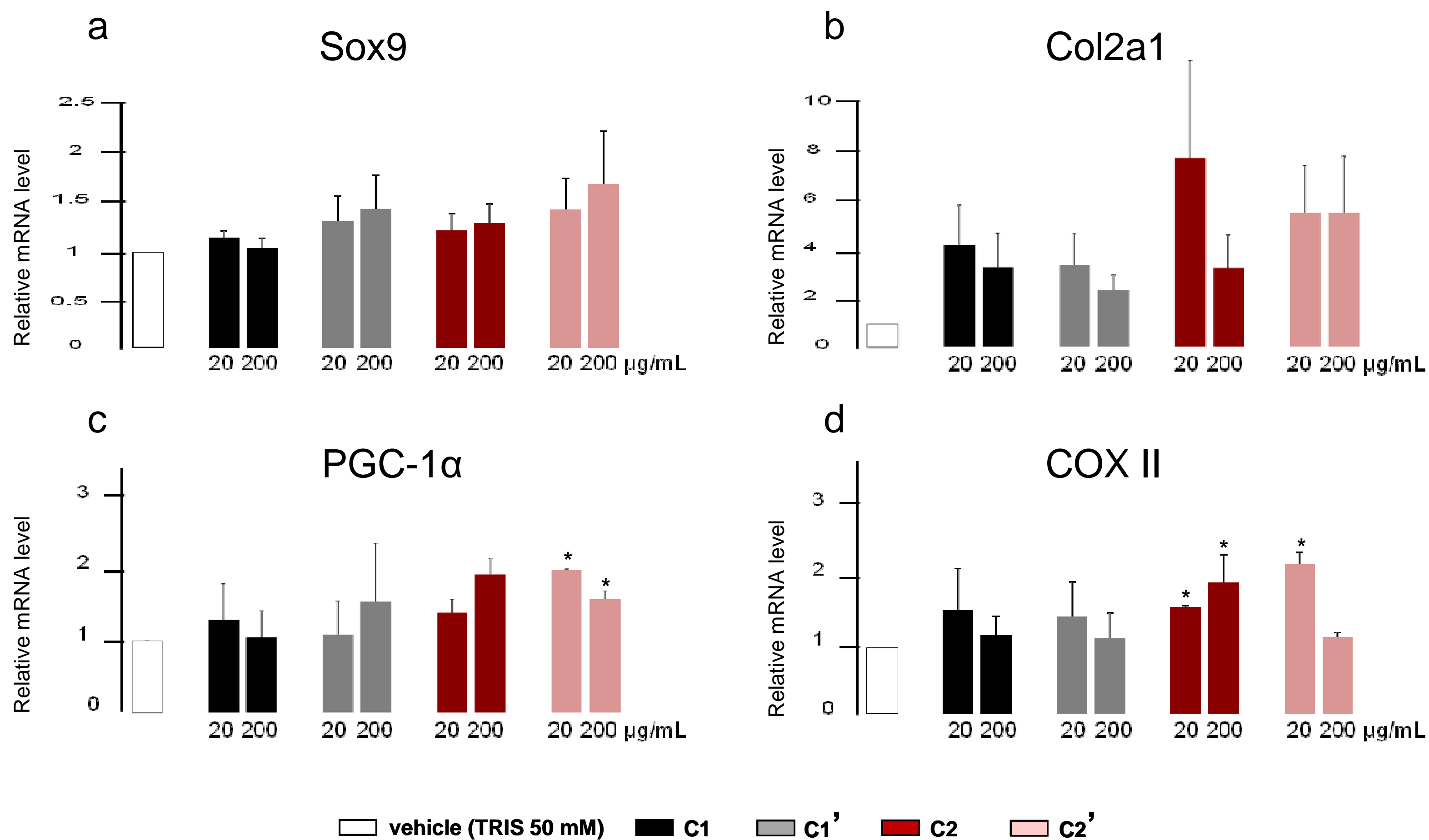
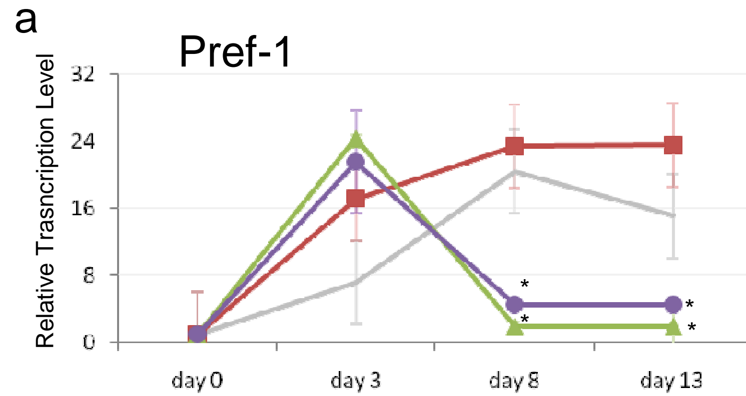
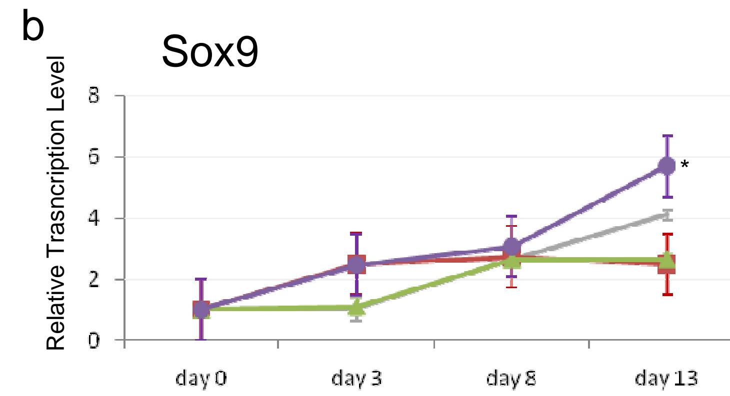
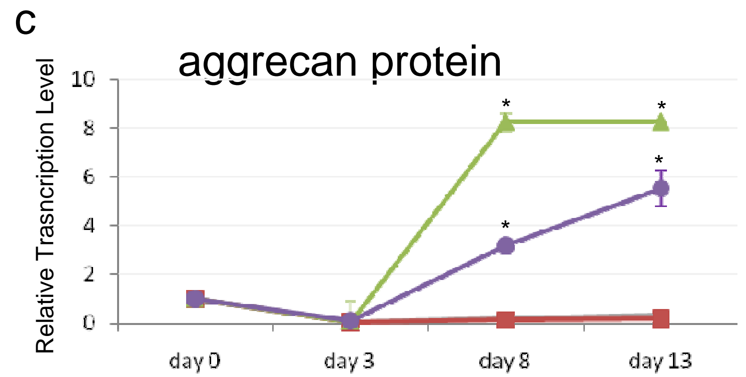
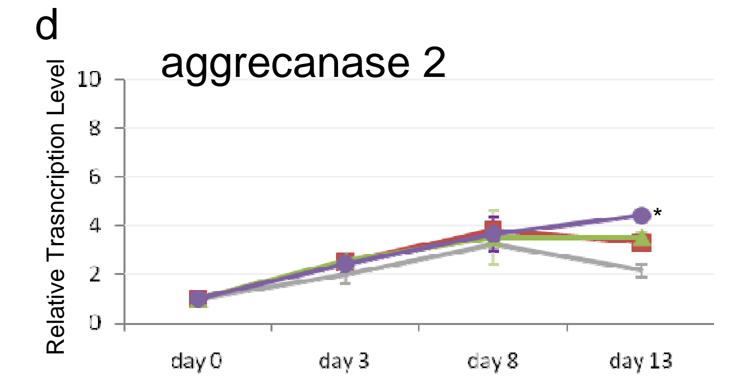


Figure 3.- Effects of compounds C1, C1', C2 and C2' on chondrocyte markers in MEFs subjected to adipogenic differentiation. MEFs were subjected to adipogenic differentiation in monolayer culture in the presence of vehicle (TRIS 50 mM) or C1, C2, C1' and C2' at 20 µg/mL and 200 µg/mL from day 0 (two-day post-confluent cells), as described in the Materials and Method section. Expression levels of Sox9 (a), Col2a1 (b), PGC-1α (c) and COX II (d) in cells at day 8 of culture were measured by RT-qPCR and normalized to β-actin. Values are the means of at least three experiments (carried out in quadruplicates) with standard error represented by vertical bars, and are expressed relative to the mean value in the vehicle-treated control cells (which was set as 1). *, $p<0.05$ in Student's *t* test, treatment *versus* vehicle.









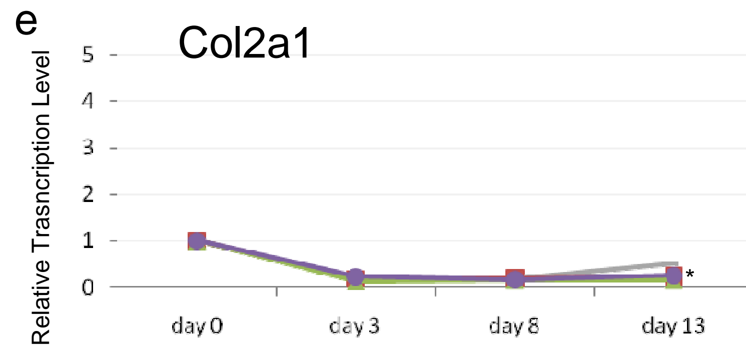
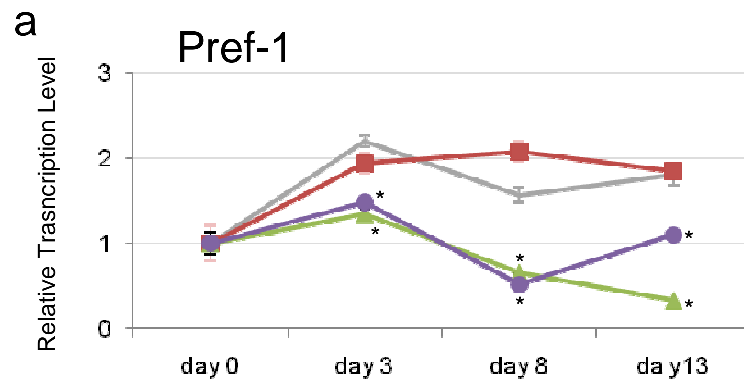
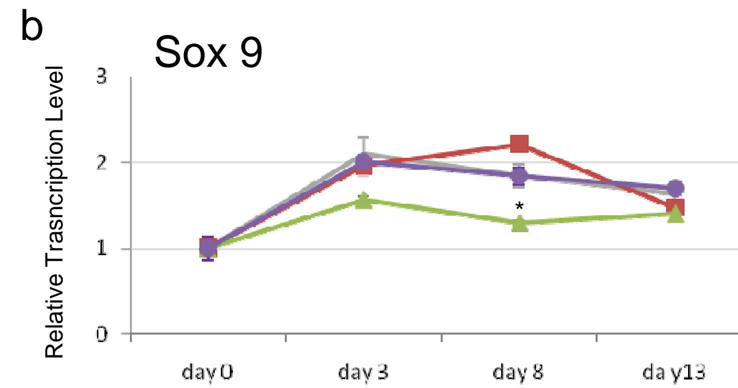
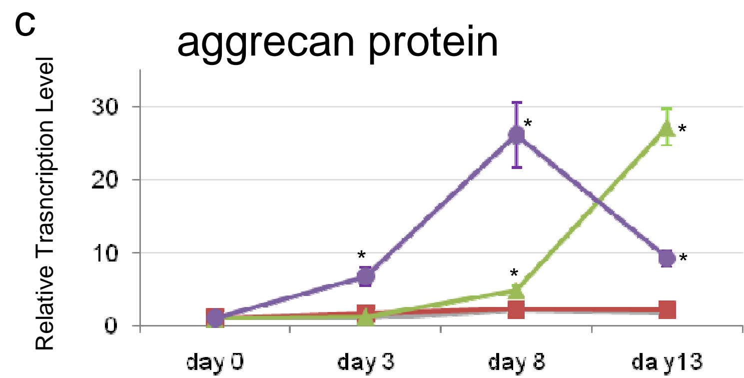


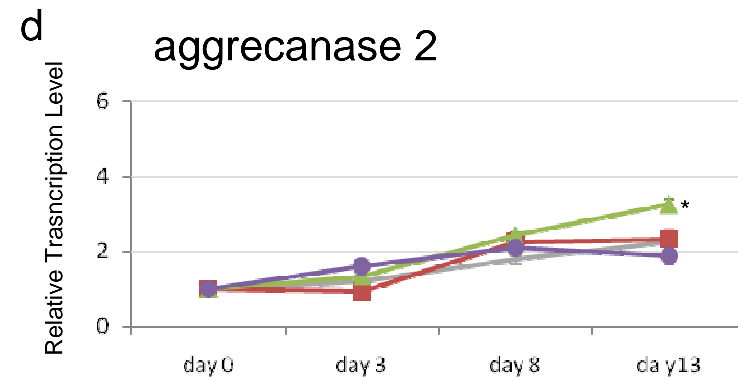
Figure 4.- Effects of compounds C1, C2 and BMP2 on the expression of chondrogenesis-related genes in MEFs in monolayer culture not subjected to adipogenic stimulation. Two-day post-confluent MEFs (day 0 of culture) were cultured in monolayer in growth medium devoid of hormonal adipogenic stimuli, in the presence of vehicle (TRIS 50 mM) or C1 (100 µg/mL), C2 (100 µg/mL) or BMP2 (100 ng/mL). Expression levels at days 0, 3, 8 and 13 of culture of Pref1 (a), Sox9 (b), aggrecan (c), aggrecanase-2 (d) and Col2a1 (e) were measured by RT-qPCR and normalized to β-actin. Values are the means of one experiment carried out in triplicates with standard error represented by vertical bars, and are expressed relative to the mean value in the vehicle-treated control cells at day 0 (which was set as 1). *, $p<0.05$ in Student's *t* test, treatment versus vehicle at each time point.

vehicle (TRIS 50 mM) — C1 100 µg/mL — C2 100 µg/mL — BMP2 100 ng/mL









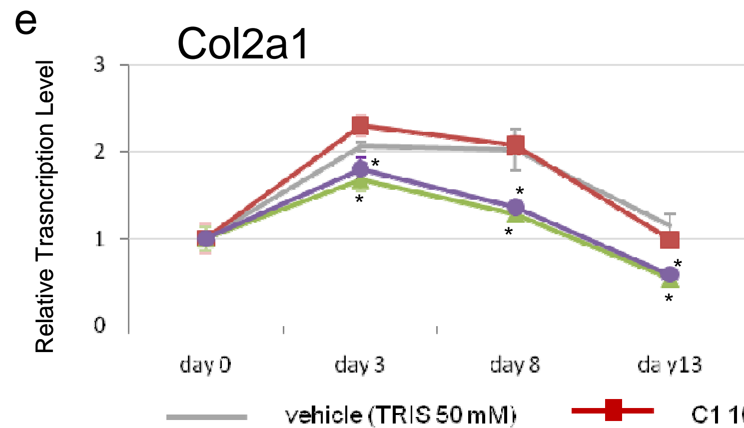


Figure 5.- Effects of compounds C1, C2 and BMP2 on on the expression of chondrogenesis-related genes in MEFS in micromass culture not subjected to adipogenic stimulation. MEFs (1.2 x 10^5 cells in 10µl drop of medium) were seeded in micromass (day 0 of culture) and cultured in growth medium devoid of hormonal adipogenic stimuli, in the presence of vehicle (TRIS 50 mM) or C1 (100 µg/mL), C2 (100 µg/mL) or BMP2 (100 ng/mL). Expression levels at days 0, 3, 8 and 13 of culture of Pref-1(a), Sox9 (b), aggrecan (c), aggrecanase-2 (d) and Col2a1 (e) were measured by RT-qPCR and normalized to β-actin. Values are the means of one experiment carried out in triplicates with standard error represented by vertical bars, and are expressed relative to the mean value in the vehicle-treated control cells at day 0 (which was set as 1). *, $p<0.05$ in Student's t test, treatment versus vehicle at each time point.

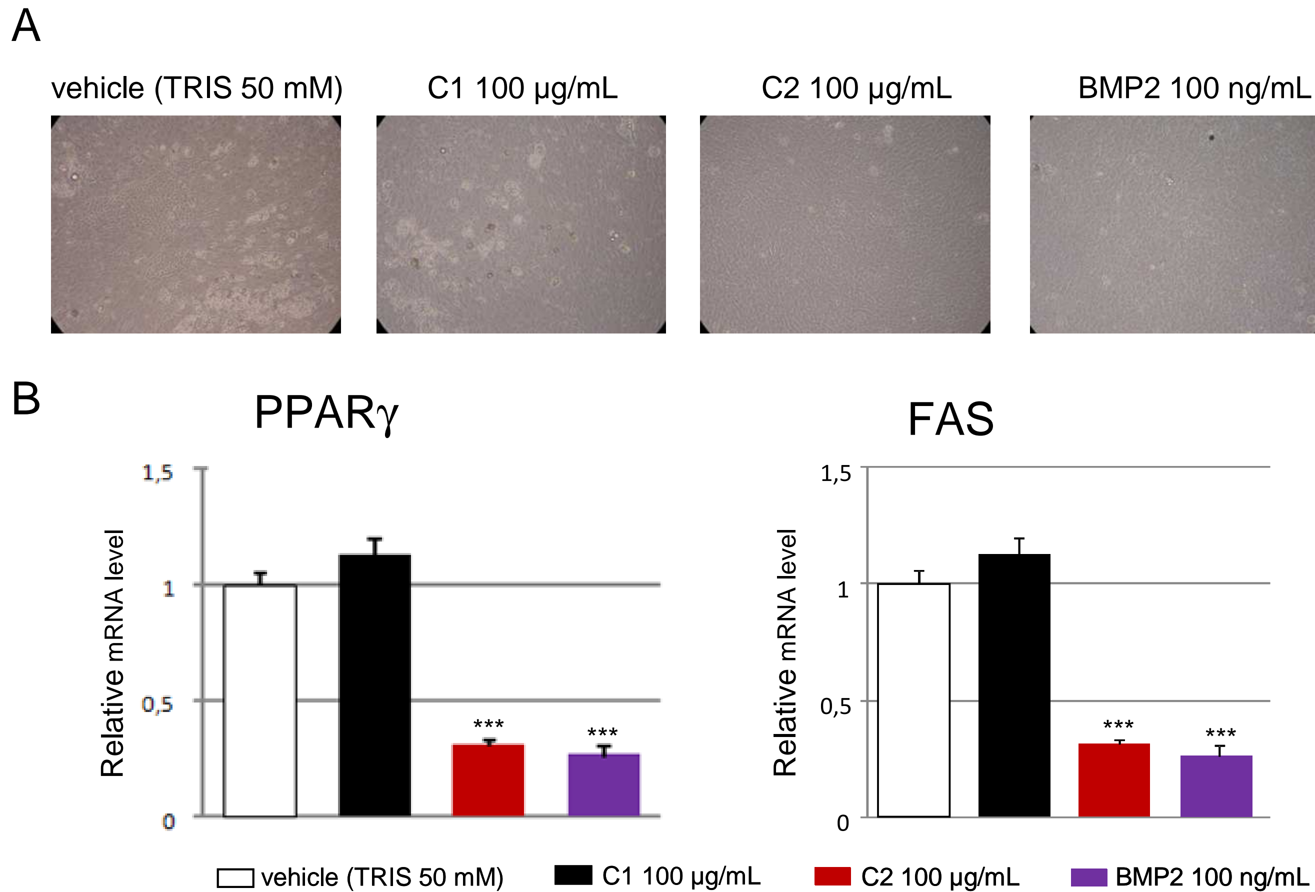


Figure 6.- Effects of compounds C1, C2 and BMP2 on spontaneous adipogenesis in MEFs in monolayer culture. Two-day post-confluent MEFs (day 0 of culture) were cultured in monolayer in growth medium devoid of hormonal adipogenic cocktail, in the presence of vehicle (TRIS 50mM) or C1 (100 µg/mL), C2 (100 µg/mL) or BMP2 (100 ng/mL) for 8 days, after which the cells were examined by phase-contrast microscopy (A) and harvested to extract total RNA. PPARγ and FAS mRNA levels were measured by RT-qPCR and normalized to β-actin (B). Values are the means of one experiment carried out in triplicates with standard error represented by vertical bars, and are expressed relative to the mean value in the vehicle-treated control cells (which was set as 1). ***, $p<0.001$ in Student's *t* test, treatment versus vehicle.

MANUSCRITO V

Molecular players at the intersection of obesity and osteoarthritis.

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Current Drugs Targets, en prensa.

Title: **Molecular Players at the Intersection of Obesity and Osteoarthritis**

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ABSTRACT

Obesity and degenerative joint disease (osteoarthritis, OA) are two multifactorial pathologies that are becoming major medical issues with the aging of the world population. The relationship of OA with obesity is complex, involving both biomechanical and metabolic links. Dysregulated production of adipose tissue-derived inflammatory mediators, hyperlipidemia, and increased systemic oxidative stress are conditions frequently associated with obesity that may favor joint degeneration. In addition, it is remarkable that many regulatory factors have been implicated in the development, maintenance and function of both adipose tissues and cartilage and other articular joint tissues. Disturbances in these factors may underlie additional links between obesity and OA. In this review, molecular players at the intersection of adipose tissue and joint cell biology − including differentiation signals and transcription factors, extracellular matrix components and remodelers, joint cell- and adipose tissue cell-derived mediators (cytokines, adipokines), hypoxia inducible transcription factors, lipids, advanced glycation end products and miRNAs − are reviewed, with emphasis on their dysregulation in obesity and OA. Knowledge of these factors may illuminate a novel, adipocentric avenue for the pathogenesis and therapy of OA and other joint diseases.

Keywords: adipose tissue, adipokines, advanced glycation end products, differentiation signals, extracellular matrix, joint tissues, lipids, miRNAs, transcription factors

INTRODUCTION

Obesity and degenerative joint disease (osteoarthritis, OA) are two multifactorial pathologies that are becoming major public health issues with the aging of the world population. Both of them represent an increasing burden from a medical, social and economic point of view. Obesity and OA are interrelated. This relationship is nowadays well recognized to extend beyond a biomechanical basis to a metabolic basis following the realization that obesity is a risk factor for OA, not only for weight-bearing joints (e.g., knees), but also for non-weight-bearing joints (e.g. hands); that reduction of body fat ameliorates OA more than reduction of body weight *per se*; and that systemic factors frequently associated with obesity might be critically involved in the development of OA, particularly generalized OA affecting multiple joints [1-6].

Obesity can be most simply defined as the presence of excess body fat as adipose tissue and is strongly associated with the metabolic syndrome (clustering of dyslipidemia, insulin resistance, hypertension and often also fatty liver in an individual). Obesity is ultimately caused by chronic energy imbalance whereby energy intake exceeds energy expenditure. Biochemical processes and systems influencing body fat content and distribution include those controlling satiety/hunger and/or thermogenesis, nutrient partitioning between tissues and catabolic *versus* anabolic pathways, and adipocyte number (e.g., preadipocyte proliferation, adipocyte differentiation or adipogenesis, adipocyte apoptosis) [7-9]. Obesity can arise basically from genetic and/or acquired alterations affecting any of these aspects. The central nervous system (CNS) plays a key role in the control of energy balance and peripheral metabolism [10]. Adipose tissue also plays an important role in this context, by producing signaling molecules (e.g., adipokines) that impinge on relevant CNS regulatory circuitries and peripheral tissue metabolism, and owing to the unique capabilities of brown adipose tissue (BAT) to dissipate energy as heat and of white adipose tissue (WAT) to store fat in a relatively "safe" manner (see Appendix for differences between WAT and BAT). When WAT, particularly subcutaneous WAT, is in shortage (e.g. in lipodystrophies), pathological accumulation of fat in other tissues may occur, thus leading to metabolic disturbances (lipotoxicity) [11]. Hence, both excess and deficiency of WAT may have severe metabolic consequences.

Osteoarthritis, in turn, has traditionally been regarded as a disease of articular cartilage, although nowadays it is viewed as a process that affects the whole joint, including the subchondral bone, synovial capsule and membrane and the periarticular (connective and muscular) tissues [12, 13]. Moreover, it has been proposed that OA, at least in its generalized form, is a systemic musculoskeletal disorder with a metabolic component [14]. The hallmarks of OA are articular cartilage breakdown, subchondral bone alterations (thickening, osteophyte formation) and synovitis (inflammation of the synovial membrane). OA is thought to arise as a response to abnormal mechanical loading, instability or injury, in conjunction with predisposing factors such as genetics, aging, and several systemic aspects. Thus, it is generally accepted that OA can result from abnormal loads on normal cartilage or normal loads on abnormal cartilage [12].

Dysregulated production of adipose tissue-secreted inflammatory mediators, hyperlipidemia, and increased systemic oxidative stress are conditions frequently associated with obesity that may also favor joint degeneration. In addition, it is remarkable that many regulatory factors have been implicated in the development, maintenance and function of both adipose tissues and cartilage and other articular joint tissues. Disturbances in these factors may underlie additional links between obesity and OA. In this review,

molecular players at the intersection of adipose tissue and joint cell biology are reviewed, with emphasis on their possible dysregulation in obesity and OA. Knowledge of these factors may illuminate novel avenues for the simultaneous prevention/treatment of these two conditions, which often coexist in afflicted individuals.

DIFFERENTIATION SIGNALS AND TRANSCRIPTION FACTORS

Adipocytes, chondrocytes and osteoblasts originate from a common multipotent mesenchymal stem cell precursor. Adipogenesis occurs late in embryonic development and in postnatal periods. Chondrogenesis and osteogenesis during skeletal development occur in early embryonic stages. The initial step in chondrogenesis is the recruitment of mesenchymal cells to future sites of skeletal development, which is followed by cellular aggregation (condensation) and the differentiation of mesenchymal cells to the chondrogenic lineage. Differentiated chondrocytes may remain as such, to form articular cartilage and function in joint development, or may undergo maturation into hyperthrophic chondrocytes to function as a template for long bone formation through endochondral ossification. Osteogenesis of other bones occurs directly from mesenchymal condensation by intramembranous ossification [15].

Distinctive, yet interacting, transcription factors play an essential role in the specification of the adipocyte, chondrocyte and osteoblast lineage. These include CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator activated receptor γ (PPARγ) for adipocytes, runt-related transcription factor 2 (Runx2) and osterix for osteoblasts, and Sry-related high-mobility-group box 9 (Sox9) for chondrocytes [16]. Interestingly, lineage-specific transcription factors can inhibit differentiation of other lineages by suppressing gene expression. For instance, PPARγ inhibits Runx2 expression and thus terminal osteoblast differentiation of mesenchymal cells [17]. Sox9, the master regulator of chondrogenic commitment and early chondrogenesis, prevents chondrocyte maturation and osteogenesis [18] and, of particular interest in the context of this review, has recently been shown to repress adipogenesis as well [19]. Modulation of lineage-specific transcription factors during development is controlled by several conserved signaling pathways which in turn are modified by cell-matrix and cell-cell interactions.

Signaling pathways involved in lineage determination from mesenchymal stem cells during development might be of relevance for both joint and adipose function and pathophysiology. First, similar pathways are likely to be implicated in cell turnover in adult tissues involving differentiation of resident adult mesenchymal stem cells, which are found in bone marrow, adipose tissues and joint-related tissues (synovium, synovial fluid) among other tissues. Second, these pathways are likely to play a role in the maintenance of cell terminal differentiated state and healthy function. In cartilage, for example, reduced Sox9 expression and related dedifferentiation of chondrocytes appears to contribute to OA pathology [15, 18]. Moreover, the hypothesis has been proposed that OA ultimately results from excessive, poorly regulated growth of musculoskeletal tissues possibly resulting from cells reverting to an earlier developmental phenotype [14, 20]. Finally, an understanding of these pathways is crucial for stem-cell based regenerative and reconstructive strategies in the areas of cartilage repair, orthopedics and aesthetics, which involve the *ex-vivo* or *in vivo* controlled differentiation of stem cells into specific cell types.

The contribution of transcription-related factors and developmental signals implicated in the differentiation and/or function of adipocytes and joint cells is compiled in Tables 1 and 2, and further addressed in this section. Importantly, most of these factors and signaling pathways have been found to be dysregulated both in obesity and OA, consistent with a role of endogenous stem cell niches in adipose tissue and joint homeostasis, remodeling and disease.

Pref-1 signaling and Sox9

Preadipocyte factor 1(Pref-1) is a protein highly expressed in preadipocytes which, in its secreted form, functions in a paracrine/autocrine manner to prevent preadipocyte differentiation through activation of mitogen activated protein kinase (MAPK) pathways [21]. Expression levels of Pref-1 markedly decrease upon differentiation of preadipocytes into adipocytes, and the repressive effect of Pref-1 on adipogenesis has been firmly established, both in adipocyte cell models and *in vivo* [22-24].

Recently, a functional link between Pref-1 and Sox9 was unveiled following the demonstration that Pref-1 inhibits adipogenesis through up-regulating Sox9 [19]. It was shown that Sox9 expression parallels Pref-1 expression during adipogenic differentiation of multipotent mesenchymal cells (mouse embryo fibroblasts) and already committed preadipose cells (3T3-L1); that constitutive overexpression of Sox9 inhibits, while knockdown of Sox9 enhances adipocyte differentiation in cell models and *in vivo*; that Pref-1 was unable to inhibit adipogenesis in cells in which Sox9 had been silenced; and that, mechanistically, Sox9 directly binds to the promoter regions of the early adipogenic transcription factors C/EBPβ and C/EBPδ to suppress their promoter activity, thus preventing adipocyte differentiation. Interestingly, by inducing Sox9, Pref-1 was also shown to promote chondrogenic induction of mesenchymal cells [19].

Sox-9 is required for mesenchymal cell condensation during development and for the expression of cartilaginous matrix proteins such as type II and IX collagens and aggrecan [18]. Sox9 expression in cartilage is reduced in individuals with OA compared with age-matched controls and down-regulated in response to pro-inflammatory cytokines favoring cartilage degradation [18]. These and other findings suggest that Sox9 has potential clinical value in the treatment of OA. The results of a number of cell and animal studies support the concept of Sox9 gene augmentation therapy in the treatment of OA, and pharmacological activation of Sox9 in chondrocytes, although not as yet reported, is also of interest in this context [18].

A putative dysregulation of Sox9 in obesity remains largely unexplored. However, it is suggested by the findings that Sox9 mRNA is expressed in the stromal vascular fraction of mouse WAT, and that its expression is reduced in WAT of high fat diet-induced and genetically obese (*ob/ob*) mice, which also display reduced WAT expression of Pref-1 and increased WAT levels of adipocyte markers compared with lean animals [19]. In view of the reported inhibitory effect of Sox9 on adipogenesis, increasing Sox9 activity in WAT could be a way of tipping the adipocyte birth-death balance in favor of reducing the number of fat cells. Overall, activation of Sox9 is emerging as a potential molecular target in the treatment of both OA and obesity.

PPARγ

Peroxisome proliferator-activated receptors (PPARs) are lipid-activated transcription factors of the nuclear receptor superfamily that play important roles during embryonic development [25] and in the transcriptional control of lipid and glucose metabolism and inflammation in adulthood [26]. PPARs form heterodimers with retinoic X receptors, and, upon ligand binding, modulate the expression of downstream target genes depending on the presence of co-activators or co-repressors. There are three known PPAR family members: PPARα, PPARδ and PPARγ each encoded by a separate gene and with different ligand specificities, tissue distribution and target genes. PPARγ is the most studied member of the PPAR family. It exists in two isoforms, PPARγ1 and PPARγ2, which derive from a single gene through alternate promoter usage and splicing. The PPARγ2 protein is expressed mainly in adipose tissues. PPARγ1 is expressed in many tissues and is the predominant form expressed in human cartilage [27].

PPARγ plays critical roles in adipose tissue biology: it is the master regulator of fat cell differentiation (adipogenesis), it promotes lipid storage in mature adipocytes by enhancing the expression of key genes in fatty acid uptake and trapping, and it is required for the survival of mature adipocytes [28, 29]. PPARγ is also involved in the control of whole-body glucose homeostasis and insulin sensitivity and is the presumed target for binding by thiazolidinediones (TZDs), a class of synthetic insulin sensitizers that are used to treat human type 2 diabetes, though at the expense of subcutaneous fat gain and other undesirable side effects. Genetic studies have confirmed an important role of PPARγ in adipogenesis, fat cell function and insulin sensitivity in humans [30].

Dysregulation of PPARγ in cartilage has been implicated in the pathogenesis of joint diseases [27, 31]. PPARγ1 expression is diminished in human OA cartilage compared with normal cartilage, and in human articular chondrocytes following exposure to inflammatory cytokines and inflammatory lipid mediators known to play a role in the development of OA, such as interleukin-1 (IL-1), tumor necrosis factor α (TNFα), and prostaglandin E_2 (PGE_2). Several studies have shown that PPARγ agonists down-regulate inflammatory and catabolic responses in chondrocytes and synovial fibroblasts, and protective effects of PPARγ agonists have been demonstrated in arthritis experimental models *in vivo*. PPARs can suppress the expression of inflammation-related genes by interacting with and blocking the activity of key transcription factors in the inflammatory response, such as nuclear factor-κB (NF-κB) and SP-1, and by other means [27]. Thus, accumulated evidence suggests that PPARγ agonists might be useful as anti-arthritic drugs, and there are ongoing clinical trials testing this concept [27, 31]. Reduced PPARγ activity in the OA joint may also contribute to abnormal growth and remodeling of subchondral bone, since recent research has unveiled an inhibitory role of PPARγ on osteoblastogenesis [32].

PPARγ coactivator 1 alpha (PGC-1α)

PGC-1α is a transcriptional coactivator for several nuclear receptors and other transcription factors involved in multiple aspects of cellular energy metabolism [33]. PGC-1α coactivates the transcription of the uncoupling protein 1 (UCP1) gene – encoding the molecular marker and thermogenic effector of brown adipocytes – and of genes related to mitochondrial biogenesis and function [34, 35]. Dysregulation of PGC-

1α in obesity and diabetes has been described. The expression of PGC-1 isoforms is reduced in skeletal muscle and WAT of obese and diabetic humans and rodents [36-40], and several human genetic studies have found polymorphisms in PGC-1 isoforms to associate with obesity and diabetes [41-44].

Interestingly, expression of PGC-1α is induced at chondrogenesis sites during mouse embryonic limb development and during chondrogenesis in human mesenchymal stem cells cultures [45]. Moreover, it has been shown that PGC-1α directly interacts with Sox9 and promotes Sox9-dependent transcriptional activity on the type II collagen gene, strongly suggesting that PGC-1α behaves as a transcriptional coactivator for Sox9 in chondrogenesis [45]. However, dysregulation of PGC-1α expression in articular chondrocytes as a factor in OA remains, to our knowledge, an unexplored issue.

Wnt signaling

Wnts (wingless-type MMTV integration site) are a family of secreted glycoproteins acting in autocrine and paracrine manners to control cell proliferation, survival, fate and behavior during embryonic development and adult tissue homeostasis and remodeling [46, 47]. Wnt proteins bind to frizzled (Fzd) receptors and LRP5/6 (low-density lipoprotein-receptor-related protein-5 or -6) co-receptors at the cell membrane initiating a signaling cascade that leads to the activation and nuclear translocation of the transcriptional regulator beta-catenin and subsequent changes in gene expression. Wnt proteins can also activate a number of additional, non-canonical, pathways independently of beta-catenin. Wnt signaling is modulated by several intracellular inhibitory proteins and extracellular antagonists. The latter include secreted frizzled-related proteins (sFRPs) and dickkopf (Dkk) family members [48]. Activation of the canonical Wnt pathway generally results in inhibition of chondrogenesis and adipogenesis and induction of osteoblastogenesis [15, 32].

There is increasing recognition that activation of Wnt signaling in musculoskeletal tissues of the joint (cartilage, subchondral bone) may represent a pathologic mechanism for OA development [14, 15, 49, 50]. An increased level of beta-catenin, certain Wnt signals (Wnt16) and the downstream Wnt signaling effector WISP-1 (Wnt-induced signaling protein 1) have been observed in degenerative cartilage, both in humans and rodents [15, 49-51]. In adult mice, conditional chondrocyte-specific activation of the beta-catenin gene [52] or adenoviral overexpression of WISP-1 in knee joints [51] leads to an OA-like phenotype. A genetic polymorphism in the Wnt inhibitor sFRP-3 that results in a protein product with a reduced ability to limit beta-catenin signaling has been found to associate with an increased susceptibility to develop OA in humans [49]. Conversely, high circulating levels of another Wnt inhibitor, Dkk-1, associated with a slow progression of hip OA in humans [49].

Endogenous Wnts (possibly Wnt10b) normally function to restrain adipogenesis by blocking the induction of PPARγ and C/EBPα in preadipose cells [53]. Adipose-specific overexpression of Wnt10b results in leanness and protects against diet-induced obesity [54] and genetic obesity [55] in transgenic mice. Polymorphisms in Wnt related genes have been associated with obesity and/or obesity-related traits in human population studies [56, 57]. Results from animal studies suggest that epigenetic reprogramming of Wnt signaling in WAT might alter susceptibility to obesity [58]. Very recently, the Wnt inhibitor sFRP5 has emerged as a potential anti-inflammatory adipokine capable of attenuating systemic metabolic dysfunction

in obesity, probably by neutralizing Wnt5a-dependent c-Jun N-terminal kinase (JNK) activation in WAT [59]. In the same report, human obese individuals with a bad metabolic profile (i.e., exhibiting increased WAT inflammation and insulin resistance) were shown to have reduced sFRP5 transcript expression in WAT biopsies compared with healthier obese individuals [59]. Overall, these and other results implicate alterations of Wnt signaling in obesity.

Wnt signaling has been suggested as a potential target in OA therapy [60] and in the therapy of obesity and associated disorders [53]. However, the complexity and widespread biological effects of Wnt signaling, which include proven roles in carcinogenesis, makes Wnt-based approaches challenging. Specific strategies are required to pinpoint targets for safe and efficient OA therapy, based on identifying the misexpression of specific Wnt proteins, Wnt inhibitors, or downstream Wnt signaling effector molecules in tissues that are important in OA and obesity.

Hedgehog signaling

The Hedgehog (Hh) signaling pathway is one of the key regulators of animal development conserved from flies to humans [61]. Secreted Hh proteins convey changes in gene expression following their interaction with specific cell membrane receptors, and they regulate the commitment of precursors to a wide array of cell fates.

Hh signaling regulates early and terminal chondrocyte differentiation as well as osteoblastogenesis and bone formation through endochondral ossification (see [62, 63]). During chondrocyte terminal differentiation, Sox9 expression decays, Runx2 expression increases, and chondrocytes become hypertrophic, remove the collagen and aggrecan matrix through the production of specific extracellular proteases, and finally die by apoptosis and are replaced by bone. In OA, articular cartilage chondrocytes undergo phenotypic and gene expression changes that are reminiscent of their end-stage differentiation in the growth plate during skeletal development, and abnormal activation of Hh signaling appears to be involved in this process [63]. Pharmacological or genetic inhibition of Hh signaling has been seen to reduce the severity of OA in mice and human cartilage explants [63]. Activation of Hh signaling may also underlie subchondral bone remodeling in OA [64]. Together, the findings point to a blockade of Hh signaling as a potential therapeutic target in OA.

Hh signaling also plays an important role in regulating adipogenesis, having been shown to inhibit white fat formation in species as diverse as flies and mice [65, 66]. Hh signals elicit this function early in adipogenesis, upstream of PPARγ, potentially diverting preadipocytes and multipotent mesenchymal precursors away from adipogenesis and toward osteogenesis [65]. Signs of reduced Hh signaling in WAT in mouse models of obesity have been reported [65] and, interestingly, mice with constitutive activation of Hh signaling in adipose tissues display WAT-specific lipoatrophy with normal BAT development, normal glucose tolerance and insulin sensitivity and no evidence of pathologic ectopic lipid accumulation [66]. The favorable metabolic profile despite WAT lipoatrophy of these transgenics has been attributed [66] to their intact capacity to burn energy in BAT and to their higher production of adiponectin, an anti-diabetic and anti-atherosclerotic adipokine. The Hh pathway could be a potential therapeutic target for adipose tissue-related disorders including obesity, in which activation of adipose Hh signaling would be pursued.

Transforming growth factor beta (TGFβ)

TGFβ is the prototypic member of a large family of structurally related pleiotropic, secreted cytokines that control differentiation, proliferation, and state of activation of many different cell types, including differentiation of mesenchymal stem cells into different lineages. TGFβ and members of the TGFβ superfamily signal through binding to specific cell membrane receptors, initiating specific intracellular transduction pathways, such as those involving the Smad transcription factors.

TGFβ is a multifunctional regulator of chondrocyte biology with dual roles in OA development [67]. In general, TGFβ is protective for articular cartilage, behaving as an anabolic and repair factor capable of stimulating extracellular matrix (ECM) synthesis by chondrocytes. However, this protective effect is lost in older animals, and there is evidence that TGFβ signaling can be deleterious for articular cartilage [67], besides being involved in osteophyte formation [68]. Dual effects of TGFβ on chondrocytes have been attributed to its ability to signal via different receptors and related Smad signaling routes [67]. In the nucleus, different activated (phosphorylated) Smads modify the expression of distinct subsets of genes. Whereas activation of the Smad2/3 route appears to potentiate anabolic responses, activation of the Smad1/5/8 route would selectively stimulate chondrocyte expression of extracellular proteases, terminal differentiation and hyperthrophy, all of which are hallmarks of OA. Remarkably, the ALK1/ALK5 ratio in cartilage is significantly increased in aging mice and in experimental models of OA, a feature that may favor TGFβ signaling via the "deleterious" Smad1/5/8 route. Stimulation of the Smad2/3 route and/or inhibition of the Smad1/5/8 route downstream of TGFβ are potential targets in OA therapy, although the possible side effects are unclear [67].

Cumulative evidence indicates an inhibitory role for TGFβ on white adipogenesis, by enhancing ECM synthesis and preventing normal changes of the ECM during adipogenesis [69, 70] and by Smad3 interacting with C/EBPs and repressing C/EBP transactivation function [71]. *In vivo*, transgenic overexpression of TGFβ results in a lipodystrophy-like syndrome, with a severe reduction of body fat in the mutant mice [72]. Quite paradoxically, TGFβ expression is increased in WAT depots of obese mice [73] and humans [74], where it closely correlates with the expression levels of plasminogen activator inhibitor-1 (PAI-1) [74]. The latter is one of the best characterized downstream targets of TGFβ in many cell systems and the most important physiological inhibitor of the fibrinolytic process that degrades fibrin clots in the blood.

Bone morphogenetic proteins (BMPs)

BMPs are cytokines of the TGFβ superfamily with wide-ranging biological activities, including the regulation of proliferation, apoptosis, differentiation and migration of mesenchymal cells. Over 20 BMPs are known and these proteins are involved in numerous cellular functions of which bone formation is just a small part. BMPs play important roles during embryonic development and are also important for the maintenance of tissue homeostasis during adult life.

BMP activity boosts all phases of chondrocyte differentiation, from mesenchymal condensation to terminal differentiation [75]. The paradigm is that BMPs are protective for articular cartilage because of their ability to promote ECM synthesis and deposition through up-regulation of Sox-9, and BMP-based

therapies hold considerable promise for effective cartilage repair and/or regeneration [75]. However, there is evidence that with aging and/or with the development of OA, articular chondrocytes may become unresponsive to growth factor stimulation. Moreover, similar to TGFβ, there is evidence that BMPs may play a role in cartilage destruction and OA progression by favoring the expression of extracellular proteases associated to chondrocyte terminal differentiation and hypertrophy [75].

There is increasing recognition that different BMPs may have differential effects on cartilage, and that not all BMPs are equally well suited for OA therapy [75]. One BMP of particular interest in this context is BMP-7, also known as osteogenic protein-1. BMP-7 has been reported to induce similar anabolic responses in normal and OA chondrocytes from young and old donors without causing chondrocyte hypertrophy [76]. Moreover, unlike other members of the BMP family, BMP-7 has anti-catabolic properties in addition to its strong pro-anabolic activity. Thus, reported effects of BMP-7 in chondrocytes include: counteraction of the inhibition of proteoglycan and hyaluronan synthesis induced by various catabolic mediators; inhibition of the endogenous expression of pro-inflammatory cytokines; blockade of basal and cytokine-induced expression of key matrix degrading enzymes; and reduction of chondrocyte apoptosis after acute cartilage trauma in a sheep model. Inhibition of transcription factors NF-κB and AP-1 are thought to be part of the underlying mechanisms responsible for the anti-catabolic activity of BMP-7 in chondrocytes [76].

BMPs have been implicated in adipogenesis [77]. Remarkably, whereas the activity of other BMP family members (such as BMP-2 and BMP-4) promotes white adipogenesis, BMP-7 behaves specifically as a brown fat inducer capable of activating by itself the full program of brown adipogenesis, including induction of UCP1 and of mitochondria biogenesis, both in cell models (primary brown preadipocytes or pluripotent C3H10T1/2 mesenchymal cells) and *in vivo* [78]. Moreover, BMP-7 knockout embryos showed a marked paucity of brown fat and an almost complete absence of UCP1, indicating an essential role of BMP-7 in the development of BAT *in vivo* [78]. Interestingly, adenoviral-mediated expression of BMP7 in adult mice resulted in a significant increase in brown, but not white, fat mass and led to increased energy expenditure and reduced weight gain [78]. Overall, up-regulation of BMP-7 is emerging as an attractive potential therapeutic target in the treatment of both obesity and OA.

Insulin-like growth factor 1 (IGF-1)

IGF-1 is the main anabolic mediator in articular cartilage, i.e. the main stimulator of matrix synthesis by chondrocytes. It is believed that in the OA cartilage IGF-1 has poor anabolic efficacy partly because of its sequestration by abnormally high levels of extracellular IGF binding proteins (IGBPs) [79]. The latter are a superfamily of homologous proteins, totaling 15 members, which modulate the availability of IGF-1 to the cells. IGBPs generally bind IGFs preventing them from binding their cognate receptors, although there are examples of positive modulation of IGF signaling by IGBPs, as well as of IGF-independent effects of IGBPs [80, 81]. IGFBP-3 is the main IGFBP secreted by OA cartilage explants [82]. Reducing IGFBP-3 activity is a potential therapeutic target in OA, and small molecules have been developed that inhibit the binding of IGF-1 to IGFBP-3 and effectively restore proteoglycan synthesis by human OA chondrocytes in culture [82]. Interestingly, in human articular cartilage IGFBP-3 is located not only extracellularly, but also

closely associated with the chondrocyte nucleus, a finding that suggests IGF-independent nuclear actions of IGFBP-3 in cartilage [83].

IGF-1 is expressed at high levels in adipose tissues, and a wealth of studies in cell and transgenic animal models indicate the IGF system impacts on adipocyte development favoring adipogenesis and is involved in the pathogenesis of obesity and insulin resistance [81]. Human epidemiological studies have suggested that, while IGFBP-1 and IGFBP-2 might be protective against the metabolic syndrome, IGFBP-3 might be a risk factor for it [80, 81], and this is in accordance with results of *in vitro* studies [84-86]. Furthermore, infusion of physiological concentrations of IGFBP-3 rapidly induced insulin resistance in rats [85], and overexpression of IGFBP-3 led to hyperglycemia, glucose intolerance and insulin resistance in transgenic mice [87]. Additionally, associations of IGFBP-3 gene polymorphisms with lipid parameters in human adolescents have been reported [88].

Overall, accumulated evidence suggests that strategies aimed at decreasing IGFBP-3 activity systemically, and specifically in cartilage and adipose tissue, could be explored in the treatment of both OA and obesity-associated cardiometabolic complications.

EXTRACELLULAR MATRIX COMPONENTS AND REMODELERS

The ECM plays a critical role in tissue development and function. ECM components serve structural roles thereby determining tissues' physical properties. In addition, the ECM can affect the bioavailability of growth factors and substrate molecules to cells. Moreover, cell-matrix and cell-cell interactions impinge on cell shape and influence gene expression and function, and hence cell differentiation and fate.

ECM remodelers include extracellular proteases belonging to three main families: (i) the classical MMPs (matrix metalloproteinases), which collectively are able to cleave collagens, glycoproteins and proteoglycans that populate the ECM of all major tissue types; (ii) the ADAM proteases (a disintegrin and metalloproteinase), whose substrates differ from MMPs and include growth factors, receptors, and cell-cell adhesion molecules; and (iii) the ADAMTS proteases (a disintegrin and metalloproteinase with thrombospondin motifs), a subgroup of ADAMs characterized by the presence of thrombospondin type I sequence repeats involved in the processing of collagen, coagulation and degradation of proteoglycans and extracellular glycoproteins [89]. Extracellular proteases comprise both secreted and membrane-anchored forms. These enzymes may impact on tissue biology by helping to modify the ECM via degradation, but also by affecting the processing and bioavailability of specific growth factors, receptors and substrates.

The ECM of cartilage and adipose tissue differ in their relative abundance, composition, and physicochemical properties. However, ECM dynamics is important in the development and homeostasis of both tissues. Erosion of articular cartilage ECM is a hallmark of OA, and the complex role that ECM dynamics plays in adipocyte biology, adipose tissue expansion and obesity is being increasingly recognized. These aspects are reviewed below.

Cartilage ECM and OA

Articular cartilage is an avascular, aneural tissue made up of a small number of chondrocytes embedded in an extensive ECM that is responsible for the functional properties of cartilage in resisting compression.

Chondrocytes occupy only about 5% of the tissue volume and are assumed to maintain the ECM by low-turnover replacement of matrix components. The two main components of cartilage ECM are type II collagen and aggrecan, the cartilage-specific proteoglycan. Aggrecan monomer consists of a core protein heavily modified with two types of sulfated glycosaminoglycans: chondroitin sulfate and keratan sulfate. Aggrecan monomers, often as many as 50 molecules, interact with single filaments of hyaluronan (a glycosaminoglycan that is a polymer of disaccharides of D-glucuronic acid and D-N-acetylglucosamine) to form proteoglycan aggregates that are further stabilized by link protein. The aggrecan-rich matrix is assembled and retained at the cell surface of chondrocytes via the interaction of hyaluronan with the hyaluronan receptor CD44.

Homeostasis of normal cartilage in adults represents a delicate balance between the synthesis and degradation of ECM components to maintain the functional integrity of the joint. In OA, both ECM synthesis and degradation are increased, but the balance is shifted towards net matrix loss, as much of the newly synthesized matrix molecules seem to be exported to the synovial fluid rather than incorporated into tissue [14]. Metalloproteases of the MMP and ADAMTS families are involved in the pathological destruction of joint tissues. They may also be involved in tissue repair, but become a part of the destructive disease process due to overexpression driven by a pathological stimulus, such as pro-inflammatory cytokines. Proteolysis of cartilage ECM constituents directly causes erosion as well as predisposing the tissue to mechanical disruption even with loading at physiologic levels. Moreover, matrix degradation products − e.g. fibronectin and collagen fragments − interact with specific cell surface receptors (integrins, CD44) in chondrocytes and synovial fibroblasts to activate catabolic intracellular signaling pathways such as the NF-κB pathway, which leads to further cartilage destruction and the establishment of a vicious circle perpetuating cartilage degeneration [90, 91].

Breakdown of aggrecan is a key early event in the development of OA that precedes and may be a prerequisite for subsequent collagenolysis. Aggrecan proteolysis is mediated by several matrix proteases, mainly aggrecanases of the ADAMTS family. ADAMTS-5 appears to be the primary enzyme responsible for aggrecan degradation in mice [92]. Work with human cartilage has suggested that ADAMTS-4 is also involved [93, 94]. Activation of ADAMTS-5 in OA seems to be dependent on its physical interaction with syndecan-4 [95], a member of a family of membrane-bound heparan sulfate proteoglycans that interact with proteinases, growth factors, cytokines, and structural ECM components through their heparan sulfate chains. Syndecan-4 expression is specifically induced in chondrocytes both in human OA and in murine models of the disease [95]. There is much interest in aggrecanases as potential therapeutic targets in OA, and selective ADAMTS-5 and ADAMTS-4 inhibitors have been synthesized in the search for new OA disease-modifying agents [96, 97]. Additionally, inhibition of syndecan-4 may represent a new strategy for the treatment of cartilage destruction based on blocking aggrecanase activation [95].

Cartilage collagenolysis in OA depends mainly on the activity of MMPs, although other enzymes, such as cathepsin K, may also be involved [98]. Synthetic inhibitors with a broad activity against MMPs have shown beneficial effects in animal models of OA [99, 100]. However, results from clinical trials in humans have been disappointing due to a lack of efficacy or safety concerns (severe musculoskeletal side effects were reported). Currently, efforts are directed to the design of specific inhibitors towards specific

MMP family members − such as MMP-13 (collagenase-3), which appears to be the main collagenolytic activity involved in cartilage erosion [101] − for selective usage. MMP activity is endogenously regulated by a family of tissue inhibitors of metalloproteinases (TIMPs), which represent another target in anti-catabolic strategies in OA [99, 100].

Cartilage ECM contains a large number of non-collagen proteins, some of which appear to be altered in OA. One of them is thrombospondin 1 (TSP-1), which has been found to be up-regulated in early OA cartilage lesions, but down-regulated in severe human OA [102]. TSP-1 normally functions to inhibit blood vessel formation (angiogenesis) and is also a major regulator of TGF-β activity due to its ability to convert latent TGFβ procytokine to the biologically active form [103, 104]. These two activities of TSP-1 may both oppose the development of OA because angiogenesis, which in OA occurs in the osteochondral junction and synovium, may accelerate inflammation and contribute to the severity of OA, while TGFβ is a potential anabolic growth for cartilage with immunosuppressive and chondroprotective effects [105]. Accordingly, intraarticular gene transfer of TSP-1 has been recently shown to suppress the disease progression of experimental OA in rats [105].

The expression of two additional non-collagen ECM proteins, COMP (cartilage oligomeric matrix protein) and SPARC (secreted protein, acidic and rich in cysteine; also known as osteonectin or BM-40) is induced in OA cartilage, possibly as an attempt to ameliorate matrix breakdown, since both proteins may function to facilitate collagen deposition. COMP is considered a marker for joint destruction and serum COMP levels are used to predict future disease progression [106]. Recent studies have identified molecular functions of COMP that include catalyzing polymerization of type II collagen fibrils that may enhance cartilage ECM stability, synthesis and assembly [106]. SPARC plays important roles in ECM assembly through its interactions with collagens [107] and might also function as a chaperone for proper intracellular trafficking of ECM proteins in chondrocytes [108, 109]. Induction of COMP [106] and SPARC [110] expression in OA chondrocytes might be part of the increased anabolic responses that occur in OA along with increased catabolic responses.

Hyaluronan − which is synthesized by chondrocytes and by the synoviocytes in the synovial membrane − plays a pivotal role in the organization and retention of cartilage ECM by serving as the core filament of the proteoglycan aggregate (see above). Moreover, hyaluronan acts in the synovial fluid as a viscous lubricant for slow joint movements and as an elastic shock absorber during rapid movements. Changes in OA include a reduction in the concentration and average molecular weight of hyaluronan in joint fluids. Decreased expression of hyaluronan synthetic enzymes and increased expression of hyaluronan degrading enzymes (hyaluronidases) have been found in the synovium of knees in arthritides [111]. There is evidence to suggest that hyaluronan degradation products (e.g. hyaluronan hexasaccharides) may boost cartilage catabolism in OA, similar to fibronectin and collagen fragments [91]. On the other hand, preclinical and clinical data suggest that intraarticular high molecular weight hyaluronan (“viscosupplementation”) may have a positive, disease-modifying activity on OA [112].

Adipose tissue ECM and obesity

Mature adipocytes are surrounded by a unique ECM that takes the form of a basement membrane composed mainly of collagens VI and IV, laminin and heparan sulfate. Collagen VI, in particular, is highly enriched in the ECM of WAT as compared to other tissues. WAT ECM provides mechanical support to maintain the structural integrity of adipocytes, in addition to participating in a variety of signaling events. Extensive ECM remodeling accompanies adipogenesis, and changes in the ECM are also required to accommodate adipocyte hyperthrophy and to sustain neovascularization during adipose tissue expansion [113, 114].

Specific extracellular proteases have been implicated as factors necessary for proper preadipocyte proliferation, adipogenesis and WAT expansion. For example, the activity of MMP-14 (a membrane-anchored collagenase) appears to be required for normal adipogenesis. In MMP-14- null mice, WAT (but not BAT) development is aborted, leaving fat depots populated by mini-adipocytes which render the null mice lipodystrophic [115]. MMP-14 activity is also required for adult WAT expansion: on a high fat diet, MMP-14 haploinsufficient mice are unable to remodel fat pad collagen architecture and display blunted weight gain [116]. This is in keeping with reports showing that treatment with broad-spectrum MMP inhibitors reduces fat mass gain induced by a high fat diet in mice, coupled with increased adipose collagen content [117, 118]. It is unclear whether the aforementioned concepts imply that MMP inhibitors could be useful in the treatment of human obesity. In fact, it is now recognized that, both in rodents and humans, increased deposition of collagens in the ECM, so-called adipose tissue fibrosis, characterizes WAT in obesity and diabetes [119, 120]. Adipose tissue fibrosis might directly contribute to a failure to further expand the tissue mass during states of positive energy balance, and has been related to the development of adipose tissue inflammation and obesity-linked metabolic derangements [119].

As a mediator of collagen deposition, the matricellular protein SPARC might contribute to limit WAT expansion but also to adipose tissue fibrosis and subsequent metabolic dysregulation in obesity. Recombinant SPARC was shown to inhibit the adipose conversion of primary murine preadipocytes by retarding morphological changes of the ECM and through activation of the Wnt/beta-catenin signaling pathway [121]. In humans, SPARC is abundantly expressed in WAT, especially subcutaneous WAT, and SPARC plasma concentration directly correlates with body mass index (BMI) and is associated with insulin resistance and diabetes complications [122]. Expression of SPARC in WAT is also enhanced in several murine models of obesity [123].

Although a dense ECM seems to exert a gross inhibitory effect on adipogenesis, specific ECM components may actually stimulate this process. For instance, there is evidence of a functional role of aggrecan in promoting early stages of adipogenesis of preadipose cells *in vitro* [124]. One potential mechanism to explain this is that cell surface heparan sulfate proteoglycans such as aggrecan can bind different lipoprotein particles and facilitate their uptake by differentiating fat cells [125]. Interestingly, aggrecan and aggrecanase (ADAMTS-4 and ADAMTS-5) mRNAs are expressed in mouse WAT, mainly in cells of the stromal-vascular fraction, and signs of increased aggrecan catabolism in WAT are present in diet-induced and genetic murine obesity [124]. It is suggested that intact aggrecan promotes adipose tissue formation in its very early stages, and is gradually down-regulated and degraded during further development, especially with obesity [124].

Adipose tissue expansion requires the parallel growth of its capillary network. Adipose tissue angiogenesis is increasingly been considered as a therapeutic target for obesity and metabolic diseases [126, 127]. Although inhibition is the usual paradigm in anti-obesity strategies, activation of adipose tissue angiogenesis might also be desirable (e.g., in BAT to promote BAT growth and thermogenic function, or in WAT when ectopic fat deposition in non-WAT tissues is the problem). Specific extracellular proteases switch on neovascularization by activation, liberation, and modification of angiogenic growth factors and degradation of the endothelial and interstitial matrix [128]. Accordingly, administration of synthetic MMP inhibitors reduces adipose tissue angiogenesis and results in reduced high fat diet-induced obesity in mice [126]. On the other hand, other ECM-related factors such as certain ADAMTS family members (e.g. ADAMTS-1 and -8) and thrombospondins (TSP-1 and TSP-2) are generally considered to be endogenous anti-angiogenic factors. In humans, TSP-1 is highly expressed in adipocytes of obese, insulin-resistant subjects, and adipose TSP-1 expression is highly correlated with markers of WAT inflammation and with the adipose expression of pro-thrombotic factor PAI-1, and is decreased following treatment with anti-diabetic drugs (TZDs) [129]. TSP-1 is expressed to higher levels in visceral than in subcutaneous fat, and it has been suggested that the physiological processes related with this gene (and with carboxypeptidase E) may contribute to adipose depot- and gender-specific differences regarding the metabolic complications of obesity [130].

Beyond direct effects on adipose tissue ECM remodeling, ECM-related factors may impact on adipose tissue expansion by acting in other tissues, e.g. in the hypothalamus to affect central circuitries regulating food intake and/or energy expenditure. For example, TIMP-1 mRNA is induced by leptin in mouse hypothalamus, and hyperphagia and obesity have been reported in mice lacking TIMP-1 [131]. As another example, syndecan-3 has been implicated in the control of food intake by interacting with the central melanocortin system [132], and syndecan-3 null mice are hypophagic and resistant to diet-induced obesity [133, 134]. Another member of the syndecan family, syndecan-1, mediates the hepatic clearance of triglyceride-rich lipoprotein remnants, and its dysregulation appears to contribute to postprandial hyperlipidemia associated with diabetes and abdominal obesity [135].

ADIPOKINES AND PRO-INFLAMMATORY PROTEIN MEDIATORS

Adipokines are adipose tissue-derived, secreted signaling proteins. White adipose tissue cells, including adipocytes, stromovascular cells and the associated immune cells, cross-talk via adipokines with the hypothalamus and other organs, which is essential to the control of energy homeostasis and metabolism. In addition, adipokines have important local (paracrine and autocrine) effects, because adipocytes express receptors for most of the signals they produce. Adipokines also play important roles in modulating inflammation and the immune response [136], and several of them have been implicated specifically in the pathophysiology of joint diseases such as rheumatoid arthritis (RA) and OA.

Some adipokines are cytokines or cytokine-like molecules and the distinction between these two categories is vague. As opposed to cytokines, which are produced by multiple cell types, and in WAT often mainly by immune cells infiltrating the tissue, adipokines are supposed to be released specifically by adipocytes. However, most (if not all) adipokines are produced also in cell types other than adipocytes. In

particular, joint tissues including synovium, osteophytes, cartilage and bone have been demonstrated to produce typical adipokines such as leptin and adiponectin [137]. Noteworthy, some joints contain articular adipose tissue, e.g. the infrapatellar fat pad of the knee joint, which is situated intracapsularly and extrasynovially and in close contact with synovial layers and articular cartilage [138]. Thus, adipokines and cytokines found in the joint may originate in adipose tissue or other non-joint tissues (and reach thereafter the joint by diffusion from the circulation) and/or be produced locally. The origin of articular adipokines and cytokines in joint diseases may have important implications for therapy (see Concluding remarks).

Main cytokines and adipokines known to be dysregulated in obesity and implicated in OA are summarized in Table 3 and briefly addressed next (see [5, 6] for recent reviews).

Pro-inflammatory cytokines

Inflammation plays an important role in the pathogenesis of both obesity and OA. A low-grade, chronic, systemic and WAT inflammation accompanies obesity, and it is thought that inflammation might be a primary cause of obesity-linked metabolic disturbances [139, 140]. Hypertrophied adipocytes and/or macrophages that selectively infiltrate WAT in obesity produce pro-inflammatory cytokines whose elevated levels, through local and distant effects, contribute to insulin resistance, hepatosteatosis (fatty liver) and increased risk markers for cardiovascular disease. The link between obesity and a pro-inflammatory state is further corroborated by the reduction of inflammatory markers in overweight subjects following weight loss. As for OA, even if it is traditionally classified as a non-inflammatory arthritis (due to low synovial fluid leukocyte count), there is increasing evidence that synovial inflammation is already common at early stages of the disease. It is now well established that, in OA, pro-inflammatory cytokines secreted by the synovial membrane, infiltrated immune cells, subchondral bone and the cartilage itself actively trigger cartilage destruction by inducing the expression of ECM degrading enzymes [5, 141]. Pro-inflammatory cytokines also boost the production of additional inflammatory mediators (such as nitric oxide and PGE_2) and of reactive oxygen species (ROS) that can induce oxidative damage, including mitochondrial damage eventually leading to chondrocyte apoptosis [142]. These effects are mediated by cytokine-dependent activation of intracellular signaling pathways such as the p38 MAPK, JNK, and NF-κB pathways.

Several mechanisms have been proposed to explain WAT inflammation associated with adipocyte enlargement, including adipocyte death, cellular (endoplasmic reticulum and mitochondrial) stress including oxidative stress, increased production of inflammatory lipid mediators and adipose tissue hypoxia. These mechanisms are not mutually exclusive and are likely to be cross-related. It has been proposed that adipocytes that reach a maximal size upon lipid loading spontaneously undergo necrosis, which is associated with increased infiltration of macrophages around the dying adipocyte [143]. Shear forces at the cell membrane-ECM interface may play a role in the death of enlarging adipocytes [119]. The infiltration of macrophages into obese WAT may also result from the release of monocyte chemoattractant protein 1 (MCP1) and other chemotactic signals (possibly including leptin) by the hypertrophied adipocytes [140]. An emerging view is that hypertrophy of adipocytes prevents proper oxygen supply to the cells creating a state of hypoxia that may promote adipocyte death and subsequent tissue inflammation [144, 145]. Additionally, hypoxia can induce oxidative stress and endoplasmic reticulum stress, which may also contribute to WAT inflammation [144, 145]. The mechanisms by which production of inflammatory

mediators in the joint is initiated are unclear, but abnormal mechanical and oxidative stresses have been implicated [12], as well as synovial tissue hypoxia [146].

Inflammatory cytokines known to be produced by adipose tissue, increased in obesity, and involved in obesity-associated metabolic disturbances such as insulin resistance and fatty liver have also been implicated in synovitis and other aspects of OA [5, 141, 147-153] (Table 3). This has led to the concept that − in addition to locally produced (joint born) cytokines − adipose tissue-derived cytokines in obesity may contribute to the cytokine storm in the joint that is a hallmark of the early stages of OA [5, 154]. The pathophysiological relevance of adipose tissue-derived signals is highlighted by the fact that WAT as a whole may be one of the largest endocrine organs in obese individuals [155].

Pro-inflammatory cytokines dysregulated in both obesity and OA include TNF-α and various interleukins such as IL-1, IL-6, IL-18 and IL-8. In particular, IL-1 and TNF-α are considered major players in cartilage breakdown [5, 141]. Strikingly, many of these cytokines − e.g. IL-1, IL-6, IL-18, as well as leptin (see below) − appear to play a physiological role in the endogenous control of energy homeostasis exerting an overall anti-obesogenic effect, in such a way that their deficiency results in obesity in mouse models [156-160]. This has led to the suggestion that up-regulation of these cytokines in WAT in obesity, i.e. adipose tissue inflammation, may serve as a feedback break to inhibit further increases in adiposity, yet at the expense of systemic derangements [139].

Pro-inflammatory cytokine activity is counteracted by several regulating factors such as IL-10, which in animal models can prevent diet-induced insulin resistance [161] and cartilage destruction induced by IL-1 and TNF-α [5]. Expression of IL-10 is increased in OA cartilage according to the degree of anatomical damage, and this up-regulation is generally viewed as an attempt to restore normal chondrocyte functions [5]. Importantly, human WAT is a regulated source of IL-10 [162] and increases in circulating levels of IL-10 after weight loss in humans have been reported [163]. These findings further reinforce the link between obesity and joint disease and contribute to explain the potential benefit of weight loss in OA patients [5].

Polymorphisms in cytokine-related genes have been found to associate with OA and obesity traits in human genetic epidemiology studies, further reinforcing the involvement of inflammatory cytokines in both conditions. For example, polymorphisms in genes of the IL-1 system (which include the IL-1 isoforms themselves, their receptors and an endogenous antagonist, the IL1 receptor antagonist or IL1Ra) have been linked to fat mass content and specifically to central obesity in humans ([164] and references therein), as well as to radiographic signs of OA [165-167].

Leptin

The most studied adipokine, leptin, is prevalently (though not solely) secreted by white adipocytes, circulates in blood at levels that correlate with the size of fat stores, and has central and peripheral effects that oppose weight gain, downstream of its interaction with specific cell surface receptors belonging to class I cytokine receptors [168]. In both rodents and humans, defective leptin signaling due to leptin deficiency (e.g. in *ob/ob* mice) or dysfunctional leptin receptors (e.g. in *db/db* mice) causes early-onset, severe, spontaneous obesity. However, the vast majority of obese subjects have very high levels of circulating

leptin in their blood and appear to be resistant to the action of leptin. The metabolic effects of leptin include reduction of food intake, stimulation of energy expenditure including BAT thermogenesis, enhancement of lipolysis in adipocytes and of fatty acid oxidation in several tissues (adipose tissues, skeletal muscle, liver), and inhibition of gluconeogenesis and lipogenesis in the liver. Most of these effects originate from the central actions of leptin on hypothalamic neurons, although direct peripheral action of leptin contributes as well [169]. Leptin also has an impact on the development of the neuronal circuitries regulating energy homeostasis [170], and animal studies indicate that oral leptin intake in early life can prevent overweight/obesity in adulthood, with an improvement of related functions such as leptin and insulin sensitivity and food preferences [171-173].

In addition to its principle role as a regulator of energy homeostasis, leptin is also involved in the immune and inflammatory response, acting mainly as a pro-inflammatory signal [174, 175], and has been specifically implicated in the pathophysiology of OA. Synovial fluid obtained from OA patients contains leptin at levels that correlate with BMI [176] but exceed those in paired serum samples [137, 177], and it has been shown that OA cartilage (and other joint tissues) produces leptin to a level that correlates with the degree of cartilage damage [176, 177].

There is evidence to suggest that increased articular leptin production in OA may represent an endogenous homeostatic, chondroprotective mechanism. Articular chondrocytes express functional leptin receptors, and cultured human chondrocytes stimulated with leptin exhibit increased proliferation and proteoglycan and collagen synthesis [178]. Intra-articular injection of leptin into the rat knee joint stimulates proteoglycan synthesis and the expression of the anabolic factors IGF-1 and TGFβ, as well as of leptin itself, in cartilage [176]. Moreover, in human OA cartilage, the pattern and level of leptin expression parallel those of IGF-1 and TGFβ, while normal cartilage does not express either leptin or growth factors, further suggesting a functional and reciprocal link [176]. However, leptin has also been reported to stimulate the expression of pro-inflammatory mediators (IL-1, IL-6, IL-18, nitric oxide, PGE_2) and extracellular proteases (MMP-9, MMP-13) in cultured chondrocytes and human cartilage explants [177, 179]. Furthermore, recombinant leptin injection into rat knee induced proteolytic enzymes in articular cartilage including ADAMTS-4 and ADAMTS-5 [180], the main aggrecanases in the pathogenesis of OA. Leptin is also likely to be involved in changes in subchondral bone in OA [5]. Importantly, a recent study showed that extreme obesity *per se* does not cause knee osteoarthritis in mutant mice with impaired leptin signaling (*ob/ob* and *db/db*), suggesting that, in mice, leptin is essential to induce OA, and other fat-derived messengers cannot surrogate leptin action [181].

On the whole, leptin seems to exert both anabolic and catabolic actions in articular cartilage, which is significant because both anabolic and catabolic activities of chondrocytes are up-regulated with the development of OA [181].

Adiponectin

Adiponectin is an adipokine with well established insulin-sensitizing and anti-atherogenic effects [182]. Adiponectin increases insulin sensitivity by stimulating glucose utilization and fatty acid oxidation in skeletal muscle and liver, and by suppressing gluconeogenesis and *de novo* lipogenesis in liver [182].

Adiponectin also exerts effects at adipose tissue level, as it is pro-adipogenic [183]. More recently, central effects of adiponectin on hypothalamic centers regulating feeding have been reported, although there have been controversial results as to whether central adiponectin stimulates [184] or suppresses [185] food intake, and the physiological importance of adiponectin in the hypothalamic regulation of energy homeostasis remains to be defined. Adipose expression and circulating levels of adiponectin, as well as the expression of adiponectin receptors (AdipoR1 and AdipoR2) in tissues, are decreased in obesity, conditions associated with insulin resistance, and cardiovascular disease [182].

An inverse correlation between adiponectin and classic markers of inflammation in plasma, such as C-reactive protein and IL-6, is found in obese and insulin-resistant subjects, and several reports have shown that adiponectin brings about a variety of anti-inflammatory activities, ranging from inhibition of inflammatory cytokine production by adipocytes and macrophages, to induction of anti-inflammatory factors, reduction of adhesion molecules expression in the vasculature, and others [175, 186]. However, in some chronic inflammatory/autoimmune diseases adiponectin may have pro-inflammatory effects and its production correlates with inflammatory markers and disease activity [175, 186]. In this context, the effects of adiponectin on joint inflammation and both RA and OA are interesting but controversial.

Adiponectin levels in serum are elevated in RA patients compared with healthy controls with a similar BMI [187, 188] and in patients with erosive compared with non-erosive OA [189]. In the synovial fluid, adiponectin levels have been reported to be higher in RA patients, in whom joint inflammation is more prominent, than in OA patients [188]. Interestingly, adiponectin levels in the synovial fluid of RA and OA patients are lower than in paired serum samples, with significant correlation noted between levels in the two sources, suggesting that the main source of articular adiponectin is peripheral adipose tissue rather than local tissues [188, 190].

A protective effect of adiponectin against cartilage degeneration has been suggested by the finding that in synovial fluid of RA patients, adiponectin levels inversely correlate with leukocyte count [188]. Moreover, joint tissues including chondrocytes express functional adiponectin receptors [190, 191], and incubation with adiponectin up-regulates the expression of the MMP inhibitor TIMP-2 and down-regulates IL-1-induced MMP-13 expression in OA chondrocytes [190]. However, there is also evidence pointing to a destructive role of adiponectin in cartilage homeostasis, since adiponectin-dependent induction of pro-inflammatory cytokines and other inflammatory mediators (e.g. IL-6, IL-8, MCP1, nitric oxide, PGE_2), MMPs (MMP-1, MMP-3, MMP-9), and vascular endothelial growth factor has been reported in human cultured chondrocytes and synovial fibroblasts [191-195]. Thus, there is controversy as to whether increased adiponectin in the diseased joint may serve to counteract local inflammation or, on the contrary, contribute to cartilage destruction.

Renin-angiotensin system

The renin-angiotensin system (RAS) converts angiotensinogen into angiotensin II, which is an important vasoconstrictor and a regulator of blood pressure and of salt and water balance. In the classical RAS, circulating renal-derived renin cleaves angiotensinogen, a protein principally synthesized in the liver, to form the inactive decapeptide angiotensin I, which in turn is converted into active angiotensin II by the action of the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) in the lungs [196]. Effects

of angiotensin II are mediated by specific cell surface receptors, notably angiotensin type 1 and 2 receptors (AT1 and AT2).

In addition to the systemic RAS that regulates blood pressure and fluid-electrolyte balance, local functional RAS have been demonstrated in many organ and tissue systems. In particular, the components of the RAS are fully represented in WAT [197, 198]. There is evidence that, with obesity, the adipose tissue RAS becomes activated and is a primary contributor to systemic concentrations of angiotensin peptides [197]. In addition, angiotensin II might exert paracrine/autocrine effects in WAT, as both AT1 and AT2 receptors have been localized to adipocytes, and effects of angiotensin II on adipocyte growth and differentiation, lipid metabolism, and expression and release of adipokines and RAS components have been reported [197]. Angiotensin II, mainly through the AT1 receptor, has also been demonstrated to promote inflammation by elaboration of cytokines and chemokines (e.g., upregulation of TNFα, IL-6 and MCP1) and increased oxidative stress [199]. In particular, angiotensin II activates NF-κB and it is itself a chemotactic factor for the recruitment of inflammatory cells. Therefore, activation of the adipose tissue RAS system in the obese state may contribute to WAT inflammation and related metabolic disorders, as well as to hypertension and other potential systemic effects − including effects on the joints − by elevating the circulating concentrations of RAS components. Furthermore, there is evidence of a pathophysiological crosstalk between the activation of RAS and the generation of advanced glycation end products in diabetic vascular complications [200].

Studies have suggested that local RAS activity can contribute to synovial inflammation and joint destruction in RA [201]. For instance, synovial fluid ACE levels and renin concentrations are higher in patients with RA than in patients with OA [201], and AT1 receptors are up-regulated in the rheumatoid synovium [202]. Whether RAS activation plays a role in OA is less clear. Interestingly, polymorphisms in the ACE gene have been associated with the risk of OA in some human population studies [203].

Angiotensin-receptor blockers are used clinically for the treatment of hypertension and cardiovascular disease. More recently, studies in experimental models of obesity have shown that AT1 receptor blockade reduces plasma glucose levels and improves insulin sensitivity [204]. There is also evidence, principally from studies in animal models, that AT1 receptor antagonists can reduce inflammatory synovitis [202]. Further studies are warranted to establish the extent to which AT1 receptor blockers may provide anti-inflammatory benefits in the context of obesity and/or OA in humans.

Resistin and visfatin

Resistin was originally identified as an adipocyte-secreted protein down-regulated by anti-diabetic drugs that causes insulin resistance in mice, although there have been subsequent conflicting findings, particularly in humans [205]. Visfatin, in turn, was claimed to be a beneficial adipokine with insulin-mimicking/sensitizing effects, although regulation of visfatin production and its physiological importance in the conditions of obesity and type 2 diabetes remains unclear [206]. Despite putative opposing effects of resistin and visfatin on insulin sensitivity, both adipokines have pro-inflammatory properties [207], and both have emerged as pro-inflammatory and pro-catabolic factors in arthritides [5, 6]. Resistin is detected in the inflamed joints of patients with OA and, especially, RA, and resistin serum levels positively correlate

with disease activity in RA [208]. In line with a catabolic role, resistin is elevated in the joint following traumatic joint injury and resistin promotes ECM degradation and release of inflammatory cytokines from articular cartilage *in vitro* [209]. Likewise, serum levels of visfatin are elevated in RA patients, in which they correlate with the severity of the disease [210], and there is evidence that visfatin is produced by human OA chondrocytes and stimulates the production of matrix degrading enzymes and PGE_2 by cultured chondrocytes [211].

More recently, newly discovered adipokines such as vaspin, omentin and apelin have been detected in the synovial fluid of RA and OA patients [212, 213]. Apelin, in particular, appears to play a catabolic role in cartilage in both *in vitro* and *in vivo* studies [214]. Nerve growth factor is another signal produced by adipose tissue that might play a role in the pathogenesis of obesity-associated OA and cardiometabolic diseases, with a pro-inflammatory effect [5, 6, 100].

HYPOXIA INDUCIBLE TRANSCRIPTION FACTORS (HIFs)

HIFs control changes in gene expression typically in response to changes in oxygen availability in the cellular environment, although their activity might respond to other forms of cellular stress besides hypoxia [215]. The HIF protein family consists of α- and β-subunit members which function by forming heterodimers. Under normoxic conditions, the α-subunit members (HIF-1α, HIF-2α and HIF-3α) undergo oxygen-dependent hydroxylation, resulting in ubiquitination and degradation by the proteosome. In contrast, under hypoxic conditions they are neither hydroxylated nor degraded and are active in the control of hypoxia-inducible genes, which include genes encoding proteins that promote angiogenesis, anaerobic metabolism and pH homeostasis.

Articular cartilage is a physiologically hypoxic tissue, and HIF-1α is required for the maintenance of anaerobic glycolysis and ECM synthesis in mature chondrocytes and as a survival factor inhibiting chondrocyte apoptosis [216, 217]. In striking contrast, the closely related HIF-2α has recently emerged as a central player promoting the development of OA [217-219]. It has been shown that HIF-2α directly induces the expression in chondrocytes of catabolic factors including MMPs and aggrecanases as well as of proteins linked to pathological endochondral ossification; that HIF-2α expression in cartilage is markedly increased in early stages of human and mouse OA; that ectopic expression of HIF-2α results in articular cartilage destruction in mice and rabbits, whereas its experimental knockdown blocks cartilage destruction; and that HIF-2α expression is induced in chondrocytes by multiple pro-inflammatory cytokines, possibly through the activation of the NF-κB pathway [218, 219]. These findings suggest that in OA cartilage there is a stress-induced increase in the activity of HIF-2α which overshadows the beneficial effects of HIF-1α, and that down-regulation of HIF-2α in the joint may represent a novel therapeutic target in OA [217].

HIFs, in particular HIF-1α, have also been implicated in adipose tissue biology and obesity. HIF-1α transactivates the human leptin gene, which is induced in response to hypoxia in human adipocytes and preadipocytes [144, 220]. In addition, a role of HIF-1α for normal BAT function, and thereof for obesity resistance, has been recently proposed, following the demonstration of an obesity-prone phenotype in mice with adipose tissue-selective inhibition of HIF-1α which is associated with reduced angiogenesis in BAT

(but not WAT) and reduced mitochondrial content in brown adipocytes [221]. Other authors have investigated the consequences of transgenic HIF-1α overexpression in adipose tissues and found that HIF-1α activation initiates a local fibrotic response with an associated increase in local inflammation [222]. Induction of adipose tissue fibrosis by activated HIF-1α is explained, at least in part, because one HIF-1α target gene encodes a lysil oxidase that catalyses cross-linking of collagen I and III to form the fibrilar collagen fibers [222]. Fibrosis in adipose tissue has previously been linked to increased stress on expanding adipocytes, and to adipocyte necrosis [119]. The results led the authors to hypothesize that, in obesity, localized WAT hypoxia and subsequent HIF-1α activation and HIF-1α-induced fibrosis could be early upstream initiator factors for subsequent adipocyte death, monocyte infiltration, local inflammation and systemic consequences thereof, such as reduced insulin sensitivity [222]. Overall, the emerging picture is that activation of HIF-1α in BAT might serve to increase thermogenesis in tissue and energy expenditure, whereas activation of HIF-1α in WAT may favor WAT dysfunction and insulin resistance.

LIPIDS

Obesity, particularly abdominal obesity, is frequently accompanied by dyslipidemia, with high circulating levels of free fatty acids, triacylglycerol and cholesterol, all of which are well-known risk factors for type II diabetes and vascular disease. Different factors underlie dyslipidemia in obesity, including aberrant lipolysis and/or fatty acid metabolism in dysfunctional adipocytes. Because lipids are likely contributors to the pathogenesis of cartilage degradation, altered lipid metabolism is a potential link between obesity and OA [1, 4, 223].

Multiple lines of evidence implicate lipids in the development of OA. Even if articular cartilage is an avascular tissue, there is evidence that circulating lipids can access chondrocytes. For instance, dietary lipid interventions in animals impact on the fatty acid composition of articular cartilage [224, 225], and in *ex vivo* experiments using cartilage explants fatty acids were shown to enter the cartilage matrix at a faster rate than albumin [226]. Excessive fat in the synovial fluid can induce arthritic changes in articular cartilage and synovial membrane, and raised concentrations of fatty acids and neutral lipids are found in cartilage from patients with OA [227, 228]. Epidemiological studies have shown serum cholesterol to be a risk factor for OA development [1, 223]. Proteomic analyses indicate that many of the proteins that are differentially expressed in OA cartilage compared with normal cartilage are related to lipid metabolism [223]. In particular, human OA chondrocytes display a reduced expression of genes regulating cholesterol efflux [228], and an increased expression of the oxidized low density lipoprotein receptor, LOX-1 [229].

Apart from effects related to hypercholesterolemia, oversupply of free fatty acids to tissues as occurs in obesity might boost the local production of inflammatory lipid mediators which may play a part in pathologies typically associated with obesity as well as in arthritides [230, 231]. The main bioactive lipids in this context are addressed below.

Eicosanoids

The n-6 polyunsaturated fatty acid (PUFA) arachidonic acid (ARA, 20:4 cis-$\Delta^{5,8,11,14}$) is the precursor of pro-inflammatory eicosanoids [232]. ARA is formed from the essential n-6 fatty acid linoleic acid (18:2 cis-

$\Delta^{9,12}$) and is abundant in cell membrane phospholipids. The first step in the production of ARA-derived eicosanoids is the release of ARA from cell membrane phospholipids by the action of phospholipase A_2, especially when inflammatory signals such as interleukins or interferons stimulate the cell. Released ARA is then converted to leukotrienes through the action of the enzyme lipooxygenase or to prostaglandin G_2 (PGG_2) through the action of cyclooxygenases. From PGG_2 other prostaglandins and thromboxanes are formed through different terminal synthases. Cycloxygenase-derived metabolites are collectively termed prostanoids. Prostanoids can function from the cell exterior in a paracrine-autocrine manner through activation of specific cell-surface G protein-coupled receptors, or they can function in an intracrine manner by serving as activating ligands of nuclear receptors such as PPARs.

Evidence from animal studies indicates that cyclooxygenase activation is crucially involved in the development of the inflammatory response in WAT of high fat diet-induced obese rats and in obesity-linked insulin resistance and fatty liver [233, 234]. In addition, prostaglandins modulate adipocyte differentiation and lipid metabolism in a rather complex manner. For instance PGE_2 inhibits white adipocyte differentiation [235], yet it can contribute to the hypertrophic enlargement of adipocytes through its anti-lipolytic activity [236]. Moreover, recent studies in animals indicate that local cyclooxygenase activity and PGE_2 in WAT may oppose obesity by favoring the recruitment of brown adipocyte precursors present in white fat depots, and thereof energy expenditure [237, 238]. Interestingly, reduced production of PGE_2 in WAT in obesity has been reported [239], a feature that could contribute to dysregulated lipolysis, enhanced white adipogenesis and reduced energy expenditure. Other ARA-derived eicosanoids, in particular PGI_2 (prostacyclin) and 15-deoxy PGJ_2, have been implicated as pro-adipogenic factors capable of inducing white adipogenesis and fat accumulation in mature adipocytes [240, 241]. In keeping with an overall pro-adipogenic effect of ARA-derived eicosanoids, several human and animal studies have shown a direct correlation between increased levels of ARA in fat depots or plasma and increased obesity or body weight [231, 242].

ARA-derived eicosanoids have been implicated in the pathogenesis of OA. The articular cartilage content of ARA correlates with OA severity [227]. The expression of microsomal PGE_2 synthase-1 (mPGES-1) − a terminal PGE_2-synthesizing enzyme − is induced in human cartilage in response to inflammatory cytokines [243] and mechanical compression [244]. Among the prostaglandins, a lot of work has focused on PGE_2, which is usually implicated as a major lipid inflammatory mediator, yet its role in chondrogenesis and OA development remains controversial. Whereas some studies have found PGE_2 to be pro-catabolic and anti-anabolic in cartilage (e.g. [245, 246]) others have found this prostaglandin to induce chondrogenesis *in vitro* and cartilage regeneration *in vivo* [247, 248]. Chondrocytes express different isoforms of the PGE_2 cell surface receptor (named EP1 to EP4), and PGE_2 appears to exert different effects through each isoform. Data suggest that PGE_2 signals catabolic effects through the EP4 receptor [245] and anabolic effects through the EP2 receptor [247, 248]. Whether the expression pattern of PGE_2 receptors in cartilage varies with OA development is unknown, but is conceivable since, for instance, up-regulation of EP1 and EP4 in articular chondrocytes following pro-inflammatory cytokine (IL-1) stimulation has been reported [249]. Other prostaglandins besides PGE_2 have been implicated in the pathogenesis of OA. In

particular, a potential role of 15-deoxy PGJ_2 favoring articular chondrocyte apoptosis has been proposed [250].

Interestingly, normal young cartilage in human and other species appears to have a unique fatty acid composition, with high levels of unusual n-9 PUFA (20:3 cis-$\Delta^{5,8,11}$) and low levels of n-6 PUFA (linoleic acid and ARA) [251]. Importantly, the high levels of n-9 fatty acid in young cartilage are progressively depleted during aging, accompanied by an increase in n-6 PUFA, a trend that is particularly pronounced in OA cartilage [251]. The significance of these findings is not fully understood, but it was suggested that, since the 20:3 n-9 fatty acid cannot be a substrate for cyclooxygenase, its accumulation in young cartilage may be important to prevent prostaglandin-induced inflammatory responses [251].

Whereas n-6 PUFA-derived eicosanoids have pro-inflammatory and pro-atherogenic effects, eicosanoids derived from the n-3 PUFAs eicosapentaenoic acid (EPA, 20:5 cis-$\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA, 22:6 cis-$\Delta^{4,7,10,13,16,19}$) have either the opposite effects or possess much lower levels of biological activity than the n-6 eicosanoids [232]. Moreover, additional classes of anti-inflammatory lipid mediators, namely resolvins and protectins, are formed from n-3 PUFAs [252]. In chondrocyte cell systems, incubation with n-3 PUFAs (particularly EPA) specifically reduced the expression of cartilage-degrading proteinases (aggrecanases, MMP-13), inflammatory cytokines (IL-1 and TNFα) and cyclooxygenase-2 [253]. Besides anti-inflammatory, n-3 PUFAs may be viewed as anti-obesogenic because they can inhibit the production of pro-adipogenic lipid mediators from n-6 PUFA in preadipocytes (by competing for the same eicosanoid producing enzymes), and because they have well-established effects reducing lipogenesis in liver and stimulating fatty acid oxidation in WAT [254]. In fact, it has been suggested that a relatively high intake of n-6 PUFA and low of n-3 PUFA, especially during early life and infancy, could be a contributory factor to current obesity pandemics; what is more, it has been noted that the n-6 PUFA/n-3 PUFA ratio has continuously and markedly increased in human breast milk and infant formulas in the last decades [255]. EPA ad DHA are abundant in fatty fish and fish oil, and can be formed in mammalian cells from the essential n-3 fatty acid α-linolenic acid (18:3 cis-$\Delta^{9,12,15}$), although only in low amounts.

Cardioprotective effects of fish oil consumption are well established, and n-3 PUFA supplementation is also viewed as a useful anti-inflammatory strategy to decrease obesity-induced insulin resistance [256]. As for effects on obesity itself, studies in rodents have consistently reported that intake of n-3 PUFA reduces adipose mass, preferentially visceral fat, and limits diet-induced obesity (see [257]). In humans, there have been studies showing that dietary fish oil consumption can increase whole-body lipid oxidation, reduce total body fat content and specifically abdominal fat content, and improve insulin sensitivity [258, 259]. In one study, dietary n-3 PUFA supplementation combined with a very low calorie intake enhanced weight loss in obese women [260]. Regarding arthritis, clinical trials have generally shown that dietary supplementation with n-3 PUFA provides modest symptomatic benefit in arthritic patients, but most of these studies have focused on RA, rather than on OA [253, 261].

Ceramide and sphingolipids

Sphingolipids are derivatives of sphingosine, an 18-carbon amino alcohol itself derived from linkage of serine and palmitic acid (from palmitoyl-CoA) through the action of the enzyme serine palmitoyltransferase [230]. Sphingolipids are structural components of cell membranes and many of them also exert signaling functions. Ceramide, a sphingolipid made up of sphingosine and a fatty acid, is an important signaling molecule on its own and a precursor for other signaling molecules including sphingosine, sphingosine-1-phosphate (S1P), and ceramide-1-phosphate. Ceramide also functions as a structural building block for complex sphingolipids such as sphingomyelin and glycosphingolipids. Sphingolipids have been implicated in diverse regulatory functions including: promotion of apoptosis (ceramide), inhibition of apoptosis and promotion of cell proliferation (S1P), and promotion of inflammation (S1P, ceramide-1-phosphate) [230]. S1P and ceramide-1-phosphate favor the production of inflammatory lipid mediators by activating cyclooxygenase-2 and phospholipase A2 in cells, respectively [230]. Moreover, S1P is produced in response to inflammatory cytokines (such as TNFα and IL-1β) and promotes further cytokine production by activating NF-κB signaling, potentially providing a positive feedback loop in the inflammatory response [230].

Tissue production of sphingolipids is increased in human and rodent obesity, probably boosted by fatty acid oversupply, and it is now recognized that aberrant production of sphingolipids plays a part in the pathophysiological changes associated with obesity [230, 262]. Sphingolipids have also been implicated in the pathophysiology of arthritides [4]. Cell-permeant ceramide increases proteoglycan degradation in cartilage explants and induces the production of MMPs and apoptosis in cultured chondrocytes [263]. There are results indicating increased local production and concentration of S1P in the inflamed joint [264, 265], and suggesting a catabolic, pro-inflammatory activity of S1P in human cartilage and synovium [264, 266]. Other authors, however, have reached the opposite conclusion that S1P might provide a pro-proliferative, anti-catabolic signal in articular chondrocytes [267, 268]. All in all, although the effects of S1P in chondrocytes require further investigation, the accumulated evidence sustains a role for sphingolipid balance in joint diseases.

Inhibition of sphingolipid synthesis (by treatment with the serine palmitoyltransferase inhibitor myriocin) increased insulin sensitivity, resolved hepatic steatosis and prevented the onset of diabetes in obese rodents [269]. Chemical inhibition of sphingosine kinase (which catalyses the synthesis of S1P from sphingosine) inhibited disease severity and reduced articular inflammation and joint destruction in a murine model of inflammatory arthritis [270]. These and other results suggest that modulation of sphingolipid levels and sphingolipid signaling pathways may represent a novel therapeutic target in pathologies associated with obesity as well as in arthritides.

Lipid peroxidation

Oxidative stress (i.e., the accumulation of ROS and other free radicals) and inflammation go hand-in-hand because oxidative stress induces the production of pro-inflammatory cytokines, and the cytokines in turn induce free radical production. Oxidative stress has been implicated both in cartilage degeneration [271] and in obesity and the metabolic syndrome [272]. One specific facet of oxidative stress is membrane lipid peroxidation, an autocatalytic process initiated by free radical attack on the double bonds of membrane

unsaturated fatty acids. A specific aldehydic product of lipid peroxidation, 4-hydroxynonenal (HNE), is increasingly recognized as a particularly important mediator and marker of cellular dysfunction and degeneration in a range of disorders including arthritis [4, 273] and the metabolic syndrome [274].

HNE acts mainly by covalently modifying (i.e. forming adducts with) different proteins. Levels of HNE are increased in tissues (blood, muscle) of obese subjects [274] and in OA joints [4, 273]. HNE can damage pancreatic beta cells, impair the ability of muscle and liver cells to respond to insulin, promote atherosclerosis by modifying lipoproteins and cause cardiac cell damage by impairing metabolic enzymes [274]. Additionally, it has been suggested that increased levels of HNE in the brain may underlie associations of obesity (or factors promoting it) with increased risk of neurodegenerative diseases such as Alzheimer's and Parkinson's [274]. In cartilage, HNE activates MMP-13 and facilitates collagen degradation by MMP-13 by forming adducts with type II collagen [273]. Moreover, a recent report indicates that interactions of articular chondrocytes with HNE-modified ECM protein can induce a plethora of changes in cell phenotype and function which may contribute to OA development, including induction of inflammatory and catabolic responses and cell death, and that these outcomes can be prevented by an HNE-trapping drug (carnosine) [275].

Exercise and dietary energy restriction reduce HNE production and may also increase cellular systems for HNE detoxification including glutathione and oxidoreductases [274]. Additionally, it has been suggested that specific dietary interventions and pharmacological strategies aimed at preventing HNE production or blocking its adverse action (e.g., treatment with low molecular weight molecules that scavenge HNE, such as carnosine analogs) may be useful in the treatment of obesity and the metabolic syndrome [274], and possibly also OA [275].

ADVANCED GLYCATION END PRODUCTS (AGEs)

AGEs are a heterogeneous group of macromolecules that are formed by the non-enzymatic glycation of proteins, lipids and nucleic acids [276, 277]. AGEs resulting from the reaction between reducing sugars and free amino groups in lysine or arginine residues of proteins have been studied most. Once they are formed, AGEs cannot be removed from the protein and only leave a tissue when the protein involved is degraded; therefore, AGE accumulation is largely determined by the rate of protein turnover, affecting mainly long-lived proteins. Humans are exposed to two main sources of AGEs: AGEs formed endogenously and AGEs originating from exogenous sources such as tobacco smoke and diet. Food processing, especially prolonged heating, has an accelerating effect on AGE formation, and a significant proportion of ingested AGEs are absorbed with food, although the relative importance of these exogenous sources of AGEs in the pathogenesis of disease remains to be determined.

AGEs interfere with normal physiology by directly modifying and covalently cross-linking proteins and other macromolecules, and by initiating cellular responses after interacting with specific proteins that bind to these chemical moieties [276]. Among the latter, the most extensively studied is the receptor for AGE (RAGE), a member of the Ig superfamily of receptors found at the cell surface. AGE modification of ECM proteins is prominent because of the low turnover rate of these proteins, and leads to structural alterations including increased stiffness and resistance to proteolytic digestion. Additionally,

through their interaction with RAGE and other accidental "receptors", AGEs activate intracellular pathways and targets such as NF-κB which induce the expression of inflammation-related genes and evoke oxidative stress. Oxidative stress in turn favors AGE formation, potentially perpetuating a vicious circle.

Via direct and receptor-dependent pathways, AGE modification of proteins and lipids has been implicated in pathologies including diabetic complications, atherosclerosis and arthritides, as well as in the aging phenotype itself [276-280]. Therapies for these pathologies based on blocking the production of AGEs or their effects are being evaluated in preclinical studies. The strategies proposed include the use of inhibitors of AGE formation (such as pyridoxamine and benfotiamine), putative cross-linking breakers, or the exploitation of soluble RAGE (sRAGE), which is an endogenous non-functional RAGE isoform that can competitively bind RAGE ligands, preventing their interaction with the cell-surface RAGE receptor, thus hindering cellular signaling [276].

The production of AGEs is enhanced in the diabetic milieu owing to chronic hyperglycemia and increased oxidative stress, and there is a substantial body of evidence linking abnormal protein and lipid glycation in vascular, renal, neural, cardiac and other cells with diabetes complications and atherosclerosis [276, 278]. Moreover, the AGEs-RAGE system could play a role in the initiation of insulin resistance and the development of diabetes itself by interfering with the complex molecular pathways of insulin signaling [281, 282]. Several studies have suggested the effects of AGEs in adipocytes might contribute to obesity-related insulin resistance and increased cardiovascular risk. Thus, in white adipocytes in culture, AGEs down-regulate leptin expression [283], attenuate insulin responsiveness [284], and induce the expression of pro-thrombotic PAI-1 [285]. These effects have been linked to the ability of AGEs to induce ROS production in adipocytes.

Studies on AGE levels in obesity are scarce. In a recent study, AGE levels in serum were not increased in human obese subjects compared with lean controls, but were found to be reduced following a low calorie diet [286]. The latter observation may plausibly be a reflection of a reduction in glycation/lipoxidation due to caloric restriction and its metabolic consequences, or it may be due to the decreased intake of food containing glycotoxins, or a combination of both. In a study in rats, high fat diet-induced obesity was found to be associated with increased AGE levels in serum and visceral tissues (heart and liver) and with signs of increased tissue damage [287].

Accumulation of AGEs in joints takes place with aging and adversely affects cartilage turnover and mechanical properties, providing a molecular mechanism by which aging contributes to the development of OA [280]. Crosslinking by AGEs increases the stiffness of the collagen network in human articular cartilage ECM, making the cartilage more brittle and increasing the susceptibility of the tissue to fatigue failure [280]. In addition, articular chondrocytes express RAGE [288] and in human OA chondrocytes AGEs can activate intracellular pathways leading to the production of pro-inflammatory mediators such as PGE_2 and nitric oxide [288-290], increase TNF-α and MMP expression [288, 291], and reduce anabolic activity [292]. Pro-inflammatory and pro-catabolic effects of AGEs in OA fibroblast-like synovial cells have also been described [293, 294].

Hypercaloric, pro-obesogenic diets rich in AGEs and poor in antioxidants are easily conceivable. By favoring obesity and/or oxidative stress and altered glucose metabolism, these diets can further promote endogenous AGE formation. Excess AGEs from exogenous and endogenous sources – along with other components and characteristics of these types of diets – can contribute to altered insulin sensitivity and to multiple derangements, including OA (Figure 1).

MicroRNAs (miRNAs)

miRNAs are a class of short, noncoding RNAs that negatively regulate gene expression by inhibition of translation or cleavage of target messenger RNAs, to which they bind through perfect or imperfect base-pairing. Hundreds of miRNAs have been identified so far and studies are linking specific miRNAs or miRNA profiles to different tissues, developmental processes and pathologies. miRNAs add another level of complexity to gene regulation, which could open novel avenues in therapeutic strategies for many pathologies.

miRNAs have been implicated in the pathogenesis of OA and obesity. These are rapidly evolving fields and only a few examples will be presented. miR-140, for instance, appears to be particularly important in cartilage: among other targets, miR-140 represses the expression of ADAMTS-5, a key aggrecanase in OA, and miR-140 expression increases during chondrogenesis and is reduced in the OA cartilage [295, 296]. Moreover, miR-140 deficient mice are prone to age-related OA-like changes, whereas transgenic mice overexpressing miR-140 in cartilage are resistant to experimentally-induced OA [297]. miR-519d has been associated with human obesity; its expression levels are increased in subcutaneous WAT of obese subjects, and it has been suggested that this increase could contribute to metabolic imbalance and subsequent adipocyte hypertrophy, because miR-519d can repress PPARα, and genes normally transactivated by PPARα are important for fatty acid oxidation [298]. Interestingly, miRNA expression profile studies have revealed that, both in mice and humans, many miRNAs up-regulated during adipogenesis *in vitro* are down-regulated in WAT in obesity (i.e., less expressed in WAT of obese *versus* lean animals/subjects) and vice versa [285, 286], suggesting that obesity leads to a loss of miRNAs that characterize fully differentiated, metabolically active adipocytes. This scenario has been related to the possible presence of an excess of small adipocytes in the obese (i.e., to the hyperplasic component of obesity) [286] and also to the action of pro-inflammatory cytokines in WAT in obesity, such as TNFα, which can interfere with proper adipocyte function [285].

Some specific miRNAs have been related to both cartilage and adipose tissue biology. In particular, miR-27a has been implicated as a negative regulator of adipogenesis by suppressing PPARγ expression [287] and as an indirect repressor of catabolic enzyme (MMP-13) expression in chondrocytes [296]. Moreover, there are results to suggest that obesity can impact on the development of OA by changing miRNA expression in cartilage. A pioneer study in the field identified through miRNA profiling 16 miRNAs that were differentially expressed in human OA cartilage compared to normal cartilage; of them, five correlated significantly with BMI, two positively and three negatively [288]. The authors then sought to identify functional miRNA-gene target pairs relating obesity with OA pathogenesis mechanisms. They provided evidence that miR-22 − one of the two miRNAs whose expression in cartilage positively

correlated with BMI − down-regulates PPARα and BMP-7 expression in normal and OA chondrocytes, and that inhibition of miR-22 blocks inflammatory and catabolic changes in OA chondrocytes [288]. Hence, this study shows that a BMI-sensitive miRNA controls genes for proteins potentially involved in the development of OA. The other miRNA found to positively correlate with BMI in the study by Iliopoulos *et al.* [288], miR-103, appears to exert a stimulatory role on adipogenesis [285].

CONCLUDING REMARKS

The relationship of OA with obesity is complex, involving both biomechanical and metabolic links and interactions between them [154] (Figure 2). Conditions frequently associated with obesity, namely dysregulated production of cytokines and adipokines in inflamed fat, increased oxidative stress (in fat and systemically) and altered lipid metabolism appear to be part of the metabolic component of OA. Importantly, the same systemic conditions underlie the links between obesity and metabolic and cardiovascular disease. Importantly, the concurrence of these conditions can lead to metabolic/cardiovascular disease and OA in the absence of obesity and, in fact, epidemiological studies have revealed an association of OA with the metabolic syndrome independently of obesity [299, 300]. Specific diet features and alterations in signaling pathways controlling cell growth and differentiation might favor both obesity and OA (Figure 2).

The recent discovery of a high turnover of adipocytes in adult human WAT (approximately 10% annually) establishes the adipocyte birth–death balance as a novel potential therapeutic target for the pharmacological intervention of obesity [301]. Likewise, there is renewed interest in treatments aimed at BAT activation, following the realization that BAT is more represented and potentially functional in humans than previously believed [302]. The implications of these novel treatments for joint health are unknown but may exist. In many instances, weight reducing strategies require increased care to avoid any adverse effects on obesity–related complications like those affecting joint health, which may arise from collateral signals generated during the weight-loss period, and also as a consequence of increased exercising during it. Additionally, within the difficulties in combating obesity, it might be more fruitful to control directly for its undesirable consequences, i.e. to shift the focus from a lean phenotype to a "healthier obese" phenotype. From this perspective, inflammation in adipose tissue and adipokines and cytokines are emerging as potential targets for treating obesity-related health problems including type II diabetes and cardiovascular risk [155, 303, 304], as well as OA [5, 6, 100].

In this article we have reviewed molecular players at the intersection of adipose tissue and articular joint cell biology, many of which are altered in both obesity and OA. Such an exercise allows for the identification of a number of potential targets of particular interest in the sense that their change in a given, common direction (either potentiation or inhibition), might be useful in the simultaneous treatment of both obesity and OA in the (many) patients co-presenting them, hence leaving open the possibility of systemic treatment. These targets are compiled in Table 4. For other putative molecular targets, the sense of the desired change is opposed for one and the other pathology, which could make the systemic approach difficult or even unfeasible, although local therapy remains possible, particularly in the case of OA, through intraarticular injection of the therapeutic agent(s). For instance, findings so far indicate that blockade of Hh

signaling in cartilage could be therapeutic in OA, whereas activation of Hh signaling in adipose tissue could be desirable in obesity. Another example is offered by adiponectin, for which there is evidence of a destructive role in cartilage homeostasis, but of anti-inflammatory effects on the vasculature and positive effects on metabolic pathways. Finally, other signaling pathways/molecules reviewed herein (e.g. Wnt signaling, TGFβ) are involved in OA and/or obesity in rather complex ways or have an intrinsic complexity which makes it difficult to make predictions as to possible therapeutic implications, which in any case would only be feasible provided specificity can be achieved. On the whole, understanding the connections between joint degeneration and excess body fat could serve to better and more efficiently combat both conditions.

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APPENDIX. Two types of adipose tissue in mammals

Mammals have two main types of adipose tissue depots: brown and white (BAT and WAT), made up mainly of white and brown adipocytes, respectively. White adipocytes have a low oxidative capacity and serve primarily to store lipids. Brown adipocytes are rich in mitochondria and function to dissipate energy as heat (adaptive thermogenesis) due to the activity of uncoupling protein 1 (UCP1), a brown adipocyte-specific protein of the inner mitochondrial membrane which has proton transport activity allowing the uncoupling of respiration from ATP synthesis. BAT activity can counteract obesity, and recent clinical data suggest that BAT is more abundant in humans than previously recognized, and that individuals with low levels of BAT are more susceptible to obesity and related metabolic complications than those with higher BAT contents [302]. In addition to forming discrete, discernible BAT depots, brown adipocytes are found interspersed among white adipocytes in WAT depots of both rodents and humans [305]. A growing body of evidence suggests that WAT and BAT are derived from distinct precursor populations during embryonic development, with brown adipocytes sharing a common precursor with skeletal muscle cells [306, 307]. Besides mature adipocytes, fat depots include a stromal-vascular fraction that contains preadipocytes and a variety of other cell types, such as vascular endothelium, vascular smooth muscle, macrophages, lymphocytes and mast cells.

Table 1. Transcription –related factors at the intersection of adipose tissue and joint cell biology

Protein	**Function**	**Effects on adipogenesis/ adipocytes**	**Changes/involvement in obesity**	**Effects on joint cell biology**	**Changes in OA**	**References**
Sox9	DNA-binding transcription factor	Inhibits adipogenesis	Down-regulated in WAT in murine obesity	Stimulates chondrogenesis; Might be important to maintain anabolic functions in mature chondrocytes	Down-regulated in OA cartilage	[18] [19]
PPARγ	DNA-binding transcription factor	Stimulates adipogenesis and lipogenesis; Modulates adipokine expression in a favorable manner	Its activity can contribute to obesity and to increased systemic insulin sensitivity	Suppresses the expression of inflammation-related and catabolic genes in chondrocytes; Inhibits osteoblastogenesis	Down-regulated in OA cartilage	[27] [28] [31] [32]
PGC-1α	Transcriptional coactivator	Stimulates brown adipogenesis (expression of UCP1 and of genes related to mitochondriogenesis)	Down-regulated in skeletal muscle and WAT of obese and diabetic subjects	Stimulates chondrogenesis by serving as a coactivator of Sox9	Unknown	[34-40] [45]

Table 2. Signaling pathways, growth factors and related proteins at the intersection of adipose tissue and joint cell biology

Signaling pathway/protein	Effects on adipogenesis/adipocytes	Involvement in obesity	Effects on joint cell biology	Involvement in OA	References
Pref-1	Inhibition of white adipogenesis	Its deficiency in null mice accelerates fat deposition; Overexpression in AT* leads to leanness but with exacerbated insulin resistance	Promotes chondrogenic induction of mesenchymal cells	Unknown	[19] [21] [22] [23] [24]
Wnt signaling	Inhibition of white and brown adipogenesis	Overexpression in AT protects against obesity without signs of lipotoxicity; Polymorphisms in related genes have been linked to human obesity	Inhibition of chondrogenesis; Induction of osteoblastogenesis	Activated in OA cartilage (canonical pathway); Polymorphisms in Wnt-related genes have been linked to human OA	[15] [32] [53]
Hh signaling	Inhibition of white adipogenesis	Constitutive activation in AT results in leanness without signs of lipotoxicity	Activates chondrogenesis, chondrocyte terminal differentiation, endochondral ossification, and osteoblastogenesis	Activated in OA cartilage	[62] [63] [65] [66]
TGFβ	Inhibition of white adipogenesis	Increased in WAT in obesity; Expression in WAT correlates with that of pro-thrombotic PAI-1	Activates chondrogenesis and chondrocyte terminal differentiation; Favors osteophyte formation	Dual effects in cartilage: it induces anabolic responses but may also induce matrix degradation during terminal differentiation, and osteophyte formation	[67] [71] [73] [74]
BMP-7	Induction of brown adipogenesis	Overexpression in adult mice reduces weight gain	Induces chondrogenesis but not chondrocyte terminal differentiation	Pro-anabolic and anti-catabolic effects in cartilage	[76] [78]
IGFBP-3	Causes insulin resistance and suppresses adiponectin expression	Possible risk factor for the metabolic syndrome	Anti-anabolic activity through sequestering of IGF-1	Increased in OA cartilage	[81] [82]

*AT, adipose tissues

Table 3. Main cytokines and adipokines at the intersection of adipose tissue and articular joint cell biology

Factor	Effects related to OA	Metabolic effects	Levels in obesity	References
IL-1	Synovitis Cartilage breakdown	Impaired beta-cell function and insulin secretion	Increased	[5] [141] [148]
TNFα	Synovitis Cartilage breakdown	Insulin resistance	Increased	[5] [141] [147]
IL-6	Synovitis Osteophyte formation	Insulin resistance in liver and adipose tissue	Increased	[5] [141] [152]
IL-18	Synovitis Chondrocyte apoptosis	Insulin resistance and pro-atherogenic effects, through down-regulation of adiponectin and by other means	Increased	[5] [149] [153]
IL-8	Synovitis	Pathogenesis of non-alcoholic fatty liver disease	Increased	[5] [141] [150] [151]
IL-10	Anti-inflammatory effects Chondroprotective effects	Anti-inflammatory effects; Prevention of diet-induced insulin resistance	Decreased	[5] [141] [161]
Leptin	Induction of cartilage IGF-1 and TGF-β expression and proteoglycan synthesis; Pro-inflammatory effects; Stimulation of cartilage MMPs; Bone matrix regulation	Reduces food intake; Increases energy expenditure; Enhances lipolysis and fatty acid oxidation in peripheral tissues; Inhibits gluconeogenesis and lipogenesis	Increased, but resistance to its effects	[5] [6]
Adiponectin	Inhibition/stimulation of inflammation; Inhibition/stimulation of cartilage MMPs	Insulin-sensitizing effects; Anti-atherogenic effects	Decreased	[5] [6]
Resistin	Stimulation of inflammation; Stimulation of cartilage MMPs	May promote insulin resistance	Increased	[5] [6]
Visfatin	Stimulation of inflammation; Stimulation of cartilage MMPs	May have insulin-sensitizing effects	Increased	[5] [6]
Angiotensin II	Pro-inflammatory action (proven in rheumatoid arthritis)	Favors hypertension; May favor adipose tissue inflammation, dyslipidemia and insulin resistance	Increased	[201] [202] [197]

Table 4. Putative molecular targets for simultaneous treatment of obesity or obesity-linked metabolic derangements (e.g. insulin resistance) and osteoarthritis

Target	**Rationale**	**References**
• Up-regulation of Sox9	Sox-9 inhibits adipogenesis and stimulates chondrogenesis. Sox-9 activity is down-regulated in the OA cartilage	[18] [19]
• Up-regulation of PGC-1α	PGC-1α stimulates both brown adipogenesis and chondrogenesis	[34] [45]
• Up-regulation of PPARγ	Activation of PPARγ increases insulin sensitivity (though at the expense of subcutaneous fat expansion) and reduces inflammatory and catabolic responses in cartilage	[27] [28]
• Up-regulation of BMP-7	BMP-7 stimulates both brown adipogenesis and chondrogenesis and has pro-anabolic and anti-catabolic effects in cartilage	[76] [78]
• Down-regulation of IGFBP-3	IGFBP-3 is a putative risk factor for metabolic syndrome, has anti-anabolic activity in cartilage, and is increased in the OA cartilage	[81] [82]
• Blockade of pro-inflammatory cytokines	The activity of pro-inflammatory cytokines has been related to both obesity-related metabolic disturbances and OA	[5]
• Up-regulation of IL-10	IL-10 is an anti-inflammatory cytokine	[5]
• Down-regulation of leptin	Hyperleptinemia and resistance to leptin are common in obesity. Leptin may exert pro-inflammatory effects in the joint.	[174] [180]
• Angiotensin II receptor type 1 (AT1) blockade	Increased local and systemic angiotensin II might be involved through AT1 in joint and adipose tissue inflammation, and in obesity-linked hypertension and insulin resistance	[202] [204]
• Inhibition of sphingolipid synthesis	Sphingolipids have been linked to both metabolic syndrome and OA	[4] [230]
• Blockade of 4-hydroxynonenal production/action	4-hydroxynonenal has been linked to both metabolic syndrome and OA	[273] [274]
• Up-regulation of miR-27a	miR-27a has been implicated as a negative regulator of adipogenesis and as a repressor of MMP-13	[296] [287]

Figure legends

Figure 1. Diagram illustrating the possible involvement of advanced glycation end products (AGEs) in the relationship between diet, obesity and osteoarthritis.

Figure 2. Conditions frequently associated with obesity, namely dysregulated production of cytokines and adipokines in inflamed fat, increased oxidative stress and altered lipid metabolism appear to be part of the metabolic component of osteoarthritis (OA), and underlie as well links between obesity and metabolic and cardiovascular disease (CVD). These conditions can lead to metabolic disease, CVD and OA independently of obesity. Specific diet features and alterations in pathways controlling cell growth and differentiation may favor both obesity and OA. Mechanical and systemic factors are likely to interact in the development of OA.

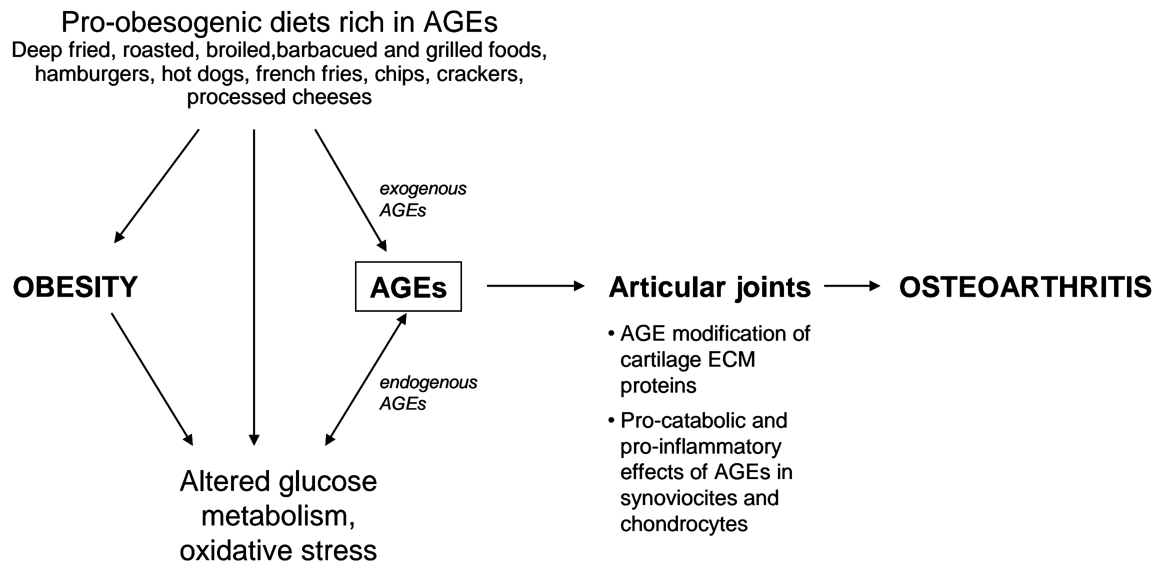

Figure 1

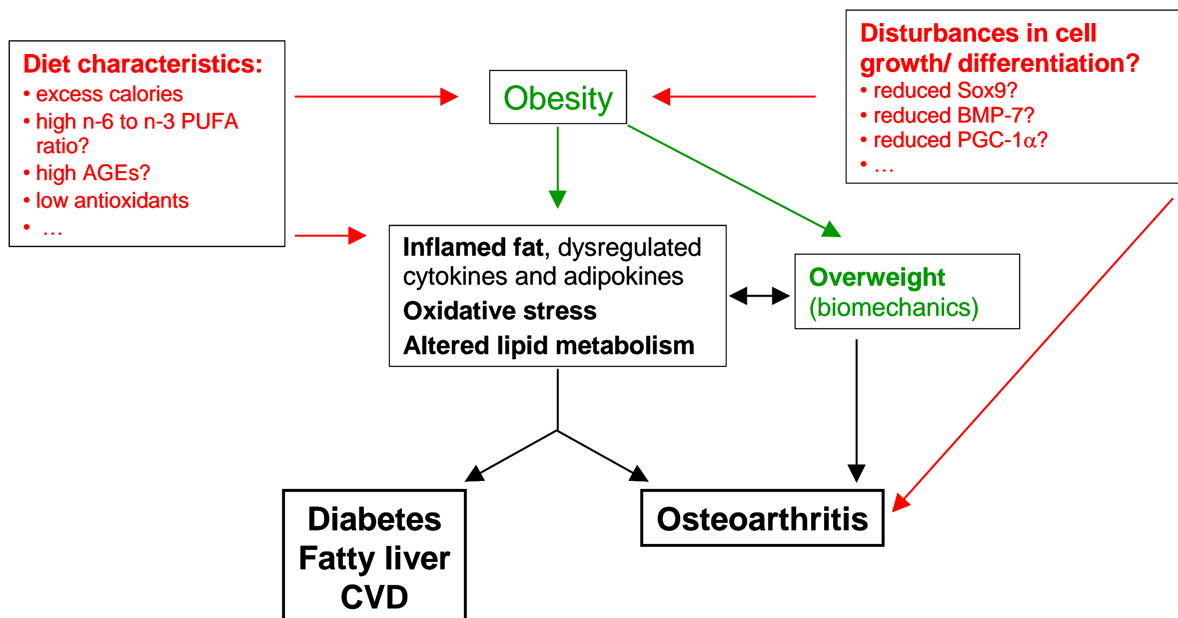

Figure 2
(color)

MANUSCRITO VI

Moderate vitamin A supplementation in early life affects later adiposity in rats.

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Moderate vitamin A supplementation in early life affects later adiposity in rats

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ABSTRACT

Introduction: Vitamin A is an essential regulator nutrient which, mainly in the form of retinoic acid, has been implicated in the control of adipocyte biology and of adiposity in adult animals. However, little is known about the impact of vitamin A intake in early life on adipose tissue development and later adiposity, which is the subject of this work.

Methods: Rat pups received during the suckling period a daily oral dose of retinyl palmitate corresponding to three times the vitamin A ingested daily from maternal milk. Control rats received the vehicle (olive oil). After weaning, control and vitamin A-treated rats were fed a normal fat diet or a high fat diet for 16 weeks. In another experiment, control and vitamin A-treated rats were sacrificed the day after weaning. Parameters related to energy metabolism, gene expression, and morphology and morphometry of white adipose tissue (WAT) were analyzed.

Results: Despite no differences in body weight gain or energy intake, vitamin A-treated rats developed higher adiposity than vehicle-treated rats on a high fat diet as indicated by body composition analysis and increased mass of WAT depots, adipocyte diameter, WAT total DNA content, leptinemia and adipose leptin expression. Vitamin A-treated neonatal rats (d 21) displayed a trend for higher percentage of smaller cells in WAT and gene expression features in WAT consistent with an increased cell proliferation potential, which correlated with a reduced expression of adipogenic markers. Evidence of increased retinoic acid-mediated transcriptional responses in tissues of vitamin A-treated neonatal rats was obtained.

Conclusion: Vitamin A in the early stages of postnatal life may condition long-term effects on body adiposity through effects on adipose tissue development, possibly mediated by retinoic acid.

INTRODUCTION

A crescent body of evidence has demonstrated that hormonal, metabolic and nutritional cues at critical periods in early life including the intrauterine life and the immediate postnatal period may determine the propensity to develop obesity and obesity-related metabolic pathologies [1, 2]. The mechanisms underlying early programming of obesity are still poorly understood, and seem to include epigenetic changes − such as DNA methylation and certain histone modifications − that can condition lasting effects on gene expression [3, 4] and effects on the development of anatomical structures crucial to the control of energy balance and energy storage, such as the brain centers involved in the control of food intake and energy expenditure [5, 6] and the adipose tissue depots themselves [7]. In this context, there is evidence that cellularity of adipose tissue depots is fixed in early life (childhood/adolescence in humans), even though a considerable turnover of adipocytes occurs at all adult ages [8], and that adipocyte number is a major determinant of body fat mass and the capacity of adipose tissue expansion [8-10].

Nutritional factors implicated in the early programming of obesity include total energy intake (with important effects of both undernutrition/caloric restriction and overnutrition) and diet macronutrient composition [1]. Specific nutrients have also been implicated, such as leptin, which is present in breast milk and absorbed by neonate rats [2]. Certain vitamins and other dietary factors that act as donors or enzyme cofactors in methyl transfer reactions potentially related to epigenetics mechanisms, such as methionine, folate and vitamin B12, have also been considered [11, 12]. In general, however, studies examining the impact of specific micronutrients on metabolic programming in general, and programming of obesity in particular, are scarce.

Vitamin A is an essential nutrient involved in a wide range of functions and with a crucial role in development through effects on gene expression and extragenomic actions. Among the many functions attributed to this vitamin, its role in the control of lipid and energy metabolism is receiving an increasing attention in the latest years [13]. Studies in cell models and adult animals indicate that vitamin A derivatives, notably retinoic acid, can impact on developmental and biochemical processes influencing mammalian adiposity including adipocyte differentiation (adipogenesis) and lipogenesis, adaptive thermogenesis, lipolysis and fatty acid oxidation in tissues [13, 14]. Treatment with retinoic acid [15-25] or retinaldehyde [26] has been shown to reduce body fat and

improve insulin sensitivity in lean and obese rodents, and genetic manipulation of various carotenoid/retinoid-metabolising enzymes and transport proteins has been shown to result in alterations in adiposity in mouse models [26-31]. Additionally, there is evidence that adiposity might be physiologically related to vitamin A status: animal studies indicate that diets low in vitamin A favor adipose tissue formation [16, 32] and, in humans, studies have reported an inadequate vitamin A status in overweight and obese individuals [33] and an inverse association between vitamin A intake and adiposity [34]. On the other hand, vitamin A supplementation as retinol or retinyl palmitate (at 40- to 50-fold the regular dose) was shown to associate with reduced WAT mass in some studies in adult rats [35, 36] and modestly counterbalanced the development of diet-induced obesity in mice [37]. The anti-adiposity action of retinoic acid has been traced to increased oxidative metabolism and energy expenditure in tissues including brown and white adipose tissues, skeletal muscle and the liver [15, 19, 21-23, 38], and to reduced peroxisome proliferator-activated receptor γ (PPARγ) levels and activity in white adipose tissue [16, 31, 39]. PPARγ is a PPAR isoform of particular relevance for adipocyte biology, since it is the master regulator of adipogenesis and is also required for lipogenesis and survival in mature adipocytes [40].

Despite the accumulated evidence linking vitamin A to the control of adiposity in adult animals, little is known about the impact of vitamin A intake in early life on adipose tissue development and later adiposity. In this work, we sought to contribute to fulfill this gap by studying the impact of a moderate vitamin A supplementation during the suckling period on the later response to a high fat diet in rats.

MATERIALS AND METHODS

Animals, diets and experimental designs

Animal protocols followed in this study were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands, and guidelines for the use and care of laboratory animals of our institution were followed.

Analysis of vitamin A concentration in milk of nursing rats during lactation

Our study design involved supplementing rat pups with a moderate amount of vitamin A during the entire suckling period. It has been reported that vitamin A concentration in rat milk doesn't significantly change throughout lactation and it is highly dependent on the

mother's vitamin A consumption [41, 42]. In order to fix the exact vitamin A amount to be used in our experiments, we first quantified by HPLC the total vitamin A (retinol) concentration in the milk of lactating rats mated at our animal house. For this, after an acclimation period of one month, three-month-old, virgin female Wistar rats rat (from Charles River Laboratories, Barcelona, Spain) were caged with a male rat. After mating, each female was placed in an individual cage. Rats were kept in a room with controlled temperature (22 ºC) and a 12-h light/dark cycle, with free access to water and a standard chow (Panlab, Barcelona, Spain; 15000 UI vitamin A/kg). After delivery, three dams were used for milk studies. Birth was defined as d 0 of lactation. Milk samples from each dam were collected on d 7, 12 and 18 of lactation. For milk collection, nursing rats were separated from their pups for 6 h to guarantee that mammary glands were full of milk. Before milking, dams were exposed to ether, and then milk was obtained from the mammary glands by manual milking and stored frozen at -20 ºC until analysis. HPLC analysis revealed that vitamin A sources in milk were free retinol (in all-trans and 13-cis form) and retinyl ester. The total vitamin A concentration (free retinol plus retinyl ester) in rat milk at d 7, 12 and 18 of lactation was, respectively, 1.15 μmol/L, 0.93 μmol/L and 1.32 μmol/L. The average concentration of the three days (1.13 μmol/L) was used in further study designs (see below).

Analysis of the effects of vitamin A supplementation during the suckling period on high fat diet-induced obesity later in life

Female Wistar rats were mated with male rats and housed as described above. At d 1 after delivery, excess pups in each litter were removed to keep 10 pups per dam, and the pups were randomly assigned into two groups: control group and vitamin A-treated group (5 pups in each group). From day 1 to 20 of lactation, between 10 and 15 μl of the vehicle (olive oil) or a solution of vitamin A as retinyl palmitate (Sigma, Madrid, Spain) was supplied orally every day to the pups in the control and the vitamin A-treated groups, respectively, using a pipette. The amount of vitamin A given was progressively adjusted throughout the suckling period to be approx. 3 times the daily vitamin A intake from maternal milk. The latter was calculated from the experimentally determined vitamin A content in rat milk (1.13 μmol/L, see above) and the estimated daily milk intake throughout the suckling period according to Kojima et al. (1998) [43]. Blood samples were taken from the pups on d 10 and d 20 between one and two hours our after treatment to verify retinyl ester absorption. On the day after weaning (d 21), control and vitamin A-

treated male rats (10 animals per group) were housed 2 or 3 animals per cage and split into two groups (5 animals per group), which were fed *ad libitum* with a normal fat (NF) diet (3.8 kcal.g^{-1}, 10% calories from fat) or a high fat (HF) diet (5.2 kcal.g^{-1}, 60% calories from fat), making a total of 4 experimental groups. Both diets are commercially available and were obtained from Research Diets, Inc., New Brunswick, NJ, USA. Male rats were used because in preliminary experiments they were found to develop more obesity upon HF diet feeding than female rats. Possible differences between litters were solved by ensuring the presence of pups from the same litter in each of the four experimental groups. Body weight and food intake were recorded between 2 and 3 times a week from d 21 on. Rectal temperature and body composition was determined at defined time points during the experiment using a digital thermometer and an Echo MRI-TM wall body composition analyzer, respectively. The animals were killed after 16 weeks on the NF or the HF diet, at d 135 of age, by decapitation, under fed conditions and within the first three hours of the light period. Tissues including brown adipose tissue (BAT), different white adipose tissue (WAT) depots – gonadal, inguinal and retroperitoneal –, gastrocnemius muscle, and liver were rapidly removed, weighted, frozen in liquid nitrogen, and stored at -70 ºC until analysis. Blood was collected, incubated at room temperature for 1 h and centrifuged at 1000g for 1 min to obtain the serum, which was stored at -20 ºC until analysis. This experiment was conducted twice, using two independent cohorts of 20 animals each.

Analysis of the effects of vitamin A supplementation during the suckling period in young rats

Male and female rats that had been treated during the suckling period with vehicle or vitamin A as described above were killed on the day after weaning (d 21) (13-16 rats per group). Blood was collected to prepare the serum and tissues including intestine, liver and WAT depots (gonadal, retroperitoneal and inguinal) were sampled as described above and stored at -70 ºC until analysis. A lengthways fragment of inguinal WAT (iWAT) was fixed by immersion in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight at 4 ºC, for histology and immunohistochemistry analysis (see below).

Oral glucose tolerance test

An oral glucose tolerance test was performed to the four groups of rats in the feeding experiment at the age of 70 and 128-130 d, to check glucose tolerance and insulin

sensitivity in an early and an advanced stage of the HF diet challenge. A load of 1- to 1.5-ml glucose (1.5 g/kg body weight) was orally given to the rats using a cannula, after overnight fasting. Blood samples were taken from the tail of the animals before glucose load at time zero and at 30, 60, 120, and 180 min thereafter. Plasma glucose levels were measured as described below. The homeostatic model assessment for insulin resistance (HOMA-IR) score was calculated from fasting insulin and glucose concentrations at time zero as previously [44].

Histology and immunohistochemistry

Fixed iWAT lobules were dehydrated, cleared and then paraffin-embedded so that the plane of section corresponded with the one of the wider surface. 3 μm-thick sections at the same level were obtained and stained with hematoxylin/eosin for morphometric analysis, performed by digital acquisition of adipose tissue areas (Digital Still Camera DXM 1200 and NIKON 6000 Eclipse Microscope). Two histological sections were counted per mouse. Immunohistochemistry of proliferating cell nuclear antigen (PCNA) was performed using a commercial anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Extraction and analysis of retinoids

Retinoids were extracted from milk and plasma under dim red safety light (600 nm). Briefly, 200 μl of plasma or milk was added to 200 μl of methanol. Then, 400 μl of acetone was added to the sample. The extraction of retinoids was repeated three times using petrolether, and the collected organic phases were dried under stream of nitrogen and redissolved in HPLC solvent. Extraction of retinoids from adipose tissue and liver tissue was performed as previously described [27, 31]. HPLC separation of retinoids and quantification of the peak integrals was perfomed as previously described [45]. Solvents for HPLC and extraction were purchased in HLPC grade from Merck (Darmstadt, Germany).

Blood parameters

Serum insulin, leptin and resistin concentrations were measured using commercial ELISA kits (from DRG Instruments GnbH, Marburg, Germany, for insulin; R&D Systems, Minneapolis, MS, USA, for leptin; and Phoenix Pharmaceutical Inc, Belmont, CA, USA, for resistin). Glucose, triacylglycerol and nonesterified fatty acids (NEFA) in serum were measured enzymatically using commercial kits and following standard procedures (kits

from Roche and R-Biopharm, Darmstadt, Germany, for glucose; Sigma, St. Louis, MO, USA, for triacylglycerol; and Wako Chemicals GmbH, Neuss, Germany, for NEFA).

Other determinations

Tissue DNA content was determined by a fluorometric method that uses 3,5-diaminobenzoic acid and total lipid content was extracted using organic solvents and quantified by weight, as previously described [19].

RNA isolation

Total RNA was extracted from tissues using Tripure Reagent (Roche, Barcelona, Spain). Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis.

Real-time quantitative PCR (RT-qPCR) analyses

Real-time polymerase chain reaction was used to measure the mRNA expression levels of: PPARγ, PPARγ coactivator 1α (PGC-1α), PPARβ/δ, PPARα, CCAAT-enhancer binding protein α (C/EBPα), lipoprotein lipase (LPL), glucose transporter 4 (GLUT4), PCNA, preadipocyte factor 1 (Pref1), Cyp26a1, ISX, uncoupling protein (UCP)1 (UCP1), UCP3, sterol regulatory element binding protein 1c (SREBP1c), fatty acid synthase (FAS), stearoyl-CoA desaturase 1 (SCD1), vascular endothelial growth factor (VEGF), 18S rRNA, β-actin and LRP10 (the latter three used as internal controls). 0.25 μg of total RNA (in a final volume of 5 μl) was denatured at 65 °C for 10 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20ºC for 15 min, 42 °C for 30 min, with a final step of 5 min at 95 °C in a Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted (1/20) cDNA template, forward and reverse primers (1 μM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primers for the different genes were obtained from Sigma (Madrid, Spain) (sequences are available upon request). Real time PCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95 ºC, followed by a total of 40 two- temperature cycles (15 s at 95 ºC and 1 min at 60 ºC). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle

(Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated according to Pfaffl (2001) [46], based on the efficiency of each reaction and the crossing point deviation of each sample versus a control and expressed in comparison with a reference gene (18S rRNA, β-actin or LRP10; similar results were obtained using either of them).

Statistical analysis

Data are presented as means ± SEM. Statistical significance of effects was assessed by two-way ANOVA or two-tailed Student's *t* test using SPSS 14.0 for windows (SPSS, Chicago, IL, USA), with threshold of significance set at $p<0.05$.

RESULTS

Vitamin A given orally as retinyl palmitate is readily absorbed by suckling rats

To verify that the exogenous retinyl palmitate given to the suckling rats was absorbed, we performed HPLC analysis of retinoids in serum taken from 10-day-old pups between one and two hours our after the treatment. As expected, serum retinyl ester concentration was significantly higher in the vitamin A-treated pups (0.619 ± 0.152 μmol/L) than in their control counterparts receiving the vehicle (0.242 ± 0.030 μmol/L) ($p<0.05$, n= 3-4 pups/group). Similar results were obtained in 20-day-old pups. Retinol levels in serum were unaffected by vitamin A supplementation, reflecting vitamin A homeostasis (see Figure 4A).

Vitamin A supplementation during the suckling period leads to increased adiposity following high fat diet feeding later in life

We first studied whether vitamin A supplementation during the suckling period could influence the development of diet-induced obesity later in life. For this, rats treated daily with vehicle or vitamin A from day 0 to day 20 of life − referred hereafter as control and vitamin A-treated rats, respectively − were fed from the day after weaning (d 21) on either a NF or a HF diet. Food intake, body weight, and body composition were regularly monitored up to d 135 of age, when the animals were euthanized. Growth curves showed, as expected, differences in body weight gain between NF and HF diet-fed rats, but, for a given diet, there were no differences in body weight gain between control and vitamin A-treated rats (Figure 1A). Likewise, cumulative energy intake was higher in the HF diet- than in the NF diet-fed rats, but did not differ between control and vitamin A-treated rats

in either diet (Figure 1B). Strikingly, despite no differences in body weight gain or energy consumption, the rats that had been supplemented with vitamin A during the suckling period developed higher adiposity on the HF diet than the control rats. This trait was already apparent by ECHO-MRI analysis after a relatively short period of HF diet feeding (40 d) (Figure 1C), and was confirmed when WAT depots were weighed following euthanization of the animals after 16 weeks on the HF diet, as the mass of all three WAT depots dissected (inguinal, gonadal and retroperitoneal) was significantly higher in the vitamin A-treated rats than the control rats (Figure 2A). Increased depot expansion was particularly pronounced for the inguinal depot (48% excess compared with 25% excess for the gonadal and 19% excess for the retroperitoneal). Overall, the adiposity index following HF diet feeding was 30% higher in the vitamin A-treated rats than the control rats (Figure 2B). No differences in adiposity were observed between vitamin A-treated and control rats on the NF diet (Figures 1C, 2A and 2B). Leptin levels in serum reflected, as expected [47], changes in body adiposity, being increased following HF diet particularly in the animals that had been supplemented with vitamin A during the suckling period (Figure 2C). The increased mass of iWAT in the HF diet-fed animals compared to the NF diet-fed animals was associated with increases in both adipocyte diameter and total tissue DNA content, suggesting both a hypertrophic and a hyperplasic component (Table 1). Remarkably, the hyperplasic component of HF-diet induced adiposity gain appeared to be more important in the vitamin A-treated rats than the control rats, as 16 weeks of HF diet feeding associated with an increase of total iWAT DNA content by a factor of 4 in the former and of 2 in the latter (Table 1).

Comparison of gene expression of selected genes in iWAT of control and vitamin A-treated rats after HF diet feeding revealed an increased expression of leptin and a tendency to higher expression levels of several genes related to lipogenesis and adipocyte growth (PPARγ, LPL, GLUT4) in the vitamin A-treated rats, in keeping with the higher depot mass of these animals (Figure 3). No significant differences between control and vitamin A-treated rats were observed regarding the mRNA expression levels after HF diet feeding of the following genes related to lipid and energy metabolism that were tested: UCP1 and PGC-1α in BAT; UCP3 and PPARβ/δ in skeletal muscle; and PPARα, SREBP1c, FAS and SCD1 in the liver (Supplemental Table 1). Likewise, no differences in rectal temperature, a surrogate marker of energy expenditure, were found between

control and vitamin A-treated rats after different periods of HF diet feeding at which it was measured (Table 2).

It has been suggested that metabolic derangements in obesity could result at least in part from the inability of adipose tissue, particularly subcutaneous fat, to further expand under conditions of sustained positive energy balance, this leading to lipid accumulation in ectopic sites [48]. This background prompted us to examine parameters related to insulin sensitivity and liver steatosis in our animals. Excess adiposity in the vitamin A-treated rats after HF diet did not associate with changes in parameters such as liver fat content, circulating levels of glucose, NEFA and triacylglycerol in the fed state, resistin levels in serum or HOMA-IR index, which were similarly affected by HF diet feeding irrespective of treatment with vehicle or vitamin A in early life (Table 2). Glucose tolerance was somewhat impaired in the more obese HF diet-fed vitamin A-treated rats as compared to the HF diet-fed control rats (see area under the curve in Table 2). Thus, in general, we did not find evidence of improvements in obesity-related complications in the more obese vitamin A-treated rats, although no gross aggravation was either evident.

Effects of vitamin A supplementation during the suckling period on retinoid levels in tissues of young and adult rats

Retinoid levels were analyzed in serum, liver and iWAT of young rats (d 21) and of adult rats (d 135), the latter after a NF or a HF diet (Figure 4). Serum retinol levels were unaffected by early life vitamin A treatment irrespective of the age or diet, reflecting vitamin A homeostasis (4A and 4D). In the young vitamin A-treated rats, retinyl ester and free retinol levels in liver (4C) and total retinol levels in iWAT (4B) were increased compared to the levels in the corresponding control littermates. In the adult vitamin A-treated rats, levels of retinyl ester in liver (4F left), but not in iWAT (4E), remained slightly higher than in the control animals. Free retinol levels in liver of adult rats were unaffected by early vitamin A treatment and were significantly reduced following high fat diet feeding (4F right).

Vitamin A supplementation during the suckling period affects adipose tissue development in young rats

Differences in HF diet-induced body fat gain between control and vitamin A-treated rats prompted us to examine treatment effects in young rats that could explain lasting effects

as observed. At the end of the treatment, at the age of 21 d, there were no differences between control and vitamin A-treated rats in body weight (control rats, 41.3 ± 0.9 g; vitamin A-treated rats, 41.3 ± 0.6 g; n= 13-16 per group), fat depot mass (combined mass of WAT depots: control rats, 0.56 ± 0.03 g; vitamin A-treated rats, 0.56 ± 0.03 g) or leptin levels in serum (control rats, 1233 ± 118 pg/mL; vitamin A-treated rats, 1167 ± 142 pg/mL). There were no differences either in liver mass (control rats, 1.55 ± 0.04 g; vitamin A-treated rats, 1.60 ± 0.04 g), indicating no major hepatotoxic effect of the moderate vitamin A dose administered. Morphometric analysis of iWAT of the young rats revealed, however, a tendency to an increased proportion of smaller adipocytes in the vitamin A-treated animals (Figure 5A and B). Gene expression analysis indicated significantly reduced levels of PPARγ mRNA (by 30%) and increased levels of PCNA mRNA (by 90%) in iWAT of vitamin A-treated rats compared with control rats (Figure 5C). Vitamin A-treated rats also displayed a tendency to reduced mRNA levels of C/EBPα, LPL and p21 in iWAT, although differences in the expression of these genes between the two groups did not reach statistical significance ($p=0.14$, $p=0.11$, and $p=0.31$, respectively; Student's t test) (Figure 5C). Pref-1 and VEGF mRNA levels were expressed to similar levels in iWAT of control and vitamin A-treated rats. Increased expression of PCNA in iWAT of vitamin A-treated young rats was confirmed by immunohistochemical analysis, which revealed more PCNA positive cells scattered among the white adipocytes in iWAT sections from these rats compared to control rats (Figure 5D, I and II). Interestingly, PCNA positive staining was observed not only in typical precursor cells (Figure 5D, III), but in the vitamin A-treated animals eventually also in cells presenting with small cytoplasmic lipid droplets, i.e. presumably already engaged in terminal adipogenic differentiation (Figure 5D, IV).

Vitamin A supplementation during the suckling period results in increased retinoic acid-mediated responses in tissues of neonatal rats

To examine whether oral vitamin A supplementation in the form of retinyl palmitate could elicit increased retinoic acid-mediated responses in tissues of neonatal rats, we compared control and vitamin A-treated rats for the expression of CYP26a1 mRNA in adipose and liver tissue and ISX in the intestine. CYP26a1 is a retinoic acid hydroxylase that is transcriptionally induced by retinoic acid in a RAR-dependent manner [49]. ISX is a gut specific transcription factor that is induced in intestinal cells by retinoic acid via RAR and functions to limit intestinal beta-carotene absorption and conversion to vitamin

A through effects on gene expression [50]. CYP26a1 mRNA levels in liver and WAT and ISX mRNA levels in intestine were all significantly higher in the vitamin A-treated rats compared with the control rats (Figure 6).

DISCUSSION

Although many studies in the latest years have dealt with the impact of vitamin A on body adiposity, few have analyzed long-term effects in this respect of vitamin A supplementation in early life. Here, we provide novel evidence that supplementation with a moderate amount of vitamin A as retinyl palmitate during the suckling period leads to increased subsequent high fat diet-induced adiposity in male rats, without changes in body weight. The pro-obesogenic effect of early vitamin A supplementation was indicated by the increased mass of fat depots, adipocyte diameter, WAT total DNA content, leptinemia and adipose leptin expression in vitamin A-treated, HF diet-fed rats as compared to their control littermates treated with the vehicle, and was reproduced in two independent cohorts of animals. Excess adiposity in the vitamin A-treated rats after HF diet was attributable mainly to increased adipocyte hyperplasia rather than to increased adipocyte hyperthrophy, and was unrelated to differences in energy intake, rectal temperature or approximates of oxidative capacity in tissues. It could possibly be related to differences in nutrient partitioning, with a greater drive to WAT in the vitamin A-treated rats, as suggested by trends to increased PPARγ, LPL and GLUT4 gene expression in iWAT of these animals as compared to control animals following HF diet feeding.

Because WAT development in the rat takes place postnatal within the first 30 days of life [51], largely coincident with the treatment period used here, and because mainly as retinoic acid vitamin A is known to affect adipogenesis (see below), we investigated whether early vitamin A supplementation could have elicited changes in the WAT of young rats at weaning. In fact, morphometric analysis revealed that vitamin A-treated young rats tended to have a higher percentage of smaller cells in iWAT than their vehicle-treated counterparts. Moreover, gene expression results pointed to an increased cell proliferation potential in the iWAT of vitamin A-treated young rats, as suggested by significantly increased PCNA mRNA levels and a trend to reduced p21 mRNA levels. PCNA is a classical marker of cell proliferation, and p21 is an inhibitor of the CDK-cyclin complexes that trigger the entry into the S phase of the cell cycle. Cell proliferation and differentiation are often mutually exclusive phenomena, and, interestingly, the

aforementioned changes in cell proliferation-related genes correlated with a reduced expression of adipogenic markers such as PPARγ, CEBPα and LPL in the iWAT of the vitamin A-treated neonatal rats. Thus, although cell proliferation was not directly measured, our results suggest that vitamin A supplementation during the active phase of early postnatal WAT development may favor reduced differentiation and the retention of a competent proliferative status, which could in turn favor the hyperplasic component of WAT development upon a subsequent stimulus in the form of a high fat diet.

In this work we supplemented rat pups orally with retinyl ester, which is the major dietary form of vitamin A from animal products. We used a moderate amount of retinyl palmitate, which did not lead to signs of vitamin A toxicity, such as liver enlargement. Our results demonstrate that excess retinyl ester was efficiently absorbed by neonatal rats, and strongly suggest that it fueled retinoic acid production in tissues including intestine, liver and, most importantly, also adipose tissue. This is suggested by our finding of increased classical retinoic acid-mediated transcriptional responses in tissues of vitamin A- treated young rats, including the induction of CYP26a1 in liver and iWAT and of ISX in the intestine [49, 50], and supported by increased vitamin A (retinol) levels in iWAT and liver of these young (d 21) animals.

Retinoic acid is known to impact in complex ways adipogenesis, a process that has been most studied *in vitro*, in established cell lines committed to the adipocyte lineage, such as 3T3-L1 cells. Dual effects of retinoic acid on adipogenesis *in vitro* have been described, from inhibition when applied at relatively high doses (0.1-10 μM) at early stages of the process [52] to promotion when applied at low doses (1 pM to 10 nM range) [53]. Furthermore, recently it has been reported that adipogenesis *in vitro* is accompanied by retinoic acid production and is impaired in cells lacking the main retinoic acid-producing enzyme in adipocytes, aldehyde dehydrogenase 1a1 [54]. Additionally, stem cell commitment into the adipocyte lineage was found to require a time-defined treatment with retinoic acid [55]. It should be stressed that the process of adipogenesis is much less well understood *in vivo*, where, together with precursor cells, nerve endings and capillaries, a continuous of adipocytes in different stages of differentiation, and possibly with different proliferative capacities, may co-exist [9]. Our results suggest that excess retinoic acid in fat depots during the active phase of WAT development may have a repressive effect on adipogenesis *in vivo*, as indicated by reduced mRNA levels of adipogenic markers, notably PPARγ. Overall, this is in keeping with reported inhibitory

effects of retinoic acid on adipogenesis *in vitro*, and with reports that expression levels of PPARγ in murine WAT depots inversely correlate with vitamin A status [16], and that retinoic acid derived from beta-carotene reduces PPARγ expression and activity in cultured adipocytes and *in vivo* in WAT of mice [31, 39]. Most importantly, our results herein suggest that inhibitory effects on adipogenesis at critical developmental stages may, paradoxically, favor subsequent adipose tissue expansion by increasing the pool of immature fat cells capable of proliferating under conditions of nutritional stimulation. The increased cell proliferation potential in WAT of vitamin A-treated young rats found in this work is in line with previous reports showing repression of the expression of p21 and the retinoblastoma protein in adipocyte cell models by retinoic acid [56] and increased thymidine incorporation in 3T3-L1 preadipocyte cultures following exposure to retinoic acid [57]. In fact, it was proposed that one of the mechanism through which retinoic acid inhibits adipogenesis is by favoring the retention of a pool of adipose cells able to re-enter the cell cycle [56]. Results presented herein strongly suggest that this effect of vitamin A derivatives may have important implications for the developmental dynamics of adipose tissue *in vivo*. Importantly, total retinol levels in iWAT of vitamin A-treated rats were elevated at an early age (d 21) but normal by d 135 of age, supporting the concept that early effects on adipose tissue development are involved in the adipose phenotype of these animals.

Our results highlight that the impact of a particular dietary compound on adiposity may greatly vary depending on the developmental time-window of exposure. Thus, whereas early vitamin A supplementation under the experimental conditions of this work led to increased body fat mass following a high fat diet, most studies conducted in older animals conclude that adipose tissue is increased in situations of vitamin A deficiency and reduced after retinoic acid administration (see the Introduction section). Nevertheless, results in this work are consistent with previous studies indicating a pro-adipogenic effect of excess vitamin A when administered at a young age. In particular, in young rats (50-70 g, age 3 weeks), short-term exposure to a high fat (cafeteria) diet was found to result in higher adiposity when the diet was enriched in vitamin A (4-fold excess dietary retinol) [58]. Interestingly, this synergic effect of vitamin A and high fat diet in adipose tissue development was concomitant with increases in the proliferation of primary preadipocytes isolated from the animals' fat depots [58]. In another study, this one in lambs, supplementation with retinyl palmitate from birth and during the whole period of

growth (until d 101 of age) under normal feed did not influence growth or adiposity, but resulted in a 30% increase in the number of adipocytes in the perirenal depot, and in smaller adipocytes in this and other fat depots [59]. Signs of increased proliferative potential in WAT of young rats treated with vitamin A during the suckling period detected in our work might be in keeping with these previous reports.

Increased adipose tissue mass is the primary phenotypic characteristic of obesity. Under conditions of a positive energy balance, it is known that the deposition of lipid initially results in increased fat cell size, but soon triggers increases in fat cell number [9]. Studies have highlighted adipose tissue cellularity as an important determinant of fat mass [8], and evidence from animal models suggest that increased adipocyte number might *per se* lead to obesity, by altering or overcoming the normal mechanisms of body weight control [10]. Thus research into factors and molecular mechanisms influencing adipose cell proliferation and differentiation *in vivo* at critical life stages represents a crucial area of research. Results in this work indicate that, in rats, a moderate excess of dietary vitamin A in the early stages of postnatal life potentiates adiposity gain in response to a high fat diet later in life possibly through effects on adipose tissue development, thus suggesting that vitamin A intake during the suckling period might be one of such factors. The results might have implications for the obesity pandemics and particularly for childhood obesity, as the vitamin A content of human milk is related to maternal vitamin A status and maternal dietary vitamin A intake during lactation [60].

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FIGURE LEGENDS

Figure 1. Growth curves (A), cumulative energy intake (B) and evolution of body fat content (C) in male rats treated with vehicle (empty symbols) or vitamin A as retinyl ester (RE, filled symbols) during the suckling period (d 1-20 of life) and fed after weaning a normal fat (NF, circles) or a high fat diet (HF, diamonds) for 16 weeks. Body fat content was determined at the indicated ages using an Echo MRI-TM wall body composition analyzer. Body weight and fat content data represent the mean ± SEM of 10 animals per group and cumulative energy intake data represent the mean ± SEM of 4 cages (2-3 animals each) per group, distributed in two separate experiments. Significant differences were tested by two-way ANOVA and two-tailed Student's *t* test ($p<0.05$): D indicates diet effect in two-way ANOVA analysis; RExD indicates interaction between RE treatment and diet in two-way ANOVA analysis; at each time point, * indicates significant differences between vehicle- and RE-treated animals and $^\#$ indicates significant differences between NF- and HF-fed animals in the two-tailed Student's *t* test post hoc analysis.

Figure 2. Vitamin A supplementation during the suckling period increases high fat diet-induced adiposity later in life. White adipose tissue (WAT) depots weight as percent of body weight (A), adiposity index (B), and leptin levels in serum (C) in male rats treated with vehicle (controls, white bars) or vitamin A as retinyl ester (RE, black bars) during the suckling period (d 1-20 of life) following 16 weeks on a normal fat (NF) or a high fat diet (HF) from the day after weaning (d 21). Data represent the mean ± SEM of 10 animals per group, distributed in two separate experiments. Significant differences were tested by two-way ANOVA and two-tailed Student's *t* test ($p<0.05$): D indicates diet effect in two-way ANOVA analysis; RExD indicates interaction between RE treatment and diet in two-way ANOVA analysis; * indicates significant differences between vehicle- and RE-treated animals and $^\#$ indicates significant differences between NF- and HF-fed animals in the two-tailed Student's *t* test post hoc analysis.

Figure 3. Gene expression of selected genes in inguinal white adipose tissue of male rats treated with vehicle (controls, white bars) or vitamin A as retinyl ester (RE, black bars) during the suckling period (d 1-20 of life) and fed after weaning a normal fat (NF) or a high fat diet (HF) for 16 weeks. Data represent the mean ± SEM of 5 animals per group and are expressed relative to the mean value in control (vehicle-treated) normal fat-fed

animals, which was set to 1. The expression levels of selected genes were analyzed by qPCR and normalized to the expression of β-actin mRNA. Significant differences were tested by two-way ANOVA and two-tailed Student's *t* test ($p<0.05$): RExD indicates interaction between RE treatment and diet in two-way ANOVA analysis; * indicates significant differences between vehicle- and RE-treated animals and # indicates significant differences between NF- and HF-fed animals in the two-tailed Student's *t* test post hoc analysis.

Figure 4. Retinoid levels in serum (A, D), inguinal white adipose tissue (B, E) and liver (C, F) of vehicle-treated control (white bars) and vitamin A-treated rats (black bars) at a young age (d 21; A, B, C), and at 135 days of age following a normal fat (NF) or a high fat diet (HF) from weaning (D, E, F). Data represent the mean ± SEM of 13-16 (A, B, C) or 5 (D, E, F) animals per group. Significant differences were tested by two-tailed Student's *t* test and two-way ANOVA ($p<0.05$): * indicates significant differences between vehicle- and RE-treated animals in the two-tailed Student's *t* test; RE indicates retinyl ester effect and D indicates diet effect in two-way ANOVA analysis. ROL, free retinol; RE, retinyl ester; Retinols, total retinol (RE plus free ROL).

Figure 5. Vitamin A supplementation during the suckling period affects white adipose tissue development in young rats. Morphometric analysis of adipocytes (A, B), analysis of genes related to cell proliferation and adipogenesis (C) and immunohistochemistry of PCNA (D) in inguinal white adipose tissue of 21 day-old rat pups treated with vehicle (controls, white bars) or vitamin A as retinyl ester (RE, black bars) during the suckling period (d 1-20 of life). Mean adipocyte area data are the mean ± SEM of 6 animals per group, and the area of at least 100 cells were measured per animal. Gene expression data are the mean ± SEM of at least 8 animals per group. Significant differences were tested by two-tailed Student's *t* test ($p<0.05$): * indicates significant differences between vehicle- and RE-treated animals. In D, representative microphotographies of PCNA immunostaining are shown: I, control rat pups; II, vitamin A-treated rat pups; III, magnification of PCNA positive precursor cells; and IV, magnification of PCNA positive cells presenting with small cytoplasmic lipid droplets in vitamin A-treated pups. PCNA positive cells are indicated with arrows.

Figure 6. Vitamin A supplementation during the suckling period results in increased retinoic acid-mediated responses in tissues of neonatal rats. Expression levels of

CYP26a1 mRNA in inguinal white adipose tissue (A), CYP26a1 in the liver (B) and ISX mRNA in the intestine (C) of 21 day-old rat pups treated with vehicle (controls, white bars) or vitamin A as retinyl ester (RE, black bars) during the suckling period (d 1-20 of life). Data represent the mean ± SEM of at least 8 animals per group. Significant differences were tested by two-tailed Student's *t* test ($p<0.05$): * indicates significant differences between vehicle- and RE-treated animals.

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Table 1. Depot weight, mean adipocyte diameter and DNA content in inguinal WAT of male rats treated with vehicle (control) or vitamin A as retinyl ester (RE) during the suckling period (d 1-20 of life) and thereafter fed for 16 weeks a normal fat or a high fat diet.

	Normal fat				High fat				
	Control		RE		Control		RE		ANOVA
depot weigth *(g)*	10.2	± 0.6	10.1	± 0.8	22.9	± 3.1 [#]	31.5	± 1.9 *,#	RExD
adipocyte diameter *(µm)*	54	± 2	64	± 8	76	± 2	97	± 7	D, RE
DNA content									
(µg DNA/g wet tissue)	343	± 94	268	± 38	334	± 27	384	± 61	
(mg DNA)	3.56	± 0.81	2.61	± 0.25	7.66	± 1.24 [#]	12.3	± 1.84 *,#	RExD

Mean adipocyte diameter data are the mean ± SEM of 3 animals per group, and for each animal at least 100 adipocytes were measured. Other data are the mean ± SEM of 5 animals per group. Significant differences were tested by two-way ANOVA and two-tailed Student's *t* test ($p<0.05$): D indicates diet effect in two-way ANOVA analysis; RExD indicates interaction between RE treatment and diet in two-way ANOVA analysis; * indicates significant differences between vehicle- and RE-treated animals and # indicates significant differences between normal fat- and high fat-fed animals in the two-tailed Student's *t* test post hoc analysis.

Table 2. Rectal temperature and parameters related to obesity-associated metabolic complications in male rats treated with vehicle (control) or vitamin A as retinyl ester (RE) during the suckling period (d 1-20 of life) and fed after weaning a normal fat or a high fat diet for 16 weeks.

	Normal fat						High fat						
	Control			RE			Control			RE			ANOVA
rectal temperature *(ºC)*													
day 80	37,5	±	0,3	37,5	±	0,1	37,0	±	0,3	38,0	±	0,2	
day 91	37,1	±	0,3	37,3	±	0,2	37,6	±	0,2	37,1	±	0,2	
day 123	36,7	±	0,4	37,3	±	0,1	36,5	±	0,3	36,6	±	0,7	
hepatic lipids *(mg/g wet tissue)*	41,3	±	4,2	41,3	±	1,5	85,6	±	9,8	87,1	±	11,3	D
glucose *(mg/dL)*	87,4	±	2,6	86,8	±	3,7	100,4	±	3,0	103,3	±	4,3	D
NEFA (mEq/L)	2,86	±	0,39	2,43	±	0,27	4,27	±	0,29	3,76	±	0,50	D
triacylglycerols *(mg/mL)*	0,275	±	0,021	0,289	±	0,046	0,222	±	0,019	0,203	±	0,017	D
resistin *(ng/mL)*	44,1	±	4,5	53,8	±	5,2	46,7	±	4,7	44,8	±	3,7	
HOMA-IR													
day 70	0,80	±	0,04	0,77	±	0,05	2,74	±	1,60	2,49	±	0,98	
day 128	1,82	±	0,47	1,56	±	0,44	3,16	±	1,08	2,91	±	0,63	D
oGTT *(area under the curve, a.u.)*													
day 70	15660	±	785	15825	±	472	18690	±	662	19145	±	500	D
day 128	17919	±	518	18227	±	519	19428	±	250	22247	±	1214 *,#	RExD

Data represent the mean ± SEM of at least 5 animals per group. Significant differences were tested by two-way ANOVA and two-tailed Student's *t* test ($p<0.05$): D indicates diet effect in two-way ANOVA analysis; RExD indicates interaction between RE treatment and diet in two-way ANOVA analysis; * indicates significant differences between vehicle- and RE-treated animals and # indicates significant differences in the *t*-test between normal fat- and high fat-fed animals in the two-tailed Student's *t* test post hoc analysis.

Supplemental Table 1. mRNA expression levels of selected genes related to lipid and energy metabolism in tissues of male rats treated with vehicle (control) or vitamin A in the form of retinyl ester (RE) during the suckling period (d 1-20 of life) and thereafter fed for 16 weeks a normal fat or a high fat diet.

	Normal fat						High fat						
	Control			RE			Control			RE			ANOVA
Brown adipose tissue													
UCP1	100	±	8	69	±	8 *	92	±	20	160	±	26 #	RExD
PGC1α	100	±	9	86	±	21	89	±	22	112	±	12	
Skeletal muscle													
UCP3	100	±	12	84	±	17	225	±	52	221	±	61	D
PPARβ/δ	100	±	11	90	±	21	113	±	14	104	±	11	
Liver													
PPARα	100	±	19	129	±	58	148	±	27	164	±	27	
FAS	100	±	22	92	±	22	41	±	26	37	±	14	D
SCD1	100	±	14	134	±	12	20	±	3	15	±	4	D
SREBP1c	100	±	16	115	±	11	93	±	10	85	±	7	

Data are the mean ± SEM of 5 animals per group and are expressed relative to the mean value of control normal fat-fed animals, which was set to 100. The expression levels of selected genes were analyzed by qPCR and normalized to the expression of 18S rRNA. Significant differences were tested by two-way ANOVA and two-tailed Student's *t* test ($p<0.05$): D indicates diet effect in two-way ANOVA analysis; RExD indicates interaction between RE treatment and diet in two-way ANOVA analysis; * indicates significant differences between vehicle- and RE-treated animals and # indicates significant differences in the *t*-test between normal fat- and high fat-fed animals in the two-tailed Student's *t* test post hoc analysis.

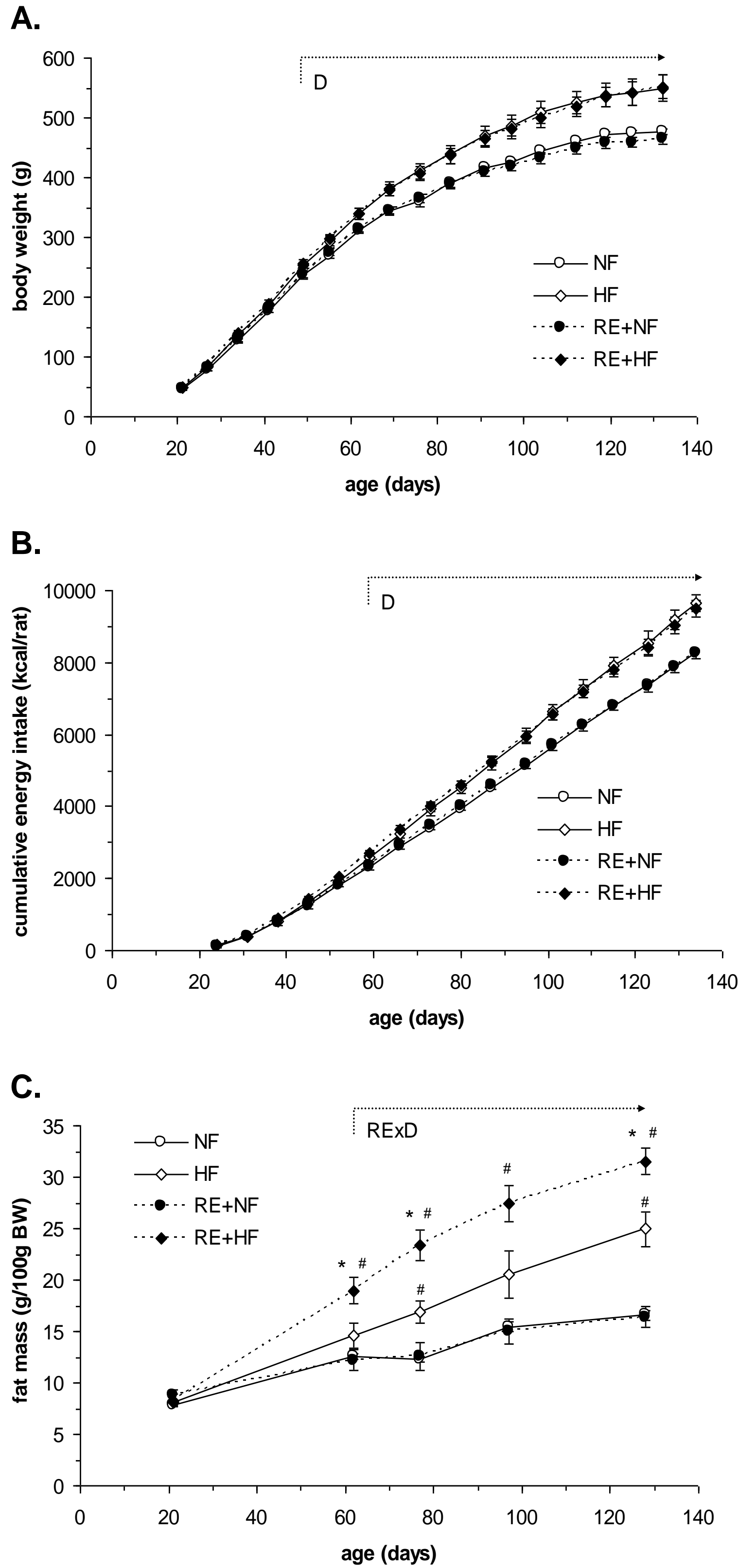

Figure 1

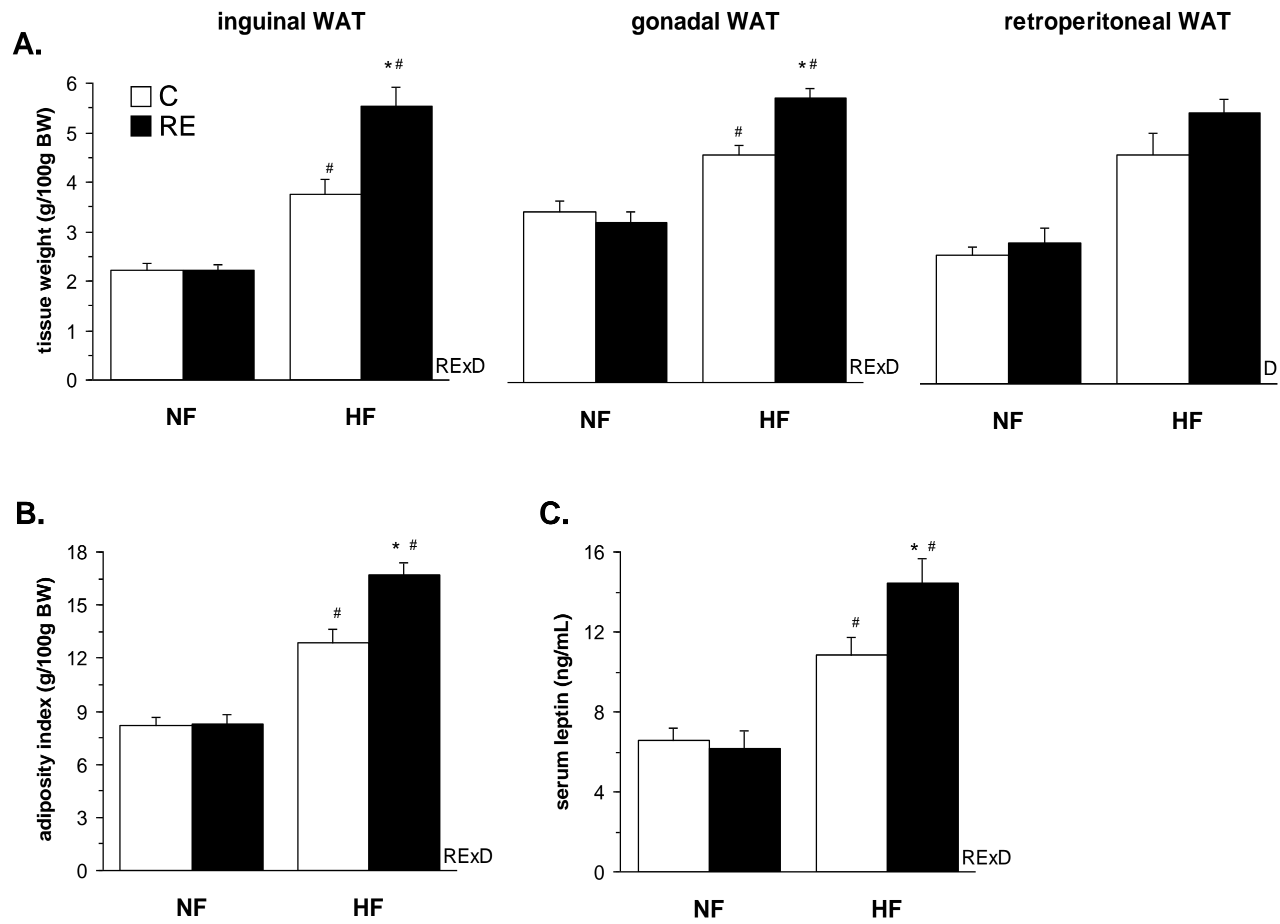

Figure 2

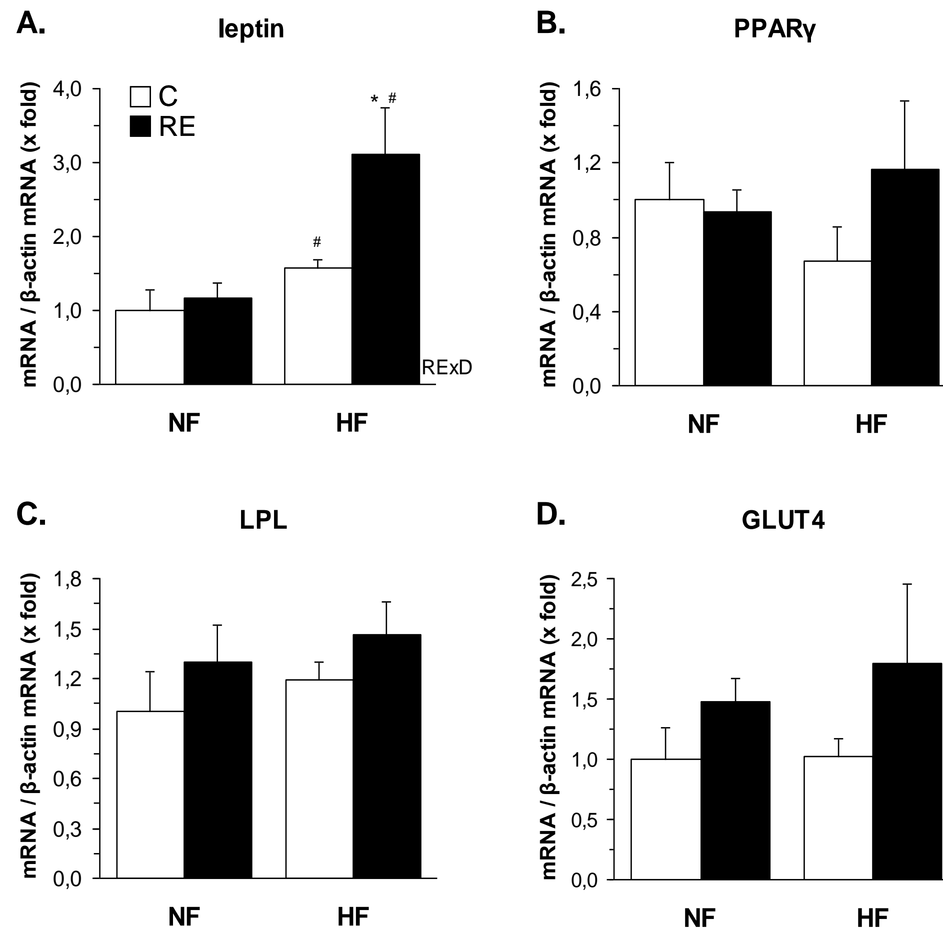

Figure 3

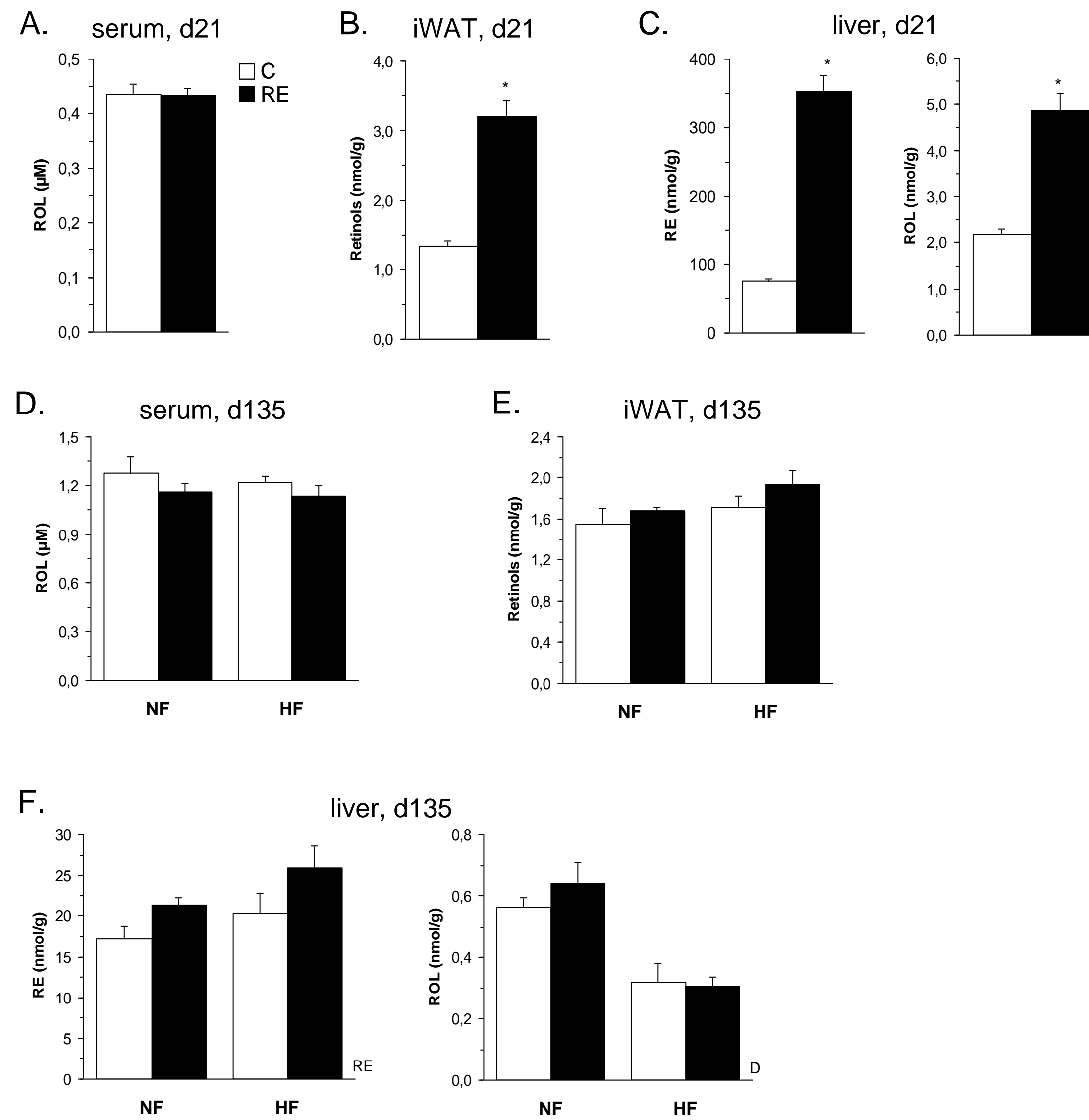

Figure 4

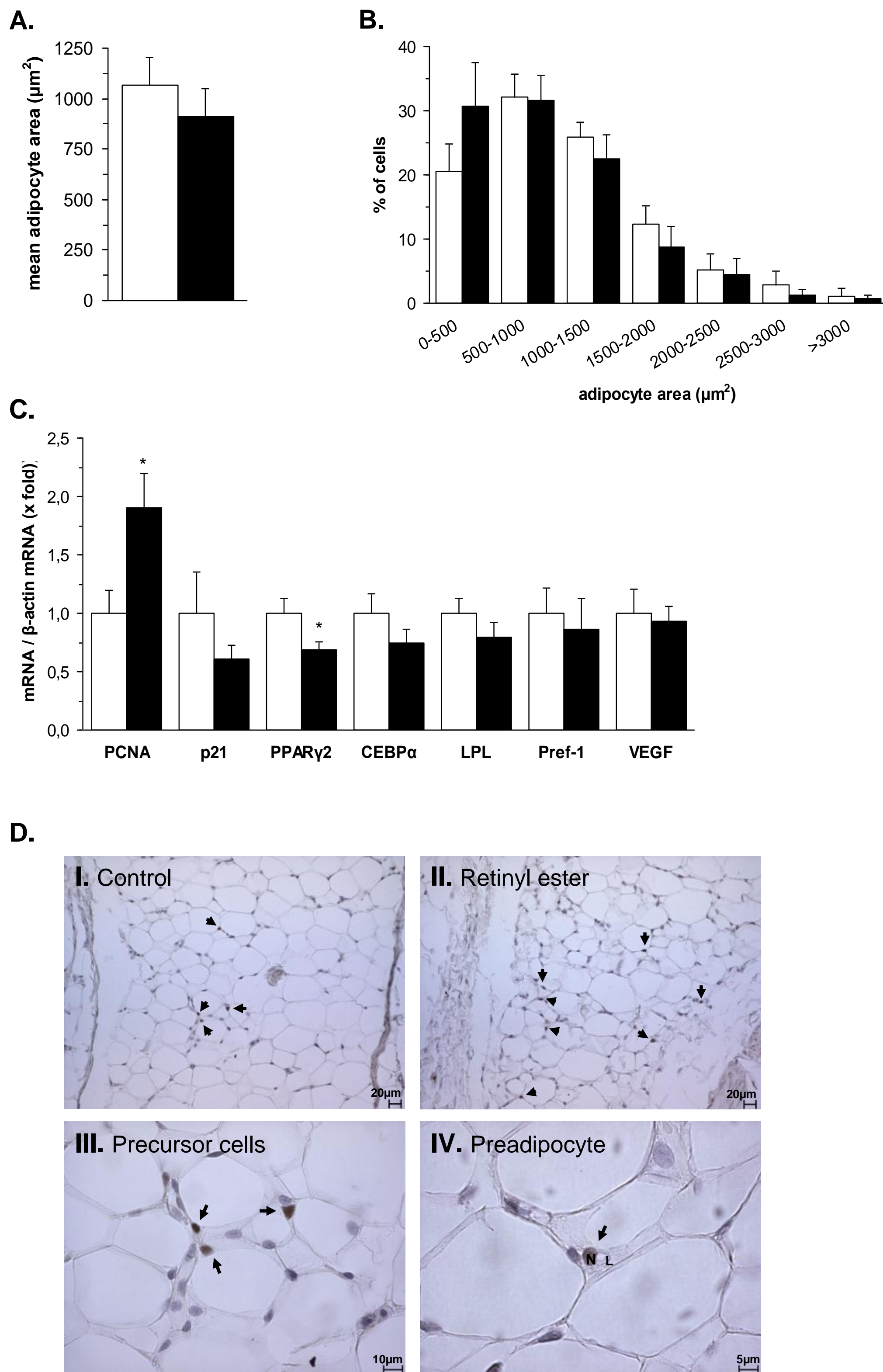

Figure 5

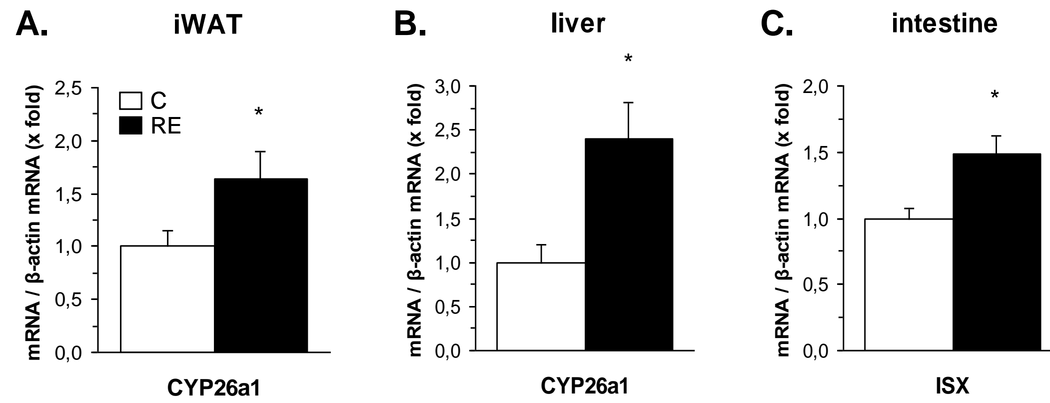

Figure 6

5. RECAPITULACIÓN

Esta tesis gira en torno a una serie de conceptos en obesidad y nutrición molecular que se han ido consolidando y han recibido una gran atención en los últimos años; el concepto de inflamación asociada a la obesidad y su importancia como mecanismo etiológico en la disminución de la sensibilidad a la insulina y otras complicaciones frecuentes en el estado obeso; el del tejido adiposo y el músculo como sitios de producción de proteínas de secreción bioactivas; el de los nutrientes como moduladores de la expresión génica (nutrigenómica); y el concepto de que la alimentación en etapas tempranas de la vida puede condicionar efectos perdurables sobre la salud y el metabolismo (programación metabólica).

En este contexto, los principales resultados de esta tesis aportan evidencia de nuevos efectos diferenciales de nutrientes específicos sobre la adipogénesis y la producción de proteínas y mediadores inflamatorios relacionados con la sensibilidad a la insulina, procesos que son considerados posibles dianas terapéuticas en la prevención y el tratamiento de la obesidad y complicaciones médicas asociadas, particularmente desde el punto de vista de la alimentación funcional. Además, en esta tesis se muestra como nutrientes en la etapa perinatal, concretamente un exceso moderado de vitamina A durante la lactancia, pueden condicionar la respuesta ulterior a una dieta rica en grasa, resultados que subrayan la importancia de una alimentación adecuada de las madres lactantes, o en su defecto, de una composición equilibrada de las leches artificiales, para prevenir la obesidad desde etapas tempranas del desarrollo.

El control de la función secretora del tejido adiposo es un campo de investigación que ha tenido un peso importante en esta tesis. Se había descrito que en el estado obeso la producción de adipoquinas está alterada (Kershaw y Flier, 2004), lo cual reforzaría un posible papel de las adipoquinas como mediadores en la patogénesis de factores de riesgo relacionados con la obesidad. Dos proteínas, la RBP y la Nampt/visfatina, fueron re-descubiertas al inicio de esta tesis como adipoquinas potencialmente relacionadas con la sensibilidad a la insulina. Estudios bioquímicos, genéticos y farmacológicos evidenciaron un papel de la RBP secretada por el tejido adiposo en el desarrollo de la resistencia sistémica a la insulina mediante mecanismos que incluyen el bloqueo de la señalización por insulina en músculo y la estimulación de la producción hepática de glucosa (Yang et al., 2005). Por su parte, la publicación donde se reportaban efectos insulino-miméticos de la Nampt/visfatina (Fukuhara et al., 2005), a la que describían como una proteína producida por el tejido adiposo visceral obeso, suscitó una fuerte controversia, hasta el punto que, debido a la irreproducibilidad de los resultados, los autores se retractaron poco tiempo después (Fukuhara et al., 2007). Sin embargo, estudios bien documentados demostrarían más adelante que Nampt/visfatina puede

actuar como una citoquina pro-inflamatoria y también modular numerosos procesos dependientes de NAD, incluyendo la secreción de insulina por las células β pancreáticas (Revollo et al., 2007b).

A partir del re-descubrimiento de RBP y Nampt/visfatina como adipoquinas, numerosos trabajos, sobre todo en humanos, estudiaron posibles asociaciones entre niveles circulantes y de expresión en el tejido adiposo de estas proteínas con la obesidad/resistencia a la insulina, con resultados contradictorios (Broch et al., 2007; Dogru et al., 2007; Friebe et al., 2011; Fukuhara et al., 2005; Janke et al., 2006; Jian et al., 2006; Lemaitre et al., 2006; Lewis et al., 2007; Pagano et al., 2006; Promintzer et al., 2007; Revollo et al., 2007b; Sandeep et al., 2007; Silha et al., 2007; von Eynatten et al., 2007; Yao-Borengasser et al., 2007). Esta controversia, junto con la relativa escasez de trabajos que evaluaran posibles cambios en la expresión de RBP y Nampt/visfatina en modelos animales de obesidad y resistencia a la insulina (Kloting y Kloting, 2005; Yang et al., 2005), nos llevó a estudiar dichos cambios en dos modelos de obesidad en ratas bien establecidos, un modelo de obesidad genética (ratas Zucker fa/fa deficientes en el receptor de leptina) y un modelo de obesidad dietética (ratas Wistar alimentadas con dieta de cafetería (**manuscrito I)**.

Las ratas Zucker fa/fa desarrollaron, como era de esperar, hiperleptinemia, hiperinsulinemia y resistencia a la insulina, mientras que las ratas con obesidad dietética desarrollaron signos de resistencia a la insulina en grado variable. Ambos modelos de obesidad presentaron, en comparación con sus correspondientes controles delgados, niveles séricos y de expresión génica en tejidos adiposos de RBP y Nampt/visfatina no alterados o reducidos. Por tanto, en conjunto, nuestros resultados indican que, en los modelos animales de obesidad estudiados, el desarrollo de resistencia a la insulina es independiente de incrementos en la concentración circulante o la expresión adiposa de RBP o Nampt/visfatina.

Llama la atención que los niveles séricos de RBP estén reducidos en las ratas obesas Zucker fa/fa comparadas con sus correspondientes controles delgados, ya que se había descrito que los niveles séricos de RBP están incrementados por un factor de 13 en ratones ob/ob deficientes en leptina (Yang et al., 2005), y hubiera sido de esperar que la deficiencia en leptina (como en los ratones ob/ob) y en su receptor (como en las ratas Zucker fa/fa) diera lugar a un mismo fenotipo RBP. De hecho, tanto en ratones ob/ob (Yang et al., 2005) como en nuestras ratas Zucker fa/fa se detecta una reducción de la expresión génica específica de RBP en tejidos adiposos, lo que sugiere que la leptina normalmente regula al alza la expresión de la RBP adiposa. Esta regulación por leptina estaría de acuerdo con el papel de la leptina como lipostato y el papel de la RBP

antagonizando la señalización pro-lipogénica por insulina en el tejido adiposo. El diferente fenotipo RBP en ratas Zucker fa/fa hiperleptinémicas y en ratones ob/ob deficientes en leptina sugiere que la leptina puede tener efectos independientes de su receptor favoreciendo la reducción de la RBP circulante (por ejemplo, su eliminación del torrente sanguíneo). Efectos opuestos de la leptina sobre la expresión adiposa y la concentración circulante de RBP, aumentando la primera y reduciendo la segunda, podrían explicar parcialmente las inconsistencias en la literatura sobre RBP en obesidad, especialmente si se tiene en cuenta que hiperleptinemia y resistencia a la leptina son condiciones que coexisten en diferentes grados en muchas formas de obesidad. Por otro lado, no puede descartarse que la regulación de la RBP sea dependiente de la especie y distinta en ratas, ratones y humanos.

Actividad investigadora previa de nuestro grupo y otros grupos ha evidenciado un papel regulador de la vitamina A, particularmente en forma de AR, sobre la biología del adipocito y la adiposidad corporal (Bonet et al., 2003; Bonet et al., 2011; Villarroya et al., 2004). En ratones, los efectos del tratamiento *in vivo* con AR incluyen un incremento de la tolerancia a la glucosa y la sensibilidad a la insulina que obedecería tanto a efectos indirectos, consecuencia de la reducción de la adiposidad corporal que propicia, como a efectos directos del AR modulando la expresión de adipoquinas. En concreto, se había demostrado al inicio de esta tesis que el AR inhibe la producción adiposa de resistina y leptina (Felipe et al., 2004; Felipe et al., 2005; Hollung et al., 2004), dos proteínas consideradas pro-inflamatorias y relacionadas con la disminución de la sensibilidad a la insulina. La RBP tiene un papel clave y bien conocido en el transporte de la vitamina A (retinol), y su regulación por vitamina A había sido muy estudiada en el hígado, su principal lugar de síntesis, pero no en el tejido adiposo, lugar de producción de la RBP relacionada con la resistencia a la insulina. Por todo ello, nos interesó explorar la regulación de la RBP por retinoides en tejidos adiposos **(manuscrito II)**.

Nuestros resultados muestran que el AR inhibe la producción de RBP a nivel transcripcional en TAB, pero no en el hígado, *in vivo* y en dos modelos celulares de adipocitos blancos, adipocitos 3T3-L1 y adipocitos derivados de MEFs, en los que dicha inhibición fue reproducida por retinal y por agonistas de los receptores de retinoides RAR y RXR. *In vivo*, el tratamiento agudo con AR se acompañó de un incremento de la sensibilidad a la insulina (con reducción del HOMA-IR), una reducción de los niveles de proteína RBP en depósitos de TAB e hígado, y un aumento drástico de la RBP circulante. Ésta última es poco probable que proceda de los tejidos adiposos, ya que el tratamiento con AR inhibió la expresión de RBP en TAB a nivel transcripcional y redujo la cantidad de RBP secretada al medio en adipocitos en cultivo; nuestros resultados,

incluyendo el inmunoanálisis de complejos de RBP en suero tras electroforesis no desnaturalizante llevado a cabo, sugieren que se trataría de RBP unida a AR de origen hepático.

En conjunto, nuestros resultados **(manuscrito II)** revelan una regulación diferencial de la RBP por vitamina A en función del tejido, disminuyendo el AR selectivamente la producción adiposa pero no la hepática, son compatibles con una papel de la RBP específicamente de origen adiposo en el desarrollo de resistencia a la insulina, y vendrían a reforzar el concepto de que vitámeros de la vitamina A pueden afectar la sensibilidad a la insulina vía efectos sobre adipoquinas. Teleológicamente, que un agente que promueve la movilización de la grasa almacenada en el TAB como es el AR suprima la expresión adipocitaria de leptina (Felipe et al., 2005; Hollung et al., 2004), resistina (Felipe et al., 2004) y RBP (manuscrito II) puede verse como un mecanismo de seguridad tendente a evitar una depleción masiva de grasa, teniendo en cuenta que estas tres adipoquinas tienen efectos paracrinos suprimiendo la actividad pro-lipogénica de la insulina en TAB (Bjorbaek y Kahn, 2004; Kim et al., 2001b; Ost et al., 2007; Steppan et al., 2001; Steppan et al., 2005).

Numerosos estudios han mostrado efectos beneficiosos del ácido oleico sobre la salud cardiovascular y la sensibilidad a la insulina y efectos adversos derivados del consumo de ácidos gras *trans* de origen industrial (AGT-PI) sobre múltiples factores de riesgo cardiovascular, incluyendo la inducción de un perfil lipídico pro-aterogénico, disfunción endotelial e inflamación (Mozaffarian, 2006; Mozaffarian et al., 2009; Teegala et al., 2009). Sin embargo, hay más controversia en relación al impacto del consumo de AGTs-PI sobre la resistencia a la insulina y el riesgo de DT2, y, además, los posibles mecanismos moleculares subyacentes han sido relativamente poco estudiados (Lovejoy et al., 2002; Mozaffarian et al., 2009; Odegaard y Pereira, 2006; Riserus et al., 2009; Tardy et al., 2008). En este contexto, nos interesó comparar los efectos directos del ácido oleico y su isómero *trans*, el ácido elaídico – el más abundante en la grasa *trans* de origen industrial – sobre la producción de mioquinas y adipoquinas potencialmente relacionadas con la inflamación, la sensibilidad a la insulina y el metabolismo **(manuscrito III)**.

Concretamente, en células musculares en cultivo comparamos la interacción de oleico y elaídico con los niveles de IL-6 e IL-15, dos proteínas secretadas por el músculo en contracción con efectos positivos sobre la sensibilidad a la insulina y efectos anti-obesogénicos (Pedersen y Febbraio, 2008), y de TNFα, un factor potencialmente patogénico en el desarrollo de la resistencia a la insulina cuyos niveles están

aumentados en músculo y tejido adiposo en el estado obeso. En adipocitos blancos 3T3-L1, analizamos los efectos de ambos ácidos grasos sobre la producción de adiponectina, una adipoquina anti-inflamatoria de efectos positivos sobre la sensibilidad a la insulina y la salud cardiovascular cuyos niveles están disminuidos en la obesidad y la resistencia a la insulina (Kondo et al., 2009; Ohashi et al., 2009; Ouchi et al., 2000; Ribel-Madsen et al., 2009; Shibata et al., 2008; Yun et al., 2009), y resistina, una proteína pro-inflamatoria e inductora de resistencia a la insulina (Lee et al., 2009; McTernan et al., 2006; Steppan et al., 2001; Won et al., 2009).

Nuestros resultados muestran efectos diferenciales del ácido oleico y el ácido elaídico sobre la producción de todas estas proteínas **(manuscrito III)**. La exposición a ácido oleico, pero no elaídico, indujo la expresión de IL-6 en las células musculares e indujo la expresión de adiponectina a la vez que reprimió la de resistina en los adipocitos en cultivo. La exposición a ácido elaídico, pero no oleico, indujo la expresión de TNFα y reprimió la expresión de IL-15 en las células musculares. En conjunto, estos resultados son compatibles con efectos beneficiosos del ácido oleico y adversos del ácido elaídico sobre la sensibilidad a la insulina previamente descritos, y describen por primera vez mecanismos moleculares que sustentan dicha relación vinculados a efectos directos diferenciales sobre la producción de adipoquinas y mioquinas.

También demostramos que la captación de glucosa estimulada por insulina en células musculares es menor si las células habían sido previamente incubadas con ácido elaídico respecto a células pre-incubadas con ácido oleico **(manuscrito III)**. Teniendo en cuenta que el músculo esquelético es responsable del 80% de la captación de glucosa estimulada por insulina (Parish y Petersen, 2005), estos resultados vendrían a confirmar un efecto adverso directo de la grasa *trans*, comparada con la cis, sobre la homeostasia de la glucosa vía reducción de su captación por células musculares.

Numerosos estudios muestran una relación inversa entre masa adiposa y masa ósea (osteoporosis) (Duque, 2008) y una relación entre desórdenes músculo- esqueléticos como la disfunción articular (osteoartritis) y la obesidad que va más allá del mero nexo biomecánico para incluir asimismo múltiples nexos metabólicos y celulares (Aspden et al., 2001; Griffin y Guilak, 2008; Hu et al., 2010; Iannone y Lapadula, 2010; Pottie et al., 2006). En este contexto, y dentro de un contrato de nuestro grupo con la empresa Bioibérica S.A. enmarcado en el programa CENIT-Pronaos, nos interesó realizar una revisión de los factores moleculares simultáneamente involucrados en el desarrollo y función de adipocitos y condrocitos, con especial énfasis en su posible alteración en la obesidad y la osteoartritis y su idoneidad como dianas terapéuticas para ambas condiciones en los (muchos) casos en que coexisten en un mismo individuo. Hemos

identificado las siguientes categorías de factores en la intersección entre obesidad y osteoartritis: señales y factores de transcripción involucrados en la diferenciación celular a partir de células multipotentes; componentes y factores de remodelación de la matriz extracelular; adipoquinas y mediadores proteicos pro-inflamatorios; factores de transcripción inducidos por hipoxia; lípidos; productos finales de la glicosilación avanzada y microRNAs **(manuscrito V)**.

Adipocitos, condrocitos y osteoblastos se cree que derivan de células mesenquimales precursoras comunes durante el desarrollo, y hay evidencia de que la diferenciación de células multipotentes en estos diferentes tipos celulares está competitivamente equilibrada. Se había descrito el papel que algunos nutrientes tenían sobre la función articular, pero no se había evaluado si estos mismos nutrientes podían tener algún efecto sobre el proceso adipogénico (la identidad de estos compuestos está sujeta a medidas de confidencialidad de cara a una posible patente). Nuestros resultados, obtenidos de la observación microscópica de la morfología celular y la tinción de triacilgliceroles intracelulares, muestran un efecto inhibidor de algunos de los nutrientes de este tipo ensayados sobre la adipogénesis en MEFs, tanto espontánea como inducida hormonalmente **(manuscrito IV)**. Al nivel molecular, algunos de los nutrientes ensayados indujeron en MEFs la expresión de factores que inhiben la adipogénesis y estimulan la condro-/osteo-génesis (Sox-9, Pref-1, PGC1α, TNFα, leptina), y reprimieron la expresión de PPARγ, un factor clave en la diferenciación de adipocitos. Además, la expresión de genes clave en la diferenciación terminal de adipocitos (CEBPα) y la lipogénesis (FAS) y adipoquinas pro-inflamatorias como la resistina y la RBP resultó inhibida por los nutrientes ensayados, al tiempo que la expresión de un componente de la matriz extracelular del cartílago como es la proteína del agregan resultó incrementada. Estos resultados sugieren que los nutrientes ensayados tienen capacidad de regular el balance de diferenciación de células multipotentes hacia unos u otros tipos celulares, inhibiendo la adipogénesis y potenciando la condrogénesis, lo que puede resultar de interés en la alimentación funcional para, simultáneamente, prevenir/tratar la obesidad y mejorar la salud articular. La regeneración del cartílago y la inhibición de la adipogénesis en la médula ósea podrían ser objetivos en la terapia de la osteoartritis (De Bari et al., 2010; Liu et al., 2009). Por su parte, la inhibición de la adipogénesis en los depósitos de TAB puede ser una diana terapéutica en el tratamiento de la obesidad complementaria a las estrategias convencionales basadas en la consecución de un balance energético negativo, especialmente en los casos de obesidad hiperplásica, más refractaria a la pérdida estable de peso/adiposidad (Spalding et al., 2008).

Finalmente, en esta tesis nos ha interesado estudiar los efectos a largo plazo de la suplementación con vitamina A durante la lactancia sobre la susceptibilidad a la obesidad dietética. Esta investigación la planteamos a la luz de diferentes considerandos. La nutrición en etapas tempranas de la vida puede tener consecuencias a largo plazo sobre la susceptibilidad a padecer obesidad y desórdenes metabólicos asociados en la edad adulta, y la vitamina A modula múltiples procesos determinantes del balance energético y la adiposidad corporal en animales adultos, incluyendo el metabolismo lipídico y energético en diferentes tejidos, y es un conocido regulador de la adipogénesis y un nutriente esencial clave en el desarrollo embrionario. Y, a pesar de todo ello, los estudios sobre los efectos de la vitamina A en etapas tempranas de la vida sobre el desarrollo de los tejidos adiposos eran escasos.

Nuestros resultados **(manuscrito VI)** muestran que ratas que recibieron una suplementación moderada de vitamina A en forma de retinil palmitato durante la lactancia (días 0 a 20 de vida) acumulan subsiguientemente más grasa corporal que sus controles en respuesta a una dieta hiperlipídica, presentando mayor leptinemia, diámetro de los adipocitos y, especialmente, mayor contenido en ADN en el TAB. La ganancia de peso y la ingesta fueron, no obstante, comparables en animales controles y tratados. Los mecanismos subyacentes a esta ganancia incrementada de adiposidad en las ratas tratadas con vitamina A estarían posiblemente relacionados con cambios en el desarrollo del TAB, que en la rata ocurre sobre todo post-natalmente durante el primer mes de vida. En concreto, en el momento del destete, en el tejido adiposo subcutáneo de las crías los niveles de ARNm de PCNA, un marcador de proliferación celular, se presentaron incrementados, mientras que los niveles de expresión de marcadores de diferenciación adipocitaria (como PPARγ y CEBPα) se presentaron disminuidos. Esto sugiere una mayor competencia proliferativa en los tejidos adiposos juveniles en las ratas tratadas con vitamina A que podría derivar, en respuesta a una rica dieta en grasa, en un mayor número de adipocitos y en consecuencia en una mayor adiposidad corporal. En conjunto, los resultados sugieren que la vitamina A en etapas tempranas de la vida puede condicionar efectos a largo plazo sobre la adiposidad corporal vía efectos sobre el desarrollo y la celularidad del tejido adiposo. Teniendo en cuenta que los niveles de vitamina A en la leche materna reflejan el estatus de vitamina A de la madre así como la ingesta materna de vitamina A, estos hallazgos pueden resultar de interés en la prevención de la obesidad y especialmente la obesidad infantil, actualmente un grave problema de salud a nivel mundial.

En definitiva, el conjunto de resultados presentados nos aportan nueva información acerca de los efectos de determinados nutrientes sobre la secreción de proteínas

bioactivas por tejido adiposo y muscular, la adipogénesis y también, administrados en etapas tempranas del desarrollo, la futura susceptibilidad a la obesidad. El conocimiento de las interacciones nutrientes-genes en procesos que regulan la adiposidad corporal y la sensibilidad a la insulina en etapas tempranas y en la edad adulta puede ayudar al diseño de nuevas estrategias para prevenir/tratar la obesidad y complicaciones médicas asociadas.

6. CONCLUSIONES

1) En ratas obesas Zucker fa/fa y ratas Wistar obesas por dieta de cafetería el desarrollo de resistencia a la insulina es independiente de incrementos en la concentración circulante o expresión adiposa de RBP y Nampt/visfatina, lo que sugiere que la desregulación de estas dos proteínas no es un factor relevante en el deterioro de la homeostasia metabólica en dichos modelos de obesidad en rata.

2) El ácido retinoico (AR), forma carboxílica de la vitamina A, inhibe la expresión de RBP a nivel transcripcional en modelos celulares de adipocitos. En adipocitos derivados de fibroblastos embrionarios de ratón (MEFs), dicha inhibición es reproducida por agonistas de receptores de retinoides (RAR y RXR) y por retinal, y se acompaña de una reducción de la RBP secretada al medio de cultivo. En ratones, el tratamiento agudo *in vivo* con AR reprime selectivamente la expresión génica de RBP en el tejido adiposo blanco, pero no en el hígado, y conlleva un incremento de la sensibilidad a la insulina. En conjunto, los resultados revelan una regulación tejido-específica de la RBP por AR, son compatibles con una papel de la RBP específicamente de origen adiposo en el desarrollo de resistencia a la insulina en ratones, y vienen a reforzar el concepto de que vitámeros de la vitamina A pueden afectar la sensibilidad a la insulina vía efectos sobre adipoquinas.

3) El ácido oleico y su isómero *trans*, el ácido elaídico, tienen efectos directos diferenciales sobre la producción de proteínas bioactivas secretadas por adipocitos y células musculares en cultivo. La exposición a ácido oleico incrementa la expresión de interleuquina-6 muscular y adiponectina adiposa, al tiempo que reduce la expresión de resistina en adipocitos. La exposición a ácido elaídico incrementa la producción de TNFα y disminuye la expresión de interleuquina-15 en células musculares. Estos resultados contribuyen a establecer un vínculo molecular entre los efectos adversos del ácido elaídico y beneficiosos del ácido oleico sobre la sensibilidad a la insulina y el riesgo cardiovascular y la función secretora de adipocitos y miocitos.

4) La exposición a ácido elaídico disminuye la captación de glucosa estimulada por insulina en células musculares en comparación con el ácido oleico, lo que estaría de acuerdo con un efecto adverso directo de la grasa *trans* sobre la homeostasia de la glucosa. A los efectos sobre la captación de glucosa estimulada por insulina podrían contribuir los cambios observados en la regulación de la expresión de mioquinas.

5) El tratamiento con determinados compuestos comúnmente vinculados a la salud de las articulaciones inhibe la adipogénesis e induce la expresión de marcadores condrogénicos en células multipotentes. En particular, en MEFs en vías de diferenciación en presencia de estímulos adipogénicos, los compuestos ensayados determinan una disminución de la acumulación de lípidos y una inhibición de la expresión de genes para proteínas involucradas en la diferenciación terminal de adipocitos y la lipogénesis (CEBPα, FAS) y proteínas potencialmente inflamatorias (RBP y resistina), así como un incremento de la expresión de señales anti-adipogénicas (leptina) y de proteínas relacionadas con la función mitocondrial y/o la condrogénesis (PGC1α, Sox-9, Col2a1). En MEFs no estimulados a diferenciarse en adipocitos, algunos de los compuestos ensayados reprimieron la adipogénesis espontánea y tuvieron efectos sobre la expresión génica similares a los de un conocido factor inductor de la condrogénesis (BMP2).

6) Existen numerosos factores moleculares alterados en la obesidad y la osteoartritis que reflejan como la pérdida de homeostasia en el estado obeso afecta negativamente al desarrollo, función y mantenimiento de las articulaciones. El conocimiento de estos factores puede ayudar a prevenir y/o tratar de forma simultánea ambas condiciones patológicas.

7) Ratas que recibieron una suplementación moderada de vitamina A como retinil palmitato durante la lactancia presentan un mayor potencial de proliferación celular en el tejido adiposo blanco en el momento del destete, con un incremento de la expresión del marcador de proliferación celular PCNA, una disminución de la expresión de marcadores adipogénicos (PPARγ, CEBPα, LPL), y una tendencia a presentar una mayor proporción de células de pequeño tamaño.

8) Ratas que recibieron una suplementación moderada de vitamina A como retinil palmitato durante la lactancia acumulan subsiguientemente más grasa corporal que sus controles en respuesta a una dieta hiperlipídica, y presentan mayor leptinemia, diámetro de los adipocitos y contenido en ADN en el tejido adiposo blanco. A esta mayor ganancia de adiposidad podría contribuir la retención de una mayor competencia proliferativa celular en el tejido adiposo, que podría derivar en una mayor hiperplasia de este tejido bajo el estímulo de una dieta rica en grasa.

7. BIBLIOGRAFÍA

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ANEXO I

ANEXO I: Índice de materiales y métodos.

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1. Animales de estudio

Las ratas empleadas eran de las cepas Wistar y Zucker (normopeso (fa/?) y obesas(fa/fa)) y los ratones eran de la cepa NMRI. Todos los animales fueron suministrados por los laboratorios Charles River (Barcelona). Durante el periodo de experimentación los animales se mantenían en el estabulario de la UIB aclimatados a 22ºC con un ciclo de doce horas de luz y doce horas de oscuridad., con acceso libre al agua y al pienso estándar (Panlab). Los ratones fueron sacrificados por asfixia mediante CO_2 .Las ratas fueron sacrificadas por decapitación mediante guillotina. Después de la disección los tejidos de interés o bien se lavaban con salino que contenía 0,1% de dietilpirocarbonato (DEPC, Sigma) para evitar la degradación del ARN y se almacenaban a -70ºC, o bien se guardaban en paraformalheído al 4% para futuros análisis morfométricos e immunohistiquímicos.

2. Cultivo de células 3T3-L1. Siembra, subcultivo, proliferación y diferenciación a adipocitos. Tratamientos.

Siembra: Partíamos de un criovial de pre- adipocitos de ratón 3T3-L1 (ATT CL-173™; LGC Deselaers SL. Barcelona, Spain), conservado en nitrógeno líquido, con aproximadamente 5 x 10^5 células en 1 ml de medio de conservación. El criovial se descongelaba rápidamente en un baño termostatizado a 37Cº y con agitación, y la suspensión de células se pipeteaba en un frasco de cultivo de 25 cm^2 al que se le añadían 4 ml de medio de crecimiento para obtener un volumen total de 5ml. Las células se dejaban proliferar en un incubador a 37Cº y 5% de CO_2.

Subcultivo (Tripsinizacion). Se aspiraba el medio de crecimiento del frasco y las células se lavaban dos veces con tampón fosfato salino *(*PBS) celular. Se añadía 1 ml de tripsina (Sigma) por frasco, se introducían los frascos cerrados en el incubador a 37ºC y se esperaba a que las células se despegasen (aproximadamente 2-3 minutos). Una vez despegadas las células, se inactivaba la tripsina añadiendo 4 ml de medio de crecimiento por frasco.

Recuento celular y siembra para experimento: Para el recuento celular se utilizaba una cámara de Neubauer y se mezclaban a partes iguales (10 µl) suspensión celular y azul tripan (Sigma). Las células se sembraban a una densidad de 5.000 células/cm^2.

Diferenciación: Dos días de después de que las células hubieran llegado a la confluencia (día 0), se sustituía el medio de crecimiento por un primer medio inductor

de la diferenciación que contenía un estímulo hormonal. Transcurridos dos días se sustituía por un medio de diferenciación suplementado con insulina durante 48 horas más. A partir de entonces se dejaban las células con medio básico que se cambiaba cada dos días, muy cuidadosamente para evitar que se despegaran las células que se iban cargando de lípidos, hasta que se observaban gotículas lipídicas en más del 95% de las células (generalmente a día 8-10), en este momento se añadían los tratamientos: retinoides o ácidos grasos disueltos en DMSO. Como controles se empleaban células tratadas con el vehículo DMSO.

Medios:

**todos los reactivos eran de Sigma a menos que se indique lo contrario*

- *Medio de crecimiento*: preparado con Dulbecco's Modified Eagle Medium (DMEM) al que se le añadía un 10% (vol/vol) de suero de feto bovino (Linus), glutamina (4 mM) y antibióticos (50 IU penicilina/ml y 50 mg estreptomicina/ml).

- *Medio de diferenciación I*: se preparaba añadiendo al medio de crecimiento las siguientes hormonas: insulina (0.175μM), isobutilmetilxantina (IBMX) (0.5mM) y dexametasona (1μM).

- *Medio de diferenciación II:* se preparaba añadiendo al medio de crecimiento insulina (175mM).

3. Cultivo de fibroblastos embrionarios de ratón (MEFs, Mouse Embryonic Fibroblasts). Obtención, siembra, proliferación y diferenciación a adipocitos y condrocitos. Tratamientos.

Obtención y siembra: Los fibroblastos de ratón se obtenían de la carcasa de embriones de 13 días (tras descartar cabeza, hígado y otros órganos).La carcasa de cada embrión se cortaba en piezas pequeñas que se resuspendían en 50 ml de tampón fosfato salino (PBS) y después se centrifugaban (300g / 5 min), este paso se repetía las veces necesarias hasta la obtención de un sobrenadante claro. Tras descartar el sobrenadante, el precipitado se resuspendían en una solución de colagenasa a 37ºC en agitación durante una hora, tras lo cual se disgregaban las células pasándolas varias veces por una jeringa. Las células ya disgregadas se resuspendían en medio de crecimiento y se sembraban a una densidad de aproximadamente 50.000 células /cm^2 y se dejaban proliferar a 37ºC 5% CO_2.

Subcultivo (Tripsinizacion). Se aspiraba el medio de crecimiento del frasco y las células se lavaban dos veces con tampón fosfato salino *(*PBS) celular. Se añadía 1 ml de tripsina (Sigma) por frasco, se introducían los frascos cerrados en el incubador a 37ºC y se esperaba a que las células se despegasen (aproximadamente 2-3 minutos). Una vez despegadas las células, se inactivaba la tripsina añadiendo 4 ml de medio de crecimiento por frasco.

Recuento celular y siembra para experimento: Para el recuento celular se utilizaba una cámara de Neubauer y se mezclaban a partes iguales (10 µl) suspensión celular y azul tripan (Sigma). Las células se sembraban a una densidad de 5.000 células/ cm^2 aproximadamente.

Diferenciación en adipocitos: Dos días de después de que las células hubieran llegado a la confluencia (día 0) se sustituía el medio de crecimiento por un primer medio inductor de la diferenciación que contenía un estímulo hormonal. Transcurridos dos días se sustituía el medio inductor de la diferenciación por un medio de crecimiento suplementado con insulina y rosiglitazona durante 48 horas más. A partir de entonces se dejaban las células con medio de crecimiento que se cambiaba cada dos días hasta que se observaban gotículas lipídicas en un 95% de las células (generalmente a día 6-8).

En los estudios de los efectos de la vitamina A sobre la producción adipocitaria de RBP4 los tratamientos (retinoides y agonistas de RAR, RXR) se añadían al medio a día 8 cuando las células estaban completamente diferenciadas en adipocitos.

En los estudios sobre los efectos de los compuestos de interés habitualmente usados para mejorar la función articular sobre la adipogénesis, los tratamientos se añadieron desde el día 0 hasta el final del experimento.

Medios:

- *Medio de crecimiento*: preparado con medio básico AmnioMAX-C1008 (Invitrogen) al que se le añadía un 7,5% (vol/vol) de medio suplementado AmnioMax (Invitrogen), glutamina (2 mM) (Sigma) y antibióticos (50 IU penicilina/ml y 50 µg estreptomicina/ml) (Sigma).

- *Medio de diferenciación I*: se preparaba añadiendo al medio de crecimiento las siguientes hormonas: insulina (0,87µM) (Sigma), isobutilmetilxantina (IBMX) (Sigma), (0.5 mM), dexametasona (1µM) (Sigma), y rosiglitazona (0.5µM) (BioVision).

- *Medio de diferenciación II*: se preparaba añadiendo al medio de crecimiento insulina (0,87µM) y rosiglitazona (0.5µM).

Diferenciación en condrocitos:

Para la diferenciación de MEFs en condrocitos se emplearon dos variantes en la metodología del cultivo: a) cultivo en monocapa y b) cultivo en tres dimensiones.

a) cultivo en monocapa. Para el cultivo en monocapa se procedía de la misma manera que la descrita anteriormente para diferenciar MEFs en adipocitos con la única salvedad que no se añadía ninguno de los inductores de la adipogénesis (insulina, rosiglitazona, IBMX, dexametasona). Dos días después de la confluencia (día 0) y hasta el final del experimento, se añadían o bien los tratamientos o bien proteína morfogénica 2 (BMP2) (Sigma), como control positivo de la chondrogenesis, a una concentración de 100 ng/ml.

b) cultivo en tres dimensiones. Para el cultivo en tres dimensiones se dejaban proliferar las células en frascos de 75 cm^2 hasta obtener el número de células total suficiente para el experimento. Después de tripsinizar, contar y centrifugar se resuspendían las células una concentración 12.000 células/µl y en placas de 24 pocillos se ponían dos gotas de 10 µl de suspensión celular (1,2 x 10^5 células por gota) lo suficientemente separadas entre ellas y del borde del pocillo. Se introducía la placa en el incubador y se dejaba 4 horas a 37ºC, para que se adhirieran las células entre ellas y a la superficie celular. De esta manera las células crecían estableciendo contactos entre ellas en tres dimensiones de forma análoga a lo que ocurre *in vivo* en la condensación mesenquimática previa a la condrogénesis. Tras sacar las placas de la estufa (día 0) se añadirán 500 µl de medio de crecimiento por pocillo, conteniendo este medio o bien los tratamientos o bien BMP2 100 ng/ml (como control positivo de la condrogénesis), estos medios se mantenían hasta el final del experimento.

4. Cultivo de células C2C12 (Mouse myoblast cell line). Siembra, proliferación, diferenciación a miocitos. Tratamientos.

Siembra: Partíamos de un criovial, conservado en nitrógeno líquido, con aproximadamente 2 x 10^5 células en 1,5 ml de medio de conservación. El criovial descongelaba rápidamente en un baño termostatizado a 37Cº, y la suspensión de células se pipeteaba en un frasco de cultivo de 25 cm^2 al que se le añadían 3,5 ml de

medio de crecimiento para obtener un volumen total de 5ml. Las células se dejaban proliferar en un incubador a 37Cº y 5% de CO_2.

Subcultivo (Tripsinizacion). Se aspiraba el medio de crecimiento del frasco y las células se lavaban dos veces con tampón fosfato salino (PBS) celular. Se añadía 1 ml de tripsina (Sigma) por frasco, se introducían los frascos cerrados en el incubador a 37ºC y se esperaba a que las células se despegasen (aproximadamente 2-3 minutos). Una vez despegadas las células, se inactivaba la tripsina añadiendo 4 ml de medio de crecimiento por frasco y se centrifugaban a 1.000 g durante 5 min. El sobrenadante se descartaba y las células se resuspendÍan en medio de crecimiento fresco.

Recuento celular y siembra para experimento: Para el recuento celular se utilizaba una cámara de Neubauer y se mezclaba a partes iguales suspensión celular y azul tripan (Sigma) (10 µl). Las células se sembraban a una densidad de 5.000 células/cm^2.

Diferenciación: Cuando las células alcanzaban un 85% de confluencia (aproximadamente transcurridas 24 horas desde su siembra) el medio de crecimiento se sustituía por medio de diferenciación (día 0) que se sustituía por medio fresco cada dos días. Transcurridos 9-10 días las células se habían diferenciado en miotubos, en este momento se añadían los tratamientos (ácidos oleico y elaídico).

Medios:

- *Medio de crecimiento*: preparado con Dulbecco's Modified Eagle Medium

(DMEM, Sigma) al que se le añadía un 10% (vol/vol) de suero de feto procedente de ternera, glutamina (3 mM) y antibióticos (50 IU penicilina/ml y 50 µg estreptomicina/ml).

- *Medio de diferenciación*: preparado con DMEM al que se le añadía un 2% (vol/vol) de suero de caballo, glutamina (3 mM) y antibióticos (50 IU penicilina/ml y 50 µg estreptomicina/ml).

5. Tinción Oil Red y valoración del contenido de triacilgliceroles.

El *Oil Red* es un colorante que tiñe específicamente triacilgliceroles y ésteres de colesterol. Se preparaba una solución stock disolviendo 0.5 g de Oil Red (Sigma) en

100 ml de isopropanol. Esta solución se diluía con agua en el momento de ser utilizada (3 vol de solución stock /2 vol agua).

Se descartaba el medio de cultivo de las células y se lavaban tres veces con PBS, tras lo cual se fijaban con paraformalheído al 4% durante una hora a temperatura ambiente. Se lavaban de nuevo con PBS y se incubaban a durante 2-3 minutos con isopropanol antes de ser teñidas con 0.5 ml (placas de 12 pocillos) de la solución Oil Red recién preparada durante una hora a temperatura ambiente. Finalmente se lavaban tres veces con agua estéril para realizar fotografías.

La determinación del contenido de triacilgliceroles intracelulares se realizaba a partir de células teñidas con Oil Red siguiendo el protocolo descrito en el artículo de Ramirez-ZacaríasJ.L; *et al.Histochemistry 97*(1992) pp.493-497.El agua estéril se evaporaba a 32ºC y se añadía isopropanol para disolver el colorante y se recogía pipeteando varias veces. Se determinaba la absorbancia a 510 nm tras haber calculado previamente la linealidad de las muestras.

6. Determinación del transporte de glucosa en modelos celulares.

La determinación del transporte de glucosa se midió mediante la utilización de 2-deoxi-glucosa (2DOG) marcada radioactivamente con tritio (^3H) en cultivos celulares de células C2C12 y adipocitos 3T3-L1.

Las células se diferenciaron en placas de 12 pocillos y se trataron durante 24 horas con los ácidos grasos de interés (ácido oleico y ácido elaídico).Después se lavaron con tampón *KRP (*Kreb's Ringer phosphate) y con 0,05% albúmina bovina sérica (BSA). Luego se les añadía el mismo tampón previamente atemperado, con o sin insulina (100 nM), dependiendo de si queríamos medir captación de glucosa estimulada con insulina o basal respectivamente, durante 15 minutos a 37ºC. Transcurrido este tiempo, las células se incubaban durante 10 minutos más a 37ºC con el buffer KRP (con o sin insulina 100 nM) al que se le añadía ^3H-2-DOG-H (3700Bq/ml en una solución de 2-DOG1 µM no marcada) y cuya actividad específica final era de 0.2 µCi/ml.

Pasado este tiempo, rápidamente, las células se lavaban 3 veces con PBS celular frío y se resuspendían con 500 µl de NaOH (0,1N) que se transfería a un expender para posterior sonicación. Dos alícuotas de 100 µl de células lisadas se pasaban a 2 nuevos ependorfs, se les añadía 1 ml de líquido de centelleo para contar radioactividad (Perkin Elmer, Shelton, CT, USA) usando el contador de líquido de

centelleo Beckman Coulter LS 6500. El resto de lisado celular (300µl) se utilizaba para la cuantificación de las proteínas totales mediante el *Kit BCA protein assay* (Pierce, USA).

-*Tampón PRK: el tampón estaba compuesto por :* 10 mM de KH_2PO_4 a pH=7.4, 136 mM de NaCl, 4.7 mM de KCl, 1.25 mM de $CaCl_2$, 1.25 mM de $MgSO_4$.

7. Determinación de metabolitos plasmáticos

La glucosa en sangre se determinó mediante el uso del medidor de glucemia Accu-Check Sensor (Roche Diagnostics).

Los niveles en suero de leptina, resistina e insulina, se determinaron mediante el uso de kits basados en el ensayo por inmuno-absorcion ligado a enzimas (ELISA), siguiendo siempre las instrucciones de las casas comerciales:

Kits:

- Leptina: Leptin Quantikine ELISA kit (R&D Systems, USA).

- Resistina: Mouse Resistin Quantikine ELISA kit (R&D Systems, USA).

- Insulina: Insulin Mouse Ultrasensitive ELISA kit (DRGInstruments GmbH,

Alemania).

La concentracion de ácidos grasos libres y triacilgliceroles se determinó utilizando kits basados en reacciones enzimático-colorimétricas:

Kits

- ácidos grasos libres: test colorimétrico enzimático NEFA-C (Wako) Este método se basa en la acilación de la coenzima A (CoA) por los ácidos grasos de la muestra en presencia acil-CoA sintetasa (ACS). CoA. El acil-CoA que se produce es oxidado en una segunda reacción por acil-CoA oxidasa (ACOD) con la generación de peróxido de hidrógeno (H_2O_2), éste último induce la condensación oxidativa del 3-metil-N-etil-N (β-hidroxietil)-anilina (MEFA) con 4 aminoantipirina para rendir un pigmento rojo-púrpura. La determinación se realizaba siguiendo las indicaciones del fabricante y la absorbancia se medía a 550nm en un espectrofotómetro de placas de ELISA *Sunrise* de TECAN.

- triacilgliceroles: test colorimétrico enzimático Sigma Diagnostics (España). Este método cuantifica la cantidad de glicerol existente en la muestra. El glicerol es secuencialmente fosforilado (glicerol quinasa) y oxidado (glicerol fosfato oxidasa) reacción que se acompaña de la producción de peróxido de hidrógeno (H_2O_2).Finalmente una peroxidasa cataliza la unión de H_2O_2 4 aminoantipirina y N-textil-N-(3-sulfopropyl)m-anisidine(ESPA) para producir un colorante (quinoneimina) que absorbe a 540. La determinación se realizaba siguiendo las indicaciones del fabricante y la absorbancia se media a 540nm en un espectrofotómetro de placas de ELISA Sunrise de TECAN.

*Los triacilgliceroles en hígado también se determinaron siguiendo este método.

8. Determinación de proteínas secretadas al medio de cultivo.

La concentración IL-6 y TNFα en medio de cultivo celular se determinó mediante el uso de kits basados en el ensayo por inmuno-absorción ligado a enzimas (ELISA), siguiendo siempre las instrucciones de las casas comerciales:

Kits:

-IL-6: Mouse IL-6 Colorimetric ELISA Kit (Pierce, Rockford, IL., USA)

-TNFα: Mouse TNF alpha Colorimetric ELISA Kit (Pierce, Rockford, IL., USA)

La concentración de RBP4 se determinó por Western-blot.

9. Determinación de los lípidos totales en hígado.

Para la determinación del contenido total de lípidos en el hígado se utilizó el método de Folch (Ramirez-Zacarias et al., 1992). Aproximadamente 0,5 g de tejido se pesaron en una balanza de precisión, se homogenizaron en una mezcla 2:1 de cloroformo (Panreac): metanol (Panreac) y posteriormente se enrasaron hasta 10 ml en un tubo de vidrio. Los restos de tejido sin homogenizar fueron descartados mediante el uso de un filtro de papel. A continuación se añadieron 2 ml de NaCl (Panreac) 0,45% (para eliminar posibles impurezas polares que se encontraran en la mezcla) y se agitó vigorosamente durante 5 min, después se eliminó la fase superior acuosa y la fase inferior se enrasó con metanol hasta 10 ml. Se añadieron 2 ml de NaCl 0.9% y, transcurridos 5 min, se eliminó de nuevo la fase acuosa y se enrasó con metanol hasta 10 ml. Este paso se repitió 2 veces. Una vez finalizado, se enrasó hasta 10 ml con cloroformo/metanol y de cada muestra se recogieron 8 ml que se

repartieron en dos tubos Folch previamente pesados (4 ml en cada uno). Se dejaron los tubos destapados a 60ºC toda la noche con el fin de evaporar todo el cloroformo/metanol y luego se volvieron a pesar los tubos con el extracto de lípidos. La medida del peso final del tubo Folch menos la medida de peso inicial indica la cantidad de lípidos totales en 4 ml y conociendo el peso exacto del tejido utilizado se puede calcular la cantidad de lípidos totales por gramo de tejido.

10. Determinación del contenido en ADN.

Para la determinación del contenido de ADN de los tejidos utilizados se siguió el protocolo basado en el artículo de Kissane J.M. y Robins, E. J.Biol Chem., (1958) 233:184-188. (Folch et al., 1957). Las muestras de tejido se homogenizaban con un homogeneizador de aspas con PBS (dilución 1:3 p:v) en frío y se centrifugaban a 500g durante 10 minutos a 4ºC, después se recogía el sobrenadante que contenía el ADN. Primero se realizaba una estimación el contenido de ADN con un espectrofotómetro Nanodrop (ND-100) a 260 nm teniendo en cuenta que una unidad de densidad óptica equivale a 35-40 µg de ADN/ml. Después se preparaban alícuotas de las muestras de tal forma que el contenido estimado de ADN entrase en el patrón. Todas las muestras se enrasaban a 100µl con PBS y se añadían 50µl de ácido diaminobenzoico 1,2 M en cada tubo; se agitaban y se incubaban durante 50 min en un baño a 60ºC.Tras dejar que las muestras se enfriaran se añadía 1 ml de HCl 1M y se leía la absorbancia en un fluorímetro a una longitud de onda de excitación de 405nm y de emisión de 505 nm.

11. Aislamiento y cuantificación de proteínas

Para la homogenización de muestras procedentes de tejido adiposo se utilizaba tampón PBS (NaCl 137 mM, KCl 2.7 mM y fosfato sódico mono/di-básico 10 mM, todos los reactivos de Panreac) con una mezcla de inhibidores de proteasas (0.6 mM de fluoruro de fenilmetilsulfonilo, 1 mg/ml de aprotinina y 1 mg/ml de leupectina, todos procedentes de Sigma). La homogenización de la muestra se realizaba con *Potter-Envelhein* en frio, después se centrifugaba el homogenado a 500g durante 10 minutos a 4ºC y se descartaba el precipitado. En este punto se podía cuantificar la proteína.

En el caso de las células se empleó PBS (manuscrito 3) o TBS (manuscrito 2) (Tris HCl pH6.8, 10% de glicerol, 2.5% dodecil sulfato sódico (SDS), 10mM DTE, 10mM B –glicerofosfato, 10mM NaF, 0.1mM Na ortovanadato e inhibidores de proteasas). Con

la ayuda de un raspador se despegaron todas las células de la superficie del frasco de cultivo y o bien se sonicaron (manuscrito 3) o se vortearon e hirvieron durante 3 minutos antes de ser tratadas con benzonasa (1µl/pocillo) durante 15 minutos.

Por último se centrifugaban los lisados obtenidos (por cualquiera de los dos métodos) a 13.000 rpm durante 3 minutos y se descartaba el precipitado. En este punto se podía cuantificar la proteína.

La cuantificación de las proteínas totales se realizaba mediante el *Kit BCA protein assay* (Pierce), que se basa en la reducción del Cu^{2+} a Cu^{1+} por parte de los enlaces proteicos en un medio alcalino (reacción de Biuret) y en la detección colorimétrica del catión utilizando ácido bicinconinico (BCA). Se utilizaba como patrón albúmina bovina sérica (BSA) disuelta en el mismo tampón de homogenización (PBS). En una placa de 96 pocillos se añadían 25 µl de muestra, blanco y recta patrón así como 200 µl de una mezcla de 50 partes de reactivo A (que contiene BCA) y una parte de reactivo B (que contiene sulfato cúprico al 4%). La placa se protegía de la luz y se mezclaba durante 30 segundos. La reacción se producía incubando a 37ºC durante 30 min. Finalmente se leía la absorbancia a 562 nm en un lector de placas modelo Sunrise (Tecam).

12. Western-Blot

La determinación de los niveles de RBP4 y Nampt/visfatina en suero, tejidos y células en cultivos se realizó por *Western Blot.*

Preparación de las muestras

Por cada 7 µg de proteína resuspendida en tampón (PBS) o (TBS) se añadía 1 µl de tampón de carga: Tris-HCl 0.5 M pH 6.8 (Panreac); 5% SDS (Sigma); 10% de glicerol (Sigma); 5% de 2-mercaptoetanol (Sigma) y 1% de azul de bromofenol (Panreac) per cada 7 µg de proteína, de manera que la proporción proteína : SDS fuera 1:4. Las muestras se calentaban en un baño hirviendo durante 2-3 minutos. Para las determinaciones de RBP4 se cargaron 20-50 µg de proteína en tejido adiposo e hígado, 3 µl de suero disueltos en PBS y 10 µl de medio de cultivo celular.

Electroforesis. Primero se preparaba el gel de acrilamida en el que se llevaba a cabo la separación de las proteínas en función de su peso molecular (*resolving gel)* mezclando: 2.5ml de Tris-HCl 1,5M pH 8,8, 0,1ml de SDS al 10% y unos volúmenes determinados de una solución al 40% de acrilamida/bis-acrilamida 37,5:1(BioRad) y

agua bidestilada hasta un total de 10 ml. El porcentaje de acrilamida variaba en función del tamaño de la proteína que debíamos separar, en el caso de Nampt/visfatina empleábamos un gel de acrilamida al 10% y en el caso de la RBP4 al 13%. Después se añadían los polimerizadores: 10 µl de TEMED (Sigma) y 100 µl de persulfato amónico al 4% (Panreac) se mezclaba y se añadía rápidamente dentro de los moldes de polimerización (Mini-protean 3, BioRad) previamente ensamblados. En la parte superior se añadía una capa de agua bidestilada para evitar la inhibición por oxígeno de la polimerización. Mientras el *resolving gel* polimerizaba se preparaba el *stacking gel* al 4% de acrilamida, cuya función era concentrar las muestras. El *stacking gel* al 4% se preparaba mezclando: 2,5 ml de Tris-HCl 0.5 M pH 6.8, 0,1 ml de SDS al 10%, 1,3 ml de acrilamida y 6,1 de agua bidestilada. Se añadían los polimerizadores ; 20 µl de TEMED y 100 µl de persulfato amónico al 4%, se mezclaba y rápidamente se introducía la mezcla en los moldes de polimerización por encima del *resolving gel,* para ello previamente se eliminaba la capa de agua bidestilada. Se introducía un peine que funciona como molde para la formación de pocillos en el *stacking gel.* El número y grosor de los pocillos variaba en función de la cantidad de muestra que necesitáramos emplear para detectar la proteína.

Se cargaban las muestras en los geles de acrilamida incluyendo el marcador de pesos moleculares (Invitrogen) y se corrían en el tampón Tris-glicina 0.5x pH 8.3 (Tris base 0.025 M, glicina 0.195 M, 0.2 % SDS) aproximadamente durante 1 h.

Transferencia: Las proteínas se trasferían a una membrana de *PolyVinyliDene Fluoride,* PVDF (BioRad) de 0.2 µm utilizando un *electroblotter* semi-seco (*Trans-blot Semi-dry Electophoretic Transfer Cell, BioRad*). La membrana de PVDF se activaba previamente sumergiéndola 5 segundos en metanol (Panreac), después se lavaba en agua bidestilada durante 2 min y finalmente se mantenía en el tampón de transferencia (48 mM Tris, 39 mM Glicina y un 20% de metanol pH 9.2) durante al menos 5 min. Sobre el cátodo de titanio se colocaban 6 trozos de papel Whatman nº 3 del mismo tamaño que la membrana, empapados en tampón de transferencia. Se eliminaban las burbujas formadas entre las capas de papel y sobre ellos se colocaba la membrana de PVDF. Después se colocaba el gel de acrilamida previamente equilibrado en tampón de transferencia y finalmente, sobre el gel, se colocaban 6 trozos más de papel Whatman nº 3 empapados en tampón de transferencia. Se colocaba el cátodo de acero y se aplicaba un amperaje constante de 0,25 A durante 30 min. Una vez transferidas las proteínas a la membrana de PVDF se procedía a la tinción de la membrana con negro amido B10 con el objetivo de verificar que se había

cargado y transferido correctamente. El exceso de tinción de negro amido B10 se eliminaba con varios lavados de agua bidestilada.

Incubación de la membrana: La membrana conteniendo la totalidad de proteínas se bloqueaba durante 1 hora con PBS-Tween (Sigma) con 5% de leche en polvo. Tras sucesivos lavados se incubaba con anticuerpo de RBP4 para ratón producido en conejos (Axxora) durante 1 hora (PBS-Tween, 0,1% de BSA, 0,1% y anticuerpo diluido 1:1000). Como anticuerpo secundario se utilizó anti IgG de conejo conjugado a un complejo estreptavidina biotinilada-peroxidasa de rábano (Sigma) diluido 1:5000 en PBS-Tween y con 0,1 µg de BSA y 2% de leche en polvo. Tras 1 h se volvía a lavar la membrana 3 veces (15, 5, y 5 min) con PBS y se procedía a la detección por quimioluminiscencia del marcaje poniendo sobre la membrana reactivo de detección (ECL)(Amersham).

Revelado y cuantificación. Se ponía sobre la membrana reactivo de detección (ECL) Amershan, después se colocaban entre transparencias y se introducían en un casete donde se exponían a películas fotográficas Hyperfilm ECL (Amersham) durante el tiempo necesario para obtener una buena señal. La imagen era captada con el transiluminador *Chemigenius* (Syngene) utilizando el programa GeneSnap (Syngene). Las densidades de las bandas se cuantificaban con el programa GeneTools (Syngene).

13. Aislamiento y cuantificación de ARN

Se utilizaban dos métodos distintos a la hora de extraer el ARN en función de la cantidad de muestra. La extracción de ARN a partir de tejidos o células en cultivo (frascos de 25 cm2, placas de 6 y 12 pocillos) se realizaba utilizando un reactivo comercial basado en fenol e isotiocianato de guanidina que permite la lisis de las estructuras celulares manteniendo la integridad del ARN (Tripure, Roche) (procedimiento I). A la hora de extraer ARN de células cultivadas en placas de 24 pocillos (monocapa o 3-D), se utilizaba un kit comercial, E.Z.N.A^{TM}. EaZy Nucleic Acid Isolation (procedimiento II) que permite extraer ARN de una forma rápida y sencilla a partir de pequeñas cantidades de muestra.

En ambos casos se siguieron las instrucciones de las casas comerciales.

Procedimiento I:

Las muestras de tejido se homogenizaban con Tripure (1 ml de Tripure por cada 100 mg de TAM, hígado o músculo y por cada 300 mg de TAB). En el caso de las células en cultivo, las células se lavaban previamente con PBS frio y se añadía a continuación el Tripure (40 µl Tripure/cm^2). Cuando se trataba de muestras adiposas se realizaba una centrifugación a 12000 g durante 10 min para eliminar la grasa. Para la separación del ARN se añadían 200/50 µl (tejido/cultivo celular) de cloroformo (Sigma) a las muestras y se agitaba vigorosamente durante 15 s. Las muestras se dejaban reposar unos 5-15 min y posteriormente se centrifugaban 15 min a 12000g y 4ºC, recogiendo a continuación la fase acuosa superior, transparente, que contenía el ARN mientras que la fase inferior, en la que se encontraban las proteínas y el ADN, se guardaba a -20ªC. Para la precipitación del ARN se añadían 500/125 µl (tejido/cultivo celular) de isopropanol (Sigma) y se agitaba ligeramente por inversión. Las muestras se dejaban reposar al menos 10 min a temperatura ambiente o a -20ºC toda la noche. Después, las muestras se centrifugaban a 12000g durante 15 min y se obtenía así un precipitado de ARN. Se retiraba el isopropanol, se añadía 1 ml de etanol al 75% (Panreac) y se agitaba vigorosamente durante 15 s. A continuación se realizaba una centrifugación, esta vez a 7600g durante 5 min, obteniéndose así el precipitado final de ARN. Una vez eliminado el etanol, el ARN se resuspendía en un volumen apropiado (entre 20 y 200 µl) de agua libre de ARNasas (Sigma).

Para facilitar la resuspensión del ARN, las muestras se podían calentar en un baño termostatizado a 60ºC. El ARN extraído se guardaba a -70ºC para su correcta conservación.

Procedimiento II:

El kit de extracción de ARN, combina la lisis con isotiocianato de guanidina con la rapidez y la purificación de las columnas con membrana de silica. Las células se lisaban con 350 µl de tampón de lisis (isotiocinato de guanidina), se añadían 350 µl de etanol al 70% para favorecer las condiciones de unión del ARN a la membrana de las columnas y se mezclaba con la pipeta. El lisado obtenido se transfería a una columna HiBind y se centrifugaba a 10.000g durante 1 min a temperatura ambiente. Se descartaba el eluyente y se añadía a la columna los tampones incluidos en el kit para la eliminación de posibles contaminantes y se centrifugaba en las mismas condiciones anteriores. Finalmente se añadían entre 30-50 µl de agua libre de ARNasas y se centrifugaba a 10.000g 2 min para eluir el ARN purificado.

Cuantificación y valoración del estado del ARN

Cuantificación: El ARN se cuantificaba por espectrofotometría (NanoDrop ND-1000), valorando la absorbancia de las muestras a 260 nm. Una unidad de absorbancia a 260 nm corresponde a 40 µg de ARN por ml. Se realizaba también una medida a 280 y 230 nm y se calculaban los siguientes ratios: 260/280 y 260/230 indicativos del grado de contaminación por proteínas y por disolventes orgánicos respectivamente. Un ARN puro tiene valores de entre 1,8-2,0 para el ratio 260/280 y de 1,8-2,2 para el ratio 260/230.

Valoración de la integridad del ARN. La integridad del ARN se comprobaba mediante la carga de 0,25 µg de ARN, previamente mezclados con un tampón de carga, en un gel de agarosa al 1% (Pronadisa) con bromuro de etidio. La visualización de dos bandas correspondientes a los ARNs ribosomales 28S y 18S son indicativos de un ARN en buen estado mientras que un difuminado de bandas indica que el ARN esta degradado.

Tampones:

- Tampón de electroforesis: TBE 0,5 ´.

- Tampón de carga: glicerol, azul de bromofenol.

14. RT-qPCR

Retro-transcripción (RT): Para la síntesis de ADN-copia del ARN previamente purificado se usaban 0,25 µµg de ARN total diluidos en 5 µl de agua libre de ARNasas (Sigma) previamente desnaturalizados a 65ºC durante 10 min. Para llevar a cabo la RT se añadían 7,5 µµl de una mezcla de RT que contenía: a) Tampón (Promega); b) 2,5 mM de $MgCl_2$ (Promega); c) 0,4 mM de nucleótidos (Invitrogen); d) 5 µM de una mezcla de hexámeros sintetizados al azar (Applied Biosystems); e) 5 µM de inhibidor de ARNasas (Applied Biosystems) y f) 2,5 U/µl de transcriptasa inversa (MuLV RT, *murine leukemia virus reverse transcriptase*, Applied Biosystems). Las condiciones de retrotranscripción eran las siguientes: 15 min a 20ºC, 30 min a 42ºC y un paso final de 5 min a 95ºC. Todas las reacciones se llevaron a cabo en un termociclador Perkin Elmer 9700.

Reacción en cadena de la polimerasa a tiempo real (qPCR): En éste caso se utilizaban 2 µl de una dilución del ADN copia (desde 1/5 a 1/50, dependiendo de la abundancia del gen problema en la muestra).

A la muestra se le añadían 4,25 µl de una mezcla de PCR que contenía: a) 0.5 µl de agua libre de ARNasa; b) 0,313 µl de cebadores en dirección 5'-3' y 3´-5' (sigma) diseñados para cada gen con el programa Primer3 (Whitehead Institute for Biomedical Research, Cambridge, MA, USA) a una concentración que dependía de la abundancia de cada ARNm, generalmente a 20µM) y c) 3,124 µl de una mezcla comercial *Power SYBR Green PCR Master Mix* (Applied Biosystems). La PCR fue llevada a cabo utilizando el sistema *StepOnePlusTM* (Applied Biosystems) con el siguiente perfil: 10 min a 95ºC, seguido por un total de 40 ciclos que incluían una desnaturalización durante 15 segundos a 95ºC seguida de una elongación de 1 min a 60ºC. Con el objetivo de verificar la pureza de los productos obtenidos, se realizó una curva de desnaturalización después de cada PCR siguiendo las instrucciones de la casa comercial. Adicionalmente, una vez finalizada la reacción se verificaba el fragmento amplificado mediante la visualización con luz ultravioleta de un gel de agarosa al 2% teñido con bromuro de etidio. Las imágenes se captaban con el programa GeneSnap y las bandas se cuantificaban con el programa GeneTools. La expresión relativa de cada ARNm se calculó de acuerdo a Pfaff, M. W., *Nucleic Acids Res. 2001 1;29(9):e45.*(Kissane and Robins, 1958) utilizando distintos genes de referencia en función del modelo celular, tejido y tratamiento (β actina, 18S, LRP10).

15. Medida de concentración de retinoides por HPLC

La cuantificación de los retinoides extraídos por HPLC (Agilent, Santa Clara, CA, EE.UU.) en fase normal se llevó a cabo siguiendo el protocolo descrito por (von Lintig and Vogt, 2000) (von Lintig and Vogt, 2000) con algunas modificaciones. Brevemente, 1mg de hígado o tejido adiposo se homogeneizó en 100 µl PBS celular, y se volvió a diluir en PBS 1:3 (vol: vol). Para la extracción se partió 70 a 100 µl de suero, leche, u homogenado de tejido a los que se le añadieron 200 µl de metanol. Después se añadieron 400 µl de acetona y la extracción se repitió dos veces usando 500 µl de hexano. Las fases orgánicas fueron secadas bajo una corriente de nitrógeno y disueltas en solvente de HPLC .Para el análisis de HPLC se empleó una columna Zorbax SB-C18 de 4,6 x 150 mm y tamaño de partícula 5 µm (Agilent). La separación cromatográfica se logró por el flujo isocrático de una solución al 10% de etilacetato: 90% de hexano con una velocidad de flujo de 1,4 ml/min. Al final del proceso los retinoides fueron detectados por UV (λ absorción 325nm). El análisis de los cromatogramas se realizó con el programa (Star Chromatography Workstation, Varian Deutschland GmbH, Darmstadt, Germany).Para la cuantificación de

cantidades molares de retinoides, las integrales de los picos fueron comparadas con cantidades conocidas de las sustancias de referencia.

16. Análisis morfométrico de tejidos

Fragmentos de tejido adiposo blanco inguinal e hígado frescos se fijaron por inmersión con paraformalheído al 4% en tampón fosfato 0,1M toda la noche a 4ºC. Las muestras se lavaron con tampón fosfato 0,1M. A continuación, se deshidrataban las muestras sumergiéndolas secuencialmente en soluciones con concentraciones crecientes de etanol (50, 75, 96 y 100%) hasta que finalmente se incuban en xileno. Después las muestras se incluían en parafina y se dejaban solidificar en bloques a temperatura ambiente. Posteriormente, los tejidos se cortaban con un micrótomo en secciones de 5 µm de grosor y se tenían con hematoxilina y eosina. Las imágenes del tejido se obtuvieron empleando un microscopio óptico (Zeiss Axioskop 2) y se analizaron con el programa Axio Vision (Carl Zeiss Imaging Solutions).

Se calculó el diámetro de 200 adipocitos por cada muestra de tejido.

Reactivos:

- Paraformalheído (Sigma)
- Tampón fosfato 0,1 M (Tampón fosfato 0.2 M diluido 1:1 en H_2O destilada)
- Tampón fosfato 0.2 M pH 7.2 (3.25 g NaH2PO4 H_2O, 11.24 g Na2HPO4 en 1 L)
- Etanol absoluto (Panreac).
- Chile (Panreac)
- Paraplast (Sigma)
- Hematoxilina (Panreac)
- Eosina (Panreac)

17. **Análisis Immunohistoquímico de tejidos**

Se contaron las células inmunoreactivas al antígeno nuclear de células en proliferación (PCNA) en tejidos adiposos inguinales de crías de ratas Wistar a día 21. Mediante el método de avidina-biotina peroxidasa (ABCLas secciones de tejido se montaron en los portaobjetos *Super-Frost/Plus* y se incubaron secuencialmente en: a) solución de tampón citrato pH 6 durante 10 min en el microondas; b) peróxido de hidrógeno al 3% en metanol durante 10 min, para bloquear la peroxidasa endógena; c) suero normal de cabra al 2% en PBS, para reducir las tinciones inespecíficas que puedan aparecer antes de la incubación con el anticuerpo primario y que incrementarían la señal de fondo. Después se incubaron con el anticuerpo primario: anticuerpo policlonal anti-PCNA de conejo

(Santa Cruz Biotechnology cat. FL-261) diluido 1:50 en PBS, toda la noche a 4ºC. Posteriormente, las muestras se incubaron a temperatura ambiente durante 30 minutos con un anticuerpo secundario biotinilado anti-conejo (Vector cat. BA 1000) diluido 1:200 en PBS. A continuación se incubaron con el reactivo ABC (Vectastain ABC , Vector) que porta la enzima peroxidasa ligada, disuelto en PBS, durante 30 min a temperatura ambiente, y después se añadió 3,3' Diaminobenzidine (DABA) incubando durante 3 minutos en oscuridad, permitiendo la actividad de la enzima peroxidasa. Finalmente, las muestras se lavaron con agua destilada, se tiñeron con hematoxilina y fueron deshidratadas con concentraciones crecientes de etanol y finalmente con xileno y se montaron con Eukitt. Paralelamente a las muestras se procesaron controles negativos por omisión del anticuerpo primario durante el proceso.

Reactivos:

- Peróxido de hidrógeno al 3% en metanol (*Sigma)
- H_20 bidestilada
- PBS, pH 7.4: NaCl (Panreac) 137 mm, KCl (Panreac) 2.7 mm y fosfato
- sódico mono-/dibasic (Panreac) 10 mm.
- Etanol absoluto (Panreac)
- DAB (Sigma)
- Reactivo ABC, Vectastain ABC kit (Vector)
- Suero normal de cabra (Vector)- Citrato de Sodio (Panreac)

18. Análisis estadístico

Para el análisis estadístico se utilizaba el programa SPSS para Windows. En función del tipo de variable y los grupos de estudio las diferencias se determinaban utilizando el análisis de la media por t-student o por análisis de la varianza (ANOVA) de medidas repetidas o univariante seguido del test de la diferencia menos significativa (Least Significant Difference, LSD) También se utilizaron las correlaciones bivariadas de Pearson. En todos los casos el nivel de confianza considerado era 95% ($P<0,05$) o superior.

19. Bibliografía del Anexo I

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