Universidad de las Islas Baleares

Departamento de Biología Fundamental y Ciencias de la Salud

Laboratorio de Biología Molecular, Nutrición y Biotecnología – Nutrigenómica

TESIS DOCTORAL

Efecto de la suplementación con calcio y CLA sobre el balance energético, su repercusión sobre la adiposidad y mecanismos implicados

Programa de doctorado de Nutrición y Metabolismo

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Palma de Mallorca, marzo de 2010

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A mis padres

y a Simón

Agradecimientos

Aquesta tesis recopila la feina del meus darrers cinc anys, que ha estat el fruit de molta il·lusió i esforç així com de llàgrimes i molts mals de cap. El que no recull però són tots aquells moments que han fet d’aquesta, una experiència inoblidable i de la que sempre guardaré un gran record. Vull aprofitar aquí per mostrar el meu agraïment, encara que sigui amb unes breus paraules, a totes aquelles persones que han format part d’aquest moments i m’han acompanyat en aquesta part del camí.

He tengut l’oportunitat de formar part d’un gran grup de científics i de persones, i per això, des d’aquí, agrair-lis a tots el suport rebut en aquests anys i per transmetre'm la seva il·lusió per la ciència. En especial voldria agrair al professor Andreu Palou i a la professora Francisca Serra el haver-me brindat aquesta oportunitat, així com, la confiança dipositada en mi en tot moment, gracies per tot.

A na Joana i na Teresa, gràcies per la vostra ajuda incondicional, sou les millors, no sé què hauria fet sense vosaltres. Al meus companys de dia a dia: en Toni - per la gran persona que ets i pels cafetons de després de dinar que ara tant enyor -, en Jaume – ningú como tu ens ha fet riure tant -, a Maria – por los buenos momentos a lo largo de todos estos años -, n’Ona – la meva compi de labo i de gimnàs, gràcies per aguantar-me -, a Ana Paula – por dejarme invadir tu mesa, fue relindo-, na Núria – mi ídolo - a tots vosaltres pels riures compartits: Aixa, Rubén, Nora, Pep, Jª Mari, Xisco.

A mis amigos: Victoria, David, Cris, Geles, Garrote, Diego, Damián, Raúl, Pili, Tomeu, Ana, Bárbara por llenar de tantos buenos momentos mi vida, por estar ahí en los buenos y malos momentos, por escuchar cuando necesitaba hablar y por saber cuando más vale no preguntar jijiji tengo mucha suerte de teneros a mi lado; sois los mejores. En especial agradecerte, a ti Victoria, por ser una persona y amiga excepcional en todos los aspectos, por mantenernos unidos, a mi también me ha encantado recorrer esta parte del camino juntas. Gracias a Xavi y a Carol por esas cenas al lado de la chimenea y algún que otro sueñecito en su sofá. A Sandra y a Celso por las escapaditas a la montaña. Y a Marga y Miguel por compartir el arte del buen comer. Gosia, thanks for everything. I really appreciated your help and your hospitality that really made me feel at home. You are a great friend and I wish you all the best.

Agradecer a toda mi familia su comprensión y apoyo, sé que no ha sido fácil entender a qué me he dedicado estos últimos años. Agradecer a mis padres su confianza en mí y el enseñarme que en la vida con esfuerzo todo se consigue. En especial a tu, mama, perquè sé que t'hagués agradat compartir aquests moments amb nosaltres, sempre et tenc present. A mi hermana, por su paciencia, por ser tan buena niña y por que continuemos haciendo tonterías juntas a pesar de que nos vayamos haciendo mayores. A Rusti, por mantenerme en forma con esos paseos a marchas forzadas. Vull agrair també a la família d'en Simó la seva acollida i l'interès mostrat per la meva feina. A todos mil gracias.

Simón, aquesta tesis és en certa part també teva perquè t'ha tocat sofrir-la de ben a prop. Mai et podré agrair abastament la teva santa paciència, el teu suport incondicional i els teus consells, sempre tan encertats. Gràcies per entendre que això es important per mi. El teu amor és el que m'ha donat forces per afrontar el moments dolents i ets sens dubte el millor que m'ha pogut passar. T'ESTIM.

GRÀCIES A TOTS ☺

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ABREVIATURAS / ABBREVIATIONS

$1,25(OH)_2D_3$	calcitriol
Acc	acetil coenzima A carboxilasa, *acetyl coenzyme A carboxylase*
AGL	ácidos grasos libres
AMPc	adenosín monofosfato cíclico, *cyclic adenosine monophosphate*
ANOVA	análisis de la varianza, *analysis of variance*
ARNm	ácido ribonucleico mensajero, *messenger ribonucleic acid*
ATP	adenosín trifosfato, *adenosine triphosphate*
BCA	ácido bicinconínico, bicinchoninic acid
BSA	albúmina sérica bovina, *bovine serum albumin*
C/EBPα	proteína alfa estimulante de unión a CCAAT, *CCAAT-enhancer-binding protein alpha*
CaSR	receptor sensor de calcio, *calcium-sensing receptor*
ChREBP	proteína de unión al elemento de respuesta a carbohidratos, *carbohydrate response element-binding protein*
CLA	ácido linoleico conjugado, *conjugated linoleic acid*
Cpt1	carnitina palmitoiltransferasa (1a, hepática; 1b, muscular), *carnitine palmitoyltransferase (1a, hepatic; 1b, muscular)*
DMEM	modificación de Dulbecco del medio basal de Eagle, *Dulbecco's modified Eagle medium*
DR-3	elemento 3 de respuesta a vitamina D, *vitamin D response element 3*
ELISA	ensayo por inmunoabsorción ligado a enzimas, *enzyme-linked immunosorbent assay*
Emr1	*epidermal growth factor module-containing mucin-like receptor 1*
FABP	proteína de unión a ácidos grasos, *fatty acid binding protein*
Fasn	ácido graso sintasa, *fatty acid synthase*
Glut4	transportador de glucosa de tipo 4, *glucose transporter type 4*
HOMA	modelo de determinación de homeostasis, *homeostasis model assessment*
HSL	lipasa sensible a hormona, *hormone-sensitive lipase*
ICAM-1	molécula de adhesión intercelular-1, *intercellular adhesion molecule-1*
IL	interleucina, *interleukin*
IMC	índice de masa corporal
iNOS	sintasa de óxido nítrico inducible, *inducible nitric oxide synthase 2*

Lpl	lipoproteína lipasa, *lipoprotein lipase*
LSD	test de la diferencia menos significativa, *least significant difference*
MAPK	proteína quinasa activada por mitógenos, *mitogen-activated protein kinase*
MCP-1	proteína quimiotáctica de monocitos, *monocyte chemoattractant protein-1*
miRNAs	microRNAs
NF-κβ	factor de transcripción nuclear kappa beta, *nuclear factor-kappa beta*
nVDR	receptor nuclear de la vitamina D, *nuclear vitamin D receptor*
OMS	organización mundial de la salud
PAI-1	inhibidor 1 del activador de plasminógeno, *plasminogen activator inhibitor type 1*
PBS	tampón fosfato salino, *phosphate buffered saline*
PCR	reacción en cadena de la polimerasa, *polymerase chain reaction*
PPAR	receptor activado por la proliferación de los peroxisomas, *peroxisome proliferator-activated receptor*
PTH	hormona paratiroidea, *parathyroid hormone*
PUFAs	ácidos grasos poliinsaturados, *polyunsaturated fatty acids*
RARα	receptor del ácido retinoico alfa, *retinoic acid receptor alpha*
RBP4	proteína fijadora del retinol 4, *retinol binding protein 4*
R-QUICKI	índice cuantitativo revisado de sensibilidad a la insulina, *revised quantitative insuline sensitivity index*
RXR	receptor X de retinoides, *retinoid X receptor*
Scd1	estearoil coenzima A desaturasa 1, *stearoyl-coenzyme A desaturase 1*
SREBP-1c	proteína de unión al elemento de respuesta a los esteroles 1c, *sterol regulatory element binding protein 1c*
SVF	fracción estromal-vascular, *stromal vascular fraction*
TAB	tejido adiposo blanco
TAG	triacilgliceroles
TAM	tejido adiposo marrón
TNFα	factor de necrosis tumoral alfa, *tumor necrosis factor alpha*
Ucp	proteína desacopladora, *uncoupling protein*
VDR	receptor de la vitamina D, *vitamin D receptor*

Tesis Doctoral, Pilar Parra Moyà

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RESUMEN

Determinados nutrientes pueden desempeñar un papel importante en la regulación del balance energético y en consecuencia, pueden integrarse en intervenciones nutricionales dirigidas a prevenir, mitigar y/o tratar la obesidad. El objetivo de la presente tesis ha sido profundizar en el conocimiento de los efectos de dos nutrientes, el calcio y el CLA, sobre la acumulación de grasa corporal y diversos aspectos del metabolismo energético.

Los resultados obtenidos muestran el efecto potencialmente beneficioso del enriquecimiento de la dieta en calcio sobre el peso corporal y la adiposidad: por una parte, contribuye a reducir el incremento de peso y la deposición de grasa en ratones alimentados con una dieta hiperlipídica y por otra, acelera la pérdida de peso cuando pasan a ser alimentados con una dieta normolipídica. No se observa un incremento en los niveles de proteína o mensajero de la Ucp1 en el tejido adiposo marrón lo que sugiere que los efectos antiobesidad del calcio deben estar mediados por mecanismos diferentes al incremento de la termogénesis adaptativa en este tejido. Por otra parte, la suplementación con calcio de fuente láctea trae aparejado un aumento de magnesio y zinc. La mayor biodisponibilidad de dichos minerales repercute en su mayor excreción en orina, sin repercutir negativamente en su retención ósea.

La reducción de adiposidad producida por el CLA puede acompañarse en el ratón, una especie particularmente sensible, de efectos adversos (esteatosis hepática, hiperinsulinemia y/o resistencia a la insulina) al utilizar dosis altas de CLA o exclusivamente el isómero *trans*-10, *cis*-12. Nuestros resultados indican que la utilización de dosis moderadas de la mezcla (50:50, *trans*-10, *cis*-12 y *cis*-9, *trans*-11) reduce la acumulación de grasa en animales alimentados con dieta hiperlipídica o normolipídica, sin promover por ello un estado inflamatorio en el tejido adiposo o una pérdida de la expresión de genes característicos del fenotipo de adipocito maduro. La sensibilidad a la insulina se mantiene y el contenido en triacilgliceroles del hígado no aumenta, en parte debido al equilibrio observado a nivel transcripcional entre la vía de oxidación de ácidos grasos y la vía de síntesis *de novo* de triacilgliceroles. El específico y elevado incremento de la expresión de Scd1 en músculo, el cual podría estar vinculado al mantenimiento de la homeostasis energética, conjuntamente con los resultados que indican que la expresión de los miRNAs puede verse modificada por el tratamiento con CLA; crean nuevas expectativas en el estudio de los efectos y mecanismos de acción del CLA.

Doctoral Thesis, Pilar Parra Moyà

Effect of supplementation with calcium and CLA on energy balance, their impact on adiposity and the mechanisms involved

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ABSTRACT

Some nutrients play a role in the regulation of energy balance and, as a result, may be incorporated in nutritional interventions addressed to prevent, mitigate and/or treat obesity. The aim of the current thesis was to gain insight into the knowledge of the effects of two nutrients, calcium and CLA, on body fat deposition and energy metabolism.

The present results demonstrate the potential beneficial effects of dietary calcium supplementation on body weight and adiposity: it contributes to reduced weight gain and fat deposition in mice fed a high-fat diet, and accelerates weight loss in overweight mice under normal-fat diet feeding. Increased Ucp1 protein or transcript levels are not observed in brown adipose tissue, which suggest that the anti-obesity effect of dietary calcium must be mediated by a mechanism different from the induction of adaptive thermogenesis in this tissue. Supplementation of diet with calcium from dairy sources entails an increase in the intake of dietary magnesium and zinc. The higher bioavailability of these minerals increases their urine excretion without negatively affecting their bone accretion.

Reduction of adiposity, induced by the use of high doses of CLA or the *trans*-10, *cis*-12 isomer alone, may be accompanied in mice, the most sensitive species, by adverse effects (hepatic steatosis, hyperinsulinaemia and/or insulin resistance). Our results indicate that the use of moderate doses of CLA mixture (50:50, *trans*-10, *cis*-12 y *cis*-9, *trans*-11) reduces fat deposition in mice fed a normal- or high-fat diet, without promoting an inflammatory state in adipose tissue or reducing mature adipocyte marker gene expression. Insulin sensitivity is preserved, as is triacylglycerol hepatic content, which remains unaltered, partly due to the balance observed at transcriptional level between oxidative and lipogenesis pathways. The specific, high increase in muscle Scd1 expression, which could be related to the maintenance of energy homeostasis, together with the results that indicate that miRNAs expression may be modulated by CLA treatment, engender novel perspectives in the research of CLA effects and mechanisms of action.

LISTADO DE PUBLICACIONES / LIST OF PUBLICATIONS

El trabajo realizado en la presente tesis ha dado lugar a la preparación de los siguientes manuscritos:

I. **Parra P**, Bruni G, Palou A, Serra F. *Dietary calcium attenuation of body fat gain during high-fat feeding in mice.* J Nutr Biochem, Vol. 19(2): 109-117 (2008).

II. Perez-Gallardo L, Gomez M, **Parra P**, Sanchez J, Palou A, Serra F. *Effect of calcium-enriched high-fat diet on calcium, magnesium and zinc retention in mice*. Br J Nutr, Vol. 101(10): 1463-6 (2009). doi:10.1017/S0007114508102446

III. **Parra P**, Serra F, Palou A. *Moderate doses of conjugated linoleic acid isomers mix contribute to lowering body fat content maintaining insulin sensitivity and a noninflammatory pattern in adipose tissue in mice*. J Nutr Biochem, Vol. 21: 107-115 (2010). doi:10.1016/j.jnutbio.2008.10.010.

IV. **Parra P**, Palou A, Serra F. *Moderate doses of conjugated linoleic acid reduce fat gain, maintain insulin sensitivity without impairing inflammatory adipose tissue status in mice fed a high-fat diet*. Nutr Metab (Lond), Vol. 7:5 (2010). doi:10.1186/1743-7075-7-5.

V. **Parra P**, Serra F, Palou A. *Functional transcriptional analysis reveals a high impact of conjugated linoleic acid on stearoyl-Coenzyme A desaturase 1 mRNA expression in gastrocnemius muscle*. Manuscrito en vías de publicación.

VI. **Parra P**, Palou A, Serra F. *Loss of fat by moderate doses of conjugated linoleic acid in mice is not accompanied by induction of hepatic lipogenic gene transcription.* Manuscrito en vías de publicación.

VII. **Parra P**, Serra F, Palou A. *Expression of adipose microRNAs is sensitive to dietary conjugated linoleic acid treatment in mice.* Manuscrito en vías de publicación.

I. Introducción

1. EL TEJIDO ADIPOSO

El tejido adiposo es un tejido conjuntivo de origen mesenquimal, mayoritariamente formado por células especializadas en la acumulación de lípidos en su citoplasma: los **adipocitos**. Sin embargo, también se encuentran otros tipos celulares como células precursoras de adipocitos (preadipocitos), pericitos, células pluripotenciales, células del sistema inmune (macrófagos, células dendríticas, mastocitos, linfocitos y granulocitos), fibroblastos y células endoteliales. El conjunto de estas células es lo que conocemos como la **fracción estromal-vascular** del tejido adiposo (SVF).

En mamíferos existen dos tipos de tejido adiposo: el tejido adiposo blanco (TAB) y el adiposo marrón (TAM) (revisado en (Cinti, 2005)). Ambos tejidos tienen capacidad para almacenar lípidos pero presentan claras diferencias morfológicas y funcionales.

1.1. El tejido adiposo marrón

El TAM está formado principalmente por **adipocitos marrones**, células que se caracterizan por almacenar los triacilgliceroles (TAG) en múltiples vacuolas, y por presentar un gran número de mitocondrias responsables del característico color del tejido. Se trata además de un tejido muy vascularizado y con una rica inervación simpática. Su principal función es la de generar calor para el mantenimiento de la temperatura corporal mediante un proceso denominado **termogénesis adaptativa**. La base molecular de la termogénesis es la actividad de la proteína desacopladora 1 (**Ucp1**), presente en la membrana interna mitocondrial de los adipocitos marrones. Esta proteína es capaz de disipar como 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 adenosín trifosfato (ATP) (revisado en (Cannon y Nedergaard, 2004)). Este proceso es vital para el mantenimiento de la temperatura corporal de los animales hibernantes y de los pequeños roedores, como también de los neonatos.

Se han descubierto otras proteínas mitocondriales homólogas a la Ucp1 (Ucp2, Ucp3, Ucp4, Ucp5, Ucp6) (Harper y Gerrits, 2004) que podrían ser mediadoras de la termogénesis adaptativa en los tejidos en que son expresadas.

Además de por el frío, la termogénesis adaptativa puede ser activada por un exceso de ingesta (termogénesis inducida por la dieta) (Rothwell y Stock, 1979), y es por ello que se ha relacionado el TAM con el mantenimiento del balance

energético y el peso corporal. La importancia de la termogénesis adaptativa en el TAM para la protección frente a la obesidad está demostrada en modelos animales (Lowell y col., 1993; Cummings y col., 1996). El incremento de la expresión de la proteína Ucp1 o bien de la actividad termogénica tiene un efecto protector, no obstante la disminución de las mismas no implica necesariamente el desarrollo de obesidad (Enerback y col., 1997). La implicación del TAM en el balance energético en humanos es menos evidente debido a que se encuentra únicamente presente en neonatos y su presencia va disminuyendo en la etapa adulta, quedando sólo vestigios de adipocitos marrones inmersos en el TAB (Oberkofler y col., 1997). Sin embargo, nuevos datos indican que el TAM podría estar presente y activo en humanos adultos (revisado en (Farmer, 2009)).

1.2. El tejido adiposo blanco

El TAB distribuido en múltiples depósitos corporales representa la principal reserva de energía a largo plazo. Se encarga de almacenar la energía sobrante en forma de TAG y de hidrolizarlos cuando se requiere energía, para obtener ácidos grasos libres (AGL) y glicerol que son liberados al torrente sanguíneo para que puedan ser utilizados por el hígado, el músculo y el TAM para su oxidación. Los **adipocitos blancos** a diferencia de los adipocitos marrones acumulan los lípidos en una única gota que ocupa la mayor parte del citoplasma.

1.3. El tejido adiposo como órgano endocrino

Para participar en el control de la homeostasis energética, el TAB expresa diferentes receptores que le permiten responder tanto a las señales hormonales, como a las del sistema nervioso autónomo. Asimismo, el TAB expresa y secreta una gran variedad de proteínas bioactivas denominadas en su conjunto **adipocitoquinas**. Las adipocitoquinas junto con otros factores no proteicos (AGL, corticosteroides, hormonas esteroides sexuales, monobutirina, prostaglandina E_2, prostaciclina, etc.) pueden actuar localmente de una forma **autocrina** y/o **paracrina**, o bien, de forma **endocrina** tener una acción a nivel sistémico (***Tabla 1***). Esta acción endocrina le permite al TAB repercutir sobre diferentes procesos biológicos relacionados con la homeostasis energética (metabolismo lipídico y de carbohidratos, control de la ingesta, termogénesis), la reproducción, la función inmune o la angiogénesis, a la vez que le permite interactuar con el resto de tejidos y órganos pudiendo influir sobre su funcionamiento (Prins, 2002).

REMODELACIÓN DE LA MATRIZ EXTRACELULAR	METBOLISMO LIPÍDICO	ANGIOGÉNESIS
Colágeno	Proteína fijadora de retinol 4 (RBP4)	Factor de crecimiento del endotelio vascular
Inhibidor 1 del activador de plasminógeno 1 (PAI-1)	Proteína transportadora de ésteres de colesterol	Monobutirina
Metaloproteinasas -2 y -9	Lipoproteína lipasa	Leptina
Inhibidor de metaloproteinasas -1 y -2	Autotaxin (fosfolipasa D)	Angiopoietinas
	Perilipina	Angiotensinógeno
	Proteína estimulante de la acilación	Apelina
	Leptina	PAI-1
		Prostaciclina
		Prostaglandina E_2

METABOLISMO ENERGÉTICO	FUNCIÓN INMUNE	
Leptina		
Adiponectina	TNFα	Lipocalina 24p3
Resistina	Factores del complemento	Glicoproteína ácida alfa 1
IL-6	Pentraxina 3	Metalotioneína
RBP4	Proteína C reactiva	Interleucinas (IL-6, IL-8, IL-1, …)
Galanina	Adipsina	Haptoglobina
Omentina	Factor amiloide sérico A3	
Vaspina	Proteína quimiotáctica de monocitos 1 (MCP-1)	
Ácidos grasos libres	Molécula de adhesión intracelular 1 (ICAM-1)	

Tabla 1. **El tejido adiposo como órgano endocrino**. Las adipocitoquinas conjuntamente con factores no proteicos pueden intervenir en una gran variedad de procesos biológicos.

Algunas de las adipocitoquinas más estudiadas debido a su relación con el metabolismo energético y la obesidad son la leptina, la adiponectina, la interleucina 6 (IL-6) y el factor de necrosis tumoral alfa (TNFα).

La **leptina** circula en sangre a concentración bastante proporcional al tamaño de las reservas grasas y tiene un papel importante en la regulación del balance energético, inhibiendo la ingesta e incrementando el gasto energético (Zhang y col., 1994). La leptina incrementa la oxidación hepática y muscular de ácidos grasos y la lipólisis en el músculo y TAB (revisado en (Muoio y Lynis Dohm, 2002)).

La **adiponectina**, secretada en gran cantidad por el TAB, circula en plasma en diferentes isoformas producidas por modificaciones post-traduccionales. Aunque su papel fisiológico no está totalmente definido, parece desempeñar un papel en la prevención de la resistencia a la insulina y de la aterosclerosis, además de tener propiedades anti-inflamatorias. La adiponectina mejora la sensibilidad a la insulina mediante la inhibición de la producción hepática de glucosa, la estimulación de la captación de glucosa por parte del músculo y mediante una mayor oxidación de los AGL tanto en el hígado como en el músculo (revisado en (Kadowaki y Yamauchi, 2005)).

La **IL-6** es una citoquina multifuncional producida por diferentes tipos celulares, siendo el tejido adiposo una fuente importante. Sus niveles circulantes y su producción en el TAB se incrementan en el estado obeso y se consideran marcadores de la resistencia a la insulina (Kern y col., 2001; Vozarova y col., 2001). Elevados niveles circulantes de esta citoquina pro-inflamatoria se asocian con un mayor riesgo de diabetes tipo 2, independientemente del peso corporal (Wannamethee y col., 2007). En el TAB, la IL-6 inhibe la actividad de la lipoproteína lipasa (Lpl), promueve la lipólisis y estimula la captación basal de glucosa. La IL-6 es producida también por el hipotálamo y parece intervenir en la regulación central de la ingesta y el gasto energético (Wallenius y col., 2002).

Los niveles circulantes de la citoquina pro-inflamatoria **TNFα** y su expresión en el TAB se ven aumentados en la obesidad tanto en modelos animales como en humanos y se correlacionan de forma positiva con la resistencia a la insulina. Ejerce además un papel importante en el control del metabolismo del adipocito mediante una acción paracrina y/o autocrina. Se han sugerido varios mecanismos por los cuales el TNFα podría regular el tamaño de los tejidos adiposos: promoción de la apoptosis de preadipocitos y adipocitos, inhibición de la adipogénesis y la lipogénesis, así como activación de la lipólisis (revisado en (Warne, 2003)). El TNFα induce también cambios en la expresión génica de los adipocitos, alterando la expresión de genes tales como la adiponectina (Ruan y col., 2002b).

2. LA OBESIDAD

El desarrollo económico producido durante las últimas décadas ha ido acompañado de cambios en el patrón de alimentación y en el estilo de vida, los cuales han contribuido a la creciente prevalencia de obesidad hasta alcanzar dimensiones globales.

En 2005 había en todo el mundo aproximadamente 1600 millones de adultos con sobrepeso (índice de masa corporal, IMC ≥ 25) y al menos 400 millones de obesos (IMC ≥ 30) según datos de la Organización Mundial de la Salud (OMS), la cual prevé un incremento en 2015, en el que se alcanzarán los 2300 millones de adultos con sobrepeso y más de 700 millones con obesidad (OMS, 2006). En España más de un 7% de la población adulta presentaba una obesidad establecida en 1987. Según los últimos datos de la Encuesta Nacional de Salud (Ministerio de Sanidad y Política Social, 2006) el porcentaje de personas con obesidad se ha incrementado, representando más del 15% de la población adulta mientras que el sobrepeso alcanza ya el 37%. Resulta especialmente alarmante la creciente tendencia también observada entre los menores donde el sobrepeso alcanza más del 18% y la

obesidad prácticamente el 9% de la población infantil (Ministerio de Sanidad y Política Social, 2006).

La obesidad es una enfermedad compleja de **origen multifactorial** cuyo desarrollo viene dado por la interacción entre el **genotipo** y el **ambiente**. Se han descrito algunos casos de obesidad monogénica en humanos en los que una mutación en un único gen puede ser responsable de la obesidad del sujeto, como sucede con los genes de la leptina (Farooqi y col., 1999) y de su receptor (Clement y col., 1998), la proopiomelanocortina (Miraglia del Giudice y col., 2001) y el receptor de melanocortina 4 (Farooqi y col., 2003). Sin embargo, en la mayoría de los casos, la etiología de la obesidad es de **origen poligénico** y es por ello que entonces se habla de susceptibilidad o predisposición genética. Considerando que es poco probable que se hayan producido grandes cambios en el material genético en los últimos años, la elevada prevalencia de obesidad y sobrepeso se explica por una combinación de la susceptibilidad genética individual con una mayor disponibilidad de más alimentos y más energéticos, junto con una disminución de la actividad física asociada al estilo de vida moderna.

La obesidad lleva asociado un aumento del riesgo a desarrollar otras enfermedades crónicas como la diabetes tipo 2, enfermedades coronarias, una mayor incidencia de determinados tipos de cáncer, complicaciones respiratorias y osteoartritis (Kopelman, 2000); lo que la convierten en una de las principales causas de mortalidad prematura y evitable. Se hace así necesaria una mayor comprensión de los procesos asociados a la obesidad y de los cambios producidos a nivel del tejido adiposo que promueven este estado patológico. Igualmente se hace necesaria la búsqueda de nuevas estrategias dirigidas a prevenir el desarrollo de obesidad y favorecer la pérdida moderada de peso (5-10%), la cual se ha visto puede ayuda a reducir, por ejemplo, los factores de riesgo cardiovascular (Van Gaal y col., 1997).

2.1. Obesidad e inflamación

En el estado obeso se han observado elevados niveles circulantes de marcadores inflamatorios que se reducen y/o normalizan con la pérdida de peso (Bastard y col., 2000; Esposito y col., 2002; Cancello y col., 2005). Por ello, la obesidad se ha asociado a un proceso inflamatorio crónico en el cual el tejido adiposo, como órgano endocrino, parece ser el principal responsable, si bien, otros tejidos metabolitamente claves pueden estar igualmente implicados.

El tejido adiposo en el estado obeso se caracteriza por una mayor expresión de adipocitoquinas y factores pro-inflamatorios, a la que parecen contribuir mayoritariamente las células de la SVF y en especial los macrófagos, cuya presencia en el tejido adiposo se encuentra aumentada (Weisberg y col., 2003; Xu y col., 2003). Algunos de los cambios a los que se encuentra sujeto el tejido adiposo durante su expansión parecen contribuir a la llegada e infiltración de dichos macrófagos (Surmi y Hasty, 2008):

a) la **hiperleptinemia**, así como el **daño endotelial** sufrido por el cambio de tamaño del tejido y la exposición a citoquinas inflamatorias y a especies reactivas de oxígeno derivadas de un mayor ambiente lipolítico, promueven en las células endoteliales la producción de moléculas como ICAM-1 o MCP-1 que favorecen la adhesión y la entrada de los monocitos en el tejido. La leptina podría actuar además directamente como quimioatrayente de monocitos (Curat y col., 2004; Gruen y col., 2007);

b) 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 que a su vez, atraen la llegada de células fagocitarias (macrófagos y neutrófilos) (Ye y col., 2007);

c) puede darse una **transdiferenciación de preadipocitos a macrófagos**. Los preadipocitos comparten con las células inmunes su capacidad para activar la vía alternativa del complemento, producir citoquinas pro-inflamatorias y fagocitar (Cousin y col., 1999; Charriere y col., 2003). Paralelamente, los macrófagos expresan genes que codifican para factores de transcripción o transportadores de ácidos grasos característicos de los adipocitos;

d) se produce un **cambio en el fenotipo de los macrófagos** presentes en el tejido adiposo. En el tejido adiposo de sujetos normopeso predominan los macrófagos de tipo M2 –activación alternativa- los cuales secretan citoquinas antiinflamatorias como la IL-10 o IL-1. Sin embargo, en el estado obeso se produce un cambio hacia macrófagos de tipo M1 -activación clásica- productores de factores inflamatorios como TNFα, IL-6, IL-12 y una mayor actividad de la sintasa de óxido nítrico inducible (iNOS) (Lumeng y col., 2007).

No se conoce con exactitud si la infiltración de macrófagos en el tejido adiposo es la causa y/o la consecuencia del estado de inflamación, sin embargo, se tienen evidencias de que la comunicación paracrina que se establece entre adipocitos y macrófagos estaría promoviendo el inicio y la perpetuación del estado leve de

inflamación que caracteriza a la obesidad. Los macrófagos cuentan con receptores de membrana, los cuales en respuesta a una mayor presencia de AGL y de adipocitoquinas inflamatorias activan vías de señalización como las del factor de transcripción nuclear kappa beta (NF-κβ) que promueven la secreción de factores inflamatorios, principalmente de TNFα (Suganami y col., 2005; Suganami y col., 2007). Estos factores inflamatorios se unen a sus respectivos receptores en la membrana de los adipocitos desencadenando la activación de diferentes proteínas quinasas activadas por mitógenos (MAPK), así como del NF-κβ, 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 (Ruan y col., 2002a; Permana y col., 2006).

La producción anormal de adipocitoquinas y el incremento de factores inflamatorios en el tejido adiposo, observados tanto con un exceso de grasa corporal (obesidad) como con una marcada reducción de la misma (lipodistrofia) (Yamauchi y col., 2001; Sevastianova y col., 2008), se considera que contribuyen a la menor sensibilidad a la insulina observada en ambos casos, en los que además la funcionalidad del tejido adiposo se ve comprometida (Garg, 2006). La respuesta inflamatoria resulta en general en un deterioro de la homeostasis metabólica y del metabolismo de la glucosa en particular y, en el estado obeso se asocia a su vez, a un aumento del riesgo a desarrollar resistencia a la insulina, diabetes y/o enfermedades cardiovasculares. La infiltración de macrófagos y la producción de factores de inflamación en el tejido adiposo parecen preceder el desarrollo de la resistencia a la insulina en modelos animales (Xu y col., 2003).

3. IMPORTANCIA DE LAS INTERACCIONES GEN-NUTRIENTE EN EL CONTROL DEL BALANCE ENERGÉTICO

Los alimentos más allá de constituir una fuente de energía y aportarnos toda una serie de nutrientes esenciales (vitaminas y minerales), además de elementos estructurales, nos ofrecen numerosas moléculas bioactivas capaces de alterar la expresión y/o estructura de nuestro genoma.

Los nutrientes pueden alterar la expresión génica directa o indirectamente. A nivel celular, los nutrientes pueden actuar como ligandos de factores de transcripción; ser metabolizados por rutas metabólicas primarias o secundarias, alterando de ese modo las concentraciones de substratos o intermediarios; o influir positiva o negativamente sobre las rutas de señalización (Kaput y Rodriguez, 2004). Determinados nutrientes pueden producir también una remodelación

cromosómica con consecuencias a corto y largo plazo sobre la expresión génica (Waterland y Jirtle, 2004).

El desequilibrio energético crónico que conduce al estado obeso es fruto de alteraciones o deficiencias en procesos bioquímicos que afectan al control de la ingesta, la eficiencia energética, la adipogénesis y/o la partición de nutrientes (Palou y col., 2000). Del mismo modo que la susceptibilidad genética individual a través de genes específicos puede influir sobre estos procesos, se ha demostrado que los nutrientes también tienen un papel regulador importante (revisado en (Palou y col., 2004)):

Regulación de la ingesta. Los macronutrientes afectan de forma diferencial a la sensación de hambre. Algunos aminoácidos (fenilalanina, triptófano) y péptidos (dipéptido fenilalanina-aspartato, macropéptido de caseína) tienen un efecto saciante por sí mismos actuando a nivel del tracto gastrointestinal, siendo algunos además precursores de neurotransmisores implicados en el control central de la ingesta (Rogers y Blundell, 1994). Los carbohidratos por su parte pueden interaccionar con receptores específicos del intestino delgado estimulando la secreción de péptidos saciantes y el retraso del vaciamiento gástrico y del tránsito intestinal (Feinle y col., 2002).

Eficiencia energética. Resulta particularmente interesante el control de la eficiencia energética mediante cambios en la termogénesis (termogénesis adaptativa). Se ha demostrado en experimentación animal y modelos *in vitro* que ciertos nutrientes como el ácido retinoico, el beta-caroteno y los ácidos grasos poliinsaturados (PUFAs) pueden incrementar la expresión y/o actividad de las proteínas desacopladoras, lo que permite la disipación de la energía en forma de calor (Sadurskis y col., 1995; Puigserver y col., 1996; Serra y col., 1999; Felipe y col., 2003).

Partición de nutrientes. Algunos compuestos promueven la canalización de los AGL hacía tejidos con mayor capacidad oxidativa como el hígado o el músculo, en detrimento de su canalización hacia el TAB donde serían preferentemente almacenados. Muchos de estos efectos parecen estar mediados a través de la regulación por nutrientes de factores de transcripción clave como la proteína de unión al elemento de respuesta a carbohidratos (ChREBP), la proteína de unión al elemento de respuesta a los esteroles (SREBP-1) o los receptores activados por la proliferación de los peroxisomas alfa (PPARα) (Jump, 2008).

Control nutricional de la adipogénesis. Algunos nutrientes como el ácido retinoico pueden tener un efecto anti-adipogénico que se explica, principalmente, por el bloqueo de la actividad de ciertos factores de transcripción adipogénicos importantes en el proceso de diferenciación de los preadipocitos (revisado en (Bonet y col., 2003)). Además, resulta especialmente interesante la capacidad potencial del ácido retinoico para remodelar el TAB hacia la adquisición de características propias del TAM, en particular la adquisición de una mayor capacidad oxidativa mediante el aumento de la expresión de enzimas oxidativas y proteínas desacopladoras (Mercader y col., 2006).

4. GENÓMICA NUTRICIONAL

Los avances tecnológicos alcanzados por la genómica funcional (transcriptómica, proteómica, metabolómica) han contribuido a la transición de una nutrición clásica hacia una nutrición molecular, con la aparición de una nueva disciplina conocida como **genómica nutricional** la cual presenta dos vertientes: la nutrigenómica y la nutrigenética. La **nutrigenómica** pretende proporcionar un conocimiento molecular sobre los componentes de la dieta que contribuyen a la salud mediante la regulación de la expresión génica. La **nutrigenética** estudia la respuesta de los individuos a la dieta en función de sus diferencias en el genoma, y como las variaciones genéticas influyen en la interacción entre dieta y enfermedad. Las variaciones genéticas individuales pueden alterar el modo en que los nutrientes son asimilados, metabolizados o almacenados por el organismo (Muller y Kersten, 2003).

En su conjunto, la genómica nutricional ofrece la posibilidad de personalizar la nutrición de acuerdo con la constitución genética del individuo, teniendo en cuenta para ello el conocimiento de las variantes genéticas que afectan al metabolismo de los nutrientes y a las dianas de los nutrientes. De este modo se pretende conseguir una intervención dietética óptima para prevenir, mitigar o curar enfermedades crónicas como la obesidad.

5. EL CALCIO

El calcio es un micronutriente de gran versatilidad metabólica. Además de su importante papel estructural como principal componente de los huesos, el calcio, en su forma ionizada (Ca^{2+}) actúa como segundo mensajero participando en procesos tan diversos como, por ejemplo, la contracción muscular, la neurotransmisión, la secreción de enzimas y hormonas, el control del crecimiento, la proliferación y la muerte celular, así como la regulación de la expresión de algunos genes.

La ingesta diaria recomendada de calcio, necesaria para cubrir las necesidades fisiológicas, varía en función de la edad y del estado fisiológico (**Tabla 2**).

Ingesta adecuada de calcio	
Edad	**mg/día**
0 – 6 meses	210
7 – 12 meses	270
1 - 3 años	500
4 - 8 años	800
9 – 18 años	1300
19 – 50 años	1000
50 – 70 años	1200
embarazo	1000
lactancia	1000

Tabla 2. Ingesta adecuada de calcio en función de la edad. (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997)

5.1. Efecto del calcio sobre el peso y la grasa corporal en humanos

El interés científico suscitado por el calcio como potencial nutriente modulador del peso corporal, tiene su origen en estudios poblacionales en los que se observa una relación positiva entre una ingesta deficiente en calcio y un mayor IMC (McCarron y col., 1984; Zemel y col., 2000).

Estudios de tipo transversal y **longitudinal** permitieron confirmar las observaciones iniciales. En la mayoría de estos estudios, aunque no en todos ellos, se señala una asociación negativa entre el peso y la grasa corporal y el consumo de lácteos y/o calcio (revisado en (Barba y Russo, 2006; Van Loan, 2009)). Esta relación entre la ingesta de calcio y la composición corporal se ha descrito para poblaciones de diferente nacionalidad, raza, grupos de edad, así como para ambos sexos. De los estudios epidemiológicos se desprende que la ingesta de calcio podría explicar el 3% de la variabilidad del peso corporal (Davies y col., 2000). La reducción de peso que se consigue es pequeña *a priori*, pero a largo plazo puede tener una repercusión positiva sobre el mantenimiento del peso corporal. No obstante, debido a la naturaleza de este tipo de estudios no se puede descartar que la asociación encontrada sea debida, por ejemplo, a la convergencia de un elevado consumo de lácteos con unos hábitos alimentarios y/o estilo de vida más saludables.

De hecho, los **estudios de intervención**, más apropiados a la hora de valorar el impacto de las dietas ricas en calcio, no parecen apoyar, en su mayoría, un efecto positivo del consumo de lácteos y/o calcio sobre la composición corporal, tal como se concluye en diferentes revisiones y metanálisis de los estudios realizados tanto en menores (Huang y McCrory, 2005; Winzenberg y col., 2007) como en adultos (Barr, 2003; Trowman y col., 2006; Lanou y Barnard, 2008). Los resultados obtenidos de los estudios de intervención son, en general, inconsistentes y en algunos casos contradictorios, observándose un aumento de peso en los grupos con mayor ingesta de calcio (Barr y col., 2000). No obstante, la mayoría de estos ensayos fueron realizados con otros fines, generalmente el de investigar el efecto del incremento del consumo de calcio sobre la masa ósea, y fueron posteriormente reanalizados en cuanto al papel del calcio sobre el peso y la adiposidad; lo que dificulta y limita las conclusiones derivadas en este sentido. Además se ha señalado que algunos factores como el IMC y la ingesta de calcio habitual de los participantes en estos estudios, puede condicionar la respuesta al incremento de calcio en la dieta. Así, en algunos ensayos clínicos en los que no se ha observado el efecto de la suplementación con calcio de dietas hipocalóricas, se habían realizado en sujetos que habitualmente ingerían cantidades moderadas de calcio, superiores a los 600 mg diarios (Shapses y col., 2004; Bowen y col., 2005).

El calcio puede tener un impacto no sólo sobre el peso corporal, sino también sobre otros aspectos metabólicos. Los estudios poblaciones señalan que un elevado consumo de lácteos y/o de calcio puede tener efectos beneficiosos sobre la hipertensión (McCarron y col., 1982; McCarron y col., 1984; Zemel y col., 2005),

los niveles de colesterol y el perfil lipídico (Denke y col., 1993; Jacqmain y col., 2003; Major y col., 2007), además de un papel protector frente al desarrollo de diabetes tipo 2, así como de enfermedades cardiovasculares (Pereira y col., 2002). En una revisión realizada sobre patrones dietarios y riesgo a presentar síndrome metabólico se señala que, a pesar de algunas inconsistencias sobre el papel protector frente a la obesidad, una elevada ingesta de lácteos se asocia generalmente con un menor riesgo a presentar componentes del síndrome metabólico (Baxter y col., 2006). En un estudio reciente acerca de la relación entre dieta y síndrome metabólico entre los participantes del estudio ARIC (Artheriosclerosis Risk in Communities), los autores destacan que el consumo de carnes y frituras promueve el desarrollo del síndrome metabólico mientras que el consumo de lácteos semi- y desnatados muestra un efecto protector (Lutsey y col., 2008).

5.2. Efecto del calcio sobre el peso y la grasa corporal en animales

Las evidencias que se tienen acerca del efecto del calcio sobre la composición corporal en animales provienen, en su mayoría, de estudios realizados por el grupo del profesor Zemel utilizando un modelo de ratón transgénico con sobreexpresión adipocitaria de la proteína agouti. Con este modelo se ha demostrado que el enriquecimiento de la dieta en calcio consigue: a) atenuar la ganancia de peso y la acumulación de grasa que se produce en animales alimentados con una dieta hiperlipídica (Zemel y col., 2000); b) promover durante un periodo de restricción calórica una pérdida más rápida de peso y grasa corporal (Shi y col., 2001); y c) disminuir la recuperación del peso perdido tras una etapa de adelgazamiento (Sun y Zemel, 2004b).

Por otra parte, los efectos beneficiosos del calcio sobre la composición corporal también se han observado en ratas (Papakonstantinou y col., 2003; Bollen y Bai, 2005). No obstante, en un estudio realizado en ratones C57BL/6J y ratas Sprague-Dawley alimentadas con una dieta normolipídica o hiperlipídica el incremento del calcio de la dieta no tiene ningún efecto sobre la ingesta, peso o grasa corporal (Zhang y Tordoff, 2004). De manera que, si bien los resultados derivados del modelo de ratón transgénico parecen respaldar el potencial papel antiobesidad del calcio, las evidencias que se tienen en otros modelos animales son escasas y no siempre señalan dicho efecto.

Los efectos del calcio sobre la composición corporal son más evidentes cuando se ha utilizado calcio proveniente de fuentes lácteas o directamente, dietas con un

aporte elevado de lácteos, tal como se extrae de estudios realizados tanto en animales como en humanos (Shi y col., 2001; Sun y Zemel, 2004b; Zemel y col., 2004). Esto sugiere un efecto debido a la presencia de otros componentes, además del calcio, en dichos alimentos como por ejemplo: péptidos bioactivos, aminoácidos de cadena ramificada o ácido linoleico conjugado (CLA), que pueden afectar al balance energético y, en consecuencia reducir el peso corporal (Teegarden y Zemel, 2003).

Además de los efectos sobre el peso corporal, las dietas ricas en calcio disminuyen la expresión de factores pro-inflamatorios como el TNFα y la IL-6 y promueven la expresión de factores antiinflamatorios como la IL-15 y la adiponectina en el tejido adiposo (Sun y Zemel, 2007), así como, consiguen reducir la producción de especies reactivas de oxígeno en el tejido adiposo subcutáneo y visceral en ratones (Sun y Zemel, 2006). De manera que el calcio, además de su papel modulador del peso corporal, puede tener efectos beneficiosos sobre la inflamación y el estrés oxidativo; factores ambos que contribuyen a la resistencia a la insulina y al síndrome metabólico.

5.3. Mecanismos implicados en el efecto del calcio sobre el peso corporal y la adiposidad

a) Disminución de la absorción de las grasas

En el tracto gastrointestinal el calcio interacciona con las sales biliares y los TAG formando jabones insolubles que limitan la absorción efectiva de grasa. Éste es uno de los mecanismos propuestos tras observar tanto en ratas (Papakonstantinou y col., 2003) como en estudios a corto plazo en humanos (revisado en (Christensen y col., 2009)) un aumento de la grasa excretada en heces en los grupos alimentados con una dieta rica en calcio.

El incremento de la excreción de grasa con las heces es considerado por algunos investigadores el principal mecanismo de acción del calcio (Papakonstantinou y col., 2003). Por el contrario, hay quienes opinan que esta pérdida energética es modesta y que si bien debe contribuir al efecto antiobesidad del calcio, no puede explicar enteramente los cambios en el peso y adiposidad observados (Zemel, 2005; Major y col., 2008).

b) Papel del calcio intracelular y la 1,25(OH)$_2$-vitamina D3

En base a los estudios realizados tanto en ratones como *in vitro*, el grupo del profesor Zemel ha propuesto un mecanismo de acción por el cual el calcio de la dieta, mediante la regulación de los niveles circulantes del calcitriol (1,25(OH)$_2$D$_3$) y la concentración intracelular de calcio en el adipocito, modularía la adiposidad (revisado en (Zemel, 2001; Zemel, 2004)).

Las hormonas calciotrópicas -hormona paratiroidea (PTH) y 1,25(OH)$_2$D$_3$- son las encargadas de la homeostasis del calcio. Ambas hormonas y en especial la 1,25(OH)$_2$D$_3$ promueven un rápido incremento de la concentración de calcio intracelular en los adipocitos humanos (Zemel y col., 2000). En el adipocito, el calcio intracelular actúa como regulador del metabolismo lipídico, por una parte promueve la síntesis *de novo* de TAG mediante el incremento coordinado de la expresión y la actividad de la ácido graso sintasa (Fasn) y por otra inhibe la lipólisis mediante la activación de fosfodiesterasas que reducen los niveles de adenosín monofosfato cíclico (AMPc) y disminuyen la fosforilación de la lipasa sensible a hormona (HSL).

Por consiguiente, la disminución de las hormonas calciotrópicas en respuesta a una dieta rica en calcio evitaría el aumento de la concentración intracelular de calcio en los adipocitos y la acumulación de grasa. Niveles más elevados de PTH y 1,25(OH)$_2$D$_3$ se han observado en personas obesas (Bell y col., 1985) lo que remarca su posible implicación en el balance energético. Al igual que se han observado concentraciones adipocitarias de calcio intracelular más elevadas entre los obesos respecto a los sujetos con normopeso (Draznin y col., 1988). Asimismo, el calcio intracelular se ha implicado en la etiopatogenia de la hipertensión arterial, la resistencia a la insulina y la dislipemia.

Los estudios del grupo del profesor Zemel han demostrado en ratones transgénicos que los niveles intracelulares de calcio en el adipocito se reducen con una dieta rica en calcio en diferentes condiciones: ganancia (Zemel y col., 2000), pérdida (Shi y col., 2001) o recuperación de peso (Sun y Zemel, 2004b). Paralelamente a la disminución de la concentración de calcio intracelular, han observado una disminución de la expresión y de la actividad de la Fasn y una activación de la lipólisis, acorde al modelo de funcionamiento que proponen (Sun y Zemel, 2004b) (***Figura 1***). Sin embargo, estos resultados no se han confirmado en ningún otro modelo animal.

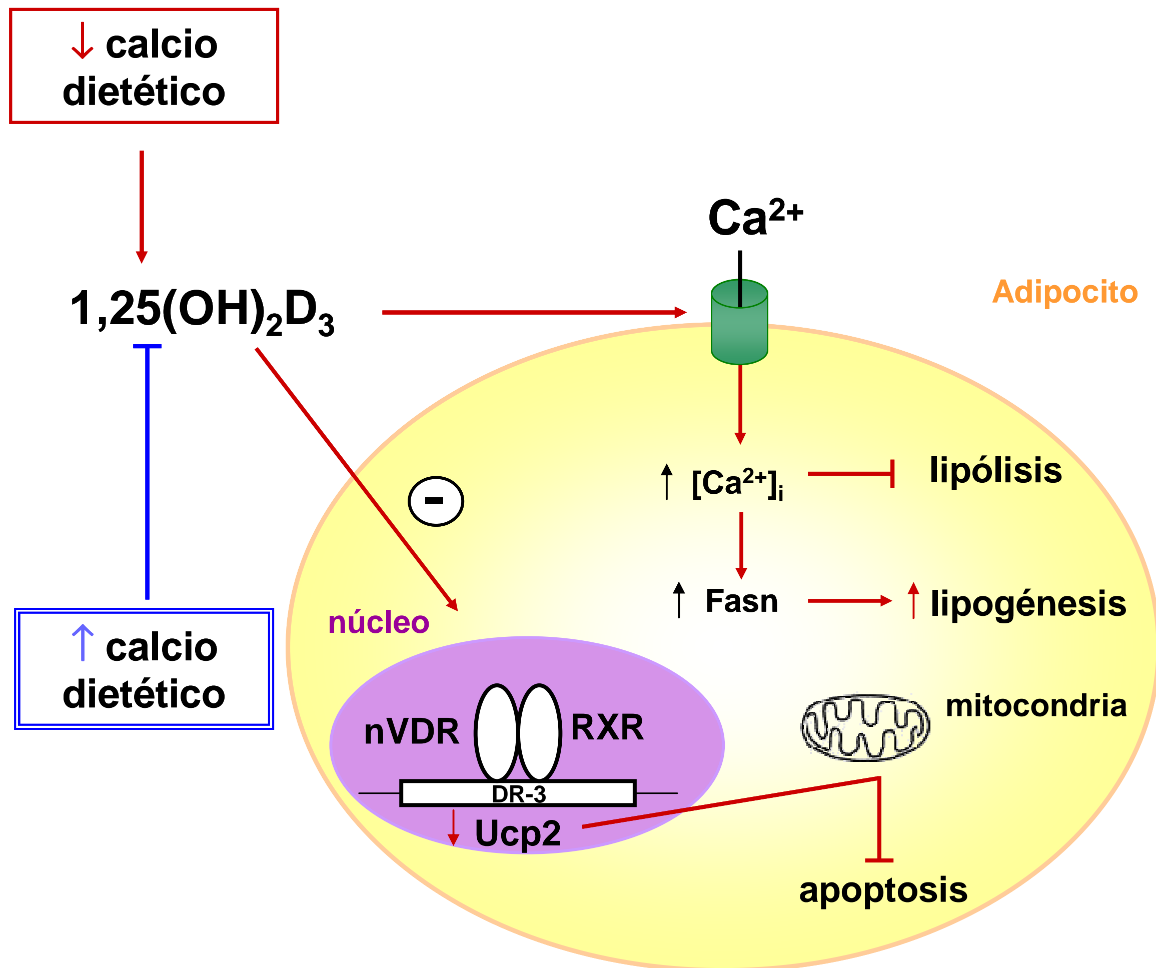

Figura 1. Implicación de la $1,25(OH)_2D_3$ y de la concentración de calcio intracelular en la modulación del tamaño de los adipocitos a través del calcio dietético. $1,25(OH)_2D_3$, 1,25-dihidroxivitamina D_3; $[Ca^{2+}]_i$, concentración de calcio intracelular; nVDR, receptor nuclear de la vitamina D; RXR, receptor X de retinoides; Ucp2, proteína desacopladora 2; Fasn, ácido graso sintasa; DR-3, elemento de respuesta a vitamina D. Adaptada de (Zemel, 2004).

c) Mayor oxidación lipídica

En humanos la ingesta de dietas ricas en calcio se ha relacionado con una mayor tasa de oxidación lipídica con independencia de la duración del estudio o del tipo de régimen alimentario (dietas isocalóricas o hipocalóricas) (Melanson y col., 2003; Gunther y col., 2005; Melanson y col., 2005; Cummings y col., 2006; Teegarden y col., 2008). Sin embargo, esta capacidad para incrementar la oxidación de ácidos grasos no se observa en todos los estudios (Boon y col., 2005; Jacobsen y col., 2005).

El incremento de la oxidación de grasas promovido por la ingesta de calcio podría estar mediada por un incremento de la proteína desacopladora Ucp2. La $1,25(OH)_2D_3$ inhibe la expresión de Ucp2 mediante el receptor nuclear de la vitamina D en adipocitos humanos *in vitro* (Shi y col., 2002). Por el contrario, la disminución de los niveles de $1,25(OH)_2D_3$, producido por el incremento de la ingesta de calcio, resulta en un incremento de la expresión de Ucp2 con un incremento paralelo de la temperatura corporal en ratones transgénicos (Shi y col., 2001). Además de su posible implicación en la termogénesis, la proteína Ucp2 está igualmente implicada en el transporte mitocondrial de ácidos grasos y su oxidación.

d) Apoptosis de los adipocitos

Aunque se tienen pocas evidencias al respecto, la supresión de la $1,25(OH)_2D_3$ podría tener también un papel pro-apoptótico, reduciendo el número de adipocitos capaces de acumular lípidos. Dosis moderadas de $1,25(OH)_2D_3$ inhiben la apoptosis en adipocitos diferenciados 3T3-L1, mientras que la supresión de $1,25(OH)_2D_3$ *in vivo* debido a la ingesta de una dieta rica en calcio tiene un efecto pro-apoptótico en ratones transgénicos (Sun y Zemel, 2004a). El efecto pro-apoptótico podría estar mediado en parte por un aumento de la acción de la Ucp2 que estimularía la apoptosis mediante la inducción del colapso de la mitocondria y la inhibición de la producción de ATP.

6. EL ÁCIDO LINOLEICO CONJUGADO

El CLA (conjugated linoleic acid) es el acrónimo utilizado para hacer referencia a un grupo de ácidos octadecadienoicos (18:2) que son isómeros del ácido linoleico y que se caracterizan por tener dobles enlaces de forma conjugada, en lugar de tenerlos separados por un grupo metileno, como ocurre con el ácido linoleico (***Figura 2***).

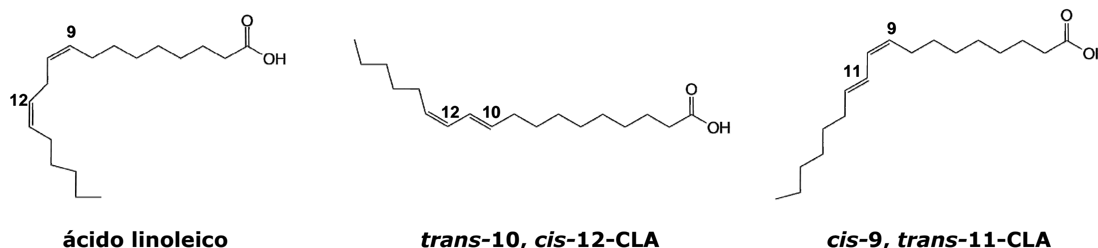

Figura 2. Estructura química del ácido linoleico y de sus isómeros *trans*-10, *cis*-12 y *cis*-9, *trans*-11.

De forma natural los encontramos predominantemente en la carne de rumiantes y en los productos lácteos, en donde pueden representar el 0,5-2% de los ácidos grasos totales. En estos productos, el isómero predominante (alrededor del 70-90%) es el *cis*-9, *trans*-11, también llamado ácido ruménico por sintetizarse en el rumen a partir de la biohidrogenación bacteriana del ácido linoleico. Otros isómeros como el *trans*-7, *cis*-9, el *trans*-9, *cis*-11 y el *trans*-11, *cis*-13 suelen estar también presentes en concentraciones intermedias, mientras que hay más de otros 20 isómeros que pueden encontrarse en pequeñas cantidades.

El CLA ha suscitado un gran interés por sus beneficios potenciales, entre los que destacan: su efecto hipocolesterolémico y antiaterogénico, su acción inmuno-estimulante, la protección que ofrece contra cierto tipo de cánceres, su función antioxidante y en particular en el contexto que nos ocupa, por su efecto sobre la composición corporal (revisado en (Benjamin y Spener, 2009; Churruca y col., 2009)).

El CLA producido para su comercialización y utilizado en complementos dietéticos para humanos es, usualmente, una mezcla relativamente rica (entre el 55 y 85%) en CLA de los dos principales isómeros bioactivos: el *trans*-10, *cis*-12 y el *cis*-9, *trans*-11, ambos presentes en proporciones equivalentes (Gaullier y col., 2002). Estas mismas mezclas, al igual que los isómeros por separado, han sido utilizadas en los estudios realizados tanto en humanos como en animales para investigar el potencial efecto antiobesidad del CLA.

6.1. Efecto del CLA sobre la composición corporal en animales

El efecto antiobesidad del CLA se ha estudiado en diferentes modelos animales, observándose en la mayoría de estudios una disminución en la acumulación de grasa corporal, a pesar de que los resultados varían entre especies. El ratón es la especie más sensible, en ellos se consigue reducir la grasa corporal entre un 50-80% (Park y col., 1997; Tsuboyama-Kasaoka y col., 2000; West y col., 2000; Terpstra y col., 2002; Tsuboyama-Kasaoka y col., 2003), mientras que en otras especies, como la rata, la disminución que se produce es tan sólo del 15-25% (Azain y col., 2000).

En algunos estudios, la reducción de grasa corporal se acompaña de un incremento de la masa magra y/o de un incremento del contenido hídrico (Park y col., 1997; DeLany y col., 1999; Park y col., 1999). Tal vez por ello, la disminución de la adiposidad no se acompaña siempre de una pérdida de peso (Park y col., 1997; Tsuboyama-Kasaoka y col., 2000; West y col., 2000; Yamasaki y col., 2003).

Por lo general, los efectos reductores del CLA sobre la grasa y/o el peso corporal se dan preferentemente en animales en período de crecimiento y al suplementar las dietas con dosis altas de CLA (> 0,5% CLA en la dieta) o mezclas de CLA con una alta concentración del isómero *trans*-10, *cis*-12; el cual parece ser el principal isómero responsable de los efectos del CLA sobre la composición corporal (Park y col., 1999; Ryder y col., 2001).

6.2. Mecanismos de acción implicados en el efecto del CLA sobre la composición corporal en animales

A pesar de no estar totalmente elucidados, se han propuesto diferentes mecanismos, tanto a nivel de todo el organismo como en el tejido adiposo en concreto, por los cuales el CLA podría ejercer su efecto antiadipogénico (revisado en (Kennedy y col., 2009)):

a) Disminución de la ingesta energética y/o incremento del gasto energético

La disminución de la ingesta puede contribuir a la pérdida de peso y/o a la menor acumulación de grasa corporal producidos con la suplementación con CLA (West y col., 1998; Ryder y col., 2001), sin embargo, no se observa en todos los casos (West y col., 2000; Sisk y col., 2001), lo que sugiere que otros mecanismos, como el incremento del gasto energético observado en algunos estudios (West y col., 1998; West y col., 2000; Ohnuki y col., 2001b; Terpstra y col., 2002) deben estar implicados en el efecto antiobesidad del CLA. Se ha sugerido que la activación

de la termogénesis podría ser la causante de un mayor gasto energético, sin embargo no siempre se han observado cambios en la expresión génica de la principal proteína desacopladora -la Ucp1- en el TAM e incluso se ha visto una disminución de la misma (West y col., 2000; Takahashi y col., 2002; Ribot y col., 2007). No obstante, se ha encontrado un incremento en la expresión de otras proteínas desacopladoras como la Ucp3 (Ribot y col., 2007) o la Ucp2 en otros tejidos (Tsuboyama-Kasaoka y col., 2000; Ryder y col., 2001; Takahashi y col., 2002; Tsuboyama-Kasaoka y col., 2003).

b) Inhibición de la proliferación y diferenciación de los preadipocitos

Los estudios *in vitro* con células 3T3-L1 muestran una inhibición en la proliferación y diferenciación de los preadipocitos (Brodie y col., 1999; Evans y col., 2000), que se acentúa al tratar con el isómero *trans*-10, *cis*-12 en lugar de con la mezcla (Evans y col., 2000; Brown y col., 2001). Estos efectos se deben en parte a la inhibición de la expresión de factores de transcripción claves en el proceso de diferenciación de los adipocitos como son el PPARγ y la proteína alfa estimulante de unión a CCAAT (C/EBPα). La disminución de la expresión génica y/o de la actividad de enzimas características del adipocito maduro, e implicadas en procesos tales como la captación de ácidos grasos (Lpl), como en el trasporte de glucosa (transportador de glucosa de tipo 4, (Glut4)), o en la acumulación y síntesis *de novo* de TAG (SREBP-1c, Fasn, acetil coenzima A carboxilasa (Acc), PPARγ) (Park y col., 1997; Tsuboyama-Kasaoka y col., 2000; Takahashi y col., 2002; Brown y col., 2003; Kang y col., 2003) contribuyen a la reducción del tamaño de los adipocitos, así como, a la reducción de su contenido lipídico observado tanto en estudios *in vitro* como *in vivo* (Azain y col., 2000; Evans y col., 2000; Brown y col., 2001; Brown y col., 2003; Brown y col., 2004).

c) Apoptosis de los adipocitos

En algunos estudios, tanto *in vitro* como *in vivo*, se observa un incremento de la apoptosis de los adipocitos. Este efecto podría estar mediado por el incremento de la expresión de TNFα que estimularía la apoptosis de los adipocitos, reduciendo así el tamaño de los depósitos grasos (Evans y col., 2000; Tsuboyama-Kasaoka y col., 2000).

d) Mayor movilización y oxidación de ácidos grasos

La disminución intracelular de TAG y glicerol, así como, el incremento de glicerol en el medio de cultivo sugieren una mayor movilización de los lípidos al tratar con CLA (Park y col., 1997; Park y col., 1999). También se han realizado estudios en 3T3-L1 con ácido oleico marcado radiactivamente observándose una mayor

oxidación de este ácido graso (Evans y col., 2002) al tratar con el isómero *trans*-10, *cis*-12, sin embargo, no se ha observado en preadipocitos humanos (Brown y col., 2003). Un menor coeficiente respiratorio (Ohnuki y col., 2001b) y una mayor expresión y/o actividad de enzimas implicadas en la β-oxidación en diferentes tejidos estarían respaldando la hipótesis de que el CLA contribuye a una mayor oxidación y utilización de los ácidos grasos (Park y col., 1997; Rahman y col., 2001; Takahashi y col., 2003).

6.3. Efectos adversos de la suplementación con CLA en animales

Los efectos adversos asociados a la suplementación con CLA –hiperinsulinemia, resistencia a la insulina y lipotoxicidad- se han observando mayoritariamente en ratones (Poirier y col., 2005a); la especie más sensible a los efectos del CLA. La aparición de estos efectos parece estar muy ligada a la administración de **altas dosis de CLA** (0,5 – 1,5% CLA en la dieta) y en especial, a la utilización del **isómero puro *trans*-10, *cis*-12**. Este tipo de tratamientos produce una rápida y a la vez, drástica pérdida de peso que conducen al animal a un estado de lipodistrofia (Tsuboyama-Kasaoka y col., 2000; Clement y col., 2002; Liu y col., 2007). Por el contrario, con la administración de mezclas de los dos principales isómeros bioactivos en dosis más bajas, se consigue una disminución de la grasa corporal, si bien menos significativa, sin presentarse efectos adversos (Ohnuki y col., 2001a; Tsuboyama-Kasaoka y col., 2003). En especies como la rata, en los que la pérdida de grasa corporal es más leve, se observan incluso efectos beneficiosos como una mayor tolerancia a la glucosa (Houseknecht y col., 1998; Ryder y col., 2001).

Hiperinsulinemia. La hiperinsulinemia observada en algunos estudios animales se debe en parte a una menor retirada y metabolización de la insulina circulante, así como, a la estimulación por parte del CLA de la hiperplasia e hipertrofia de las células beta pancreáticas (Poirier y col., 2005b), aumentándose así la capacidad para producir y secretar insulina.

Resistencia a la insulina. Los niveles de leptina y adiponectina, dos adipocitoquinas importantes en el mantenimiento de la homeostasis de la glucosa, se reducen de forma considerable en el estado de lipodistrofia inducido por el CLA, produciéndose en consecuencia una menor sensibilidad a la insulina. La disminución de sus niveles, tal como se ha observado en ocasiones, puede producirse transcurridos pocos días del inicio del tratamiento y de forma previa a la reducción de la adiposidad, sugiriendo la existencia de otros mecanismos de regulación independientes del tamaño de los depósitos grasos (Poirier y col., 2005b). Poco a

poco, se van teniendo evidencias de que la presencia de algunas moléculas inflamatorias como el TNFα o la IL-6 en el medio de cultivo pueden tener un efecto inhibidor de la expresión de la adiponectina en adipocitos 3T3-L1 (Fasshauer y col., 2002; Fasshauer y col., 2003). Así, el desarrollo de un estado inflamatorio en el seno del tejido adiposo podría contribuir en parte al desarrollo de la resistencia a la insulina producida por la suplementación con CLA. El isómero *trans*-10, *cis*-12 produce, en diferentes modelos *in vitro*, una mayor secreción de moléculas pro-inflamatorias como la IL-6, la IL-8 o el TNFα y una menor sensibilidad a la insulina (Chung y col., 2005). *In vivo*, la administración de dosis altas de la mezcla de CLA o del isómero *trans*-10, *cis*-12 promueve una mayor infiltración de macrófagos en el tejido adiposo y una mayor producción de moléculas pro-inflamatorias (Poirier y col., 2006; Liu y col., 2007). Por el contrario, con el isómero *cis*-9, *trans*-11 se consigue mejorar el perfil inflamatorio del tejido adiposo en el estado obeso (Moloney y col., 2007).

Lipotoxicidad. Se considera que la acumulación ectópica de lípidos puede producirse como mecanismo compensatorio y protector ante la incapacidad que presenta el tejido adiposo para captarlos y almacenarlos. El hígado parece ser el primer destino de estos lípidos no acumulados en el tejido adiposo al tratar con CLA, lo que produce hepatomegalia y esteatosis. A su vez, se observa en el hígado la inducción de genes que se expresan poco en condiciones normales y que están relacionados con la captación de ácidos grasos y la lipogénesis (Clement y col., 2002; Tsuboyama-Kasaoka y col., 2003).

6.4. Efecto del CLA sobre la composición corporal en humanos

El consumo medio de CLA presente en la dieta europea está en torno a los 150-200 mg/día (Ritzenthaler y col., 2001), aunque estos aportes son muy variables y dependen de los hábitos alimentarios y del origen de los alimentos. En la mayoría de estudios en humanos las dosis de CLA utilizadas corresponden a una suplementación de 3-3,4 g de CLA mezcla por día.

El efecto antiobesidad del CLA observado en animales es menos evidente en humanos. Además las diferencias en el diseño experimental -composición en isómeros de las mezclas utilizadas, dosis (de 1 a 6,8 g CLA mezcla/día), duración de la intervención, así como las características de los sujetos (normopesos, con sobrepeso, obesos, con síndrome metabólico)- contribuyen a la disparidad de resultados obtenidos y dificultan el dilucidar la efectividad del CLA sobre la composición corporal. No obstante, en un reciente meta-análisis de 18 estudios en

humanos se señala que el CLA produce una modesta, pero significativa, reducción de la grasa corporal del orden de 0,09 kg/semana que, aunque aparentemente pueda parecer pequeña en un principio, tiene un importante papel preventivo si tenemos en cuenta que la ganancia media de peso al año en un adulto es de 0,4 kg, es decir unos 0,008 kg por semana (Whigham y col., 2007). En este meta-análisis se describe también un efecto sobre la reducción de grasa dependiente de la dosis de CLA, así como un efecto de la duración del tratamiento, observándose una pérdida lineal de grasa durante los 6 primeros meses; si bien los estudios de intervención a más largo plazo son escasos. La reducción de grasa corporal parece producirse preferentemente en mujeres y en determinadas zonas corporales como son las piernas, aunque también hay una tendencia a dismuir la grasa abdominal (Gaullier y col., 2007). El incremento de la masa no grasa con la ingesta de CLA también se ha observado en unos pocos casos (Kamphuis y col., 2003; Gaullier y col., 2007).

Aunque en menor número, también se han realizado estudios para investigar el papel del CLA en el mantenimiento del peso tras un periodo de adelgazamiento sin que se hayan encontrado efectos significativos (Kamphuis y col., 2003; Whigham y col., 2004; Larsen y col., 2006).

A pesar de que en la mayoría de estudios realizados en humanos no se han observado efectos perjudiciales severos, en algunos estudios la aparición de factores de riesgo como el incremento de marcadores de inflamación (proteína C reactiva) y de peroxidación lipídica (isoprostanos), o bien un cambio desfavorable del perfil lipídico (Basu y col., 2000; Riserus y col., 2002b), han despertado algunas dudas sobre la seguridad alimentaria del CLA. Además también se ha descrito un aumento de la resistencia a la insulina especialmente en estudios de corta duración (Moloney y col., 2004) y/o con la administración de un único isómero. Así por ejemplo, se ha observado una disminución de la sensibilidad a la insulina con la administración del isómero *trans*-10, *cis*-12 en sujetos obesos con síndrome metabólico (Riserus y col., 2002a) y con el isómero *cis*-9, *trans*-11 en sujetos obesos (Riserus y col., 2004). En cambio, la mezcla de ambos isómeros no altera la sensibilidad a la insulina en sujetos con síndrome metabólico (Riserus y col., 2002a) e incluso la mejora en individuos sedentarios (Eyjolfson y col., 2004). Sin embargo, son mayoría los estudios en los que no se han observado cambios en los niveles de glucosa o insulina en ayuno, como tampoco en la sensibilidad a la insulina (Whigham y col., 2007).

II. OBJETIVOS / AIMS

Los objetivos de esta tesis se enmarcan dentro de una de las principales líneas de investigación de nuestro grupo: el impacto de determinados nutrientes sobre los procesos reguladores del balance energético y su potencial efecto en la prevención del desarrollo de la obesidad. En esta tesis se ha querido profundizar concretamente en los efectos de dos nutrientes: el calcio (1er objetivo) y el CLA (2º objetivo) sobre la acumulación de grasa corporal y diversos aspectos del metabolismo energético.

Los objetivos concretos planteados en esta tesis han sido los siguientes:

1er objetivo:

- Comprobar el efecto reductor del incremento de calcio dietario sobre la deposición de grasa en ratones alimentados con una dieta hiperlipídica (manuscritos I y II).
- Determinar el efecto del enriquecimiento en calcio de una dieta normolipídica sobre el peso y la grasa corporal en ratones con sobrepeso (manuscrito I).
- Analizar la posible implicación de la termogénesis adaptativa en el efecto del calcio sobre la grasa corporal (manuscritos I y II) y los efectos de una mayor ingesta de calcio sobre la fijación ósea (manuscrito II).

2º objetivo:

- Caracterizar el efecto de la suplementación oral de dosis moderadas de una mezcla equimolar de los isómeros bioactivos del CLA (*trans*-10, *cis*-12 y *cis*-9, *trans*-11) sobre el peso y la grasa corporal, la sensibilidad a la insulina, los niveles séricos de adipocitoquinas y el perfil inflamatorio del tejido adiposo en ratones alimentados con una dieta normolipídica (manuscrito III) o con una dieta hiperlipídica (manuscrito IV).
- Discriminar mediante el análisis transcripcional de genes claves de diferentes rutas metabólicas, las posibles dianas de acción implicadas en el efecto antiobesidad del CLA en los principales tejidos involucrados en la homeostasis energética: músculo (manuscrito V) y tejido adiposo e hígado (manuscrito VI). Analizar la capacidad potencial de la suplementación con CLA en la modulación de la expresión de microRNAs (miRNAs) (manuscrito VII).

El trabajo experimental se ha llevado a cabo en el Laboratorio de Biología Molecular, Nutrición y Biotecnología –Nutrigenómica- de la Universidad de las Islas Baleares, dirigido por el Profesor Andreu Palou. Se ha completado la formación del doctorando en técnicas de biología molecular con una estancia de tres meses en el laboratorio de la Dr. Aldona Dembinska-Kiec en el Departamento de Bioquímica Clínica. The Jagiellonian University, Medical College (Cracovia, Polonia) 2007. Esta estancia fue subvencionada por la red de excelencia europea NuGO (The European Nutriegenomics Organization) y por el Gobierno de España.

The aims of this PhD Thesis are one of our group's lines of research: the study of the impact of nutrients on regulatory processes concerning energy balance and their potential effect in preventing obesity. This Thesis was focused on the effects of two nutrients: calcium (1st aim) and CLA (2nd aim) on body fat accumulation and energy metabolism regulation.

The specific aims of this Thesis were as follows:

1st aim:

- To evaluate the effectiveness of a calcium-enriched diet in reducing fat accumulation in mice receiving a high-fat diet (manuscripts I and II).
- To determine the effect of a calcium-enriched standard-fat diet on body weight and adiposity in overweight mice (manuscript I).
- To assess the potential role of adaptive thermogenesis in the effects of calcium on body fat (manuscripts I and II) and to assess the effects of increased calcium intake on mineral bone accretion (manuscript II).

2nd aim:

- To characterize the effects of an oral supplementation with moderate doses of an equimolar mixture of the bioactive isomers of CLA (*trans*-10, *cis*-12 and *cis*-9, *trans*-11) on weight and body fat, insulin sensitivity, plasma adipokines, and adipose tissue inflammatory profile in mice fed a standard-fat diet (manuscript III) or a high-fat diet (manuscript IV).

- To discriminate by transcriptional analysis of key genes from different metabolic pathways, potential targets involved in the antiobesity effect of CLA in the main tissues involved in energy homeostasis: skeletal muscle (manuscript V), adipose tissue and liver (manuscript VI). To analyze the potential effect of CLA supplementation on modulating microRNAs expression in adipose tissue (manuscript VII).

The experimental work was carried out in the Laboratory of Molecular Biology, Nutrition and Biotechnology -Nutrigenomics- in the University of the Balearic Islands, directed by the Professor Andreu Palou. The doctoral training was complemented with a stay in the laboratory of Dr. Aldona Dembinska-Kiec in the Department of Clinical Biochemistry, The Jagiellonian University Medical College (Krakow, Poland) for three months. This stay was funded by the European Network of Excellence NuGO (The European Nutriegenomics Organization) and the Government of Spain

III. PLANTEAMIENTO EXPERIMENTAL

En este apartado se describe el planteamiento experimental utilizado en el desarrollo de la presente tesis. Las técnicas utilizadas se encuentran detalladas en el apartado VII de Materiales y Métodos.

En los diferentes estudios *in vivo* se han utilizado ratones machos C57BL/6J, de cinco semanas de edad (20-21 g) en el momento de inicio de los diferentes experimentos, suministrados por Charles River (Barcelona). Esta cepa presenta una alta susceptibilidad a la obesidad inducida por la dieta y desarrolla hiperglicemia e hiperinsulinemia con dietas ricas en grasa. Se trata así de una cepa frecuentemente utilizada para el estudio de la enfermedad cardiovascular, la diabetes y la obesidad.

Durante el periodo de experimentación los animales se mantuvieron estabulados a una temperatura de 22ºC y un ciclo de 12 h de luz y 12 h de oscuridad, con libre acceso al agua y a su dieta correspondiente.

Experimento I: Diseño y modelos utilizados en el estudio del calcio

En el estudio del efecto de las dietas enriquecidas en calcio sobre la composición corporal se plantearon dos modelos experimentales en los que se varió el contenido lipídico de la dieta y el contenido en calcio (***Figura 3***).

Se utilizó una dieta hiperlipídica y una dieta normolipídica en las que un 43% y un 12% respectivamente de sus calorías procedían de grasa. Las dietas fueron formuladas por Research Diet (New Brunswick) y su composición se encuentra detallada en el manuscrito I.

Respecto al contenido en calcio, se usaron dos tipos de dieta, una dieta con un contenido de calcio estándar (0,4% en peso) y una dieta enriquecida con un contenido en calcio tres veces superior al de la dieta sin suplementar (1,2% en peso). El calcio con el que se enriqueció las dietas provenía en un 42% de leche en polvo desnatada, ajustándose en cada caso el contenido de caseína para mantener la proporción proteica equivalente entre las diferentes dietas (20% del contenido calórico).

En el primer experimento (**Exp. Ia**), se quiso determinar el efecto del enriquecimiento de la dieta en calcio en condiciones que favorecen la aparición de sobrepeso y/u obesidad. Los animales se agruparon en dos grupos experimentales (n= 6) que fueron alimentados durante dos meses con la dieta hiperlipídica suplementada o no con calcio.

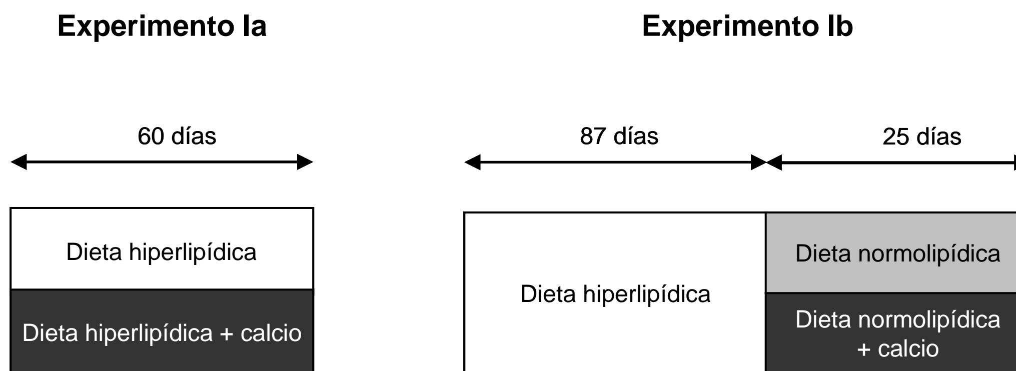

Figura 3. Diseños experimentales utilizados en el estudio del efecto del calcio.

En el segundo experimento (**Exp. Ib**) nos planteamos analizar el efecto del calcio sobre la pérdida de peso. Para ello se alimentaron los animales durante 87 días con la dieta hiperlipídica (no suplementada con calcio) con el fin de que desarrollasen sobrepeso/obesidad. Posteriormente, pasaron a ser alimentados con la dieta normolipídica durante 25 días y fueron asignados a dos grupos experimentales (n= 6) en función de si la dieta estaba o no enriquecida en calcio.

Durante el transcurso de los dos estudios se realizó el seguimiento de la ingesta y del peso de los animales (3 veces por semana). Al finalizar los mismos, los animales fueron sacrificados por decapitación y en condiciones de alimentación al comienzo del periodo de luz. Se procedió a la recogida de los tejidos de interés que fueron pesados y lavados con suero salino que contenía un 0,1% de dietil pirocarbonato para su correcta conservación para el análisis transcriptómico. Finalmente los tejidos se guardaron a -70ºC hasta su posterior análisis.

Para caracterizar el efecto del enriquecimiento del calcio dietario se analizó la expresión de una serie de marcadores relacionados con la obesidad y/o el estatus metabólico en tejido adiposo (blanco y marrón) y muscular. En concreto, se analizaron los niveles de expresión y/o de proteína de proteínas desacopladoras (Ucp1, Ucp2 y Ucp3); receptores nucleares (RXRα, receptor del ácido retinoico alfa (RARα)); marcadores del metabolismo lipídico (C/EBPα, leptina, resistina); del metabolismo del calcio (receptor de la vitamina D (VDR), receptor sensor de calcio (CaSR), estaniocalcina) y de la capacidad transportadora de ácidos grasos a la mitocondria (carnitina palmitoiltransferasa 1b, Cpt1b).

Siguiendo este mismo modelo experimental se realizó un experimento en paralelo en colaboración con el Departamento de Bioquímica, Biología Molecular y Fisiología de la Universidad de Valladolid, en el que adicionalmente se procedió a la recogida, dos veces por semana de la orina que fue guardada a -20ºC para la

posterior valoración de la excreción urinaria de calcio, magnesio y zinc. Finalizado el estudio, también se recogió el fémur para la determinación ósea de estos mismos minerales y se determinó la composición tisular del hígado (contenido proteico, glucógeno, lípidos saturados y concentraciones de colesterol y TAG), así como, la concentración de TAG y grasas saturadas en el tejido adiposo epididimal.

Experimento II: Diseño y modelos utilizados en el estudio del CLA

En el estudio del efecto del CLA sobre la composición corporal se plantearon dos experimentos *in vivo* con diferentes dosis y tiempos de tratamiento así como con dietas con diferente contenido lipídico (***Figura 4***).

Para los tratamientos se utilizó la mezcla comercial de CLA conocida como Tonalin® TG 80 que nos fue proporcionada por Cognis. El Tonalin consiste en una fórmula de TAG con un 80% de CLA que procede de aceite de cártamo y que contiene los dos principales isómeros bioactivos del CLA: *trans*-10, *cis*-12 y *cis*-9, *trans*-11 en cantidades equimolares (50:50).

Respecto a las dietas se utilizó una dieta normolipídica y una dieta hiperlipídica en las que un 10% y un 45% respectivamente de las calorías procedían de la grasa. Ambas dietas fueron adquiridas a Research Diet (New Brunswick) en cuya página web se puede encontrar su composición detallada (referencia de las dietas: dieta normolipídica D12450B[1]; dieta hiperlipídica D12451[2]).

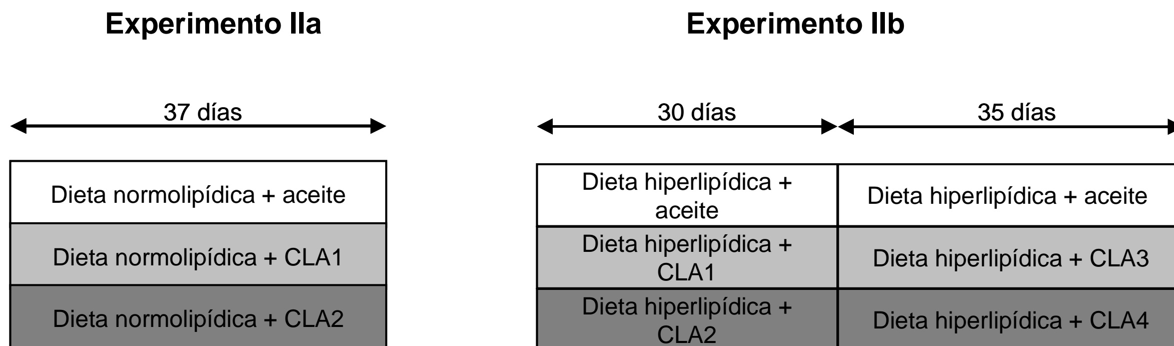

Figura 4. Diseños experimentales utilizados en el estudio del efecto del CLA.

Los grupos CLA1 recibieron diariamente una dosis equivalente a 0,15 g CLA/kg peso corporal y los grupos CLA2 recibieron diariamente 0,5 g CLA/kg peso corporal. La nomenclatura CLA3 y CLA4 indica que se suministró una dosis doble a la inicialmente administrada (CLA1 y CLA2 respectivamente).

[1] http://www.researchdiets.com/pdf/Data%20Sheets/D12450B.pdf
[2] http://www.researchdiets.com/pdf/Data%20Sheets/D12451.pdf

En un primer experimento (**Exp. IIa**) se quiso determinar el efecto del tratamiento con dos dosis diferentes de CLA en animales alimentados con una dieta normolipídica durante 37 días. Los animales se agruparon en tres grupos experimentales (n= 12): el grupo CLA 1 y CLA2 recibieron una dosis oral de CLA correspondiente a 0,15 y 0,5 g CLA/kg animal respectivamente, mientras que el grupo control recibió una dosis calórica equivalente de aceite de girasol.

En el segundo experimento (**Exp. IIb**) se quiso determinar el efecto del tratamiento con dos dosis diferentes de CLA en animales alimentados con una dieta hiperlipídica. Se definieron tres grupos experimentales (grupo control, CLA3 y CLA4) (n= 8). Durante el primer mes se administraron las mismas dosis que se habían utilizado en el experimento IIa, y a partir de ese momento, cada grupo pasó a recibir una dosis doble durante los 35 días restantes del estudio.

Durante el transcurso de ambos experimentos se realizó el seguimiento de la ingesta y del peso de los animales. Después de 30 días de tratamiento se les practicó la prueba de tolerancia a la insulina y se recogió sangre de la cola para determinar la concentración plasmática de glucosa, insulina y adiponectina en el **Exp. IIa** y de glucosa, adiponectina y leptina en el **Exp. IIb**.

Al finalizar los estudios, los animales fueron anestesiados y sacrificados en condiciones de alimentación en el **Exp. IIa**, o bien en condiciones de ayuno (10 h) en el **Exp. IIb**. Se realizó una punción cardiaca para la recogida de sangre y se procedió a la disección y extracción de los tejidos de interés de forma similar a como previamente se describe en el estudio del calcio.

Con el fin de analizar la fracción celular implicada en la respuesta inflamatoria observada en el tejido adiposo con determinados tratamientos con CLA, en muestras de tejido adiposo fresco se procedió a la separación de los adipocitos de las otras células presentes en el tejido. Así, a partir del tejido adiposo epididimal se aislaron los adipocitos maduros y la SVF para la posterior determinación, mediante la reacción en cadena de la polimerasa (PCR), de los niveles de expresión de citoquinas y factores inflamatorios (IL-6, TNFα, iNOS, MCP-1, epidermal growth factor module-containing mucin-like receptor 1 (Emr1)); adipocitoquinas (adiponectina, leptina, RBP4) y genes del metabolismo adipocitario (PPARγ, Glut4) (***Tabla 3***).

Al final del tratamiento se caracterizó el perfil circulatorio, determinándose la concentración de diferentes metabolitos plasmáticos: adipocitoquinas (adiponectina, leptina y resistina), TAG, AGL e insulina, además del glicerol y la glucosa en el **Exp. IIb**. También se determinaron los TAG hepáticos.

	Exp. IIa	Exp. IIb
Adipocitos maduros	adiponectina, leptina, MCP-1, RBP4, Glut4, PPARγ2	adiponectina, leptina, MCP-1
SVF	PPARγ2, IL-6, TNFα, iNOS, Emr1, MCP-1,	IL-6, TNFα, iNOS, Emr1, MCP-1

Tabla 3. Genes estudiados por PCR a tiempo real en cada una de las fracciones aisladas del tejido adiposo epididimal en el Exp. IIa y IIb.

A nivel tisular se caracterizaron los efectos del CLA sobre la expresión de factores de transcripción y enzimas clave implicados en las principales vías metabólicas relacionadas con el mantenimiento de la homeostasis energética (TAB, TAM, hígado y músculo) (***Tabla 4***).

En el tejido adiposo retroperitoneal se analizó también el efecto potencial del tratamiento con CLA sobre la expresión de diferentes miRNAs, los cuales introducen un nuevo nivel de regulación génica en eucariotas. Se seleccionaron cinco miRNAs (miR-143, miR-103, miR-107, miR-221 y miR-222) que se han relacionado recientemente con la diferenciación adipocitaria y la obesidad.

Estudio *in vitro* (Exp. IIc)

Utilizamos el cultivo de células musculares de ratón C2C12 para determinar el efecto del CLA sobre la expresión de dos genes lipogénicos: Fasn y estearoil coenzima A desaturasa 1 (Scd1). Las células C2C12 son una línea celular miogénica murina que se diferencia rápidamente dando lugar a amplios miotubos contráctiles que expresan características propias del músculo esquelético.

Las células C2C12 se diferenciaron a miotubos y se trataron entonces con una concentración 10 μM de una mezcla equimolar de los isómeros de CLA: *trans*-10, *cis*-12 y *cis*-9, *trans*-11 (disueltos en etanol y conjugados con albúmina sérica bovina (BSA)) en ausencia o presencia de insulina (100 nM) durante 24 h en un medio libre de suero. Las células consideradas control se incubaron con una cantidad proporcional de etanol y BSA utilizada en los tratamientos. El experimento se realizó un total de tres veces (n= 3-5 en cada experimento).

	Transporte sustratos	Metabolismo glucídico	Marcadores miogénicos	Lipólisis	Oxidación	Adipogénesis/ lipogénesis
TAB	Glut4, Lpl			Pnpla2, HSL	PPARα, Ucp2, Cpt1a, Cpt1b	C/EBPα, PPARγ, SREBP-1c, Fasn, Scd1
TAM					PPARα, Ucp2, Ucp1, Cpt1a, Cpt1b	SREBP-1c, PPARγ, Fasn, Scd1
HÍGADO					PPARα, PPARδ, Ucp2, Cpt1a, Fgf21, Acox1	PPARγ, SREBP-1c, Acc1, Fasn, Scd1
MÚSCULO	Cd36, Lpl, Glut4	Pdk4, Hexoquinasa 2	MyHC-b, MyHC-IIa, MyHC-IIb, MyHC-IIx/d		PPARα, PPARδ, ERRα, PGC-1α, PGC-1β Cpt1b, Acox1, MCAD, Ucp3,	SREBP-1c, Acc1, Acc2, Fasn, Scd1

Tabla 4. Genes estudiados por PCR a tiempo real en el TAB retroperitoneal, TAM interescapular, hígado y músculo de los experimentos IIa y IIb.

IV. RESULTADOS Y DISCUSIÓN / RESULTS AND DISCUSSION

MANUSCRITO I

Dietary calcium attenuation of body fat gain during high-fat feeding in mice

Parra P, Bruni G, Palou A, Serra F.

J Nutr Biochem, Vol. 19(2): 109-117 (2008)

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Available online at www.sciencedirect.com
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Journal of Nutritional Biochemistry 19 (2008) 109–117

Journal of Nutritional Biochemistry

Dietary calcium attenuation of body fat gain during high-fat feeding in mice

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Received 6 November 2006; received in revised form 10 January 2007; accepted 17 January 2007

Abstract

Human epidemiological studies have supported the hypothesis that a dairy food-rich diet is associated with lower fat accumulation, although prospective studies and intervention trials are not so conclusive and contradictory data exist in animal models. The purpose of this study was to assess the effects on body weight and fat depots of dairy calcium (12 g/kg diet) in wild-type mice under ad libitum high-fat (43%) and normal-fat (12%) diets and to gain comprehension on the underlying mechanism of dairy calcium effects.

Our results show that calcium intake decreases body weight and body fat depot gain under high-fat diet and accelerates weight loss under normal-fat diet, without differences in food intake.

No differences in gene or protein expression of UCP1 in brown adipose tissue or UCP2 in white adipose tissue were found that could be related with calcium feeding, suggesting that calcium intake contributed to modulate body weight in wild-type mice by a mechanism that is not associated with activation of brown adipose tissue thermogenesis. UCP3 protein but not gene expression increased in muscle due to calcium feeding. In white adipose tissue there were effects of calcium intake decreasing the expression of proteins related to calcium signalling, in particular of stanniocalcin 2. CaSR levels could play a role in decreasing cytosolic calcium in adipocytes and, therefore, contribute to the diminution of fat accretion.

Results support the anti-obesity effect of dietary calcium in male mice and indicate that, at least at the time-point studied, activation of thermogenesis is not involved.
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Keywords: Dietary calcium; Energy expenditure; Adipose tissue; Thermogenesis; UCP1; UCP2

1. Introduction

Epidemiological studies have shown a potential association between dairy food consumption and body weight regulation. In particular, human data from cross-sectional studies support the hypothesis that a dairy food-rich diet is associated with lower fat accumulation in both adults and children; although prospective studies and intervention trials have yielded nonconclusive results (for a recent review, see Ref. [1]). A great amount of work has been developed during recent years looking to provide evidence for a potential biological mechanism for this association. Using transgenic mice expressing agouti protein in adipose tissue under the control of the aP2 promoter, Zemel [2] demonstrated that high calcium diets reduce fat accretion and weight gain and increase thermogenesis in animals maintained at identical caloric intakes. However, no evidence for higher energy expenditure has been found in Wistar rats and the decrease in body weight and fat content have been at least partially attributed to faecal fat loss due to the formation of calcium soap [3]. Moreover, recent papers in C57BL/6J mice and also in Sprague-Dawley or Wistar rats do not support the hypothesis of dietary calcium in regulating energy metabolism and obesity [4,5]. Therefore, contradictory data exist concerning the effects of dietary calcium on body weight and energy metabolism in animal models. The aim of this work was to assess the effects on

Abbreviations: ANOVA, analysis of variance; au, arbitrary units; BAT, interscapular brown adipose tissue; C/EBPα, CCAAT enhancer binding protein α; CaSR, calcium sensing receptor; CPT-1b, carnitine palmitoyltransferase1b muscle; RARα, retinoid alpha receptor α; RXRα, retinoid X receptor α; SDS, sodium dodecyl sulphate; SSC, saline sodium citrate buffer; STC2, stanniocacin 2; UCP-1, UCP-2, UCP-3, uncoupling proteins 1, 2 and 3 isoforms; VDR, vitamin D receptor; WAT, white adipose depots.

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doi:10.1016/j.jnutbio.2007.01.009

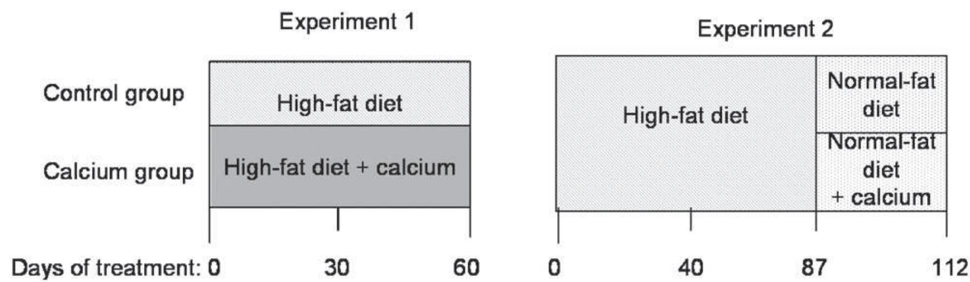

Fig. 1. Experimental design and dietary treatment followed by animals. In Experiment 1, male mice were fed a high-fat diet (control group) or the same diet supplemented with calcium (calcium group). In Experiment 2, animals were initially fed a high-fat diet (for 86 days) and then switched to a normal-fat diet either with normal (control group) or higher calcium content (calcium group).

body weight and body fat of dairy calcium in normal mice under high-fat and normal-fat diets and to gain comprehension about the potential involvement of thermogenic mechanisms, particularly in high-fat fed animals, which could also contribute to a better knowledge of the effect of calcium-rich diets in humans.

2. Methods and materials

2.1. Animals

Five-week-old male mice (C57BL/6J) from Charles River (Barcelona) were housed in groups of three in plastic cages, acclimated to 22°C with a 12-h light/12-h dark cycle and had free access to the diet and water. Animals were incorporated into one of the following experiments (see Fig. 1):

Experiment 1: Animals were fed ad libitum with a high-fat diet (43%) (control group, $n=6$) or with this diet supplemented with calcium (12 g/kg) (calcium group, $n=6$) for 2 months.

Experiment 2: Animals were fed ad libitum with a high-fat diet (43%) for 86 days to make them obese. Then, animals were fed with a standard, normal-fat, diet (12%) (control group, $n=6$) or with this normal-fat diet supplemented with calcium (calcium group, $n=6$) (12 g/kg) to the end of experiment.

All experimental procedures were performed according to the National and Institutional Guidelines for Animal Care and Use at the University of the Balearic Islands.

2.2. Diets

Diets were prepared by Research Diets Inc. (New Brunswick) and presented as pellets to the animals. The detailed diet composition is listed in Table 1. All diets provided 20% of calories from protein. In calcium diets, composition was aimed to supply 42% calcium from nonfat dry milk and the amount of purified casein was adjusted accordingly. The calcium content was 1.03 g/kcal in control diets and 3.10 g/kcal in the supplemented diets. Normal-fat diets provided 12% of calories from lard and soybean oil and were increased to 43% in high-fat diets. Carbohydrate was supplied as sucrose and cornstarch. The gross energy density of these formulations was calculated to be 3.8 kcal/g in normal-fat and 4.5 kcal/g in high-fat diets.

2.3. Determinations

2.3.1. Food intake and body weight

Food intake of animals was followed three times a week by weighing the amount of food left to the animals (per cage) and the remaining food on the following day. Experiment 1 was performed twice (the second time in parallel with Experiment 2). Body weight and food intake were available from 12 animals per group for Experiment 1 and from 6 animals per group for Experiment 2. Body weight was recorded for each individual animal at the same time as food intake.

Table 1
Diet composition in grams and expressed as a percentage of the calorie content

Diet composition	High fat		Normal fat	
	Control	Calcium	Control	Calcium
Ingredient (g)				
Casein, 80 mesh	212	50	214	50
DL-Methionine	3	3	3	3
Sucrose	350	142	400	439
Corn starch	0	0	250	0
Cellulose	50	50	50	50
Soybean oil	66	62	32	30
Lard	120	120	20	20
t-Butylhydroquinone	0.014	0.014	0.014	0.014
Mineral mix S10022B	7	7	7	7
Calcium carbonate	10	17.4	10	17.4
Potassium phosphate, monobasic	8	0	8	0
Potassium citrate, 1 H_2O	1.6	1.6	1.6	1.6
Vitamin mix V10037	10	10	10	10
Choline bitartrate	2.5	2.5	2.5	2.5
Milk, nonfat, dry	0	400	0	400
kcal (%)				
Protein	19.6	19.8	19.8	19.8
Carbohydrate	37.2	37.2	68.2	67.8
Fat	43.2	43.0	12.1	12.4
kcal/g	4.61	4.48	3.84	3.76
Calcium content (mg/kcal)	1.03	3.10	1.03	3.10

2.3.2. Tissue sampling and determinations

At the end of each experiment, animals were sacrificed and liver, kidneys, muscle, interscapular brown adipose tissue (BAT) and white adipose depots (WAT) were rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at −70°C.

Total RNA and protein was extracted from tissue samples from six animals of Experiment 1 using Tripure reagent (Roche, Barcelona, Spain) according to the instructions provided by the supplier. Protein concentration was determined by the BCA assay (Pierce) using a BSA standard supplied with the kit.

2.3.3. Western blots

UCP-1, UCP-2, UCP-3, C/EBPα, retinoid X receptor (RXRα), retinoid alpha receptor (RARα), vitamin D receptor (VDR) and calcium sensing receptor (CaSR) were determined by Western blotting. β-Actin was determined in some gels to confirm equal protein load charge between samples.

The procedure was as follows: 30–100 μg of protein was heat denatured in sample buffer [62.5 mM Tris, 5% sodium dodecyl sulphate (SDS), 10% v/v glycerol, 5% v/v β-mercaptoethanol, 0.0025% bromophenol blue] and electrophoresed on 10% SDS-polyacrylamide gel electrophoresis according to Laemmli as previously described [6]. Proteins were then electrotransfered (semi-dry electroblotter) to nitrocellulose membrane 0.45 μm (Bio-Rad, Madrid, Spain), and Ponceau-S (0.1% in 5% acetic acid; Sigma) staining was performed to confirm equal loading/transfer. Following transfer, membranes were blocked with 5% or 1% milk in PBS-Tween 20 for 1 h or overnight, respectively. Then they were incubated with the primary antibody with 0.1% BSA in PBS-Tween 20 for 1 h. The primary antibodies used were rabbit anti-mouse UCP-1 (1:1000–1:3000, Alpha Diagnostic International); rabbit anti-mouse UCP-2 (1:1000, Alpha Diagnostic International); guinea pig anti-human UCP-3 (1:5000, Linco Research, Inc); rabbit polyclonal anti-rat C/EBPα (1:4000, Santa Cruz Biotech); rabbit polyclonal anti-human RXRα (1:2000–1:3000, Santa Cruz Biotech); rabbit polyclonal anti-human RARα (1:200–1:1000, Santa Cruz Biotech); rat monoclonal anti-VDR (1:1300, Sigma); rabbit polyclonal anti-rat CaSR (1:100–1:1000, Abcam); and anti-β actin mouse monoclonal antibody (1:1000, Abcam). Membranes were then washed with PBS-Tween 20 and incubated with the secondary peroxidase-linked anti-rat IgG or anti-rabbit IgG antibody (Amershan Biosciences, Barcelona, Spain) or anti-guinea pig (Linco Research) diluted 1:5000 with 0.1% BSA in PBS-Tween 20. The immunocomplexes were revealed using an enhanced chemiluminescence detection system (ECL, Amershan Biosciences, Barcelona, Spain) and visualized by exposure to sensitive films (Hyperfilm ECL, Amershan Biosciences). The films were scanned in ChemiGenius (SynGene) using the software GeneSnap (6.03 version), and the bands were quantified using GeneTools version 3.04 (SynGene).

2.3.4. Northern blots

Twenty- to 30 μg of total RNA, denatured with formamide/formaldehyde, was fractionated by agarose gel electrophoresis as previously described [7]. The RNA was then transferred onto a Hybond Nylon membrane in 20× SSC (saline sodium citrate buffer: 1× SSC in 150 mM NaCl, 15 mM sodium citrate, pH 7.0) by capillary blotting for 16 h and fixed with UV light [7]. The specific mRNAs were detected by a chemiluminescence-based procedure, using antisense oligonucleotide probes (Table 2) synthesised commercially (TIB MOLBIOL) and labelled at both ends with a single digoxigenin ligand. Prehybridization was at 42°C for 15 min in DIG-Easy Hyb (Roche). Hybridization was at 42°C overnight in DIG-Easy Hyb containing 35 ng/ml of the oligonucleotide probe. Then, hybridized membranes were washed twice for 15 min at room temperature with 2× SSC/0.1% SDS, followed by two 15-min washes at 48°C with 0.1× SSC/0.1% SDS. After 1-h blocking at room temperature with a blocking reagent (Roche), the membranes were incubated first with antidigoxigenin-alkaline phosphatase conjugate (Roche) and then with the chemiluminescent substrate CDP-Star (Roche). Finally, membranes were exposed to Hyperfilm ECL (Amersham Biosciences, Barcelona, Spain). The films were scanned and quantified as described for the Western films. Finally, blots were stripped by 10-min exposure to boiling 0.1% SDS and re-probed for other mRNA or finally for 18S rRNA detection, to check the loading and transfer of RNA during the blotting.

2.3.5. Reverse transcriptase–polymerase chain reaction analysis of carnitine palmitoyltransferase1b muscle mRNA

Carnitine palmitoyltransferase1b muscle (CPT-1b) mRNA expression was assessed by reverse transcription–PCR assays using β-actin expression as an internal control in muscle. In brief, 1 μg of total RNA was denatured at 65°C for 10 min and reverse transcribed to cDNA using MuLV reverse transcriptase (according to Applied Biosystems's procedure) at 20°C for 15 min, 42°C for 30 min, with a final step of 5 min at 95°C in a Perkin Elmer 2400 Thermal Cycler (Norwalk, CT, USA). Two microlitres of the RT product was used for PCR amplification following the "hot

Table 2
Probes used for Northern blot hybridization

Probe	Gene
5′-GTTGGTTTTATTCGTGGTCTCCCAGCATAG-3	UCP-1
5′-GGCAGAGTTCATGTATCTCGTCTTGACCAC-3′	UCP-2
5′-GACTCCTTCTTCCCTGGCGATGGTTCTGTAGG-3	UCP-3
5′-TCCCACGAGCCACAGGCAGAGCCACAGGAGCAGC-3′	Resistin
5′-GGTCTGAGGCAGGGAGCAGCTCTTGGAGAAGGC-3′	Leptin
5′-CCCTCGCTCACCCTTGGCACCTCTGTTGGC-3	STC-2
5′-CTCGATAATGTCAGCCATCGCGGTGGCCTG-3′	CaSR
5′CGCCTGCTGCCTTCCTTGGATGTGGTAGCCG-3′	18S rRNA

start PCR" method and using the TAQ DNA polymerase in buffer B (Promega, Barcelona, Spain). The sample was first denatured at 95°C for 105 s and then PCR was carried out using the following parameters: 95°C for 15 s, 56°C for 15 s and 72°C for 30 s. Twenty-two cycles were used for both CPT-1 and β-actin. The amplification was finished by a final extension step of 7 min at 72°C. Primers for the CPT-1b gene were as follows: f5′ -AAGGGTAGAGTGGG-CAGAGG -3′ and r5′-GCAGGAGATAAGGGT-GAAAGA-3′, and for the β-actin gene were as follows: f5′-GTGGTGGTGAAGCTGTAGCC-3′ and r5′-ACGGGCATTGTGATGGACTC-3′. The expected size of the products was 222 bp for the CPT-1b gene and 165 bp for the β-actin gene, which were visualized by electrophoresis in a 2% agarose gel containing ethidium bromide and verified by using a DNA 100-bp ladder. The bands in the gel were quantified as described above. The signal for CPT-1b mRNA was normalized to the signal of the housekeeping gene β-actin, and the results were expressed as the CPT-1b/β-actin mRNA ratio.

2.4. Statistics

Data are presented as mean values±S.E.M. Differences between groups were assessed by two-way analysis of

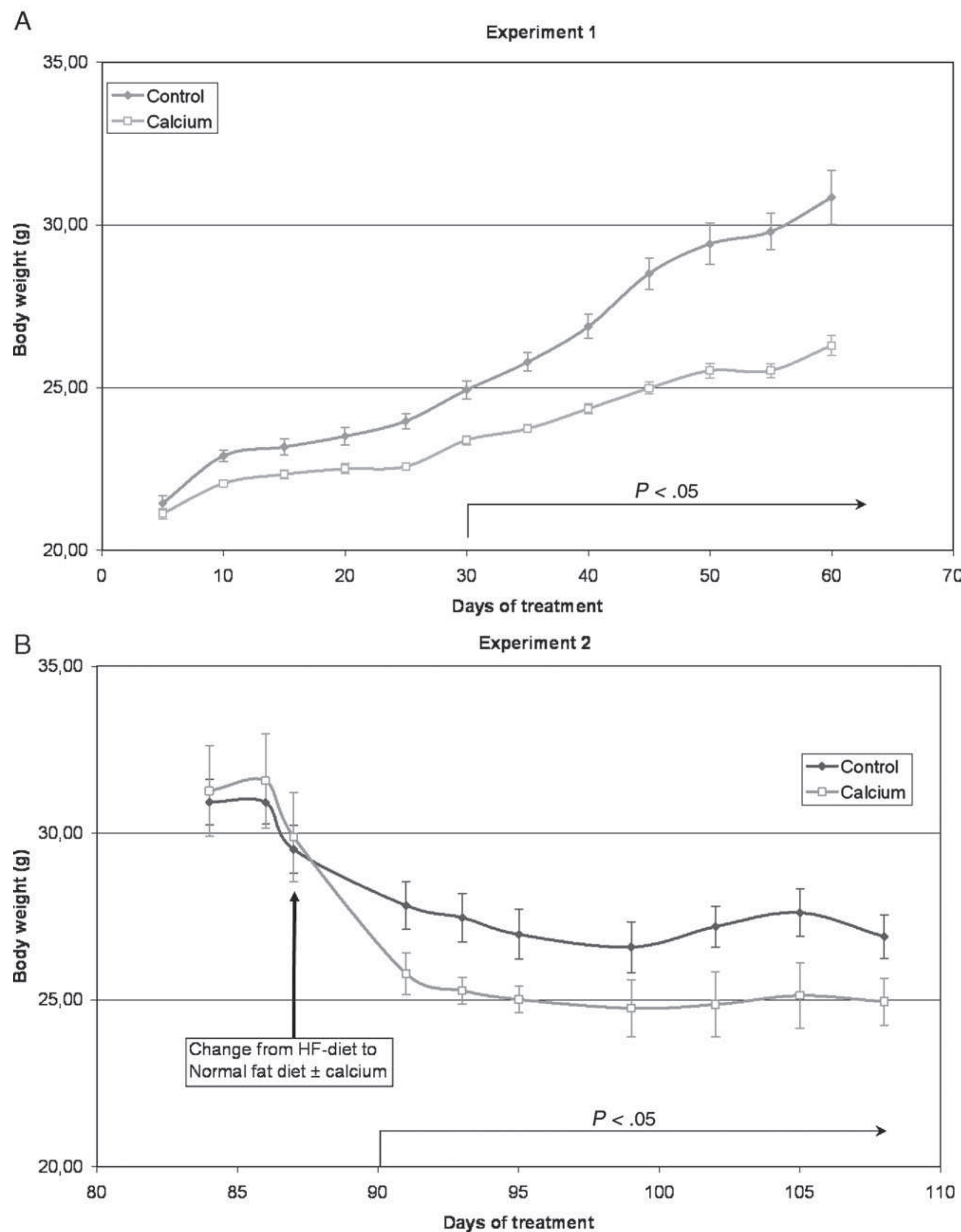

Fig. 2. Body weight evolution of animals from Experiments 1 (A) and 2 (B) during dietary treatment. In (A), animals were fed ad libitum a high-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium). In (B), after 86 days of high-fat feeding, animals were switched to a normal-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium) ($n=12$ for Experiment 1 and $n=6$ for Experiment 2). Body weights were significantly different between groups from Day 30 onwards (ANOVA) in (A) and from Day 90 onwards (ANOVA) in (B).

Table 3
Body and tissue weights in mice at the end of dietary treatments

Diet	Experiment 1 (n=12)		Experiment 2 (n=6)	
Group	High fat		Normal fat	
	Control	Calcium	Control	Calcium
Body weight (g)	31.1±1.0	26.8±0.4*	27.1±0.5	26.4±0.5
Liver (g)	1.28±0.03	1.18±0.03*	1.03±0.09	1.17±0.1
Kidneys (mg)	356±9	357±11	369±2	361±2
White adipose (g)				
Inguinal	0.822±0.07	0.305±0.02*	0.373±0.04	0.389±0.04
Epididymal	1.26±0.11	0.584±0.05*	0.580±0.07	0.585±0.04
Mesenteric	0.689±0.08	0.456±0.03*	0.507±0.04	0.532±0.04
Retroperitoneal	0.492±0.047	0.179±0.022*	0.182±0.03	0.155±0.008
Sum	*3.27±0.3*	*1.52±0.1**	*1.64±0.1*	*1.66±0.09*
Brown adipose (mg)	203±17	109±6*	158±5	143± 1

Statistically significant differences between groups were determined by Student's t test (*P<.05).

variance (ANOVA) or by Student's t-test with the level of significance set at P<.05. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL, USA).

Data obtained by Western and Northern blot have been referred to the values of control animals and are expressed in arbitrary units (au) with respect to milligrams of total tissue protein or total RNA, respectively.

3. Results

3.1. Body and tissue weights and food intake

3.1.1. Experiment 1

Although food consumption did not differ between groups (11.3±0.4 kcal/day in the control group and 11.4±0.4 kcal/day in the calcium group), the calcium group showed a lower rate of body weight gain (Fig. 2A) during the period of high-fat feeding. The amount of food eaten elicited a calcium intake of 11.6±0.4 mg Ca/day in control animals and 34.2±1.1 mg Ca/day in calcium-fed animals (P<.05). Body weight differences were statistically significant from Day 30 onwards (P<.05), and, at the end of the treatment, body weight in the calcium group was 14% lower than in controls (P<.05) (Table 3). All adipose tissue depots were significantly reduced in high-calcium-fed animals (Table 3). In fact, the sum of fat pads decreased by half; mesenteric was the least affected, showing a decrease of 34% in weight, and the greatest decrease was seen in the retroperitoneal fat pad (64%). Interscapular brown fat depot (BAT) followed a similar trend, being 46% lower in calcium-fed animals than in controls. Kidney weight was not affected by the treatment, and liver weight was slightly but significantly lower (P<.05) in calcium-fed animals than in control animals (Table 3).

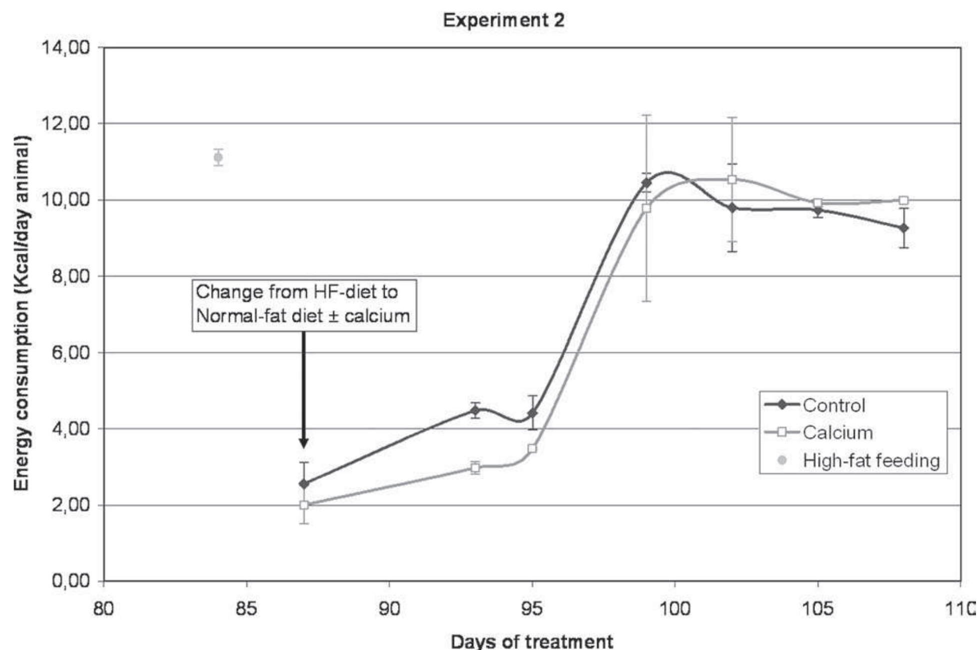

Fig. 3. Energy intake in animals from Experiment 2, fed on a high-fat diet and switched (at Day 86) to a normal-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium) (n=6 animals per group).

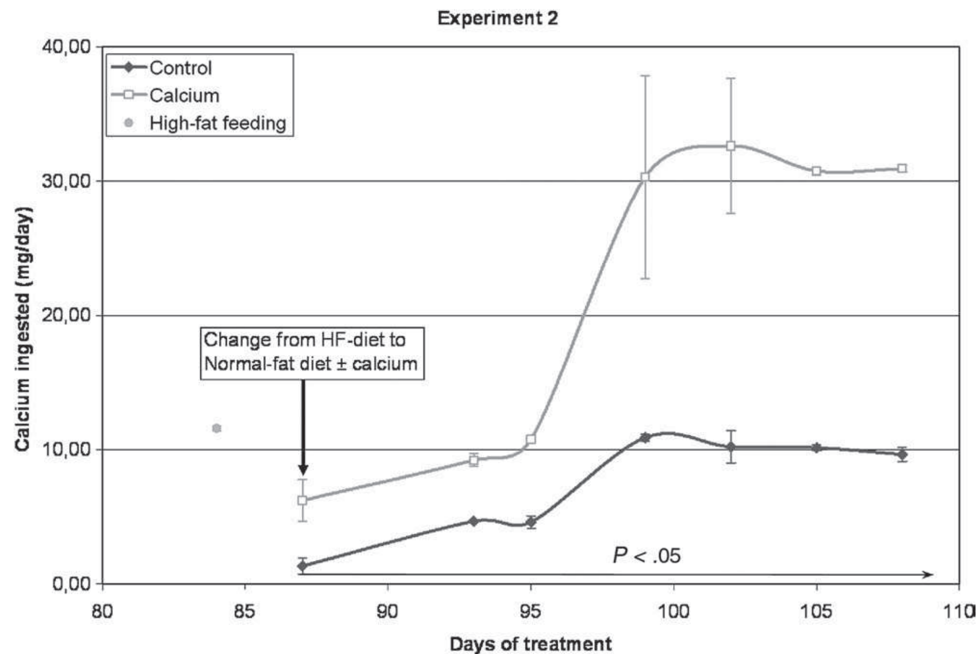

Fig. 4. Calcium intake in animals from Experiment 2, fed on a high-fat diet and switched (at Day 86) to a normal-fat diet either with a calcium content of 1.03 g/kcal (control) or 3.10 g/kcal (calcium) (n=6 animals per group). Calcium intake was significantly different between groups from Day 87 onwards (ANOVA).

3.1.2. Experiment 2

During the period of high-fat feeding, food intake did not differ between the groups and provided 11.1±0.2 kcal/day and 11.6±0.2 mg Ca/day on average. Substitution of the high-fat diet by the normal-fat diet was accompanied by a drastic reduction in food intake in both groups and took around 14 days to reach a steady level (Fig. 3). Once food intake was stabilised, there were no significant differences between the groups in terms of calorie content (C: 10.1±0.2 kcal/day; Ca: 9.9±0.4 kcal/day) from Day 100 onwards. However, in calcium-fed animals, the calcium intake was higher from the first day, they achieved the previous calcium intake level faster and their calcium level rose to a steady level three times higher than that of controls. By the end of the experiment, calcium intake was stabilised at 10.6±0.4 mg Ca/day in controls and 30.8±0.8 mg Ca/day (P<.05) in calcium animals (Fig. 4).

3.2. Tissue gene and protein expression

3.2.1. Brown adipose tissue

Feeding animals with a high-calcium diet (Experiment 1) was accompanied by a drastic reduction in the mRNA expression of leptin in brown adipose tissue (Table 4). UCP1 showed a nonstatistically significant reduction in both protein and mRNA expression (61% and 52%, respectively). No changes were observed in the expression of RXRα, C/EBPα, VDR or resistin. By contrast, RARα expression showed increased levels in calcium-fed animals (Table 4).

3.2.2. Muscle

UCP3 mRNA levels were not different between groups (100±15, control; 68±13, calcium), but protein levels were increased by 63% (P<.05) in calcium-fed animals. CPT-1b mRNA levels were not different between groups (100±4, control; 83±7, calcium). No differences were observed in protein levels of VDR (100±6, control; 96±7, calcium), RARα (100±8, control; 114±5, calcium), RXRα (100±30 control; 96±9, calcium) or CaSR (100±11, control; 93±7, calcium) by calcium diet.

3.2.3. White adipose

Protein expression of VDR (100±35, control; 106±13, calcium), resistin (100±14, control; 108±29, calcium) and UCP2 (100±20, control; 92±15, calcium) was not different between groups. The same was seen for UCP2

Table 4
Protein and RNA expression of target factors in brown adipose tissue from animals fed on a high-fat diet (control) and supplemented with calcium (calcium)

Brown adipose	High-fat diet	
	Control	Calcium
Protein (au/mg protein)		
VDR	100±13	103±7
RARα	100±12	176±24*
RXRα	100±19	115±22
C/EBPα	100±13	112±16
UCP1	100±21	61±9
mRNA (au/mg RNA)		
UCP1	100±24	52±18
Resistin	100±30	117±9
Leptin	537±18	Not detected*

Data have been referred to the values of control animals (set at 100%) and are expressed in arbitrary units with respect to milligrams of total tissue protein or total RNA.
n=6 animals for each group. Statistically significant differences between groups were determined by Student's t test (*P<.05).

mRNA (100±13, control; 82±7, calcium). Levels of C/EBPα (100±32, control; 54±17, calcium) and CaSR proteins (100±9, control; 56±11, calcium) and leptin mRNA (100±23, control; 59±8, calcium) showed a decrease in calcium-fed animals, but did not attain statistical significance. Staniocalcin-2 mRNA was decreased in calcium-fed animals (100±10, control; 68±7, calcium; $P<.05$).

4. Discussion

4.1. Body weight and food intake

Our results support the hypothesis that calcium intake contributes to combat obesity in mice. High-fat diet produces an increase in body weight that is counteracted by feeding ad libitum a high-calcium diet from a dairy source (42% of calcium from milk). The anti-obesity effect was particularly seen during the weight-gain phase (Experiment 1) as has been described in other animal models [2–4,8]. Furthermore, during the slimming phase (Experiment 2), calcium supplementation also played a role against obesity, contributing to a faster rate of body weight loss.

Our results indicate that no aversion to food is present; calcium animals are eating the same amount of food as the corresponding controls, irrespective of calcium or fat content in the diet. Probably because we are using moderately high calcium diets (0.4% in control and 1.2% in calcium-fed animals) and from a dairy source, which seems to be the best source concerning this aspect [9]. It is remarkable that there was a drastic decrease in food intake observed when the high-fat diet was replaced with a normal-fat diet (Experiment 2, Fig. 3). After eating a palatable diet, animals do not like the not-so-tasty normal-fat diet [10]. Therefore, the decrease in food intake seems to be the major factor responsible for the initial reduction in body weight seen in both groups (control and calcium) after changing the dietary regime. Since no differences in calorie load were found, it is suggestive to assume that higher calcium intake played a role in the accelerated body weight loss seen in the high-calcium group.

In Experiment 1, calcium intake was accompanied by lower liver weight. This has also been found in rats fed a high-calcium diet [3] and actually reflects lower body fat content, as adipose fat pads were reduced by 53%. Interestingly, the fat content in high-fat, high-calcium-fed animals (Experiment 1) was similar to that found in both groups of animals from Experiment 2 (control and calcium-fed animals), suggesting the presence of underlying mechanisms directed to modulate fat content close to 'control' values.

Altogether, these results support the anti-obesity effect of dietary calcium in male mice. Dairy calcium contributed to the attenuation of body weight and fat gain during high-fat feeding and also to the faster slimming rate in animals switched from a high-fat diet to a normal-fat one under ad libitum conditions.

To gain insight into the molecular mechanism involved in the decrease of fat accretion by dietary calcium, particularly during the high-fat feeding (Experiment 1), we first tested the hypothesis that uncoupling proteins could be involved in participation in activation of adaptive thermogenesis.

UCP1 is expressed in brown adipocytes and constitutes the main thermogenic effector in small rodents (for review, see Ref. [11]). UCP1 levels, assessed at the level of transcription and translation, were not different between calcium and control animals (Table 4). In fact, we found a decrease in UCP1 expression (61% in protein and 52% in mRNA levels), although animal variability and/or sample size did not sustain statistical significance between calcium and control animals. Furthermore, the decrease in UCP1 expression correlates with the increased levels of RARα as we and others have previously described, that UCP1 induction goes in parallel with a decrease in RARα during BAT differentiation [12,13]. Therefore, our data did not indicate an activation of thermogenesis in BAT, but a decreased diet-induced thermogenesis in high-calcium-fed animals.

In addition to UCP1, other uncoupling proteins such UCP2 and UCP3 may also act as uncouplers of oxidative phosphorylation and, therefore, contribute to modulating metabolic efficiency. In contrast to UCP1, UCP3 is predominantly expressed in skeletal muscle, and although it needs further assessment, its primary physiological role may be involved in mitochondrial handling of fatty acids [14]. Our data showed increased UCP3 protein levels (but not mRNA) in high-calcium-fed animals, which could have a role in protecting mitochondria against fatty acid accumulation and might help to maintain muscular fat oxidative capacity. However, CPT-1b expression, which is a key factor in mitochondrial fatty acid oxidation, did not indicate a higher rate of fat oxidation in calcium-fed animals.

Concerning modulation of UCP2, our data do not indicate an activation of its transcription in WAT as has been seen in aP2-agouti transgenic mice fed on a high-dietary calcium diet which shows increased UPC3 transcription in muscle too [15]. These activations have been linked to the reduction found in ROS production by high-calcium diet feeding, indicating a role of mitochondrial uncoupling in counteracting oxidative stress. However, our data on uncoupling proteins suggest high-calcium feeding in wild-type mice is not accompanied by activation of thermogenesis, maybe because there is no need to counteract oxidative stress as previously seen in this animal model [15] and it is closer to the trend of inhibition of thermogenesis found in other animal models of dietary obesity [16].

Gene expression parameters related to adipose tissue metabolism (C/EBPα, resistin, leptin) and/or calcium signalling (STC-2, VDR, CaSR) were measured in adipose tissue. C/EBPα is the initiation marker of the signalling cascade for adipocyte differentiation, responsible for the expression of adipocyte-specific genes and is expressed late in the differentiation program [17,18]. Our data show that C/

EBPα is not significantly altered by dietary calcium either in BAT or in WAT.

Resistin is expressed in brown and white adipocytes [19,20] and participates in the regulation of energy homeostasis (see Ref. [21] for review). At least in rodents, resistin plays an important role in the development of insulin resistance (see Ref. [22] for review). In this paper, we have shown that the expression of resistin in brown and white adipocytes is not affected by dietary calcium intake and/or body fat content. Therefore, the slimming effect of dietary calcium under high-fat feeding does not contribute to impair insulin signalling as has been observed with other nutrients such as CLA in particular conditions [23].

Leptin, an important signalling factor in obesity, shows a pattern well correlated with the diminution of body fat stores in high-calcium animals, and the disappearance of its expression in BAT is remarkable.

We studied the expression of STC2, which shares limited sequence similarity with an antihypercalcemic hormone first discovered in fish [24,25] and is widely expressed in mammals [26]. There is only limited information available on STC2 expression and function, but a role in mammal calcium homeostasis has been proposed [26], and, recently, a novel function for STC2 in protecting neuronal cells from oxidative stress and hypoxia has also been reported [27]. Although the exact function of STC2 in adipose cells has not been previously studied, whether this could be related to oxidative stress, the reduction found in its expression in WAT of high-calcium-fed animals is well correlated with the pattern of UCP2 shown in these animals. Therefore, these data also support the fact that induction of oxidative stress is not present in adipose cells of wild-type mice fed a high-fat-enriched calcium diet.

VDR is a transcription factor belonging to the superfamily of steroid/thyroid hormone receptors. Traditionally associated with calcemic activities, VDR is also known to be involved in cell proliferation, differentiation and immunomodulation using RXR as obligate partner (see Ref. [28] for review) and, recently, a role in modulating adipogenesis has been associated with VDR [29]. The data do not show any difference in adipose tissues or in muscle of high-calcium-fed mice. Therefore, the loss of fat accretion in high-calcium-fed animals does not seem to be modulated by a mechanism involving VDR, at least in the tissues studied.

CaSR is a G protein-coupled receptor that was originally thought to be mainly involved in calcium homeostasis, as the primary regulator of PTH secretion in response to changes in circulating calcium [30]. We found lower PTH serum levels in high-calcium-fed animals (data not shown), which are in agreement with higher levels of extracellular calcium that would inhibit parathyroid hormone secretion [31]. On other hand, several studies mainly made by the group of Zemel [32] have already demonstrated that increased intracellular calcium in adipocytes results in stimulation of lipogenic gene expression and suppression of lipolysis, which achieve adipocyte lipid filling and increased adiposity. Therefore, activation of CaSR in adipocytes could mediate an increase in intracellular calcium that could be responsible for triggering signalling cascades that would influence adipogenesis and triglyceride storage in fat cells. Our data demonstrate expression of CaSR in WAT and muscle of mice. No activation of CaSR was found in adipose tissue (if there was any effect, there is a nonstatistically significant reduction of its expression), suggesting that no increased cytosolic calcium is present in adipocytes, in accordance with data from other high-calcium-fed animal models [15,33]. The molecular basis involving the decrease in intracellular calcium could be related to CaSR, as antagonists used in other cell types have been shown to mimic this effect [34]. Altogether, our data suggest that modulation of CaSR in adipocytes could attenuate the intracellular calcium levels and, thus, contribute to the pattern of reduced fat accretion seen in high-calcium-fed animals.

It can be concluded that dietary calcium has a role in modulating body weight in wild-type male mice. Dairy calcium contributed to the attenuation of body weight gain during high-fat feeding. Furthermore, a faster slimming rate was also observed when animals were switched from a high-fat diet to a normal-fat one high in calcium content under ad libitum conditions. However, the effect of calcium on body weight is not due to a decrease in food intake; neither can it be explained by activation of BAT thermogenesis. The known effect of dietary calcium decreasing intestinal bioavailability of fat [3] may contribute to counteract any effect of calcium on thermogenesis. CaSR levels could play a role in decreasing cytosolic calcium in adipocytes and therefore contribute to the diminution of fat accretion. No evidence of oxidative stress is present in this animal model and the role of STC-2 in adipocytes, particularly in this aspect, would need further research. Finally, our data point out that wild-type mice do not follow the metabolic adaptations found in the aP2-agouti transgenic model under high-fat, high-calcium feeding and indicate that activation of uncoupling proteins does not seem to be the main factor responsible for the slimming effect of high-calcium diets.

Acknowledgments

This work was supported by grants from the Spanish Government (BFI2003-04439, AGL2004-07496). P. Parra is a recipient of a fellowship from the Spanish Government.

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MANUSCRITO II

Effect of calcium-enriched high-fat diet on calcium, magnesium and zinc retention in mice

Pérez-Gallardo L, Gómez M, Parra P, Sánchez J, Palou A, Serra F.

Br J Nutr, Vol. 101(10): 1463-6 (2009)

British Journal of Nutrition (2009), **101**, 1463–1466
doi:10.1017/S0007114508102446
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Effect of calcium-enriched high-fat diet on calcium, magnesium and zinc retention in mice

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(Received 6 May 2008 – Revised 14 August 2008 – Accepted 2 September 2008 – First published online 6 November 2008)

The aim of this work was to assess the effects of a high-fat diet enriched in Ca, which accompanies lower body fat deposition, on mineral depots, as well as to assess the potential role of adaptive thermogenesis in mice. Male mice were fed *ad libitum* a high-fat (43 %) diet with a Ca content of 4 g/kg from calcium carbonate (control group) or 12 g/kg (42 % from milk powder and the rest from calcium carbonate) (Ca group) for 56 d. Body weight, food intake and urine were periodically collected. Tissue samples were collected when the mice were killed and the composition was determined. Expression of uncoupling proteins was determined by Western blotting. Mineral content was measured by flame atomic absorption spectrometry. Lower body weight gain and fat accretion was found in the Ca group. This could not be attributable to lower gross energy intake or to activation of adaptive thermogenesis. Although significant urine mineral loss was found in the Ca group, preservation of mineral depots in bone was observed. Our data support the fact that adding more Ca to the diet, using a combination of calcium carbonate plus milk powder containing among other things higher Zn and Mg, contributes to counteracting obesity and improving lipid metabolism.

Dietary calcium: Zinc: Magnesium: Obesity

Observational and epidemiological data have shown that a low-Ca diet may be a risk factor for obesity development, and beneficial aspects of milk components on metabolic syndrome are gaining strength[1,2], even though intervention trials have yielded inconsistent results to date[3].

The molecular mechanisms responsible for the impact of dairy products on body weight and fat have been studied in animal models with important contributions from the group of Zemel[4]. Using a transgenic animal model (over-expressing agouti protein under the control of the aP2 promoter), Zemel and co-workers showed that dietary Ca could influence fat deposition by direct modulation on adipocyte metabolism, increasing thermogenesis and lipolysis and decreasing lipogenesis following a high-Ca diet[5,6]. However, in normal mice, activation of thermogenesis does not seem to be responsible for the lower rate of weight gain seen with a high-fat diet enriched with dairy Ca[7].

Minerals have been suggested to beneficially modulate cardiovascular risk factors[8,9]. However, there are few data available concerning dietary mineral interactions. The aim of the present work was to assess the effects of a high-fat diet enriched in Ca on body fat and mineral bone retention, particularly on Ca, Mg and Zn, as well as to assess the potential involvement of adaptive thermogenic mechanisms in mice.

Experimental methods

Animals and diets

Twelve male mice (C57BL/6J) (Charles River, Spain) weighing approximately 21 g were housed in groups of three and kept in a single metabolic cage throughout the experiment under controlled conditions. After a 1-week adaptation, mice received *ad libitum* either a control diet or a high-Ca diet (Research Diets Inc., New Brunswick, NJ, USA) for 56 d. Both diets provided the same digestible energy content (19 kJ/g), 20 % kJ as protein (casein) and 43 % kJ as fat (lard and soyabean oil). In addition, the high-Ca diet supplied 1·2 % (w/w) in Ca (from calcium carbonate and milk powder), three times higher than control group (0·4 % w/w, exclusively from calcium carbonate). High-Ca diet was aimed to supply 42 % Ca from non-fat dry milk which also provided higher levels of Mg (1·09 g/kg) and Zn (0·05 g/kg) than the control diet (Mg, 0·57 g/kg; Zn, 0·03 g/kg). Food and water intake and body weight were recorded weekly.

Abbreviations: BAT, brown adipose tissue; UCP, uncoupling protein.

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All experimental procedures were performed according to the national and institutional guidelines for animal care and use at the university.

Sampling

Urine was collected twice a week from each cage, and measured and stored at $-20°C$ for posterior analysis. Animals were killed by decapitation at the end of the experiment; tissues were dissected, weighed, rinsed with saline containing 0·1 % diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at $-70°C$ until analysis.

Analytical procedures

Ashed femur samples and aliquots of urine were measured after appropriate dilution by flame atomic absorption spectrometry (Perkin-Elmer 272). Liver glycogen was extracted[10] and the released glucose was determined by enzymatic assay[11]. Lipids were extracted[12] and TAG and cholesterol content were determined using commercial kits from Biotécnica 2000. Liver protein content was determined from homogenised samples in PBS[13].

Muscle and brown adipose tissue (BAT) proteins were extracted using Tripure reagent (Roche, Barcelona, Spain). Protein concentration was determined by the bicinchoninic acid assay (Pierce) using a bovine serum albumin standard. Uncoupling protein 1 (UCP-1) in BAT and UCP-3 in muscle were determined by Western blotting[7]. β-Actin was determined in representative gels to confirm equal protein load charge between samples. The immunocomplexes were revealed using an enhanced chemiluminescence detection system (ECL™; Amersham Biosciences, Barcelona, Spain) and visualised by exposure to sensitive films (Hyperfilm™ ECL; Amersham Biosciences). The films were scanned in a ChemiGenius (SynGene) using the software GeneSnap version 6.03, and the bands were quantified using GeneTools version 3.04 (SynGene).

Statistical analysis

The effect of Ca treatment on body weight was assessed by ANOVA followed by *post hoc* analysis. Comparison between control and Ca animals for the rest of the variables was assessed by Student's *t* test. The analysis was performed using the SPSS program for Windows version 14 (SPSS, Chicago, IL, USA). Urine mineral data from control and Ca animals were analysed on each day of treatment; no differences were observed throughout the period, and therefore data are presented as the average of all samples collected. The level of significance was set at $P<0·05$. Data are presented as means with their standard errors.

Results

Food consumption did not differ between groups during the period studied 52·3 (SEM 1·7) kJ/d in the control group and 53·1 (SEM 2·1) kJ/d in the Ca group. The amount of food eaten implies an intake of 10·8 (SEM 0·3) mg Ca/d, 1·5 (SEM 0·04) mg Mg/d and 0·08 (SEM 0·002) mg Zn/d in the control group and 46·8 (SEM 1·2) mg Ca/d, 4·2 (SEM 0·1) mg Mg/d and 0·2 (SEM 0·05) mg Zn/d in the Ca group. No difference in water consumption was seen between groups 4·27 (SEM 0·08) ml/d in controls and 4·42 (SEM 0·05) ml/d in the Ca group.

Body weights were significantly different between groups from week 6 onwards ($P=0·017$) and, by the end of the study, Ca-fed animals weighed less than control mice (Fig. 1). During the treatment, the control group gained 7·61 (SEM 0·7) g, whereas animals fed the high-Ca diet gained 4·41 (SEM 0·2) g ($P=0·003$).

Adipose tissue weights were significantly smaller in mice fed the high-Ca diet than in controls, mesenteric was the least affected (50 %) and the retroperitoneal the highest (69 %) compared with controls. BAT was also 40 % lower in the Ca group than in the control group (Table 1). Saturated lipid concentration and TAG were determined in epididymal adipose tissue and were not different between the control and Ca group (data not shown).

Liver weight was not affected by dietary treatment and the same was seen for glycogen, proteins, saturated lipid and cholesterol concentrations in liver. However, TAG levels were reduced by half in the Ca group.

UCP-1 levels in BAT were not different between control and Ca-fed animals (100 (SEM 11·2) % in control group; 110 (SEM 15·4) % in Ca group). The same pattern was seen in muscle UCP-3 levels (100 (SEM 10·5) % in control; 89·1 (SEM 11·7) % in Ca group).

No significant differences were observed with respect to the volume of urine eliminated daily (control 0·77 (SEM 0·05) *v*. Ca 0·89 (SEM 0·07) ml/d, $P=0·19$). However, Ca (control 5·18 (SEM 1·0) *v*. Ca 603 (SEM 79) μg/d, $P=2·3 \times 10^{-6}$), Mg (control 0·50 (SEM 0·1) *v*. Ca 494 (SEM 70) μg/d, $P=5·3 \times 10^{-6}$) and Zn (control 21·5 (SEM 1·7) *v*. Ca 89·2 (SEM 6·0) μg/d, $P=3·6 \times 10^{-8}$) urine excretion were significantly higher in the Ca group than in controls. To assess mineral deposition, femur composition was analysed and no statistically significant effects either on femur weight or on Ca, Mg or Zn content at the end of treatment were found (data not shown).

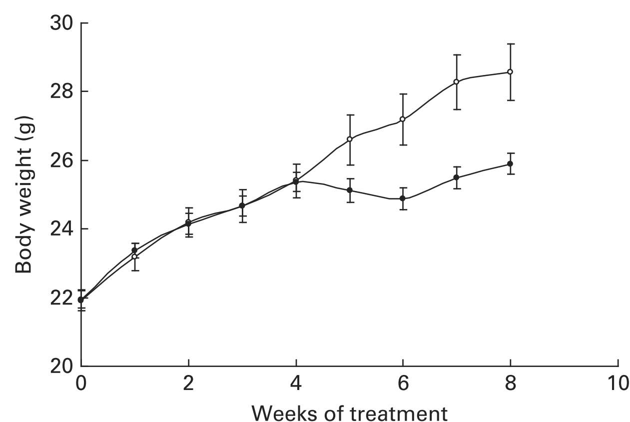

Fig. 1. Body weight evolution of animals fed either the control (○) or the high-calcium (●) diet for 56 d. Values are means with their standard errors depicted by vertical bars (six animals for each group). Body weights were significantly different from week 6 onwards ($P=0·017$), determined by repeated-measures ANOVA.

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Table 1. Fat depot weights and liver weight and composition in mice fed either the control or the high-calcium diet for 56 d*

(Mean values with their standard errors)

	Control diet (*n* 6)		Calcium diet (*n* 6)		
	Mean	SEM	Mean	SEM	*P*†
Adipose tissue weight (mg)					
Epididymal	826	130	381	9·7	0·009
Inguinal	533	55	222	9·3	0·002
Mesenteric	302	42	151	15	0·015
Retroperitoneal	267	33	81	6·9	0·002
Interscapular brown fat	85	6·5	51	2·4	0·001
Liver weight (g)	1·15	0·07	1·18	0·02	0·66
Glycogen (mg/g)	20·3	1·3	18·0	1·9	0·33
Protein (mg/g)	49·0	4·1	49·5	3·9	0·93
Saturated lipids (mg/g)	6·36	0·5	6·20	0·5	0·85
Cholesterol (μg/g)	175	12	180	15	0·77
TAG (μg/g)	995	11	473	91	0·005

* For details of procedures and diets, see Experimental methods.
† Statistically significant differences between groups were determined by Student's *t* test.

Discussion

The aim of the present work was to assess the effects of high-fat diet enriched in Ca, which has been previously associated with a lower body fat deposition, on Ca, Mg and Zn bone retention, as well as on thermogenic capacity in normal mice.

In accordance with previous results[7], a reduction in body weight gain (by 12·7 %) was observed following the treatment with a high-Ca diet, which was also accompanied by lower fat deposition affecting all the adipose depots. In addition, hepatic lipid profile was not altered in the Ca group and the lower TAG content, associated with diminished hepatic lipogenesis, was in accordance with the lower fat accretion seen in adipose tissues.

No differences in food intake and in the expression of UCP-1 in BAT or in muscle UCP-3 were found, indicating that lower fat accretion in high-Ca-fed animals was not accompanied by lower energy intake either by activation of BAT or muscle thermogenesis, in accordance with results previously found in normal animals[7,14] but not in a largely characterised transgenic model[15].

Urine mineral excretion (Ca, Mg and Zn) was increased in the Ca group and, to a certain extent, this could contribute to compromised mineral bioavailability. For example, high Ca to Mg dietary ratio has been proposed to pose a risk for Mg deficiency[16] although, in practice, high-Ca diets have not been demonstrated to affect Mg retention in the long term[17]. Furthermore, Ca, especially in the presence of phytate, may have an inhibitory effect on Zn absorption[18] and a reduction in tibia Zn content has been reported in rats fed on high-Ca diet[19], interestingly this does not seem to happen in the present experimental conditions. The preservation of Ca, Mg and Zn content in femur, despite the higher urine excretion, supports the fact that body levels of these minerals are not compromised in the Ca group, at least during the period studied.

In conclusion, the present results support a lower body weight gain and lower body fat accretion in adult mice fed a high-fat diet enriched with dairy Ca with respect to those fed with standard Ca levels. This could not be attributable to a lower gross energy intake or to activation of thermogenesis in BAT or muscle. Although significant urine mineral loss was found in Ca-fed animals, the presence of dairy Ca was accompanied by preservation of mineral depots in bone. Further characterisation of cellular metabolism of these minerals would be necessary to attain total comprehension of their role in diminishing fat accretion. Nevertheless, the present data support the fact that adding more Ca to the diet, using a combination of milk powder containing among other things higher Zn and Mg, contributes to counteract obesity and improves lipid metabolism in high-fat-fed mice.

Acknowledgements

This work was supported by grants from the Spanish Government (AGL2004-07 496 and AGL2006-04 887) and European Commission (European Nutrigenomics Organisation, NUGO-FP6-506360). CIBER de 'Fisiopatología de la Obesidad y Nutrición' is an initiative of ISCIII. P. P. is a recipient of a fellowship from the Spanish Government. M. G. took care of animal handling, sample collections and analytical determinations. J. S. contributed to animal dissection and tissue sample collection. P. P. performed protein extraction and Western determinations. A. P., F. S. and L. P.-G. equally contributed to the conception and design of the study, interpretation of data and drafting of the manuscript. The authors declare no conflict of interest.

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MANUSCRITO III

Moderate doses of conjugated linoleic acid isomers mix contribute to lowering body fat content maintaining insulin sensitivity and a noninflammatory pattern in adipose tissue in mice

Parra P, Serra F, Palou A

J Nutr Biochem, Vol. 21: 107-115 (2010)

ARTICLE IN PRESS

Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry xx (2009) xxx–xxx
Journal of Nutritional Biochemistry

Moderate doses of conjugated linoleic acid isomers mix contribute to lowering body fat content maintaining insulin sensitivity and a noninflammatory pattern in adipose tissue in mice☆

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Received 8 July 2008; received in revised form 8 October 2008; accepted 31 October 2008

Abstract

Conjugated linoleic acid (CLA) modulates body composition, especially by reducing adipose tissue. However, despite the increasing knowledge about CLA's beneficial effects on obesity management, the mechanism of action is not yet fully understood. Furthermore, in some human studies fat loss is accompanied by impairment in insulin sensitivity, especially when using the *trans*-10,*cis*-12 isomer. The aim of this work was to study the effects of moderate doses of CLA on body fat deposition, cytokine profile and inflammatory markers in mice. Mice were orally treated with a mixture of CLA isomers, *cis*-9,*trans*-11 and *trans*-10,*cis*-12 (50:50), for 35 days with doses of CLA1 (0.15 g CLA/kg body weight) and CLA2 (0.5 g CLA/kg body weight). CLA had discrete effects on body weight but caused a clear reduction in fat mass (retroperitoneal and mesenteric as the most sensitive depots), although no other tissue weights were affected. Glucose and insulin were not altered by CLA treatment, and maintenance of glucose homeostasis was observed even under insulin overload. The study of gene expression (Emr1, MCP-1, IL-6, TNFα, PPARγ2 and iNOS) either in adipocytes and/or in the stromal vascular fraction indicated that CLA does not lead to the infiltration of macrophages in adipose tissue or to the induction of expression of pro-inflammatory cytokines. The use of a mixture of both isomers, as well as moderate doses of CLA, is able to induce a reduction of fat gain without an impairment of adipose tissue function while preserving insulin sensitivity.
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Keywords: Conjugated linoleic acid; Insulin sensitivity; Macrophage infiltration; Mice; Adipose tissue

1. Introduction

Conjugated linoleic acid (CLA) has been tested extensively for its ability to modify body composition in humans. In particular, the mixture containing mainly the two bioactive isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA (see Ref. [1] for a review) reduces body fat at the same time as preserving lean body mass [2–4]. Supplementation with the two bioactive CLA isomers is mainly associated with beneficial effects such as stimulation of immune response [5,6] and improvement of insulin sensitivity [7] and lipid metabolism [8]. However, studies on type 2 diabetics showed conflicting results [9,10] and some studies on healthy obese subjects have found an increase in markers of lipid peroxidation [11]. Furthermore, when supplemented with the isomers separately, particularly at high concentration of either the *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA, increased insulin resistance in obese subjects by these single isomers has been reported [12–14], whereas the mixture of both bioactive isomers did not affect the insulin sensitivity of subjects with the metabolic syndrome [12] and improved it in sedentary men [7]. Additionally, supplementation with CLA triggers a fat loss which is associated with insulin resistance, robust hyperinsulinemia and massive steatosis

☆ This work was supported by grants from the Spanish Government (AGL2004-07496, AGL2006-04887). Our laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, European Union Contract No. FP6-506360). P. Parra is a recipient of a fellowship from the Spanish Government. CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative from ISCIII.

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0955-2863/$ – see front matter © 2009 Published by Elsevier Inc.
doi:10.1016/j.jnutbio.2008.10.010

[15,16] in sensitive animal models such as mice. Antiadipogenic effects of CLA and liver steatosis have been attributed to the *trans*-10,*cis*-12 CLA isomer [16,17]. Furthermore, proinflammatory cytokine concentrations were recently found to increase in white adipose tissue of mice treated with *trans*-10,*cis*-12 CLA associated with a recruitment of macrophages in the adipose tissue [18].

Reported adverse effects of CLA in mice, which are the more sensitive species, are accompanied by important losses of fat, thus driving an excess of fatty acids to the liver and fatty acids also competing with the flux of glucose to other peripheral tissues, a situation that, together with selective effects of CLA on lypolisis and lipogenesis, may give the impression of loss of insulin sensitivity and, perhaps, a proinflammatory state. However, this is not the situation seen in humans, in whom very modest effect on fat loss has been demonstrated while preserving muscle mass. The aim of this study was to analyze the effects of a supplementation with moderate doses of bioactive isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA in mice fed with a standard-fat diet, an animal model particularly sensitive to the potential adverse effects of CLA. Our interest was to use the bioactive compounds usually tested in humans and a range of doses comparable to the doses assayed in humans. In addition, it was our interest to test the potential infiltration of macrophages in adipose tissue and to analyze the profile of expression of cytokines.

2. Methods and materials

2.1. Animals

Male mice (C57BL/6J) from Charles River (Barcelona, Spain) were housed in groups of four in plastic cages, acclimated to 22°C with a 12-h light/12-h dark cycle. Animals were fed *ad libitum* with a standard diet (D12450B) (Research Diets Inc, New Brunswick, Canada) which contains 10% calorie content as fat (25 g/100 g soybean oil and 20 g/100 g lard), 70% calorie content as carbohydrate (315 g/100 g corn starch, 35 g/100 g maltrodextrin 10, 350 g/100 g sucrose and 50 g/100 g cellulose) and the remaining 20% as protein (casein and L-cystine). Food intake and body weight were recorded every 3 days during the experiment. Fresh food was provided to the mice biweekly. At 30 days of treatment, animals were starved for 3 h and tail blood samples were obtained to perform plasma determinations and were then submitted to the insulin tolerance test (ITT). One week after, the animals were sacrificed under feeding conditions.

All experimental procedures were performed according to both national and institutional guidelines for animal care and use.

2.2. Conjugated linoleic acid treatment

The CLA used was Tonalin TG 80 derived from safflower oil (kindly provided by Cognis). Tonalin is composed of triglycerides containing approximately 80% CLA, with a 50:50 ratio of the active CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12.

Mice weighing 20±0.2 g were randomly assigned to three experimental oral treatments: sunflower oil (control), CLA1 or CLA2 for 37 days. Two different doses of CLA were assessed in this study: CLA1 (0.15 g CLA/kg body weight) and CLA2 (0.50 g CLA/kg body weight), taking as reference the weight of the animals at the beginning of the experiment. Therefore, animals received a daily amount of Tonalin equivalent to 3 mg CLA/animal in the CLA1 group and 10 mg/animal in the CLA2 group. An adequate amount of commercial sunflower oil was given to the animals to achieve isocaloric load between groups.

2.3. Insulin tolerance test

Insulin tolerance test was carried out after 30 days of oral treatment. Glucose concentration was determined from tail blood samples. Then, recombinant human insulin (Humulin R; Eli Lilly, Spain), previously diluted in 0.9% saline, was intraperitoneally injected (0.8 U/kg body weight). Subsequent blood samples were taken from the tail tip, and glucose was directly measured at 15, 30, 60, 90 and 120 min postinjection using an Accu Check Sensor (Roche Diagnostics, Barcelona, Spain).

2.4. Sacrifice and tissue sampling

Mice were anesthetized by intraperitoneal injection of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight) and blood collected by cardiac puncture. Liver, pancreas, gastrocnemius, stomach, brown and white adipose depots were rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at −70°C. Blood collected by cardiac puncture with heparinized syringe and needle (0.2% heparin diluted with saline; Sigma) was centrifuged at 2500 rpm for 10 min at 4°C and plasma obtained was stored at −70°C for later analysis.

2.5. Plasma analysis

Adiponectin and insulin plasma concentrations were measured using a rat/mouse adiponectin enzyme-linked immunosorbent assay (ELISA) kit (Phoenix Europe GmbH, Karlsruhe, Germany) and Insulin Mouse Ultrasensitive ELISA (DRG Instruments GmbH, Marburg, Germany), respectively. Resistin and leptin plasma concentrations were also assessed by ELISA using the following commercial kits: Mouse Resistin Quantikine ELISA Kit and Mouse Leptin Quantikine (R&D Systems, Minneapolis, MN, USA). Commercial enzymatic colorimetric kits were used for the determination of plasma nonesterified fatty acids (NEFAs; Wako Chemicals GmbH, Neuss, Germany) and circulating concentrations of triglycerides (Sigma Diagnostics, Madrid, Spain).

2.6. Isolation of mature adipocytes and stromal vascular fraction from epididymal fat depots

Fresh epididymal white adipose tissue was minced into small pieces and placed in sterile plastic tubes with Krebs-Ringer buffer containing 25 mM $NaHCO_3$, 11 mM glucose, 25 mM Hepes, pH 7.4, 2% bovine serum albumin (BSA) (Fraction V, Sigma Diagnostics) and 1.5 mg/ml collagenase type I (Gibco-Invitrogen, Prat de Llobregat, Spain). The ratio between adipose tissue mass and incubation solution was 1:4 (w/v). The tissue suspension was incubated at 37°C with gentle shaking for 45–60 min. Once digestion was completed, samples were passed through a sterile 250-μm nylon mesh (Sefar America, Inc., Depew, NY, USA). The suspension was centrifuged at 200×*g* for 10 min, the pelleted cells were collected as stromal vascular fraction (SVF) and the floating cells were considered the mature adipocyte-enriched fraction. The latter was washed twice with Krebs-Ringer-bicarbonate-Hepes-BSA buffer and centrifuged as above. The SVF was resuspended in erythrocyte lysis buffer consisting of 0.154 M NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA, and incubated at room temperature for 10 min. The erythrocyte-depleted SVF was centrifuged at 400×*g* for 5 min, the pellet was resuspended and washed twice in Krebs-Ringer-bicarbonate-Hepes-BSA buffer and centrifuged at 400×*g* for 5 min. After washing and short centrifugation steps, both samples, the SVF and the mature adipocyte fraction, were resuspended and disrupted by adding the buffer provided by the RNA extraction kit used afterwards (see below) and kept at −70°C.

2.7. Real-time quantitative polymerase chain reaction analysis

Total RNA was extracted using an RNAeasy Mini Kit from Qiagen (Barcelona, Spain). RNA was quantified using the NanoDrop Spectrophotometer ND-1000. Real-time polymerase chain reaction (real-time PCR) was used to measure mRNA expression levels of target genes. Aliquots of 0.5 μg of total RNA (in a final volume of 10 μl) were denatured at 90°C for 1 min and then reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 42°C for 60 min, with a final step of 5 min at 99°C in a Perkin-Elmer 9700 Thermal Cycler (PerkinElmer, Wellesley, MA, USA). Real-time PCR was completed using the LightCycler System with SYBR Green I (Roche Diagnostic GmbH, Mannheim, Germany). Primer sequences were as follows: *Adiponectin* forward, 5′-GCTCAGGATGCTACTGTTG-3′; *adiponectin* reverse, 5′- TCTCACCCTTAGGACCAAG-3′; *leptin* forward, 5′-TTGTCACCAGGATCAATGACATTT-3′; *leptin* reverse, 5′- GACAAACTCAGAATGGGGT-GAAG-3′; serum retinol-binding protein 4 (*RBP4)* forward, 5′-ACTGGGGTGTAGCCTCCTTT-3′; *RBP4* reverse, 5′-GGTGTCGTAGTCCGTGTCG-3′; glucose transporter type 4 (*Glut4)* forward, 5′-GGCATGCGTTTCCAGTATGT-3′; *Glut4* reverse, 5′-GCCCCTCAGTCATTCTCATC-3′; peroxisome proliferator-activated receptor gamma 2 (*PPARγ2*) forward, 5′-GGTGAAACTCTGGGAGATTC-3′; *PPARγ2* reverse, 5′-TAATAAGGTGGAGATGCAGG-3′; monocyte chemotactic protein-1 (*MCP-1)* forward, 5′-GCTCTCT-CTTCCTCCACCAC-3′; *MCP-1* reverse, 5′-GCTTCTT-TGGGACACCTGCT-3′; epidermal growth factor module-containing mucin-like receptor 1 (*Emr1*) forward, 5′-TTTCCTCGCCTGCTTCTTC-3′; *Emr1* reverse, 5′-CCCCGTCTCTGTATTCAACC-3′; interleukin-6 (*IL-6*) forward, 5′-TGGGAAATCGTGGAAATGAG-3′; *IL-6* reverse, 5′-GAAGGACTCTGGCTTTGTCTT-3′; tumor necrosis factor alpha (*TNFα*) forward, 5′-CGTCGTAG-CAAACCACCAA-3′; *TNFα* reverse, 5′-GAGAACCTGG-GAGTAGACAAGG-3′; inducible nitric oxide synthase (*iNOS*) forward, 5′-GGCAGCTACTGGGTCAAAGA-3′; *iNOS* reverse, 5′-TCTGAGGGCTGACACAAGG-3′; *18s* forward, 5′-CGCGGTTCTATTTTGTTGGT-3′; *18s* reverse, 5′-AGTCGGCATCGTTTATGGTC-3′. All primers were obtained from Sigma. Each PCR was performed in a total volume of 8 μl, made from diluted cDNA template, forward and reverse primers (1 μM each), and SYBR Green I master mix (including Taq polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye and dNTP mix). In order to verify the purity of the products, a melting curve was produced after each run by increasing the temperature of the reaction mixtures up to 95°C, by 0.1°C/s, starting at 55°C for 10 s. PCR products were also analyzed by electrophoresis in an ethidium bromide-stained agarose gel to check that a single amplicon of the expected size was indeed obtained. Relative quantification of a target gene was calculated based on efficiency and the crossing point deviation of an unknown sample vs. a control, and expressed in comparison to the reference housekeeping gene *18s* [19]. Target gene mRNA expression normalized by the internal control *18s* was expressed relative to the control group. Data were expressed using both mRNA concentration in each cellular fraction and total mRNA content.

2.8. Statistical analysis

Data are presented as means±S.E.M. Repeated measure analyses of variance was used to determine differences in body weight gain. One-factor ANOVA was used to determine the significance of the differences in tissue weights, plasmatic concentrations of metabolites, mRNA abundances and levels with different treatments. If there was a significant difference, a least significant difference (LSD) test was used to determine the particular effect that caused that difference. $P<.05$ was statistically significant, and different superscripts discriminate differences between groups. The analysis was performed using the SPSS program for Windows version 14 (SPSS, Chicago, IL, USA).

3. Results

3.1. Body and tissue weights, food intake

CLA treatment was accompanied by a decrease in total fat mass (Table 1), particularly with the higher dose of

Table 1
Tissue weights (g) in mice supplemented with CLA

	Control	CLA1	CLA2
White adipose tissues			
Epididymal	0.365±0.008[a]	0.363±0.016[a]	0.240±0.020[b]
Retroperitoneal	0.092±0.007[a]	0.051±0.004[b]	0.036±0.004[c]
Mesenteric	0.230±0.012[a]	0.195±0.012[b]	0.158±0.010[c]
Sum	0.662±0.021[a]	0.609±0.027[a]	0.433±0.029[b]
Brown adipose tissue	0.120±0.005[a]	0.123±0.005[a]	0.088±0.004[b]
Pancreas	0.136±0.013	0.149±0.009	0.152±0.007
Gastrocnemius	0.261±0.013	0.276±0.006	0.271±0.012
Liver	1.061±0.029	1.077±0.037	1.142±0.033
Stomach	0.101±0.003	0.106±0.005	0.109±0.003

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 9–12 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).

CLA (35% lower adipose tissue weights in CLA2 vs. control group; $P<.001$). Although minor effects were seen on the rate of body weight change during the treatment, body weight gain was lower in CLA-treated groups (Fig. 1) (repeated measure analyses of variance: $P<.05$, effect of treatment), which was associated with smaller fat accumulation. Mesenteric and epididymal fat pads were respectively 31% and 34% lower in CLA2 animals vs. controls. Specific sensitivity was seen in the retroperitoneal fat pad, which was diminished twofold (61% lower) compared with the other depots. Treatment with CLA1 also affected the size of the retroperitoneal depot (45% lower) and to a lesser extent the mesenteric (15% lower) with respect to the controls. Neither the weights of liver, pancreas, stomach nor the gastrocnemius muscle was affected by treatment.

The dietary energy intake during the study was not significantly different between groups (286±5.1 in control, 271±30.6 in CLA1 and 280±7.1 kcal/animal in the CLA2 group).

3.2. Insulin tolerance test, homeostasis model assessment and plasmatic factors

Plasma concentrations of glucose and insulin were not statistically different between groups after 3 h of starvation (Table 2). Treatment with CLA did not alter the response to an ITT either measuring the change in plasmatic glucose concentrations (data not shown) or the area under the curve (681±37 in control, 665±44 in CLA1 and 734±39 mmol of glucose/min per liter in the CLA2 group). The homeostasis model assessment index (2.94±0.22 in control, 3.12±0.42 in CLA1 and 3.51±0.32 in the CLA2 group) was not different between groups either.

Plasma adiponectin concentrations in CLA1 were close to control values, whereas in the CLA2 group they were lower than those of control and CLA1 ($P<.001$, Table 2) under both fasting and feeding conditions. Concerning leptin concentrations, no significant effect in CLA groups was seen with respect to the control. However, leptin concentrations were lower in CLA2 with respect to the CLA1 group ($P<.01$, Table 2). Resistin concentrations were not affected by CLA treatment at the lower dose, whereas they showed a diminution in the CLA2 group. The treatment with CLA did not alter the plasma concentrations of glycerol (data not shown), NEFAs or triglycerides (Table 2).

3.3. Gene expression in adipocytes and SVF

CLA treatment showed a tendency to increase mRNA concentration in the SVF and particularly in mature

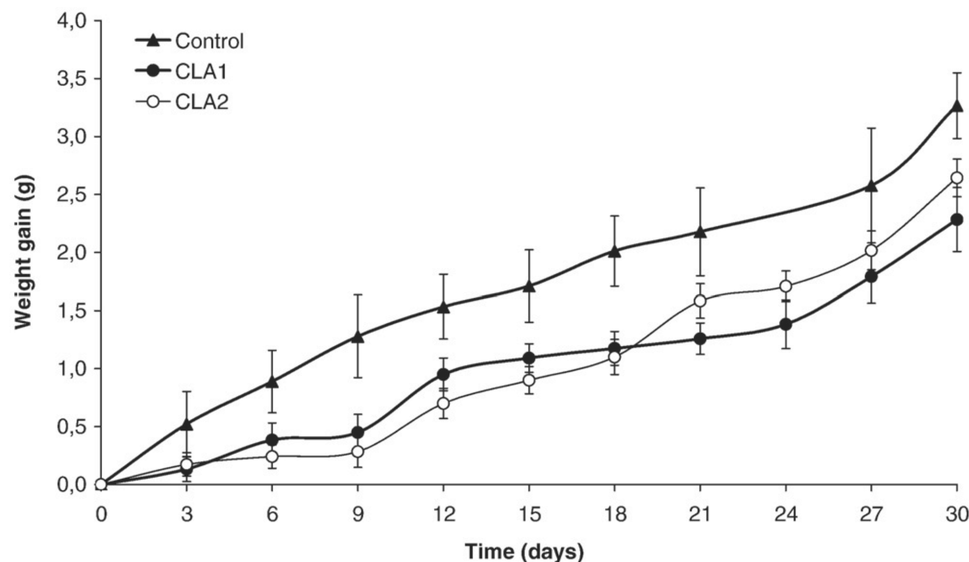

Fig. 1. Effects of CLA on body weight gain in mice. Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 9–12 mice. Repeated measures analyses of variance of decreases in body weight gain in CLA-treated animals were significant ($P<.05$). No differences between doses were found.

Table 2
Effects of CLA treatment on plasmatic concentrations of metabolites in mice

	Control	CLA1	CLA2
Fasting conditions (3h)			
Glucose (mmol/L)	6.7±0.3	6.8±0.2	7.2±0.3
Insulin (μg/L)	0.41±0.07	0.43±0.10	0.49±0.04
Adiponectin (μg/ml)	21.02±0.82^{a}	25.09±2.42^{a}	16.01±0.91^{b}
Feeding conditions			
Adiponectin (μg/ml)	22.62±0.70^{a}	23.75±1.06^{a}	16.43±0.85^{b}
Leptin (ng/ml)	4.84±0.53^{ab}	7.04±0.87^{a}	3.78±0.50^{b}
Leptin/adiponectin	0.22±0.02	0.30±0.03	0.22±0.03
Resistin (ng/ml)	23.33±0.96^{a}	25.99±1.38^{a}	19.16±0.70^{b}
Insulin (μg/L)	0.16±0.04	0.17±0.03	0.18±0.03
NEFAs (mg/dl)	16.66±3.25	13.33±2.58	13.03±2.12
Triglycerides (mg/ml)	0.79±0.09	0.70±0.08	0.67±0.06

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 8–12 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).
Samples from fasting conditions were bled from the tail tip from conscious animals. Samples from fed animals were obtained by cardiac puncture from anesthetized animals. Leptin/adiponectin ratio has been multiplied by a factor of 10^3.

Table 4
Effects of CLA supplementation on relative expression of target mRNA in mature adipocytes and SVF in mice

	Control	CLA1	CLA2
Mature adipocytes			
Adiponectin	100±4^{a}	71±9^{b}	57±3^{b}
Leptin	100±19^{a}	136±1^{a}	57±6^{b}
RBP4	100±7^{a}	126±6^{b}	87±4^{a}
Glut4	100±6^{a}	94±5^{a}	71±4^{b}
PPARγ2	100±1^{a}	80±4^{ab}	63±7^{b}
MCP-1	100±2^{a}	64±8^{b}	28±7^{c}
Stromal vascular fraction			
IL-6	100±17	98±15	72±20
TNFα	100±13	70±8	79±13
iNOS	100±15	71±4	59±11
Emr1	100±18	90±11	97±9
MCP-1	100±17	101±17	89±17
PPARγ2	100±17	72±5	68±12

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 6–8 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).

adipocytes where it attained statistical significance (Table 3). This is of special relevance because of the minor size of adipose depots in CLA-treated mice.

Mature adipocytes of animals treated with CLA showed a lower expression of most of the target mRNA determined and, particularly, at the highest treated dose — adiponectin (57%, $P<.001$), leptin (57%, $P<.038$), Glut4 (71%, $P<.01$), PPARγ2 (63%, $P<.01$) and MCP-1 (28%, $P<.001$) — with respect to the control group (Table 4). However, in the CLA1 group, only the decrease in adiponectin (29% lower, $P<.05$) and MCP-1 (36% lower, $P<.05$) attained statistical significance. The CLA1 group also showed an increase (by 26%, $P<.01$) of RBP4 expression vs. control. No effects of CLA treatment on gene expression were appreciated on the SVF, either on PPARγ2, inflammatory factors (IL-6, TNFα, iNOS), macrophage marker (Emr1) or on the main recruitment macrophage factor MCP-1 (Table 4). Taking into account the important change in adipose size by the CLA treatment and the variation in total mRNA concentration in epididymal adipose tissue, the values of mRNA gene expression were referred to the total mRNA content in the depot studied. Under this new perspective, gene expression data showed a slightly different profile than above. In mature adipocytes, only leptin increased in CLA1 (twofold, $P<.05$) and the tendency to decrease MCP-1 expression was maintained (ANOVA: $P<.05$, effect of treatment); the rest of the values were similar between CLA-treated animals and controls (Fig. 2). In SVF, no changes were observed by CLA, although a nonstatistically significant tendency in the reduction of mRNA expression of TNFα and iNOS was seen (Fig. 3).

Table 3
mRNA levels (μg/g tissue) in mature adipocytes and SVF cells isolated from epididymal adipose tissue in CLA-treated mice

	Control	CLA1	CLA2
Adipocytes	12±1^{a}	15±3^{ab}	23±4^{b}
Stromal vascular fraction	12±1	16±2	17±1

Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. Data are means±S.E.M. of 6–8 mice. Means in a row without a common letter differ, $P<.05$ (one-factor ANOVA followed by LSD test).

4. Discussion

In this study, we have demonstrated that supplementation with moderate doses of an equimolar mix of *cis*-9, *trans*-11 and *trans*-10,*cis*-12 CLA in mice fed with a standard-fat diet allows for the maintenance of insulin sensitivity and does not lead to the infiltration of macrophages in adipose tissue or to the induction of expression of pro-inflammatory cytokines.

The commercial product Tonalin was selected as the source of dietary CLA because most human studies designed to assess the effects on body weight and/or composition are done using this or other available products sharing its characteristics (i.e., containing equimolar mix of both bioactive CLA isomers). Mice have been described as an animal model particularly sensitive to potential adverse effects of CLA administration [15,20], and the *trans*-10,*cis*-12 isomer seems to be the most adverse [18,21,22]. However, most animal studies have been performed with diets supplemented with 0.5–1.5% CLA, combining either a mixture of the two active isomers or only one of them (for review, see Refs. [17,23,24]). Our focus was to assess in mice the effects of the CLA used in humans but at

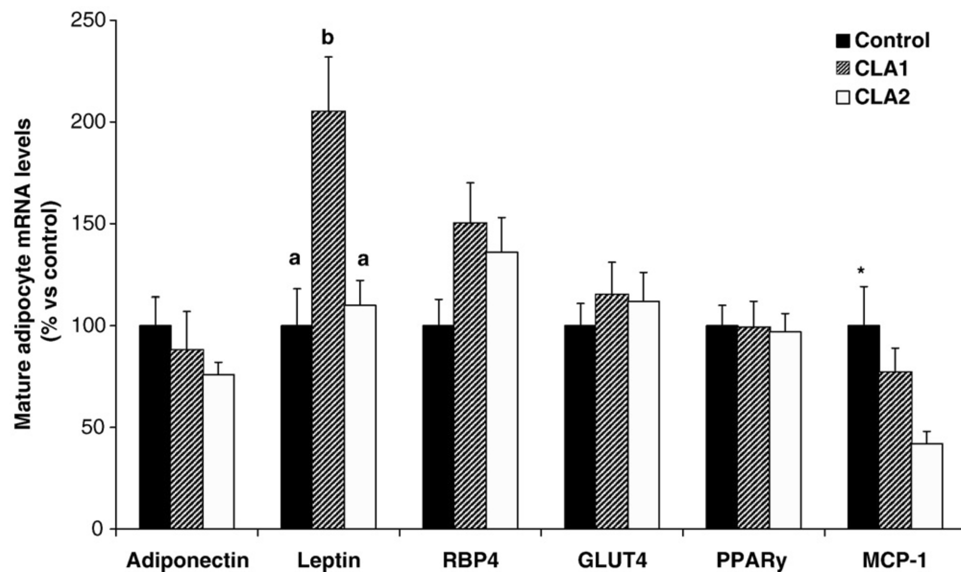

Fig. 2. Contribution of mature adipocytes isolated from epididymal fat depot to the expression of target mRNA in CLA-treated mice. Initial data, derived from equal total amount of RNA (shown in Table 4), were referred to the total mRNA content in the depot. Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. mRNA expression of the control group was designated as 100% to normalize the mRNA expression of CLA-treated groups to this value. Error bars represent means±S.E.M. (n=5–8). By one-factor ANOVA only leptin gene expression increased in CLA1 (ANOVA followed by LSD test, bars without a common letter differ, P<.05), and a significant tendency to decrease MCP-1 gene expression by treatment was seen (*). The rest of the genes were not affected by CLA treatment.

lower doses than those usually used in mice. When this amount is referred to the weight of the animals, this implies a daily dose that is around 50 times higher (1% CLA) than doses successfully used in human trials [1]. Our approach was to test a range of doses (150 and 500 mg CLA/kg per day, in CLA1 and CLA2, respectively) that are about 3–10 times (per kilogram of body weight) the 3.4 g CLA/day usually recommended for use in humans (assuming a body weight of 70 kg). In this way, taking into account that mice are the most sensitive specie [25], it is intended here to assess the efficacy of the treatment in mice, aiming to moderately reduce body fat content without showing some of the adverse effects reported. In addition, we were interested in doses of CLA below the nonobserved adverse effects levels, seeking for the

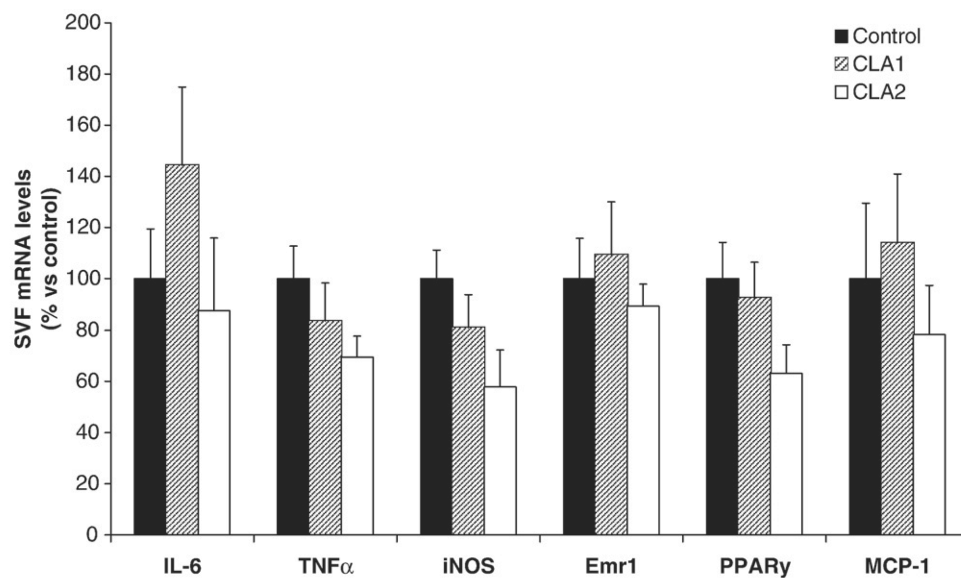

Fig. 3. Contribution of SVF isolated from epididymal fat depot to the expression of target mRNA in CLA-treated mice. Initial data, derived from equal total amount of RNA (shown in Table 4), were referred to the total mRNA content in the depot. Two doses of CLA were assayed: CLA1=0.15 g CLA/kg body weight and CLA2=0.50 g CLA/kg body weight. mRNA expression of the control group was designated as 100% to normalize the mRNA expression of CLA-treated groups to this value. Error bars represent means±S.E.M. (n=6–8). By one-factor ANOVA, CLA treatment did not affect gene expression.

compromise between safety and efficacy, which has been estimated to correspond to a CLA intake of about 500 mg/kg per day [20].

As expected, we found discrete effects on body weight. However, CLA caused a clear reduction in fat mass at the doses tested and no other tissue weights were affected. Contrary to other studies in mice using higher doses [21], no lipodystrophy was caused, even when the treatment was prolonged for more than 1 month, and the modest fat reduction found resembled what is seen in human trials [1]. Retroperitoneal and mesenteric tissues were the most sensitive adipose tissues, which showed decreased weight at the lower CLA dose used, while mobilization of epididymal fat pad required the higher dose of CLA. These results are in accordance with the finding that body fat reduction by CLA is also region specific in humans [3]. The referred human study included mainly women, and the CLA was more active in decreasing fat in the legs, while showing also a tendency to reduce abdominal fat when considering both sexes. Here we show that abdominal fat was the most sensitive depot to the action of CLA in male mice.

Increased inflammatory properties of adipose tissue macrophages recruited during CLA supplementation have been recently reported [18], and these cells are a considerable source of TNFα and IL-6 that can induce insulin resistance in adipocytes [26]. Adipocytes are able to synthesize and secrete the chemokine MCP-1, a recruiting factor for circulating monocytes [27], as well as nonfat cells present in adipose tissue [28,29]. These and other data are giving strong support to the fact that adipose tissue is a potent source of interleukins and other cytokines, but the majority of this release is due to nonfat cells in the adipose tissue [30]. In order to establish the specific role of both adipocytes and nonfat cells, under CLA supplementation, adipose tissue cellular fractioning and separation were performed and both types of cells were independently analyzed for expression of inflammatory factors and related markers. Epididymal fat depot was chosen as a representative adipose tissue because it has been reported that macrophage content and transcripts expression are comparable between different adipose depots in mice [31], and it is the largest among those easily distinguished anatomically, allowing then to minimize the number of animals.

Isolation of mRNA from epididymal fat revealed the presence of a higher amount in adipocytes from the CLA2 group. In PCR determinations, we performed gene amplification from samples containing an equal amount of total RNA in the different groups studied. Therefore, these results represented the gene abundance from samples containing the same amount of total RNA, but they could reflect a different number of adipocytes and this distinction can be of particular significance when CLA causes a reduction of fat depots together with an increase in total RNA content. To interpret our data in a more physiologically meaningful way, we calculated the changes in gene expression referred to the total tissue contribution and also in the conventional way, as transcript per gram of tissue, to be able to compare our data with previous references. Adiponectin and leptin gene expression in adipocytes showed a similar trend to that seen in related studies [18]. However, this profile changed — for example, leptin increase was magnified in the CLA1 group and adiponectin decrease was attenuated in CLA-treated mice — when the contribution of the depot to the whole organism was taken into account. From this angle, expression levels of Glut4 transporter and RBP4 protein in adipocytes were compatible with a status of insulin sensitivity, in close accordance with the other biomarkers studied. Our results point to the importance of taking into account not only the specific gene expression but also the total contribution of the organ of interest in CLA studies.

Feeding high doses of CLA to mice has been associated with impairment of insulin sensitivity [15,16,21,22,32], which has been attributed to a rapid and significant reduction of adipose tissue and a sharp decline in insulin-sensitizing adipokines such as leptin and adiponectin [22]. In this study, glucose and insulin were not altered by CLA treatment and maintenance of glucose homeostasis was observed even under insulin overload, suggesting that the fat loss, although moderate, was slow enough to keep up the metabolic control on insulin sensitivity.

Although higher leptin expression was found in epididymal adipocytes from CLA1 animals, the plasma concentration in CLA-treated animals was not different from control animals (although CLA2 showed lower concentration than CLA1 animals). Although we cannot rule out opposite changes in other synthesizing organs, in any case, plasma concentration was in accordance with the moderate reduction seen in fat mass [33], which was more efficient at the higher dose of CLA. Another aspect in our study concerns the circulating concentrations of inflammatory adipokines. Leptin and resistin are considered pro-inflammatory cytokines [34,35], while adiponectin seems to have an anti-inflammatory role [36] and decreases in association with insulin resistance, type 2 diabetes and visceral adiposity [37]. Therefore, the lack of change (in CLA1 group) or the reduction (in CLA2 group) in plasma resistin contributes, together with leptin, to preserving an anti-inflammatory profile in the CLA-treated mice. Concerning the role of adiponectin, recent studies have shown an improvement in glucose/insulin metabolism without increasing plasma adiponectin [38] and, in general, plasma concentrations correlate well with expression in adipose tissue [37]. In our animals, plasma adiponectin decreased only at the higher dose of CLA tested, whereas expression in epididymal adipocytes showed a tendency to decrease following a dose–response pattern without attaining statistical significance. Interestingly, the leptin/adiponectin ratio is not altered by CLA treatment at the doses tested in our study. This could suggest that the decrease of leptin (accompanying fat loss) was in concordance with lower adiponectin levels, reaching a novel set point between these two cytokines that still corresponds to the range of normality (seen by weight loss, maintenance of insulin sensitivity, lack of macrophage

infiltration and nonexpression of inflammatory markers). Our results are in close agreement with a recent study that, using ob/ob mice fed with 1.5% CLA, found a depletion of both leptin and adiponectin and this is accompanied by insulin resistance and macrophage infiltration. However, when mice received a daily injection of leptin, depletion of adiponectin is not so strong and there is neither insulin resistance nor macrophage infiltration, although they find hepatic steatosis [39]. Authors suggest that a basal level of both leptin and adiponectin may be critical to maintaining energy homeostasis, and our doses tested seem to fulfill this requirement.

A number of studies show that adipose tissue contains macrophages that participate in the inflammatory changes found in obesity and contribute to insulin resistance and hepatic steatosis [31,40–43]; as mentioned above, a recent paper has also found that nutritional supplementation with *trans*-10,*cis*-12-CLA induces local infiltration of macrophages in adipose tissue and induction of pro-inflammatory cytokines and MCP-1 expression [18]. Interestingly, mice supplemented with a *cis*-9,*trans*-11-CLA diet show a reduction in macrophage infiltration and a marked down-regulation of several inflammatory markers in adipose tissue [44]. Therefore, it was of great interest to assess the inflammatory role of potentially infiltrated macrophages in adipose tissue of mice fed with both active isomers, replicating conditions of human CLA treatments (lower doses, both isomers and in mice subjects as sensitive species), whereas to assess the specific role of each isomer per se under these conditions was out of the scope of the present study.

MCP-1 is a critical factor in the recruitment of macrophages to sites of injury and inflammation [45]. Genetic deficiency of MCP-1 and its receptor chemokine (C-C motif) receptor 2 (CCR2) reduces macrophage accumulation in adipose tissue of high fat-fed obese mice and partially protects against the development of obesity. On the other hand, mice overexpressing MCP-1 have increased numbers of macrophages in adipose tissue along with increased insulin resistance [46,47]. The expression of MCP-1 showed a decrease by CLA treatment in isolated mature adipocytes. This was in accordance with the lack of macrophage recruitment in adipose tissue, reflected by Emr1 and MCP-1 markers in SVF. Furthermore, the absence of higher pro-inflammatory cytokine expression (IL-6 and TNFα), PPARγ2 and iNOS induction in SVF was a result indicative of an absence of macrophage infiltration and inflammation in CLA-treated animals.

In summary, the use of moderate doses of the mixture of the two main active isomers may be preferable to the use of the single compounds when considering inflammatory potential and insulin tolerance. When given together as a mixture, the antagonistic role of *cis*-9,*trans*-11-CLA [44] and *trans*-10,*cis*-12-CLA [18] may compensate each other, resulting in changes in adiposity without detrimental effects. In our study, CLA treatment was able to attenuate body fat deposition in mice fed with a standard diet, without impairment of insulin sensitivity and in the absence of pro-inflammatory outcomes in adipose tissue. Conversely, an anti-inflammatory status in adipose tissue seems to be promoted by CLA which may help in the preservation of normal adipose tissue function without adverse consequences.

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MANUSCRITO IV

Moderate doses of conjugated linoleic acid reduce fat gain, maintain insulin sensitivity without impairing inflammatory adipose tissue status in mice fed a high-fat diet

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Nutr Metab (Lond), Vol. 7:5 (2010)

Moderate doses of conjugated linoleic acid reduce fat gain, maintain insulin sensitivity without impairing inflammatory adipose tissue status in mice fed a high-fat diet

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Abstract

Background: The enrichment of diet with nutrients with potential benefits on body composition is a strategy to combat obesity. Conjugated linoleic acid (CLA) due its beneficial effects on body composition and inflammatory processes becomes an interesting candidate, since the promotion and impairment of obesity is closely linked to a low-grade inflammation state of adipose tissue. Previously we reported the favourable effects of moderate doses of CLA mixture on body composition and inflammatory status of adipose tissue in mice fed a standard-fat diet. In the present study we assessed the potential beneficial effects of CLA mixture (*cis*-9,*trans*-11 and *trans*-10,*cis*-12, 50:50) in mice fed a high-fat diet.

Methods: Two doses were assayed: 0.15 g (CLA1) and 0.5 g CLA/kg body weight (CLA2) for the first 30 days of the study and then animals received a double amount for another 35 days.

Results: The lowest dose (CLA1) had minor effects on body composition, plasma parameters and gene expression. However, a clear reduction in fat accumulation was achieved by CLA2, accompanied by a reduction in leptin, adiponectin and non-esterified fatty acids (NEFA) plasma concentrations. Insulin sensitivity was maintained despite a slight increase in fasting glucose and insulin plasma concentrations. The study of gene expression both in adipocytes and in the stromal vascular fraction (SVF) suggested that CLA may reduce either the infiltration of macrophages in adipose tissue or the induction of expression of pro-inflammatory cytokines.

Conclusion: In conclusion, the use of moderate doses of an equimolar mix of the two main CLA isomers reduces body fat content, improves plasma lipid profile, maintains insulin sensitivity (despite a moderate degree of hyperinsulinaemia) without the promotion of inflammatory markers in adipose tissue of mice fed a high-fat diet.

Background

CLA refers to a group of positional and geometric isomers of linoleic acid and has been extensively studied due to its potential beneficial effects in several diseases including cancer, atherosclerosis, diabetes and obesity [1, 2]. It has been suggested that the anti-carcinogenic and anti-atherosclerosis effect of CLA stems from its anti-inflammatory properties. One of the mechanisms proposed by which CLA could exert its anti-inflammatory effect is by the negative regulation of gene expression of inflammation mediators [3]. The increased size of adipose depots in obesity is related to a certain degree of inflammation which may be involved in the pathophysiology of obesity-associated disorders. This low-grade inflammatory state has been associated with the progressive infiltration of macrophages into adipose tissue, which may be the main source of pro-inflammatory cytokines and associated insulin resistance in obesity [4, 5]. Furthermore, most studies conducted in animals demonstrated that CLA reduces body fat mass [6] with the *trans*-10,*cis*-12 CLA isomer mainly responsible for this effect [1, 7]. Therefore CLA, due to its beneficial potential effects on both body composition and inflammation, becomes an interesting nutritional strategy in the treatment of obesity. However, in some studies conducted in mice –the most sensitive species– fat loss triggered by CLA was accompanied by deleterious side effects such as insulin resistance, hyperinsulinaemia and liver steatosis [8-11]. Both beneficial and detrimental effects of CLA supplementation are more modest or less evident in human studies. However, a recent meta-analysis of human studies supports a modest effect of CLA reducing body fat [12].

Controversial results about the anti-inflammatory properties of CLA also exist. *In vitro* data demonstrate that *trans*-10, *cis*-12 CLA activates NFκB- and ERK1/2-dependent IL-6, IL-8, and TNFα production, which impairs adipogenic gene expression and glucose

uptake [13]. Furthermore, supplementation with *trans*-10,*cis*-12 CLA promotes macrophage infiltration into adipose tissue, contributing to adipose tissue inflammation and insulin resistance [14]. In contrast, treatment with *cis*-9,*trans*-11 CLA reduces macrophage infiltration and attenuates the inflammatory profile of obese adipose tissue [15]. Interestingly, we have shown that the use of moderate doses of an approximately equimolar mixture of both main CLA isomers achieves a modest reduction of fat gain, ameliorates macrophage infiltration into adipose tissue and expression of pro-inflammatory cytokines, therefore, contributing to preserve adipose function [16].

In the present study, we analyze the potential effects of moderate doses of CLA mixture on body composition and insulin sensitivity, as well as on adipose tissue inflammatory profile in mice fed a high-fat diet.

Methods

Animals

Male mice (C57BL/6J) from Charles River (Barcelona, Spain) were housed in groups of four in plastic cages, acclimated to 22ºC with a 12 h light/12 h dark cycle. Animals were fed *ad libitum* with a high-fat diet (D12451, Research Diets Inc, New Brunswick) which contains 45% calorie content as fat, 35% calorie content as carbohydrate and the remaining 20% as protein. Food intake and body weight were recorded every three days during the experiment. Total calories consumed was measured for each cage and expressed as the average of the two cages per group. Fresh food was provided to the mice biweekly. At 30 days of treatment, animals were starved for 3 h, tail blood samples were obtained to perform plasma determinations and were then submitted to the insulin tolerance test (ITT). 35 days later, animals were sacrificed under fasting conditions (10 h).

All experimental procedures were performed according to both national and institutional guidelines for animal care and use.

CLA Treatment

The CLA used was Tonalin ® TG 80 derived from safflower oil (kindly provided by Cognis). Tonalin is composed of triglycerides containing approximately 80% CLA with a 50:50 ratio of the active CLA isomers *cis*-9,*trans*-11 and *trans*-10,*cis*-12.

Mice weighing 20 ± 0.2 g (5-week-old) were randomly assigned to three experimental oral treatments: sunflower oil (control group, n=8), CLA1 (n=8) or CLA2 (n=8) for 65 days. For the first 30 days, two doses of CLA were assessed: CLA1 (0.15 g CLA/kg body weight) and CLA2 (0.50 g CLA/kg body weight), taking the weight of the animals at the beginning of the experiment as a reference. After 30 days of treatment and until the end of the experiment, the corresponding dose of each group was doubled. Therefore, animals received a daily amount of Tonalin equivalent to 3 mg CLA/animal in CLA1 group and 10 mg/animal in CLA2 group for the first 30 days and 6 mg CLA/animal in CLA1 group and 20 mg/animal in CLA2 group for the last 35 days of treatment. An adequate amount of commercial sunflower oil was given to the animals to achieve isocaloric load between groups.

Insulin tolerance test

ITT was performed on day 30 of the study after 3 h fast. Recombinant human insulin (Humulin R; Eli Lilly, Spain), previously diluted in 0.9% saline, was intraperitoneally injected (0.8 U/kg body weight). Blood glucose concentration was determined from tail blood samples before and at 15, 30, 60, 90, and 120 min postinjection using an Accu Check Sensor (Roche Diagnostics, Barcelona, Spain). The area under the curve for each

mice was calculated using the KaleidaGraph software version 3.0 (Synergy Software, Reading, PA, U.S.A.), and the mean value ± SEM calculated for each group.

Sacrifice and tissue sampling

Mice were anaesthetised by intraperitoneal injection of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight) and blood was collected by cardiac puncture. Liver, brown and white adipose depots were rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at -70ºC. Blood collected by cardiac puncture with heparinized syringe and needle (0.2% heparin diluted with saline, Sigma, Madrid, Spain) was centrifuged at 1000 g for 10 min at 4°C and plasma obtained was stored at -70ºC for later analysis.

Plasma analysis

Adiponectin and insulin plasma concentrations were measured using a rat/mouse adiponectin ELISA kit (Phoenix Europe GmbH, Karlsruhe, Germany) and Insulin Mouse Ultrasensitive ELISA kit (DRGInstruments GmbH, Marburg, Germany) respectively. Resistin and leptin plasma concentrations were also assessed by ELISA using the following commercial kits: Mouse Resistin Quantikine ELISA kit and Mouse Leptin Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA). Commercial enzymatic colorimetric kits were used for the determination of plasma NEFA (Wako Chemicals GmbH, Neuss, Germany) and circulating concentrations of triglycerides (Sigma Diagnostics, Madrid, Spain).

Hepatic triglyceride quantification

A sample of liver (200–300 mg) was homogenized in PBS (1:2, wt:v) using a polytron homogenizer. Homogenates were centrifuged at 500*g* for 10 min and the supernatant was used for the quantification. Total triglyceride levels were measured using a commercial enzymatic colorimetric kit following standard procedures (Sigma Diagnostics, Madrid, Spain).

Isolation of mature adipocytes and SVF from epididymal fat depots

Fresh epididymal white adipose tissue was digested with collagenase and after filtration and washing steps the SVF and the mature adipocyte-enriched fraction were obtained following the protocol previously described [16].

RT-PCR reaction analysis

Total RNA from mature adipocytes and SVF were extracted using the RNAeasy Mini Kit from Qiagen (Barcelona, Spain). RNA was quantified using the NanoDrop®Spetrophotometer ND-1000. RT-PCR was used to measure mRNA expression levels of target genes. Aliquots of 0.5 μg of total RNA (in a final volume of 10 μL) were denatured at 90°C for 1 min and then reverse-transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 42°C for 60 min, with a final step of 5 min at 99°C in a Perkin-Elmer 9700 Thermal Cycler (PerkinElmer, Wellesley, MA). RT-PCR was completed using the LightCycler System with SYBR Green I (Roche Diagnostic Gmbh, Mannheim, Germany). Primer sequences are listed in Table 1. All primers were purchased from Sigma (Madrid, Spain). Each PCR was performed in a total volume of 8 μL, made from diluted cDNA template, forward and reverse primers (1 μmol/L each), and SYBR Green I master mix (including Taq

polymerase, reaction buffer, $MgCl_2$, SYBR Green I dye, and dNTP mix). In order to verify the purity of the products, a melting curve was produced after each run by increasing the temperature of the reaction mixtures up to 95ºC, by 0.1°C/s, starting at 55ºC for 10 s. PCR products were also analyzed by electrophoresis in an ethidium bromide-stained agarose gel to check that a single amplicon of the expected size was indeed obtained.

The relative quantification of each target gene (*adiponectin*, *leptin*, *MPC1*, *Emr1*, *IL-6*, *TNFα*, and *iNOS*) was calculated based on efficiency and the crossing point deviation of an unknown sample versus a control, and normalized by the expression of the reference housekeeping gene *18S* rRNA [17]. Results from CLA treated groups were expressed as fold induction relative to the control group. Data were expressed using both mRNA concentration in each cellular fraction and total mRNA content.

Statistical analysis

Data are presented as means ± SEM. Repeated-measures ANOVA was used to determine differences in body weight gain. One-way ANOVA was used to determine the significance of the differences in tissue weights, plasma concentrations of metabolites, mRNA abundance and levels with different treatments. If there was a significant difference, a Least Significant Difference (LSD) test was used to determine the particular effect that caused that difference. $P<0.05$ was statistically significant, and different superscripts discriminate differences between groups. The analysis was performed using the SPSS program for Windows version 14 (SPSS, Chicago, IL, USA).

Results

Body and tissue weights and energy intake

After 30 d of CLA treatment, no evident effects on the rate of body weight gain were observed (Figure 1). Since circulating leptin levels are proportional to overall adipose mass rather than body weight, plasma leptin concentration was also determined at this time-point and no differences between groups were found. As previously suggested, fat content in diet could determine the effectiveness of CLA doses [18]. Therefore, considering that the amount of CLA administered didn't seem to have a significant effect on body fat content, we decided to double the doses from day 30 onwards. Accordingly, mice started to receive 251 mg CLA/kg body weight and day in CLA1 group and 414 mg CLA/kg body weight and day in CLA2 group until the end of the experiment.

Then, body weight reached at the end of the treatment was still not statistically different between control and CLA supplemented animals. However, lower body weight gain was observed during the treatment by CLA (Figure 1) (repeated-measures ANOVA: $P<0.05$, effect of time × treatment) and the effects were more noticeable from day 30 onwards, with doubled doses, and in the CLA2 group, in which the increase in body weight gain for the last 30 days of study was 39% lower than in control group.

In the course of the study, no differences in total energy consumed were found between groups (2428 ± 119 in control, 2263 ± 104 in CLA1 and 2382 ± 5 kJ/animal in CLA2 group, each group n = 8). Adiposity was significantly reduced with the highest dose of CLA (47% lower vs. control group, $P<0.001$) and weights of epididymal, retroperitoneal and brown adipose tissue were significantly lower in this group (Table 2). This effect was more marked in retroperitoneal (67% lower) and epididymal (56% lower) depots than in brown adipose tissue (20% lower) while mesenteric depot was not

affected at all. CLA1 group experienced only a significant reduction in retroperitoneal fat depot.

Liver weight and triglyceride content

There was no effect of CLA treatment on the weight of liver (0.876 ± 0.03 in control, 0.932 ± 0.025 in CLA1 and 0.970 ± 0.052 g in CLA2 group, n = 8). No changes in hepatic triglyceride content were observed after CLA treatment (50.49 ± 2.98 in control, 55.66 ± 2.12 in CLA1 and 50.72 ± 3.36 mg triglycerides/g liver, n = 8).

Plasma parameters

Plasma glucose, adiponectin and leptin concentrations were not different between groups after 30 days of treatment (Table 3).

Adiponectin and leptin concentrations were significantly decreased with the highest dose of CLA at the end of the study (Table 3). No significant differences in circulating resistin concentration were found between groups and the same was seen concerning plasma triglycerides. NEFA concentration decreased in both CLA treated groups while plasma glycerol concentration decreased only in CLA2 group (Table 3). Insulin concentration increased with CLA treatment ($P<0.01$) (16% and 25% in CLA1 and CLA2 group, respectively) and CLA2 group presented higher fasting glucose concentration than CLA1 ($P<0.05$) (Table 3).

ITT and calculated indices

No differences between groups were observed in the ITT carried out at day 30 of treatment, either measuring the change in plasma glucose concentration (data not shown) or the area under the curve (756 ± 41 in control, 794 ± 46 in CLA1 and 913 ±

81 mmol glucose · min/L in CLA2 group, n = 5-6). The calculated homeostatic model assessment for insulin resistance (HOMA-IR) was higher in CLA2 group at the end of the study (Table 3) (*P*<0.001). However, the calculated revised quantitative insulin sensitivity check index (R-QUICKI) showed no differences between groups.

Gene expression in adipocytes and SVF

Adiponectin and leptin mRNAs were dose-dependently reduced by CLA treatment in mature adipocytes (*P*<0.001). MCP1 mRNA expression was reduced with the highest dose with respect to both control and CLA1 groups, while it was increased in adipocytes from CLA1 with respect to both control and CLA2 groups (Table 4).

No effects of CLA treatment on iNOS and MCP1 gene expression were appreciated on the SVF (Table 4). The highest CLA dose achieved a reduction in IL-6 gene expression (*P*<0.05) and an increase in Emr1 (*P*<0.001) with respect to both control and CLA1 groups. Meanwhile TNFα gene expression was increased in SVF of CLA2 animals with respect to the control group (*P*<0.01) (Table 4).

Interestingly, CLA treatment showed a tendency to increase RNA yield, particularly in the adipocyte fraction where it attained statistical significance (Table 5). This is of special relevance because of the minor size of adipose depots in mice treated with CLA. For this reason, gene expression in mature adipocytes (Figure 2) and SVF (Figure 3) was referred to the total RNA content of the respective epididymal fraction in order to attain a closer physiological view of the endocrine function of the fat depot and its potential for macrophage recruitment. Under this novel perspective, gene expression data showed a slightly different profile than above, whereas MCP1 adipose gene expression was unaffected, adiponectin and leptin decreased only with the highest dose of CLA in mature adipocytes (Figure 2), therefore, total contribution of mature

adipocytes reflected in a better way, the plasma levels of the two adipocytokines. Concerning the expression profile in cells from the SVF, the decrease in IL-6 gene expression with the highest dose was maintained (Figure 3) and the same tendency was now evident in CLA1 group. Furthermore, TNFα and Emr1 gene expression were not affected by CLA treatment, while iNOS gene expression decreased with both doses of CLA. A reduction of MCP1 gene expression, although not statistically significant, was observed with CLA2 dose in relation to control group.

Discussion

Mice constitute an animal model particularly sensitive to potential deleterious side effects of CLA such as insulin resistance, hyperinsulinaemia and liver steatosis [7-11]. Most previous studies have made use of enriched diets, containing between 0.5 and 1.5% CLA (for review, see [6, 19]) which in this animal model, supply a daily dose around 50 times higher than those successfully used in human trials [12, 20]. Therefore, the adverse side effects seen in mice could be due to the use of large doses rather than the use of optimal doses which would reduce body fat content without showing any of the adverse effects reported. According to this hypothesis, we have previously reported that mice fed with a standard-fat diet and treated with moderate doses of the commercial product Tonalin® - an equimolar mix of *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers - show reduced body weight gain and lower fat depots without any of the adverse effects associated with CLA treatments [16]. Therefore, similar doses of the CLA mixture were used in the current study, in order to assess their efficacy in animals with susceptibility to diet-induced body weight gain and exposure to a high-fat diet.

A slight effect on reducing body weight gain with CLA treatment was observed by the end of the study. In fact, the lower dose of CLA reduced only the retroperitoneal fat

depot, which seems to be the most sensitive to CLA effects [16, 21, 22], whereas the highest dose also reduced the size of the epididymal depot, supporting the fact that CLA effects are tissue-specific as seen in humans [23]. In brief, the administration of CLA reduces the gain of weight and fat observed in control group, suggesting that the administration of CLA may mitigate the effects of an obesity-promoting environment.

A reduction in adiposity is usually associated with improved insulin sensitivity and plasma adipocytokine profile, but this is not so clear when the fat loss is caused by CLA supplementation. While some studies have shown beneficial effects in rat models [24-26] and in mice [16, 25], several have observed harmful effects of CLA on insulin sensitivity, particularly in mice [8, 9, 11, 14, 27]. Here, the higher dose of CLA caused a reduction in both, plasma leptin and adiponectin concentrations (Table 3); which could be attributed to the reduction of fat depots, the main synthesizing organs, and also to the reduction in absolute terms of its gene expression (Figure 2). Deregulation in the production of these two adipocytokines has been observed in both obese and lipodystrophy states [28] and has been proposed to contribute to the impairment of insulin sensitivity [8]. Lipodystrophy may occur in mice treated with high doses of CLA, due to its higher sensitivity to the CLA-induced reduction in body fat [11, 29, 30]. In these conditions, the drastic plasma reduction of leptin and adiponectin associated with CLA treatment, induces fatty liver and hyperinsulinaemia, not through the direct induction of hepatic lipid synthesis and insulin resistance, but because of the scarcity of the adipose tissue [30]. Interestingly, the doses of CLA tested here were associated with an important reduction in body fat, but without reaching the lipodystrophy status. Although fat loss was accompanied by a moderate degree of hyperinsulinaemia (25% increase) it was far from the 300-400% increase found in other studies using higher doses [8, 10, 31]. No hepatic steatosis or liver enlargement was observed and it was

accompanied by maintenance of insulin sensitivity, as particularly indicated by ITT and R-QUICKI, despite the higher HOMA-IR index. In fact, R-QUICKI has been described as more accurate than HOMA-IR, as surrogate marker to assess insulin sensitivity incorporating the level of fasting NEFA together with insulin and glucose levels [32, 33]. In consequence, the decreased circulating concentrations of leptin and adiponectin promoted by CLA treatment were consistent with maintenance of glucose-insulin homeostasis, as seen in normal-fat fed mice, where CLA causes fat loss, decreases leptin and goes in hand with lower adiponectin levels, reaching a novel set point between these two circulating adipocytokines, which is associated with the maintenance of insulin sensitivity and a decrease in the expression of inflammatory markers in adipose tissue [16]. The relative amount of these two adipocytokines is likely to be more important than their absolute concentrations. Thus, for example, lipoatrophy-associated insulin resistance can be completely reversed by the combination of adiponectin and leptin, but only partially by either adiponectin or leptin alone [28].

Concerning the effects of CLA on the inflammatory profile of adipose tissue, supplementation with CLA may induce inflammatory gene expression in adipocytes and promote macrophage infiltration into adipose tissue showing isomer specific dependence as seen for *trans*-10,*cis*-12 CLA [13, 14, 34, 35] but not for *cis*-9,*tras*-11 CLA [15] either for the mix of both isomers under normal fat diet [16].

In accordance with the minor outcome on fat reduction, minor effects on gene expression were also seen in the group that received the lowest dose of CLA, whereas the highest dose of CLA had a major impact on adipose and SVF gene expression profile. Expression of MCP1, a chemoattractant protein which promotes recruitment of macrophages into adipose tissue and, therefore, inflammatory responses in obesity [36], was decreased in adipocytes (Figure 2) and showed the same tendency in SVF of CLA2

group (Figure 3). This was accompanied by a reduction in the expression of pro-inflammatory mediators such as IL-6 and iNOS and unaltered expression of both TNFα and the macrophage marker Emr1. Proinflammatory cytokines have been shown to promote adipocyte delipidation and impair insulin signaling [13, 37, 38]. In fact, *trans*-10,*cis*-12 CLA was reported to induce IL-6 secretion which seemed to be, at least in part, responsible for the isomer-mediated suppression of PPARγ target gene expression and impairment of insulin sensitivity in mature human adipocytes [13]. Collectively, our data suggested that, particularly at the highest dose tested, CLA supplementation may ameliorate the inflammatory state in obesity, attenuating macrophage infiltration and/or activation into adipose tissue, as seen in animals fed with a standard-fat diet [16] but not with higher doses [10] or by administration of the single *trans*-10,*cis*-12 CLA isomer [14].

Conclusion

In conclusion, an equimolar mix of the two main CLA isomers, at a moderate dose, was able to mitigate body fat accumulation by high fat feeding, and in contrast to studies with larger doses of CLA and particularly with pure *trans*-10,*cis*-12 isomer, this was associated with an improvement of the lipid profile in plasma and maintenance of insulin sensitivity, despite a moderate degree of hyperinsulinaemia, which was far from the 3-4 fold increase observed with higher doses and *trans*-10,*cis*-12 isomer. Furthermore, in our experimental conditions, CLA seems to ameliorate the inflammatory profile in adipose tissue, causing a reduction in the expression of MCP1, the main macrophage recruitment factor, and a decrease in the expression of the pro-inflammatory mediators iNOS and IL-6.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

PP was responsible for animal care, experimental work, acquisition of data, statistical analysis, and manuscript preparation. She has also collaborated in study design and interpretation of data. FS and AP have equally contributed to the conception and design of the study, interpretation of data and drafting of the manuscript.

All authors read and approved the final manuscript.

Acknowledgements

This work was supported by a grant from the Spanish Government (AGL2006-04887). Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, European Union Contract No. FP6-506360). P. Parra is a recipient of a fellowship from the Spanish Government. CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative from ISCIII.

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Figures

Figure 1 - Effects of CLA on body weight gain in mice

Mice received a daily dose of CLA equivalent to 3 mg CLA/animal in CLA1 group and 10 mg/animal in CLA2 group for the first 30 d and 6 mg CLA/animal in CLA1 group and 20 mg/animal in CLA2 group for the last 35 d of treatment. Data are means ± SEM of 8 mice. Repeated-measures analysis of variance of body weight gain associated with CLA treatment was significant with respect to the control ($P<0.05$). No differences between doses were found. x2dose: indicates the point from which the double dose was given.

Figure 2 - Contribution of mature adipocytes isolated from epididymal fat depot to the expression of target mRNA in CLA treated mice

Epididymal adipose tissue was digested by collagenase and then separated into mature adipocytes and stromal vascular fraction. Expression levels of target genes of each fraction were measured by real time PCR and normalized by the internal housekeeping gene *18S* rRNA. Expression data in adipocytes, derived from equal amount of RNA (Table 4), were referred to the total RNA content in the adipocyte fraction. Data, means ± SEM of 7-8 mice, are represented as fold induction over control group. Mean values with unlike letters are significantly different ($P<0.01$); ANOVA followed by LSD test.

Figure 3 - Contribution of SVF cells isolated from epididymal fat depot to the expression of target mRNA in CLA mice

Epididymal adipose tissue was digested by collagenase and then separated into mature adipocytes and stromal vascular fraction. Expression levels of target genes of each fraction were measured by real time PCR and normalized by the internal housekeeping gene *18S* rRNA. Expression data in the stromal vascular fraction (SVF), derived from equal amount of RNA (Table 4) were referred to the total RNA content in SVF. Data, means ± SEM of 6-8 mice, are represented as fold induction over control group. Mean values with unlike letters are significantly different ($P<0.01$); ANOVA followed by LSD test.

Tables

TABLE 1 - Gene-specific primer sequences used in real-time PCR amplification

Gene	Primer sequence (5' → 3')	Product length (bp)	Primer efficiency
Adiponectin	F: GCTCAGGATGCTACTGTTG R: TCTCACCCTTAGGACCAAG	255	1.9
Leptin	F: TTGTCACCAGGATCAATGACATTT R: GACAAACTCAGAATGGGGTGAAG	106	1.9
MCP1	F: GCTCTCTCTTCCTCCACCAC R: GCTTCTTTGGGACACCTGCT	208	1.8
Emr1	F: TTTCCTCGCCTGCTTCTTC R: CCCCGTCTCTGTATTCAACC	222	1.8
IL-6	F: TGGGAAATCGTGGAAATGAG R: GAAGGACTCTGGCTTTGTCTT	249	1.9
TNFα	F: CGTCGTAGCAAACCACCAA R: GAGAACCTGGGAGTAGACAAGG	145	1.7
iNOS	F: GGCAGCTACTGGGTCAAAGA R: TCTGAGGGCTGACACAAGG	172	1.8
18S	F: CGCGGTTCTATTTTGTTGGT R: AGTCGGCATCGTTTATGGTC	219	1.9

F, forward; R, reverse. Target genes: adiponectin; leptin; monocyte chemotactic protein-1 (*MCP1*); epidermal growth factor module-containing mucin-like receptor 1 (*Emr1*); interleukin-6 (*IL-6*); tumor necrosis factor alpha (*TNFα*); inducible nitric oxide synthase (*iNOS*). *18S* rRNA was used for normalization.

TABLE 2 - Adipose tissue weights in mice supplemented with CLA

	Control	CLA1	CLA2
White adipose tissues			
Epididymal (g)	0.644 ± 0.048[a]	0.628 ± 0.057[a]	0.284 ± 0.022[b]
Retroperitoneal (g)	0.212 ± 0.030[a]	0.129 ± 0.015[b]	0.069 ± 0.007[c]
Mesenteric (g)	0.250 ± 0.020	0.262 ± 0.022	0.233 ± 0.018
Sum (g)	1.107 ± 0.089[a]	1.018 ± 0.091[a]	0.586 ± 0.042[b]
Brown adipose tissue (g)	0.118 ± 0.007[a]	0.126 ± 0.007[a]	0.095 ± 0.003[b]

Weights of white adipose tissues from different anatomical locations and brown adipose tissue of mice treated with a daily dose of CLA equivalent to 3 mg CLA/animal in CLA1 group and 10 mg/animal in CLA2 group for the first 30 d and 6 mg CLA/animal in CLA1 group and 20 mg/animal in CLA2 group for the subsequent 35 d of treatment. Data are expressed in grams and are the means ± SEM of 8 mice. Means in a row without a common letter differ, $P<0.05$ (ANOVA followed by LSD test).

TABLE 3 - Effects of CLA treatment on plasma concentration of metabolites in mice

	Control	CLA1	CLA2
30 days of treatment			
Glucose (mmol/L)	8.2 ± 0.2	7.6 ± 0.2	8.0 ± 0.3
Adiponectin (μg/ml)	13.41 ± 1.35	13.78 ± 0.67	13.64 ± 1.14
Leptin (ng/ml)	3.11 ± 0.88	2.08 ± 0.35	1.62 ± 0.27
65 days of treatment			
Glucose (mmol/L)	4.33 ± 0.23[ab]	4.15 ± 0.14[a]	4.90 ± 0.21[b]
Adiponectin (μg/ml)	17.33 ± 1.05[a]	16.87 ± 0.84[a]	11.64 ± 1.25[b]
Leptin (ng/ml)	2.09 ± 0.39[a]	2.57 ± 0.45[a]	0.45 ± 0.10[b]
Resistin (ng/ml)	15.27 ± 1.04	15.90 ± 0.91	14.32 ± 0.93
NEFAs (mg/dl)	26.00 ± 1.80[a]	19.36 ± 1.96[b]	14.44 ± 1.47[b]
Glycerol (mg/ml)	0.19 ± 0.02[a]	0.15 ± 0.02[a]	0.06 ± 0.02[b]
Triglycerides (mg/ml)	0.61 ± 0.03	0.60 ± 0.05	0.51 ± 0.08
Insulin (pmol/L)	15.95 ± 0.35[a]	18.50 ± 0.87[b]	19.89 ± 0.80[b]
Leptin / adiponectin ratio	0.12 ±0.02[a]	0.16 ± 0.03[a]	0.04 ±0.01[b]
HOMA-IR	0.42 ± 0.02[a]	0.47 ± 0.02[a]	0.60 ± 0.04[b]
R-QUICKI	0.46 ± 0.01	0.48 ± 0.01	0.49 ± 0.01

At 30 d of treatment and after 3 h fast, glucose, adiponectin and leptin plasma concentrations were determined from tail blood samples. The rest of plasmatic metabolites were determined at the end of the study (65 days of treatment) after 10 h fast and from blood samples collected by cardiac puncture. Data are means ± SEM of 8 mice at 65 d of treatment; of 3-8 mice for leptin and adiponectin on day 30 and of 7 mice for glucose on day 30. Means in a row without a common letter differ, $P<0.05$ (ANOVA followed by LSD test).

TABLE 4 - Relative expression of target mRNAs in mature adipocytes and stromal vascular fraction in mice treated with CLA

	Control	CLA1	CLA2
Mature adipocytes			
Adiponectin	1.00 ± 0.05^{a}	0.72 ± 0.04^{b}	0.39 ± 0.02^{c}
Leptin	1.00 ± 0.07^{a}	0.57 ± 0.05^{b}	0.16 ± 0.01^{c}
MCP1	1.00 ± 0.09^{a}	1.28 ± 0.10^{b}	0.71 ± 0.06^{c}
Stromal Vascular Fraction			
IL-6	1.00 ± 0.09^{a}	0.96 ± 0.10^{a}	0.61 ± 0.04^{b}
TNFα	1.00 ± 0.11^{a}	1.47 ± 0.18^{ab}	1.87 ± 0.30^{b}
iNOS	1.00 ± 0.08	0.81 ± 0.05	1.24 ± 0.20
Emr1	1.00 ± 0.11^{a}	1.46 ± 0.11^{a}	2.64 ± 0.28^{b}
MCP1	1.00 ± 0.13	1.40 ± 0.18	1.23 ± 0.11

Epididymal adipose tissue was digested by collagenase and then separated into mature adipocytes and stromal vascular fraction. Expression levels of target genes of each fraction were measured by real time PCR and normalized by the internal housekeeping gene 1*8S* rRNA. The results, mean ± SEM of 6-8 mice/group, are expressed as fold induction over control group. Means in a row without a common letter differ, $P<0.05$ (ANOVA followed by LSD test).

TABLE 5 - Total RNA yields obtained from mature adipocytes and stromal vascular fraction in CLA treated mice

	RNA yield (μg RNA / g of epididymal depot)		
	Control	**CLA1**	**CLA2**
Mature adipocytes	5.5 ± 0.6[a]	9.8 ± 0.7[b]	8.8 ± 0.8[b]
Stromal vascular fraction	8.8 ± 0.4	6.1 ± 1.2	11.0 ± 2.5

Epididymal adipose tissue was digested by collagenase and then separated into mature adipocytes and stromal vascular fraction. RNA extracted from each fraction was quantified and referred per gram of epididymal adipose tissue weight. Data are expressed in μg RNA per g of epididymal tissue and are the means ± SEM of 7-8 mice/group. Means in a row without a common letter differ, $P<0.05$ (ANOVA followed by LSD test).

Figure 1

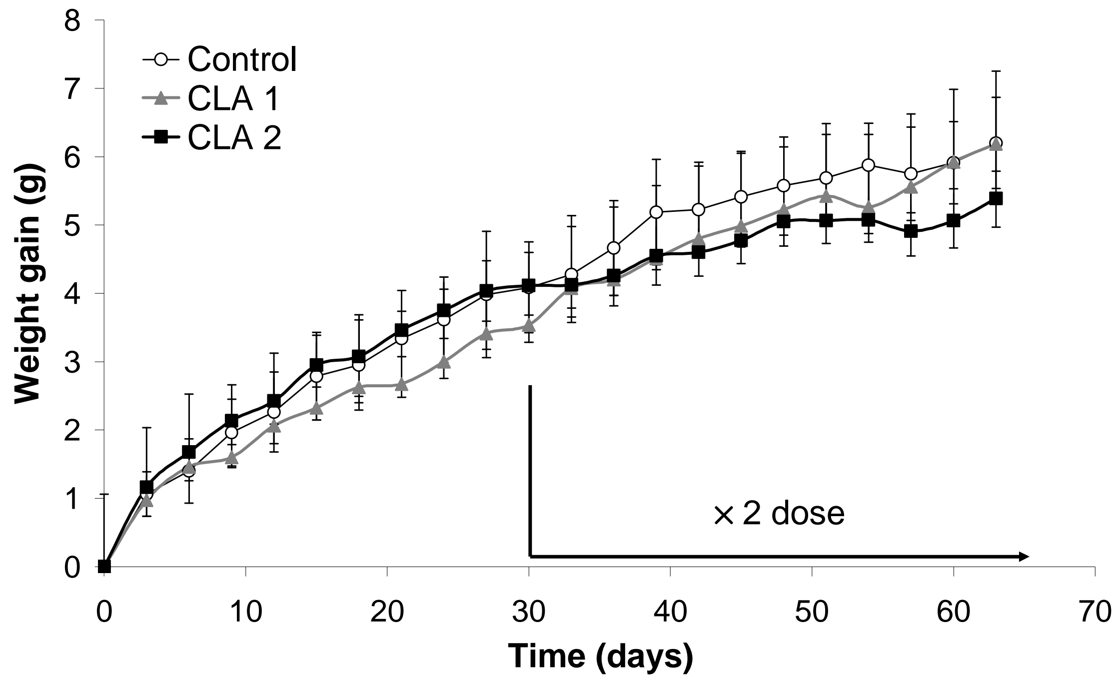


Figure 2

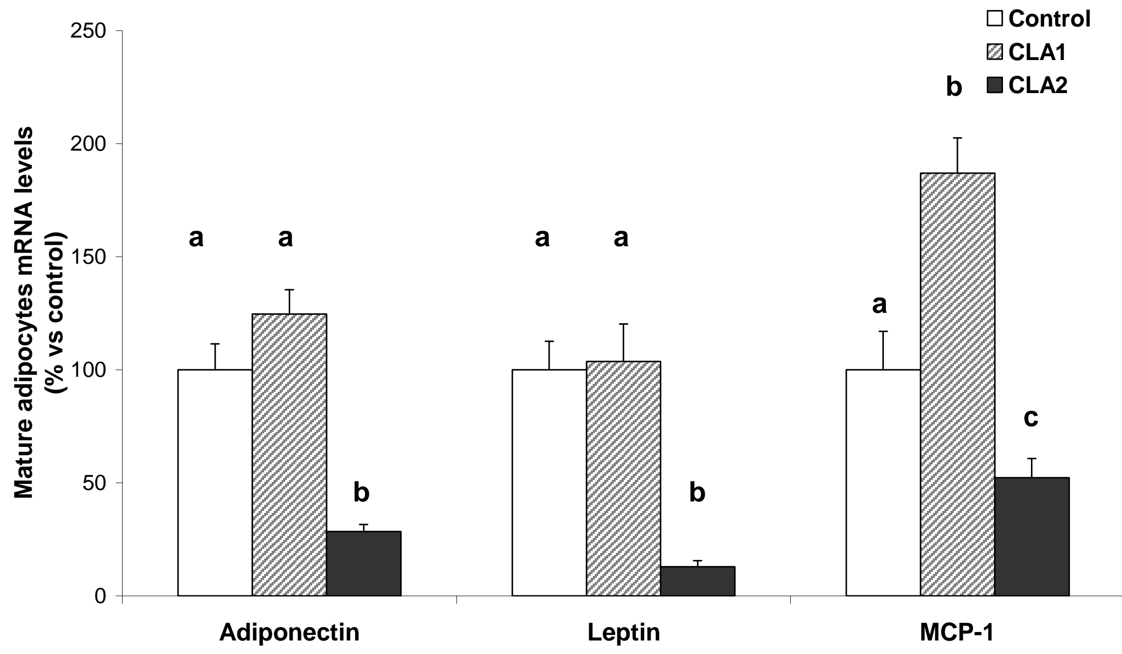
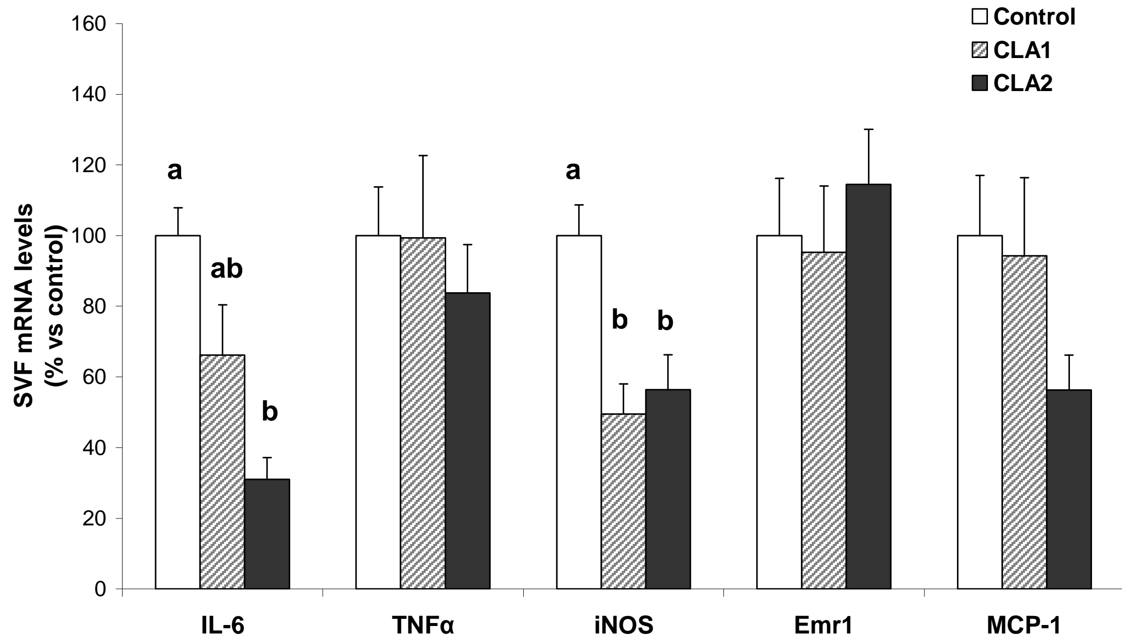


Figure 3



MANUSCRITO V

Transcriptional analysis reveals a high impact of conjugated linoleic acid on stearoyl-Coenzyme A desaturase 1 mRNA expression in gastrocnemius muscle

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Manuscrito en vías de publicación

Transcriptional analysis reveals a high impact of conjugated linoleic acid on stearoyl-Coenzyme A desaturase 1 mRNA expression in gastrocnemius muscle

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Running title: **Impact of conjugated linoleic acid on stearoyl-Coenzyme A desaturase 1 expression in muscle**

This work was supported by grants from the Spanish Government (AGL2006-04887 and AGL2009-11277). Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, European Union Contract No. FP6-506360). P. Parra is a recipient of a fellowship from the Spanish Government. CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative from ISCIII.

Keywords: Conjugated linoleic acid, Scd1, lipogenesis, muscle

Abbreviations: Acc1, acetyl-Coenzyme A carboxylase alpha; *Acc2*, acetyl-Coenzyme A carboxylase beta; *Acox1*, acyl-Coenzyme A oxidase; *ANOVA*, analysis of variance; *Cd36*, CD36 antigen; *CLA*, conjugated linoleic acid; *DMEM*, dulbecco's modified Eagle's medium; *Elovl6*, elongation of long-chain fatty acids family member 6; *ERRα*, estrogen related receptor alpha; *Exp1*, experiment 1; *Exp2*, experiment 2; *FABP*, fatty acid binding protein; *Fasn*, fatty acid synthase; *BSA*, fatty acid-free bovine serum albumin; *Glut4*, glucose transporter type 4; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *CHO*, high-sucrose diet; *LSD*, least Significant Difference; *Lpl*, lipoprotein lipase; *MCAD*, medium chain acyl-Coenzyme A dehydrogenase; *Cpt1b*, muscle carnitine palmitoyltransferase 1b; *MyHC-IIx/d*, myosin heavy polypeptide 1; *MyHC-IIa*, myosin heavy polypeptide 2; *MyHC-IIb*, myosin heavy polypeptide 4; *MyHC-b*, myosin heavy polypeptide 7 beta; *PPARα*, peroxisome proliferator activator receptor alpha; *PPARδ*, peroxisome proliferator activator receptor delta; *PGC-1α*, peroxisome proliferative activated receptor gamma coactivator 1 alpha; *PGC-1β*, peroxisome proliferative activated receptor gamma coactivator 1 beta; *Pdk4*, pyruvate dehydrogenase kinase, isoenzyme 4; *real-time PCR*, real-time polymerase chain reaction; *SEM*, standard error of the mean; *Scd1*, stearoyl-Coenzyme A desaturase 1; *SREBP-1c*, sterol regulatory element binding factor 1; *Ucp3*, uncoupling protein 3.

ABSTRACT

Conjugated linoleic acid (CLA) has the potential to modulate body composition, particularly decreasing body fat accumulation, in both animals and humans. Most of the studies have been focused on the underlying mechanisms in adipose tissue and less attention has been paid to other significant organs in energy homeostasis such as muscle. In this study, we have examined the potential implication of skeletal muscle in the lowering fat effect of CLA observed in mice after a treatment with moderate doses of an equimolar mixture of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers. In a first experiment, mice were fed with a standard-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/day for 37 days. In a second experiment, mice were fed with a high-fat diet for 65 days. For the first 30 days mice received the same doses of CLA described above and, from that time onwards, animals received double dose. Gene expression of key proteins involved in energy metabolism processes (fatty acid transport and oxidation; regulation of lipid and carbohydrate utilization, fiber types composition, and thermogenesis) was determined. Results didn't support an enhancement of β-oxidation in skeletal muscle to contribute to the antiobesity effect of CLA, but, an interesting increase in transcriptional expression of lipogenic genes, particularly Scd1, was observed in both experiments. Our data, contribute to demonstrate that moderate doses of CLA causes a strong induction of muscle Scd1 expression which would be responsible for increased production of palmitoleate, and as confirmed in animal models of targeted disruptions of genes involved in lipogenesis, this specific fatty acid would contribute to sort out maintenance of insulin sensitivity under both standard and high-fat feeding.

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of positional and geometric conjugated isomers of linoleic acid that exhibits a number of biological actions (for a review see (Bhattacharya et al. 2006)). The beneficial effects of CLA on body weight and adiposity have been extensively studied and particularly, those mixtures containing both, the isomer *trans*-10, *cis*-12 and the *cis*-9, *trans*-11. Various mechanisms of action have been proposed, most of them having place in the adipose tissue: decreased lipid synthesis, increased lipolysis, decreased preadipocyte proliferation and differentiation, and increased apoptosis have been reported in the literature (for a review see (Wang et al. 2004)). Thus, most of the studies have been focused on adipose tissue, which seems to be the main target of CLA action, and less attention has been paid to other organs and tissues, such as muscle, which also play an important role in energy metabolism.

Skeletal muscle is one of the most metabolically demanding tissues. It may account for 50% of energy expenditure and 75% of glucose disposal (Baron et al. 1988), and consequently, has a significant influence in nutrient partitioning, insulin sensitivity, and the blood-lipid profile. Moreover, skeletal muscle presents a notable metabolic flexibility in response to physiological and pathophysiological conditions. Concerning the impact of CLA, there are some evidences of its anabolic properties on lean body mass in animals (Park et al. 1997; DeLany et al. 1999; Ostrowska et al. 1999; Peters et al. 2001), which is of interest since body weight loss often results in the loss of lean body mass. However, the specific effects of CLA on the skeletal muscle and the mechanism of action are still unclear (Park et al. 1997; Martin et al. 2000; Rahman et al. 2001; Ealey et al. 2002; Takahashi et al. 2002; Zabala et al. 2006).

We have previously reported that moderate doses of an equimolar mix of *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers reduces body fat accumulation and is accompanied by an amelioration of the inflammatory profile in adipose tissue in both, mice fed a standard-fat (Parra et al. 2009) and a high-fat diet (Parra et al. 2009). In the present work, we have been interested in assessing the potential contribution of skeletal muscle to the fat-lowering effect observed with CLA treatment, analysing the profile of gene expression of key proteins involved in energy and lipid metabolism in gastrocnemius muscle.

MATERIALS AND METHODS

Animals and diets

Male C57BL/6J mice from Charles River (Barcelona, Spain) weighing 20 ± 0.2 g were housed in groups of four in plastic cages and maintained on a 12-h light:dark cycle at 22ºC. These mice were used in two independent experiments with different dietary regimens. In Experiment 1 (Exp1) animals were fed *ad libitum* with a standard diet (D12450B, Research Diets Inc, New Brunswick) which contains 10% calorie content as fat. In Experiment 2 (Exp2) animals were fed *ad libitum* with a high-fat diet (D12451, Research Diets Inc, New Brunswick) which contains 45% calorie content as fat. Food intake and body weight were recorded every three days during the experiments. Fresh food was provided to the mice biweekly.

All experimental procedures were performed according to both national and institutional guidelines for animal care and use.

CLA Treatment

Mice were orally treated with CLA (Tonalin® TG 80 derived from safflower oil, kindly provided by Cognis). Tonalin is composed of triglycerides containing approximately 80% conjugated linoleic acid with a 50:50 ratio of the active CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12. Control animals received an adequate amount of commercial sunflower oil to achieve isocaloric load between groups.

In Exp1, mice were randomly assigned to three experimental groups (n= 12 each group) for 37 days: control (sunflower oil), CLA1 (3 mg CLA/day) or CLA2 (10 mg CLA/day).

In Exp2, mice were also assigned to three oral treatments (n= 8 each group) for 65 days: control (sunflower oil), CLA3 or CLA4. For the first 30 days, animals received the

same doses of CLA used in Exp1, from that time onwards animals received double dose. Therefore, animals received a daily amount of 6 mg CLA in CLA3 and of 20 mg in CLA4 group for the last 35 days of treatment.

Sacrifice and tissue sampling

Mice from Exp1 were sacrificed under feeding conditions and from Exp2 were fasted for 10 h before sacrifice. At the beginning of the light cycle, animals were anaesthetised by intraperitoneal injection of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). Gastrocnemius muscle was rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at -70ºC.

Myoblast culture and treatment

The murine skeletal muscle cell line C2C12 was cultured in the growth medium Dulbecco's modified Eagle's medium (DMEM) (Sigma, Madrid, Spain) supplemented with 10% fetal bovine serum (Invitrogen), 3 mM L-glutamine (Sigma, Madrid, Spain), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Sigma, Madrid, Spain). Cells were maintained at 37°C in humidified 95% air and 5% CO_2 atmosphere. To induce differentiation, C2C12 myoblasts were platted at an initial density of 3×10^4 cells/cm^2 in 12-well culture dishes for 24 h, allowing them to reach 80% confluence; then, growth media was replaced with DMEM containing antibiotics, 3 mM L-glutamine, and 2% horse serum; referred to as differentiation media. Treatments were performed in differentiated C2C12 myotubes after 9 days in differentiation media.

Trans-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers (>98%) obtained from Matreya were dissolved in ethanol. A mixture 200 mmol/L of both isomers was conjugated to fatty

acid-free bovine serum albumin (BSA) with a final molar ratio of CLA:BSA of 5:1. At day 9, differentiated C2C12 myotubes were treated for 24 h with CLA mixture (10 μM) in the absence or in the presence of insulin (100 nM) (Sigma, Madrid, Spain) in serum-free differentiation media. Control cells received the proportional volume of ethanol and BSA. All experiments were performed three times. Following incubation, RNA was extracted from myotubes as described below.

RNA extraction

Total RNA from gastrocnemius and C2C12 cells was extracted using Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop®Spetrophotometer ND-1000 (NadroDrop Technologies, Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis.

mRNA expression analysis

Real-time polymerase chain reaction (real-time PCR) was used to measure mRNA expression levels of target genes. 0.5 μg of total RNA (in a final volume of 10 μL) was denatured at 90°C for 1 min and then reverse-transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 42°C for 60 min, with a final step of 5 min at 99°C in a Perkin-Elmer 9700 Thermal Cycler (PerkinElmer, Wellesley, MA). Real-time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95ºC, followed by a total of 40 temperature cycles (15 s at 95ºC and 1 min at 60ºC). Each PCR was performed in a total volume of 6.25 μl, made from diluted cDNA template, forward and reverse primers (1 μM each), and Power SYBER Green PCR

Master Mix (Applied Biosystems, CA, USA). Primer sequences are listed in Table 1. All primers were purchased from Sigma (Madrid, Spain). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. PCR products were also analyzed by electrophoresis in an ethidium bromide-stained agarose gel to check that a single amplicon of the expected size was indeed obtained. The relative quantification of each target gene was calculated based on efficiency and the crossing point deviation of an unknown sample versus a control. Data were normalized relative to the levels of the housekeeping genes β-actin and GAPDH. Results from CLA treated groups were expressed as relative fold induction relative to the control group.

Statistical analysis

Data are presented as means ± SEM. One-factor ANOVA was used to determine the significance of the differences between groups. If there was a significant difference, a Least Significant Difference (LSD) test was used to determine the particular effect that caused that difference. $P < 0.05$ was statistically significant, and different superscripts discriminate differences between groups. The analysis was performed using the SPSS program for Windows version 16 (SPSS, Chicago, IL, USA).

RESULTS

Effects of CLA supplementation on the expression of metabolic biomarkers in gastrocnemius muscle

Changes in mRNA levels of the peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC-1α) were not observed in either of the experiments after CLA treatment (Table 2 and 3). Peroxisome proliferative activated receptor gamma coactivator 1 beta (PGC-1β) mRNA levels increased in groups treated with CLA in Exp1 (Table 2) but not in Exp2 (Table 3) ($P < 0.05$). Myosin heavy-chain isoforms expression, typically used as an indicator of the composition of the different fiber types in muscle, showed no differences due to CLA treatment in either of the experiments (Table 2 and 3).

Analysis of the expression of transcription factors – estrogen related receptor alpha (ERRα), peroxisome proliferator activator receptor alpha (PPARα) and delta (PPARδ) - which are known regulators of lipid and carbohydrate metabolism in muscle were not affected by CLA treatment in either of the experiments (Table 2 and 3). In accordance, the same pattern was seen on mRNA levels of biomarkers of glucose metabolism (glucose transporter type 4 (Glut4), pyruvate dehydrogenase kinase, isoenzyme 4 (Pdk4) and Hexoquinase 2), fatty acid transport and oxidation (CD36 antigen (Cd36), lipoprotein lipase (Lpl), muscle carnitine palmitoyltransferase 1b (Cpt1b), acyl-Coenzyme A oxidase (Acox1), medium chain acyl-Coenzyme A dehydrogenase (MCAD)) or uncoupling protein 3 (Ucp3) expression (Table 2 and 3).

Interestingly, mRNA levels of genes involved in *de novo* lipogenesis were increased by CLA treatment and influenced by dietary fat content. In Exp1, under a standard-fat diet, mRNA levels of acetyl-Coenzyme A carboxylase alpha (Acc1) and beta (Acc2) were not affected by CLA treatment and the highest dose of CLA produced a slight but

statistically significant increase in the transcript of sterol regulatory element binding factor 1 (SREBP-1c) ($P< 0.05$) respect both, the control and CLA1 groups (Figure 1A). However, in animals receiving a high-fat diet (Exp2), an increase in Acc1 ($P< 0.01$) was observed with the higher dose of CLA respect to the control group (Figure 1B) and no changes on the expression of SREBP-1c and Acc2 were seen. Interestingly, both dietary conditions were associated with induction of fatty acid synthase (Fasn) (1.9 times ($P< 0.05$) in standard-fat and 1.6 times ($P< 0.05$) in high-fat) in the groups receiving the highest dose of CLA, respect to both, the respective control and the group treated with the lowest dose (Figure 1A and B).

A remarkable increase in the expression of stearoyl-Coenzyme A desaturase 1 (Scd1) mRNA was seen in all the groups treated with CLA and, particularly under the high-fat diet. Therefore, in animals fed the standard-fat diet, Scd1 was higher in CLA groups, reaching statistically significance with the highest dose (1.5-fold induction, $P< 0.05$) in comparison with the control group (Figure 1A). In Exp2, under the high-fat diet, a dose-dependent strong induction of Scd1 mRNA was observed (3.2-fold induction in CLA3 ($P< 0.001$) and 8.6-fold ($P< 0.001$) in CLA4 groups with respect to control group).

Effects of CLA treatment on Fasn and Scd1 gene expression in C2C12 cells

Differentiated C2C12 myotubes incubated with CLA for 24h, showed an increase in the expression of both Fasn and Scd1 mRNA ($P< 0.001$) in presence of insulin but not in cells treated with CLA or insulin alone (Figure 2).

DISCUSSION

In previous studies, we have shown that moderate doses of CLA are able to decrease body fat accumulation and modulate the inflammatory profile of adipose tissue with a minor impact on insulin sensitivity in mice (Parra et al. 2009; Parra et al. 2009). At this step, the interest has been to assess whether skeletal muscle is a target tissue of CLA action and whether it is involved in the body fat lowering effect of CLA observed in our previous studies, analyzing muscle gene expression of selected proteins involved in glucose and lipid homeostasis using the same experimental design, that is, moderate doses of isomer mix of CLA under either standard or high-fat diet.

PGC-1α and PGC-1β are members of a small family of transcriptional co-activators that influence metabolic pathways in muscle and other tissues by co-activating a number of transcription factors including PPARγ, PPARα, and ERRα. Although we found an increase in PGC-1β induced by CLA treatment in standard-fat conditions, it did not have any impact on the expression of the associated transcription factors. Because, recent studies in rodents suggest that PGC-1α and PGC-1β influence the composition of fibre types in skeletal muscle (Mortensen et al. 2006; Arany et al. 2007) we explored that possibility. However, analysis of gene expression of myosin heavy-chain isoforms did not show any effect of CLA irrespective of the type of diet (Table 2 and 3).

Although evidences are limited (Park et al. 1997) (Rahman et al. 2001; Zabala et al. 2006) and conflicting results have also been obtained (Martin et al. 2000), enhancement of fat transport and oxidation in muscle has been proposed to contribute to the CLA-induced changes in body composition. In either of our experimental conditions, no major changes in gene expression of fatty acid transport and fatty acid oxidation proteins or in key regulators of lipid and carbohydrate metabolism have been observed. Thus, our transcriptional results, *a priori*, didn't support an enhancement of β-oxidation

in the skeletal muscle to contribute to the antiobesity effect of CLA observed in these mice.

By contrast, and unexpectedly, genes involved in lipogenesis were significantly activated by CLA treatment. Muscle has not been normally considered a lipogenic tissue, but some evidences suggest that *de novo* lipogenesis can indeed take place in muscle and to be modulated by factors influencing nutritional status (Aas et al. 2004; Guillet-Deniau et al. 2004). In our experimental conditions, Acc1, which is the isoform that synthesizes the pool of malonyl-CoA that is used for *de novo* lipogenesis, was increased in Exp2 and no affected in Exp1 by CLA treatment whereas the expression of SREBP-1c, which is a master regulator of lipogenic/glycolitic genes, was increased in Exp1 by CLA. In accordance with this profile, Acc2, which is expressed in 'nonlipogenic' tissues such as skeletal and cardiac muscle and is thought to control the pool of malonyl-CoA that regulates fatty acid oxidation, was unaltered in both situations (Figure 1).

Interestingly, the effect of CLA increasing Fasn (up to 2-fold induction) and mostly Scd1 (up to 8-fold) gene expression is a very consistent effect, common to both experimental conditions. In addition, the increase in Scd1 was more pronounced in Exp2 (high-fat feeding) than in Exp1 (standard-fat feeding) suggesting a clear impact of conditions (metabolic status of the animals, diet composition, duration of treatment and doses of CLA) on the potential action of CLA. In fact, the results from cell culture gave support to this view as the increase in Fasn and Scd1 mRNA levels were attained with the combined effect of CLA and insulin but not with CLA or insulin alone.

Scd1 is the Δ9 desaturase that catalyzes the conversion of saturated fatty acids into monounsaturated fatty acids (Enoch et al. 1976). As Scd1 is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids, it becomes a critical control point

regulating hepatic lipogenesis and lipid oxidation (Dobrzyn et al. 2005). A common function of desaturases in organisms is to maintain the optimal fatty acid composition of membranes since alterations in the composition might induce modification in lipid signalling molecules and/or alter the activities of multiple enzymes and proteins (Hulbert et al. 2005). Effect of CLA on Scd1 expression has been previously reported in hepatocytes and adipocytes, where in general is associated to decreased transcription and/or activity (Lee et al. 1998; Bretillon et al. 1999; Choi et al. 2000; Park et al. 2000; Choi et al. 2001). However, studies addressing the effects on muscle are scarcer and show contradictory results, which could be a reflection of the CLA isomer used in combination with the dietary fat content and composition (Demaree et al. 2002; Kang et al. 2004). A lower Δ9 desaturase index (Viswanadha et al. 2006; Zabala et al. 2006) or no changes (Demaree et al. 2002; Kang et al. 2004) have been found in muscle of CLA treated animals. These results on this index pattern (which is assessed by determining the ratio of palmitoleate/palmitate and oleate/stearate) are, in principle, difficult to reconcile with our data where up to 8-fold increase in muscle Scd1 activity was found, but would fit if the product of Scd1 is not incorporated into muscle cell membranes but released to circulation in our experimental setting. Recently, Cao and co-workers (Cao et al. 2008) have found that palmitoleate (C16:1 n-7) is an important signalling lipid hormone, which they designate as a 'lipokine', as in their animal model (fatty acid binding protein (FABP) 4 and FABP5 null mice) the source of this unique fatty acid is *de novo* synthesis in the adipose tissue. This double knock-out mouse displays a remarkable state of insulin sensitivity and is protected from metabolic deterioration induced by a high-fat diet (Maeda et al. 2005). In wild-type animals, a high-fat diet suppresses *de novo* synthesis of fatty acids in adipose tissue, leading to decreased production and concentration of palmitoleate, which contributes to insulin resistance,

whereas in the absence of those lipid chaperones, a high-fat diet does not suppress fatty acid synthesis and circulating palmitoleate levels remains high, and this is linked to the preservation of insulin sensitivity (Cao et al. 2008; Olefsky 2008). Our results are suggestive that CLA would contribute to increase palmitoleate production in muscle cells, through activation of Scd1, that would be released to plasma and exert a regulatory action on the interplay between muscle, liver and adipose tissue, concerning lipid partitioning and insulin sensitivity. Furthermore, Scd1 overexpression in L6 myotubes prevenst the accumulation of lipotoxic lipid metabolites such as ceramide and diacylglycerol and also protects from fatty acid-induced insulin resistance (Pinnamaneni et al. 2006). Finally, a recent paper on mice deficient in elongation of long-chain fatty acids family member 6 ($Elovl6^{-/-}$) also highlights the potential role of palmitoleate as a fatty acid likely responsible for sustained insulin sensitivity (Matsuzaka et al. 2007). Metabolic profile of $Elovl6^{-/-}$ mice is consistent with the absence of elongation of C16 fatty acids to C18; hepatic concentrations of stereate and oleate (18:1 n-9) are lower, whereas those of palmitate and palmitoleate are heightened, as compared to wild-type mice. These trends result even more prominent in livers of mice fed a fat-free, high-sucrose diet (CHO) that enhances endogenous hepatic fatty acid synthesis. The increase was significant enough to make palmitoleate, which is usually a minor fatty acid class, the second most abundant fatty acid present in livers of CHO-fed mice. Furthermore, $Elov6^{-/-}$ deficiency increased liver and, resultantly, plasma palmitoleate levels and under a high-fat, high-sucrose diet this animal model showed a nearly normal response to insulin whereas this was markedly reduced in the wild-type indicating that Elovl6 deletion may be protective against hepatic lipotoxicity and insulin resistance (Matsuzaka et al. 2009). All together, animal models of targeted disruptions of genes involved in lipogenesis highlight the relevance of endogenous synthesis of particular

fatty acids, specifically of palmitoleate, in response to dietary inputs and how this has a potential role to modulate lipid partitioning and insulin resistance.

Our data showed that muscle didn't contribute to the CLA antiobesity effect through induction of oxidative genes expression. However, an unexpectedly strong induction of Scd1 (up to 8 fold) was observed in both experimental conditions, which shed some light on the little understood muscle's response to CLA treatment but that will need further studies to assess its physiological implications.

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FIGURE LEGENDS

Figure 1. Effect of CLA on lipogenic gene expression in gastrocnemius muscle.

(A) In Exp1, mice were fed a standard-fat diet and received a daily dose of CLA equivalent to 3 mg CLA/animal in CLA1 group and 10 mg/animal in CLA2 group for 37 days. (B) In Exp2, mice were fed a high-fat diet and received a daily dose of CLA equivalent to 3 mg CLA/animal in CLA3 group and 10 mg/animal in CLA4 group for the first 30 days and 6 mg CLA/animal in CLA3 group and 20 mg/animal in CLA4 group for the additional 35 days of treatment. Data are means ± SEM of 8-12 mice in Exp1 and 5-8 mice in Exp2. Mean values with unlike letters are significantly different ($P < 0.05$); ANOVA followed by LSD test.

Figure 2. Effect of CLA on Fasn (A) and Scd1 (B) gene expression in C2C12 myotubes.

Fully differentiated myotubes were incubated at day 9 with a CLA mixture (10 μM) in the absence or in the presence of insulin (100 nM) for 24 in serum-free differentiation media. Data are expressed as mean ± SEM of three independent experiments yielding essentially the same results (n= 3-5 in each experiment). Mean values with unlike letters are significantly different ($P < 0.001$); ANOVA followed by LSD test.

TABLES

Table 1. Gene-specific primer sequences used in real-time PCR amplification.

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
β-actin	TACAGCTTCACCACCACAGC	TCTCCAGGGAGGAAGAGGAT
Acc1	GAACCCGAAACTCCCAGAAC	GAAGACCACTGCCACTCCA
Acc2	TTCCAGATGCTAATGGGTTG	GCAGGTCCAGTTTCTTGTGTT
Acox1	TGGTGAAGAAGATGAGGGAGT	AGCAAGGTGGGCAGGAAC
Cd36	GTCCTGGCTGTGTTTGGA	GCTCAAAGATGGCTCCATTG
ERRα	CAAGGAGGGTGTGCGTCT-	CGTTCACTGGGGCTGTCT
Fasn	TTCGGTGTATCCTGCTGTCC	TGGGCTTGTCCTGCTCTAAC
Glut4	GGCATGCGTTTCCAGTATGT	GCCCCTCAGTCATTCTCATC
GAPDH	ATTCAACGGCACAGTCAAGG	GTGGTTCACACCCATCACAA
Hexokinase 2	CAGCCTAGACCAGAGCATCC	CGCATCTCTTCCATGTAGCA
Lpl	CCTGATGACGCTGATTTTGT	TATGCTTTGCTGGGGTTTTC
MCAD	GGATTAGGGTTTAGTTTTGAGTT	CCAAAGAATTTGCTTCAATAG
M-CPTI	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG
MyHC-IIx/d	CTAAAGGCAGGCTCTCTCACT	TAGCATCCACCACAAACACC
MyHC-IIa	CACCTTCTCGTTTGCCAGTA	ATTCGCTCCTTTTCGGACTT
MyHC-IIb	GCCTGCCTCCTTCTTCATCT	TCGCTCCTTTTCAGACTTCC
MyHC-b	GAGACGGTGGTGGGTTTGTA	CTTTCTTTGCCTTGCCTTTG
PGC-1α	CATTTGATGCACTGACAGATGGA	CCGTCAGGCATGGAGGAA
PGC-1β	ACTGGATGAAGGCGACACAC	GCTTGCTGTTGGGGAGGA
PPARα	CGTTTGTGGCTGGTCAAGTT	AGAGAGGACAGATGGGGCTC
PPARδ	GCCTCGGGCTTCCACTAC	TCCGTCCAAAGCGGATAG
Pdk4	TCCTTCACACCTTCACCACA	AAAGAGGCGGTCAGTAATCC
Scd1	GGAAATGAACGAGAGAAGGTG	CCGAAGAGGCAGGTGTAGAG
SREBP-1c	CAGCGGTTTTGAACGACA	GCCAGAGAAGCAGAAGAGAAG
Ucp3	GGAGGAGAGAGGAAATACAGAGG	CCAAAGGCAGAGACAAAGTGA

Target genes: β-actin; acetyl-Coenzyme A carboxylase alpha (Acc1); acetyl-Coenzyme A carboxylase beta (Acc2); acyl-Coenzyme A oxidase (Acox1); CD36 antigen (Cd36); estrogen related receptor alpha (ERRα); fatty acid synthase (Fasn); glucose transporter type 4 (Glut4); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); hexokinase 2; lipoprotein lipase (Lpl); medium chain acyl-Coenzyme A dehydrogenase (MCAD); muscle carnitine palmitoyltransferase 1b (M-CPTI); myosin heavy polypeptide 1 (MyHC-IIx/d); myosin heavy polypeptide 2 (MyHC-IIa); myosin heavy polypeptide 4 (MyHC-IIb); myosin heavy polypeptide 7 beta (MyHC-b); peroxisome proliferative activated receptor gamma coactivator 1 alpha (PGC-1α); peroxisome proliferative activated receptor gamma coactivator 1 beta (PGC-1β); peroxisome proliferator activator receptor alpha (PPARα); peroxisome proliferator activator receptor delta (PPARδ); pyruvate dehydrogenase kinase, isoenzyme 4 (Pdk4); stearoyl-Coenzyme A desaturase 1 (Scd1); sterol regulatory element binding factor 1 (SREBP-1c); uncoupling protein 3 (Ucp3). β-actin and GAPDH were used for normalization.

Table 2. Relative changes in the expression of genes encoding transcription factors, myogenic markers and proteins involved in energy metabolism in gastrocnemius muscle of mice treated with CLA and fed a standard-fat diet.

Experiment 1 (standard-fat diet)

	Control	**CLA1**	**CLA2**
Transcriptional co-activators			
PGC-1α	1.00 ± 0.11	0.87 ± 0.04	1.11 ± 0.10
PGC-1β	1.00 ± 0.06[a]	1.21 ± 0.05[b]	1.24 ± 0.05[b]
Contractility/myogenic markers			
MyHC-b	1.00 ± 0.17	0.98 ± 0.10	0.91 ± 0.08
MyHC-IIa	1.00 ± 0.09	0.98 ± 0.06	1.02 ± 0.06
MyHC-IIb	1.00 ± 0.05	0.96 ± 0.03	0.96 ± 0.04
MyHC-IIx/d	1.00 ± 0.06	1.01 ± 0.04	1.14 ± 0.10
Transcription factors			
ERRα	1.00 ± 0.05	0.95 ± 0.05	0.90 ± 0.04
PPARα	1.00 ± 0.10	0.88 ± 0.04	0.90 ± 0.06
PPARδ	1.00 ± 0.03	0.99 ± 0.02	1.07 ± 0.03
Fatty acid metabolism			
Cd36	1.00 ± 0.07	0.82 ± 0.04	0.88 ± 0.09
Lpl	1.00 ± 0.06	0.97 ± 0.03	0.98 ± 0.05
M-CPTI	1.00 ± 0.18	1.22 ± 0.09	1.14 ± 0.09
Acox1	1.00 ± 0.04	0.95 ± 0.02	1.05 ± 0.04
MCAD	1.00 ± 0.11	1.01 ± 0.09	1.21 ± 0.07
Glucose metabolism			
Glut4	1.00 ± 0.06	1.11 ± 0.05	1.10 ± 0.04
Pdk4	1.00 ± 0.10	0.79 ± 0.07	0.84 ± 0.10
Hexokinase 2	1.00 ± 0.05	0.94 ± 0.03	1.00 ± 0.04
Energy expenditure			
Ucp3	1.00 ± 0.09	0.86 ± 0.07	1.03 ± 0.10

RNA was isolated from a homogenate of a whole gastrocnemius muscle. Expression levels of target genes were measured by real-time PCR and normalized by two internal housekeeping genes: β-actin and GAPDH. The results, mean ± SEM of 8-12 mice/group, are expressed as fold induction over control group. Means in a row without a common letter differ, $P < 0.05$ (ANOVA followed by LSD test).

Table 3. Relative changes in the expression of genes encoding transcription factors, myogenic markers and proteins involved in energy metabolism in gastrocnemius muscle of mice treated with CLA and fed with a high-fat diet.

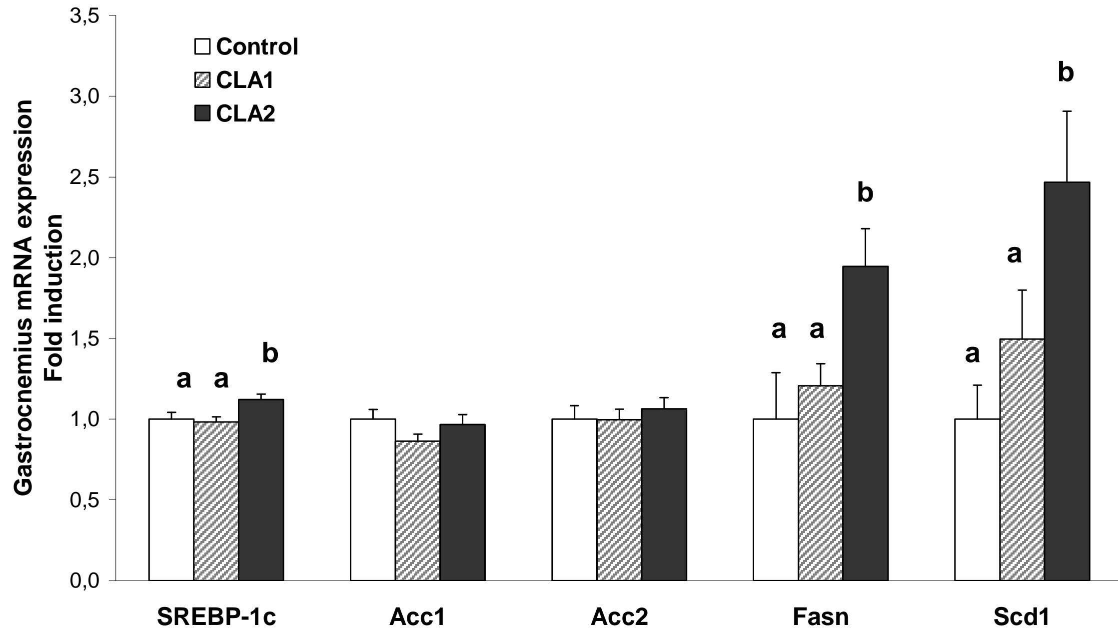
Experiment 2 (high-fat diet)

	Control	**CLA1**	**CLA2**
Transcriptional co-activators			
PGC-1α	1.00 ± 0.05	1.00 ± 0.05	1.05 ± 0.05
PGC-1β	1.00 ± 0.12	1.13 ± 0.10	1.01 ± 0.12
Contractility/myogenic markers			
MyHC-b	1.00 ± 0.08	0.95 ± 0.07	0.87 ± 0.08
MyHC-IIa	1.00 ± 0.09	1.07 ± 0.05	1.08 ± 0.07
MyHC-IIb	1.00 ± 0.12	1.26 ± 0.10	1.14 ± 0.05
MyHC-IIx/d	1.00 ± 0.07	0.88 ± 0.03	0.88 ± 0.05
Transcription factors			
ERRα	1.00 ± 0.02	0.89 ± 0.06	0.87 ± 0.05
PPARα	1.00 ± 0.08	1.06 ± 0.07	1.05 ± 0.09
PPARδ	1.00 ± 0.04	1.02 ± 0.06	1.04 ± 0.06
Fatty acid metabolism			
Cd36	1.00 ± 0.06	1.05 ± 0.07	0.99 ± 0.06
Lpl	1.00 ± 0.10	0.96 ± 0.07	1.03 ± 0.04
M-CPTI	1.00 ± 0.04	0.94 ± 0.03	0.93 ± 0.04
Acox1	1.00 ± 0.05	0.98 ± 0.04	0.96 ± 0.03
MCAD	1.00 ± 0.08	0.96 ± 0.06	1.08 ± 0.04
Glucose metabolism			
Glut4	1.00 ± 0.06	0.90 ± 0.05	0.89 ± 0.03
Pdk4	1.00 ± 0.04	0.89 ± 0.05	0.84 ± 0.06
Hexokinase 2	1.00 ± 0.08	1.05 ± 0.06	1.02 ± 0.03
Energy expenditure			
Ucp3	1.00 ± 0.05	0.90 ± 0.09	0.90 ± 0.05

RNA was isolated from a homogenate of a whole gastrocnemius muscle. Expression levels of target genes were measured by real-time PCR and normalized by two internal housekeeping genes: β-actin and GAPDH. The results, mean ± SEM of 5-8 mice/group, are expressed as fold induction over control group. Means in a row without a common letter differ, $P < 0.05$ (ANOVA followed by LSD test).

Figure 1

(A)



(B)

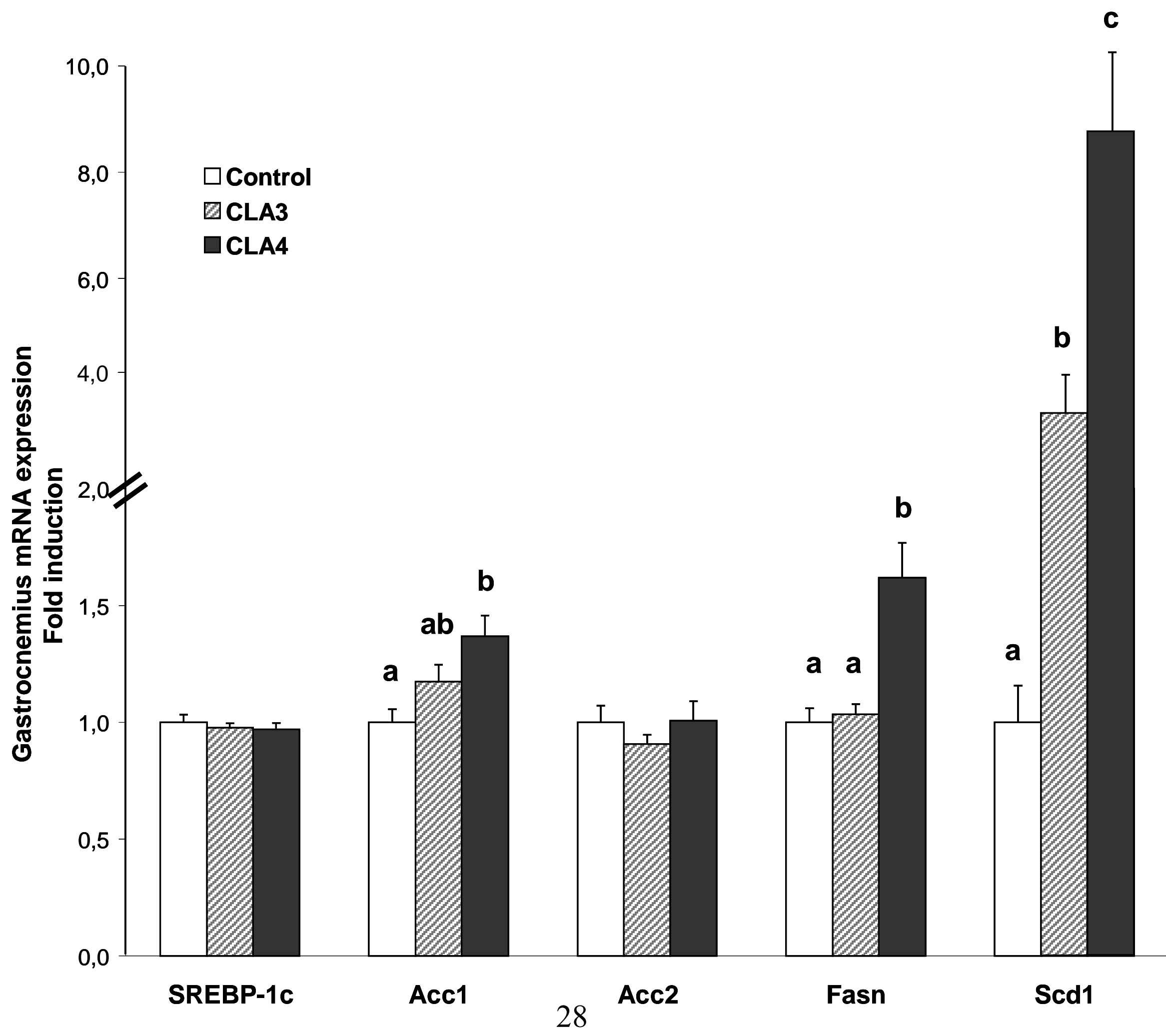
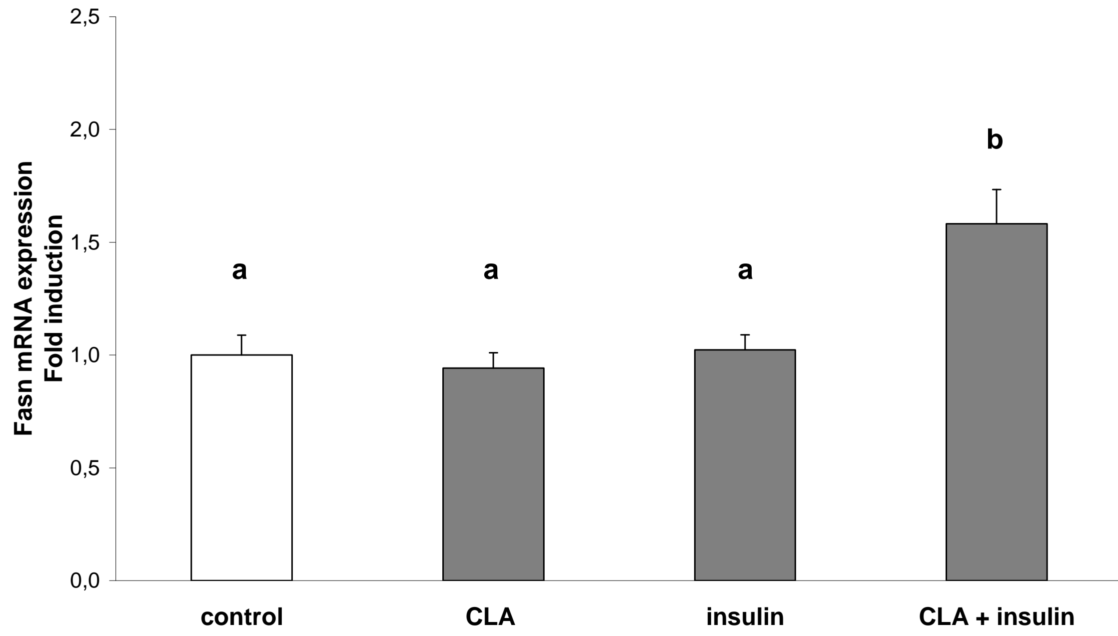
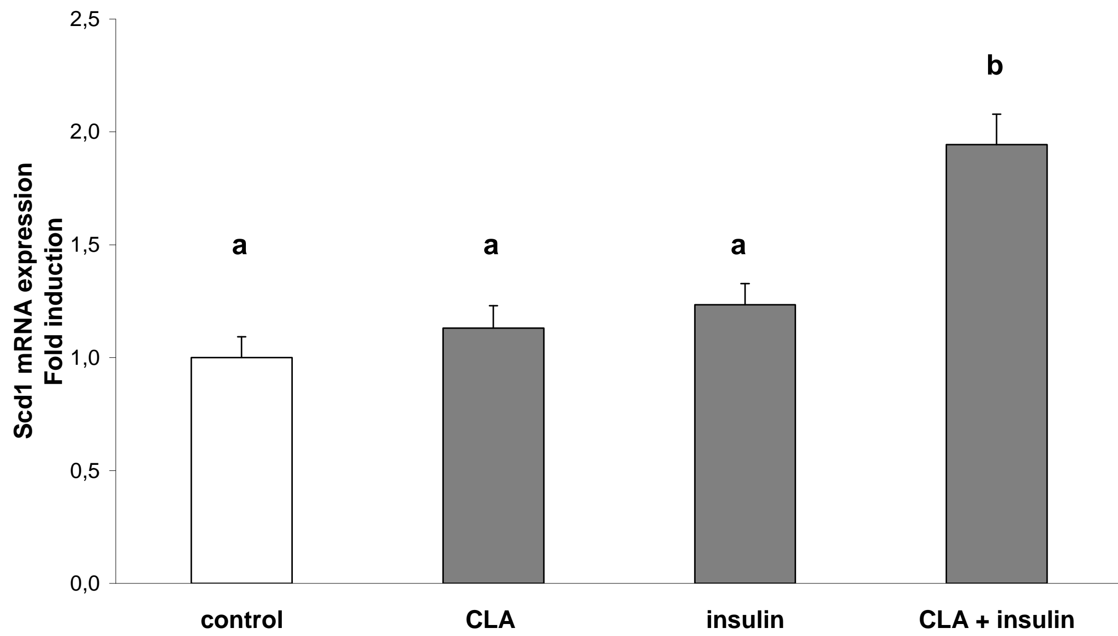


Figure 2

(A)



(B)



MANUSCRITO VI

Loss of fat by moderate doses of conjugated linoleic acid in mice is not accompanied by induction of hepatic lipogenic gene transcription

Parra P, Palou A, Serra F.

Manuscrito en vías de publicación

Loss of fat by moderate doses of conjugated linoleic acid in mice is not accompanied by induction of hepatic lipogenic gene transcription

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This work was supported by grants from the Spanish Government (AGL2006-04887 and AGL2009-11277). Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, European Union Contract No. FP6-506360). P. Parra is a recipient of a fellowship from the Spanish Government. CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative from ISCIII.

Keywords: Conjugated linoleic acid, stearoyl-CoA desaturase, fatty acid oxidation, fatty acid synthesis, hepatic steatosis

Abbreviations: Acc1, acetyl-Coenzyme A carboxylase alpha; Acox1, acyl-Coenzyme A oxidase; ANOVA, one-factor analysis of variance; BAT, brown adipose tissue; C/EBPα, CCAAT/enhancer binding protein alpha; Cpt1a, liver carnitine palmitoyltransferase 1a; Cpt1b, muscle carnitine palmitoyltransferase 1b; Faskol, liver-specific fatty acid synthase knockout mice ; Fasn, fatty acid synthase; Fgf21, fibroblast growth factor 21; Glut4, glucose transporter type 4; HC/HF diet, high-carbohydrate/high-fat diet; HSL, hormone sensitive lipase; Lpl, lipoprotein lipase; LSD, least significant difference; Pnpla2, patatin-like phospholipase domain containing 2; PPAR, peroxisome proliferator activator receptor alpha (α), delta (δ), gamma (γ); real-time PCR, real-time polymerase chain reaction; rWAT, retroperitoneal white adipose tissue; Scd1, stearoyl-Coenzyme A desaturase 1; SEM, standard error of the mean; SREBP-1c, sterol regulatory element binding factor 1; Ucp, uncoupling protein.

ABSTRACT

We have previously reported that moderate doses of a mixture of CLA isomers under, either standard- or high-fat diet, decrease fat accumulation, ameliorate adipose inflammatory status and keep insulin sensitivity in the absence of hepatic lipid accretion in mice, which is accompanied by a strong induction of muscle Scd1 gene expression. In the present study, we have examined the effects of CLA on expression of fatty acid metabolism genes in adipose tissues and liver, to assess whether tissue specific regulation of lipogenic/oxidative potential was present and could explain the preservation of glucose-insulin homeostasis found. In a first experiment, mice were fed with a standard-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/day (CLA1 and CLA2 group, respectively) for 37 days. In a second experiment, mice were fed with a high-fat diet for 65 days. For the first 30 days mice received the same doses of CLA described above and, from that time onwards, animals received double dose. In rWAT, activation of UCP2 but unaltered Cpt1 expression was found in Exp1 and, with high fat feeding, increases were attenuated and the marker of mitochondrial transport capacity (Cpt1) was further diminished. Despite reduced fat mass, markers of fatty acid or glucose input were not altered and the same was seen concerning expression of adipogenic markers; interestingly no activation of oxidation or lipogenic gene expression was observed but induction by CLA of Fasn and particularly of Scd1 was present in both experimental settings. BAT in CLA treated groups was not showing a expression profile of active thermogenesis and showed a lipid oxidation potential resembling rWAT; concerning lipogenic potential, this was reduced in Exp1 animals whereas in Exp2, showed the pattern of induction of Scd1, and at less extent, of Fasn as in rWAT. In liver, the lipid oxidation potential was unaltered or showed a tendency to decrease by CLA in both experiments; interestingly, lipogenic potential was

decreased by CLA under standard-fat diet and attenuated under high-fat diet. Therefore, loss of fat induced by moderate doses of CLA in mice was not associated with induction of hepatic lipogenic gene transcription, particularly Fasn and Scd1, but with preservation of lipogenic capacity in adipose tissues (white and brown).

INTRODUCTION

We have shown that moderate doses of isomer mixture of CLA under, either standard- or high-fat diet, are effective in decreasing fat accumulation, ameliorate adipose inflammatory status and, contrary to what is usually described with higher dietary doses of CLA, keep insulin sensitivity in mice (Parra et al. 2009; Parra et al. 2009). Furthermore, a functional transcriptional analysis revealed a high impact of CLA on stearoyl-Coenzyme A desaturase (Scd1) mRNA expression in gastrocnemius muscle (Parra P manuscript under preparation).

Scd1 is a key enzyme regulating lipogenesis and lipid oxidation, catalyzes the synthesis of monounsaturated fatty acids, particularly oleate (C18:1 n-9) and palmitoleate (C16:1 n-7) and its regulation is of considerable physiological importance since its activity affects membrane fluidity, lipid metabolism and obesity (Miyazaki et al. 2003). Many developmental, dietary, hormonal and environmental factors also regulate Scd1 expression. High-carbohydrate fat-free diets induce hepatic Scd1 mRNA expression whereas polyunsaturated fatty acids inhibit Scd1 mRNA transcription and activity in the liver (Ntambi 1992; Waters et al. 1996; Kim et al. 1999). Therapeutic manipulation of Scd1 has been proposed as beneficial in the treatment of obesity and metabolic syndrome (Dobrzyn et al. 2005; Paton et al. 2009). Although the mechanisms by which some of the metabolic modulators regulate Scd1 are not totally understood (Miyazaki et al. 2003), the recent development of related transgenic mice has given some clues to this aspect. Mice with a global knockout of Scd1 ($Scd1^{-/-}$ mice) show decreased lipogenic gene expression and increased β-oxidation and are protected from diet-induced obesity and insulin resistance when fed a high-carbohydrate/high-fat diet (HC/HF diet) (Cohen et al. 2002; Ntambi et al. 2002; Dobrzyn et al. 2004). In agreement, inactivation of Scd1 gene protects against liver steatosis caused by high-fat diet (Kang et al. 2004).

Inhibition of Scd1 using antisense oligonucletoides in both liver and adipose tissues also prevents many of the HC/HF-diet metabolic complications, including hepatic steatosis and hyperglycemia (Jiang et al. 2005; Gutierrez-Juarez et al. 2006).

Liver-specific fatty acid synthase (Fasn) knockout mice (Faskol) results in mutant mice that possess similar phenotype than control mice when fed chow diet, but intriguingly, the lack of Fasn - which is believed to be a determinant of the maximal capacity of liver to synthesise fatty acids by *de novo* lipogenesis- does not protect against the development of fatty liver, but rather exacerbated it (Chakravarthy et al. 2005). This mouse model gives support to the novel concept that 'new fat' synthesised via Fasn activity would specifically activate a pool of nuclear receptors (e.g. peroxisome proliferator activator receptor alpha (PPARα)) and would in turn lead to enhanced β-oxidation (Chakravarthy et al. 2005). Since Scd1 is downstream of Fasn, the effects of Fasn inhibition may in part occur via reduced flux through Scd1. The above findings lead to the idea that the protective effect on hepatic steatosis seems to be found when a combined modulation in both lipogenic rates and β-oxidation pathway occurs *in vivo* and that Fasn and Scd1 are important determinants of metabolic status involving fatty liver development.

Concerning the effects of CLA on Scd1, although the majority of reports show that treatment with mixed isomers, and more specifically with the isomer *trans*-10, *cis*-12, decreases either the activity, which results in a decreased ratio of palmitoleic to palmitic acid and of oleic to stearic acid, and/or the abundance of Scd1 in a number of cells such as human breast cancer cell (Choi et al. 2002), hepatocytes (Choi et al. 2001) and preadipocytes (Brown et al. 2003), and in murine adipocytes (Choi et al. 2000) and hepatocytes (Lee et al. 1998; Park et al. 2000); there are also studies in which CLA

treatment do not change or even increase Scd1 expression (Peters et al. 2001; Kang et al. 2004).

Therefore, the aim of this work has been to get insight on the impact of moderate doses of CLA in the expression of Scd1 in liver and adipose tissues and its association with the lipogenic/oxidative potential of animals fed either a standard- or a high-fat diet, that show lower body fat content, a pattern of maintenance of insulin sensitivity and up to 8-fold increased expression of Scd1 in muscle.

MATERIALS AND METHODS

Animals and diets

Male C57BL/6J mice from Charles River (Barcelona, Spain) weighing 20 ± 0.2 g (5-week-old) were housed in groups of four in plastic cages and maintained on a 12-h light:dark cycle at 22ºC. These mice were used in two independent experiments with different dietary regimens. In Experiment 1 (Exp1) animals were fed *ad libitum* with a standard diet (D12450B, Research Diets Inc, New Brunswick) which contains 10% calorie content as fat. In Experiment 2 (Exp2) animals were fed *ad libitum* with a high-fat diet (D12451, Research Diets Inc, New Brunswick) which contains 45% calorie content as fat. Food intake and body weight were recorded every three days during the experiments. Fresh food was provided to the mice biweekly.

All experimental procedures were performed according to both national and institutional guidelines for animal care and use.

CLA Treatment

Mice were orally treated with CLA (Tonalin® TG 80 derived from safflower oil, kindly provided by Cognis). Tonalin is composed of triglycerides containing approximately 80% conjugated linoleic acid with a 50:50 ratio of the active CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12. Control animals received an adequate amount of commercial sunflower oil to achieve isocaloric load between groups.

In Exp1, mice were randomly assigned to three experimental groups (n= 12 each group) for 37 days: control (sunflower oil), CLA1 (3 mg CLA/day) or CLA2 (10 mg CLA/day).

In Exp2, mice were also assigned to three oral treatments (n= 8 each group) for 65 days: control (sunflower oil), CLA3 or CLA4. For the first 30 days, animals received the

same doses of CLA used in Exp1, from that time onwards animals received double dose. Therefore, animals received a daily amount of 6 mg CLA in CLA3 and of 20 mg in CLA4 group for the last 35 days of treatment.

Sacrifice and tissue sampling

Mice from Exp1 were sacrificed under feeding conditions and from Exp2 were fasted for 10 h before sacrifice. At the beginning of the light cycle, animals were anaesthetised by intraperitoneal injection of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). Liver, retroperitoneal white adipose tissue (rWAT) and brown adipose tissue (BAT) were rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid and stored at -70ºC.

Hepatic triglyceride quantification

A sample of liver (200–300 mg) was homogenized in phosphate buffered saline (1:2, wt:v) using a polytron homogenizer. Homogenates were centrifuged at 500*g* for 10 min and the supernatant was used for the quantification. Total triglyceride levels were measured using a commercial enzymatic colorimetric kit following standard procedures (Sigma Diagnostics, Madrid, Spain).

RNA extraction

Total RNA from liver, rWAT and BAT was extracted using Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop®Spetrophotometer ND-1000

(NadroDrop Technologies, Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis.

mRNA expression analysis

Real-time polymerase chain reaction (real-time PCR) was used to measure mRNA expression levels of target genes. 0.5 µg of total RNA (in a final volume of 10 µL) was denatured at 90°C for 1 min and then reverse-transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 42°C for 60 min, with a final step of 5 min at 99°C in a Perkin-Elmer 9700 Thermal Cycler (PerkinElmer, Wellesley, MA). Real-time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95ºC, followed by a total of 40 temperature cycles (15 s at 95ºC and 1 min at 60ºC). Each PCR was performed in a total volume of 6.25 µl, made from diluted cDNA template, forward and reverse primers (1 µM each), and Power SYBER Green PCR Master Mix (Applied Biosystems, CA, USA). Primer sequences are listed in Table 1. All primers were purchased from Sigma (Madrid, Spain). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. PCR products were also analyzed by electrophoresis in an ethidium bromide-stained agarose gel to check that a single amplicon of the expected size was indeed obtained. The relative quantification of each target gene was calculated based on efficiency and the crossing point deviation of an unknown sample versus a control. Data were normalized relative to the levels of the housekeeping genes: 18S. Results from CLA treated groups were expressed as relative fold to control group.

Statistical analysis

Data are presented as means ± standard error of the mean (SEM). One-factor analysis of variance (ANOVA) was used to determine the significance of the differences between groups. If there was a significant difference, a Least Significant Difference (LSD) test was used to determine the particular effect that caused that difference. $P < 0.05$ was statistically significant, and different superscripts discriminate differences between groups. The analysis was performed using the SPSS program for Windows version 16 (SPSS, Chicago, IL, USA).

RESULTS

Experiment 1

rWAT: Markers of fatty acid input (lipoprotein lipase (Lpl)) and lipolysis (patatin-like phospholipase domain containing 2 (Pnpla2) and hormone sensitive lipase (HSL)) in adipose tissue were not altered by CLA treatment; although a higher expression in the glucose transporter type 4 (Glut4) was seen at the lower CLA dose (CLA1) respect to both CLA2 and control groups ($P<0.01$) (Table 2). A potential increase in the lipid oxidation was observed (Fig. 1a): PPARα expression was higher at both doses ($P<0.05$) and, uncoupling protein 2 (Ucp2) only attained statistical significance at the highest dose ($P<0.001$) (Fig. 1a). However, liver (Cpt1a) and muscular (Cpt1b) carnitine palmitoyltransferase isoforms, were not altered at either dose (Fig. 1a). Transcription factors related to lipogenesis in adipose tissue (peroxisome proliferator activator receptor gamma (PPARγ) and sterol regulatory element binding factor 1 (SREBP-1c) were unaltered by the treatment (Fig. 1b). Similar trend compared to the control group was observed by CCAAT/enhancer binding protein alpha (C/EBPα), although expression in CLA1 was higher than in CLA2 group ($P<0.01$) (Fig. 1b). Interestingly, expression of two lipogenic markers as Fasn and Scd1 were statistically significant higher in CLA1 group and recovered control values in CLA2 (Fig. 1b).

BAT: Ucp1, the characteristic uncoupling protein in BAT, was not altered by CLA treatment, whereas Ucp2 compared with control values shown decreased expression in CLA1 group and increased expression in CLA2 ($P<0.001$) indicating different regulatory signals in function of the dose and metabolic conditions (Fig. 2a). Concerning expression of PPARα, which controls fatty acid oxidation, a dose-response decrease was observed ($P<0.001$) which was accompanied by a similar pattern in Cpt1b although only reached statistically difference with the highest dose ($P<0.001$, respect to

control group) (Fig. 2a). No changes in Cpt1a expression were found (Fig. 2a). PPARγ, which promotes lipid storage, and SREBP-1c, which is a master regulator of lipogenic related genes, were both unaffected by the lowest dose (CLA1) but showed a tendency to decrease by CLA2, which was statistical significant for PPARγ ($P<0.001$) (Fig. 2b). In accordance with this pattern, Fasn and Scd1 showed a decrease which attained statistical significance at the highest CLA dose ($P<0.01$) (Fig. 2b).

Liver: No changes in hepatic triglyceride content were observed after CLA treatment (6.82 ± 0.44 in control, 5.75 ± 0.27 in CLA1 and 5.86 ± 0.41 in CLA2 group mg triglycerides / g liver, n= 8). A dose-response decrease in the expression of PPARα and fibroblast growth factor 21 (Fgf21) was observed by CLA treatment but only CLA2 was statistically different from control group ($P<0.01$) (Fig. 3a). Similarly, a tendency to decrease Cpt1a expression in a dose-dependent manner was observed but without statistically significance (Fig. 3a). Ucp2 showed decreased expression by CLA treatment, at both doses ($P<0.01$) (Fig. 3a). PPARδ and acil-Coenzyme A oxidase (Acox1) expression levels did not change by CLA treatment (Fig. 3a). Lower lipogenic potential (Fig. 3b) was indicated by the decreased transcriptional factors SREBP-1c and PPARγ at both doses of CLA ($P<0.01$ and $P<0.05$ respectively), this was accompanied by a tendency to decrease Acc1 and Fasn together with a significant reduction in Scd1 expression at both doses ($P<0.05$).

Experiment 2

rWAT: Expression of biomarkers of fatty acid and glucose input in adipose tissue (Lpl, HSL, Pnpla2 and Glut4) were unaffected by CLA treatment (Table 3). Markers of fatty acid oxidation: PPARα, Cpt1a and Ucp2 were unaltered or even decreased such as Cpt1b, particularly at the highest dose ($P<0.01$) (Fig. 4a). Transcriptional factors

typically induced during adipogenesis (PPARγ, C/EBPα and SREBP-1c) were unaffected by CLA treatment (Fig. 4b). However, as seen in Exp1, Fasn and Scd1 were also induced at the lowest dose of CLA ($P<0.001$ and $P<0.01$ respectively), with a tendency to recover control values at the highest dose (Fig. 4b).

BAT: Modulation of Ucps expression by CLA showed the same pattern under high-fat feeding (Exp2) (Fig. 5a) as found under standard-fat feeding (Exp1). Expression levels of PPARα and Cpt1a were unaltered but a dose-response decrease was observed in Cpt1b ($P<0.01$) (Fig. 5a). PPARγ expression was unchanged whereas SREBP-1c decreased by CLA treatment ($P<0.05$) (Fig. 5b). Fasn and Scd1 showed an increase in CLA groups, although only attained statistical significance for Scd1 with the highest dose compared with control group ($P<0.01$) (Fig. 5b).

Liver: A decrease in the expression of Fgf21 was observed by CLA treatment (Fig. 6a). Ucp2 and PPARδ expression increased at the lowest dose of CLA and recovered control values at the highest dose (Fig. 6a). This was accompanied by a decrease in PPARα at the highest dose of CLA but no changes in Cpt1a and Acox1 (Fig. 6a). Unaltered lipogenic potential was indicated by SREBP-1c, Acc1, Fasn and Scd1 at both doses of CLA tested (Fig. 6b). Compared to controls, a tendency to increase mRNA levels of PPARγ in CLA treated groups was observed although did not reach statistical significance (Fig. 6b).

DISCUSSION

Using this animal model, we have previously found that dietary CLA is able to decrease body fat accumulation and modulate the inflammatory profile of adipose tissue with a minor impact on insulin sensitivity and in the absence of hepatic steatosis in mice (Parra et al.; Parra et al. 2009). In addition, we have shown that CLA causes a strong induction of muscle Scd1 which potentially would be responsible for increased production of palmitoleate, and would contribute to sort out maintenance of insulin sensitivity under both standard-fat and high-fat feeding (Parra P manuscript under preparation). Our present results outline the fact that moderate doses of CLA are able to modulate the expression of lipogenic/fatty oxidation markers in a tissue specific manner associated to the metabolic status of the animal. Interestingly, the high induction of Scd1 found in muscle (Parra P manuscript under preparation) in both experimental settings was not found either in adipose tissue or in liver, which highlights that this strong upregulation was not casual but it was produced in response to CLA impact on energy homeostasis. Therefore, differential regulation of Scd1 and Fasn in muscle, adipose tissue and liver, might play a role in tissues interplay and could contribute to prevent excess fat deposition in adipose tissue and to the maintenance of liver homeostasis.

Fat mass reduction by CLA is well established in mice and different mechanisms explaining de-lipidating effects have been proposed. A number of *in vitro* and *in vivo* studies have found that mainly *trans*-10, *cis*-12 isomer is consistently associated with a reduction in the expression and/or activity of Glut4 (Takahashi et al. 2002; Tsuboyama-Kasaoka et al. 2003; House et al. 2005) and Lpl (Park et al. 1997; Park et al. 1999; Lin et al. 2001; Xu et al. 2003) resulting in reduced glucose and fatty acid uptake; in association, several studies reported decreased lipogenesis (Tsuboyama-Kasaoka et al. 2000; Xu et al. 2003; LaRosa et al. 2006). Increased fatty acid oxidation (Park et al.

1997; House et al. 2005; LaRosa et al. 2006) together with upregulation of uncoupling proteins by CLA, indicators for energy expenditure, have also been reported (Peters et al. 2001; Ealey et al. 2002; Roche et al. 2002; Kang et al. 2004; House et al. 2005). In our experimental setting conditions, the potential input of glucose or fatty acids into adipose tissue, measured by expression of Glut4 and Lpl was unaffected by CLA feeding either under standard- or high-fat diet. Expression of enzymes involved in fatty acid oxidation as Cpts, were mainly decreased or unaffected. Although, an induction of Ucp2 was found in BAT and rWAT (only significant in Exp1) at the highest dose of CLA, but no induction of Ucp1 was found in BAT. Upregulation of Ucp2 seems to be sufficient to deal with the control and mobilization of fat stores at the appropriate level in these experimental conditions.

The reduction of fat observed in our experiments takes place far from the lipodystrophy reported in some stronger CLA treatments in mice, in which the rapid and drastic reduction of fat depots is commonly accompanied by a compensatory fat accumulation in liver and even an increase in hepatic lipogenesis to metabolize glucose (Nadler et al. 2001) that finally results in hepatic steatosis and increased risk of lipotoxicity (Tsuboyama-Kasaoka et al. 2000; Clement et al. 2002; Tsuboyama-Kasaoka et al. 2003; Javadi et al. 2004; Poirier et al. 2005; Yanagita et al. 2005). Supporting this idea, we didn't observe an increase in hepatic fat content. Equilibrium between hepatic lipogenic capacity and fatty acid oxidation appeared to exist in both experiments. However we can't rule out that prolonged CLA treatment would produce an unbalance and induce liver steatosis as previously has been reported (Javadi et al. 2004). For example, this would be feasible in the high-fat fed animals treated with the highest dose of CLA. These animals did not show hepatic fat accumulation, but a small degree of hyperinsulinemia and higher HOMA-IR (but normal R-QUICKI) was present (Parra et

al. 2009), indicating that insulin sensitivity was still under metabolic control but the conditions could be more stressful here than in the other groups studied which have all these parameters unaltered.

Interestingly, CLA treatment resulted in a tissue-specific modulation of Scd1 that was closely associated to the Fasn expression. Recently, the group of Cao (Cao et al. 2008), using a mice lacking chaperone fatty acid binding protein, have suggested that palmitoleate, the main Scd1 product, produced and released by the adipose tissue suppresses hepatic lipogenic rates through a specific inhibition of Scd1 in liver whereas improves insulin sensitivity in muscle. The Elovl6$^{-/-}$ mice model, which is unable to elongate C16 fatty acids to C18 and therefore, shows increased hepatic concentrations of palmitate and palmitoleate, also highlights the role of palmitoleate as a fatty acid potentially responsible for sustained insulin sensitivity in this animal model (Matsuzaka et al. 2009). Therefore, beyond *de novo* fatty acid synthesis, Scd1 seems to play a role in the maintenance of insulin sensitivity. Giving support to this idea, rosiglitazone treatment increases Scd1 activity indexes and gene expression in humans which is associated with improvement in insulin sensitivity (Riserus et al. 2005). Notably, the increase in the Scd1 activity index after rosiglitazone treatment is particularly marked in patients with the P467L PPAR-γ mutation, which present a lipodystrophic phenotype including insulin resistance and fatty liver, all of which improve after rosiglitazone treatment (Savage et al. 2003).

Whether the expression or activity of Scd1 in adipose tissue may impact on insulin sensitivity or liver metabolism has not been directly raised in previous CLA studies, but there are indirect evidences of this fact. For example, Scd1 and Fasn mRNA expression in adipose tissue of mice decrease as the *trans*-10, *cis*-12 content of the diet increases; in parallel, liver weight and fat content increase and Δ9 index for liver indicates linear

enhancement of Scd1 activity (Viswanadha et al. 2006). In this context, mice treated with a low dose of *trans*-10, *cis*-12 isomer show reduced fat but preserved Scd1 gene expression and upregulated Fasn and Scd2 (Scd2 prefers stearic acid (18:0) to palmitic acid (16:0) as a substrate compared to Scd1) expression in adipose tissue, whereas the liver weight and the hepatic fat content are not altered (Kang et al. 2004). Accordingly, in our experimental conditions, CLA treatment was able to maintain or to increase Fasn and Scd1 expression in adipose tissue whereas no induction in liver was found.

In summary, the fat loss triggered by moderate doses of isomers mix of CLA was not associated with activation of lipid oxidation gene expression in adipose tissue. No induction of thermogenesis was found in BAT, which showed a lipid gene expression pattern resembling the characteristics of white fat. Interestingly, a balance of the hepatic lipogenic/oxidative pathways was observed, which together with the induction of Fasn and/or Scd1 in adipose tissue, may contribute to prevent fat accumulation in the liver. We hypothesize that tissue-specific expression of Scd-1 would contribute in the insulin sensitivity and liver homeostasis preservation since its product, the palmitoleate, has been reported to play a role in interorgan crosstalk of regulation of metabolism

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FIGURE LEGENDS

Figure 1: Effects of CLA supplementation on expression of genes encoding enzymes involved in fatty acid oxidation (A) and lipogenesis (B) in the rWAT of mice fed a standard-fat diet.

Mice were fed *ad libitum* a standard-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/animal·day (CLA1 and CLA2 group, respectively) for 37 days. Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* A- mRNA levels of genes involved in fatty acid oxidation. B- mRNA levels of genes involved in lipogenesis. The results, mean ± SEM of 9-12 mice/group, are expressed as fold induction over control group. Mean values with unlike letters are significantly different ($P<0.01$); ANOVA followed by LSD test.

Figure 2: Effects of CLA supplementation on expression of genes encoding enzymes involved in fatty acid oxidation (A) and lipogenesis (B) in the BAT of mice fed a standard-fat diet.

Mice were fed *ad libitum* a standard-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/animal·day (CLA1 and CLA2 group, respectively) for 37 days. Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* A- mRNA levels of genes involved in fatty acid oxidation. B- mRNA levels of genes involved in lipogenesis. The results, mean ± SEM of 9-12 mice/group, are expressed as fold induction over control group. Mean values with unlike letters are significantly different ($P<0.05$); ANOVA followed by LSD test.

Figure 3: Effects of CLA supplementation on expression of genes encoding enzymes involved in fatty acid oxidation (A) and lipogenesis (B) in the liver of mice fed a standard-fat diet. Mice were fed *ad libitum* a standard-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/animal·day (CLA1 and CLA2 group, respectively) for 37 days. Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* A- mRNA levels of genes involved in fatty acid oxidation. B- mRNA levels of genes involved in lipogenesis. The results, mean ± SEM of 6-7 mice/group, are expressed as fold induction over control group. Mean values with unlike letters are significantly different (P<0.05); ANOVA followed by LSD test.

Figure 4: Effects of CLA supplementation on expression of genes encoding enzymes involved in fatty acid oxidation (A) and lipogenesis (B) in the rWAT of mice fed a high-fat diet. Mice were fed *ad libitum* a high-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/animal·day (CLA3 and CLA4 group, respectively) for one month and received a respective double dose for the last 35 days. Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* A- mRNA levels of genes involved in fatty acid oxidation. B- mRNA levels of genes involved in lipogenesis. The results, mean ± SEM of 7-8 mice/group, are expressed as fold induction over control group. Mean values with unlike letters are significantly different (P<0.05); ANOVA followed by LSD test.

Figure 5: Effects of CLA supplementation on expression of genes encoding enzymes involved in fatty acid oxidation (A) and lipogenesis (B) in the BAT of mice fed a high-fat diet. Mice were fed *ad libitum* a high-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/animal·day (CLA3 and CLA4 group, respectively) for one month and received a respective double dose for the last 35 days. Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* A- mRNA levels of genes involved in fatty acid oxidation. B- mRNA levels of genes involved in lipogenesis. The results, mean ± SEM of 7-8 mice/group, are expressed as fold induction over control group. Mean values with unlike letters are significantly different ($P<0.05$); ANOVA followed by LSD test.

Figure 6: Effects of CLA supplementation on expression of genes encoding enzymes involved in fatty acid oxidation (A) and lipogenesis (B) in the liver of mice fed a high-fat diet. Mice were fed *ad libitum* a high-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/animal·day (CLA3 and CLA4 group, respectively) for one month and received a respective double dose for the last 35 days. Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* A- mRNA levels of genes involved in fatty acid oxidation. B- mRNA levels of genes involved in lipogenesis. The results, mean ± SEM of 6-8 mice/group, are expressed as fold induction over control group. Mean values with unlike letters are significantly different ($P<0.05$); ANOVA followed by LSD test.

TABLES

Table 1. Gene-specific primer sequences used in real-time PCR amplification.

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
Acc1	GAACCCGAAACTCCCAGAAC	GAAGACCACTGCCACTCCA
Acox1	TGGTGAAGAAGATGAGGGAGT	AGCAAGGTGGGCAGGAAC
C/EBPα	AGGTGCTGGAGTTGACCAGT	CAGCCTAGAGATCCAGCGAC
Cpt1a	GCTCGCACATTACAAGGACAT	TGGACACCACATAGAGGCAG
Cpt1b	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG
Fasn	TTCGGTGTATCCTGCTGTCC	TGGGCTTGTCCTGCTCTAAC
Fgf21	ACAGATGACGACCAGGACAC	AGGCTTTGACACCCAGGATT
Glut4	GGCATGCGTTTCCAGTATGT	GCCCCTCAGTCATTCTCATC
HSL	TCACGCTACATAAAGGCTGCT	CCACCCGTAAAGAGGGAACT
Lpl	CCTGATGACGCTGATTTTGT	TATGCTTTGCTGGGGTTTTC
Pnpla2	TGTGGCCTCATTCCTCCTAC	AGCCCTGTTTGCACATCTCT
PPARα	CGTTTGTGGCTGGTCAAGTT	AGAGAGGACAGATGGGGCTC
PPARδ	GCCTCGGGCTTCCACTAC	TCCGTCCAAAGCGGATAG
PPARγ	AGACCACTCGCATTCCTTTG	TCGCACTTTGGTATTCTTGG
Scd1	GGAAATGAACGAGAGAAGGTG	CCGAAGAGGCAGGTGTAGAG
SREBP-1c	CAGCGGTTTTGAACGACA	GCCAGAGAAGCAGAAGAGAAG
Ucp1	GGCATTCAGAGGCAAATCAG	GCATTGTAGGTCCCCGTGTA
Ucp2	GGTCGGAGATACCAGAGCAC	ATGAGGTTGGCTTTCAGGAG
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC

Target genes: *Acc1*, acetyl-Coenzyme A carboxylase alpha; *Acox1*, acyl-Coenzyme A oxidase; *C/EBP*α, CCAAT/enhancer binding protein alpha; *Cpt1a*, liver carnitine palmitoyltransferase 1a; Cpt1b, muscle carnitine palmitoyltransferase 1b; *Fasn*, fatty acid synthase; *Fgf21*, fibroblast growth factor 21; *Glut4*, glucose transporter type 4; *HSL*, hormone sensitive lipase; *Lpl*, lipoprotein lipase; *Pnpla2*, patatin-like phospholipase domain containing 2; *PPAR*, peroxisome proliferator activator receptor alpha (α), delta (δ), gamma (γ); *Scd1*, stearoyl-Coenzyme A desaturase 1; *SREBP-1c*, sterol regulatory element binding factor 1; *Ucp*, uncoupling protein. *18S* was used for normalization.

Table 2. Relative changes in gene expression in rWAT of mice fed a standard-fat diet and treated with CLA.

Experiment 1 (standard-fat diet)

	Control	CLA1	CLA2
Glut4	1.00 ± 0.05[a]	1.29 ± 0.07[b]	0.84 ± 0.10[a]
Lpl	1.00 ± 0.09	1.05 ± 0.05	1.02 ± 0.09
Pnpla2	1.00 ± 0.13.	1.24 ± 0.07	1.02 ± 0.08
HSL	1.00 ± 0.11	1.02 ± 0.09	0.97 ± 0.11

Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* The results, mean ± SEM of 7-12 mice/group, are expressed as fold induction over control group. Means in a row without a common letter differ, $P < 0.05$ (ANOVA followed by LSD test).

Table 3. Relative changes in gene expression in rWAT of mice fed a high-fat diet and treated with CLA.

Experiment 2 (high-fat diet)

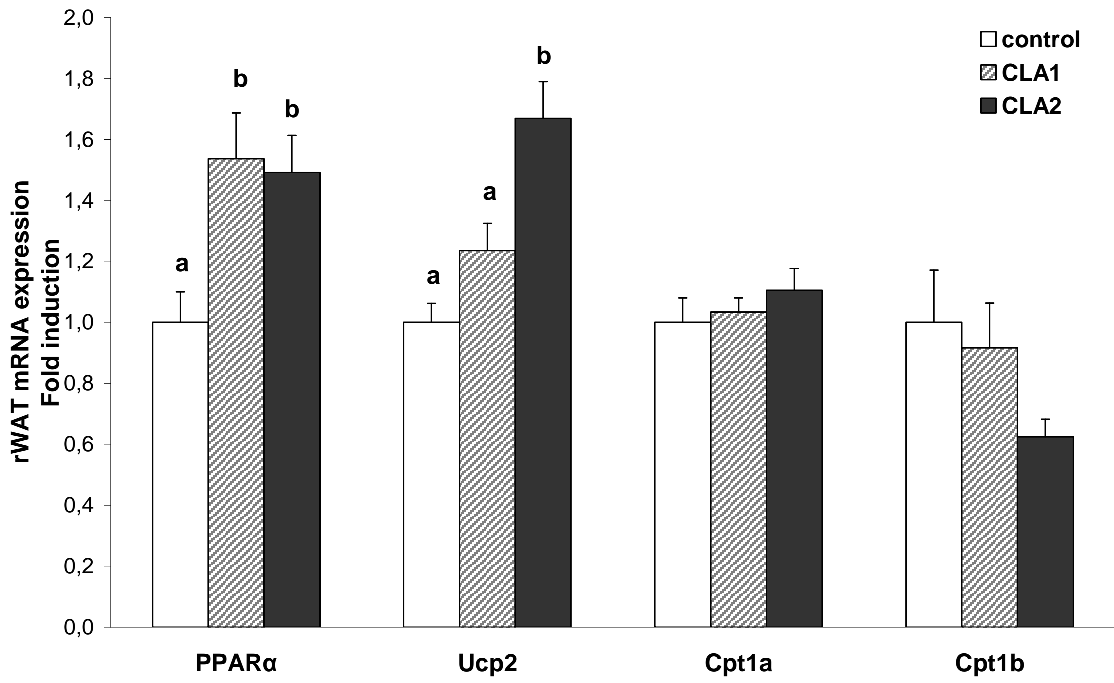
	Control	**CLA3**	**CLA4**
Glut4	1.00 ± 0.12	1.17 ± 0.10	1.12 ± 0.10
Lpl	1.00 ± 0.05	0.90 ± 0.06	1.00 ± 0.09
Pnpla2	1.00 ± 0.08	0.77 ± 0.06	0.96 ± 0.14
HSL	1.00 ± 0.11	1.12 ± 0.08	0.89 ± 0.08

Mice were fed *ad libitum* a high-fat diet and daily treated with an oral dose of CLA mixture for 65 days.

Expression levels of target genes were measured by real-time PCR and normalized by the internal housekeeping gene: *18S.* The results, mean ± SEM of 7-8 mice/group, are expressed as fold induction over control group. Means in were not significantly different at $P < 0.05$ using ANOVA.

Figure 1

a)



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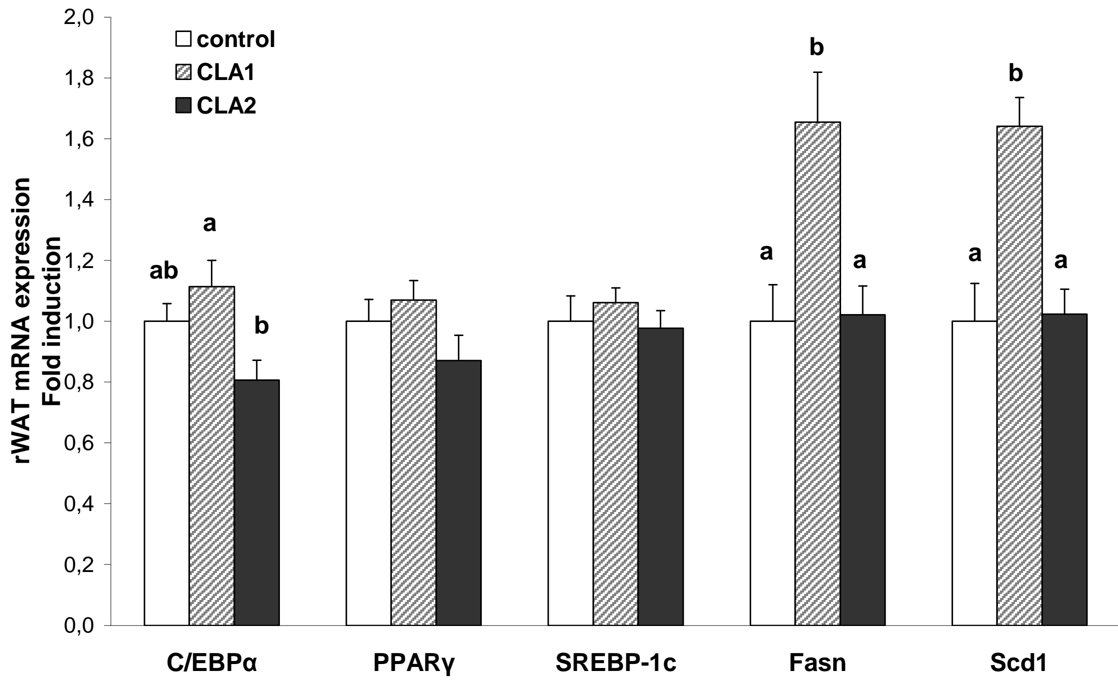

Figure 2

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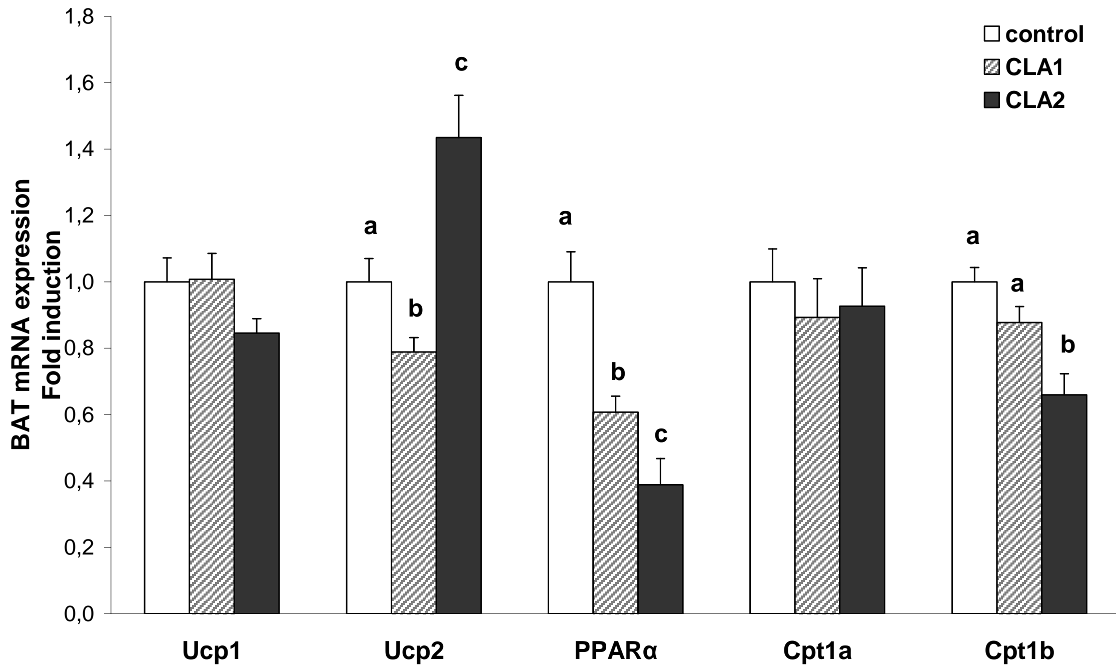

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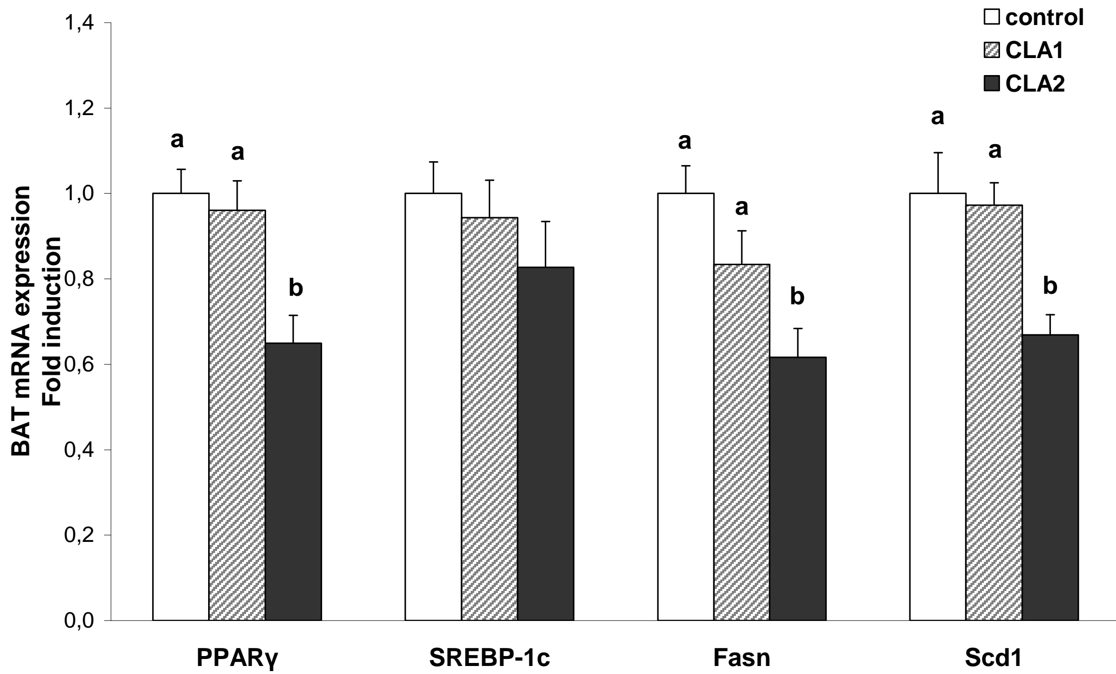

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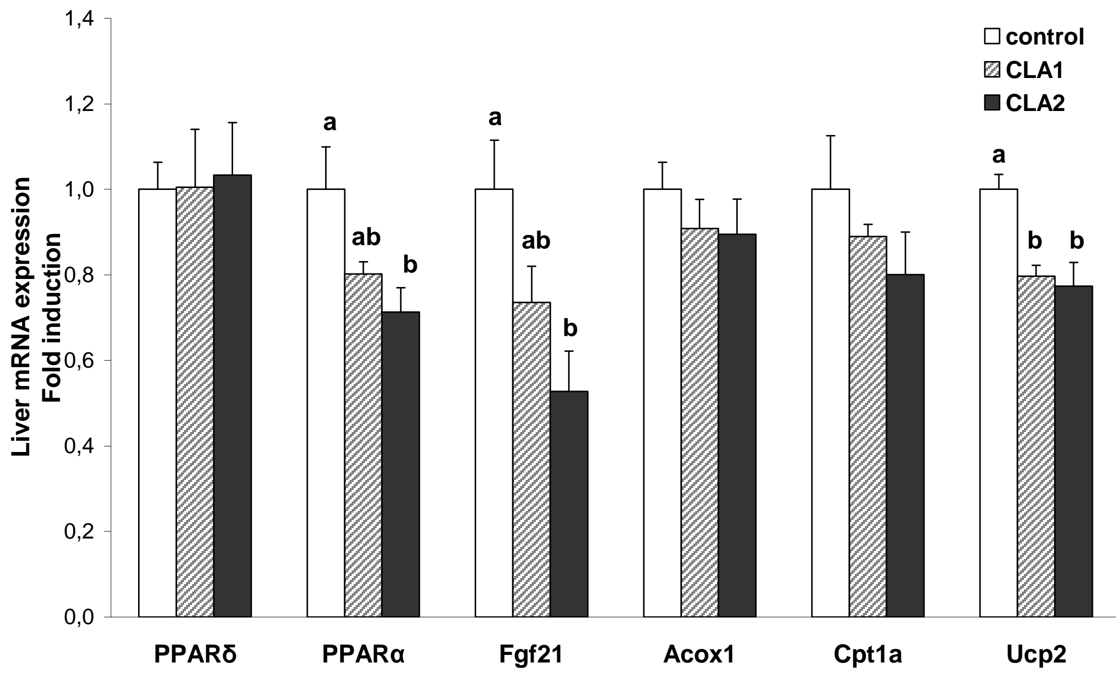

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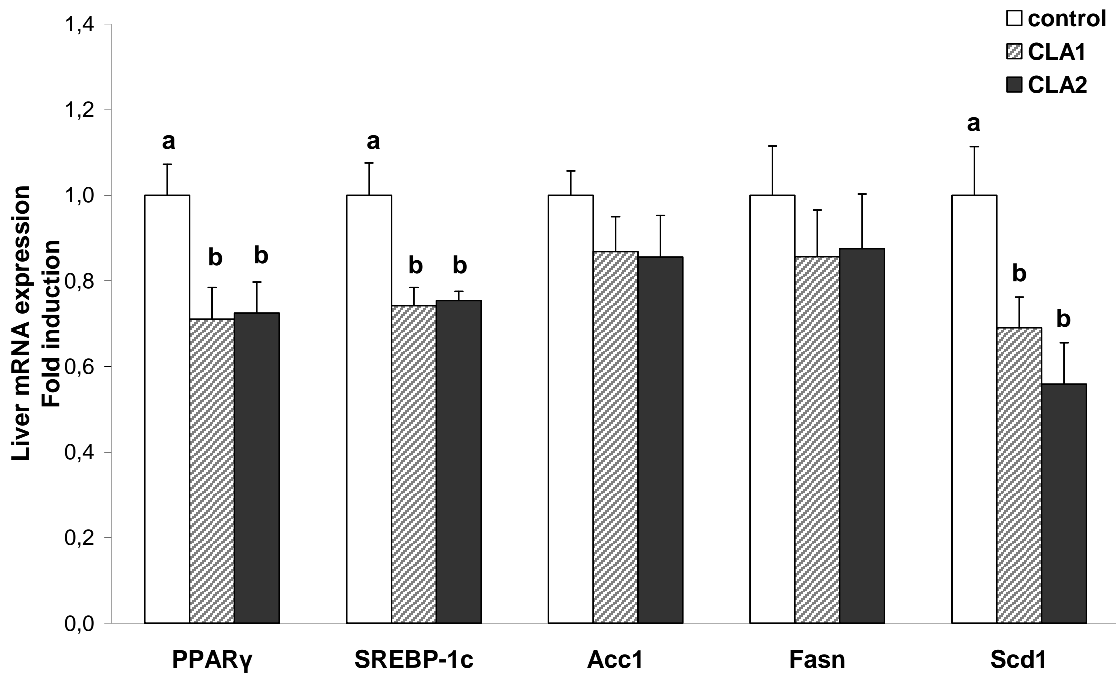

Figure 4

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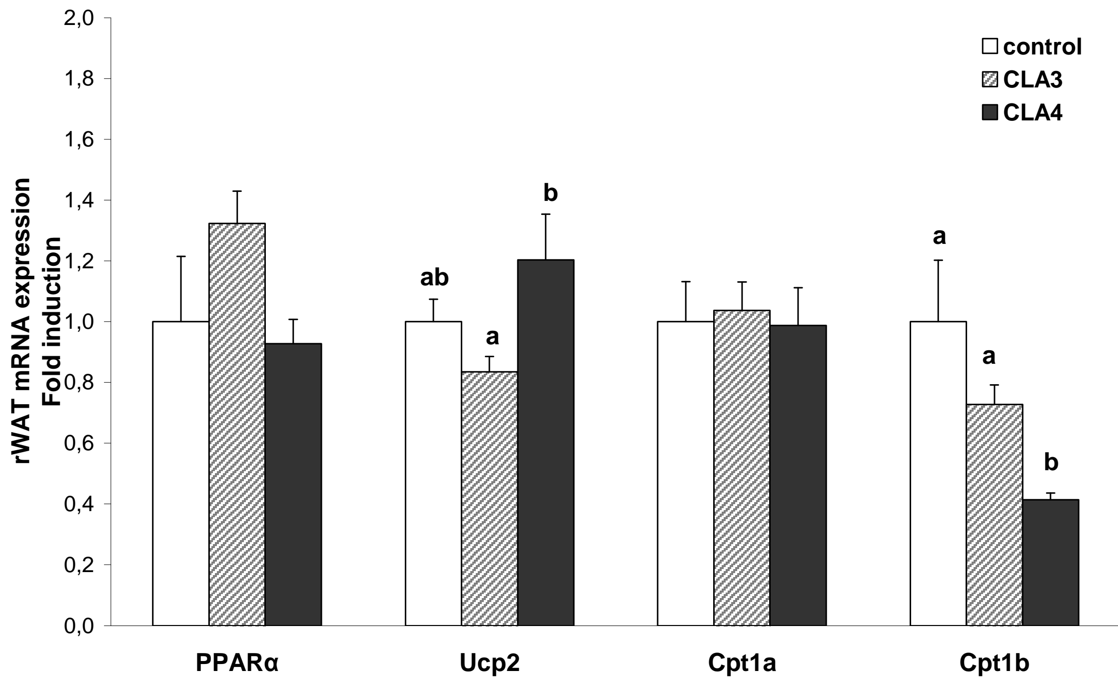

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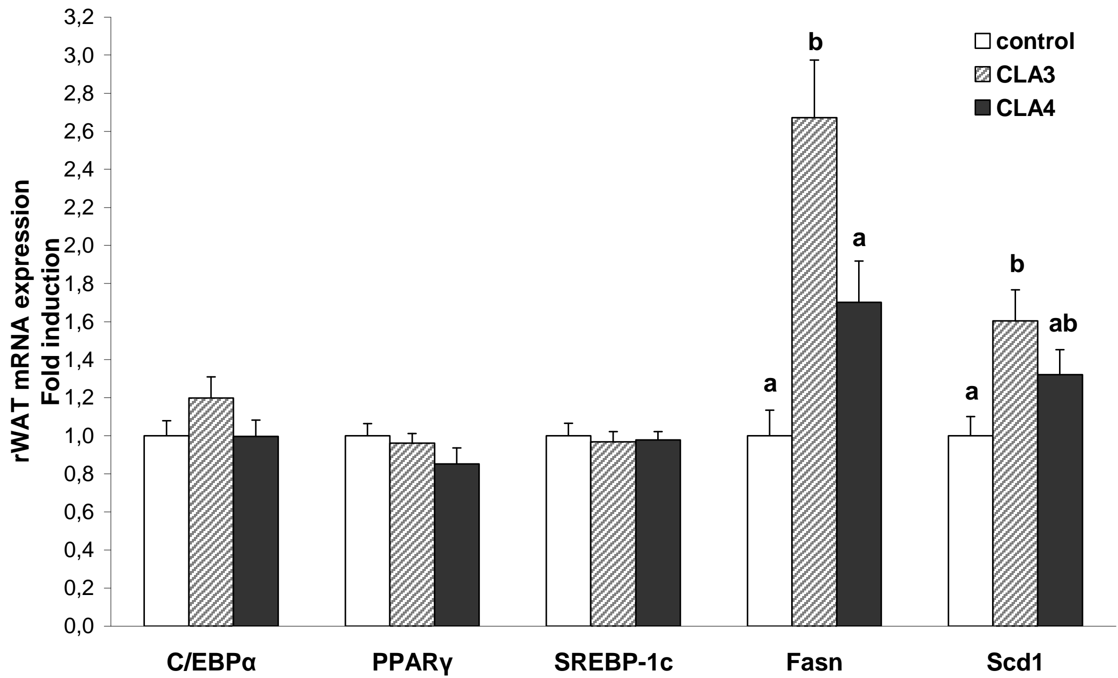
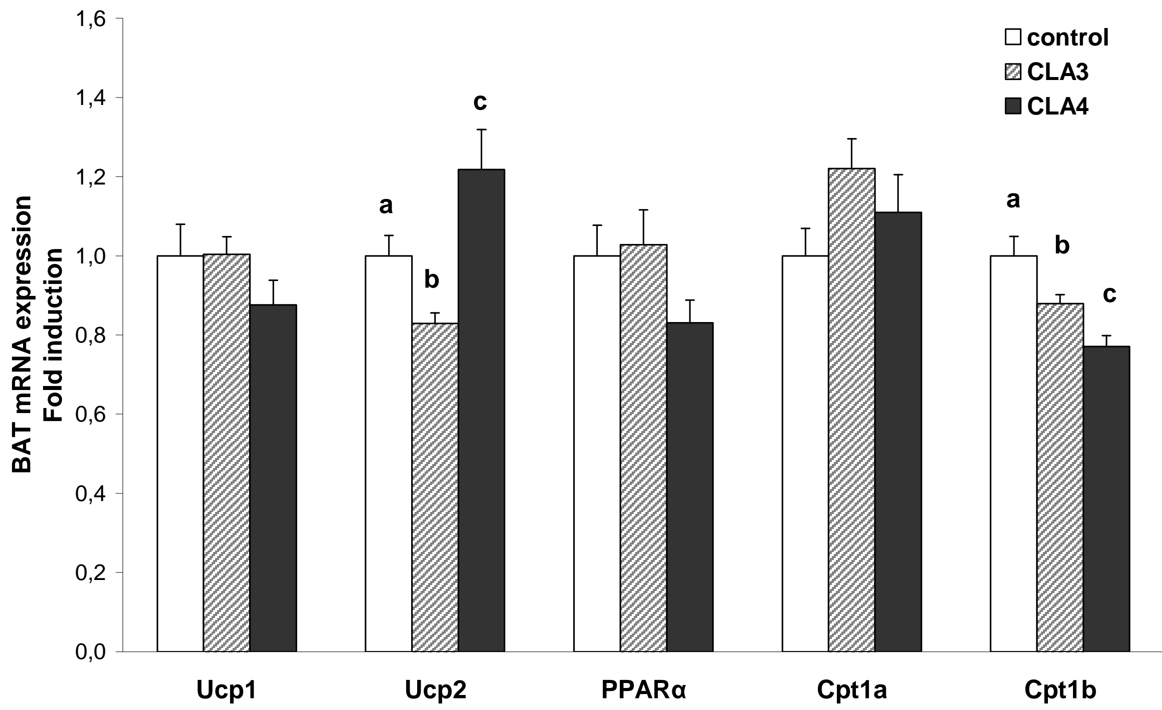


Figure 5

a)



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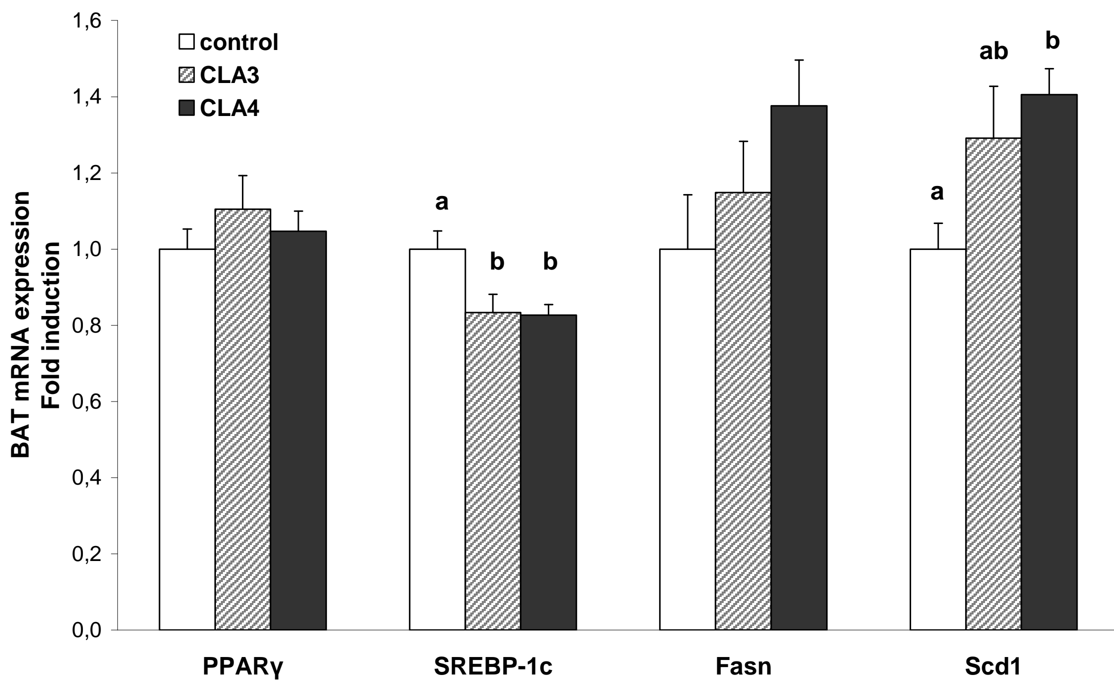
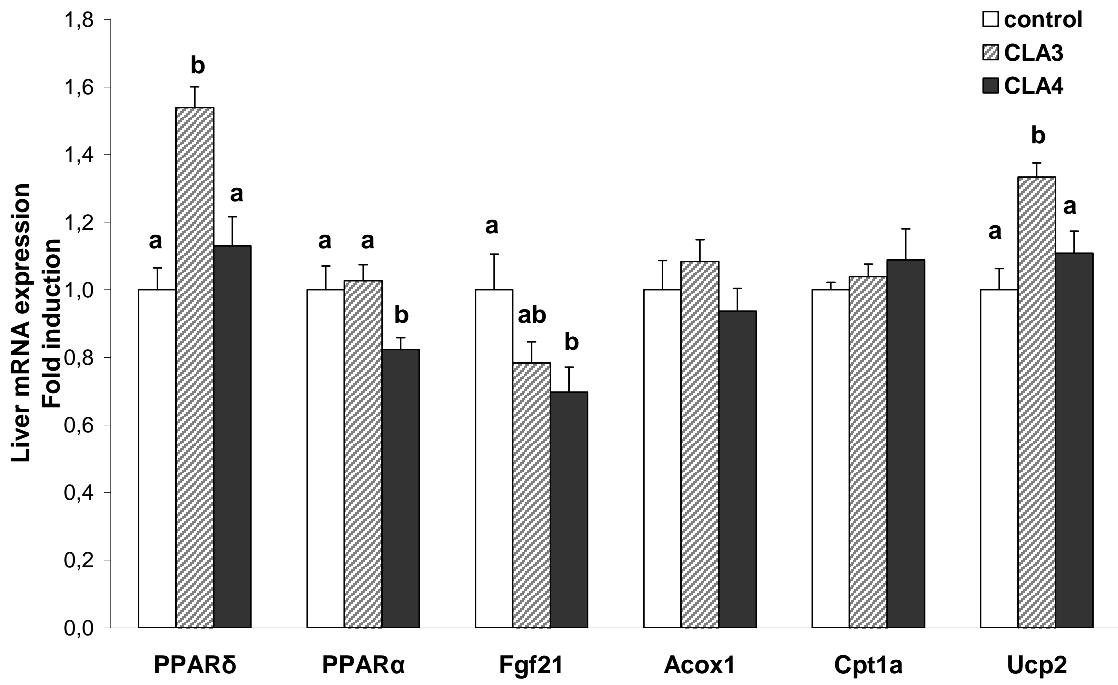


Figure 6

a)



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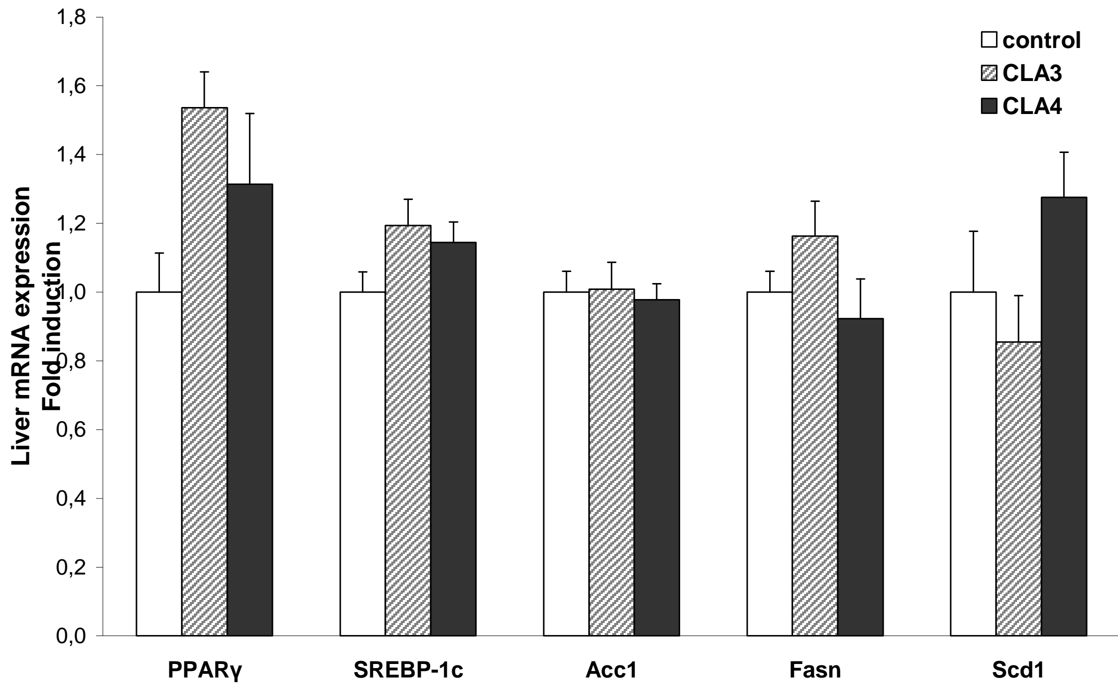

MANUSCRITO VII

Expression of adipose microRNAs is sensitive to dietary conjugated linoleic acid treatment in mice

Parra P, Serra F, Palou A.

Manuscrito en vías de publicación

Expression of adipose microRNAs is sensitive to dietary conjugated linoleic acid treatment in mice

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This work was supported by the grants from the Spanish Government (AGL2006-04887 and AGL2009-11277). Our Laboratory is a member of the European Research Network of Excellence NuGO (The European Nutrigenomics Organization, European Union Contract No. FP6-506360). P. Parra is a recipient of a fellowship from the Spanish Government. CIBER de Fisiopatología de la Obesidad y Nutrición is an initiative from ISCIII.

Keywords: Conjugated linoleic acid, microRNA, adipocyte, obesity

Abbreviations: *ap2*, adipocyte fatty acid binding protein; *C/EBPα*, CCAAT/enhancer binding protein alpha; *CLA*, conjugated linoleic acid; *Cpt1b*, carnitine palmitoyltransferase 1b (muscle); *Exp1*, experiment 1; *Exp2*, experiment 2; *Fasn*, fatty acid synthase; *Glut4*, glucose transporter type 4; *HSL*, hormone sensitive lipase; *Lpl*, lipoprotein lipase; *miRNAs*, microRNAs; *PANK*, panthothenate kinase; Pnpla2, patatin-like phospolipase domain containing 2; *PPARγ2*, peroxisome proliferator-activated receptor gamma 2; *pri-miRNA*, primary-miRNAs; *real-time PCR*, real-time polymerase chain reaction; *RISC*, RNA-induced silencing complex; *rWAT*, retroperitoneal white adipose tissue; *Scd1*, stearoyl-Coenzyme A desaturase 1; *TNFα*, tumor necrosis factor; *Ucp2*, uncoupling protein 2.

ABSTRACT

Investigation of microRNAs (miRNAs) in obesity, their genetic targets and influence by dietary modulators is of great interest because may potentially identify novel pathways involved in this complex metabolic disorder and influence future therapeutic approaches. In this study we aimed to determine whether miRNAs expression may be influenced by conjugated linoleic acid (CLA), treatment that is currently used for fat loss. We determined retroperitoneal adipose tissue expression of five miRNAs related to adipocyte differentiation (miRNA-143) and lipid metabolism (miRNA-103 and -107) and altered in obesity (miRNA-221 and -222). In a first experiment, mice were fed with a standard-fat diet and orally treated with sunflower oil (control group) and 3 or 10 mg CLA/day for 37 days. In a second experiment, mice were fed with a high-fat diet for 65 days. For the first 30 days, mice received the same doses of CLA described above and, from that time onwards, animals received a double dose. Our results showed that expression of selected miRNAS was modified in response to CLA treatment and metabolic status. Interestingly, a strong correlation was observed between miR-103 and-107 expression, as well as miR-221 and -222 in either of the experiments. Moreover, changes in miRNAs expression correlated with several adipocyte gene expressions, thus: miR-103 and -107 correlated with genes involved in fatty acid metabolism whereas miR-221 and miR-222 correlated with the expression of adipocytokines. In accordance, to minor changes observed in miR-143 expression, no differences in expression of adipogenic markers were observed. Although to elucidate the functional implications of miRNAs is beyond the scope of this study, these findings provide the first evidence that miRNA expression may be influenced by dietary manipulation and therefore, may involve a novel mechanism by which CLA exerts its de-lipidating effects, offering a new perspective for future therapeutic use of CLA.

INTRODUCTION

In the last decade, a novel class of RNA regulatory genes known as microRNAs (miRNAs) has been found to introduce a new level of gene regulation in eukaryotes (Lewis et al. 2005). miRNAs are transcribed as long primary-miRNAs (pri-miRNA) that encode a single miRNA or a cluster of miRNA species. Genomic mapping has revealed that pri-miRNA species are encoded within noncoding genomic sequences as well as in introns or, less frequently exons, of protein-coding genes. The processing of pri-miRNAs is initiated in the nucleus and is further continued in the cytoplasm giving rise to a 19–22 bp long mature miRNA. The mature miRNA is then incorporated into a protein complex, the RNA-induced silencing complex (RISC), where the miRNA strand anneals to the 3' untranslated regions of target mRNAs to promote mRNA degradation or translational repression, but in some cases, increases its translational activity (Vasudevan et al. 2007). The versatility of miRNA-mediated gene regulation is evidenced by the finding that individual miRNAs can target hundreds of genes while individual mRNAs can be targeted by multiple miRNAs, allowing for enormous complexity and flexibility in their regulatory potential (Krek et al. 2005; Shalgi et al. 2007; Baek et al. 2008; Selbach et al. 2008).

Many miRNAs are conserved across species and intervene in a variety of physiological processes including growth, differentiation, development and energy metabolism. The first evidence for participation of miRNAs in lipid metabolism came from a study in *Drosophila melanogaster*, in which miR-14 was identified as necessary for normal fat metabolism (Xu et al. 2003). In mammals, miRNAs have been shown to modulate adipocyte differentiation (Esau et al. 2004; Kajimoto et al. 2006; Sun et al. 2009; Xie et al. 2009), cholesterol and lipid homeostasis in liver (Esau et al. 2006; Krutzfeldt et al. 2006) as well as insulin secretion and signalling (Poy et al. 2004; He et al. 2007).

Recent papers have observed an association between the expression of specific miRNAs and obesity (Takanabe et al. 2008; Kloting et al. 2009; Nakanishi et al. 2009) supporting the fact that miRNAs may play a role in the pathological development of obesity and also leading to the hypothesis that miRNAs may represent a new class of adipogenic regulators with potential therapeutic interest against obesity. However, no effects on the impact of supplementation with nutrients that modulate body composition in the expression of miRNAs have been described yet.

Among nutrients, conjugated linoleic acid (CLA), which refers a group of positional and geometric isomers of linoleic acid, has been reported to reduce fat deposition both in animals (reviewed in (Wang et al. 2004)) and, to a lesser extent, in humans (reviewed in (Whigham et al. 2007)), and in consequence, is used as dietary supplement for weight loss.

We have recently shown that moderate doses of equimolar mix of the two main active isomers are associated to lower fat accretion in mice either under standard-fat (Parra et al. 2009) and high-fat diet (Parra et al. 2009) without inducing liver steatosis and keeping insulin sensitivity. In a step further, the purpose of the present study has been to assess whether miRNAs could play a role in the novel steady state induced by CLA. Consequently, expression levels of selected miRNAs (miR-143, miR-103, miR-107, miR-221 and miR-222) which seem to be related to adipose biology were studied in adipose tissue of mice treated with CLA.

METHODS AND MATERIALS

Animals and diets

Male C57BL/6J mice from Charles River (Barcelona, Spain) weighing 20 ± 0.2 g were housed in groups of four in plastic cages and maintained on a 12-h light:dark cycle at 22ºC. These mice were used in two independent experiments with different dietary regimens. In Experiment 1 (Exp1) animals were fed *ad libitum* with a standard diet (D12450B, Research Diets Inc, New Brunswick) which contains 10% calorie content as fat. In Experiment 2 (Exp2) animals were fed *ad libitum* with a high fat diet (D12451, Research Diets Inc, New Brunswick) which contains 45% calorie content as fat. Food intake and body weight were recorded every three days during the experiments. Fresh food was provided to the mice biweekly.

All experimental procedures were performed according to both national and institutional guidelines for animal care and use.

CLA Treatment

Mice from both experiments were orally treated with CLA. The CLA used was Tonalin ® TG 80 derived from safflower oil (kindly provided by Cognis). Tonalin is composed of triglycerides containing approximately 80% conjugated linoleic acids with a 50:50 ratio of the active CLA isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12.

In Exp1, mice were randomly assigned to three experimental groups (n= 12 each group): sunflower oil (control), CLA1 or CLA2 group. Two different doses of CLA were assayed in this study: CLA1 (0.15 g CLA/kg body weight) and CLA2 (0.50 g CLA/kg body weight), taking as reference, the weight of the animals at the beginning of the experiment. Therefore, animals received a daily amount of Tonalin equivalent to 3

mg CLA/animal in CLA1 group and 10 mg/animal in CLA2 group for 37 days. Control animals received an isocaloric dose of commercially available sunflower olive oil.

In Exp2, mice were also assigned to three experimental oral treatments (n= 8 each group): sunflower oil (control), CLA3 or CLA4 group for 65 days. For the first 30 days the same doses of CLA used in Exp1 were assayed, then after 30 days of treatment and until the end of the experiment, the dose of each group was doubled. Therefore, animals received a daily amount of Tonalin equivalent to 6 mg CLA/animal in CLA3 and 20 mg/animal in CLA4 groups for the last 35 days of treatment. An adequate amount of commercial sunflower oil was given to the animals to achieve an isocaloric load between groups.

Sacrifice and tissue sampling

Mice from Exp1 were sacrificed under feeding conditions and from Exp2 were fasted for 10h before sacrifice. Mice were anaesthetized by intraperitoneal injection of a mixture of xilacine (10 mg/kg body weight) and ketamine (100 mg/kg body weight). Retroperitoneal white adipose tissue (rWAT) was rapidly removed, weighed, rinsed with saline containing 0.1% diethyl pyrocarbonate (Sigma, Madrid, Spain), frozen with nitrogen liquid, and stored at -70ºC.

RNA extraction

Total RNA from rWAT was extracted using Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop®Spectrophotometer ND-1000 and its integrity confirmed by agarose gel electophoresis.

miRNAs expression analysis

Total RNA was diluted to 2.5 ng/µl. For each miRNA, 2 µl of these dilutions was reverse-transcribed in 4 µl reaction mix (TaqMan® MicroRNA Reverse Transcription kit, Applied Biosystems) and 1.5 µl of the miRNA-specific reverse-transcription primers provided with the TaqMan® MicroRNA Assay (Applied Biosystems). For the reverse transcription a Perkin-Elmer 9700 Thermal Cycler (PerkinElmer, Wellesley, MA) was used with the following conditions: 16ºC for 30 min; 42ºC for 30 min and 85ºC for 5 min. 2 µl of miRNA-specific cDNA from this reaction was amplified with the TaqMan Universal PCR master mix and the respective specific probe provided in the TaqMan® MicroRNA Assay (Applied Biosystems) (Table 1). PCR was performed in an Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems). Amplification initiated at 95ºC for 10 min followed by 40 cycles consisting of denaturation at 95ºC for 15 sec and anneling and extension at 60ºC for 1 min. Relative quantification of a target gene was calculated based on efficiency and the crossing point deviation of an unknown sample versus a control, and expressed in comparison to a reference gene used to normalize cDNA (U6 small nuclear RNA). miRNAs expression levels were calculated relative to the values in the respective control group.

Gene expression in retroperitoneal adipose tissue

Real-time polymerase chain reaction (real-time PCR) was used to measure mRNA expression levels. Total RNA was reverse transcribed to cDNA as previously described (Parra et al. 2009). Each PCR was performed in a total volume of 6.25 µl, made from diluted cDNA template, forward and reverse primers (1 µM each), and Power SYBER Green PCR Master Mix (Applied Biosystems). All primers were obtained from Sigma

(Madrid, Spain) and their sequences are listed in Table 2. In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. PCR products were also analyzed by electrophoresis in an ethidium bromide-stained agarose gel to check that a single amplicon of the expected size was indeed obtained. 18S ribosomal RNA was used as invariant control. Relative quantification of target genes was calculated as mentioned above.

Statistical analysis

Data are presented as means ± SEM. One-factor ANOVA was used to determine the significance of the differences between groups. If there was a significant difference, a Least Significant Difference (LSD) test was used to determine the particular effect that caused that difference. $P < 0.05$ was statistically significant, and different superscripts discriminate differences between groups. Linear relationships between key variables were tested using Pearson's correlation coefficients. The analysis was performed using the SPSS program for Windows version 16 (SPSS, Chicago, IL, USA).

RESULTS

Adipose tissue miRNA expression

CLA was shown to modulate the expression of selected miRNA in adipose tissue in specific manner, reflecting the impact of the type of diet, the metabolic status and the dose of CLA (Figure 1). The lowest dose of CLA did not cause any change in miR-143 expression, either in Exp1 or Exp2. However, the highest doses of CLA produced a decrease in each experimental design (by 20%) although only reached statistically significance in Exp1, when comparing CLA treated groups ($P < 0.05$). No changes in miR-103 were observed by CLA treatment in either of the experiments. In Exp1, miR-107 showed a dose-dependent decrease in its expression, which attained statistical significance at the highest dose (CLA2) respect to both CLA1 and control group ($P < 0.05$). Interestingly, under high-fat diet (Exp2) miR-107 showed a different expression pattern depending on the dose. A 40% increase ($P < 0.05$) respect to the control value was found with the lowest dose (CLA3 group) in contrast with the 46% decrease ($P < 0.01$) with respect to the control group that was observed at the highest dose (CLA4). miR-221 expression was not affected in Exp1 and, only the highest dose of CLA in Exp2 (CLA4) produced a significant increase of miR-221 with respect to both, CLA3 and control groups ($P < 0.01$). Concerning miR-222 expression, a tendency to increase expression with the dose was seen in Exp1, although only the 75% increase produced in CLA2 group was statistical significant compared to the control group ($P < 0.01$). In Exp2, miR-222 expression increased with the highest dose (CLA4 group) but did not reach statistical significance.

Expression levels of adipogenic marker genes

Since the miRNAs selected were mainly related with adipogenesis, we analyzed by RT-PCR expression levels of adipogenic marker genes such as glucose transporter type 4 (Glut4), lipoprotein lipase (Lpl), peroxisome proliferator activator receptor gamma 2 (PPARγ2) and CCAAT/enhancer binding protein alpha (C/EBPα). In Exp1 under a stardard-fat diet (Table 3) no differences in Lpl or the transcription factor PPARγ2 were observed by CLA treatment. Glut4 showed a statistically significant increase with the lowest dose (CLA1) respect to both CLA2 and control group ($P < 0.01$). C/EBPα slightly decreased with the highest dose but only reached statistically significance when comparing with the lowest dose (CLA1 group) ($P < 0.01$). CLA treatment did not produce changes in the expression of these genes in Exp2 (Table 4).

Relationship within miRNAs expression

We further tested the hypothesis that miRNAs are expressed in a coordinated manner as response to the remodelation of adipose tissue by CLA treatment. Data concerning the correlations within their expression levels confirmed that the expression of miRNAs was tightly correlated, particularly under normal-fat feeding (Exp1) (Table 5). In fact, expression of all miRNAs tested was significant and highly correlated within themselves, except for the case of miR-222 which was only correlated with miR-103 and miR-221. This strong association was partially lost in Exp2, with CLA treatment under high-fat feeding, in which the only correlations maintained were between miR-103 and miR-143; mir103 and miR-107; and miR-221 and miR-222 (Table 6).

Correlations between miRNAs and adipocyte gene expression

Significant correlations between the expression of miRNAs and the expression of genes contributing to the adipocytes phenotype were also identified by CLA treatment. In Exp1, miR-143 correlated positively with adiponectin ($r = 0.357$, $P < 0.05$) and leptin expression ($r = 0.358$, $P < 0.05$); miR-103 was correlated with two key markers of lipid metabolism, fatty acid synthase (Fasn, $r = 0.378$, $P < 0.05$) and muscle carnitine palmitoyltransferase 1b (Cpt1b, $r = 0.404$, $P < 0.05$); miR-107 correlated with genes involved in fatty acid oxidation such as uncoupling protein 2 (Ucp2, $r = -0.339$, $P < 0.05$) and Cpt1b ($r = 0.467$, $P < 0.01$) as well as with C/EBPα ($r = 0.370$, $P < 0.05$). miR-222 showed significant correlations with an array of genes related to adipocyte metabolism including Glut4 ($r = -0.400$, $P < 0.05$); genes related to lypolisis such as hormone sensitive lipase (HSL, $r = -0.379$, $P < 0.05$) and patatin-like phospolipase domain containing 2 (Pnpla2, $r = -0.346$, $P < 0.05$); lipogenesis such as PPARγ2 ($r = -0.396$, $P < 0.05$), Fasn, $r = -0.416$, $P < 0.05$), stearoyl-Coenzyme A desaturase 1 (Scd1, $r = -0.392$, $P < 0.05$); fatty acid oxidation as Ucp2 ($r = 0.525$, $P < 0.01$); and adipocytokines: adiponectin ($r = -0.385$, $P < 0.05$) and tumor necrosis factor alpha (TNFα, $r = 0.644$, $P < 0.01$). miR-221 was also specifically correlated with the expression of TNFα ($r = 0.385$, $P < 0.05$) (Table 7). In Exp2, miR-107 expression in adipocytes was significantly correlated with the expression of Lpl ($r = -0.428$, $P < 0.05$), PPARα ($r = 0.494$, $P < 0.05$) and TNFα ($r = -0.510$, $P < 0.05$), additionally under high-fat feeding, remained significant the correlation found by CLA under normal-fat feeding between miR-107 and Cpt1b ($r = 0.466$, $P < 0.05$). The high number of correlations with miR-222 was not found in Exp2. Correlation found for miR-221 and TNFα in Exp1 was maintained in Exp2 ($r = 0.434$, $P < 0.05$) and was also identified a significant negative correlation between adiponectin and miR-221 ($r = -0.444$, $P < 0.05$).

DISCUSSION

We have previously reported that the use of moderate doses of CLA reduces body fat accretion in mice maintained on different dietary regimen (Exp1 and Exp2) (Parra et al. 2009; Parra et al. 2009). In both experiments, the rWAT was the most sensitive to the effects of CLA, decreasing up to 67% under CLA treatment in comparison with controls. Therefore, this adipose tissue was selected to assess whether CLA treatment could produce any impact in miRNAs expression levels. Several mechanisms by which CLA decreases fat mass have been proposed such as increased energy expenditure, regulation of genes encoding for enzymes involved in lipid synthesis, promotion of adipocyte apoptosis or decreased preadipocyte proliferation and differentiation (reviewed in (Wang et al. 2004; House et al. 2005)). The complex response to CLA in adipose tissue is likely to take place through a CLA-mediated modulation of major metabolic regulators which are not totally known. In agreement with this assumption, the present study demonstrated that changes in miRNA expression occurred in adipose tissue after CLA treatment, suggesting a novel level of regulation by which CLA might exert its effects.

Emerging evidences suggest that specific miRNAs contribute to the regulation of adipocytes differentiation and in consequence may play a key role in the pathological development of obesity (Esau et al. 2004; Lin et al. 2009; Xie et al. 2009). As potential targets of the action of CLA, we focused the study on five selected miRNAs (miR-143, miR-103, miR-107, miR-221 and miR-222) which, at some extent, have been shown to be involved in adipocyte differentiation and/or associated with obesity.

MiR-143 has been the first miRNA associated with regulation of adipocyte differentiation. Its expression increases in differentiating adipocytes and antisense oligonucleotides against miR-143 inhibit human cultured adipocytes differentiation and

lead to a decrease in triglyceride accumulation and the downregulation of PPARγ2, adipocyte fatty acid binding protein (ap2) and Glut4 (Esau et al. 2004), although this has not been found in 3T3-L1 cells (Kajimoto et al. 2006). Later on it was demonstrated that in the mesenteric fat, miR-143 expression was upregulated in mice fed a high-fat diet and this was associated with an elevated body and mesenteric fat weight as well as with markers of adipocytes differentiation (Takanabe et al. 2008). Accordingly, a decrease in expression of miR-143 by CLA would justify a lower adipogenic capacity and therefore, would contribute to the decrease of fat stores observed in adipose tissue with CLA treatment. However, our data showed small changes, if any, in the expression levels of miR-143. Only the highest CLA dose assayed in each experiment caused a slight decrease of miR-143 expression, but not significantly different from controls. In accordance, gene expression of adipogenic markers such as Glut4, Lpl, PPARγ2 and C/EBPα was not decreased by CLA treatment in either of the experiments, suggesting that the body fat lowering effect of CLA observed in our conditions was unlikely a consequence of a reduction in the adipogenic capacity.

Other aspect of interest concerns to miR-103 and miR-107 regulation by CLA. Some miRNAs originate from introns of known genes; in this case, they can be co-transcribed with the 'parent' mRNAs or independently (Rodriguez et al. 2004; Baskerville et al. 2005; Lin et al. 2006; Ying et al. 2006). For example, miR-107, miR-103-1 and miR-103-2 reside in the sense orientation in intron 5 of the three panthothenate kinase (PANK) gene family members, PANK1, PANK3 and PANK2, respectively. A computational study predicts that this family of miRNAs affects multiple target genes in metabolic pathways in a manner that points to a coordinated function with the PANK genes, which are central players in regulating intracellular Co-enzyme A (Vallari et al. 1987; Rock et al. 2000; Wilfred et al. 2007). This has been further supported by

experimental data showing that induction of miR-103 and -107 during *in vitro* adipogenesis is accompanied by induction of the respective PANK gene expression (Xie et al. 2009). We found high and significant correlations between the expression of miR-103 and -107 in both experimental settings suggesting coordinated expression of both miRNAs. Interestingly, in Exp1, miR-107 was correlated with markers of oxidative pathway and miR-103 was correlated with the expression of two limiting enzymes in fatty acid metabolism, such as Fasn and Cpt1b, suggesting that CLA intake might contribute, at some extent, to modulate the role of these two miRNAs in canalizing Acil-CoA derivatives to lipogenesis and/or to oxidation. However, in Exp2 this situation was different. Although expression levels of miR-103 did not change by CLA treatment, the associations of its expression with phenotype markers of adipocytes were not found. Concerning expression of miR-107, CLA altered the profile of its expression in both experiments and this resulted in novel associations with adipocyte gene expression, only the association with Cpt1b was sustained in both experimental designs. The above data points out that the potential role of modulation of miRNAs *in vivo* is more complex that *in vitro* assays and in addition, it is affected by a number of external factors such as obesity, dietary treatment, metabolic status, etc.

Additionally, a recent report has described an inverse relation of several miRNAs expression during adipogenesis and obesity. A set of miRNAs (including miR-103, miR-107 and miR-143) are induced during adipogenesis, which may have a role in accelerating fat cell development, and then are downregulated in the obese state (Xie et al. 2009). Conversely, other set of miRNAs follows the opposite response pattern, for example miR-222 and miR-221, which are decreased during adipogenesis but upregulated in obese adipocytes (Xie et al. 2009). In agreement with this pattern, we found high and significant correlation between the expressions of these two miRNAs in

both experiments, but contrary to the above, both miRNAs did not follow a parallel regulation of its expression under CLA treatment. Although little is known about the adipocyte biology of both miRNAs, treatment of differentiated 3T3-L1 adipocytes for 24h with TNFα has been shown to induce expression of both miRNAs, which has been associated with the role of TNFα, as a major macrophage-produced cytokine involved in chronic inflammation (Xie et al. 2009), being largely responsible for inducing insulin resistance in obese adipose tissue (Cawthorn et al. 2008). Our data points out that CLA was able to counteract the joint expression profile of this two miRNAs observed in obese state, where both miRNAs increase simultaneously (Xie et al. 2009) and, therefore this is associated with adipocyte dysfunction and insulin resistance (Xie et al. 2009), which was not observed in our study using moderate doses of CLA (Parra et al. 2009; Parra et al. 2009). Furthermore, the pattern of correlations found between miRNA-221, -222 and adipocyte markers, highlights the interplay between these two miRNAs in the regulation of genes involved with fatty acid metabolism and, particularly with the expression of adipocytokines, as TNFα and its response to dietary CLA.

Investigation of miRNAs in obesity, their genetic targets and influence by dietary modulators is of great interest because may potentially identify new pathways involved in this complex metabolic disorder and influence future approaches to the treatment of obesity (Heneghan et al. 2009). Although to elucidate the functional implications of miRNAs is beyond the scope of this study, our data showed that specific miRNAs are sensible to dietary manipulation and reflected the metabolic changes that take place under CLA treatment. These results are the basis for novel ways in the research on CLA field. However, further studies will be needed to understand the role of miRNAs in CLA action and, to assess the potential of miRNA profiles to predict nutritional status in which CLA might trigger beneficial effects.

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FIGURE LEYENDS

Figure 1. Relative expression of miRNAs in rWAT of mice maintained on different dietary regimen and treated with CLA.

After CLA treatment, expression levels of miRNAs were measured by real-time PCR in rWAT of mice fed a standard-fat diet **(A)** or a high-fat diet **(B).** Data are means ± SEM of n=10-11 mice in Exp1 and n= 6-8 mice in Exp2. Mean values with unlike letters are significantly different ($P < 0.05$); one-factor ANOVA followed by LSD test.

TABLES

Table 1. Targeted miRNA assay sequences.

miRNA	Target sequence (5' → 3')
hsa-miR-103	AGCAGCAUUGUACAGGGCUAUGA
hsa-miR-107	AGCAGCAUUGUACAGGGCUAUCA
hsa-miR-143	UGAGAUGAAGCACUGUAGCUC
hsa-miR-221	AGCUACAUUGUCUGCUGGGUUUC
hsa-miR-222	AGCUACAUCUGGCUACUGGGU

Table 2. Gene-specific primer sequences used in real-time PCR amplification.

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
C/EBPα	AGGTGCTGGAGTTGACCAGT	CAGCCTAGAGATCCAGCGAC
Cpt1b	GCAAACTGGACCGAGAAGAG	CCTTGAAGAAGCGACCTTTG
Fasn	TTCGGTGTATCCTGCTGTCC	TGGGCTTGTCCTGCTCTAAC
Glut4	GGCATGCGTTTCCAGTATGT	GCCCCTCAGTCATTCTCATC
HSL	TCACGCTACATAAAGGCTGCT	CCACCCGTAAAGAGGGAACT
Lpl	CCTGATGACGCTGATTTTGT	TATGCTTTGCTGGGGTTTTC
Pnpla2	TGTGGCCTCATTCCTCCTAC	AGCCCTGTTTGCACATCTCT
PPARα	CGTTTGTGGCTGGTCAAGTT	AGAGAGGACAGATGGGGCTC
PPARγ	AGACCACTCGCATTCCTTTG	TCGCACTTTGGTATTCTTGG
Scd1	GGAAATGAACGAGAGAAGGTG	CCGAAGAGGCAGGTGTAGAG
Ucp2	GGTCGGAGATACCAGAGCAC	ATGAGGTTGGCTTTCAGGAG
TNFα	CGTCGTAGCAAACCACCAA	GAGAACCTGGGAGTAGACAAGG
18S	CGCGGTTCTATTTTGTTGGT	AGTCGGCATCGTTTATGGTC

Target genes: *C/EBPα*, CCAAT/enhancer binding protein alpha; Cpt1b, muscle carnitine palmitoyltransferase 1b; *Fasn*, fatty acid synthase; *Glut4*, glucose transporter type 4; *HSL*, hormone sensitive lipase; *Lpl*, lipoprotein lipase; *Pnpla2*, patatin-like phospholipase domain containing 2; *PPAR*, peroxisome proliferator activator receptor alpha (α), gamma (γ); *Scd1*, stearoyl-Coenzyme A desaturase 1; *Ucp*, uncoupling protein. *18S* was used for normalization.

Table 3. Relative expression of adipogenic genes in rWAT of mice fed a standard-fat diet and treated with CLA.

Experiment 1 (standard-fat diet)

	Control	CLA1	CLA2
Glut4	100 ± 5[a]	129 ± 7[b]	84 ± 10[a]
Lpl	100 ± 9	105 ± 5	102 ± 9
C/EBPα	100 ± 6[ab]	111 ± 9[a]	81 ± 6[b]
PPARγ2	100 ± 7	107 ± 6	87 ± 8

The expression levels of adipocyte differentiation markers (Glut4, Lpl, C/EBPα, and PPARγ2) were analyzed by RT-PCR. Data are means ± SEM of 8-12 mice. Means in a row without a common letter differ, $P < 0.05$; one-factor ANOVA followed by LSD test.

Table 4. Relative expression of adipogenic genes in rWAT of mice fed a high-fat diet and treated with CLA.

Experiment 2 (high-fat diet)

	Control	CLA3	CLA4
Glut4	100 ± 12	117 ± 10	112 ± 10
Lpl	100 ± 5	90 ± 6	100 ± 9
C/EBPα	100 ± 8	120 ± 11	100 ± 9
PPARγ2	100 ± 6	96 ± 5	85 ± 8

The expression levels of adipocyte differentiation markers (Glut4, Lpl, C/EBPα, and PPARγ2) were analyzed by RT-PCR. Data are means ± SEM of 7 mice. No statistically significant differences were found between groups.

Table 5. Pearson correlation coefficients for miRNA expression levels in rWAT of mice fed a standard-fat diet and treated with CLA.

Experiment 1 (standard-fat diet)

	miR-143	miR-103	miR-107	miR-221	miR-222
miR-143		0.677**	0.915**	0.622**	0.249
miR-103			0.696**	0.654**	0.393*
miR-107				0.589**	0.197
miR-221					0.787**

The comparison between miRNAs expression levels in rWAT was done by the Pearson Chi-square test. Statistical significance (2-tailed) $P < 0.05$ (*), $P < 0.01$ (**).

Table 6. Pearson correlation coefficients for miRNA expression levels in rWAT of mice fed a high-fat diet and treated with CLA.

Experiment 2 (high-fat diet)

	miR-143	**miR-103**	**miR-107**	**miR-221**	**miR-222**
miR-143		0.754**	0.418	-0.033	0.123
miR-103			0.563**	0.183	0.312
miR-107				-0.269	0.011
miR-221					0.831**

The comparison between miRNAs expression levels in rWAT was done by the Pearson Chi-square test. Statistical significance (2-tailed) $P < 0.05$ (*), $P < 0.01$ (**).

Table 7. Summary of significant correlations between expression levels of adipocyte markers and of miRNA in mice fed with a standard-fat diet and treated with CLA.

Experiment 1 (standard-fat diet)

	miR-143	miR-103	miR-107	miR-221	miR-222
Glut4					-0.400*
C/EBPα			0.370*		
PPARγ2					-0.396*
Fasn		0.378*			-0.416*
Scd1					-0.392*
Pnpla2					-0.346*
HSL					-0.379*
Cpt1b		0.404*	0.467**		
Ucp2			-0.339*		0.525**
Adiponectin	0.357*				-0.385*
Leptin	0.358*				
TNFα				0.385*	0.644**

The comparison between miRNAs and adipocyte gene expression in rWAT was done by the Pearson Chi-square test. Statistical significance (2-tailed) $P < 0.05$ (*), $P < 0.01$ (**).

Table 8. Summary of significant correlations between expression levels of adipocyte markers and of miRNA in mice fed with a high-fat diet and treated with CLA.

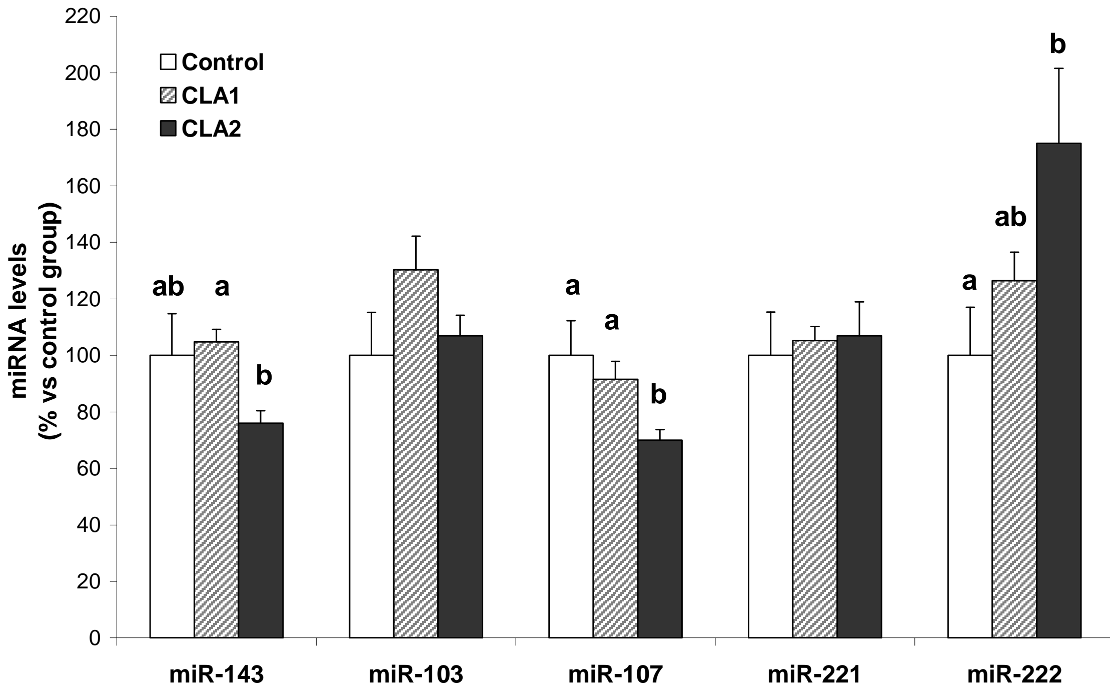
Experiment 2 (high-fat diet)

	miR-143	miR-103	miR-107	miR-221	miR-222
Lpl			-0.428*		
PPARα			0.494*		
Cpt1b			0.466*		
Adiponectin				-0.444*	
TNFα			-0.510*	0.434*	

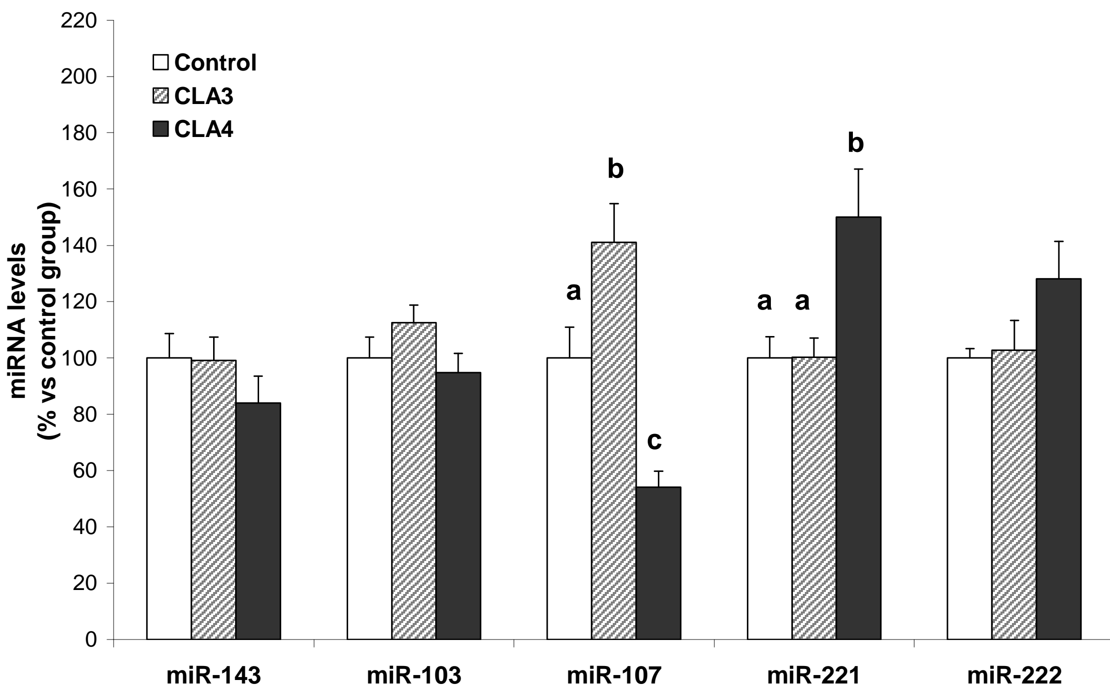
The comparison between miRNAs and adipocyte gene expression in rWAT was done by the Pearson Chi-square test. Statistical significance (2-tailed) $P < 0.05$ (*), $P < 0.01$ (**).

Figure 1

A



B



V. Recapitulación / Recapitulation

El eje central de la presente tesis doctoral ha sido el estudio del potencial efecto del calcio y el CLA sobre la modulación del balance energético, profundizando en su repercusión sobre la adiposidad y aspectos relacionados con el mantenimiento de la homeostasis.

I. Efecto del calcio dietario

Los estudios poblacionales muestran que las personas con una ingesta deficiente en calcio o productos lácteos presentan un mayor IMC y a la larga, una mayor predisposición a ganar peso y grasa corporal. Sin embargo, en los estudios de intervención no siempre se observa una disminución de la adiposidad al incrementar el contenido en calcio de la dieta (Van Loan, 2009). En el momento en que se inicia el presente trabajo las evidencias experimentales que se tienen, a favor del efecto modulador del calcio sobre la adiposidad, provienen en su mayoría de la utilización de un modelo de ratón transgénico con sobreexpresión adipocitaria de la proteína agouti bajo el promotor del gen aP2 (proteína adipocitaria que une ácidos grasos) que condiciona su expresión constitutiva en adipocitos (Shi y col., 2001). La proteína agouti es una antagonista de los receptores de la melanocortina (Tatro, 1996) los cuales participan en numerosos procesos fisiológicos como la regulación de la ingesta y el gasto energético (Voisey y col., 2003). Esta proteína estimula además el incremento de calcio intracelular (Jones y col., 1996; Kim y col., 1997) que se ha visto puede regular el metabolismo lipídico en los adipocitos (Xue y col., 1998). De manera que la respuesta de este modelo animal a las dietas enriquecidas en calcio podría estar condicionada por sus características intrínsecas. Los estudios de los que se dispone al iniciar este trabajo son escasos y en ocasiones contradictorios (Zhang y Tordoff, 2004; Paradis y Cabanac, 2005), no quedando clara la eficacia de las dietas enriquecidas en calcio en modelos animales. Es por ello, que nos planteamos estudiar el efecto del calcio en una cepa de ratón no transgénica (C57BL/6J) con predisposición al engorde con dieta hiperlipídica.

En nuestro planteamiento experimental (**Exp. Ia**) observamos que al enriquecer en calcio la dieta hiperlipídica, se atenuaba el desarrollo de obesidad inducida por la dieta. Se observó una menor ganancia de peso corporal y una reducción en el tamaño de los depósitos grasos, lo que en su conjunto reflejó una menor deposición de grasa.

En ratones con sobrepeso inducido por la ingesta de una dieta hiperlipídica, el cambio a una dieta normolipídica (**Exp. Ib**) en condiciones de libre ingesta, resultó en una pérdida de peso que se produjo de forma más rápida cuando la dieta había

sido suplementada con calcio. Sin bien, al finalizar el estudio no se encontraron diferencias en el peso corporal o en el peso de los diferentes tejidos adiposos entre los grupos experimentales.

En ambos modelos, los efectos producidos por el enriquecimiento de la dieta en calcio sobre la composición corporal no pudieron ser atribuidos a una disminución de la ingesta calórica. Se quiso determinar entonces si el efecto antiobesidad que se había observado podría estar mediado por un incremento de la termogénesis adaptativa. Se estudiaron los niveles de proteína y de mensajero de las proteínas desacopladoras predominantes en el TAM (Ucp1), TAB (Ucp2) y músculo esquelético (Ucp3) (**Exp. Ia**). No se encontraron cambios en los niveles de mensajero y de proteína de la Ucp1 y Ucp2, si bien, se observó una tendencia a disminuir en el caso de la Ucp1; principal proteína efectora de la termogénesis en roedores. Sin embargo, en el grupo alimentado con la dieta enriquecida en calcio se observó un incremento de los niveles proteicos de Ucp3 pero no así de su mensajero. No obstante, en un estudio paralelo realizado en colaboración con el Departamento de Bioquímica, Biología Molecular y Fisiología de la Universidad de Valladolid utilizando el mismo modelo experimental (**Exp. Ia**) no se observó ningún cambio a nivel proteico o de expresión de ninguna de las proteínas desacopladoras estudiadas. A diferencia de la proteína Ucp1, existe cierta controversia acerca del papel que pueden tener las proteínas Ucp2 y Ucp3 en la regulación del metabolismo energético ya que se considera que su capacidad desacopladora está relacionada con otras funciones fisiológicas (Krauss y col., 2005). Así, por ejemplo, entre las funciones que se le atribuyen a la Ucp3 está la de proteger a la mitocondria de una acumulación excesiva de ácidos grasos, así como la de mantener o promover la capacidad oxidativa del músculo. Una mayor tasa de oxidación podría contribuir a una menor deposición de grasa en los tejidos adiposos, sin embargo con el enriquecimiento de la dieta en calcio no se observó un incremento de la expresión de la Cpt1b, enzima clave en la regulación de la oxidación de ácidos grasos al controlar su entrada al interior de la mitocondria. Conforme a estos resultados, el efecto antiobesidad del calcio, *a priori*, no pareció deberse a una mayor tasa de oxidación de los ácidos grasos.

Debido a que la mayoría de evidencias existentes indican que los efectos sobre la adiposidad son más pronunciados cuando se utiliza calcio procedente de fuentes lácteas, se formuló una dieta de acuerdo a las características descritas en los estudios con los ratones transgénicos aP2-agouti (Shi y col., 2001; Sun y Zemel, 2004), utilizando calcio procedente de leche desnatada. El enriquecimiento en calcio va asociado también a un aumento de la presencia relativa de otros minerales, los

cuales se ha sugerido pueden tener también un efecto beneficioso sobre la grasa corporal (Vaskonen, 2003). Sin embargo, en algunos estudios se sugiere que el calcio puede interaccionar con minerales como el magnesio o el zinc, interfiriendo sobre su absorción y finalmente sobre su estatus en el organismo (Seelig, 1994; Wood y Zheng, 1997), aunque también hay estudios en los que no se observan efectos adversos sobre la biodisponibilidad mineral (Hardwick y col., 1991; McKenna y col., 1997; Krebs, 2000; Lonnerdal, 2000). Por este motivo, en el experimento realizado en colaboración con el grupo de la Universidad de Valladolid, se determinó la excreción urinaria de dichos minerales, así como su retención ósea. Los resultados indicaron que a pesar de que se había producido una mayor eliminación en orina de dichos minerales, probablemente como consecuencia de su mayor ingesta, su contenido en hueso no se vio negativamente afectado por ello.

En resumen, los resultados obtenidos mostraron el efecto beneficioso del enriquecimiento de la dieta en calcio, procedente de la leche, sobre la adiposidad y el peso corporal en ratones no transgénicos; efectos que parecieron ser mediados por procesos diferentes al de la inducción de la termogénesis adaptativa en el TAM.

II. Efecto de la suplementación oral de CLA

El efecto del CLA sobre la reducción de la grasa corporal está ampliamente demostrado en diferentes modelos animales, pero es en ratones donde el CLA induce una mayor pérdida de adiposidad. Debido probablemente a esta mayor sensibilidad, el CLA puede inducir un estado de lipodistrofia en esta especie, que puede acompañarse de otros efectos adversos como esteatosis hepática o resistencia a la insulina; lo que ha llevado a cuestionarse la seguridad de su utilización en humanos. Sin embargo, estos efectos adversos aparecen en su mayoría al suplementar las dietas con dosis altas de CLA (0,5-1,5%) (Tsuboyama-Kasaoka y col., 2000; Tsuboyama-Kasaoka y col., 2003; Poirier y col., 2005; Liu y col., 2007), o bien, al utilizar exclusivamente el isómero *trans*-10, *cis*-12 (Clement y col., 2002; Roche y col., 2002; Poirier y col., 2006).

En nuestros planteamientos experimentales (**IIa** y **IIb**) abordamos el estudio de la administración de CLA en dosis más bajas a las habitualmente utilizadas en otros trabajos. Nos interesó determinar, por una parte, su capacidad para reducir el peso y/o la adiposidad corporal en animales con diferente régimen alimentario y en especial, su repercusión sobre la sensibilidad a la insulina y la expresión de genes claves de la vía de la lipogénesis y de la oxidación, reguladoras ambas del contenido lipídico de los tejidos. Utilizamos para ello una de las mezclas comerciales

de CLA empleadas en humanos (Tonalín) y se optó por la administración oral de una única dosis diaria de forma similar a la posología en humanos.

Efecto del CLA sobre el peso y la adiposidad corporal

Con las dosis utilizadas, la tendencia a ganar peso durante el transcurso de los 30 primeros días de tratamiento fue menor entre los ratones tratados con CLA y alimentados con una dieta normolipídica (**Exp. IIa**) pero no así, en aquellos que habían recibido una dieta hiperlipídica (**Exp. IIb**). Se tienen evidencias de que el contenido lipídico de la dieta puede condicionar la efectividad de las dosis de CLA (Tsuboyama-Kasaoka y col., 2003) pero también, de que sus efectos se producen de forma más evidente sobre la adiposidad, no observándose siempre una disminución del peso corporal. Así pues, se determinaron los niveles circulantes de leptina a día 30, como medida indirecta del tamaño de las reservas grasas, que no variaron entre los grupos experimentales (**Exp. IIb**). Los resultados, en su conjunto, sugerían que las dosis utilizadas en el **Exp. IIb** no debían ser suficientes para contrarrestar el efecto de la dieta hiperlipídica. Se decidió entonces duplicar las respectivas dosis de cada grupo en el modelo **IIb** y continuar el tratamiento durante un mes más.

Finalizados ambos experimentos, el peso de los ratones tratados con CLA no fue diferente al de sus respectivos grupos control, a pesar de la tendencia observada durante el transcurso del tratamiento. No obstante, el efecto del CLA sobre la adiposidad fue mucho más acusado, observándose una reducción en el peso de los tejidos adiposos recogidos (TAM, retroperitoneal, epididimal y mesentérico) especialmente con la dosis más alta utilizada en cada uno de los experimentos. El menor tamaño de los depósitos grasos señaló una menor acumulación de grasa que no pudo atribuirse a diferencias en la ingesta.

Efecto del CLA sobre la sensibilidad a la insulina

La disminución de grasa corporal suele acompañarse de una mejora de la sensibilidad a la insulina, aunque no siempre se observa cuando la reducción de la adiposidad es producida por la ingesta de CLA. En este sentido, los ratones tratados con CLA en el **Exp. IIa** no presentaron cambios en la sensibilidad a la insulina, a pesar de que se produjera en el grupo tratado con la dosis más alta una disminución en los niveles circulantes de leptina (22%) y adiponectina (27%); dos adipocitoquinas consideradas claves en el mantenimiento de la sensibilidad a la insulina (Yamauchi y col., 2001). De forma similar, no se observó resistencia a la

acción de la insulina en el **Exp. IIb**, si bien los test de tolerancia a la insulina se practicaron al mes de iniciar los tratamientos y, en el caso del **Exp. IIb**, las dosis utilizadas hasta ese momento parecían no haber sido efectivas a nivel de modificación del peso corporal. Por ello, se consideró oportuno caracterizar el modelo de determinación de homeostasis (HOMA) al finalizar el **Exp. IIb**. Los grupos tratados con CLA presentaron niveles más altos de insulina en ayuno respecto al grupo control, aunque muy por debajo de la acusada hiperinsulinemia observada en otros estudios (Tsuboyama-Kasaoka y col., 2000; Ide, 2005; Liu y col., 2007). Por otra parte, si bien el índice HOMA indicó una pérdida de sensibilidad a la insulina en el grupo tratado con la dosis más alta, el índice cuantitativo revisado de sensibilidad a la insulina (R-QUICKI) no mostró diferencias entre los grupos tratados con CLA y el grupo control. En el índice R-QUICKI además de la insulina y la glucosa en ayuno se tienen en cuenta los niveles de AGL y se considera más preciso que el índice HOMA a la hora de valorar la resistencia a la insulina (Perseghin y col., 2001; Borai y col., 2007). En todo caso, solamente en el grupo tratado con la dosis más alta del **Exp. IIb** se presentó ligeramente comprometida la sensibilidad a la insulina. En este sentido, el perfil de adipocitoquinas circulantes fue menos favorable: adiponectina y leptina disminuyeron en un 33 y un 78% respectivamente. A diferencia de lo que se observó en el **Exp. IIa**, dicha reducción no parece ser únicamente consecuencia de la reducción del tamaño de los adiposos, principales productores, sino que se produjo también una disminución de su expresión, particularmente drástica en el caso de la leptina (87%); lo que podría haber contribuido al deterioro potencial en la sensibilidad a la insulina en dichos animales.

En resumen, las dosis de CLA utilizadas redujeron la acumulación de grasa de forma dosis dependiente, sin alterar la sensibilidad a la insulina, en especial en las condiciones experimentales del modelo **IIa**. No obstante, en las condiciones del modelo **IIb** y con la dosis más alta, la sensibilidad a la insulina pareció estar ligeramente comprometida.

Efecto del CLA sobre el tejido adiposo

La hiperinsulinemia y la resistencia a la insulina que se produce en algunos estudios al tratar con CLA, se dan comúnmente en otros modelos de lipodistrofia, así como también en el estado obeso; situaciones *a priori* opuestas pero que tienen en común el deterioro de la funcionalidad del tejido adiposo (Garg, 2006). En el caso de la obesidad, la disminución de la sensibilidad a la insulina se ha relacionado con el desarrollo de un proceso inflamatorio en el tejido adiposo, en el que las

células que conforman la SVF y en especial los macrófagos, parecen tener un papel clave (Xu y col., 2003). De forma similar, se ha visto que el isómero *trans*-10, *cis*-12 promueve la infiltración y aumenta el número de macrófagos en el tejido adiposo, induciendo además la secreción de factores inflamatorios que contribuyen al estado de resistencia a la insulina (Poirier y col., 2006). Por el contrario, el isómero *cis*-9, *trans*-11 reduce la presencia de macrófagos y atenúa el estado inflamatorio del estado obeso (Moloney y col., 2007). En nuestras condiciones experimentales no se observó un incremento de los niveles de mensajeros para la proteína MCP-1 o proteínas marcadoras de la presencia de macrófagos como Emr1, lo que sugirió que el CLA no había promovido la infiltración de macrófagos, y en concordancia tampoco se observó un aumento de la expresión de factores pro-inflamatorios (IL-6, iNOS y TNFα) que incluso en determinados casos disminuyeron. Así pues, el efecto individual de los isómeros pareció contrarrestarse al utilizar una combinación equimolar de los mismos, si bien, la dosis parece tener un papel determinante (Liu y col., 2007). De este modo y en concordancia con el mantenimiento de la sensibilidad a la insulina, el tratamiento con CLA no resultó en el desarrollo de un ambiente pro-inflamatorio en el tejido adiposo.

A pesar de la reducción en el tamaño de los depósitos grasos, y a diferencia de otros estudios, no se observaron cambios a nivel transcripcional que señalen una menor entrada de sustrato al interior celular (glucosa y AGL), así como tampoco una disminución en la expresión de genes característicos del adipocito maduro (PPARγ, C/EBPα) entre los que se incluyen también los genes lipogénicos (SREBP-1c, Fasn y Scd1) que incluso vieron incrementada su expresión en determinados grupos con el tratamiento de CLA. El incremento de la expresión de la proteína desacopladora Ucp2 podría mediar, al menos en parte, la reducción de grasa observada, aunque su papel en la disipación de energía no está claro.

La compleja respuesta que genera el CLA en el tejido adiposo parece estar mediada por la modulación de elementos claves en el metabolismo energético. En este sentido observamos que determinados miRNAs, recientemente relacionados con la diferenciación adipocitaria y la obesidad, en respuesta al tratamiento con CLA o bien, al ambiente metabólico generado, presentaron un patrón de expresión diferencial. Aunque se desconocen las dianas intracelulares de estos miRNAs, en el caso concreto de miR-103 y -107, un estudio computacional sugiere que podrían encontrarse en las vías relacionadas con el acetil-CoA y el metabolismo lipídico (Wilfred y col., 2007). En este sentido, encontramos numerosas correlaciones entre la expresión de estos dos miRNAs y la expresión de genes relacionados con el metabolismo de los ácidos grasos. De igual modo, miR-221 y -222, los cuales se

han descrito aumentados en el estado obeso (Xie y col., 2009), correlacionaron en nuestro estudio con la expresión de diferentes adipocitoquinas. Los miRNAs representan un nuevo nivel en la regulación génica (Lewis y col., 2005), pudiendo afectar al control del metabolismo (Krutzfeldt y Stoffel, 2006). En consecuencia y conforme a nuestros resultados, los miRNAs y las vías metabólicas en que se hallan implicados, ofrecen una nueva perspectiva en el estudio de los mecanismos de acción del CLA.

Efecto del CLA sobre el músculo esquelético y el hígado

Las dietas suplementadas con CLA pueden inducir esteatosis hepática, aunque éste parece ser un fenómeno limitado a los ratones (Tsuboyama-Kasaoka y col., 2000; Tsuboyama-Kasaoka y col., 2003; Poirier y col., 2005). En hamsters y ratas la suplementación de la dieta con CLA produce hepatomegalia, pero ésta parece ser consecuencia de una hipertrofia y/o hiperplasia y no de una acumulación de grasa (de Deckere y col., 1999; O'Hagan y Menzel, 2003; Macarulla y col., 2005). Se considera que la acumulación de lípidos en el hígado es una respuesta compensatoria a la incapacidad del tejido adiposo para captarlos y almacenarlos, especialmente cuando se produce un estado de lipodistrofia, no obstante también se ha observado la activación de genes lipogénicos en el hígado en respuesta al tratamiento con CLA (Clement y col., 2002; Javadi y col., 2004; Yanagita y col., 2005). En los dos planteamientos experimentales realizados observamos una ligera tendencia a aumentar, si bien no fue significativa, el peso del hígado en los grupos tratados con CLA pero que no pareció responder a un aumento de la concentración de TG hepáticos, que incluso disminuyeron ligeramente, aunque de forma no significativa, en los grupos tratados con CLA en el **Exp. IIa**. Conforme a la concentración hepática de TG, el estudio de la expresión de genes oxidativos (PPARα, acil coenzima A oxidasa 1 (Acox1), Cpt1a y Ucp2) y de genes implicados en la acumulación y síntesis *de novo* de ácidos grasos y TAG (PPARδ, PPARγ, SREBP-1c, Acc1, Fasn y Scd1) señaló un equilibrio entre la vía oxidativa y la vía lipogénica que, en todo caso no se vieron inducidas.

Debido a la acción específica del CLA sobre la grasa corporal, la mayoría de estudios se han centrado en investigar los posibles mecanismos que tienen lugar en el tejido adiposo. Sin embargo, otros tejidos metabólicamente activos como el músculo podrían también contribuir al efecto antiobesidad del CLA. Con el fin de discernir posibles vías del metabolismo energético afectadas por el tratamiento con CLA, realizamos un estudio transcripcional en el músculo esquelético centrándonos en la expresión de genes clave. No se observaron cambios que indicaran efectos

sobre la composición y/o características de las fibras musculares, así como tampoco se observó un incremento de la expresión de genes implicados en la β-oxidación que hubieran podido explicar, al menos en parte, la reducción de adiposidad. Sin embargo, y contrariamente a lo que cabría esperar, se observó un incremento considerable en la expresión de Fasn y en especial de Scd1 con la dosis más alta utilizada en cada uno de los experimentos. Con el cultivo de células musculares de ratón C2C12 (**Exp. IIc**), conseguimos la reproducción de dicho fenómeno *in vitro*, si bien, en las condiciones realizadas el efecto fue más atenuado; de manera que el aumento de la expresión de Scd1 se produce en presencia de CLA pero seguramente requiere de otros factores propios del ambiente metabólico generado *in vivo*. La expresión de Scd1 no se vio incrementada en el hígado mientras que en el tejido adiposo retroperitoneal se produjo un aumento en los grupos tratados, en este caso, con la dosis más baja de CLA, si bien, el incremento producido no fue comparable al alcanzado en el músculo esquelético donde llegó a ser del orden de ocho veces superior en el grupo tratado con la dosis más alta en el **Exp. IIb** (animales alimentados con dieta hiperlipídica). El papel fisiológico del incremento específico y considerable de la expresión de Scd1 está todavía por determinar, sin embargo, en los últimos años se ha sugerido que el palmitoleato, principal producto de la acción de la Scd1, puede participar en el mantenimiento de la sensibilidad a la insulina (Cao y col., 2008; Matsuzaka y Shimano, 2009). Las dietas hiperlipídicas inhiben la síntesis *de novo* de TG en el tejido adiposo pero en el modelo de ratón transgénico utilizado por Cao y colaboradores (knockout para las proteínas de unión a ácidos grasos 4 y 5, FABP4 y FABP5) esta inhibición no sólo no se produce, sino que los animales además no desarrollan resistencia a la insulina, ni esteatosis hepática. El fenotipo protegido de los efectos adversos de una dieta hiperlipídica se ha asociado a los elevados niveles circulantes de palmitoleato que presentan estos animales.

En conclusión, la utilización de dosis moderadas de la mezcla de isómeros de CLA redujo la acumulación de grasa corporal sin que se promoviera un estado inflamatorio en el tejido adiposo que podría haber contribuido a la aparición de resistencia a la insulina. No se observó acumulación hepática de TG y el tejido adiposo mantuvo un fenotipo de expresión característico del adipocito maduro. La modificación de varios miRNAs relacionados con el metabolismo adipocitario y el elevado incremento de la expresión de Scd1 específicamente en músculo crean nuevas expectativas en el estudio de los efectos y mecanismos de acción del CLA.

The major point of this PhD Thesis was the study of the potential role of calcium and CLA in the modulation of energy balance and to gain insight into their effects on adiposity and maintenance of homeostasis.

I. Effects of dietary calcium

Epidemiological studies show that people with inadequate intake of calcium or dairy products have a higher BMI, and are more prone to gain weight and body fat. However, a reduction of adiposity is not always observed in intervention trials after increasing dietary calcium intake (Van Loan, 2009). At the beginning of this Thesis, experimental evidence in favour of the modulatory effect of calcium on adiposity derived mostly from the use of a transgenic mouse model (aP2-agouti) which overexpresses agouti protein in adipose tissue under the control of aP2 promoter (aP2, a fatty acid-binding protein) (Shi et al., 2001). Agouti is an antagonist of melanocortin receptors (Tatro, 1996) which participate in many physiological processes such as control of food intake and energy expenditure (Voisey et al., 2003). Moreover, agouti protein increases intracellular calcium (Jones et al., 1996; Kim et al., 1997), which controls adipocyte lipid metabolism (Xue et al., 1998). Thus, the response of this animal model to calcium-enriched diets could be determined by its own intrinsic features. At the beginning of this work, available studies were scarce and often contradictory (Zhang and Tordoff, 2004; Paradis and Cabanac, 2005), so the effectiveness of calcium-enriched diets was not clear. For this reason we decided to study the effect of calcium in a non-transgenic mouse strain (C57BL/6J) with susceptibility to diet-induced obesity.

In our experimental approach (**Exp. Ia**), dietary calcium supplementation attenuated diet-induced obesity by a high-fat diet. Lower body weight gain and reduced fat depots were observed, reflecting lower fat deposition.

In overweight mice, the switch from a high-fat diet to a standard-fat one under *ad libitum* conditions (**Exp. Ib**) produced a faster slimming rate in the calcium supplemented group. However, no differences in body weight or size of fat depots were found between experimental groups by the end of the study.

In both experiments, effects of calcium on body composition could not be attributed to a reduction in energy intake. Therefore, we tested whether increased thermogenesis could be involved in the anti-obesity effect of calcium. We studied, at protein and transcript levels, the main uncoupling proteins in brown adipose tissue (Ucp1), white adipose tissue (Ucp2) and skeletal muscle (Ucp3) (**Exp. Ia**). Although no changes in Ucp1 and Ucp2 at protein and transcript levels were

observed, there was a tendency to decrease Ucp1, the main thermogenic effector in small rodents. However, increased Ucp3 protein levels were observed in skeletal muscle of mice fed the calcium-enriched diet. Conversely, using the same experimental model (**Exp. Ia**), in a parallel study conducted in collaboration with the Department of Biochemistry, Molecular Biology and Physiology of the University of Valladolid, no changes at protein or transcript levels were observed for any of the uncoupling proteins studied. Unlike Ucp1, there is some controversy concerning the role of Ucp2 and Ucp3 in energy balance since their uncoupling activity has been related to other physiological functions (Krauss et al., 2005). For instance, the protection of mitochondria from excessive fatty acid accumulation, as well as maintenance or promotion of fatty acid oxidation capacity in skeletal muscle have been attributed to Ucp3. A higher oxidation rate would contribute to reduce body fat deposition. However, the enrichment of diet with calcium did not increase the expression of Cpt1b, a key regulatory enzyme in mitochondrial fatty acid oxidation that controls entry of fatty acids into the mitochondria. According to these results, the anti-obesity effect of calcium, *a priori*, did not seem to be produced by increased rate of fatty acid oxidation.

Since most evidence indicates that when dairy products are the source of calcium, the effect on adiposity is more pronounced, we formulated a diet with calcium from skimmed milk, as previously used in aP2-agouti transgenic mice (Shi et al., 2001; Sun and Zemel, 2004). Dairy calcium supplementation is associated with a relative increase of other minerals, which has also been suggested to have beneficial effects on body fat (Vaskonen, 2003). Some studies describe that calcium may interact with minerals like magnesium and zinc, reducing their absorption and affecting their body balance (Seelig, 1994; Wood and Zheng, 1997), although detrimental effects on mineral bioavailability are not observed in other studies (Hardwick et al., 1991; McKenna et al., 1997; Krebs, 2000; Lonnerdal, 2000). For this reason, in the experiment conducted in collaboration with the group of the University of Valladolid, urine mineral excretion and mineral bone retention of calcium, magnesium and zinc were assessed. The results indicate that mineral content in bone was preserved, despite higher urine excretion, probably produced as a result of their greater intake.

In summary, our findings show that dietary calcium supplementation from a dairy source have beneficial effects on adiposity and body weight in non-transgenic mice by a mechanism different from an activation of brown adipose tissue thermogenesis.

II. Effects of oral CLA treatment

The benefits of CLA on adiposity have been widely demonstrated in different animal models, but it is in mice where CLA induces a greater loss of body fat. Probably due to this higher sensitivity, CLA can induce a lipodystrophy state in this species that may be accompanied by other adverse effects such as hepatic steatosis or insulin resistance; this has contributed to raise concern as to the safety of dietary CLA in humans. Most of the adverse effects are normally associated with the supplementation of diets with high doses of a mixture of the two main isomers of CLA (0,5-1,5%) (Tsuboyama-Kasaoka et al., 2000; Tsuboyama-Kasaoka et al., 2003; Poirier et al., 2005; Liu et al., 2007), or with the pure *trans*-10, *cis*-12 isomer (Clement et al., 2002; Roche et al., 2002; Poirier et al., 2006).

Our experiments (**IIa** y **IIb**) pursued the study of the administration of doses lower than those commonly used in previous works. We were interested in analyzing the ability of moderate doses of CLA to reduce weight and/or body fat in mice with different dietary regimes and particularly, their impact on insulin sensitivity and/or expression of key genes in the lipogenesis and oxidation pathways, since both processes control tissue lipid content. We used a commercial CLA mixture used in humans (Tonalín) and administrated a single daily dose to mice similar to the dosage in humans.

CLA effect on body weight and adiposity

For the first 30 days of the study, a tendency to reduce body weight gain was observed in mice treated with moderate doses of CLA and fed with a standard-fat diet (**Exp. IIa**) but not in those under high-fat feeding (**Exp. IIb**). There is some evidence that fat content of diet may influence the effectiveness of CLA doses (Tsuboyama-Kasaoka et al., 2003) and also, that the effects are more obvious on adiposity but are not always accompanied by a reduction of body weight. Consequently, we determined plasma leptin levels on day 30, as an indirect measure of adipose depot size, which were not different between experimental groups (**Exp. IIb**). Taken as a whole, our results suggest that the doses used in **Exp. IIb** would not be able to counteract obesity induced by a high-fat diet. Therefore we decided to double the doses of each group in **Exp. IIb**, and continue the treatment for another month.

Despite a tendency towards a reduction in body weight gain during the study, final body weight in CLA treated groups was not different from their respective control groups by the end of both experiments. Conversely, CLA effect on adiposity

was more pronounced. The weight of adipose depots (brown adipose tissue, retroperitoneal, epididymal and mesenteric) was reduced, particularly with the higher CLA dose, in each experiment. The smaller size of adipose tissues indicated a reduction in body fat that could not be attributed to differences in energy intake.

CLA effect on insulin sensitivity

A reduction in adiposity is usually associated with improved insulin sensitivity, but this is not so clear when the fat loss is produced by CLA. In this regard, mice from **Exp. IIa** and treated with CLA maintained insulin sensitivity despite the reduction in plasma leptin (22%) and adiponectin (27%) concentrations observed with the highest dose. Leptin and adiponectin are two adipokines considered as key players involved in the maintenance of insulin sensitivity (Yamauchi et al., 2001). Similarly, insulin resistance was not observed in **Exp. IIb**. However, since insulin tolerance tests were performed on day 30 of the study and the doses used until that day in **Exp. IIb** did not seem to have an effect on body composition, we deemed appropriate to calculate HOMA index at the end of **Exp. IIb**. CLA-treated groups showed higher fasting insulin plasma concentrations compared to control group but were far from the marked hyperinsulinaemia observed in other studies with higher CLA doses (Tsuboyama-Kasaoka et al., 2000; Ide, 2005; Liu et al., 2007). Moreover, although the HOMA index suggested an impairment of insulin sensitivity in animals treated with the highest dose, the R-QUICKI index did not show differences between groups (**Exp. IIb**). The R-QUICKI index incorporates fasting free fatty acid concentrations together with fasting insulin and glucose levels and is considered a more accurate indicator for assessing insulin resistance than HOMA (Perseghin et al., 2001; Borai et al., 2007). Either way, insulin sensitivity was slightly compromised in animals treated with the highest dose in **Exp. IIb**. In this sense, circulating adipokines profile was less favourable: adiponectin and leptin concentration decreased by 33 and 78% respectively. Unlike **Exp. IIa**, the decrease in plasma adipokine seemed to be produced not only by the reduction of adipose depots, the main source, but also by a decrease in their expression, which was particularly drastic for leptin (87%) and could have contributed to deteriorate insulin sensitivity in animals treated with the highest dose in **Exp. IIb**.

In summary, moderate doses of a mixture of the two main isomers of CLA produce a dose-dependent reduction in body fat content, without affecting insulin sensitivity, particularly under **Exp. IIa** conditions. However, under **Exp. IIb** conditions and with the highest dose, insulin sensitivity seems to be slightly deteriorated.

CLA effect on adipose tissues

Hyperinsulinaemia and insulin resistance triggered by CLA in some studies is commonly found in animal models of lipodystrophy, as well as in the obese state. Lipodystrophy and obesity are two opposite situations *a priori*, but they have in common the deterioration of adipose tissue function (Garg, 2006). In obesity, decreased insulin sensitivity has been related to the development of an inflammatory process within adipose tissue, in which cells from SVF, especially macrophages, are presumed to play a key role (Xu et al., 2003). Similarly, *trans*-10, *cis*-12 isomer promotes the infiltration and presence of macrophages in adipose tissue, and also induces secretion of pro-inflammatory factors that may contribute to the state of insulin resistance (Poirier et al., 2006). By contrast, the *cis*-9, *trans*-11 isomer reduces the presence of macrophages and attenuates the obesity-related inflammatory state in adipose tissue (Moloney et al., 2007). In our experimental conditions, transcript levels of MCP-1 or Emr1 were not induced in SVF suggesting CLA did not promote the recruitment of macrophages into adipose tissue and this was consistent with the unaltered expression of pro-inflammatory factors (IL-6, iNOS y TNFα) which in certain groups even declined. Thus, the effect of the individual CLA isomers appeared to be counteracted by the use of an equimolar mixture of both isomers, although the dose is also relevant (Liu et al., 2007). In accordance with preservation of insulin sensitivity, CLA treatment did not result in the development of a pro-inflammatory state in adipose tissue.

Unlike other studies, the reduction in adipose size was not accompanied by changes in expression of the genes involved in the entry of glucose or fatty acids into the adipocytes or by a decrease in the expression of the genes characteristic of mature adipocytes (PPARγ, C/EBPα) including lipogenic genes (SREBP-1c, Fasn and Scd1) which even increased their expression in some groups treated with CLA. The increased expression of Ucp2 might, at least in part, mediate the fat reduction, although its role in energy dissipation remains unclear.

The complex response to CLA in adipose tissue is likely to take place through a CLA-mediated modulation of major metabolic regulators. We observed that selected miRNAs, recently related to adipocyte differentiation and obesity, showed a different expression pattern in response to CLA treatment and/or the new metabolic status achieved. Although the targets of these miRNAs are still unknown, in the specific case of miR-103 and -107 a computational study predicts that they might be found in the pathways related to acetyl-CoA and lipid metabolism (Wilfred et al., 2007). In this regard, we found a number of correlations between the expression of miR-103 and -107 in adipose tissue and the expression of genes related to fatty

acid metabolism. Similarly, miR-221 and -222, which were found increased in the obese state (Xie et al., 2009) correlated with the expression of different adipocytokines. miRNAs introduce a novel level in gene regulation (Lewis et al., 2005) and may contribute to metabolic control (Krutzfeldt and Stoffel, 2006). Therefore, and according to our results, miRNAs offer a new perspective in the study of mechanisms of CLA action.

Effect of CLA on skeletal muscle and liver

CLA supplementation may induce hepatic steatosis, although this phenomenon appears to be limited to mice (Tsuboyama-Kasaoka et al., 2000; Tsuboyama-Kasaoka et al., 2003; Poirier et al., 2005). In hamsters and rats, dietary supplementation with CLA produces hepatomegaly that seems to be consequence of hypertrophy and/or hyperplasia but not of fat accumulation (de Deckere et al., 1999; O'Hagan and Menzel, 2003; Macarulla et al., 2005). Lipid accumulation in the liver is considered a compensatory response to the inability of adipose tissue to take up and store it, particularly in a lipodystrophy state. However, induction of lipogenic genes has also been observed after CLA treatment in some studies (Clement et al., 2002; Javadi et al., 2004; Yanagita et al., 2005). In both experimental approaches of our study, a slight tendency to increase liver weight in CLA-treated group was observed, although it did not reach statistical significance and did not seem to be produced by an increase of hepatic TAG content that even decreased, non-significantly, in animals treated with CLA in **Exp. IIa**. In agreement with the TAG hepatic content, the study of the expression of oxidative genes (PPARα, Acox1, Cpt1a y Ucp2) and genes involved in the accumulation of fatty acid and the synthesis *de novo* of TAG (PPARδ, PPARγ, SREBP-1c, Acc1, Fasn y Scd1) pointed towards a balance between hepatic lipogenic and oxidative capacity, which in no case was induced.

Due to the specific action of CLA on adiposity, most studies have focused on the potential mechanisms that occur in adipose tissue. However, other metabolically active tissues, such as muscle, may contribute to the antiobesity effect of CLA. With the aim of discerning potential metabolic pathways altered by CLA treatment, we conducted a transcriptional study in skeletal muscle. No changes in the composition and/or characteristics of the muscle fibers were observed. No increase in oxidative gene expression which could explain, at least in part, the lower accumulation of body fat was found. However, contrary to what was expected, there was a considerable increase in Fasn and Scd1 gene expression, especially with the highest dose used in each experiment. We reproduced this phenomenon *in vitro* using

murine C2C12 myoblast (**Exp. IIc**), although in those conditions the induction achieved was lower than *in vivo*. *In vitro* Scd1 expression increased with CLA treatment but other factors generated *in vivo* are probably required for a stronger induction. Interestingly, Scd1 expression did not increase in liver while in retroperitoneal adipose tissue it increased in groups treated with the lower dose of CLA, although the induction was not comparable to that achieved in skeletal muscle, where it became up to 8-fold in animals from **Exp. IIb** treated with the highest dose (under high-fat feeding). The physiological role of the specific, strong induction of Scd1 expression is still undetermined. Over the last few years, it has been suggested that palmitoleate, the main product of Scd1, might be involved in maintaining insulin sensitivity (Cao et al., 2008; Matsuzaka and Shimano, 2009). High-fat diets inhibit *de novo* lipogenesis in adipose tissue but not in the animal model used by Cao and co-workers (FABP4 and FABP5 null mice). Moreover, this double knock-out mouse displays a remarkable state of insulin sensitivity and is protected from the metabolic alteration induced by a high-fat diet such as hepatic steatosis. This phenotype, protected from metabolic deterioration induced by a high-fat diet, has been associated with the high circulating levels of palmitoleate shown by these animals.

In conclusion, the use of moderate doses of a mixture of CLA isomers reduced body fat accumulation without the promotion of an inflammatory state in adipose tissue which might contribute to decrease insulin sensitivity. Hepatic lipid accumulation was not observed and adipose tissue maintained a proper mature adipocyte-specific gene expression. The changes produced in expression of miRNAs related to adipocyte metabolism and the strong, specific induction of Scd1 in skeletal muscle provide new possibilities in the study of the effects and mechanisms of action of CLA.

VI. CONCLUSIONES / CONCLUSIONS

1. El enriquecimiento de la dieta en calcio reduce la ganancia de peso y la acumulación de grasa que se produce en ratones alimentados con una dieta hiperlipídica. Los efectos sobre la composición corporal no son debidos a una reducción de la ingesta, ni son mediados por un incremento de la termogénesis adaptativa en el tejido adiposo marrón. Por otra parte, en ratones con sobrepeso por dieta hiperlipídica, la pérdida de peso que se produce cuando son alimentados con una dieta normolipídica, se acelera si la dieta está suplementada con calcio.

2. La suplementación de la dieta con calcio procedente de la leche conlleva un incremento en el contenido de otros minerales como el magnesio y el zinc, lo que a su vez produce un aumento de su excreción urinaria, pero que no repercute negativamente sobre su contenido óseo.

3. La administración de dosis moderadas de una mezcla equimolar de los isómeros de CLA: *cis*-9, *trans*-11 y *trans*-10, *cis*-12, en ratones alimentados con una dieta normolipídica o hiperlipídica, atenúa la acumulación de grasa corporal y no altera el perfil inflamatorio del tejido adiposo.

4. Las dosis de CLA ensayadas mantienen la sensibilidad a la insulina, en particular en los ratones alimentados con dieta normolipídica, sin embargo aparece ligeramente comprometida cuando las condiciones son metabólicamente más severas (dosis de CLA más altas, dieta hiperlipídica y tratamiento más largo).

5. El tratamiento con CLA no induce a nivel transcripcional la expresión de enzimas involucradas en la oxidación de ácidos grasos en el músculo esquelético que contribuirían al efecto anti-obesidad del CLA. En cambio, se produce una fuerte inducción de la expresión de Scd1 cuyo producto, el palmitoleato, podría ser un factor clave en el mecanismo de acción del CLA.

6. El tratamiento con CLA, en nuestras condiciones experimentales, no promueve la acumulación hepática de lípidos. En este sentido, el estudio transcripcional de genes hepáticos claves en la oxidación de ácidos grasos y en la síntesis *de novo* de triacilgliceroles muestra un equilibrio entre ambos procesos, lo que contribuiría al mantenimiento del contenido lipídico de este tejido.

7. La menor acumulación de grasa producida por el CLA no parece responder a una pérdida de la funcionalidad del tejido adiposo, el cual mantiene intacta la expresión de genes adipocitarios característicos del fenotipo de adipocito maduro.

8. El CLA modifica, en función de la dosis, dieta y estatus metabólico del animal, el perfil de expresión de varios miRNAs relacionados con el metabolismo adipocitario. La expresión de miR-103 y -107 se correlaciona con la expresión de genes implicados en el metabolismo lipídico; mientras que la expresión de miR-221 y -222 correlaciona con la expresión de adipocitoquinas. Estos resultados plantean una nueva vía en el estudio de los mecanismos de acción del CLA, así como, en el de las estrategias dietéticas dirigidas a prevenir y/o tratar la obesidad.

1. Calcium-enriched diet reduces body weight gain and fat deposition in mice fed with a high-fat diet. Modulation of body composition is not produced by a decrease in energy intake or an activation of brown adipose tissue thermogenesis. Moreover, normal-fat diet supplemented with calcium accelerates weight loss in high-fat diet-induced overweight mice.

2. Dietary calcium supplementation from dairy sources involves a greater intake of other minerals such as magnesium and zinc, which contributes to increase their urine excretion without negative consequences on their bone accretion.

3. Moderate doses of an equimolar mixture of the two isomers of CLA: *cis*-9, *trans*-11 y *trans*-10, *cis*-12, attenuate body fat deposition without promoting inflammatory state in adipose tissue of mice fed a normal- or high-fat diet.

4. At the CLA doses used, insulin sensitivity is preserved, particularly in mice fed a normal-fat diet but appears to be slightly altered by more severe metabolic conditions (higher CLA doses, high-fat diet and longer treatment).

5. CLA treatment does not induce, at transcriptional level, expression of enzymes involved in skeletal muscle fatty acid oxidation that would contribute to the CLA anti-obesity effect. However a strong induction of Scd1 gene expression is produced and its product, palmitoleate, could be a novel key factor in the mechanism of action of CLA.

6. Under our experimental conditions, CLA treatment does not promote hepatic fat accumulation. In this sense, the transcriptional study of the key hepatic genes involved in fatty acid oxidation and *de novo* lipogenesis shows a balance between both processes which would contribute to maintain the fat content of this tissue.

7. Body fat lowering effect of CLA is not produced by a loss of adipose tissue function, which maintains mature adipocyte-specific gene expression.

8. Depending on dose, diet and animal's metabolic status, CLA modifies gene expression of several miRNAs related to adipocyte metabolism. Expression of miRNAs -103 and -107 correlates with those of genes involved in lipid metabolism, whereas miR-221 and -222 correlates with adipocytokine gene expression. These results provide a novel pathway for the study of the mechanisms of CLA action, as well as of dietary strategies addressed to prevent and/or counteract obesity.

VII. MATERIALES Y MÉTODOS

Cultivo de células C2C12

Descongelación. El criovial conservado en nitrógeno líquido se descongelaba rápidamente en un baño termostatizado a 37ºC y su contenido (1,5 ml), con aproximadamente $2x10^5$ células, se resuspendía rápidamente en un frasco de 25 cm^2 con 3,5 ml de medio de crecimiento (volumen final 5 ml). Las células se mantenían en un incubador a 37ºC y 5% de CO_2.

Subcultivo (Tripsinización). Se aspiraba el medio de crecimiento del frasco y se lavaba cuatro veces con tampón fosfato salino (PBS) celular. Se añadía la tripsina (5 ml de Tripsina-EDTA (Sigma) por frasco de 25 cm^2) y se dejaba actuar durante 1 min a 37ºC. Una vez despegadas las células, la tripsina se inactivaba añadiendo 4 ml de medio de crecimiento fresco y se centrifugaban a 1000 g durante 5 min. El sobrenadante se retiraba y las células se resuspendían en medio de crecimiento.

Recuento celular. Para el recuento celular se utilizaba una cámara de Neubauer y 10 µl de una mezcla, a partes iguales, de suspensión celular y azul tripán (Sigma).

Diferenciación. Las células se sembraban a una densidad de 30.000 células/cm^2. Cuando las células se encontraban a un 85% de confluencia (aproximadamente transcurridas 24 h de su siembra) el medio de crecimiento se sustituía por medio de diferenciación (día 0) que se remplazaba por medio fresco cada dos días. A día 9 las células, ya diferenciadas a miotubos, eran tratadas durante 24 h y se recogían a día 10.

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 mg 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 mg estreptomicina/ml).

Recogida y procesamiento de la sangre

La sangre se recogía directamente de la cola mediante un pequeño corte, o bien, mediante punción cardiaca utilizando respectivamente capilares y jeringas heparinizadas (0,2 % heparina diluida con suero salino, Sigma). Para la obtención del plasma la sangre se centrifugaba a 1000 g durante 10 min y a 4ºC. Posteriormente el plasma se guardaba a -70ºC.

Determinación de metabolitos plasmáticos

La concentración plasmática de leptina, adiponectina, resistina e insulina se determinaba mediante el uso de kits basados en el ensayo por inmunoabsorción ligado a enzimas (ELISA), siguiendo siempre las instrucciones de las casas comerciales.

Kits:

- *Leptina*: Leptin Quantikine ELISA kit (R&D Systems, USA).
- Adiponectina: adiponectin ELISA kit (Phoenix Europe GmbH, Alemania).
- *Resistina:* Mouse Resistin Quantikine ELISA kit (R&D Systems, USA).
- *Insulina*: Insulin Mouse Ultrasensitive ELISA kit (DRGInstruments GmbH, Alemania).

La concentración de glicerol, TAG y AGL se determinó utilizando kits basados en reacciones enzimático-colorimétricas:

Kits:

- *TAG* y *glycerol*:(Sigma Diagnostics, España).
- *AGL*: NEFA (Wako Chemicals GmbH, Alemania).

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

Test de tolerancia a la insulina

El pienso de la jaula se retiraba 3 h antes del inicio de la prueba, y se añadía de nuevo una vez finalizada. Primeramente se realizaba un pequeño corte en el extremo de la cola para poder determinar la concentración de la glucosa mediante el uso de tiras incluidas en el medidor de glucemia Accu Check Sensor (Roche Diagnostics). A continuación, se les inyectaba intraperitonealmente una solución de insulina (Humulin R; Eli Lilly) preparada con suero salino a una concentración de 0,8 U/kg peso corporal. La concentración de glucosa se determinaba a los 15, 30, 60, 90 y 120 min tras la inyección de insulina.

Aislamiento a partir de tejido adiposo de la fracción estromal y los adipocitos maduros

Los adipocitos maduros y la fracción estromal se aislaron del tejido adiposo epididimal de ratones C57BL/6J. El tejido se recogía y se lavaba de forma inmediata con el tampón Krebs-bicarbonato-Hepes-BSA (tampón Krebs) previamente atemperado a 37ºC. A continuación, el tejido se troceaba en pequeños trozos para favorecer su digestión, para la cual el tejido se transfería a un tubo y se añadía una solución de colagenasa (4 ml solución de colagenasa/g tejido) con la que se dejaba incubando unos 45 min aproximadamente en un baño a 37ºC con agitación suave. El tejido digerido se filtraba utilizando unos filtros de nylon (tamaño de poro 200 µm) previamente lavados con abundante solución salina y montados sobre pequeños embudos de vidrio; de este modo se conseguía separar las células del tejido conectivo y restos de tejido no digerido que podían volver a ser incubados con colagenasa. A continuación la suspensión de células se mezclaba y se centrifugaba 10 min a 200g.

El *sobrenadante*, en el que se encontraban los adipocitos maduros, se aspiraba y se transfería a otro tubo al que añadíamos 10 ml de tampón Krebs y centrifugábamos 5 min a 400 g. Este lavado se repetía en otras dos ocasiones para finalmente retirar la capa superior con los adipocitos maduros.

El *precipitado* con la fracción estromal se resuspendía en un tampón de lisis de eritrocitos y se incubaba a temperatura ambiente durante 10 min. A continuación se centrifugaba 5 min a 400 g, se retiraba el sobrenadante y se resuspendía de nuevo con 10 ml de tampón Krebs. Este lavado se repetía en otras dos ocasiones para finalmente quedarnos con la fracción estromal precipitada.

Tampones:

- *Tampón Krebs-bicarbonato-Hepes-BSA*: tampón Krebs (NaCl 126 mM (Panreac), KCl 2,5 mM (Panreac), $CaCl_2$ 2,5 mM (Sigma), $MgCl_2$ 1,2 mM (Panreac); NaH_2PO_4 1,2 mM (Panreac)) al que se le añadía $NaHCO_3$ 25 mM (Panreac), glucosa 11 mM (MERCK), Hepes 25 mM (Sigma) y BSA al 2% (Sigma). Ajustar el pH a 7,4.

- *Solución de colagenasa*: 1,5 mg de colagenasa tipo I (Invitrogen) por ml de tampón Krebs-bicarbonato-Hepes-BSA.

- *Tampón de lisis de eritrocitos*: NH_4Cl 0,154 M (Panreac), $KHCO_3$ 10 mM (Panreac) y EDTA 0,1 mM (MERK). Ajustar el pH a 7,4.

Extracción del ARN

Se utilizaban dos métodos distintos a la hora de extraer el ARN en función del tipo de muestra, escogiendo en cada caso el que ofrecía mejores resultados en cuanto a cantidad y calidad del ARN extraído. La extracción de ARN a partir de tejidos o células en cultivo 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 aisladas, concretamente de adipocitos maduros y células de la fracción estromal aislados del tejido adiposo epididimal, se utilizaba un kit comercial, RNeasy Mini Kit (Qiagen), (*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 frío 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 en frío 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 -20ºC. 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 100 μl) de agua libre de ARNasas (Sigma) con EDTA 10 mM (Merck) –cuando el ARN se utilizaba para northen blot– o sólo con agua si se iba a utilizar para RT-PCR. 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, RNeasy, combina la lisis con isotiocianato de guanidina con la rapidez y la purificación de las columnas con membrana de sílica. Las células se lisaban con 600 µl de tampón de lisis (isotiocinato de guanidina), se añadían 600 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 "RNeasy Mini spin" y se centrifugaba a 8000g durante 15 s 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 1 min a 8000g para eluir el ARN purificado. El eluido se podía volver a pasar por la columna con el fin de concentrar la muestra.

Cuantificación y valoración del estado del ARN

Cuantificación del ARN. 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 0,5 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 está degradado.

Tampones:

- *Tampón de electroforesis*: TBE 0,5 ×.
- *Tampón de carga*: glicerol, azul de bromofenol.

Northen blot

Los ARN mensajeros (ARNm) de interés se determinaron por Northen blot utilizando sondas oligonucleotídicas (Genotek) específicas, marcadas en ambos extremos con digoxigenina.

Electroforesis. 30 µg de ARN total, mezclados con 18 µl de reactivo de desnaturalización (conteniendo formamida y formaldehído), se incubaban 15 min en un baño termostatizado a 60ºC. A estas muestras se les añadía el tampón de carga y se fraccionaban por electroforesis en un gel de agarosa (Pronadisa) al 1,2% conteniendo 1x MOPS y 18% de formaldehído, usando 1x MOPS como tampón de electroforesis. El gel se corría a 100 V hasta que las muestras salían de los pocillos de carga y posteriormente se dejaba a 70 V durante 3 h.

Transferencia. Tras la electroforesis, el ARN se transfería a una membrana de nylon (Roche) cargada positivamente, por capilaridad en 20x SSC durante 16 h. Después, el ARN se fijaba a la membrana por tratamiento con luz ultravioleta en las dos caras, usando un *GS Gene linker* (BioRad). Para comprobar el estado del ARN las membranas se lavaban con ácido acético al 5% (Panreac) y luego se teñían con azul de metileno al 0,04% con 0,5 M de acetato sódico, lavándolas a continuación con agua estéril para eliminar el exceso de colorante, hasta que se veían las bandas de ARN ribosómico 18S y 28S con claridad. Tras la tinción, las membranas se dejaban 5 min agitando en tampón de lavado de hibridación.

Hibridación. El ARNm de interés era detectado por un procedimiento basado en quimioluminiscencia, utilizando sondas oligonucleotídicas específicas marcadas en ambos extremos con digoxigenina. Las membranas se prehibridaban con *Dig Easy Hyb* (Roche) durante 15 min a 42ºC, y se hibridaban toda la noche con *Dig Easy Hyb* conteniendo la sonda oligonucleotídica (todas a una concentración de 35 ng/ml, a excepción del 18S que se usaba a una concentración de 70 pg/ml). Antes de utilizar las sondas éstas se incubaban 10 min en un baño a 68ºC, se filtraban utilizando un filtro de 0,45 µm (Sigma) de diámetro de poro y se enfriaban rápidamente en agua-hielo.

Las sondas de ADN complementario se diseñaban de forma que no tuvieran reactividad cruzada con ningún otro ARNm presente en la especie estudiada, lo cual se comprobaba con el programa BLAST.

Las membranas hibridadas se lavaban dos veces durante 15 min a temperatura ambiente con 2x SSC/0,1% SDS y, posteriormente, otras dos veces durante 15 min a 48ºC con 0,1x SSC/0,1% SDS. A continuación se lavaban 5 min con tampón de

lavado inmunológico y después se bloqueaban durante 1h con el reactivo de bloqueo.

Detección. Para la detección inmunológica, las membranas se incubaban 30 min con un anticuerpo antidigoxigenina conjugado con fosfatasa alcalina (Roche), disuelto en tampón de bloqueo en una proporción 1:20000. A continuación las membranas se lavaban 15 min con tampón de lavado inmunológico, se equilibraban durante 5 min en tampón de detección y, finalmente, se añadía sustrato quimioluminiscente CDP-Star (Roche) disuelto en tampón de detección en proporción 1:100. Las membranas 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.

Análisis. Para el análisis las membranas se escaneaban en un densitómetro Agfa DUOSCAN y se cuantificaban con el programa *Kodak 1D Image Analysis Software 3.5* (Eastman Kodak Co.).

Una misma membrana se utilizaba para la detección de distintos ARNm, para ello, las membranas se deshibridaban y se volvían a hibridar en las condiciones ya mencionadas. Para deshibridar las membranas, éstas se lavaban con agua estéril y, a continuación, se añadían 100 ml de SDS 0,1% hirviendo, dejándolas incubar durante 10 min. Si las membranas no se volvían a hibridar, se guardaban a 4ºC con 2x SSC en bolsas selladas.

Reactivos y tampones:

- *Reactivo de desnaturalización*: 1x MOPS, 17,5% formaldehído al 37% (Sigma), 50% formamida (Sigma).
- *Tampón de carga*: 50% de glicerol (Sigma), 50% agua y 2,5 mg/ml de azul de bromofenol (Panreac).
- *MOPS 10x*, pH 7: 200 mM MOPS (Sigma), 50 mM acetato sódico (Panreac), 10 mM EDTA (Merck).
- *SSC 20x*, pH 7: 3 M NaCl (Panreac) y 0,3 M citrato sódico 2-hidrato (Panreac).
- *Azul de metileno*: 0,5 M acetato sódico (Panreac) y 0,04% azul de metileno (Panreac).
- *Tampón de lavado de hibridación* para 500 ml: 10 ml de 1M de Na_2HPO_4 (Panreac) a pH 7,5, 1 ml de 0,5 M EDTA (Merck) a pH 8, 5 g de SDS (Sigma).

- *Tampón maleico*, pH 7,5: 0,10 M ácido maleico (Sigma) y 0,15 M NaCl (Panreac).
- *Tampón de lavado inmunológico*: 0,3% Tween-20 (Sigma) en tampón maleico.
- *Reactivo de bloqueo*: reactivo de bloqueo (Roche) al 1% en tampón maleico.
- *Tampón de detección*, pH 9,5: 100 mM Tris-HCl (Sigma) y 100 mM NaCl (Panreac).

Todos los reactivos se autoclavaban, a excepción de las soluciones de SDS que se esterilizaban por filtración.

RT-PCR

Retrotranscripción (RT): Para la detección de los ARNm de interés se usaban 0,5 µg de ARN que se llevaban a un volumen total de 10 µl con agua y se desnaturalizaban 1 min a 90ºC. Para llevar a cabo la RT se añadían 10 µl de RT-mix preparada para que las concentraciones finales en cada tubo de reacción fueran: 1x buffer (Promega), 2,5 mM de $MgCl_2$ (Promega), 0,4 mM de nucleótidos (Invitrogen), 5 µM de "random hexamers" (Applied Biosystems), 5 µM de inhibidor de ARNasas (Applied Biosystems) y 2,5 U/µl de transcriptasa inversa (MuLV RT, murine leukemia virus reverse transcriptase, Applied Biosystems). Las condiciones de retrotranscripción eran: 60 min a 42ºC y un paso final de 5 min a 99ºC. Todas las reacciones se llevaron a cabo en un termociclador Perkin Elmer 9700.

Reacción en cadena de la polimerasa (PCR) estándar: Se utilizaban 2 µl de producto de RT al que se añadían 23 µl de una mezcla para PCR que contenía: 0,2 µl de Taq DNA polimerasa (Promega), 2,5 µl de $MgCl_2$ 2,5 mM, 2,5 µl *buffer* 10x, 2 µl de solución de nucleótidos 2,5 mM, los cebadores para el gen de interés y para el gen de referencia a una concentración variable y el volumen restante se completaba con agua libre de ARNasas. La reacción de PCR se llevaba a cabo en un termociclador Perkin Elmer 9700 y consistía en una desnaturalización a 94°C durante 105 s y un número determinado de ciclos (que dependía de la abundancia del ARNm de interés, en nuestro caso 22 ciclos) consistentes en: desnaturalización (15 s, 95°C), hibridación (15 s, a una temperatura que dependía de los cebadores utilizados, en nuestro caso 56ºC) y elongación (30 s, 72°C); y una elongación final (72°C) de 7 min.

La visualización de los productos de PCR se realizaba mediante una electroforesis en gel de agarosa (Pronadisa) al 2-3% en tampón tris-borato-EDTA al 0,5%, teñido con 0,03 mg/ml de bromuro de etidio (Sigma). Se añadían 5 µl de tampón de carga (50% de glicerol (Sigma), 50% de agua, 2,5 mg/ml de azul de bromofenol (Panreac)) a 10 µl del producto de PCR y la mezcla se cargaba en el gel. Además, se cargaba un marcador de pesos moleculares (100 pares de bases, Invitrogen) para verificar el tamaño de los productos obtenidos. Las bandas se separaban aplicando 90 voltios en la fuente de electroforesis. Las imágenes se captaban con el programa GeneSnap y las bandas se cuantificaban con el programa GeneTools.

PCR a tiempo real. En este caso se utilizaban 2 µl del producto de RT diluido (entre 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: 0,5 µl de agua libre de ARNasas, 0,313 µl de cada cebador (a una concentración que dependía de la abundancia de cada ARNm en un determinado tejido) y 3,125 µl de una mezcla comercial *Power SYBR Green PCR Master Mix* (Applied Biosystems). Para realizar la PCR se utilizaba el sistema *StepOnePlus™* (Applied Biosystems) con el siguiente perfil: 10 min a 95ºC, seguido por un total de 40 ciclos de temperatura donde había 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 realizaba una curva de desnaturalización después de cada PCR realizada, siguiendo las instrucciones de la casa comercial. Además, una vez finalizada la reacción se verificaba el tamaño del fragmento amplificado en un gel de agarosa al 2% teñido con bromuro de etidio que se visualizaba con luz ultravioleta.

RT-PCR para la determinación de la expresión de los miRNAs

El ARN extraído con Tripure, conforme se explica en el apartado de aislamiento, se llevaba a una concentración de 2,5 ng/µl. Para cada miRNA se necesitaba realizar una RT en la que se utilizaba 2 µl de la dilución de ARN y se añadían 4 µl de la mix (TaqMan® MicroRNA Reverse Transcription kit, Applied Biosystems) más 1,5 µl de unos primers de RT específicos para cada miRNA, incluidos en el TaqMan® MicroRNA Assay (Applied Biosystems). Para la transcripción se utilizaba un termociclador Perkin-Elmer 9700 (PerkinElmer, Wellesley, MA) con las siguientes condiciones: 30 min a 16ºC, seguido de 30 min a 42ºC y finalmente 5 min a 85ºC.

Finalizada la RT, 2 µl del DNA complementario específico de cada miRNA se utilizaban para la reacción de PCR utilizando en este caso sondas TaqMan (Applied Biosystems) conjuntamente con la mezcla comercial TaqMan Universal PCR master mix (Applied Biosystems). La reacción de PCR se realizó utilizando el sistema de Applied Biosystems StepOnePlus™ Real-Time PCR en las siguientes condiciones: 95ºC durante 10 min, seguido de 40 ciclos (desnaturalización a 95ºC durante 15 s, hibridación y extensión 60ºC durante 1 min).

Cuantificación de proteínas

Las proteínas se aislaban a partir de la fase orgánica obtenida en el proceso de aislamiento y separación del ARN con Tripure (Roche) siguiendo las instrucciones de la casa comercial.

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 bicinconínico (BCA). Se utilizaba como patrón BSA disuelto en el mismo tampón utilizado para las proteínas (PBS o 1% SDS). 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 s. 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).

Western blot

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) que se introducía rápidamente dentro de los moldes de polimerización (Protean II Xi de BioRad) que previamente habíamos ensamblado. Por encima se añadía un poco de agua destilada para favorecer la polimerización y conseguir un borde regular. Mientras el resolving gel polimerizaba se preparaba el stacking gel, que nos servía para concentrar las muestras antes de iniciar su separación. Una vez polimerizado el resolving gel, se retiraba con cuidado el agua y se añadía el stacking gel, seguidamente se introducía el peine y se dejaba polimerizar.

- *Resolving gel*: acrilamida/bis-acrilamida al 10% (Pronadisa), 0,1% SDS, 0,375 M Tris-SDS a pH 8,8 (0,75 M Tris base (Sigma), 0,2% SDS (Sigma)), 0,05% -V/V- TEMED (Sigma), 0,05% persulfato amónico (Panreac).
- *Stacking gel*: acrilamida/bis-acrilamida al 3%; 0,1% SDS, 0,125 M Tris-SDS a pH 6,8 (0,25 M Tris base (Sigma), 0,2% SDS (Sigma)), 0,1% TEMED -V/V-, 0,05% persulfato amónico (Panreac).

Preparación de las muestras. Entre 30-100 µg de proteína se desnaturalizaban añadiendo aproximadamente 1 µl de tampón de carga por cada 7 µg de proteína, de manera que la proporción proteína:SDS fuera 1:4. A continuación las muestras se calentaban en un baño hirviendo durante 2 minutos y ya estaban listas para ser cargadas en los geles de acrilamida, reservando un carril para el marcador de pesos moleculares (Invitrogen). Las muestras se corrían en el tampón Tris-glicina 0,5x a pH 8,3 a 70 V durante tota la noche.

- *Tampón de carga*: 0,5 M Tris-HCl (Sigma) a pH 6,8, 5% SDS (Sigma), 10% de glicerol (Sigma), 5% de 2-mercaptoetanol (Sigma), 1% de azul de bromofenol (Panreac).
- *Tris-glicina 0,5x* a pH 8,3: 0,025 M Tris base (Sigma), 0,195 M glicina (Sigma), 0,02 % SDS (Sigma).

Transferencia. Cuando el frente llegaba al borde inferior del gel se paraba la electroforesis y las proteínas se transferían a una membrana de nitrocelulosa de 0,45 µm utilizando el sistema semi-dry-electroblotter. Para ello se mojaban las placas de grafito del electroblotter y se colocaban diferentes hojas de papel Whatman nº3 sobre una de ellas, evitando la formación de burbujas y/o arrugas, en el siguiente orden: 6 hojas mojadas en el tampón 0,3 M Tris, 3 hojas mojadas en el tampón 0,025 M Tris, la membrana de nitrocelulosa mojada en el tampón 0,025 M Tris, el gel de poliacrilamida de la electroforesis realizada, 9 hojas mojadas en el tampón del cátodo, y finalmente se colocaba la segunda placa de grafito y se conectaba el sistema a una intensidad constante de 200 mA durante 2 h.

- *Tampón Tris 0,3 M a pH 10,4*: 0,3 M de Tris-base (Sigma), 20% de metanol absoluto (Panreac).
- *Tampón Tris 0,025M a pH 10,4*: 0,025 M de Tris-base (Sigma), 20% de metanol absoluto (Panreac).
- *Tampón del cátodo a pH 9,4*: 0,09% de β-Alanina (Sigma), 0,46% de Tris-base, 20% de metanol absoluto (Panreac).

Detección. Una vez transferidas las proteínas a la membrana ésta se bloqueaba durante 1 h con PBS-Tween y 5% de leche en polvo. Después se realizaban 3 lavados, de 15, 5 y 5 min, respectivamente y una incubación de 1 h con 15 ml de PBS-Tween con 0,1% de BSA (Sigma) y 0,1% de azida sódica (Sigma) y con el anticuerpo primario adecuado.

Anticuerpos:

- anticuerpo de conejo anti Ucp1 de ratón (Alpha Diagnostic International) diluido 1:1000–1:3000.
- anticuerpo de conejo anti Ucp2 de ratón (Alpha Diagnostic International) diluido 1:1000.
- anticuerpo de cobaya anti Ucp3 de ratón (Linco Research Inc) diluido 1:5000.
- anticuerpo de conejo anti C/EBPα de rata (Santa Cruz Biotech) diluido 1:4000.
- anticuerpo de conejo anti RXRα de humano (Santa Cruz Biotech) diluido 1:2000–1:3000.
- anticuerpo policlonal de conejo anti RARα (Santa Cruz Biotech) diluido 1:200–1:1000.
- anticuerpo monoclonal de rata anti VDR (Sigma) diluido 1:1300.
- anticuerpo policlonal de conejo anti CaSR de rata (Abcam) diluido 1:100–1:1000.

Después de una hora de incubación con el respectivo anticuerpo primario se realizaban 3 lavados de 15, 5 y 5 min, respectivamente con PBS-Tween. A continuación se incubaba 1 h con el anticuerpo secundario anti-IgG apropiado, conjugado a un complejo estreptavidina biotinilada-peroxidasa de rábano (Sigma) diluido 1:5000 en PBS-Tween y con 0,1 µg de BSA.

Tras 1h se volvía a lavar la membrana 3 veces (15, 5, y 5 min) y se procedía a la detección por quimioluminiscencia del marcaje, poniendo sobre la membrana el reactivo de detección (ECL de Amersham). Las membranas se colocaban entre transparencias y se introducían en un casete donde se exponían a películas fotográficas Hyperfilm ECL de Amersham durante el tiempo necesario para obtener una buena señal.

Reactivos:

- *PBS para electroforesis a pH 7,5*: 0,564% de NaCl (Panreac), 0,296% de NaH_2PO_4 (Panreac), 1,15% de Na_2HPO_4 anhidro (Panreac).
- *Tampón PBS-Tween*: 1% de Tween 20 (Sigma) en PBS para electroforesis.

Análisis. Las membranas se escaneaban en un densitómetro Agfa DUOSCAN, y se cuantificaban con el programa Kodak 1D image Analysis Software 3.5 (Eastman Kodak Co.).

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.

VIII. BIBLIOGRAFÍA / BIBLIOGRAPHY

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