UNIVERSIDAD DE NAVARRA

FACULTAD DE FARMACIA

SUPLEMENTACIÓN DE LA DIETA CON ÁCIDO LIPOICO COMO MEDIDA PARA COMBATIR LA OBESIDAD Y LA INSULINO-RESISTENCIA: ESTUDIO DE LOS MECANISMOS IMPLICADOS

Pedro Luis Prieto Hontoria

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Fdo. Pedro L Prieto-Hontoria

El presente trabajo ha sido realizado bajo nuestra dirección en el Departamento de Ciencias de la Alimentación, Fisiología y Toxicología y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

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"El trabajo del científico se parece a la perforación de un pozo: es turbia al principio, más luego se clarifica"

“La recompensa del trabajo bien hecho, es la oportunidad de hacer más trabajo bien hecho” (Jonas Edward Salk)

A mis padres y familiares

A Marta

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En esta parte de la tesis siempre uno se convierte en injusto porque se olvida, por no tener registro escrito, de las muchas personas que contribuyeron a que este trabajo salga a la luz. La tesis no es solo su escritura, sino la investigación, la gesta del proyecto en sí, el desarrollo de la idea y muchas cosas más. La felicidad por la obra terminada, y el alivio por poner punto y final a una parte de tu vida, resultan en agradecimientos muchos más que retóricos hacia quienes la han hecho posible. Pero la felicidad y el alivio son estados de ánimo, efímeros, y podrían arrojar dudas sobre la durabilidad del sentimiento de gratitud que generan. No se dude del mío. La deuda que tengo con las personas que enumeraré a continuación rebasa sobradamente su relación puntual con este proyecto de investigación.

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del mundo por haber sido tallado por vuestra mano, por haber compartido vuestros amplísimos conocimientos, filosofía positiva y vuestra gran amistad. Habéis logrado que integre en mí una forma de entender las cosas, de disfrutar de mis seres queridos y de luchar por mis sueños, que hacen que cada día se abran nuevas puertas en mi vida. Y conociendo que no tengo talentos especiales para la investigación, habéis sabido sacar de mi gran curiosidad por las cosas, incluidos los mecanismos moleculares y lo mejor de mí persona. GRACIAS POR TODO y sois las mejores sin ninguna duda.

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Aunque este proyecto tiene mucho de trabajo individual, casi solitario, mis compañeras/os en el departamento han aligerado ese sentimiento notablemente, y siempre he encontrado en ellos apoyo y ayuda cuando les he necesitado. Es fácil imaginar lo afortunado que he sido pudiendo comentar mi trabajo y hacer consultas con decenas de especialistas por el simple procedimiento de recorrer escasos metros hasta la puerta de "ordenadores, 215, 234". Aún a riesgo de provocar agravios comparativos, la extensión de estas líneas obliga a evitar una relación exhaustiva, por lo que me limitaré a expresar mi agradecimiento a todos, sin distinciones, mencionando específicamente a algunos por sus contribuciones. Ha sido un camino largo y duro en el que, algunas veces, la fijación por lograr tus objetivos te hace olvidar la importancia del contacto humano. Sin embargo, como en todas las actividades de la vida, siempre al final hay algunos criterios que te permiten priorizar y es por ello que debo resaltar mis agradecimientos para algunas personas.

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Como dice el poeta libanés Gibran Khalil, *"para entender el corazón y la mente de una persona, no te fijes en lo que ha hecho, no te fijes en lo que ha logrado sino en lo que aspira a hacer"*. Espero que hayáis comprendido mi corazón y mi mente en algunos momentos, en los cuales me ha costado expresar un agradecimiento que es lo que deseaba y no lo hice.

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ABREVIATURAS

aa- Aminoácidos

ACC- AcetilCoA carboxilasa

Acrp30- *Adipocyte complement-related protein of 30Kds*

ADHL- Ácido dihidrolipoico

AESAN- Agencia Española de Seguridad Alimentaria y Nutrición

AGL- Ácidos grasos libres

AgRP- *Agouti related peptide*/Péptido relacionado con Agouti

AMP- Adenosin di-fosfato

AMPK- *AMP-activated proteína kinase*/Quinasas activadas por AMP

ANG- Angiotensinógeno

apM1- *adipose Most abundant gene trancript 1*

ASP- *Acetylation stimulating proteína*/Proteína estimulante de la acetilación

ATCC- *Amerycan Type Culture Collection*

ATG- Alteración de la tolerancia a la glucosa

ATP- Adenosin tri-fosfato

BSA- *Bovine serum albumin*/ Albumina sérica bovina

C- Citosina

CEEA- Comité de ética para experimentación animal

CIFA- Centro de Investigación en Farmacobiología Aplicada

CMKLR1- *Chemokine-Like receptor 1*

CRH-*Corticotropin releasing hormone*/Hormona liberadora de corticotropina

CSB- *Calf serum bovine*/Suero bovino de ternero

DHLA- *Dihidrolipoic acid/*Ácido dihidrolipoico

DM2- Diabetes mellitas tipo 2

DMEM- *Dulbecco´s Modified Eagle Medium*

DORICA- Dislipemia, Obesidad y Riesgo Cardiovascular

ECA- Enzima convertidota de la angiotensina

ECV- Enfermedad Cardiovascular

ELISA- *Enzyme-Linked ImmunoSorbent Assay*

eNOS- *Endothelial nitric oxide synthase*/Sintasa endotelial de oxido nitrico

ERK- *Extracellular signal-regualted kinase*/Señal extracelular regulada por quinasa

FSB- Foetal serum bovine/Suero Fetal Bovino

G- Guanina

GALP- *Galanin like peptide*

GBP28- *Gelatin-binding protein-28*

GSK- *Glycogen synthase kinase/* Glúcogeno sintasa quinasa

GVA- Gastroplastía vertical anillada

HDL- *High density lipoprotein*/Lipoproteína de alta densidad

HMW- *High molecular weight*/Peso molecular alto

HOMA- *Homeostasis Model Assesment* /Índice de resistencia a la insulina

HRP- *Horseradish peroxidase*/Peroxidasa de rábano

IAC- Índice de adiposidad corporal

IASO- *International Association for the Study of Obesity*/Asociación internacional del Estudio de la Obesidad

IBMX- Isobutilmetilxantina

IL- Interleuquina

IL-1RA- *Interleukin 1 receptor antagonist*

IMC- Índice de masa corporal

IRS- *Insulin receptor substrate*/Sustrato receptor de la insulina

JNK- *c-Jun N-Terminal kinase*/c-Jun terminal quinasa

Kcal- Kilocalorías

LA- *Lipoic acid/*Ácido lipoico

LASY- *Lipoic acid synthase/*Ácido lipoico sintasa

LDL- *Low density lipoprotein*/Lipoproteína de baja densidad

LMW- *Low molecular weight*/Peso molecular bajo

MAPK-*Mitogen-activated protein kinase*/Proteina quinasa activada por mitogenos

MCH- *Melanin-concentrating hormone*/Hormona concentradora de melanina

MC4R- *Melanocortin 4 receptor*/Receptor de la melanocortina 4

MEM- *Minimun Essential Medium*

MIF- *Macrophage inhibitory factor*/Factor inhibidor de macrofagos

MMW- *Medium molecular weight*/Peso molecular medio

MSH- *Melanocyte-stimulating hormone*/Hormona estimuladora de melanocitos

Napmt- *Nicotinamide phosphoribosyl transferase*

NPY- Neuropéptido Y

NO- *Nitric oxide*/Oxido nítrico

OLEFT- *Otsuka Long-Evans Tokushima Fatty*/Ratas genéticamente obesas

OMS- Organización Mundial de la Salud

PAI- *Plasminogen activator inhibitor*/Inhibidor del activador del plasminógeno

PBEF- *Pre-B cell colony enhancing factor*/Factor de crecimiento pre-B

PI3K- *Phosphoinositide-3-kinase*/Fosfotidilinositol 3-quinasa

PVDF- Polivinilidenofluoruro

Rº- Radicales libres

RARRES- *Retinoic Acid Receptor Responder*

RI- Resistencia insulinica

ROS- *Reactive Oxigen Species*/Especies reactivas de oxigeno

SEEDO- Sociedad Española para el Estudio de la Obesidad

SM- Síndrome Metabólico

SNC- Sistema Nervioso Central

SREBP2- *Sterol Regulatory Element-Binding Protein-2*

T- Timina

TAB- Tejido adiposo blanco

TG- Triglicéridos

TIG- *Tarazone-Induced gene 2*

TNF-α- Factor de necrosis tumoral alfa

TZD- Tiazolinedionas

UCP- Proteína desacoplante

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INTRODUCCIÓN

1.1.- LA OBESIDAD

1.1.1.- Etiopatogenía de la obesidad

El concepto de obesidad ha sufrido diversas interpretaciones en cuanto a su relación con la salud a lo largo de la historia. En la actualidad, la obesidad se define como una enfermedad crónica multifactorial caracterizada por un aumento de la masa grasa, y por lo tanto del peso corporal, como consecuencia de un balance energético positivo mantenido en el tiempo. Esta enfermedad afecta a un gran porcentaje de la población en países desarrollados, abarcando todas las edades, sexos y condiciones sociales. Cabe destacar que un exceso de peso no significa obligatoriamente obesidad, ya que el exceso de peso puede deberse a diversas causas ajenas a la obesidad, ya sea un individuo con una masa muscular elevada, o bien, como sucede en algunas enfermedades como la insuficiencia cardiaca en las que se retiene gran cantidad de agua (Barbany y Foz, 2002).

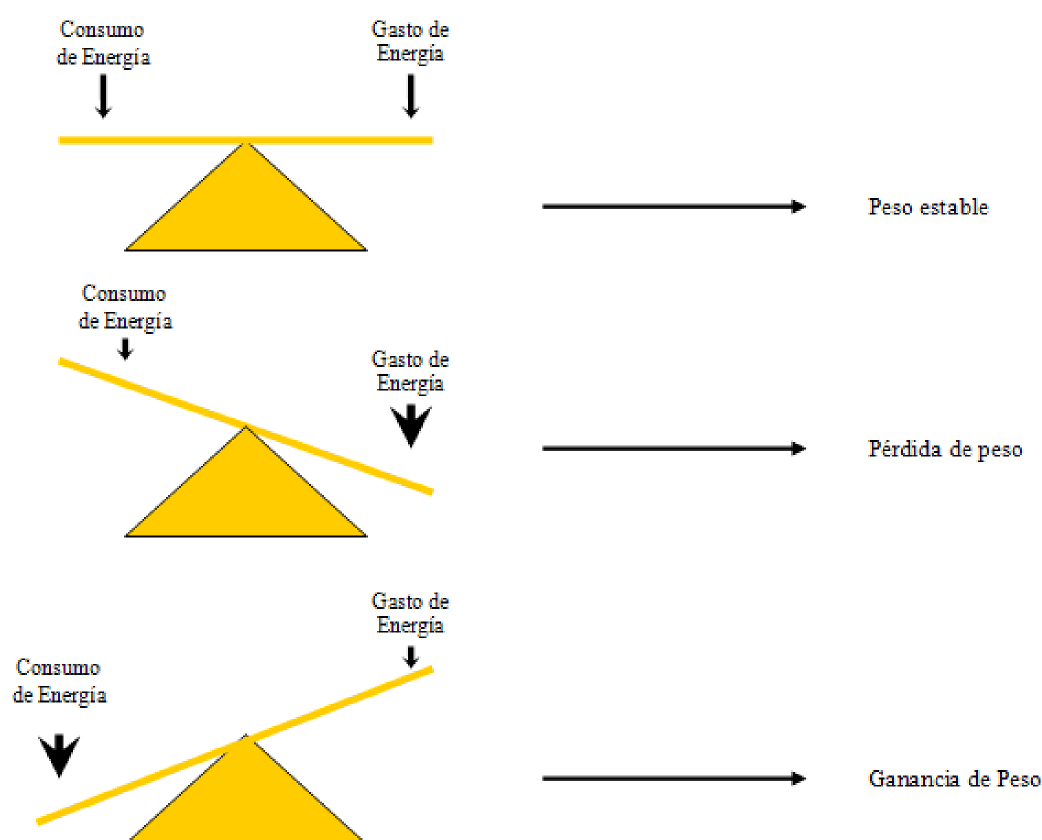

Figura 1.- Cambios en el peso corporal determinados por el equilibrio entre el consumo de energía (calorías de alimentos) y el gasto de energía (calorías consumidas).

Existen diversos criterios en función de los cuales se puede clasificar la obesidad. El más utilizado es el criterio cualitativo. Este criterio se basa en el Índice de Masa Corporal (IMC) o Índice de Quetelet (Quetelet 1835). Se trata de un índice que relaciona el peso del individuo expresado en kilogramos con la altura de dicho individuo, expresada en metros elevados al cuadrado. Dicho índice es utilizado por la mayoría de estudios epidemiológicos y recomendado por diversas sociedades médicas y organizaciones de salud internacionales para su uso clínico, dada su reproducibilidad, facilidad de utilización y capacidad de reflejar adiposidad en la mayoría de la población (Salas-Salvado *et al.* 2007). De acuerdo con el Consenso SEEDO´2007 esta clasificación queda establecida tal y como se muestra en la siguiente tabla:

Tabla 1. Criterios SEEDO para definir la obesidad en grados según el IMC en adultos.

Categoría	Valores límite del IMC (kg/m^2)
Peso insuficiente	< 18,5
Normopeso	18,5-24,9
Sobrepeso grado I	25,0-26,9
Sobrepeso grado II (preobesidad)	27,0-29,9
Obesidad de tipo I	30,0-34,9
Obesidad de tipo II	35,0-39,9
Obesidad de tipo III (mórbida)	40,0-49,9
Obesidad de tipo IV (extrema)	>50

Recientemente se ha definido un índice alternativo al IMC es el Índice de adiposidad corporal (IAC) que, refleja de una forma mucho más precisa el porcentaje de grasa acumulada y es útil tanto en hombres como en mujeres de distintas razas. IAC = [(diámetro de la cadera en centimetros)/((altura del individuo en metros)^{1,5}-18)]. Este nuevo índice se ha creado a partir del examen de 1.700 estadounidenses de origen mexicano cuya grasa corporal se evaluó a través de densitometría. Con los datos de la densitometría, los investigadores intentaron obtener la misma información a través de la combinación de distintas variables: como el peso, la altura, el sexo la edad, o el perímetro abdominal y de cadera. El citado índice de adiposidad parecía ser la fórmula más precisa para evaluar la grasa corporal pero, para estar seguros, los investigadores probaron la herramienta en una segunda muestra de población, esta

vez estadounidenses de origen africano y obtuvieron una concordancia en las cifras muy elevada. Este nuevo índice es interesante pero debe demostrarse su utilidad en otros trabajos (Bergman *et al*. 2011).

Otros criterios para la clasificación de la obesidad son los siguientes:

- Criterio celular: según el número y tamaño de los adipocitos (Moreno *et al*. 1997):
  - Hiperplásica.- Si se debe a un aumento del número de células. Este tipo de obesidad suele aparecer en épocas de crecimiento, considerándose responsable de la obesidad infantil y del adolescente.
  - Hipertrófica.- Si lo que se produce es un aumento del tamaño celular, en particular de los adipocitos, siendo este tipo el principal responsable de la obesidad en el adulto.

- Criterio anatómico: según la cantidad así como la localización predominante de los depósitos grasos. De esta forma se pueden distinguir al menos tres tipos diferenciados de obesidad (Bouchard *et al* .1998).

  - Obesidad difusa o de clase I.- Caracterizada por el exceso de grasa corporal total sin que se produzca una concentración específica de tejido adiposo en alguna región corporal.

  - Obesidad central o de clase II.- También denominada obesidad "androide" por considerarse típica de varones y caracterizada por el exceso de grasa subcutánea en la región abdominal (tipo manzana) y en el tronco. Es una obesidad fundamentalmente hipertrófica, la grasa es visceral y altamente sensible a la lipólisis inducida por catecolaminas. Esta obesidad suele asociarse a intolerancia a la glucosa, hipertrigliceridemia, diabetes mellitus tipo 2, hipertensión y enfermedad arterial coronaria.

- Obesidad glúteo-femoral o de clase III.- Típica de mujeres también denominada obesidad "ginoide", la cual se caracteriza porque la grasa se localiza preferentemente en la región glúteo femoral (tipo pera). Es una obesidad hiperplásica y poco sensible a la lipólisis. Suele asociarse a litiasis biliar, tromboflebitis e hiperinsulinemia.

Obesidad Androide **Obesidad Ginoide**

Figura 2.- Tipos de obesidad según criterio anatómico.

1.1.2.- Prevalencia de la Obesidad

La obesidad es una enfermedad crónica que se ha convertido en uno de los problemas de salud más graves de las sociedades occidentales (Powers *et al*. 2007). De hecho, ha sido catalogada por la Organización Mundial de la Salud (OMS) como *"Epidemia del siglo XXI"* y otros autores la han denominado la *"Globesidad"* y/o *"Diabesidad"* por las dimensiones que ha adquirido a lo largo de las últimas décadas y por su impacto sobre la morbimortalidad, la calidad de vida y el gasto sanitario (Sorensen *et al*. 2010; González-Zapata *et al*. 2007; Bray 2004). La prevalencia de la obesidad ha aumentado y continúa incrementándose de forma alarmante en nuestra sociedad, así como en países de economía en transición o en países no desarrollados, adquiriendo proporciones epidémicas (Finucane *et al*. 2011; Low *et al*. 2009). La obesidad aumenta sustancialmente no sólo el riesgo de sufrir/padecer diabetes y enfermedad cardiovascular, sino también ciertos tipos de cáncer (Prieto-Hontoria *et al*. 2011) y otras enfermedades altamente prevalentes en nuestros días, de manera

que la obesidad se ha convertido en la segunda causa de mortalidad prematura y evitable después del tabaco. Los pacientes con obesidad mórbida presentan también un aumento de la mortalidad total y sufren una gran estigmatización social y discriminación, ya que esta condición mórbida muchas veces no es considerada como una verdadera enfermedad. El coste económico que implica la obesidad, según la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN), es de unos 5.000 millones de euros anuales (actualizado en Noviembre de 2010), lo que supone casi el 7% del gasto sanitario (Consenso SEEDO´2007 Sociedad Española para el Estudio de la Obesidad). Según la OMS, en 2005 había en todo el mundo 1.600 millones de personas mayores de 15 años y 20 millones de menores de 5 años con sobrepeso, y 400 millones de personas con obesidad. Se estima que en el año 2015 habrá, aproximadamente, 2.300 millones de personas adultas con sobrepeso y más de 700 millones con obesidad. Los últimos análisis (2010) de la IASO (Asociación Internacional del Estudio de la Obesidad) estiman que aproximadamente 1000 millones de adultos tienen sobrepeso y 475 millones son obesos. La IASO estima que 200 millones de niños en edad escolar sufren sobrepeso u obesidad, de los cuales 40-50 millones están clasificados como obesos. En la Europa de los 27 miembros, aproximadamente el 60% de adultos y el 20% de los niños en edad escolar tienen sobrepeso u obesidad. Traducido en números esto implica aproximadamente 260 millones de adultos y 12 millones de niños que tienen sobrepeso y/u obesidad en Europa.

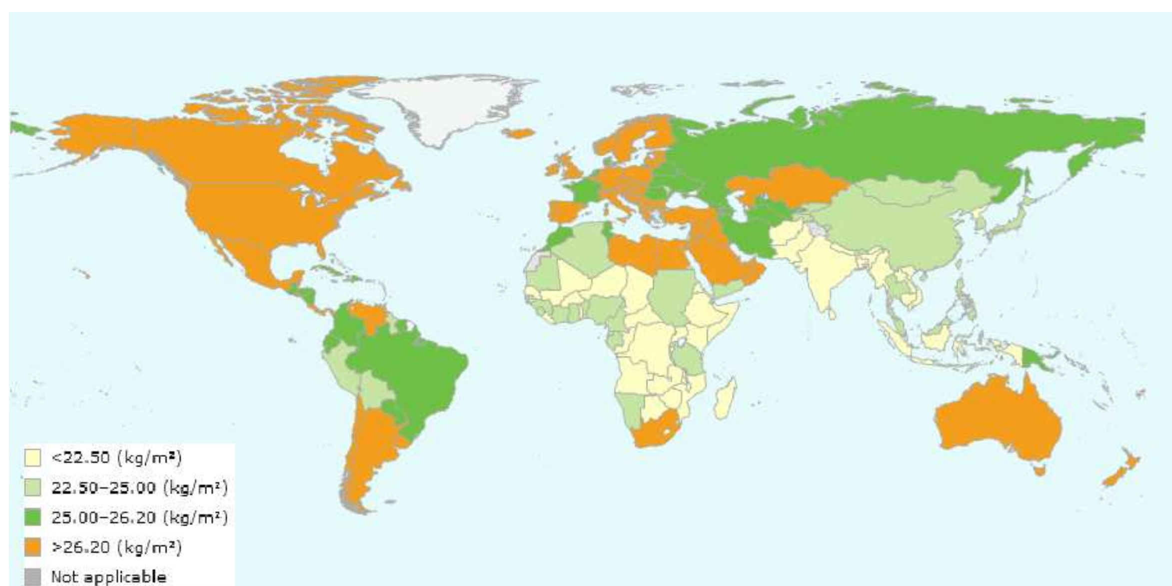

Figura 3.- Valores promedio de IMC de varones jóvenes (≥ 20 años) en distintos países (Finucane *et al.* 2011).

La prevalencia de obesidad en la población adulta (25-60 años) en España se estima en un 15,5%, y es más elevada en mujeres (17,5%) que en los varones (13,2%), según el estudio DORICA (Dislipemia, Obesidad y Riesgo Cardiovascular) (Aranceta *et al.* 2004). Además durante la edad adulta, la prevalencia de obesidad incrementa con la edad, y esta relacionado inversamente con el nivel educativo y la situación socioeconómica (Aranceta *et al.* 2001). Así, las mujeres con 45 o más años con un bajo nivel educativo son el subgrupo con mayor prevalencia de obesidad. Entre los factores que influyen en esta mayor prevalencia de obesidad destacan, por un lado, los ligados al estilo de vida: mayor sedentarismo, menor consumo de frutas y verduras, así como el incremento del aporte calórico a expensas de grasas o de alcohol. En la tabla 2, se muestra la prevalencia de obesidad según grupos de edad en nuestro país.

Tabla 2. Prevalencia de obesidad en la población española por grupos de edad y sexo (Consenso SEEDO, 2007.

	Varones (%)	Mujeres (%)	Total (%)
Población infanto juvenil [a]			
2-9 años	16,3	11,6	14,0
10-17 años	18,5	9,1	13,9
18-24 años	12,6	14,9	13,7
Población adulta [b]			
25-34 años	7,1	4,8	5,9
35-44 años	11,7	12,2	12,0
45-54 años	16,9	26,4	22,0
55-60 años	21,5	34,2	29,5
Población >65 años [c]			
Ancianos no institucionalizados [d]	31,5	40,8	36
Ancianos institucionalizados [e]	20,5	21,7	21

[a] Resultados del estudio enKid (Serra-Majen, 2003), para calcular la prevalencia de obesidad se han considerado los valores específicos por edad y sexo del percentil 97 del IMC, utilizando las tablas de Orbegozo confeccionadas por Hernández y col 1988.
[b] Resultados del estudio DORICA (Aranceta *et al.* 2004),se definió obesidad a valores de IMC por encima de 30 kg/m^2.
[c]Se utilizaron para definir obesidad, valores de IMC por encima de 30 kg/m^2.
[d]Ancianos no institucionalizados (Gutiérrez Fisac *et al.* 2004).
[e]Ancianos institutionalizados: (Aranceta *et al*. 2004).

Asimismo, se han observado diferencias significativas en la prevalencia de obesidad en las diferentes regiones españolas y entre sexos (Figura 4). En el sexo femenino la prevalencia de obesidad es alta en el Noroeste (21,8%), en el Sur (20%) y en las Islas Canarias (22,2%) comparando con el Noreste y Centro (15,3%). La distribución de la obesidad en el subgrupo de sexo masculino sigue un patrón similar, con una prevalencia mayor en el Sur (18,5%) y Noroeste (19%) comparando con el Noreste (8,5%) y Centro (9,31%).

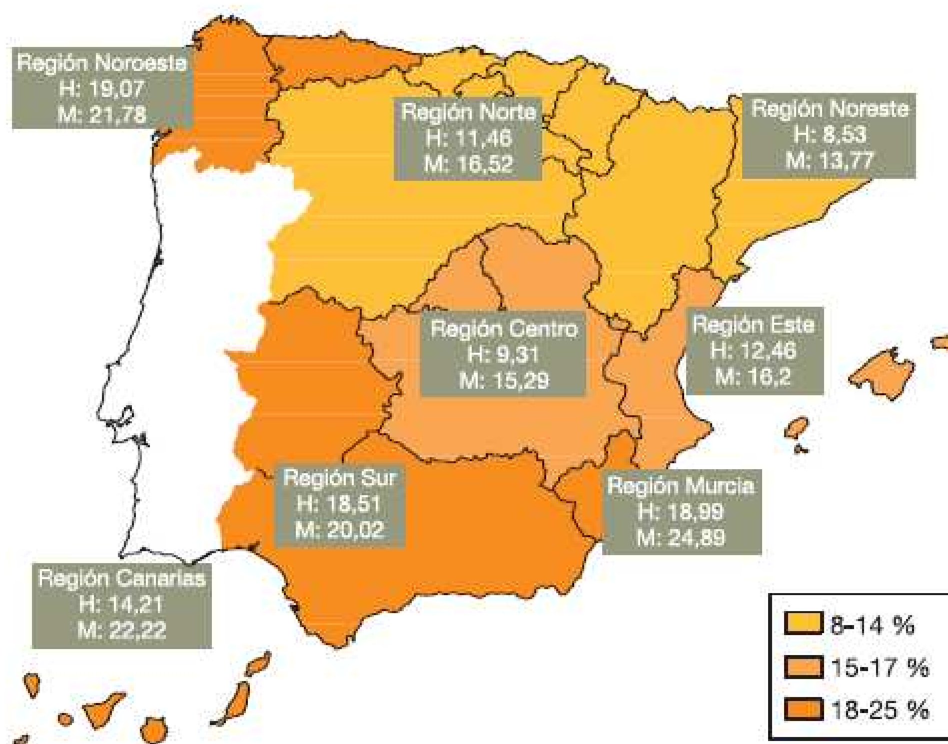

Figura 4.- Distribución de la prevalencia de obesidad (%) (IMC>30) por áreas geográficas y sexo. Estudio DORICA (2005).

Estos datos de prevalencia de obesidad han sido corroborados por la Encuesta Nacional de Salud 2006 publicada por el Ministerio de Sanidad Español, aunque se ha observado un ligero incremento en las cifras anteriormente citadas. Así, del total de la población adulta, un 37,80% padece sobrepeso y un 15,56% padece obesidad en España. Un estudio recientemente publicado (Febrero 2011) por la fundación THAO y llevado a cabo en 7 comunidades autónomas, muestra que un 29,3% de los niños españoles sufre sobrepeso u obesidad. Una cifra que se desglosa en una tasa del 21,1% de sobrepeso y del 8,2% cuando se contabiliza a menores obesos con edades

comprendidas entre los 3 y los 12 años. Un dato muy significativo es el elevado índice de obesidad en pequeños de sólo 3-5 años (8,4%).

Por último, es interesante destacar que según la OMS (Organización Mundial de la Salud) la prevalencia de obesidad (IMC≥25) y su estimación para años venideros es aún mayor, así para el año 2015 las cifras serán de un 60% de hombres y 52,5% de mujeres con obesidad en España.

1.1.3.- Complicaciones clínicas de la obesidad

La importancia clínica de la obesidad radica en sus complicaciones tal y como se ha señalado previamente. La obesidad aumenta el riesgo de desordenes metabólicos, alteraciones cerebrovasculares, respiratorias, osteoarticulares e incluso determinados tipos de cáncer (Prieto-Hontoria *et al*. 2011; Salas-Salvadó *et al*. 2007). La obesidad también produce un estigma social importante debido a la marginación social asociada a la depresión y a un aumento en la tasa de suicidio (Sánchez-Villegas *et al*. 2010; Babio *et al*. 2009; Salas-Salvadó *et al*. 2007).

En este sentido, el acúmulo de grasa corporal, principalmente en la región abdominal, está asociado a un mayor riesgo de desarrollar diabetes tipo 2 (DM2), enfermedad cardiovascular (ECV), y aumenta precozmente el desarrollo de complicaciones metabólicas como dislipemias, hipertensión arterial, hiperinsulinemia, hiperglucemia y resistencia insulínica (RI), a partir de los cuales posteriormente se caracterizaría el síndrome metabólico (SM) (Fox *et al*. 2007).

Los desórdenes clínicos asociados con la obesidad se generan bien como consecuencia directa del aumento de la masa grasa o bien de manera secundaria, debido a que los obesos presentan una actividad secretora del tejido adiposo alterada que puede afectar al funcionamiento de distintos sistemas (Bray, 2004). El clínico debe realizar un esfuerzo en determinar las patologías asociadas al exceso ponderal, y especialmente aquellas susceptibles de mejoría tras la pérdida de peso (Consenso SEEDO, 2007). A continuación se resumen las alteraciones más comúnmente asociadas a la obesidad.

- Enfermedad cardiovascular arterioesclerótica: Cardiopatía isquémica; Enfermedad cerebrovascular.

- Alteraciones cardiorrespiratorias: Insuficiencia cardíaca congestiva; Insuficiencia ventilatoria; Síndrome de apneas obstructivas del sueño.

- Alteraciones metabólicas: Resistencia a la insulina y diabetes tipo 2; Hipertensión arterial; Dislipemia aterógena; Hiperuricemia.

- Alteraciones en la mujer: Disfunción menstrual; Síndrome de ovarios poliquísticos; Infertilidad; Aumento del riesgo perinatal.

- Digestivas: Colelitiasis; Esteatosis hepática; Esteatohepatitis no alcohólica, cirrosis; Reflujo gastroesofágico, hernia de hiato.

- Músculo-esqueléticas: Artrosis; Lesiones articulares; Deformaciones óseas.

- Otras alteraciones: Insuficiencia venosa periférica; Enfermedad tromboembólica; Cáncer; Alteraciones cutáneas (estrias, acantosis nigricans, hirsutismo); Alteraciones psicológicas y psicosociales; Disminución en la calidad de vida; Trastornos del comportamiento alimentario.

1.1.4.- Tratamiento de la obesidad

Para tratar a una persona obesa es necesario conocer, en primer lugar, el agente desencadenante de la enfermedad, ya que no todos los obesos siguen un mismo patrón. Esto se debe a que la obesidad no se origina por un único factor (Das, 2010). Hay personas obesas en las que la ingesta exagerada de alimentos les induce un aumento excesivo del tejido adiposo, mientras que en otras, determinados trastornos metabólicos desencadenan la enfermedad. Debido a ello resulta necesario estudiar de forma individualizada cada caso (Marti *et al.* 2010).

En general, puede decirse que se deben conseguir reducciones de peso pequeñas y paulatinas, siendo suficiente la pérdida de un 5 a un 10% del peso. Se considera muy importante el mantenimiento de la reducción, ya que las oscilaciones de peso son perjudiciales. Con esta reducción de peso se puede conseguir una mejoría en las comorbilidades asociadas a la obesidad así como en la calidad de vida del paciente obeso (Barbany y Foz, 2002).

Ciertamente resulta muy complicado establecer pautas fijas para una población donde los hábitos de vida, las necesidades, las condiciones, los gustos y los objetivos que pueden ser alcanzados son muy heterogéneos. Por esta razón no podemos hablar de una dieta ideal en todos los casos, aunque de forma general puede decirse que es necesario que satisfaga todas las necesidades nutricionales, que permita un control del peso ideal y que, en la medida de lo posible, resulte atractiva para la persona. Es necesario, en definitiva, que la dieta sea equilibrada, que contenga todos los nutrientes necesarios y que sea personalizada (Nutrigenética- Nutrigenómica) (Strobush *et al.* 2011; Marti *et al.* 2010; McCabe-Sellers *et al*. 2008; Palou, 2007).

Sin embargo, el tratamiento nutricional de la obesidad se basa principalmente en la consecución de un balance energético negativo, mediante la reducción de la ingesta a través de dietas hipocalóricas y cambios en el estilo de vida (Abete *et al*. 2006). La restricción energética de 500-1000 kcal/día respecto a la dieta habitual se traduce en una pérdida ponderal de 0,5-1,0 kg/semana (Avenell *et al*. 2004). Esta restricción no debería comportar un aporte calórico por debajo de 1.000-1.200 kcal/día en mujeres y 1.200-1.600 kcal/día en varones (Rubio *et al*. 2007).

En los últimos años se han establecido distintas pautas dietéticas con el fin de conseguir pérdidas de peso efectivas y sostenibles a largo plazo. En un principio se hipotetizó que la alta ingesta de grasa era la causante exclusiva del aumento en la prevalencia de la obesidad, lo que llevó al diseño de dietas hipocalóricas basadas principalmente en la disminución de la ingesta lipídica. Sin embargo, se observó que las dietas con menor contenido en grasa producían modestas pérdidas de peso a corto plazo, y fácilmente recuperable en la mayoría de los casos (Best y Grainger, 2007; Abete *et al*. 2006). Sin embargo, otros estudios se han centrado en el contenido en hidratos de carbono de las dietas, ya que parecían ser los responsables de los

desequilibrios hormonales (elevados niveles de insulina) que conducían a una ingesta continuada, dificultando la adherencia a la dieta (Van Dam y Seidell, 2007). Así, la restricción en la ingesta de los hidratos de carbono se estableció como una nueva estrategia nutricional para el control de la obesidad (Malik y Hu, 2007). Este tipo de dietas han puesto de manifiesto una pérdida de peso y la regulación de los niveles de insulina, sin embargo tampoco aseguraban el mantenimiento del peso perdido a largo plazo (Nordmann *et al*. 2006; Stern *et al*. 2004).

Asimismo, se demostró que ciertos alimentos o sustancias presentes en los mismos, podrían influir específicamente en el tratamiento y prevención de la obesidad (Rodríguez *et al*. 2005). En este sentido el concepto de índice glucémico comenzó a tener gran importancia. Así, se postuló que el consumo de alimentos de alto índice glucémico podía conducir a una excesiva ingesta calórica mediante el aumento rápido de la concentración de glucosa en sangre y la posterior disminución de la misma como consecuencia de la secreción de hormonas reguladoras (Ludwig, 2002). Este rápido descenso de la glucosa sanguínea provoca a su vez el aumento de la ingesta (Melanson *et al*. 1999) favoreciendo el almacenamiento de nutrientes frente a la oxidación energética de los mismos (Pereira, 2006). Varios estudios han registrado efectos beneficiosos de la ingesta de alimentos de bajo índice glucémico sobre la sensación de hambre y la ingesta energética a corto plazo (Raben, 2002; Pereira *et al*. 2004). Además, se han observado que dietas de bajo índice glucémico mejoran la regulación del metabolismo energético (Agus *et al*. 2000). De manera que la baja concentración postprandial de sustratos podría favorecer toda una serie de adaptaciones neuroendocrinas dirigidas al mantenimiento del gasto energético (Pereira *et al*. 2004). Así, los efectos beneficiosos de las dietas de bajo índice glucémico sobre la regulación del peso corporal parecen estar mediados por efectos sobre la sensación de hambre y saciedad, así como sobre el metabolismo energético (Pereira, 2006). Asimismo, el alto contenido en fibra de las dietas se estableció como un factor determinante en la regulación del peso corporal (Slavin, 2003). La ingesta de alimentos con alto contenido en fibra favorecían la pérdida de peso y el mantenimiento del mismo, así como la mejora de los niveles de colesterol, gracias a una mayor sensación de saciedad tras las comidas (Rodríguez *et al*. 2005). Además, es conocido que la fibra soluble forma geles en contacto con el agua, retrasando el vaciado gástrico y la absorción de nutrientes, favoreciendo a su vez la disminución del índice glucémico de la dieta (Pereira, 2006). La fibra insoluble por el contrario,

es fermentada por las bacterias intestinales dando lugar a toda una serie de compuestos entre los cuales destacan los ácidos grasos de cadena corta, a los que se les ha relacionado con menores niveles de colesterol (Slavin, 2003).

Por otro lado, el tipo de grasa incluida en las dietas de pérdida de peso es otro factor nutricional a tener en cuenta (Rodríguez *et al*. 2005). Una ingesta elevada de ácidos grasos saturados se halla estrechamente relacionada con la incidencia de enfermedades cardiovasculares, mientras que los ácidos grasos insaturados tendrían un papel protector frente a este tipo de transtornos (Slavin, 2003). Una de las formas de regular el peso corporal a través del consumo de ácidos grasos omega-3 puede ser explicada a través del control sobre el apetito. En este sentido, un estudio realizado en adultos sanos ha relacionado las dietas enriquecidas en pescado con bajos niveles de leptina, independientemente de la grasa corporal, lo que sugiere una disminución de la resistencia a la leptina (Mori *et al*. 2004). Diversos autores proponen que los ácidos grasos poliinsaturados inhiben la síntesis de lípidos y disminuyen la producción del malonilCoA por supresión de la expresión de genes implicados en la lipólisis y además se induce la termogénesis por incremento de las UCP-3 (Rodríguez *et al*. 2005). Así, los ácidos grasos omega-3 pueden ser esenciales para el mantenimiento del balance energético y la prevención de la resistencia a la insulina asociada a la obesidad (Mori *et al*. 1999).

En la actualidad están tomando cierta relevancia en el tratamiento dietético de la obesidad las dietas con alto contenido en proteínas y bajo contenido en hidratos de carbono, a pesar de la existencia de datos insuficientes para poder recomendarlas indiscriminadamente en el tratamiento de la obesidad (Erlanson-Albertsson y Mei, 2005). Estos regímenes dietéticos han sido propuestos como una alternativa a las dietas convencionales con el fin de prevenir el riesgo de sobrepeso, enfermedades cardiovasculares y diabetes mellitus tipo 2 (Noakes *et al.* 2005). En algunos casos, este tipo de dietas se asemeja a las dietas cetogénicas, ya que el alto aporte proteico se suele acompañar de un alto consumo de grasas. En este contexto, Skov y colaboradores (1999) observaron una pérdida de peso de 8,9 kg vs 5,1 kg en sujetos que siguieron una dieta alta en proteínas (25%) y una dieta con un contenido en proteínas normal (12%), respectivamente, durante un período de 6 meses. El fundamento de esta intervención nutricional se basa en la reducción de la ingesta de

hidratos de carbono, aumentando el consumo de proteínas con el fin de reducir la secreción de insulina, ya que los hidratos de carbono son el mayor estímulo para la secreción de la misma. Además, se favorece la oxidación de grasa y se produce un estado de acidosis que provoca la supresión del apetito (Bravata *et al.* 2003).

Por otro lado, las proteínas tienen una alta capacidad termogénica y son el macronutriente de mayor índice de saciedad, por lo que contribuyen con el descenso del apetito y reducción de la ingesta energética (McMillan-Price y Brand-Miller, 2004). En un estudio nutricional se comparó el efecto de una dieta hiperproteica (27% proteínas) frente una dieta convencional sobre la pérdida de peso y composición corporal en sujetos hiperinsulinémicos con sobrepeso durante un período de 12 semanas de restricción energética y 4 semanas de mantenimiento (Farnsworth *et al.* 2003). La pérdida de peso alcanzada en ambos grupos fue semejante (alrededor de 8 Kg) al igual que la pérdida de masa grasa (-6,9 ± 0,4 Kg). Sin embargo, los sujetos que siguieron la dieta con alto contenido en proteínas preservaron mejor la masa libre de grasa y además, disminuyeron más los niveles de triglicéridos. Por otra parte, Parker y colaboradores (2002) realizaron un estudio similar en el que analizaron el efecto de dos dietas del mismo tipo que en el caso anterior (hiperproteica *vs* alto contenido en hidratos de carbono), durante 8 semanas de restricción energética y 4 semanas de mantenimiento. La pérdida de peso conseguida fue similar en ambos grupos, sin embargo, las mujeres que siguieron la dieta de alto contenido en proteínas perdieron mayor cantidad de grasa total y visceral en comparación con las mujeres del grupo de alto contenido en hidratos de carbono. Además los niveles de LDL-c disminuyeron más con la dieta de alto contenido en proteínas, a pesar de que la ingesta de ácidos grasos saturados fue similar en ambos grupos. Un reciente estudio de Te Marenga y colaboradores (2011) analizó el efecto de dos dietas en mujeres durante 8 semanas: una era alta en proteínas (30 %) con un 40% de hidratos de carbono frente a otra dieta con un 50 % de hidratos de carbono, 20 % de proteínas y 35 gramos de fibra, observando una mayor pérdida de peso, 4,5 Kg, en el grupo con una dieta alta en proteínas frente a una pérdida de 3,3 Kg en el grupo dietético que siguió una dieta alta en fibra. Así, otro estudio de Johnstone y colaboradores (2011) observaron que una dieta hiperproteica (30 % proteínas) y baja en hidratos de carbono (4 %) provocaba una mayor pérdida de peso 6,75 Kg frente a la misma dieta hiperproteica pero con un 35

% de hidratos de carbono que causó una pérdida de peso de 4,32 Kg en varones obesos.

En este contexto, numerosas investigaciones tratan de determinar la influencia específica de los diferentes macro y micronutrientes presentes en los alimentos sobre la regulación del peso corporal. No sólo se debe tener en cuenta la distribución de los hidratos de carbono, lípidos y proteínas, sino el tipo de restricción energética y la inclusión de determinados alimentos con el fin de potenciar la ingesta de determinados nutrientes o factores nutricionales que puedan facilitar el seguimiento y la adherencia a las dietas para la pérdida y mantenimiento del peso corporal.

Paralelamente a una regulación alimentaria resulta imprescindible la práctica de actividad física adecuada, en la que el individuo se sienta a gusto y pueda realizarla de forma sistemática al menos tres veces por semana y durante unos 30 minutos. Así mismo, es importante incentivar la práctica de actividad física no programada en cualquier grupo de edad. Si bien es cierto, en el caso de los niños resulta muy importante el fomento de hábitos que incorporen la actividad física, tanto programada como no programada en su estilo de vida cotidiano.

Existen otro tipo de intervenciones terapéuticas como son:

- Tratamiento farmacológico.- Su indicación debe regirse por los siguientes criterios:
  - No debe utilizarse como terapia aislada, sino de forma complementaria a las terapias básicas de plan de alimentación, actividad física y cambios en el estilo de vida.
  - Indicación limitada a pacientes con un IMC > 30 Kg/m^2 ó > 27 Kg/m^2 si se asocian comorbilidades mayores y cuando no se han alcanzado los objetivos de pérdida peso únicamente con los cambios en el estilo de vida. Actualmente disponemos de una única opción farmacológica aprobada para su empleo en obesidad:

- Orlistat.- Es un potente inhibidor de las lipasas gástrica y pancreática que impide la hidrólisis de los triglicéridos provenientes de la dieta, reduciendo su absorción en una proporción del 30%, lo que contribuye a desarrollar un balance calórico negativo (Guerciolini, 1997).

- Cirugía bariátrica.- Disponemos de evidencias de que la cirugía bariátrica es un tratamiento eficaz a largo plazo respecto a otras medidas convencionales en el tratamiento de la obesidad mórbida, reduciendo de manera significativa las comorbilidades asociadas a la obesidad (Maggard *et al*. 2005; Sjostrom *et al*. 2004; Sjöström *et al*. 1999; Pories *et al*. 1992). Existe unanimidad en que las indicaciones deben quedar limitadas a sujetos con IMC > 40 kg/m^2 o IMC > 35 kg/m^2 en presencia de comorbilidades mayores, pero también asumiendo ciertos requisitos de estabilidad psicológica y compromisos para el seguimiento por parte del paciente para garantizar un éxito a largo plazo.

  Actualmente existen diferentes técnicas quirúrgicas para el tratamiento de la obesidad mórbida, que se pueden agrupar en 3 categorías:

  - Técnicas restrictivas: Se incluyen la gastroplastia vertical anillada (GVA) y la banda gástrica ajustable.
  - Técnicas mixtas: Con componente restrictivo y malabsortivo, están representadas por el by-pass gástrico, que es actualmente la técnica de referencia en cirugía bariátrica.
  - Técnicas malabsortivas: Son aquellas indicadas en sujetos con IMC > 45 kg/m^2, ofreciendo buena calidad de vida, con pérdidas importantes de peso a largo plazo (70-85% del exceso de peso), pero con un coste metabólico importante originado por deficiencias nutricionales serias que hay que vigilar de cerca.

- Cirugía primaria endoluminal.- Esta modalidad consiste en reducir la capacidad del estómago por vía oral sin necesidad de abrir ninguna vía de acceso como en la cirugía bariátrica. El procedimiento endoscópico

consiste en realizar pliegues en el estómago y suturarlos para reducir su tamaño, de esta forma la capacidad del estómago queda limitada y el paciente queda saciado al ingerir una pequeña cantidad de alimentos. A las 24 horas de la intervención los pacientes pueden hacer una vida relativamente normal y un mes después, los pacientes podrán ingerir una dieta equilibrada, Esta cirugía está recomendada para pacientes con IMC entre los 34 y 40 kg/m^2 que han fracasado con los tratamientos dietéticos. También puede ser adecuada para aquellos pacientes a los que el balón intragástrico o la cirugía bariátrica no les haya funcionado o que no quieran someterse a cirugías mayores. Es una técnica muy novedosa (2010/2011) en Europa y Estados Unidos se han realizado 74 intervenciones mediante esta técnica con resultados altamente satisfactorios: con una reducción media del 32% del exceso de peso durante los 6 primeros meses posteriores a la operación.

- ❖ Balón intragástrico.- Esta modalidad terapéutica consiste en la colocación endoscópica de un balón relleno de suero salino o aire en la cavidad gástrica, con la finalidad de reducir sensiblemente la capacidad para la ingestión de alimentos y en consecuencia se alcanzan pérdidas promedio de 15 a 20 kg durante un período temporal de 6 meses (Genco *et al.* 2008). Técnica destinada a casos excepcionales, de aplicación limitada en el tiempo, donde han fracasado medidas intensivas dietéticas y farmacológicas y al mismo tiempo las opciones quirúrgicas pueden representar un riesgo no asumible por el paciente.

- ❖ Banda gástrica.- Consiste en la colocación mediante cirugía laparoscópica de una banda ajustable de silicona alrededor del estómago, cuya capacidad disminuirá hasta un tamaño de 25-30cc. Con ello, se sentirá muy rápidamente saciado y le será fácil perder peso. La colocación de bandas gástricas ajustables ofrecen una mínima morbilidad con resultados excelentes (Egan *et al.* 2010).

- ❖ Marcapasos gástrico.- El sistema denominado *abiliti*, consiste en la colocación de un electrodo de estimulacion bipolar tunelizado en la

curvatura menor gástrica, en la capa seromuscular de la pared gástrica -mediante cirugía laparoscópica- junto a un sensor de detección de alimentos, que al detectar alimentos ingeridos envía impulsos eléctricos de baja energía al electrodo de estimulación, lo que provoca en el paciente una distensión precoz del estómago y que permite reducir la ingesta debido a una sensación de saciedad producida por un retraso en el vaciamiento gástrico y/o alteraciones de la motilidad intestinal (Chen, 2004). En la actualidad, este tipo de terapéutica debe enmarcarse en el ámbito de los ensayos clínicos controlados. En marzo de 2011 se han colocado los primeros marcapasos en nuestro país. El dispositivo cuenta con un sistema de conexión inalámbrico (*wi-fi*) que transmite la información recogida por el sensor donde se refleja con detalle el consumo de comida del paciente. Con esa información, el dietista-nutricionista diseña el programa alimentario y el ejercicio. De esta forma también se facilita la reeducación del paciente en hábitos nutricionales. Los estudios clínicos realizados indican que tras un año del implante se logra una reducción de entre el 35 y el 40 % del exceso de peso.

1.2.- TEJIDO ADIPOSO

Durante muchos años, el tejido adiposo se ha considerado como el mayor reservorio energético en animales superiores y en humanos. Sin embargo, el tejido adiposo también cumple una importante función como órgano endocrino ya que produce un elevado número de moléculas bioactivas, generalmente llamadas adipoquinas, que participan en la regulación del metabolismo energético. Además, el tejido adiposo interviene en diversos procesos como son la regulación de la temperatura corporal, la protección mecánica y el aislamiento térmico (Ahima, 2006; Fantuzzi, 2005; Fruhbeck *et al*. 2001).

El tejido adiposo es un tipo de tejido conectivo especializado cuyas células más características son los adipocitos. Además, también está formado por otros tipos celulares, que constituyen la fracción estroma-vascular, como son las células precursoras de adipocitos o pre-adipocitos, células sanguíneas, endoteliales, fibroblastos y células inmunitarias (Cinti, 2005).

Se pueden distinguir dos tipos de tejido adiposo: el tejido adiposo blanco y el tejido adiposo pardo o marrón. Ambos tienen capacidad de metabolizar y almacenar lípidos, pero presentan claras diferencias en cuanto a su morfología, distribución, expresión génica, así como en su función.

El tejido adiposo marrón posee adipocitos de menor tamaño que los blancos, con multitud de pequeñas gotas lipídicas (multiloculares) en su interior y un gran número de mitocondrias cuyos citocromos le dan a este tejido su coloración característica (Figura 5) (Cannon y Nedergaard, 2004). Las funciones del tejido adiposo marrón derivan de su capacidad de disipar la energía en forma de calor en detrimento de la formación de ATP, a través de una vía de conductancia de protones mediada por la proteína desacoplante 1 (UCP1) (Cannon y Nedergaard, 2004). Por lo tanto, el tejido adiposo marrón está implicado en todos los procesos de termogénesis inducida por la temperatura y la dieta e interviene en la termorregulación, es decir, en la homeostasis de la temperatura corporal. De hecho, este tejido contribuye al mantenimiento del balance energético aumentando el gasto de energía tras el consumo de dietas ricas en grasas (Moreno y Martínez, 2002; Lafontan y Berlan, 1993).

El tejido adiposo blanco es el órgano específico que almacena la energía en forma de grasa. Los adipocitos que forman el tejido adiposo blanco presentan una morfología diferente en comparación a los adipocitos procedentes del tejido adiposo pardo (Figura 5) y, cuantitativamente, constituyen entre uno y dos tercios del número total de células presentes en dicho tejido. Los adipocitos del tejido adiposo blanco tienen una morfología más redondeada y son de mayor tamaño. Además, presentan una gran vacuola central (unilocular) formada por los lípidos acumulados en forma de triglicéridos, que constituyen un 95 % del tamaño del adipocito. Poseen un núcleo excéntrico y lateralizado y presentan un menor número de mitocondrias en comparación al tejido adiposo marrón. En el citoplasma perinuclear se encuentra un aparato de Golgi pequeño, un retículo endoplasmático rugoso poco desarrollado y ribosomas libres (Hansen y Kristiansen, 2006; Cinti, 2005).

El tejido adiposo blanco es el de mayor abundancia en humanos siendo la grasa visceral y subcutánea los mayores depósitos grasos del tejido adiposo blanco en mamíferos superiores. Sin embargo, existen diferencias en cuanto a su estructura, localización e importancia metabólica entre ambos depósitos grasos por lo que su funcionalidad y tamaño puede variar frente a distintas situaciones fisiopatológicas (Arner, 1995). Los depósitos de grasa subcutánea se encuentran entre el músculo y la piel y los dos principales sitios de acumulación son en la region glúteo-femoral. En cambio, la grasa visceral se encuentra envolviendo órganos internos como el corazón, páncreas e hígado entre otros (Bjorntorp, 1991), y está más relacionada con el desarrollo de alteraciones metabólicas y cardiovasculares (Pearson, 2002).

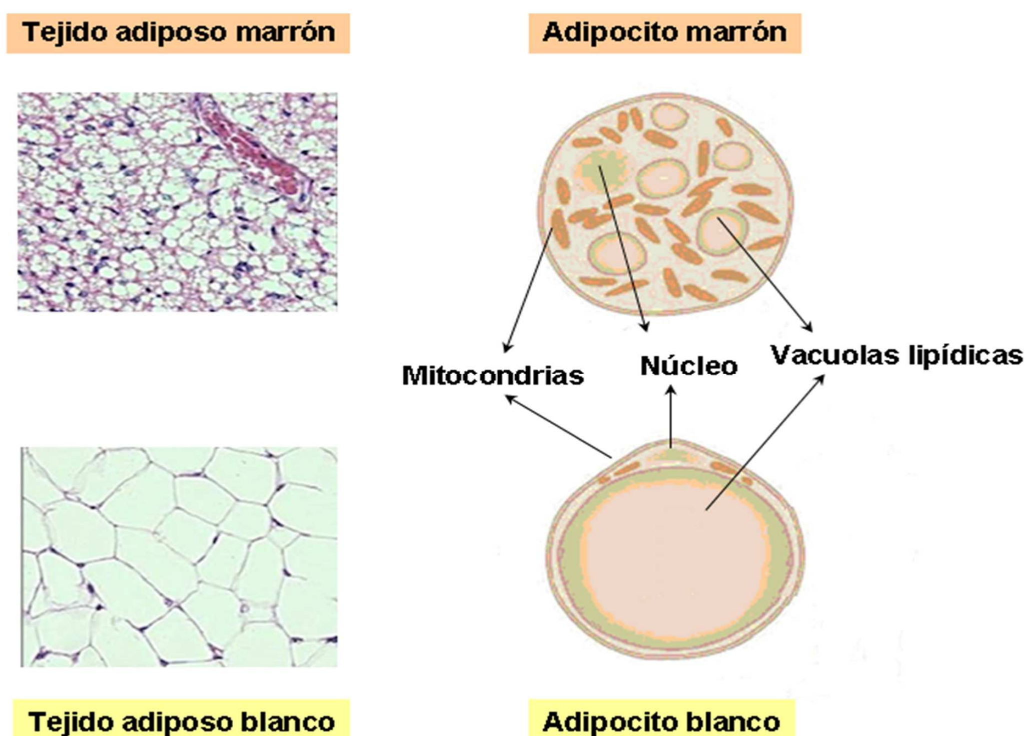

Figura 5.- Dibujo representativo de las principales diferencias morfológicas entre un adipocito de tejido adiposo blanco y de tejido adiposo pardo.

El aumento de grasa corporal que acompaña la obesidad puede ser debido a un aumento en el número y/o en el tamaño de los adipocitos. Es bien conocida la capacidad del adulto de generar nuevas células grasas en respuesta a dietas con alto contenido en carbohidratos y grasas. La adquisición de células grasas parece ser, además, un proceso irreversible (Moreno y Martínez, 2002). En este sentido,

Spalding y colaboradores (2008) mostraron que el número de adipocitos es un factor determinante para cuantificar la masa grasa en adultos siendo constante en adultos obesos o delgados, aún después de la pérdida de peso, sugiriendo que el número de adipocitos es estable desde la niñez y la adolescencia. Sin embargo, se ha observado que el 10% de los adipocitos es renovado cada año en adultos, sugiriendo una regulación del número de adipocitos en la edad adulta.

1.2.1.- Tejido adiposo como órgano secretor

Como se ha indicado anteriormente la idea tradicional que se tenía del tejido adiposo como reservorio pasivo para el almacenamiento de energía ha dejado de ser válida, al conocerse que el tejido adiposo es capaz de expresar y secretar una gran variedad de moléculas bioactivas, conocidas como adipoquinas, que pueden actuar tanto a nivel local (autocrina/paracrina) como a nivel sistémico (endocrino) (Rasouli y Kern, 2008; Fantuzzi, 2005; Frühbeck *et al.*, 2001) y están implicadas en la regulación de numerosos procesos fisiológicos (Rosen y Spiegeleman, 2006). Sin embargo, el tejido adiposo también tiene enzimas, factores de crecimiento, produce hormonas y receptores. Por todo ello, podríamos decir que el tejido adiposo aparte de ser un órgano necesario para el almacenamiento y liberación de energía, también contiene la maquinaria necesaria para permitir la comunicación con distintos órganos incluido el sistema nervioso central (SNC). Así pues, a través de una importante red interactiva, el tejido adiposo se ve implicado en una gran variedad de procesos biológicos como el metabolismo energético y diversas funciones neuroendocrinas e inmunes (Kershaw y Flier, 2004).

Tal y como se ha mencionado anteriormente, además de adipocitos, en el tejido adiposo también hay tejido conectivo, nervioso, células estromales y células inmunitarias. Por ello, aunque los adipocitos secretan un gran número de moléculas, otras proteínas pueden ser también secretadas por la fracción no adipocítica del tejido adiposo (Fain *et al*. 2004).

El número de adipoquinas que se conoce en la actualidad es superior a 50 (Ouchi *et al*. 2011; Rasouli y Kern, 2008; Rosen y Spiegelman, 2006), y las podemos clasificar como se muestra a continuación:

- ❖ Moléculas asociadas con la regulación de la ingesta/apetito y del gasto energético: leptina, adiponectina y adipsina (Havel, 2004; Meier y Gressner, 2004; Napolitano *et al*. 1994).

- ❖ Moléculas asociadas con la sensibilidad a la insulina y el metabolismo glucidico y lipídico: adiponectina, resistina, factor de necrosis tumoral α (TNF-α), apelina y visfatina, RBP-4, ZAG, omentina, vaspina, chemerina, ANGPTL2, lipocalina 2, CXCL5, SFRP5 (Ouchi *et al*. 2010; Chavey *et al*. 2009; Tabata *et al*. 2009; Wada *et al*. 2008; Aeberli *et al*. 2007; Bozaoglu 2007; Wang *et al*. 2007; Yang *et al*. 2006; Boucher *et al*. 2005; Bao *et al*. 2005; Fukuhara *et al*. 2005; Wolf *et al*. 2004; Yamauchi *et al*. 2002; Hu *et al*. 1996; Hotamisligil *et al*. 1993).

- ❖ Citoquinas relacionadas con el sistema inmune y la inflamación: TNF-α (Hotamisligil *et al*. 1995), la familia de las interleuquinas (IL-1 β, IL-6, IL-8, IL-10), MCP-1 y las proteínas reactantes de fase aguda como la haptoglobina, proteína C reactiva y el amiloide sérico (Fantuzzi, 2005; Trayhurn y Wood, 2004; Hotamisligil, 1999; Xing *et al.* 1998).

- ❖ Moléculas asociadas a la función vascular: angiotensina, PAI-1 y el factor de crecimiento endotelial vascular VEGF entre otros (Karlsson *et al*. 1998).

Sin embargo, conviene destacar que estas adipoquinas actúan de forma integral y coordinada en la regulación de los diversos procesos fisiológicos. Los principales tejidos diana donde se expresan receptores de estas adipoquinas son, además del propio tejido adiposo, el hígado, músculo esquelético, cerebro, sistema reproductor, sistema inmune y sistema cardiovascular.

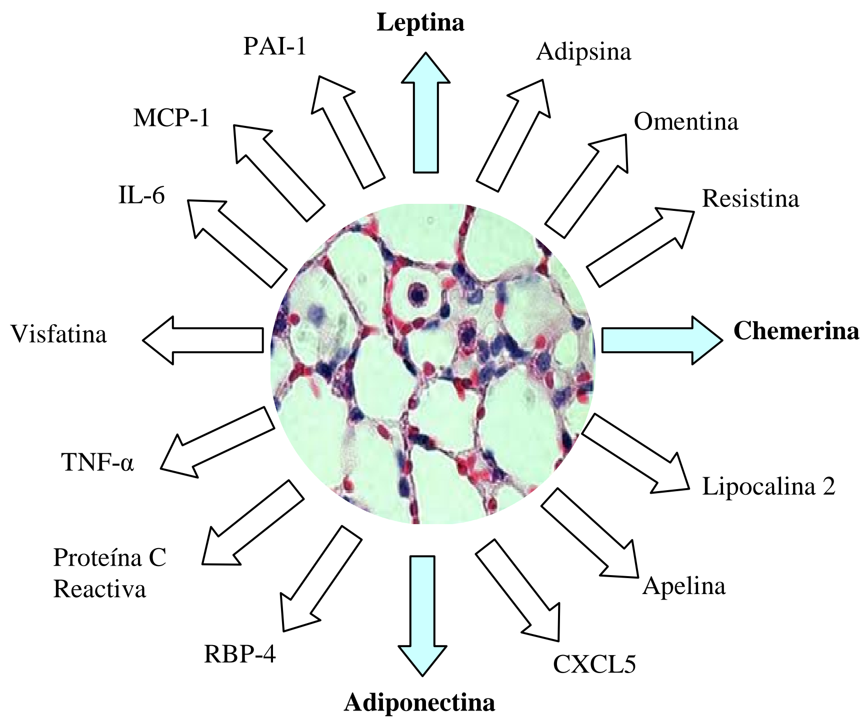

Figura 6.- Dibujo representativo de algunas de las adipoquinas secretadas por el tejido adiposo. En negrita se señalan aquellas que han sido estudiadas en el presente trabajo.

1.2.1.1.- Leptina

La leptina es una proteína de 16 kDa (Figura 7) que es segregada principalmente por el tejido adiposo blanco y que actúa en los centros nerviosos hipotalámicos controlando la ingesta y regulando el gasto energético (Ahima *et al.* 1996; Schwartz *et al.* 1996).

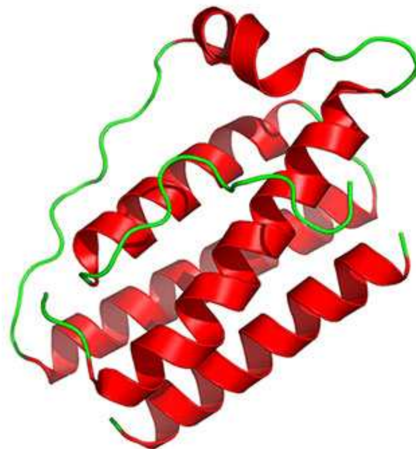

Figura 7.- Estructura tridimensional de la leptina.

En humanos, el gen de la leptina (*ob*) está localizado en el cromosoma 7q31.3 y consta de tres exones separados por dos intrones, estando la región codificadora de la leptina en los exones 2 y 3 (He *et al.* 1995; Isse *et al.* 1995). La leptina es traducida como un fragmento de 167 aminoácidos (aa) con una secuencia señal de 21 aa que se escinde antes de pasar a sangre como proteína de 146 aa. Su secuencia está muy conservada, observándose una gran homología entre la leptina de diferentes especies. Así, la leptina humana posee un 84% de homología con la de ratón y un 83% con la de rata (Zhang *et al.* 1994). Su estructura terciaria consta de cuatro hélices α y dos láminas β con un puente disulfuro simple entre los residuos 96 y 146 que parece ser necesario para su actividad (Grasso *et al.* 1997) y con similitud estructural con las citoquinas (Zhang *et al.* 1997; Madej *et al.* 1995).

La leptina, producto del gen *ob*, se produce principalmente en el tejido adiposo blanco aunque también es secretada a menor nivel por el tejido adiposo marrón (Marti *et al.* 1999). Además, se ha demostrado su secreción en otros órganos como son la placenta y el cordón umbilical, donde la leptina puede actuar como un factor de crecimiento o como una señal del estado nutritivo y energético entre la madre y el feto asegurando así un adecuado aporte alimentario (Cervero *et al.* 2006). Entre otros órganos que también secretan leptina se encuentra el estómago (Cinti *et al.* 2001). También es producida por el músculo esquelético (Wang *et al.* 1998), la glándula pituitaria de rata y ratón (Jin *et al.* 2000; Morash *et al.* 1999), las células epiteliales mamarias (Smith-Kirwin *et al.* 1998) y el hígado (Chen *et al.* 1999).

En 1995 se identificó el receptor de la leptina, al que se llamó OB-R (Tartaglia *et al.* 1995). El gen del receptor humano de la leptina se localiza en el cromosoma 1q31 y está constituido por 20 exones. Se han descrito varias isoformas que se diferencian por el dominio intracelular, denominadas OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re y OB-Rf (Lee *et al.* 1996). La isoforma, OB-Re carece de región transmembrana y citoplasmática, lo cual le confiere solubilidad, mientras que la isoforma OB-Rb presenta el dominio intracelular más largo. Ésta isoforma junto con la OB-Ra son las únicas capaces de captar la señal y por lo tanto, poseen capacidad de mediar los efectos fisiológicos de la leptina (Murakami *et al.* 1997; Chen *et al.* 1996). OB-Rb está localizado principalmente en el hipotálamo, región muy relacionada con la regulación de la ingesta y por tanto del peso corporal (Fei *et al.* 1997).

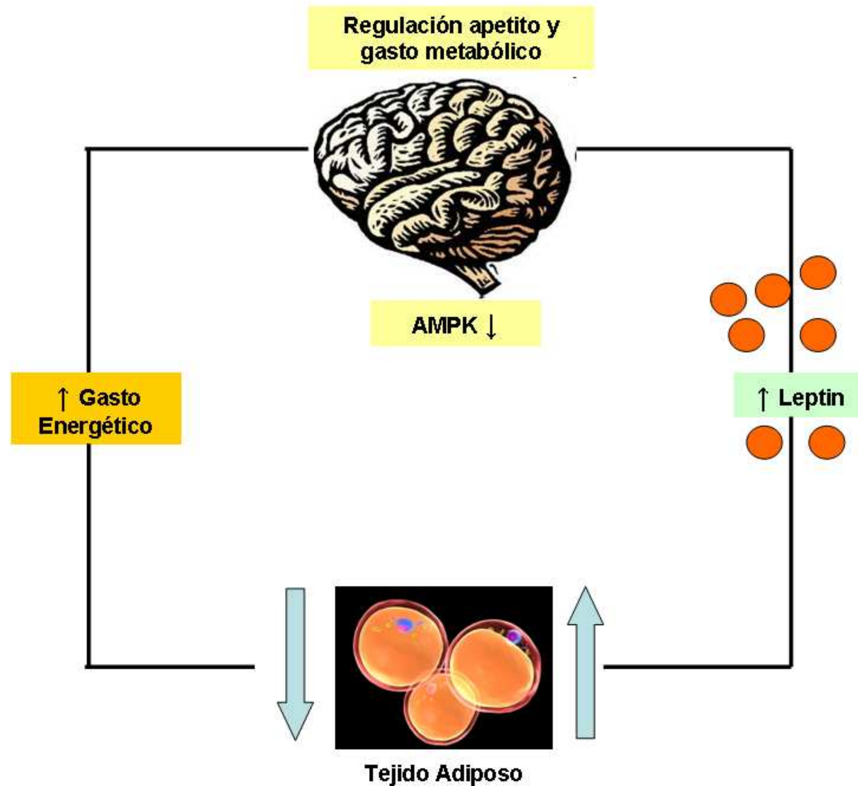

Figura 8.- Funcionamiento y papel de la leptina.

Leptina y regulación del peso corporal

El papel de la leptina en la regulación del peso corporal quedo evidenciado por el hecho de que los ratones *ob/ob* que carecen de leptina presentan obesidad severa (Zhang *et al.* 1994). La leptina producida fundamentalmente por los adipocitos, informa al cerebro sobre el estado de los depósitos grasos del organismo (Campfield *et al.* 1995). Así, en aquellas situaciones de balance energético positivo, se produce un aumento en los niveles de leptina lo que provoca una inhibición de la ingesta y un aumento del gasto energético como mecanismo compensatorio para tratar de contrarrestar esa excesiva ganancia de peso corporal. Por el contrario, en situaciones de balance energético negativo como es el caso del ayuno, se produce una caída de los niveles de leptina que llevaría a un aumento en la ingesta y un descenso del gasto energético como mecanismo compensatorio para tratar de almacenar la energía en el organismo ante una situación de escasez de alimentos (Ahima *et al.* 1996). En este sentido existe controversia en cuanto a la función principal de la leptina. Se ha propuesto que su papel primario no parece que sea

evitar la obesidad, sino prevenir la muerte en caso de falta de alimento. De hecho, en los últimos años se ha planteado si la leptina es realmente una señal de saciedad o más bien una señal de ayuno (Yadav *et al.* 2011; Ahima y Osei, 2004; Hukshorn y Saris, 2004).

Papel de la leptina en la regulación de la ingesta

La administración exógena de leptina a ratones *ob/ob* (carentes de leptina y con obesidad) demostró ser capaz de reducir la hiperfagia que presentan estos animales (Pelleymounter *et al.* 1995). Además, el hecho de que la leptina sea capaz de inhibir la ingesta cuando es administrada centralmente en el SNC a unas concentraciones en las que no presenta efectos al ser administrada periféricamente, demuestra el papel fundamental que desempeña el cerebro en mediar las acciones de la leptina sobre la regulación del balance energético (Havel, 2000). En este sentido, se sabe que la leptina es capaz de inhibir la expresión a nivel hipotalámico de diferentes neuropéptidos orexigénicos como el Neuropéptido Y (NPY) y la AgRP (péptido relacionado con Agouti), potentes estimuladores de la ingesta (Marti *et al.* 1999). También inhibe a la MCH (hormona concentradora de melanina), a orexinas y a la galanina. Por el contrario, la leptina es capaz de estimular la secreción de péptidos anorexigénicos estimulando a las neuronas POMC/CART, que liberan α-MSH (hormona α-estimuladora de melanocitos), la cual actúa a través del receptor de la melanocortina 4 (MC4R) que, a su vez, inhibe la ingesta. También es capaz de estimular la secreción de otros péptidos como la CRH (hormona liberadora de corticotropina), GALP (*galanin-like peptide*) y neurotensina (Sahu, 2004) los cuales tienen funciones como estimulantes del apetito y mediadores de las acciones de la leptina. En los últimos años se ha descubierto que la leptina impide las acciones positivas que la grelina ejerce sobre las neuronas productoras de NPY (Kohno *et al.* 2003).

Se podría concluir que las acciones de la leptina en el hipotálamo son mediadas a través de la regulación de la expresión y/o actividad de una serie de péptidos orexigénicos y anorexigénicos (Solomon *et al.* 2006; Sahu, 2004).

Efectos de la leptina sobre el gasto energético

En ratones *ob/ob* se ha encontrado que la pérdida de peso observada tras la administración de leptina no es únicamente debida a una disminución de la ingesta, puesto que paralelamente se produce también un incremento de la tasa metabólica del animal, acompañada de una elevación en el consumo de oxígeno, la termogénesis y también la actividad locomotora y la lipólisis (Frühbeck *et al.* 1998; Caro *et al.* 1996; Martínez *et al.* 1996; Halaas *et al.* 1995). Además, diversos estudios han demostrado que la leptina es capaz de estimular la expresión de la UCP1 del tejido adiposo pardo, la cual es la principal responsable de la termogénesis facultativa (Tajima *et al.* 2005). A diferencia de lo obtenido en los trabajos con roedores *ob/ob*, los estudios de administración exógena de leptina llevados a cabo con humanos resultan contradictorios, ya que mientras Rosenbaum y colaboradores (2002) observaron un incremento en el gasto energético de pacientes sometidos a una restricción calórica tras la administración de leptina a bajas dosis, otros autores no percibieron alteraciones en el metabolismo y gasto energético (Hukshorn *et al*. 2003; Farooqi *et al.* 2002).

Leptina y Obesidad

El papel de la leptina en la patogénesis de la obesidad parece estar relacionado con los niveles circulantes de la hormona (Yu *et al.* 2011; Marti *et al.* 2005). Al ser la leptina una hormona reguladora del peso corporal, cualquier alteración tanto en su producción como en la sensibilidad conducirían a diferencias en el peso de los individuos.

Las mutaciones en el gen *ob* son poco frecuentes. Montague y colaboradores (1997) identificaron dos primos de familias de alta consanguinidad y origen pakistaní con obesidad de aparición precoz y severa hiperfagia: una niña de 8 años y 86 kg de peso y un niño de 2 años y 29 kg de peso. Resultó que los niños no tenían niveles detectables de leptina sérica al ser homocigotos para la delección o pérdida de un nucleótido G (guanina) en el codon 133, lo que ocasiona un codon de finalización prematuro, y por tanto, una proteína truncada afuncional. Después de este estudio se

encontraron otras dos familias, también de origen Pakistaní, que poseían la misma mutación, y presentaban el mismo fenotipo. Asimismo, Strobel y colaboradores (1998) describieron en una familia turca tres parientes con obesidad extrema, hiperfagia y deficiencia en leptina. Se observó que eran portadores de un polimorfismo en homocigosis por el cual se cambiaba una C (citosina) por una T (timina) en el codon 105, lo cual daba lugar a un cambio de una arginina por triptófano en la secuencia de la leptina. Recientemente en el año 2011, se ha descrito un caso de obesidad monogénica de un niño chino de cuatro años con sólo un metro de estatura y que pesa ya 62 kilos y podría recibir tratamiento con una inyección de leptina.

En general, se ha observado que la administración de leptina en humanos deficientes de leptina induce un descenso en la ingesta y pérdida de peso (Rosebaum *et al.* 2002). Existe un pequeño porcentaje de la población obesa (10%) que presenta niveles plasmáticos normales de leptina e incluso que presenta niveles disminuidos de esta hormona (deficiencia parcial) (Maffei *et al.* 1995), por lo que en estos casos la obesidad podría deberse a una escasa producción de leptina por parte del tejido adiposo, y por tanto podrían beneficiarse de una administración de esta hormona (Meier y Gressner, 2004; Ioffe *et al.* 1998). Sin embargo, y a pesar de lo expuesto anteriormente, muy pocos casos de obesidad en humanos cursa con bajas o inexistentes niveles de leptina, más bien todo lo contrario, aparecen concentraciones muy elevadas de esta hormona proporcionales a la masa grasa, lo que parece sugerir una resistencia a esta hormona, lo que facilitaría el desarrollo de la obesidad tal y como se ha sugerido previamente (Frühbeck *et al.* 2001). Se han descartado por tanto en estos casos las terapias basadas en leptina, debido a que las mutaciones descritas sólo afectan a un número pequeño de personas a nivel mundial.

En resumen, las diferencias en la sensibilidad frente a la leptina y/o en su producción pueden explicar las variaciones en el peso de los individuos. Los niveles circulantes de leptina se encuentran aumentados en sujetos obesos y son proporcionales a la masa grasa corporal, siendo este hecho considerado como una señal de reserva energética del organismo, que se asocia a una situación de resistencia a la misma (Friedman, 2011). Asimismo, se ha observado que los niveles de leptina disminuyen cuando se produce una pérdida de peso (Klempel y Varady

2011) tras la realización de un *bypass* gástrico o por una intervención dietética (de Luis *et al.* 2008; Rosenbaum y Leibel 1999).

Regulación de la leptina

Los niveles de leptina circulantes se encuentran también sujetos a regulación nutricional, aumentando con la ingesta y disminuyendo en periodo de ayuno, sin que se produzcan cambios en la adiposidad (Coleman y Herrmann, 1999).

Muchos estudios, tanto *in vivo* (Cusin *et al.* 1995; Saladin *et al.* 1995 y 1996) como *in vitro* (Pérez-Matute *et al.* 2003; Hardie *et al.* 1996; Leroy *et al.* 1996; Wabitsch *et al.* 1996) han puesto de manifiesto que la insulina incrementa la expresión del gen *ob* y la secreción de leptina. Diferentes estudios han sugerido que el aumento de la producción de leptina estimulada por insulina está mediado por la utilización de glucosa en el tejido adiposo. En este sentido, estudios en cultivos primarios de adipocitos de rata mostraron que la inhibición del transporte de glucosa por la 2-deoxi-D-glucosa o por la floretina, así como la inhibición de la glucólisis por NaF o por el iodoacetato, reducían de manera concentración dependiente la secreción de leptina, incluso en presencia de insulina (Mueller *et al.* 1998). Todos estos datos sugieren que es la utilización de glucosa estimulada por insulina más que la insulina *per se* la que modula la expresión y secreción de la leptina. Además, estudios *in vivo* en humanos parecen confirmar esta hipótesis (Wellhoener *et al.* 2000).

No obstante, el transporte de glucosa al interior del adipocito no parece ser el factor determinante en el aumento de la secreción de leptina mediado por insulina, sino que son los cambios en el metabolismo de la glucosa los que parecen afectar a la secreción de leptina. Así, se ha sugerido que la secreción de leptina es inversamente proporcional a la cantidad de glucosa que es metabolizada anaeróbicamente a lactato (Pérez-Matute *et al.* 2007; Pérez-Matute *et al.* 2005; Moreno-Aliaga *et al.* 2002; Mueller *et al.* 2000). Por el contrario, se ha sugerido que un incremento en la oxidación de glucosa a CO_2 activaría la producción de leptina (Havel *et al.* 1999). Shirai y colaboradores (2004), propone que son las concentraciones de malonil Co-A que se generan en el interior del adipocito tras la captación de glucosa y durante su oxidación las que condicionan la estimulación de la producción de leptina.

El efecto inhibitorio sobre la secreción de leptina mediado tanto por la 2-deoxi-D-glucosa como por el NaF se observó también sobre la expresión del gen *ob* incluso en presencia de altas concentraciones de insulina. Se ha sugerido que esta regulación de la expresión del gen de la leptina tiene lugar a nivel transcripcional. Así, varios experimentos han observado una activación significativa del promotor de la leptina en la región -101 a -83 (región de respuesta a Sp1), en presencia de glucosa e insulina (Moreno-Aliaga *et al.* 2001; Fukuda e Iritani, 1999), activación que se vio inhibida por el tratamiento con 2-deoxi-D-glucosa (Moreno-Aliaga *et al.* 2001). Más recientemente, se ha descrito que Sp1 se encuentra relacionado con la estimulación de la actividad transcripcional del promotor de leptina por el metablismo de la glucosa mediado por insulina. De hecho, un inhibidor específico de dicho factor de transcripción provocó una inhibición en la secreción de leptina inducida por glucosa e insulina (Moreno-Aliaga *et al.* 2007). Otros estudios, relacionan las vías de señalización ERK1/2 y PI3K en la fosforilación de Sp1 (Reisinger *et al.* 2003; Zheng *et al.* 2001). Por otra parte, se ha demostrado que la insulina induce a una sobre-regulación de la leptina en células de cáncer de mama mediada por PI3K y ERK1/2 y que depende de la interacción de Sp1 con regiones específicas del promotor de leptina (Bartella *et al.* 2008).

Algunos estudios han demostrado que la leptina puede ser regulada por los macronutrientes de la dieta. En este sentido, se ha observado que una dieta alta en grasa o alta en fructosa, se ha relacionado con una mayor resistencia a la leptina (Peréz-Matute *et al.* 2007) mediante un aumento de SOCS3 o disminución de la fosforilación de STAT (Scarpace *et al.* 2009; Shapiro *et al.* 2008; Howard *et al.* 2006). Sin embargo, acidos grasos de la familia de los omega-3 como el ácido eicosapentoenoico incrementa los niveles de secreción de leptina, lo cuál podría ser beneficioso en aquellas dietas de adelgazamiento donde bajan los niveles de leptina (Peréz-Matute *et al.* 2007 y 2005).

1.2.1.2.- Chemerina

La chemerina, también conocida como *tarazone-induced gene 2* (TIG2) o *retinoic acid receptor responder* (RARRES2) es una nueva adipoquina relacionada con el metabolismo y la inmunidad (Bozaglou *et al*. 2007; Roh *et al*. 2007). Se han identificado sus propiedades como proteína quimioatrayente que sirve como ligando del receptor CMKLR1 (*Chemokine-like receptor 1*) (ChemR23 o DEZ) lo cual refuerza su potencial papel en la función inmune (Zabel *et al*. 2006; Zabel *et al*. 2005; Wittamer *et al*. 2004). Se ha observado un aumento en la expresión y secreción de chemerina en adipocitos maduros mientras que su expresión en preadipocitos es ínfima, y se ha descrito su papel regulador en la adipogenesis y en el metabolismo del adipocito maduro (Bozaglou *et al*. 2007; Goralski *et al*. 2007). De hecho la ausencia de chemerina modifica la expresión de genes importantes en el metabolismo de la glucosa y los lípidos, incluyendo GLUT4, DGAT2, la leptina y la adiponectina. Varios estudios recientes han hallado una clara asociación entre la obesidad e inflamación y chemerina (Bauer *et al*. 2011; Ernst *et al*. 2010; Parlee *et al*. 2010; Bozaglou *et al*. 2007).

La chemerina se sintetiza como una pre-proproteína con 163 aa, que es secretada como proproteína de 143 aa (18 kDa) tras la proteolisis de un péptido señal (Meder *et al*. 2003; Wittamer *et al*. 2003). Esta proproteína es de baja actividad biológica y requiere de un procesamiento posterior en su extremo C-terminal mediado por plasmina, carboxypeptidasas o serin-proteasas de las cascadas de coagulación, fibrinolisis e inflamación (Zabel *et al*. 2006; Meder *et al*. 2003; Wittamer *et al*. 2003). Así, el procesamiento proteolítico de chemerina es un mecanismo regulador clave que puede determinar las concentraciones sistémicas y locales de chemerina bioactivas. Es interesante, que muchas de las actividades biológicas de la chemerina son en su mayor parte reproducidas por pequeños péptidos que son idénticos o pequeñas variaciones de los aminoácidos 9-15 del extremo C-terminal de la chemerina 20-157 (Cash *et al*. 2010; Shimamura *et al*. 2009; Cash *et al*. 2008; Wittamer *et al*. 2004). Esto puede sugerir que la porción restante de la proteína es prescindible y carente de función, pero se necesitan más experimentos para determinar si la región N-terminal está involucrada en la formación de los complejos de multímeros de chemerina o en la interacción de chemerina con GPR1 y CCRL2.

Los niveles de chemerina activa en plasma y suero son de 3,0 y 4,4 nM respectivamente en los seres humanos y de 0,6 y 0,5 nM en roedores (Zabel *et al*. 2006). De acuerdo con las propiedades proinflamatorias, la chemerina activa es abundante en el líquido ascítico de pacientes con cáncer de ovario (1,8-7 nM) y en el líquido sinovial de pacientes con artritis (22 nM) (Wittamer *et al*. 2003).

Por otra parte, se han descrito que los niveles séricos de chemerina presentan ritmo circadiano en roedores (Parlee *et al*. 2010).

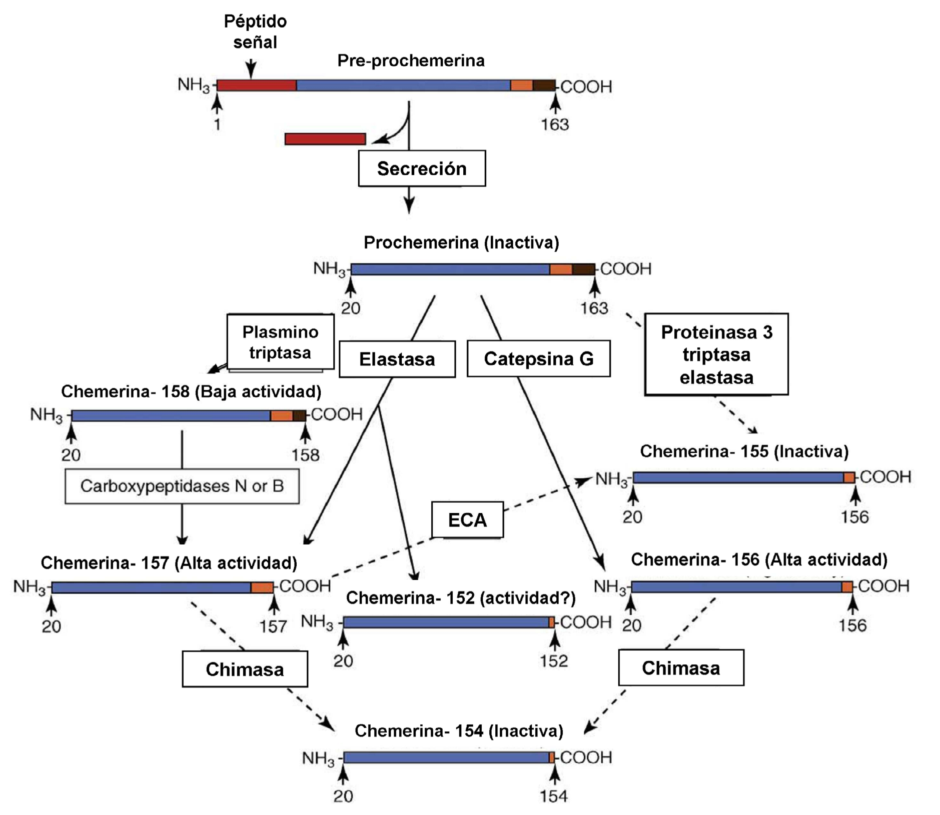

Figura 9.- Proceso proteolítico de la chemerina. Chemerina se produce como una pre-proproteína, pre-prochemerina (1-163), que requiere excisión N-terminal de un péptido señal antes de que se secrete como una proteína precursora inactiva, prochemerina (20-163). Posteriormente existe un procesamiento proteolítico extracelular en el extremo carboxi de prochemerina que expone las zonas bioactivas. Catepsina G rompe siete aminoácidos C-terminal de prochemerina (chemerina-156), elastasa es capaz de romper seis (chemerina-157), ocho (chemerina-155) u once (chemerina-152), la plasmina rompe rompe cinco (chemerina-158) y triptasa cinco (chemerina-158) u ocho (chemerina-155). Varias divisiones pueden ser necesarias para activar chemerina, la plasmino triptasa da chemerina con baja actividad (chemerina-158), y una segunda división por carboxipeptidasas N o B da una chemerina altamente activa (chemerina-157). Chemerina-156 y -157 puedan dar como resultado una chemerina-154 inactiva por la actuación de chimasas. Chemerina-157 puede también verse afectada por ECA (Enzima convertidora de la Angiotensina) para producir chemerina-155 inactiva. El número (por ejemplo, -157) se refiere a la posición terminal de aminoácidos de la proteína. Las flechas continuas representan las vías de activación; flechas discontinuas representan las vías de inactivación. (Modificado de Ernst *et al.* 2010)

Chemerina y Obesidad

La capacidad de promover la quimiotaxis de las celulas dendriticas inmaduras y macrófagos a través de la activación de CMKLR1 fue una de las primeras funciones que se atribuyeron a la chemerina (Wittamer *et al*. 2003). CMKLR1 se expresa en varios tipos de células inmunes conocidas por acumularse en el tejido adiposo de personas con obesidad (Parolini *et al*. 2007; Zabel *et al*. 2006 y 2005). Además, se observó una elevada expresión de CMKLR1 y chemerina en tejido adiposo blanco tanto en muestras de ratones y ratas como de humanos, identificando la chemerina como una nueva adipoquina con funciones paracrinas y autocrinas (Bozaglou *et al.* 2007; Goralski *et al.* 2007).

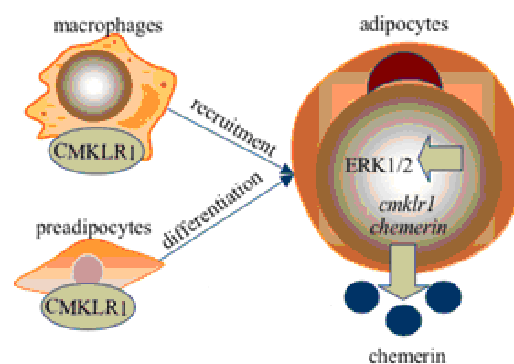

Figura 10.- El papel de Chemerina y CMKLR1 en tejido adiposo (Tomado de Goralski *et al.* 2007).

La expresión y secreción de chemerina aumenta con la adipogenesis, y es inversamente proporcional a la expresión de CMKLR1. Así, a día 9 de diferenciación existen altos niveles de secreción y expresión de chemerina, pero no de CMKLR1 donde su máxima secreción y expresión es en preadipocitos (Bauer *et al*. 2011; Muruganandan *et al*. 2010; Goralski *et al*. 2007). Bauer y colaboradores (2011) observaron en un estudio muy reciente una elevación de los niveles de chemerina en adipocitos hipertrofiados y en un estado de inflamación crónico sugieriendo la implicación de la activación de SREBP2 (*Sterol Regulatory Element-Binding Protein 2*) en la elevación de chemerina (de hecho se ha localizado el elemento de respuesta SREBP2 en el promotor de la chemerina). Además, han demostrado que la chemerina atrae al tejido adiposo varios tipos de células inmunes que contribuyen a la inflamación de este tejido en obesidad. Por otra parte, se ha demostrado que la chemerina es clave en la vía angiogénica e induce a la angiogenesis *in vitro*

(Bozaglou *et al*. 2010; Kaur *et al*. 2010). Por tanto, la elevada expresión y secreción de chemerina durante la adipogénesis, podría influir también en el crecimiento del tejido adiposo mediante la inducción de angiogénesis y el aumento de la vascularización de dicho tejido.

Estudios recientes en modelos animales han demostrado unos niveles circulantes elevados de chemerina en ratones obesos y diabéticos (Ernst *et al*. 2010; Parlee *et al*. 2010). En humanos también se han relacionado los niveles de secreción y expresión de chemerina con el desarrollo de obesidad y síndrome metabólico. Un estudio en la población mexicano-estadounidense muestra elevados niveles de chemerina en suero de pacientes con diabetes tipo 2 en comparación con los controles normoglucémicos, y en sujetos obesos y con sobrepeso en comparación con los normopeso (Bozaglou *et al*. 2009). Los niveles plasmáticos de chemerina se correlacionan positivamente con el índice de masa corporal, así como con niveles basales de glucosa e insulina, triglicéridos y colesterol total, mientras que guardan una correlación negativa con niveles de HDL-colesterol (Bozaglou *et al*. 2009). Otro estudio en humanos de diferentes etnias de Mauritania, ha demostrado que los niveles séricos de chemerina están elevados significativamente en sujetos con sobrepeso y/u obesidad, y que después de ajustar por sexo y edad, se correlacionaron positivamente con la circunferencia de la cintura, el índice cintura/cadera, el HOMA (*Homeostasis Model Assesment*) y los triglicéridos, mientras que guardaban asociación negativa con las HDL-colesterol (Bozaglou *et al*. 2007). También en sujetos caucásicos se encontró que aquellos con síndrome metabólico presentaban unos niveles significativamente superiores de chemerina en suero en comparación con sujetos sanos, en correlación con los niveles de glucosa, triglicéridos, presión arterial sistólica y diastólica (Stejskal *et al*. 2008). Además, la secreción de chemerina en explantes de tejido adiposo reveló una mayor secreción de esta adipoquina en aquellos procedentes de personas obesas frente a las normopeso (Sell *et al*. 2009). Otros estudios también han demostrado niveles más altos de chemerina en pacientes con diabetes mellitus tipo 2 y obesidad, así como una correlación positiva entre niveles séricos de chemerina, resistina, leptina, proteína C reactiva, TNF-α e IL-6 (Weigert *et al*. 2010; Lehrke *et al*. 2009).

Además, se ha descrito una reducción de los niveles séricos de chemerina tras la pérdida de peso en pacientes obesos sometidos a cirugía bariátrica en relación a la disminución del IMC y la masa grasa (Ress *et al*. 2010; Sell *et al*. 2010). También se han observado niveles elevados de secreción y expresión de chemerina en tejido adiposo subcutáneo y omental de mujeres con síndrome de ovario poliquístico (Tan *et al*. 2009).

Aunque está claro que los niveles séricos de chemerina están elevados en la obesidad, los posibles mecanismos de regulación de la expresión de chemerina no están claros y son objeto todavía de estudio. En este contexto, se ha demostrado que la insulina incrementa de forma dosis-dependiente la secreción de chemerina en el tejido adiposo en estudios *in vivo* y en explantes de tejidos (Tan *et al*. 2009). La IL-1β, una citoquina proinflamatoria asociada con resistencia a la insulina, induce la expresión y secreción de chemerina de forma dosis-dependiente en adipocitos 3T3-L1 (Kralisch *et al*. 2009). Se ha sugerido que la mayor expresión de chemerina tras el tratamiento con IL-1β, podría estar modulada por el factor nuclear kB, JNK2, p44/42MAPK, y PI3K (Kralisch *et al*. 2009). El TNF-α, otra citoquina proinflamatoria asociada con resistencia a la insulina, también aumenta los niveles séricos de chemerina *in vivo,* y aumenta la síntesis y secreción de chemerina en adipocitos 3T3-L1 (Parlee *et al*. 2010). Estos hallazgos son compatibles con el hecho de que la hiperinsulinemia y niveles elevados de citoquinas proinflamatorias se asocian con la obesidad. Otros estudios han examinado la relación entre el receptor activador del proliferador de peroxisoma (PPAR)γ y la expresión de chemerina, sugiriendo que la activación del PPARγ se asocia con niveles elevados de expresión de chemerina en la adipogenésis (Muruganandan *et al*. 2010; Vernochet *et al*. 2010; Roh *et al*. 2007). Por el contrario, la activación del PPARγ por tiazolinedionas (TZD) en los adipocitos maduros muestra una reducción en la expresión de chemerina (Vernochet *et al*. 2009).

Chemerina y metabolismo de la glucosa

Los elevados niveles plasmáticos de chemerina en sujetos con obesidad y diabetes tipo 2 han llevado a sugerir que la chemerina pueda estar implicada en la desregulación del metabolismo de la glucosa y la hiperinsulinemia que discurre a menudo asociada a esta patología (Tan *et al*. 2009). En estudios *in vitro* con adipocitos 3T3-L1 se han observado resultados contradictorios de la chemerina sobre la captación de glucosa. Así, un estudio muestra que el tratamiento con chemerina (10 µM, durante 49 horas) causó una disminución en la estimulación de la captación de glucosa mediada por la insulina (Kralisch *et al*. 2009), mientras que otro estudio describe un aumento en la señalización de la insulina y la captación de glucosa mediada por la misma tras la preincubación con chemerina (100 ng/ml, durante 12 horas) (Takashashi *et al*. 2008). Las diferentes concentraciones, duración del tratamiento y las condiciones experimentales (ausencia o presencia de suero en el medio) pueden contribuir a la divergencia de los resultados obtenidos. Otros estudios posteriores apoyan la capacidad de la chemerina para inducir intolerancia a la glucosa e insulino-resistencia. Así, Sell y colaboradores (2009) observaron que el tratamiento de cultivos primarios de células músculo-esqueléticas humanas con chemerina (60 nM, durante 24 h) causó un aumento en la fosforilación de IRS1 en los residuos de serina y una menor estimulación de la captación de glucosa mediada por la insulina. También demostraron una disminución de la fosforilación de Akt, de la glucógeno sintasa quinasa (GSK)3α y la GSK3β así como una activación de ERK1/2, y la subunidad p65 de NFκ-B. Otro estudio demostró que el tratamiento con chemerina aumenta la intolerancia a la glucosa en ratones obesos y diabéticos, pero no en ratones normoglucémicos sugiriendo que la chemerina puede contribuir a agravar los desórdenes del metabolismo glucídico asociados a obesidad y diabetes tipo 2 (Ernst *et al*. 2010).

1.2.1.3.-Adiponectina

La adiponectina es una adipoquina producida mayoritariamente por el tejido adiposo y en menor cantidad por miocitos cardiacos, células musculares y células endoteliales (Havel, 2004). Fue identificada por cuatro grupos de investigadores distintos al mismo tiempo. Inicialmente, se determinó la expresión y secreción de esta adipoquina en adipocitos murinos 3T3-L1 y 3T3-F442A antes y después de la diferenciación y fue denominada Acrp30 (*Adipocyte complement-related proteín of 30kDa*) (Scherer *et al.* 1995). Independientemente, fue clonada y llamada AdipoQ por Hu y colaboradores (1996). El homólogo humano fue aislado por secuenciación aleatoria a gran escala de cDNA de tejido adiposo humano por Maeda y colaboradores (1996) quienes lo denominaron apM1 (*adipose Most abundant gene transcript 1*). Por último, también fue aislada y purificada del plasma humano como GBP28 (*gelatin-binding protein-28*) (Nakano *et al.* 1996).

El gen de la adiponectina está formado por 3 exones y 2 intrones (Figura 11A), y está regulado principalmente por PPARγ, C/EBP y ADD1, los factores de transcripción claves en la adipogenésis (Park *et al.* 2004; Gustafson *et al.* 2003).

La adiponectina es una proteína perteneciente a la familia del complemento 1q, que consta de 244 aa (30 kDa) en humanos y de 247 aa en ratón, organizados en 4 dominios en el caso de los humanos (Tsao *et al.* 2002). El primer dominio es un péptido señal de 18 aa (en ratones es de 17aa) situado en la zona amino-terminal que permite la secreción de la hormona al exterior de los adipocitos; el segundo dominio es una región corta no homóloga de 28 aa que varía entre especies; el tercer dominio es un dominio de colágeno constituido por 22 tripletes Gly-X-Pro o Gly-X-Y; y por último, un dominio globular en la región carboxi-terminal. Comparte una alta homología con el colágeno tipo VIII y X y con el componente del complemento C1q (Figura 11B). Cabe destacar que la secuencia terciaria del dominio globular tiene una alta similitud con el TNF-α, a pesar de no ser parecidas en su estructura primaria (Kadowaki y Yamauchi, 2005). La adiponectina circula en plasma en diferentes isoformas producidas por modificaciones post-transduccionales (hidroxilaciones y glicosilaciones), las cuales se ensamblan formando trímeros, hexámeros y estructuras oligoméricas de mayor orden (Figura 11C) (Chandran *et al.* 2003). Los trímeros,

hexámeros y complejos de alto peso molecular de adiponectina activan diversos mecanismos de señalización por unión a sus receptores específicos, lo que lleva a sugerir que la oligomerización es un factor clave para, al menos, algunas de las acciones biológicas de la adiponectina (Hug *et al.* 2004). Asimismo, el dominio globular de adiponectina, producido por corte proteolítico, se encuentra también en plasma a concentraciones fisiológicas significativas y posee actividad biológica. La adiponectina monomérica no se ha detectado en la circulación sanguínea y su presencia parece confinada al adipocito (Chandran *et al.* 2003).

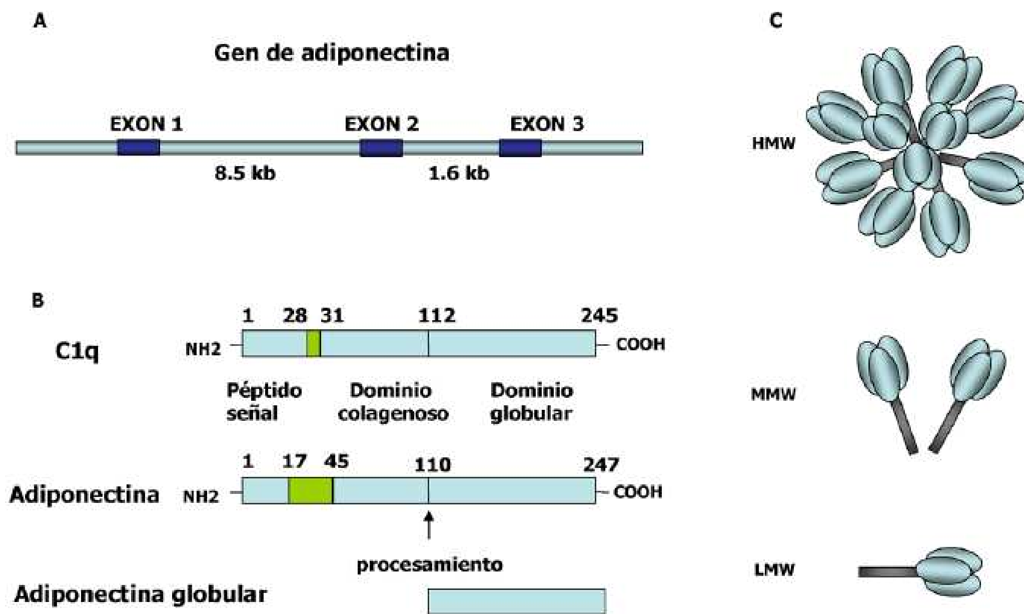

Figura 11.- (A) Esquema del gen de adiponectina de ratón. (B) Estructura y dominios de la Acrp30/Adiponectina. (C) Formación del multímero de adiponectina. LMW: peso molecular bajo, MMW: peso molecular medio, HMW peso molecular alto. (Modificado de Tilg y Moschen, 2006; Blackman, 2006; Chandran *et al.* 2003)

Varios estudios han sugerido que los complejos de adiponectina de elevado peso molecular son la forma activa de la proteína (Pajvani *et al.* 2004) y también se ha observado que mutaciones relacionadas con el desarrollo de diabetes en humanos afectaban a la oligomerización de las moléculas de adiponectina (Waki *et al.* 2003).

Los valores medios de adiponectina circulante en plasma en pacientes obesos son de unos 3,7 µg/ml mientras que en pacientes con normopeso se alcanzaban valores más altos en torno a los 8,9 µg/ml (Arita *et al.* 1999). La adiponectina es, por tanto, muy abundante en plasma humano, representando un 0,01% de las proteínas totales del plasma. Esta concentración es mil veces superior a los niveles plasmáticos de otras hormonas como la leptina o la insulina (Stefan y Stumvoll, 2002). Además, los niveles de esta adipoquina son más elevados en mujeres que en hombres, tal y como ocurre con la leptina (Kajikawa *et al.* 2011; Ayonrinde *et al.* 2011; Hotta *et al.* 2000).

Yamauchi y colaboradores (2003a) clonaron e identificaron los genes que codificaban para los receptores de adiponectina, denominados AdipoR1 y AdipoR2. Estos receptores se localizan en diversos tejidos y se encuentran en el ser humano en los cromosomas 1p36 y 12p13 respectivamente. El receptor AdipoR1 se expresa ubicuamente pero es más abundante en músculo esquelético, mientras que el AdipoR2 se expresa preferentemente en el hígado y en menor cuantía en páncreas y tejido adiposo. Diversos estudios han sugerido que AdipoR1 es el receptor para la adiponectina globular mientras que AdipoR2 es el receptor para la forma larga de adiponectina (Yamauchi *et al.* 2003b). Además, se ha observado una elevada expresión de ARNm de ambos receptores en las células β-pancreáticas tanto en humanos como en roedores, siendo estos niveles de expresión similares a los observados en hígado y mayores de los que se producen en músculo (Iwabu *et al.* 2010; Kharroubi *et al.* 2003). Ambos receptores presentan aproximadamente un 67% de homología entre ellos y parece que poseen afinidad diferente en la unión de la adiponectina (Yamauchi *et al.* 2007; Goldfine y Kahn, 2003).

Adiponectina, obesidad y alteraciones asociadas: resistencia insulínica y enfermedad cardiovascular

Diversos estudios tanto en modelos animales como en humanos han observado que la expresión y secreción de adiponectina se encuentran disminuidas en procesos relacionados con estados de inflamación, incluyendo la obesidad, la resistencia a la insulina, y la diabetes mellitus tipo 2, así como en la enfermedad coronaria (Alokail *et al.* 2011; Brethauer *et al.* 2011; Kishida *et al.* 2011; Kim *et al.* 2007; Adamczack *et al.* 2003; Hotta *et al.* 2000; Arita *et al.* 1999; Hu *et al.* 1996).

Así, estudios en ratones genéticamente obesos (*ob/ob*) demostraron que la expresión de adiponectina en el tejido adiposo blanco de estos animales era inferior a la observada en ratones no obesos (Hu *et al.* 1996). Además, un estudio prospectivo con monos *Rhesus* mostró que la reducción de la concentración plasmática de adiponectina era paralela al desarrollo de resistencia insulínica y diabetes (Hotta *et al.* 2001). Con objeto de profundizar en el estudio del papel fisiológico de la adiponectina, se generó un modelo de ratón *knockout* para adiponectina. Estos ratones, presentaban síndrome metabólico, insulino-resistencia, intolerancia a la glucosa, hipertensión e hiperlipidemia (Ouchi *et al.* 2003; Kubota *et al.* 2002; Maeda *et al.* 2002). Por el contrario, ratones transgénicos con elevados niveles de adiponectina circulante presentan mejoras en la sensibilidad a la insulina (Combs *et al.* 2004). Estas observaciones sugerían que los niveles bajos de adiponectina pueden participar en la patogénesis de la resistencia a la insulina y la diabetes en animales.

En la especie humana también parece existir una clara relación negativa entre los niveles circulantes de adiponectina, el tamaño de los depósitos grasos y el desarrollo de resistencia insulínica. Diversos estudios han confirmado la existencia de una correlación negativa entre los niveles de adiponectina y el IMC, la masa grasa corporal, la grasa intrabdominal y el índice cintura-cadera (Stefan *et al.* 2002). Asimismo, se ha observado que los niveles de adiponectina se incrementan cuando se produce una pérdida de peso tras by-pass gástrico o por intervención dietética (Bruun *et al.* 2003; Faraj *et al.* 2003). Por otra parte, la hipoadiponectinemia se ha correlacionado con la hiperinsulinemia, resistencia a la insulina y la diabetes tipo 2 (Kashyap *et al.* 2010; Matsuzawa, 2010; Weyer *et al.* 2001). Se ha demostrado

también que varios polimorfismos en el gen de adiponectina están asociados con la obesidad y la resistencia a la insulina (Jee *et al.* 2010; Tabatabaei-Malazy *et al.* 2010; Chandran *et al.* 2003; Diez y Iglesias, 2003). Los niveles de adiponectina aumentan cuando la sensibilidad a la insulina mejora, ya sea por una disminución del peso corporal o por tratamientos con drogas sensibilizadoras de la insulina (Esposito *et al.* 2011; Zulian *et al.* 2011; Joya-Galeana *et al.* 2011; Chandran *et al.* 2003; Díez e Iglesias, 2003).

Diferentes estudios han descrito una disminución en los niveles de adiponectina plasmática en pacientes con enfermedad cardiovascular (Al-Daghri *et al.* 2011; Persson *et al.* 2010; Hotta *et al.* 2000; Kumada *et al.* 2003), hipertensión (Celoria *et al.* 2010; Ohashi *et al.* 2006; Shatat *et al.* 2009; Ouchi *et al.* 2003) o síndrome metabólico (Matsuzawa, 2010; Whitehead *et al.* 2006; Trujillo y Scherer, 2005). En este sentido se ha observado que la incidencia de muerte cardiovascular es mayor en pacientes con bajos niveles de adiponectina circulante en comparación con otros que poseen elevados niveles (Zoccali *et al.* 2002). Yamauchi y colaboradores (2003b) observaron cómo la administración del dominio globular de la adiponectina mejoraba la aterosclerosis en dos modelos clásicos de esta patología. Todos estos hechos apoyan la hipótesis de que la adiponectina puede actuar como un factor de protección del sistema cardiovascular (Fortuño *et al.* 2003). Además la adiponectina sérica se relaciona de forma negativa con la concentración de colesterol total, LDL-colesterol, triglicéridos (TG), y ácido úrico y de una manera positiva con las concentraciones de HDL-colesterol (Yamamoto *et al.* 2002). Por último, un estudio con pacientes que siguieron una dieta muy baja en calorías, demostró una correlación negativa entre los niveles de adiponectina y diferentes variables del síndrome metabólico, sugiriendo que la adiponectina tiene un papel protector frente a este síndrome (Garaulet *et al.* 2004).

Por otra parte se ha observado que la expresión de los receptores de adiponectina se encuentra disminuida en el músculo esquelético de pacientes con diabetes mellitus tipo 2 (Debard *et al.* 2004). Además se ha establecido una correlación entre la expresión génica de los receptores de adiponectina y la sensibilidad a la insulina en poblaciones de mejicanos americanos no diabéticos con o sin historia familiar de diabetes tipo 2 (Civitarese *et al.* 2004). Staiger y

colaboradores (2004) encontraron una correlación positiva entre la expresión de adipoR1 en músculo esquelético humano y las concentraciones plasmáticas de insulina, péptido C, triglicéridos y colesterol, mientras que la expresión de adipoR2 sólo se correlacionó con las concentraciones plasmáticas de triglicéridos. En un estudio posterior, se estableció una correlación positiva de la expresión de ambos receptores en el músculo esquelético humano con la composición corporal y la sensibilidad a la insulina (Blüher *et al.* 2006). Existen también varios estudios que asocian ciertos polimorfismos de los receptores con una mayor susceptibilidad de padecer diabetes tipo 2 (Kim *et al.* 2009; Wang *et al.* 2009; López-Bermejo *et al.* 2008; Vaxillaire *et al.* 2006; Damcott *et al.* 2005)

En base a todo lo anterior, diversos autores han hipotetizado que la disminución de los niveles de adiponectina plasmática (hipoadiponectinemia), condicionada por factores genéticos y/o ambientales que favorecen el desarrollo de obesidad como el consumo de una dieta alta en grasas, junto con una disminución en la sensibilidad de sus receptores y en las acciones de la adiponectina, favorecen el desarrollo de la resistencia insulínica, la diabetes tipo 2, el síndrome metabólico y la ateroesclerosis (Kishida *et al.* 2011; Lau y Muniandy, 2011; Polyzos *et al.* 2011; Qiao *et al.* 2011; Ryu *et al.* 2010; Sandhya *et al.* 2010; Kadowaki y Yamauchi, 2005; Yamauchi *et al.* 2003a). Por el contrario, la elevación de los niveles de adiponectina contribuiría a prevenir estas patologías.

Mecanismos de acción de la Adiponectina

Fruebis y colaboradores (2001) describieron en ratones C57BL/6J que la administración de adiponectina globular disminuye los niveles plasmáticos de ácidos grasos libres e incrementa la oxidación de los mismos en el músculo, lo cual explica el mayor gasto energético y la consiguiente pérdida de peso. Estos hallazgos se corroboraron con el estudio de Qi y colaboradores (2004) donde observaron que la administración de adiponectina, vía intracerebrovascular, causaba una pérdida de peso por una estimulación del gasto energético, sin afectar la ingesta de comida. Diversos trabajos han demostrado que en músculo esquelético, la adiponectina incrementa la expresión de moléculas implicadas en el transporte y oxidación de

ácidos grasos (CD36 y acetil CoA carboxilasa) y en la pérdida de energía en forma de calor lo que conduce a una disminución en el contenido de triglicéridos en el músculo (Yamauchi *et al.* 2002). Por otra parte, se ha determinado que la resistencia a la insulina en ratones lipoatróficos podía revertirse por la combinación de dosis fisiológicas de adiponectina y leptina, aunque sólo parcialmente por la administración de adiponectina o leptina solas (Yamauchi *et al.* 2001).

Los efectos insulino-sensibilizadores de la adiponectina se describieron por primera vez por dos grupos independientes en el año 2001 (Berg *et al.* 2001; Yamauchi *et al.* 2001). Así, se observó que la administración exógena de adiponectina mejoró significativamente la insulino-resistencia y la hipertrigliceridemia provocada por la ingesta de una dieta alta en grasa (Yamauchi *et al.* 2001). Esto sugiere que los bajos niveles de adiponectina plasmática observados en obesidad (hipoadiponectinemia) están implicados en el desarrollo de resistencia insuliníca sistémica. Otro trabajo observó en ratones *ob/ob* que la administración intraperitoneal de adiponectina disminuia los niveles de glucosa en sangre y aumentaba significativamente la sensibilidad a la insulina en hepatocitos (Berg *et al.* 2001). Otros trabajos han observado que la administración de adiponectina provocó una disminución de la glucemia basal al inhibir la expresión de enzimas gluconeogénicas y la producción endógena de glucosa a nivel hepático en un modelo de ratón con diabetes tipo 2 (Combs *et al.* 2001).

Por otra parte, se ha sugerido que la adiponectina puede mediar algunos efectos cardioprotectores e insulino-sensibilizadores a través de sus propiedades antiinflamatorias. Se ha demostrado que la adiponectina podría inhibir la señalización de NF-kB (Xu *et al.* 2011; Wang *et al.* 2010; Zoico *et al.* 2009; Ajuwon y Spurlock, 2005; Ouchi *et al.* 2000) y reducir la actividad fagocitaria así como la producción de TNF-α en cultivos de macrófagos (Yokota *et al.* 2000). La adiponectina también induce una mayor producción de citoquinas antiinflamatorias IL-10 y IL-1RA (*IL-1 receptor antagonist*) en leucocitos humanos (Wolf *et al.* 2004). Por otra parte, en ratones deficientes de adiponectina se ha observado un incremento en los niveles de mRNA de TNF-α en el tejido adiposo y mayores niveles circulantes de TNF-α (Maeda *et al.* 2002).

La Figura 12 resume los mecanismos por los que la adiponectina ejerce sus efectos en hígado y músculo (Phillips y Kung, 2010; Chandran *et al.* 2003). En hígado, aumenta la sensibilidad a la insulina, aumenta la oxidación de ácidos grasos y reduce la producción hepática de glucosa (Yamauchi *et al.* 2002). Por otra parte la adiponectina estimula la captación de glucosa y la oxidación de ácidos grasos en músculo (Dyck, 2009; Fruebis *et al.* 2001). Por otro lado, estudios *in vivo* demostraron que el tratamiento con adiponectina incrementó los niveles de expresión génica de PPARα en hígado, lo que sugiere un aumentó en la β-oxidación de ácidos grasos y el gasto energético por activación de dicho factor de transcripción (Yamauchi *et al.* 2001). Además, parece que los efectos de la adiponectina están mediados a través de la activación de AMPK tanto en hígado como en músculo (Yamauchi *et al.* 2002). De hecho, la activación de AMPK por la adiponectina conduce primero a la inhibición por fosforilación de la Acetil CoA carboxilasa (ACC), en segundo lugar a la estimulación de la oxidación de ácidos grasos, y a la captación de glucosa y en tercer lugar a la producción de lactato en miocitos (Tomas *et al.* 2002; Yamauchi *et al.* 2002). En este contexto, también se ha sugerido la implicación de AMPK en la estimulación de la captación de glucosa inducida por la adiponectina en adipocitos primarios de rata (Wu *et al.* 2003). También, en la pared vascular inhibe la adhesión monocitaria mediante la disminución de la expresión de moléculas de adhesión, inhibe también la transformación de macrófagos en células espumosas, y disminuye la proliferación de células del músculo liso (Ouchi *et al.* 2001). Además, la adiponectina aumenta la producción del óxido nítrico en células endoteliales y estimula la angiogénesis (Eren *et al.* 2009; Shibata *et al.* 2004). Todos estos efectos son mediados mayoritariamente a través del aumento de la fosforilación del receptor de la insulina, la activación de AMPK, y la modulación de la vía de del factor nuclear κB (Kadowaki y Yamauchi, 2011; Ramanjaneya *et al.* 2011; Liu *et al.* 2010; Fisslthaler y Fleming, 2009; Ouchi *et al.* 2004; Shibata *et al.* 2004; Tomas *et al.* 2002). Por último, un estudio reciente propone que los efectos de la adiponectina pueden estar mediados por una estimulación de la actividad de la ceramidasa (Holland *et al.* 2011).

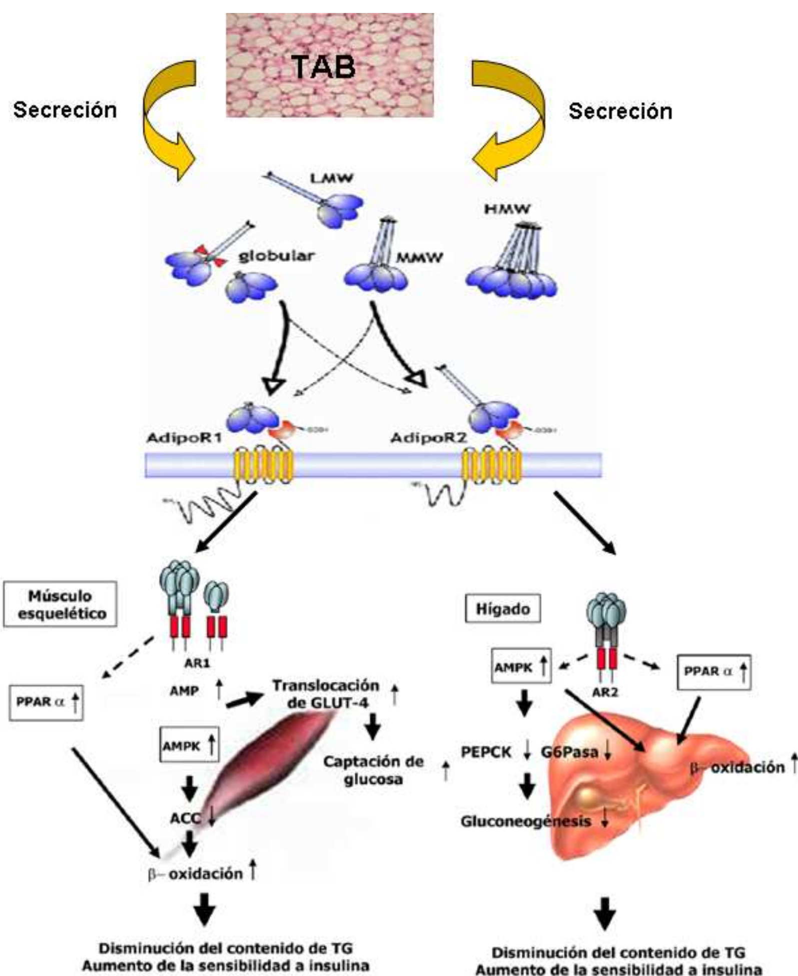

Figura 12.- Mecanismos de acción de la adiponectina (Modificado de Blackman, 2006; Tilg y Moschen, 2006; Chandran *et al.* 2003)

En resumen, debido a las implicaciones de la adiponectina en los mecanismos reguladores del peso corporal, del metabolismo glucídico y lipídico, así como la patología cardiovascular, esta molécula parece tener un papel fundamental en la prevención de la patogénesis del síndrome metabólico, tal y como ha sido sugerido por varios autores (Chiarugi y Fiaschi, 2010; Matsuzawa *et al.* 2010; Ryo *et al.* 2004).

Regulación de la adiponectina

En cuanto a los factores implicados en la regulación de la síntesis de adiponectina, se ha descrito la capacidad de la insulina de modular los niveles de secreción de dicha adipoquina aunque los estudios realizados muestran resultados contradictorios. Algunos estudios han mostrado que la insulina estimulaba tanto la secreción (Scherer *et al.* 1995) como la expresión de adiponectina (Halleux *et al.* 2001) en adipocitos 3T3-L1, mientras que otros han observado una inhibición de los niveles de mRNA de adiponectina tras el tratamiento con insulina en la misma línea celular (Fasshauer *et al.* 2002).

Un estudio reciente demuestra que existe una regulación con los ciclos circadianos de la expresión de adiponectina y de sus receptores (adipoR1 y adipoR2) en tejido adiposo subcutáneo y visceral de pacientes con obesidad morbida (Gómez-Abellán *et al.* 2010).

Por otro lado, el TNF-α, una de las moléculas implicadas en la resistencia a insulina, inhibe tanto la expresión como la secreción de adiponectina en adipocitos (Berg y Scherer, 2005; Fasshauer *et al.* 2002; Kappes y Loffler, 2000). Además, diversos autores han sugerido que los elevados niveles de TNF-α podrían estar vinculados, al menos en parte, a la hipoadiponectinemia asociada a la obesidad (Iglesias *et al.* 2004). Diversos trabajos han observado que el tratamiento con agentes con propiedades insulino-sensibilizadoras como las tiazolindinedionas (TZD), incrementan los niveles plasmáticos de adiponectina (Park *et al.* 2011; Pfützner *et al.* 2008; Iwaki *et al.* 2003; Combs *et al.* 2002; Maeda *et al.* 2001). Además, diversos estudios han observado una correlación directa entre la expresión génica y activación de PPAR-γ con la producción de adiponectina (Oster *et al.* 2010; Banga *et al.* 2009; Takasawa *et al.* 2008; Lihn *et al.* 2005; Moller y Berger, 2003).

1.3.- OBESIDAD, INFLAMACIÓN Y ESTRÉS OXIDATIVO

En los últimos años, varios estudios han sugerido que la obesidad se asocia a un proceso inflamatorio crónico de bajo grado que se caracteriza por una producción elevada de algunas citoquinas, de reactantes de fase aguda y por la activación de la señalización de las rutas de inflamación (Hotamisligil, 2003). Además, este estado inflamatorio asociado con la obesidad ha sido propuesto como el nexo de unión entre la obesidad y varios de los desórdenes que se asocian con la misma como la resistencia a la insulina, la enfermedad cardiovascular y el síndrome metabólico (Ouchi *et al.* 2011; Balistreri *et al.* 2010; Tilg and Moschen, 2006; Dandona *et al.* 2004). Aunque el estado inflamatorio asociado a obesidad se ha considerado una consecuencia de la misma, algunos estudios han sugerido que la obesidad pudiera ser secundaria a este estado proinflamatorio (Moreno-Aliaga *et al.* 2005; Engstrom *et al.* 2003; Das, 2001).

En este sentido, se ha demostrado que la endotoxemia metabólica modifica el tono inflamatorio, lo cual desencadena el incremento del peso corporal y el desarrollo de diabetes. Asimismo, diversos trabajos de investigación han sugerido que niveles elevados de algunas proteínas plasmáticas sensibles a la inflamación están asociadas con la futura ganancia de peso corporal (Engstrom *et al.* 2003) y con el desarrollo de otras patologías asociadas a la obesidad como la hipercolesterolemia (Engstrom *et al.* 2007) y la diabetes (Engstrom *et al.* 2005).

La existencia de un estado inflamatorio en el tejido adiposo y su relación con la obesidad y complicaciones asociadas fue demostrado por primera vez por Hotamisligil y colaboradores (1993). Este estudio demostró la producción del factor de necrosis tumoral (TNF-α) por parte del tejido adiposo. El TNF-α es una citoquina proinflamatoria cuya expresión aumenta en los adipocitos de los animales obesos. En este mismo estudio, se observó que la neutralización de dicho factor mediante la administración de un anticuerpo soluble de la citoquina conduce a una mejora en la sensibilidad a la insulina en estos animales. Estudios posteriores han reforzado la hipótesis de que la obesidad esté asociada a un estado pro-inflamatorio de bajo grado. Se han observado elevados niveles plasmáticos de mediadores inflamatorios producidos en el tejido adiposo, así como en otros tejidos, como son la IL-6, la

proteína C reactiva, MIF (*macrophage inhibitory factor*) y TNF-α entre otros en pacientes obesos (Dandona *et al.* 2004; Kern *et al.* 2001; Pradhan *et al.* 2001). Además, los niveles de IL-6 se han correlacionado positivamente con el desarrollo de insulino-resistencia en estudios *in vitro* e *in vivo* (Bastard *et al*. 2002). De hecho, la IL-6 bloquea la señalización de la insulina en adipocitos y hepatocitos de forma similar al TNF-α (Lagathu *et al*. 2003; Senn *et al*. 2002).

Se ha sugerido que el mecanismo que determina que el tejido adiposo de los sujetos obesos produzca una mayor cantidad de moléculas relacionadas con la inflamación podría ser intrínseco al propio tejido adiposo, siendo la hipoxia uno de los factores desencadenantes (Trayhurn y Wood, 2004), aunque también contribuye una mayor infiltración del tejido por macrófagos (Wellen y Hotamisligil, 2003). En este sentido, diversos trabajos apuntan a que los macrófagos presentes en el tejido adiposo pueden ser una fuente importante de citoquinas inflamatorias y que además, pueden regular la actividad secretora de los adipocitos (Weisberg *et al.* 2003; Xu *et al.* 2003), lo que puede contribuir a agravar el estado pro-inflamatorio y por tanto, la insulinoresistencia (Figura 13).

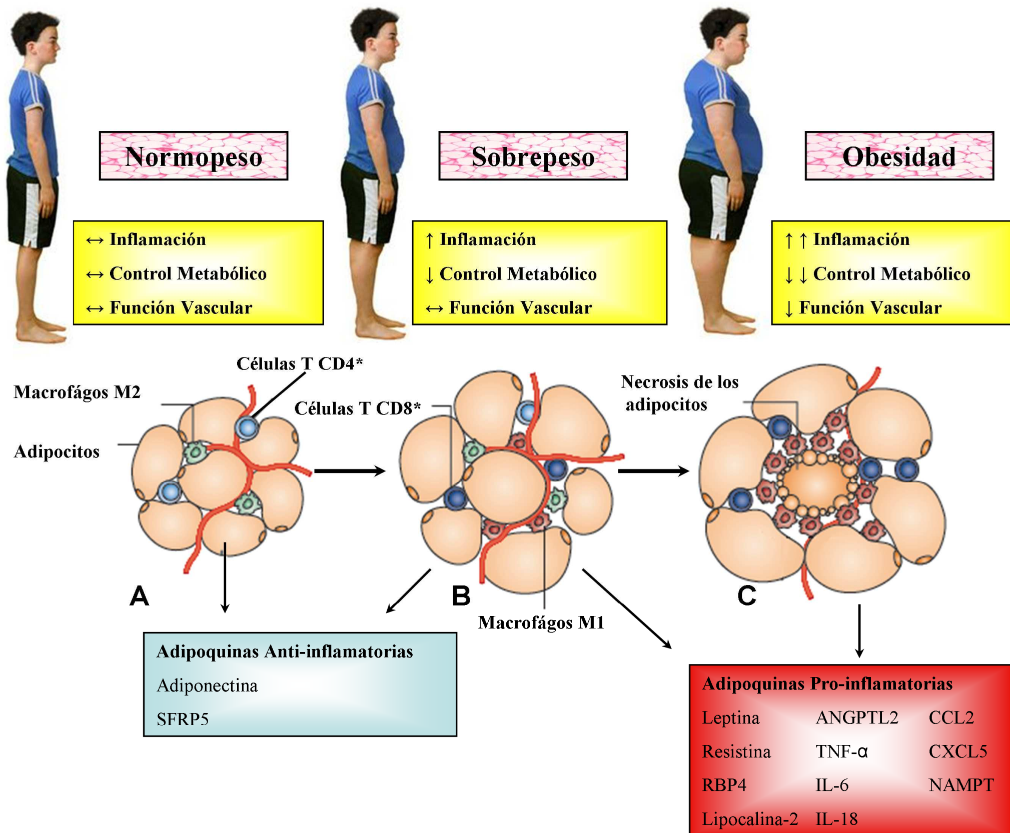

Figura 13.- Esquema del proceso inflamatorio asociado a la obesidad (Modificado de Ouchi *et al*. 2011). El tejido adiposo está compuesto por adipocitos y por la fracción estroma-vascular, en la que se incluyen los macrofágos. Conforme el ser humano va desarrollando obesidad, se produce una hipertrofia de los adipocitos por el aumento en la acumulación de triglicéridos. (A) El tejido adiposo de una persona con normopeso, posee una baja activación de las células inmune y una correcta función vascular. (B) Sin embargo, a medida que los adipocitos se hipertrofian se produce una disfunción secretora del tejido adiposo que facilita la atracción y activación de macrófagos M1, los macrófagos M2 (antiinflamatorios) están en mayor proporción en el tejido adiposo de una persona sana. (C) En estados de obesidad, el tejido adiposo genera gran cantidad de factores pro-inflamatorios, como la leptina, resistina. RBP4, lipocalina-2, ANGPTL2, TNF-α, IL-6, IL-18, CCL-2, CXCL5, NAMPT. Además, la disfunción del tejido adiposo puede estar asociada a mayores niveles de necrosis de adipocitos, colocándose los macrófagos M1 alrededor de las células muertas.

Además de tener en cuenta el papel del adipocito *per se*, es importante considerar su localización. Así, se debe mencionar que la grasa subcutánea no parece tener el mismo impacto que la grasa visceral sobre el proceso inflamatorio asociado a la obesidad, ya que la pérdida de peso y de grasa subcutánea mediante liposucción no siempre se relaciona con una reducción en los marcadores inflamatorios (Klein *et al.* 2004), mientras que la pérdida de peso, de grasa visceral mediante dieta (Nicoletti *et al.* 2003; Heilbronn *et al.* 2001) y/o tras *“bypass”* gástrico (Kopp *et al.* 2003) se asocia a una reducción en los niveles circulantes de IL-6 y proteína C reactiva, lo cual parece sugerir que es la grasa visceral la que juega un papel más importante en la inflamación y por tanto, con las alteraciones asociadas a este estado inflamatorio.

Por otra parte, diversos estudios realizados tanto en modelos animales como en seres humanos han puesto de manifiesto una relación entre el proceso de inflamación y de estrés oxidativo en la obesidad (Garcia-Diaz *et al.* 2007; Vincent y Taylor, 2006).

El estrés oxidativo se define como una alteración del balance entre los mecanismos prooxidantes y antioxidantes del organismo. Este desequilibrio se puede producir, por un incremento en la tasa de producción de radicales libres y especies reactivas de oxígeno (ROS) que conducen a daño celular, tanto a nivel del ADN como de otros componentes celulares como proteínas (Sies *et al.* 2005), aunque también puede ser debido a un descenso de los mecanismos antioxidantes de defensa del organismo (tanto enzimáticos como no enzimáticos) (Molnar *et al.* 2004).

Se ha propuesto que el estrés oxidativo podría ser el nexo de unión entre la obesidad y sus comorbilidades asociadas tales como la resistencia insulínica y la patología cardiovascular (Vincent y Taylor, 2006; Keaney *et al.* 2003) (Figura 14). En este contexto, se ha sugerido que el estrés oxidativo pudiera inducir directamente una respuesta inflamatoria a través de la activación de factores de transcripción tales como NF-κB, por radicales libres de oxígeno (Asehnoune *et al.* 2004), así como estimular la producción de citoquinas pro-inflamatorias como TNF-α (Brown *et al.* 2004).

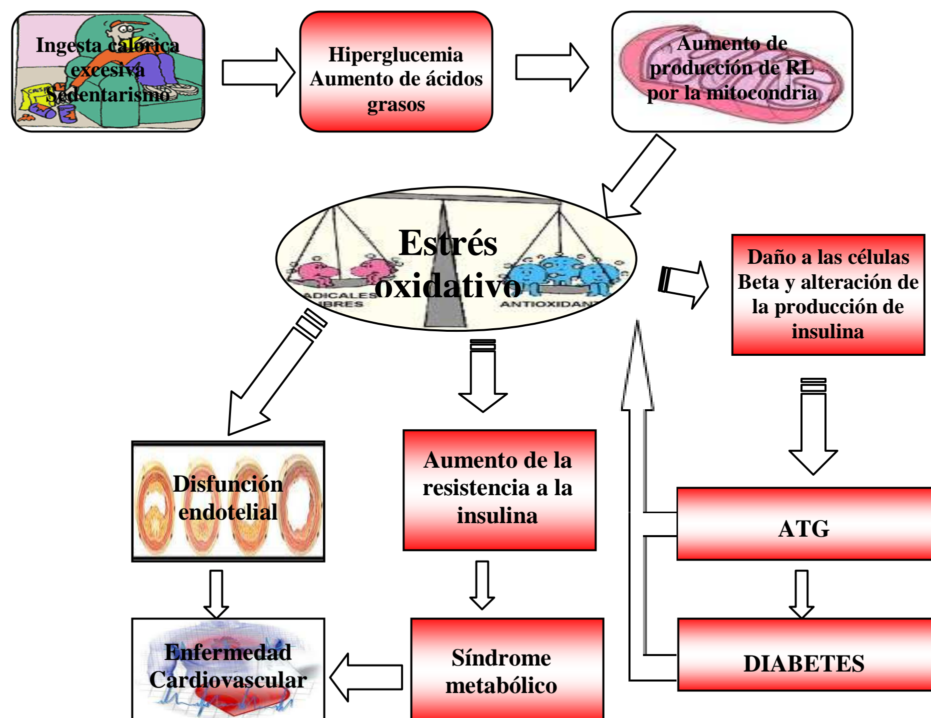

Figura 14.- Causas de estrés oxidativo y enfermedades relacionadas.

RL: Radicales libres; ATG: Alteración de la tolerancia a la glucosa

Park y colaboradores (2007) demostraron que el estrés oxidativo en el tejido adiposo en sujetos obesos se asocia a un aumento de las señales inflamatorias y a secreción anormal de adipoquinas, lo que da lugar a diversas alteraciones metabólicas. También Furukawa y colaboradores (2004) observaron un incremento en el estrés oxidativo del tejido adiposo de roedores obesos y la asociación de este estado oxidativo con el desarrollo de resistencia a la insulina.

Son diversas las causas que pueden dar lugar a un incremento en el estrés oxidativo en el tejido adiposo en obesidad. En este contexto, se ha publicado que la distribución de macronutrientes de la dieta puede afectar al estrés oxidativo y provocar cambios inflamatorios. Así, un modelo de ingesta crónica de una dieta hipercalórica podría inducir estrés oxidativo, conducir a un estado proinflamatorio (Aljada *et al.* 2004; Tailor y Granger, 2003) y por tanto, a obesidad.

Por otra parte, se ha observado que la restricción calórica y proteica reduce la formación de radicales libres y ROS e inhibe la acumulación de biomarcadores oxidativos en modelos animales y, además, tiene propiedades antiinflamatorias (Gredilla y Barja, 2005; Skrha *et al.* 2005; Chung *et al.* 2001; Dandona *et al.* 2001).

En este sentido y teniendo en cuenta que el estrés oxidativo podría funcionar como desencadenante y agravante del estado inflamatorio asociado a la obesidad, resulta lógico sugerir que la suplementación de la dieta con ingredientes con propiedades antioxidantes podrían prevenir y frenar, a diferentes niveles, el desarrollo de la obesidad y sus patologías asociadas (Valdecantos *et al.* 2009; Vincent y Taylor, 2006; Sies *et al.* 2005).

1.4.- ÁCIDO LIPOICO

El ácido lipoico (LA) es un ácido graso de cadena corta (8 átomos de carbono) con dos grupos sulfidrilo. Es una molécula soluble tanto en agua como en lípidos. Existen dos tipos: el denominado R-LA (que es la forma natural) y el S-LA (que es la forma sintética) (Estrada *et al.* 1996).

Al principio fue clasificado como una vitamina pero posteriormente se encontró que era sintetizado por los seres humanos y otros animales (Carreau, 1979). De hecho, el hígado es capaz de producirlo a través de la enzima ácido lipoico sintasa (LASY) (Padmalayam *et al.* 2009; Cakatay, 2006). Además, el LA puede llegar al organismo mediante la alimentación, ya que diversos vegetales y animales contienen bajas cantidades de R-LA en forma de lipolisina (Lodge *et al.* 1997). Las principales fuentes vegetales son espinacas, brócoli y tomates. También está presente en cereales integrales y en la levadura de cerveza. Además, el LA puede encontrarse en el hígado, corazón y riñón de los animales (Moini *et al.* 2002a).

El LA es un antioxidante de bajo peso molecular, absorbido fácilmente a partir de la dieta o de una dosis oral, capaz de cruzar la barrera hematoencefálica, y de ser incorporado a los diferentes tejidos así como de ser excretado (Bustamante *et al.* 1998). En la actualidad se conoce la íntima conexión del LA con el metabolismo

celular y el estado redox (Packer *et al.* 1997). Se trata de un importante cofactor de varias enzimas bioenergéticas mitocondriales (Smith *et al.* 2004). De hecho, el LA es esencial para el metabolismo oxidativo normal y desempeña una función vital como cofactor en las reacciones de la deshidrogenasa mitocondrial (Gilgun-Sherki *et al.* 2002). Además, tiene actividad antioxidante en su forma oxidada y también en su forma reducida como ácido dihidrolipoico (DHLA) (Figura 15). De hecho, el LA es convertido rápidamente a su forma reducida, la cual se regenera a partir del ciclo redox con otros antioxidantes como las vitaminas C y E.

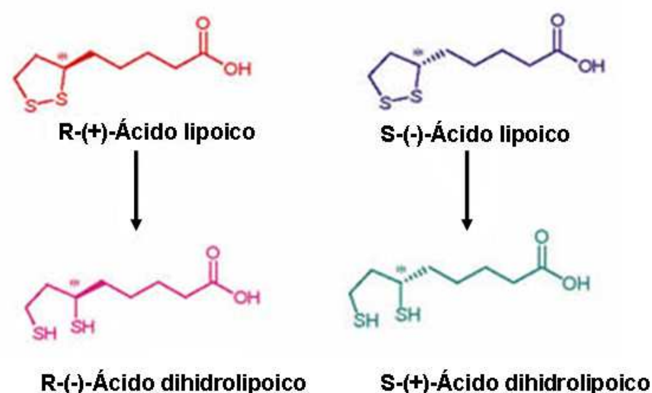

Figura 15.- Estructuras del Ácido Lipoico y Ácido dihidrolipoico (forma reducida).

Tanto el DHLA como el LA pueden quelar metales y formar complejos estables con el cobre, manganeso y zinc (Sigel *et al.* 1978). Además, el DHLA puede regenerar otros antioxidantes. También el LA rescata los radicales hidroxilo o superóxido (Suzuki *et al.* 1991) y los radicales peroxilo (Kagan *et al.* 1992). Además aumenta los niveles del glutatión intracelular, un importante antioxidante (Packer *et al.* 1997; Scholich *et al.* 1989) y los niveles de la coenzima Q10 (Kagan *et al.* 1990).

Debido a su versatilidad, algunos autores han denominado el LA como el *antioxidante universal* (Packer *et al.* 1995). Se han realizado numerosos estudios experimentales y clínicos en los que se ha sugerido que el LA puede ser útil o potencialmente útil como agente terapéutico en diferentes patologías como la diabetes (Cummings *et al.* 2010; Lee y Dugoua *et al.* 2010; Ziegler *et al.* 2004), obesidad (Koh *et al.* 2011; Carbonelli *et al.* 2010), disfunción endotelial (Xiang *et al.* 2011; Heinisch *et al.* 2010), enfermedades neurodegenerativas (Alzheimer,

Parkinson) (Cho *et al.* 2011; Di Stefano *et al.* 2010; Maczurek *et al.* 2008; Bharat *et al.* 2002), envenenamiento por metales pesados o contaminación por radiación (Motawi *et al.* 2010; Ramachandran *et al.* 2010; Suh *et al.* 2005) y la infección por VIH (Jariwalla *et al.* 2008).

El estrés oxidativo se ha relacionado con la disfunción endotelial y se ha demostrado como el LA mejora el estado redox del plasma y la vasodilatación mediada por óxido nítrico (NO) (Sena *et al.* 2007). Diferentes estudios observan que el LA juega un papel importante en la activación de eNOS (Federici *et al.* 2002; Montagnani *et al.* 2002). Un estudio *in vitro* en células humanas endoteliales demostró que el tratamiento con LA aumenta significativamente la síntesis de NO (Visioli *et al.* 2002). Además, un ensayo donde se administró oralmente LA (300 mg/día durante 4 semanas) mostró una mejora en la vasodilatación en pacientes con síndrome metabólico (Sola *et al.* 2005).

La justificación para el uso terapéutico del LA contra la hipertensión deriva de su capacidad para aumentar los niveles de GSH en tejidos y prevenir daños de grupos sulfidrilo mediante la modificación de canales de Ca^{2+}. Estudios en roedores hipertensos observaron una normalización de la presión arterial sistólica y los niveles citosolicos de Ca^{2+} tras una suplementación de la dieta con LA (Vasdev *et al.* 2007; Louhelainem *et al.* 2006; Vasdev *et al.* 2003). Otro estudio en roedores asocia la restauración de la actividad de la glutatión peroxidasa con la normalización en la producción del anión superóxido tras la suplementación con LA (El Midaoui *et al.* 2005). Clínicamente, la administración de LA (en combinación con acetil-L-carnitina) mostró una disminución en la presión sistólica en pacientes hipertensos con síndrome metabólico (McMackin *et al.* 2007).

En este sentido Bilska y colaboradores (2008) observaron en un modelo animal que 35 mg/Kg/día de LA disminuyen los niveles de ROS en todos los tejidos y los niveles de malondialdehido en el corazón, riñón e hígado. Por otra parte se observó que un tratamiento con dosis de 100 mg/Kg/día de LA presenta los mismos efectos que la dosis mas baja.

1.4.1.- Ácido lipoico y resistencia insulinica

Varios estudios tanto en modelos experimentales como clínicos sugieren la mejora en la sensibilidad de insulina después de un tratamiento con LA, tal y como se ha comentado también en las páginas anteriores (Streeper *et al.* 1997; Jacob *et al.* 1996 y 1995).

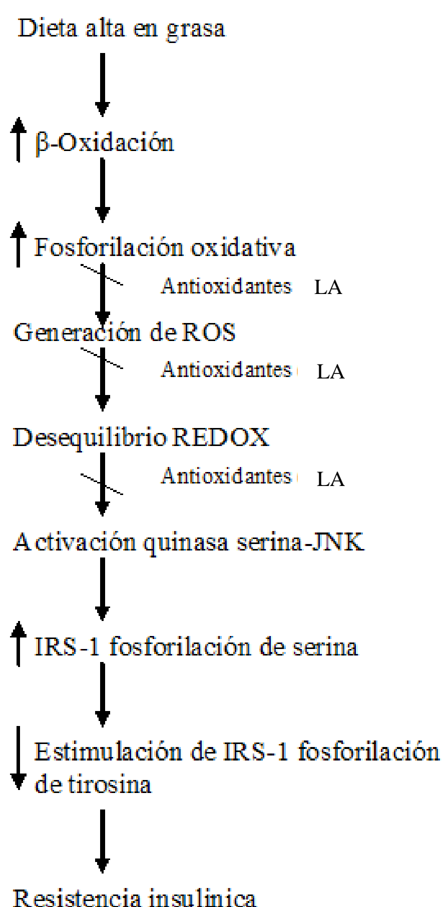

Figura 16.- Mecanismos de actuación del LA sobre la resistencia insulinica. (Modificado de Vinayagamoorthi *et al.* 2008).

En este sentido, Jacob y colaboradores (1995) observaron un aumento significativo en la estimulación de la insulina por mayor disponibilidad de glucosa en sujetos con el tipo 2 diabetes tras una dosis parenteral de 1000 mg/día de LA. Konrad y colaboradores (1999) evaluaron el efecto del LA sobre la sensibilidad a la insulina y el metabolismo de la glucosa en individuos delgados y sujetos obesos con diabetes tipo 2. Así, la administración de una dosis oral de 600 mg/día de LA dos veces al día durante cuatro semanas mejoró el metabolismo de la glucosa en individuos obesos

con un incremento en los niveles séricos de piruvato. Packer y colaboradores (2001) observaron que la administración de un suplemento de LA de 1200 mg/día durante 4 semanas mejoraba la sensibilidad a la insulina en pacientes diabéticos tipo 2.

Además, de estos estudios/ensayos en humanos, cabe destacar que las propiedades insulino-sensibilizadoras del LA se han estudiado también en modelos animales. Estos estudios han puesto de manifiesto la capacidad del LA de regular el metabolismo glucídico y la señalización de insulina en orgános claves como el músculo e hígado, así como la secreción de insulina por el páncreas.

Song y colaboradores (2005) observaron que el tratamiento con LA en ratas OLEFT (0,5% peso/peso) disminuía los niveles de glucosa, reducía el peso corporal, protegía la destrucción de las células β pancreáticas y su hipertrofía, e inducía un descenso de la acumulación de triglicéridos en el músculo esquelético y en los islotes pancreáticos, lo cual sugiere que el tratamiento con LA previene la aparición diabetes mellitus tipo 2 en ratas obesas porque reduce la acumulación de lípidos en diferentes tejidos no adiposos y mejora la sensibilidad a la insulina.

El estudio de Targonsky y colaboradores (2006) en islotes de Langerhans y en la línea celular MIN6 procedente de un insulinoma, corrobora que el LA actúa sobre las células β pancreáticas. De hecho, este trabajo demuestra que el tratamiento crónico con LA activa la AMPK en islotes pancreáticos, y reduce la secreción de insulina. En cuanto a los efectos agudos del tratamiento con dicho ácido, se observó también una inhibición de la secreción de la insulina causada por una disfunción mitocondrial mediada por ROS.

Por otra parte, el estudio de Henriksen y colaboradores (2006) sugirió que la combinación de ejercicio físico con un tratamiento con LA mejora la acción de la insulina sobre el músculo esquelético y el transporte de glucosa, en un modelo animal obeso e insulino resistente. El mecanismo de acción del LA esta mediado por la activación del receptor de la insulina, IRS-1 y fosfatidilinositol 3-quinasa (PI-3K), que causa un aumento de GLUT-4 y un mayor transporte de glucosa en el músculo

Otro de los mecanismos propuestos que podría mediar los efectos beneficiosos del LA en la diabetes tipo 2 es su capacidad para mejorar la captación de glucosa estimulada por la insulina por el adipocito. Yaworsky y colaboradores (2000) observaron en células 3T3-L1 que un tratamiento con LA (2,5 mmol/L) estimula la captación de glucosa llevando a cabo una redistribución de GLUT 1 y GLUT 4 en la membrana plasmática y activando la vía de señalización de la insulina. Estos resultados fueron corroborados por Konrad y colaboradores (2001) quienes sugieren que este efecto del LA es mediado por la estimulación de la p38 MAPK (proteína quinasa activada por mitógenos). Además, Moini y colaboradores (2002b) observaron que el tratamiento con LA de células adipocitarias 3T3-L1 (250 μM) aumenta y modula la captación de glucosa, cambiando el estado redox intracelular y actuando principalmente sobre el receptor de la insulina

En cuanto a los mecanismos por los que el LA mejora la sensibilidad a la insulina, Vinayagamoorthi y colaboradores (2008) observaron que una suplementación de la dieta alta en grasa con antioxidantes incluido el LA (0,5% peso/peso) preserva el sistema de defensa frente a radicales libres, inhibe la activación de la vía JNK (el estrés oxidativo sensible a JNK esta activado en diabéticos) y la fosforilación del residuo serina del sustrato receptor de la insulina 1 (IRS-1) tal y como se observa en la Figura 16.

Por otro lado, varios agonistas de PPARγ, como las tiazolidinedionas, se han utilizado para el tratamiento de la diabetes al mejorar la sensibilidad a la insulina. El tratamiento con estos agonistas de PPARγ lleva, sin embargo, a un aumento en la adiposidad y en la ganancia de peso corporal. Por ello, la capacidad del LA para inhibir la adipogenesis inducido por insulina y el agonista de PPARγ troglitazona, sugiere que la administración de LA junto con los anteriormente citados agonistas de PPARγ podría ser beneficioso en la mejora a la sensibilidad de insulina sin inducir un incremento en el peso corporal (Cho *et al.* 2003). Así mismo, demostraron que un tratamiento con LA estimula la captación de glucosa en células músculares y adiposas mediante la activación de la vía de señalización IR/Akt.

1.4.2.- Ácido lipoico y obesidad

Varios estudios de los últimos años han descrito los potenciales efectos beneficiosos del LA sobre la obesidad y sus complicaciones asociadas como la resistencia a la insulina o las enfermedades cardiovasculares. Así, Kim y colaboradores (2004) observaron que la suplementación de la dieta con este ácido (0,5 % peso/peso) reducía el peso corporal, la grasa visceral así como los niveles plasmáticos de glucosa, insulina, ácidos grasos libres y leptina en ratas genéticamente obesas (OLEFT). Se demostró que estos efectos se debían en parte a un incremento del gasto energético debido a un incremento en la expresión de la UCP1 en el tejido adiposo blanco y marrón, así como a una reducción de la ingesta, debida a la inhibición ejercida por el LA sobre la AMPK hipotalámica. En este mismo trabajo también se demostró que los efectos reductores del peso corporal del ácido lipoico eran reversibles ya que tras la finalización del tratamiento se observó una recuperación del peso en las ratas. Por último, este estudio también demostró que los efectos del LA sobre la ingesta de comida y el metabolismo energético son similares a los de la leptina aunque no dependen de ella.

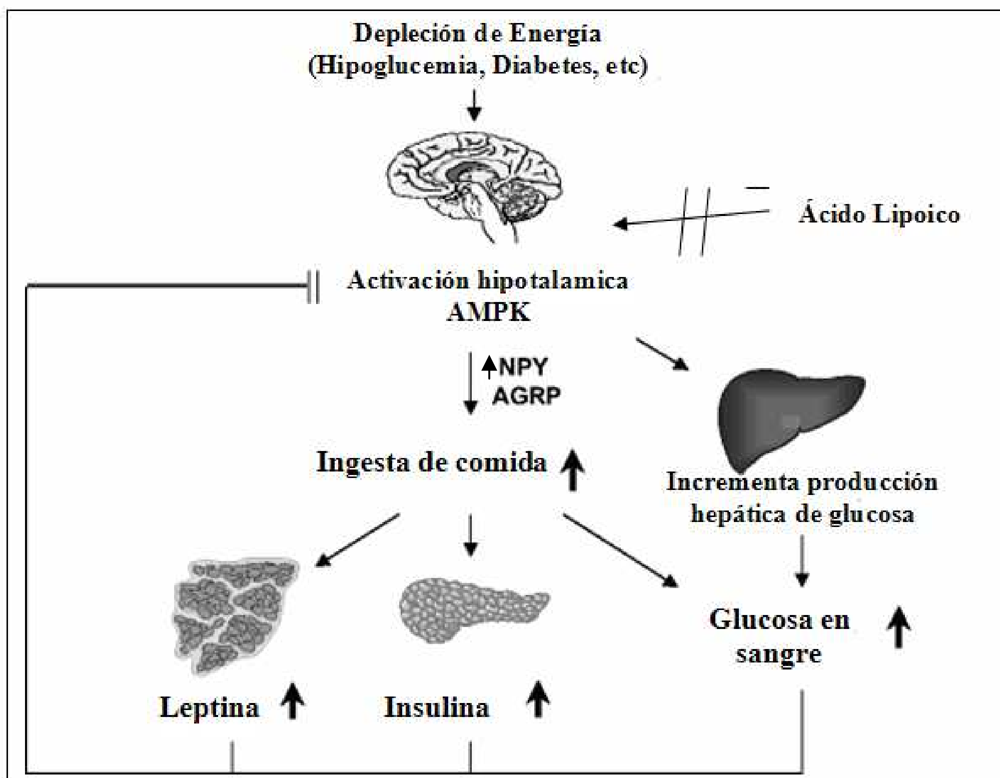

Figura 17.- El papel de la 5´- AMPK (quinasas activadas por AMP) en el hipotálamo (Modificada de Kim y Lee, 2005).

Estos resultados fueron corroborados en un trabajo de Shen y colaboradores (2005), en el cual la suplementación de la dieta durante 3 semanas con LA (0,5 y 1,0 % peso/peso) causó una pérdida de peso y de grasa gonadal, debida en parte a la menor ingesta de comida en ratones macho C57BL/6J. Este estudio sugiere que la suplementación con LA puede inhibir la activación de la AMPK en el músculo *post mortem*.

Un mecanismo que podría estar involucrado en la disminución del tamaño de los depósitos grasos es un aumento de la lipólisis. En este sentido Hamano (2006) demostró que el LA en pollos aumenta tanto la lipólisis como la sensibilidad a la insulina tras la suplementación de la dieta con LA (400 mg/kg) durante 5 semanas, sin embargo, no hay estudios que hayan analizado en profundidad los potenciales efectos lipolíticos del LA sobre los adipocitos.

Wang y colaboradores (2010) demostraron en ratones macho C57BL/6 (24 meses de edad) que una administración de α-LA en el agua de bebida (0,75 %) durante un mes, provoca una menor ingesta de comida, y causa una pérdida de peso acompañada de una menor ganancia de tejido adiposo y de masa magra. Observaron que el grupo tratado con LA tuvo un mayor gasto energético y aumentó la sensibilidad a la insulina. El incremento observado en el gasto energético en el músculo esquelético tras el tratamiento con LA pareció estar mediado, en parte, por el aumento de la biogénesis mitocondrial mediante un aumento de AMPK y PGC-1α.

Otro de los mecanismos que podría estar involucrado en la reducción del tamaño de los depósitos grasos inducido por el LA es una inhibición de la diferenciación adipocitaria, tal y como ha sido descrito por Cho y colaboradores (2003). Este estudio demostró que el LA (250-500 μM) inhibe la adipogénesis y diferenciación de preadipocitos 3T3-L1 paralelamente a una menor expresión de los genes aP2 y LPL, los cuales sólo se expresan en adipocitos plenamente maduros. Estos efectos inhibitorios del LA sobre la diferenciación adipocitaria parecen ser mediados por la reducción de los niveles de PPARγ y C/EBPα, así como por una activación de las MAPK.

Huong e Ide (2008) demostraron que el tratamiento con LA durante 21 días reduce la lipogénesis hepática, así como de los niveles séricos de lípidos. Estos efectos están causados por la inhibición de la actividad de varias enzimas hepáticas implicadas en la síntesis de ácidos grasos y desaturación de los mismos (glucosa 6-fosfato deshidrogenasa, enzima málica, enzima piruvato quinasa, ATP- citrato liasa y acido graso sintetasa). De hecho, esta disminución se observó a partir de una concentración de LA de 1 g/Kg de peso corporal con la excepción de la enzima málica, cuyo descenso empezó a ser significativo a partir de una dosis de 2,5 g/Kg. Esta reducción de la actividad enzimática se acompañó de una disminución en los niveles de RNAm de otras proteínas hepáticas (Acetyl- CoA carboxilasa, spot 14, adiponutrina, Δ^5-desaturasa, Δ^6-desaturasa, stearoyl- CoA desaturasa 1) también relacionadas con la lipogénesis y la desaturación de ácidos grasos. La disminución observada fue nuevamente dosis-dependiente siendo un descenso significativo a partir de un tratamiento con LA de 2,5 g/Kg.

Yang y colaboradores (2008) corroboran los efectos previamente descritos por Huong e Ide, (2008) al demostrar que una suplementación de la dieta alta en grasa con LA (0,1 %) durante 6 semanas en ratones C57BL/6 provoca una disminución en la peroxidación lipídica, disminuyendo los niveles de colesterol total, LDL-colesterol y triglicéridos e incrementando los niveles de HDL-colesterol. En el mismo estudio se realizó un análisis de la expresión génica en el hígado mediante *microarray*, demostrando que la suplementación con LA incrementa la expresión de los genes relacionados con la beta-oxidación de ácidos grasos mientras que disminuye la expresión de los genes que participan en la síntesis de colesterol. Los autores de estos trabajos sugieren que el LA puede prevenir la dislipemia provocada por la dieta alta en grasa, pero este trabajo también sugiere que el LA disminuye el estrés oxidativo mediante la neutralización de ROS al incrementar la expresión de enzimas “scavengers” que atrapan los radicales libres.

Carbonelli y colaboradores (2010) han estudiado por primera vez los efectos del LA sobre la obesidad en humanos. En su trabajo, participaron 1127 personas (445 hombres y 682 mujeres) de entre 18 y 60 años, que fueron divididas en tres grupos en función de su IMC: grupo de individuos con normopeso, individuos con sobrepeso y finalmente individuos con obesidad. Estos pacientes fueron tratados durante 4 meses

con 800 mg/día de LA. Transcurrido el periodo de tratamiento se observó un descenso del peso corporal de entre el 7 % y el 9 %, así como del perímetro de cintura, la presión arterial y de las citoquinas pro-inflamatorias en plasma tanto en pacientes con sobrepeso como en aquellos con obesidad sin distinción de género. Así, los autores concluyen que el LA es un candidato ideal para la terapia de la obesidad y sus comorbilidades asociadas, aunque todavía son necesarios más estudios que demuestren el papel y la eficacia del tratamiento con LA. También el estudio de Koh y colaboradores (2011), en 360 individuos obesos con diabetes mellitus tipo 2, hipertensión e hipercolesterolemia, ha observado que la administración de LA (1200-1800 mg/día) junto a una dieta hipocalórica durante 20 semanas provoca una modesta pérdida de peso solo significativa en el grupo tratado con la dosis más alta de LA.

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HIPÓTESIS Y OBJETIVOS

La obesidad se asocia a un estado inflamatorio crónico de bajo grado que contribuye al desarrollo de las complicaciones metabólicas asociadas a la misma como la insulino-resistencia y la diabetes tipo 2. Teniendo en cuenta que el estrés oxidativo podría funcionar como desencadenante y agravante del estado inflamatorio asociado a la obesidad, se ha sugerido que la suplementación de la dieta con ingredientes con propiedades antioxidantes podrían prevenir el desarrollo de obesidad y sus patologías asociadas (Vincent y Taylor, 2006). En este sentido, estudios previos han demostrado que el ácido lipoico (LA) es un antioxidante con potenciales efectos beneficiosos sobre el metabolismo energético y la obesidad (Koh *et al.* 2011; Carbonelli *et al.* 2010; Yang *et al.* 2008; Shen *et al.* 2005; Song *et al.* 2005; Kim *et al.* 2004). Respecto a los mecanismos implicados en las propiedades antiobesidad del LA, se han demostrado tanto sus efectos anorexigénicos como su capacidad de estimular el gasto energético (Kim *et al.* 2004). Además de sus acciones centrales, el LA también actúa a nivel periférico regulando no solo la producción de insulina a nivel de los islotes pancreáticos, sino también la sensibilidad a la misma, y el metabolismo glucídico y lipídico en órganos metabólicos claves tales como el hígado y el músculo (Henriksen *et al.* 2006; Targonski *et al*. 2006; Song *et al.* 2005).

El tejido adiposo desempeña funciones claves en la obesidad. Así, es evidente la importancia del metabolismo del tejido adiposo blanco y de los factores secretados por el mismo (ácidos grasos y adipoquinas) en el desarrollo de la obesidad y resistencia a la insulina sistémica (Christodoulides *et al.* 2009). El tejido adiposo es un importante órgano endocrino que produce numerosas moléculas llamadas adipoquinas que desempeñan un papel fundamental en la homeostasis energética y en el metabolismo lipídico y glucídico, y cuya producción se encuentra alterada en situaciones como la obesidad. De hecho, se ha demostrado que esta patología se acompaña de cambios en la síntesis y secreción de adipoquinas tales como la leptina, adiponectina, y chemerina, lo que se ha relacionado con el desarrollo de resistencia insulínica sistémica y complicaciones metabólicas asociadas. Algunos estudios han descrito las propiedades antiadipogénicas del LA, lo que podría contribuir a sus acciones reductoras de la adiposidad (Cho *et al.* 2003). Sin embargo, apenas existe información sobre las acciones del LA sobre la secreción de adipoquinas por el tejido adiposo. Tampoco se conocen las acciones periféricas del LA sobre la absorción

intestinal de glucosa, y que podrían ser de gran interés en la prevención de la obesidad y diabetes mellitus tipo 2. El estudio de estas acciones es pertinente ya que cualquier reducción de la absorción de glucosa a nivel intestinal disminuiría los niveles postprandiales de la misma y podría prevenir o retrasar el desarrollo de obesidad, diabetes y complicaciones relacionadas.

La hipótesis del presente trabajo es que el LA pudiera ejercer sus efectos antiobesidad y antidiabetes a través de la regulación de la producción de adipoquinas por el tejido adiposo así como mediante la regulación del transporte intestinal de azúcares.

Por ello, el objetivo general del presente trabajo fue investigar el papel preventivo de la suplementación de la dieta con LA sobre el desarrollo de la obesidad en un modelo animal de obesidad inducido por una dieta alta en grasa, así como los potenciales mecanismos implicados fundamentalmente a nivel de tejido adiposo y del intestino utilizando también para ello diversos modelos experimentales *ex vivo* e *in vitro*.

Por todo ello, los **objetivos específicos** del presente trabajo fueron:

1.- Determinar los efectos del tratamiento con LA sobre la ganancia de peso, la composición corporal y diferentes parámetros del metabolismo glucídico, tanto en ratas alimentadas con una dieta estándar como en ratas a las que se induce obesidad por una dieta alta en grasa.

2.- Investigar el efecto del LA sobre la absorción intestinal de azúcares.

3.- Analizar si los efectos del LA *in vivo* están mediados por cambios en la expresión y secreción de tres adipoquinas relacionadas con el control del peso corporal, la sensibilidad a la insulina y la inflamación: leptina, adiponectina, y chemerina.

4.- Identificar los posibles mecanismos moleculares que subyacen a las acciones del LA sobre la secreción de dichas adipoquinas en cultivos de adipocitos.

MATERIAL Y MÉTODOS

3.1.- ESTUDIOS *"IN VIVO"*

3.1.1.- Animales de experimentación

Los animales empleados para este estudio fueron ratas (*Rattus Norvergicus*) Wistar macho con un peso medio inicial de 211,76 gramos (6 semanas de edad). Estos animales se obtuvieron del animalario del Centro de Investigación en Farmacobiológica Aplicada (CIFA) de la Universidad de Navarra. Los animales se mantuvieron en jaulas de propileno de 19 x 43 x 26 cm provistas de una rejilla de acero inoxidable (tres-cuatro animales por jaula) bajo condiciones controladas de luz con ciclos de 12 h de luz/oscuridad, una temperatura de 22 ± 2ºC y humedad relativa de 55 ± 10%. Todos los procedimientos experimentales fueron realizados de acuerdo con las Guías Nacionales e Institucionales de Cuidado y Uso de Animales y con la aprobación del Comité de Ética para la Experimentación Animal (CEEA) de la Universidad de Navarra. Antes de comenzar el estudio los animales fueron sometidos a un período de adaptación a estas condiciones de cinco días.

3.1.2. Dietas

En este estudio se utilizaron dos dietas diferentes tanto en su composición como en la cantidad de energía aportada por cada una de ellas. Así, se empleó una dieta alta en grasa para inducir sobrepeso y obesidad en los animales, en comparación con la dieta control o dieta estándar de laboratorio. En la mayor parte de los grupos la ingesta de ambas dietas fue *ad libitum* y los animales tuvieron libre acceso al agua.

La dieta de referencia (Harlam Teklad Global Diets) denominada "dieta control" aportaba una cantidad de energía metabolizable de 310 Kcal/100 g de peso seco, contribuyendo las proteínas en un 16,7 %, los hidratos de carbono en un 78,6 % y en un 4,6 % los lípidos.

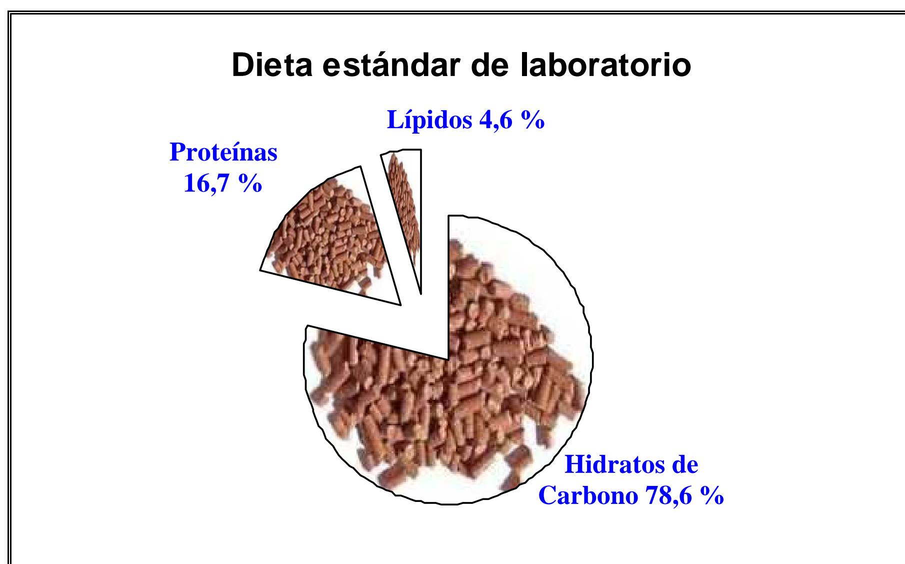


Figura 18.- Distribución de macronutrientes de la dieta de referencia.

La dieta alta en grasa (OpenSource diets Research Diets Inc) es una dieta hipercalórica cuyo contenido energético es de 524 Kcal/100 g, siendo el 20 % aportado por proteínas, el 20 % por los carbohidratos y el 60 % por los lípidos.

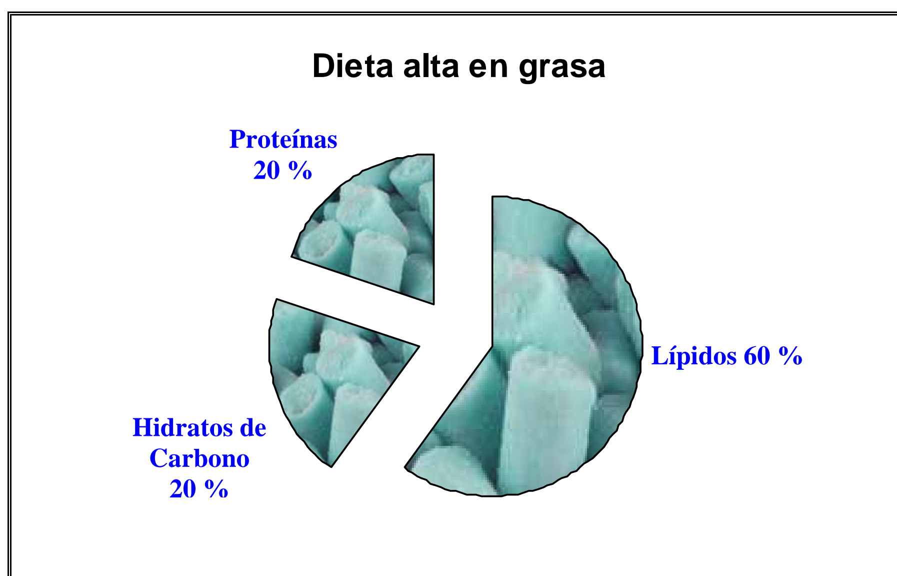


Figura 19.- Distribución de macronutrientes de la dieta alta en grasa.

3.1.3. Diseño experimental *in vivo*

El presente estudio fue diseñado para evaluar los posibles efectos preventivos de la administración de LA sobre la obesidad y la resistencia insulínica en animales sometidos a una dieta estándar y en animales en los que se indujo obesidad con la dieta alta en grasa previamente mencionada.

El LA (sintético; ≥ 99% pureza; Sigma Aldrich, Alemania) se administró mezclado con las diferentes dietas en una proporción de 0,25 g/100 g de comida (Kim *et al.* 2004). El tratamiento duró 8 semanas.

Los 54 animales del estudio se distribuyeron en seis grupos experimentales de acuerdo a la tabla 3.

Tabla 3.- Distribución de los grupos experimentales

Grupo	Dieta	Número de animales
Control	Referencia *ad libitum*	10
CLIP	Referencia *ad libitum* + LA (0,25 g / 100 g)	10
PF - CLIP	Referencia restringida	6
Obeso	Alta en grasa *ad libitum*	10
OLIP	Alta en grasa *ad libitum* + LA (0,25 g / 100 g)	12
PF - OLIP	Alta en grasa restringida	6

Como puede observarse, además de los 4 grupos alimentados *ad libitum*, se incluyeron también dos grupos *Pair-Fed*. A estos dos grupos (PF-CLIP y PF-OLIP) se les administró únicamente la cantidad de alimento ingerida por el grupo CLIP y OLIP respectivamente, pero sin suplementar con LA. Suponen, por tanto, un control para poder identificar si las diferencias que se observan entre los grupos de animales son debidas únicamente a cambios en la ingesta o por el contrario, a efectos directos del LA.

El peso corporal y la ingesta fueron evaluados cada dos o tres días durante los 56 días que duró el tratamiento. Los animales se mantuvieron en ayunas las 12 horas previas al sacrificio y posteriormente se sacrificaron por decapitación. De todos ellos se extrajeron los diferentes depósitos de tejido adiposo blanco (epididimal, subcutáneo, mesentérico y retroperitoneal), el tejido adiposo pardo, y el hígado, riñón, bazo, corazón, hipotálamo, músculo gastrocnemio y músculo tibialis. Estos órganos se pesaron y se congelaron rápidamente en N_2 líquido. Después se conservaron a -80 ºC hasta su posterior utilización. Además, se recogió la sangre de cada animal para la obtención del suero y/o plasma sanguíneo, así como los eritrocitos necesarios para las determinaciones bioquímicas y hormonales posteriores. Por último, se recogieron muestras para análisis histológicos de los diferentes órganos.

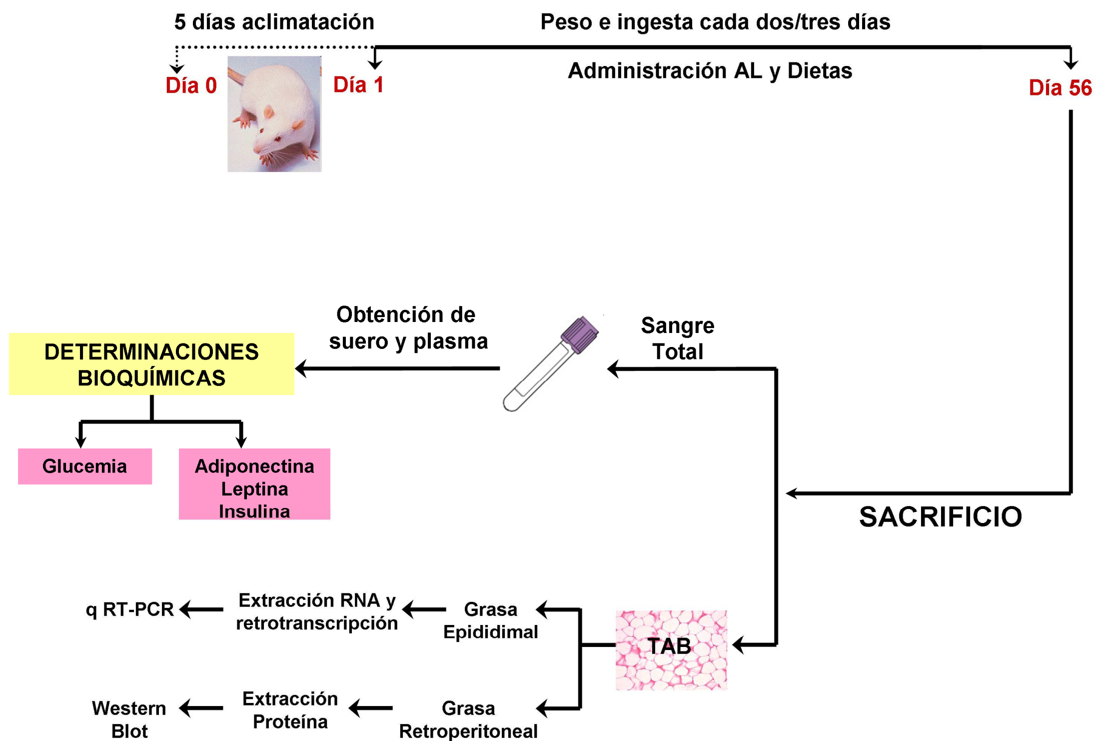


Figura 20.- Diseño del estudio

3.1.4.- Determinaciones bioquímicas en sangre

Se determinaron los niveles de glucosa, presentes en el suero y/o plasma de los animales del estudio mediante métodos enzimáticos utilizando el autoanalizador Cobas - Mira (Roche Diagnostic, Basel, Suiza), según protocolos establecidos en el Departamento de Ciencias de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra. También se determinaron los niveles de insulina, leptina y adiponectina mediante la técnica ELISA.

3.1.4.1.- Obtención de las muestras sanguíneas

La extracción de las muestras sanguíneas se realizó tras doce horas de ayuno y se recogió en Tubos Vacuntainer (Becton Dickinson, Gran Bretaña).

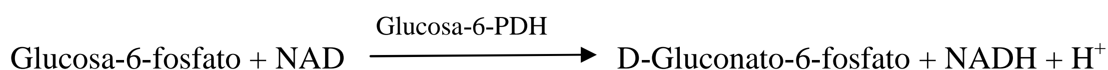
La sangre obtenida fue centrifugada a 3500 rpm (2200 *g*) durante 15 minutos a 4 ºC para separar el suero sanguíneo.

3.1.4.2.- Pruebas mediante autoanalizador

La glucemia fue analizada mediante métodos enzimáticos utilizando el autoanalizador Cobas - Mira (Roche Diagnostic, Basel, Suiza), según protocolos establecidos en el Departamento de Ciencias de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra tal y como se ha comentado anteriormente.

3.1.4.2.1.- Glucosa

La determinación de glucosa en suero se realizó mediante el método de colorimétria enzimática (Glucose HK CP, ABX Pentra) basado en la siguiente reacción catalizada por la glucosa peroxidada:



3.1.4.3.- Determinaciones hormonales mediante ELISA

Los niveles circulantes de insulina, leptina y adiponectina se analizaron mediante ELISA (Lequin, 2005; VanWeemen y Schuurs, 1971). Los kits empleados para ello fueron:

- Leptina: Rat Leptin ELISA kit (Millipore y Linco Research Inc, Estados Unidos).
- Insulina: Rat Insulin ELISA kit (Linco Research Inc., Estados Unidos).
- Adiponectina: Rat Adiponectin ELISA kit (Linco Research Inc. Estados Unidos).

3.1.4.3.1.- Fundamento

El fundamento teórico del ELISA es muy similar en todos los kits utilizados. La molécula a medir presente en el suero de rata se une a un anticuerpo policlonal primario que está pegado a la base del pocillo de la placa que se utiliza para el análisis, quedando de esta manera inmovilizado en el pocillo de la placa (primera reacción). Después de lavar, se añade a los pocillos un anticuerpo secundario biotinilado y se procede a la incubación, uniéndose el anticuerpo secundario al complejo ya formado en la primera reacción (segunda reacción). Después de volver a lavar, se añade un conjugado formado por la peroxidasa de rábano (HRP) y la estreptavidina (tercera reacción) y se realiza una nueva incubación. La HRP conjugada con estreptavidina reconoce y se une al anticuerpo secundario biotinilado. Después de lavar, se añade a los pocillos el sustrato colorimétrico de la enzima y se procede a una nueva incubación durante la cual se genera una reacción colorimétrica que se detiene añadiendo una solución de parada. La intensidad del color se mide a la longitud de onda especificada por el fabricante en cada kit.

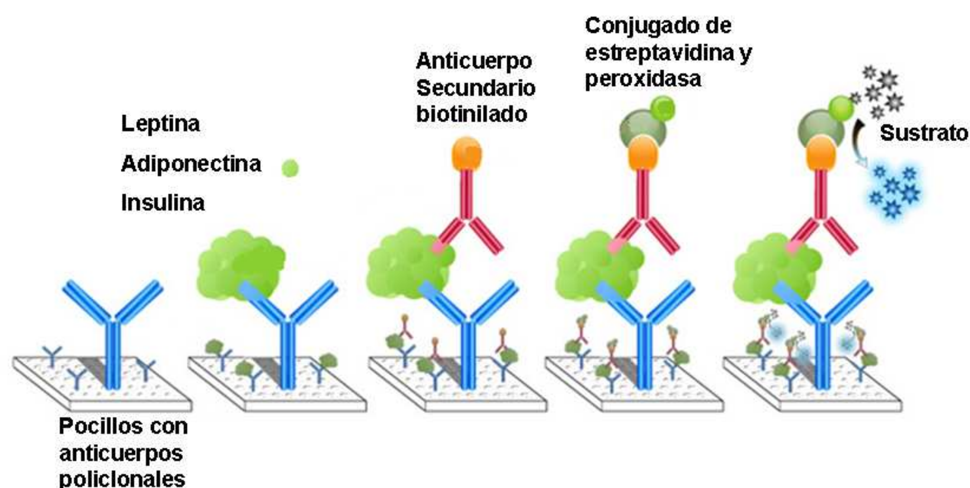


Figura 21.- Esquema de la determinación de hormonas mediante ELISA

3.1.4.3.2.- Cálculo de los resultados

Para calcular la concentración de las diferentes hormonas presentes en las muestras, se realizó una curva de calibrado representando los valores de absorbancia obtenidos a partir de soluciones estándar de concentraciones conocidas. Para normalizar los datos, se restó a cada muestra el valor de la absorbancia del blanco. La concentración de cada muestra se calculó por interpolación en la curva obtenida al representar las absorbancias frente a las concentraciones conocidas de las muestras estándar. Una vez obtenidos los valores de concentración, se multiplican por el factor de dilución correspondiente en caso necesario.

3.1.4.4.- HOMA-IR

El índice de resistencia a la insulina *homeostasis model assesment* (HOMA-IR), permite realizar estimaciones de resistencia insuliníca y función de las células beta mediante las concentraciones de la glucosa y la insulina plasmáticas en ayunas. El índice se calculó según la fórmula propuesta por Matthews y colaboradores (1985):

Índice HOMA-IR = [Glucosa (mmol) x Insulina (µUI/ml)/22,5]

3.1.5.- Determinación de la fosforilación de AMPK mediante western blot

El análisis de los niveles de AMPK total y fosforilada (Thr172) se llevó a cabo mediante la técnica Western Blot. La técnica utiliza un gel de electroforesis para separar proteínas nativas o desnaturalizadas en función de su estructura tridimensional o de la longitud del polipéptido. Las proteínas son transferidas a una membrana, habitualmente de nitrocelulosa o polivinilidenofluoruro (PVDF), donde son detectadas mediante anticuerpos específicos contra la proteína diana.

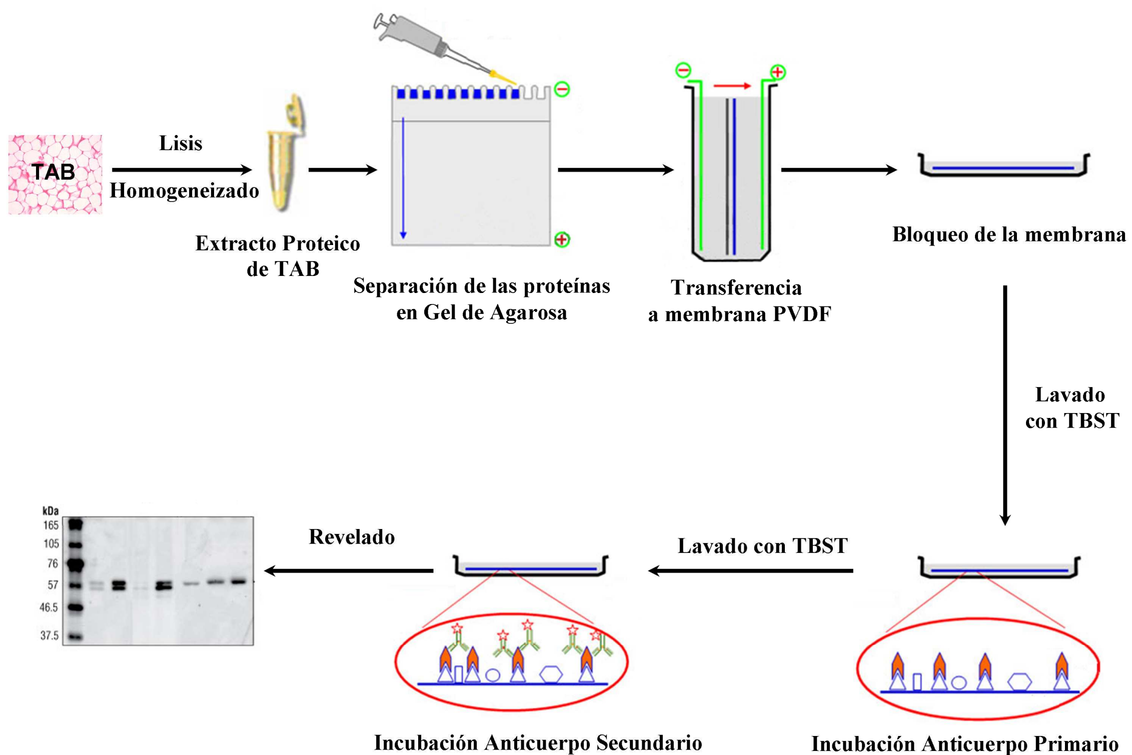


Figura 22.- Esquema general de la técnica de Western-Blot

3.1.5.1.- Preparación de las muestras

Para la obtención de los extractos proteicos del tejido adiposo retroperitoneal tras los 56 días de tratamiento, se partió de 0,3 gramos de tejido retroperitoneal a los que se adicionaron 250 μL del buffer de lisis que contenía: Tris HCl (pH 7,5) 50 mM; NaCl 150 mM, EDTA 5 mM; NaF 2 mM; Octilglicina 6 mM; Desoxicolato de Sodio 0,25%; Ortovanadato de sodio 2 mM, Cóctel inhibidor de proteasas 1 (Sigma-

Aldrich) 1%; Tritón x100 1% y Agua de biología molecular o autoclavada. Posteriormente se procedió a la sonicación del tejido por ultrasonidos (intensidad 2; 20 ciclos) hasta lograr un homogeneizado completo. Todo el proceso se hace con la muestra en frío para evitar que las altas temperaturas puedan dañar las proteínas. Tras la sonicación de la muestra con el buffer de tejido, se procedió a centrifugar 10 minutos a 13.400 x *g* a 4 ºC, y los sobrenadantes obtenidos (extractos proteicos) fueron congelados a -80 ºC.

3.1.5.2.- Electroforesis y electrotransferencia

Las proteínas se separaron mediante SDS-PAGE, en un gel desnaturalizante de acrilamida al 12%, a una intensidad de 120 voltios durante una hora (Laemmli, 1970). Se utilizó como marcador de peso molecular el Prestained SDS-PAGE Standards, Low Range (Bio Rad Laboratories).

Las proteínas fueron transferidas desde el gel a una membrana de PVDF en un tampón que contenía: 25 mM de Tris base, 192 mM de glicina y 20% de metanol y aplicando una corriente de 300 mA durante un mínimo de 45 min. El sistema fue montado tal y como se detalla en la Figura 23.

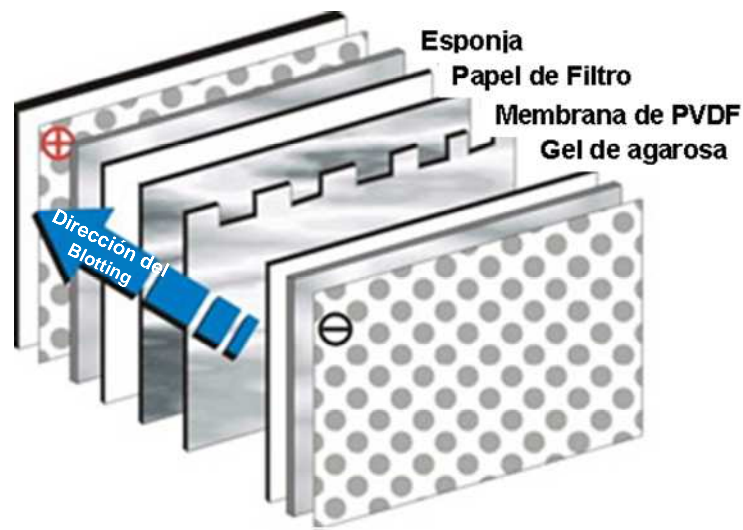


Figura 23.- Sistema de electrotransferencia de proteínas.

3.1.5.3 Inmunoblot

Una vez transferidas las proteínas a las membranas de PVDF, éstas se bloquearon durante 2 horas a temperatura ambiente en buffer TBS-T (200 mM Tris base; 1,5 M NaCl; 0,15% Tween 20) con 1% de BSA, con el fin de evitar uniones inespecíficas.

Tras el bloqueo, las membranas se lavaron con TBS-T tres veces y se incubaron con los anticuerpos primarios específicos diluidos en buffer TBS-T con 1% de BSA durante toda la noche a 4 ºC y en agitación.

Tabla 4. Listado de anticuerpos utilizados.

	Fabricante	**Ref.**	**Especificidad**
Anti-AMPK	Cell signaling technology	2532	*Rabbit anti mouse*
Anti-AMPK fosfo Thr172	Cell signaling technology	2535	*Rabbit anti mouse*
Anticuerpo Secundario	Bio Rad	170/6515	*Goat anti rabbit*

Posteriormente, y tras realizar tres lavados con TBS-T, las membranas se hibridaron con un anticuerpo secundario adecuado. Después de un nuevo ciclo de lavados, se reveló la membrana con el kit *Super signal revelation solution* (Pierce*, Rock- ford, Ill., USA*) según el protocolo del fabricante. Tras 10 minutos de exposición a los reactivos, se procedió al revelado de las películas radiográficas *Hyperfilm* (Amersham), exponiendo a distintos tiempos según la intensidad de bandas obtenidas. Como control de carga se utilizó el anticuerpo β-actina.

Los resultados se analizaron por densitometría mediante el GS-800 calibrated densitometer (Bio Rad Laboratories).

Para volver a hibridar los Western blots, los anticuerpos se eliminaron de las membranas utilizando un buffer específico [62,5 mM Tris-HCl (pH=6.8), 2% SDS y 100 mM 2-mercaptoetanol] e incubando durante 30 min a 50 ºC. Posteriormente, se lavaron las membranas y tras asegurarse de que la señal original se había eliminado, se volvieron a hibridar con el anticuerpo frente a la proteína sin fosforilar (AMPKα, Sp1 y Akt) incubando durante toda la noche. Posteriormente, las bandas se volvieron a visualizar como se ha comentado anteriormente.

3.1.6.- Determinación de la expresión de genes

El análisis de la expresión génica de las diferentes adipoquinas se realizó mediante PCR a tiempo real, tras la extracción del RNA total y su posterior retrotranscripción.

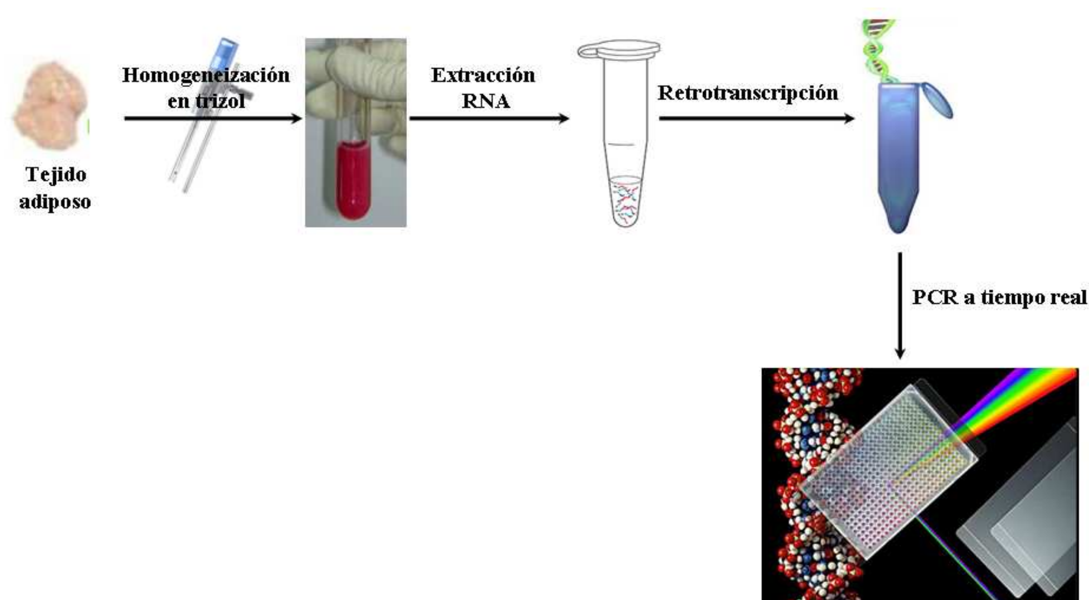


Figura 24.- Análisis de la expresión génica.

3.1.6.1 Extracción y cuantificación de RNA

La extracción del RNA total se llevó a cabo siguiendo el método del TRIZOL® (Invitrogen), que consiste en una solución monofásica de fenol e isotiocianato de guanidina. Se trata de una modificación del procedimiento de Chomczynski y Sacchi (1987). Durante esta homogenización, el Trizol mantiene la integridad del RNA, mientras que rompe las células y disuelve los componentes celulares. Con este método, se puede partir de pequeñas cantidades de tejidos, obteniéndose un RNA total libre de contaminación por proteínas y DNA

3.1.6.1.1.- Procedimiento

Para la realización de la extracción del RNA total se adicionaron a 0,4 g de grasa epididimal 4 ml de Trizol® en tubos de polipropileno previamente autoclavados, y con ayuda de un ultraturrax se homogeneizó el tejido. Posteriormente se agitaron vigorosamente durante 1 min, y tras un periodo de incubación de 5 min a temperatura ambiente se centrifugaron a 12.000 x *g* durante 10 min a 4 ºC, para eliminar los principales restos celulares.

A continuación se añadieron 800 µl (200 µl por ml de Trizol añadido) de cloroformo y se agitaron las muestras hasta conseguir la completa distribución del cloroformo (Sigma-Aldrich) sobre la fase del Trizol. Tras esperar 2-3 min, se volvió a centrifugar las muestras esta vez durante 20 min, para así separar una fase orgánica (fase inferior donde se encuentra el DNA) y una interfase (proteínas) y una fase acuosa (que supone un 60% y donde se encuentra el RNA).

Tras recoger la fase superior acuosa se le añadió 2 ml (500 µl por cada ml de Trizol añadido) de alcohol isopropílico. Posteriormente se añadieron 5 µl de Glycoblue (Ambion, Austin, USA) con el fin de identificar mejor el pellet de RNA que se obtuvo tras una centrifugación a 12.000 x *g* durante 20 min a 4 ºC.

Con objeto de lavar el RNA precipitado, se decantó el sobrenadante dejando intacto el pellet y se añadieron 1,5 ml de 75% etanol-H_2O-DEPC centrifugándolo posteriormente a 12.000 x *g* durante 5 min. Tras un nuevo lavado, se retiró todo el sobrenadante y se dejó secar el pellet a temperatura ambiente durante 2 min.

El RNA fue resuspendido en la solución RNA secure (Ambión, Austin, USA) utilizando un volumen de 20 µl por muestra, y se incubó durante 10 min en baño seco a 55-65 ºC para disolver el pellet e inactivar las RNAsas. Se enfrió en hielo y se procedió a la cuantificación del RNA total presente en cada muestra así como su grado de pureza respecto a proteínas y sales minerales utilizando el espectrofotómetro Nanodrop ND1000 (Thermo Scientific, Wilminton, DE, USA) .

3.1.6.2.- Tratamiento con DNAsa y retrotranscripción

De forma previa a la retrotranscripción se realizó un tratamiento con el *DNAfree kit* (Ambion, Austin, USA) con la finalidad de obtener un RNA mas puro y libre de contaminaciones con DNA genómico.
Para ello, se tomaron 2 µg de RNA, que se incubaron durante 30 min a 37 ºC en presencia de la enzima DNAsa, la cual degrada los restos de DNA genómico presentes en la muestra.

Seguidamente y tras la inactivación de la enzima con el *DNAsa Inactivation reagent,* se procedió a realizar la retrotranscripción de cada una de las muestras, que consiste en la obtención de DNA complementario (cDNA) a partir del RNA total obtenido para el posterior análisis de la expresión génica mediante PCR cuantitativa a tiempo real (qRT-PCR).

Para cada muestra se realizó la retrotranscripción de 2 µg de RNA utilizando la enzima retrotranscriptasa inversa (M-MLV, Invitrogen) en presencia de la enzima inhibidora de ribonucleasas (RNasinTM, Promega) en la siguiente proporción: 4 µl de buffer 5x, 2 µl DTT, 1 µl RNasin y 1 µl M-MLV.

Las muestras se incubaron 10 min a 25 ºC, 60 min a 37 ºC y finalmente 15 min a 70 ºC. Las muestras de cDNA obtenido se alicuotaron y se guardaron a -80 ºC hasta su utilización.

3.1.6.3.- Determinación de los niveles de expresión génica mediante la técnica q RT-PCR

La determinación de los niveles de expresión génica se realizó mediante PCR a tiempo real, que es un método semi-cuantitativo basado en la actividad 5´exonucleasa de la *Taq* polimerasa.

Mediante esta técnica, se puede determinar en tiempo real la amplificación del gen de estudio utilizándose otro fragmento de DNA (sonda) complementario a una parte intermedia del DNA que se quiere amplificar. Dicha sonda lleva acoplada una molécula fluorescente (*reporter*) y otra molécula que inhibe la fluorescencia (*quencher*). De esta forma, cuando la molécula fluorescente es desplazada por la enzima Taq polimerasa, dicha molécula se libera y emite fluorescencia al ser iluminada con un láser. La cuantificación de la fluorescencia emitida durante cada ciclo de PCR será proporcional a la cantidad de DNA que se está amplificando. El detector fotométrico junto con un programa especial, monitoriza el incremento en la emisión del fluorocromo. El algoritmo normaliza la señal a un patrón interno (ΔRn) y automáticamente calcula la línea de corte del ciclo (Threshold-C_T) cuando el ΔRn alcanza diez veces la desviación estándar de la línea base.

Los datos se obtuvieron como valores C_T (ciclo en el cual la señal de fluorescencia emitida se encuentra considerablemente por encima de los niveles de amplificación inespecífica y es inversamente proporcional al número de copias iniciales de la muestra). Después se determinaron los valores de ΔC_T (ΔC_T= C_T del gen en estudio - C_T del gen de referencia) para cada muestra. Los cambios en la expresión del gen se calcularon por el método de $2^{-\Delta\Delta CT}$ (Livak y Schmittgen, 2001).

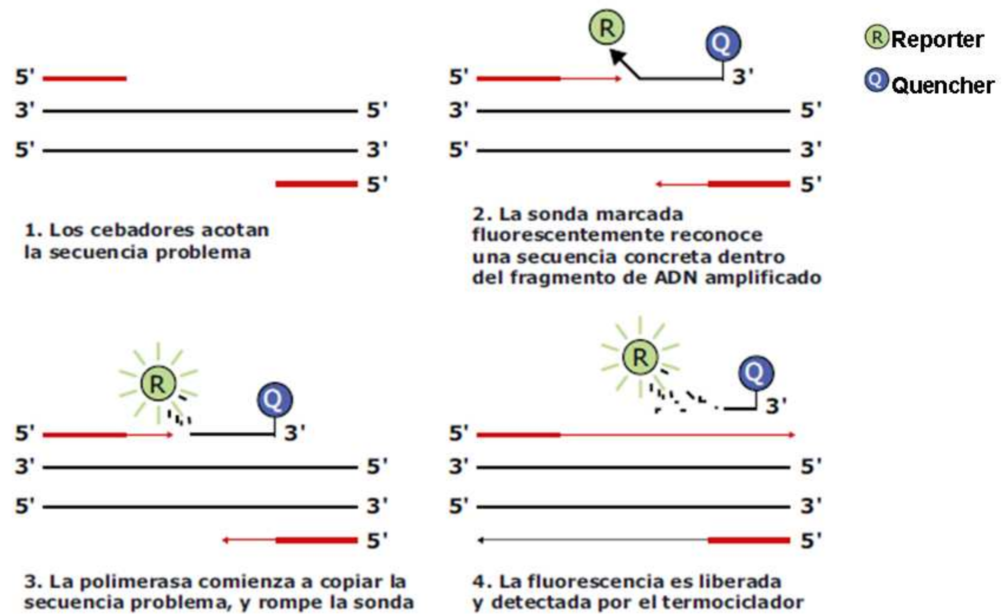


Figura 25.- Esquema de la determinación de la expresión génica mediante PCR a tiempo real.

3.1.6.3.1.- Procedimiento

Inicialmente, se realizó una curva estándar de validación para cada cebador-sonda con diluciones seriadas de varias muestras para asegurar que el final de la reacción (tanto para muestras control, como para los distintos tratamientos) se encontraba en la parte media de la curva exponencial de amplificación. Se utilizaron 5,5 µl de cDNA, 4 µl de Taqman Universal PCR Master Mix (Applied biosystems) y 0,5 µl de cada cebador-sonda por cada muestra. También se ensayaron diversos genes de referencia (*house keeping genes*) para normalizar los datos (18s, ciclofilina, β-actina, Ubiquitina C, y Gliceraldehido 3-fosfato deshidrogenasa), siendo elegidos aquellos que presentaron una menor variación entre las distintas muestras y tratamientos. En este trabajo, los genes en estudio se refirieron a los genes β-actina y ciclofilina.

Los reactivos para el análisis de expresión génica de los distintos genes en estudio, así como los genes de referencia son prediseñados y obtenidos de *Applied Biosystems* (Foster City, EEUU), y las condiciones experimentales se ajustaron a las indicaciones del fabricante. La detección y amplificación de los genes específicos se llevó a cabo con el sistema de detección de secuencias ABI PRISM 7000HT y ABI PRISM 7900HT (Secuence Detection System, Applied Biosystems).

Tabla 5. Listado de genes y las correspondientes sondas utilizadas para el análisis de la expresión génica.

Nombre	Ref sonda Taqman	Gene symbol	Especie
Leptina	Rn00565159_m1	Lep	*Rattus norvegicus*
Adiponectina	Rn00595250_m1	Adipoq	*Rattus norvegicus*
Ciclofilina	Rn00690933_m1	Ppia	*Rattus norvegicus*
Ubiquitina C	Rn01789812_g1	Ubc	*Rattus norvegicus*
Gliceraldehido 3-fosfato deshidrogenasa	Rn99999916_s1	Gapdh	*Rattus norvegicus*
18s	Hs99999901_s1	18s	*Homo sapiens*
β-actina	Rn00667869_m1	Actb	*Rattus norvergicus*

3.1.7.- Captación *ex vivo* de azúcares en anillos intestinales de rata

Para estimar el efecto de la suplementación con ácido lipoico sobre la absorción intestinal de azúcares se utilizó la técnica de acumulación en anillos intestinales evertidos, inicialmente descrita por Crane y Mandelstam (1960).

3.1.7.1.- Medios y reactivos

Se utilizó como medio de incubación, la solución salina Krebs-Ringer-Tris (KRT) de composición (en mM): NaCl 140; $KHCO_3$ 10; KH_2PO_4 0,4; K_2HPO_4 2,4; $CaCl_2$ 1,2; $MgCl_2$ 1,2. El pH del medio se ajustó a 7,4.

Se añadió al medio de incubación α-Metilglucosido, a la concentración final de 1 mM, y 0,01 μCi/ml de [U-^{14}C]-Glucopiranosido (Perkin Elmer Inc.) de actividad específica 303 mCi/mmol.

3.1.7.2.- Procedimiento

Se extrajo la porción inicial del yeyuno de cada animal de experimentación (decapitado tras los 56 días del estudio), y se sumergió rápidamente en un vaso de precipitados que contenía suero fisiológico (NaCl 0,9%) frío a 4 ºC. El comienzo del yeyuno se identificaba por proximidad al ligamento de Treitz. Con la ayuda de una jeringa, se lavó el intestino con suero, con el fin de eliminar los posibles restos de contenido que podían permanecer en su interior. Con unas pinzas, se retiraban cuidadosamente los restos de grasa y de mesenterio adheridos a la capa serosa. A continuación, se evertía el tubo intestinal y se cortaba en anillos de aproximadamente 0,3-0,5 cm de longitud y 10-30 mg de peso. Estos anillos se mantuvieron en suero fisiológico frío, desechándose aquellos que presentaban placas de Peyer.

Se distribuyeron 5-8 anillos intestinales en viales que contenían 10 ml de medio de incubación (KRT) en el que se disolvía el sustrato en estudio a la concentración deseada y al que se adicionaba trazas del mismo marcado radiactivamente.

La incubación se realizó a 37,0 ± 0,1 ºC en un baño termostático con selector de temperatura y motor de agitación controlable (Unitronic 320 OR, Selecta). En todos los casos, se acoplaba a cada vial una salida de una bombona de oxígeno puro para permitir la oxigenación constante del medio durante el periodo de de incubación.

Antes de introducir los anillos en los viales, se tomaron muestras iniciales de 200 μl del medio de incubación (medio inicial) por duplicado. Las muestras recogidas se dispusieron en viales apropiados y se añadieron 2 ml de líquido de centelleo (Ecoshcint H Itisa), para determinar la radiactividad emitida en cpm (cpmi) en un contador β (Wallac 1409, Pharmacia). Luego, se repartieron al azar los anillos intestinales en los viales y se dispusieron en el baño termostático para comenzar la incubación.

Transcurrido el tiempo de incubación (10 min), se retiraron los viales del baño, se lavaron los anillos en KRT frío, se secaron con cuidado sobre papel de filtro húmedo y se pesaron individualmente en una balanza de precisión para determinar el peso fresco (Pf). Finalmente, permanecieron durante 15 horas en la nevera, en tubos eppendorf con 0,5 ml de HNO_3 0,1 M. El ácido nítrico provoca la desnaturalización de las proteínas de membrana, alterando su permeabilidad, lo que permite la salida del sustrato marcado radiactivamente contenido en el tejido.

Tras 24 horas, se tomaron muestras por duplicado de 200 µl del medio desnaturalizante, a las que se añadieron 2 ml de líquido de centelleo para determinar la radiactividad emitida (cpmtj).

El cálculo de la cantidad de sustrato captado por cada anillo se obtuvo mediante la siguiente fórmula:

$$\mu\text{mol sustrato/g tej. húmedo} = \frac{\text{cpmtj}}{\text{cpmi}} \times \frac{[\text{sustrato}] \times Vt \times Vi}{Vtj \times Pf}$$

[sustrato]= concentración en mM de sustrato en el medio de incubación

Pf= peso fresco del tejido en gramos

cpmtj= cuentas por minuto del medio desnaturalizante

cpmi= cuentas por minuto del medio inicial

Vt= volumen de ácido nítrico (0,5 ml)

Vi= volumen de muestra tomada del medio inicial (0,2 ml)

Vtj= volumen de muestra tomada del medio desnaturalizante (0,2 ml)

3.2.- ESTUDIOS *"IN VITRO"*

3.2.1.- Cultivo de la linea celular 3T3-L1

3.2.1.1.- Fundamento teórico

Las fibroblastos de la línea celular murina 3T3-L1 son células capaces de transformarse en adipocitos en presencia de un cóctel hormonal adecuado y han sido ampliamente utilizados en los últimos años, tanto para el estudio de la adipogénesis como de la fisiología del adipocito maduro, incluyendo su metabolismo, su función secretora y las vías de señalización intracelular (Vigilanza *et al.* 2011; Meissburger *et al.* 2011; Suzuki *et al.* 2011; Moreno-Aliaga *et al.* 2002; Green y Kehinde, 1979).

3.2.1.2.- Material

- *Dulbecco´s Modified Eagle Medium (*DMEM), *High Glucose* (4,5 g/L) (Gibco-Invitrogen Corporation)
- Suero Bovino de ternero (CSB) (Gibco-Invitrogen Corporation)
- Suero Fetal Bovino (FSB), *Heat Inactivated* (Gibco-Invitrogen Corporation)
- Penicilina/Estreptomicina (Gibco-Invitrogen Corporation)
- Insulina (Sigma-Aldrich)
- Isobutilmetilxantina (IBMX) (Sigma-Aldrich)
- Dexametasona (Sigma-Aldrich)

3.2.1.3.- Procedimiento experimental

La línea celular 3T3-L1 se obtuvo de *American Type Culture Collection* (ATCC, Rockville). Las células se cultivaron en medio DMEM alto en glucosa (4,5 g glucosa/L) y suplementado con L-glutamina y piruvato. Al medio se le añadió suero bovino de ternero (CSB) al 10% y estreptomicina/penicilina al 1%.

Cuando las células alcanzaron el 100% de confluencia, se indujo la diferenciación de los preadipocitos a adipocitos. Para ello, se cultivaron las células en medio de diferenciación DMEM alto en glucosa suplementado con 10% de suero fetal bovino (FSB), estreptomicina/penicilina al 1%, insulina (10 μg/mL), IBMX (0,5 mM) y dexametasona (1 μM) durante 48 horas. Pasado ese tiempo, se retiró el medio de diferenciación y las células se cultivaron durante otras 48 horas en medio post-diferenciación (DMEM con FSB al 10%, estreptomicina/penicilina al 1% y 10 μg/mL de insulina).

A partir del día 4 postdiferenciación las células se cultivaron con DMEM suplementado con 10% FSB y estreptomicina/penicilina al 1% hasta el día 7-8 de postdiferenciación en el cual aproximadamente el 100% de las células ya habían alcanzado tanto la morfología como la funcionalidad típica de adipocitos maduros, con lo cual pudieron ser utilizadas como modelo *in vitro* de adipocitos. Todo el proceso de crecimiento y diferenciación de la línea celular 3T3-L1 se llevó a cabo en un incubador a 37 ºC y 5% de CO_2.

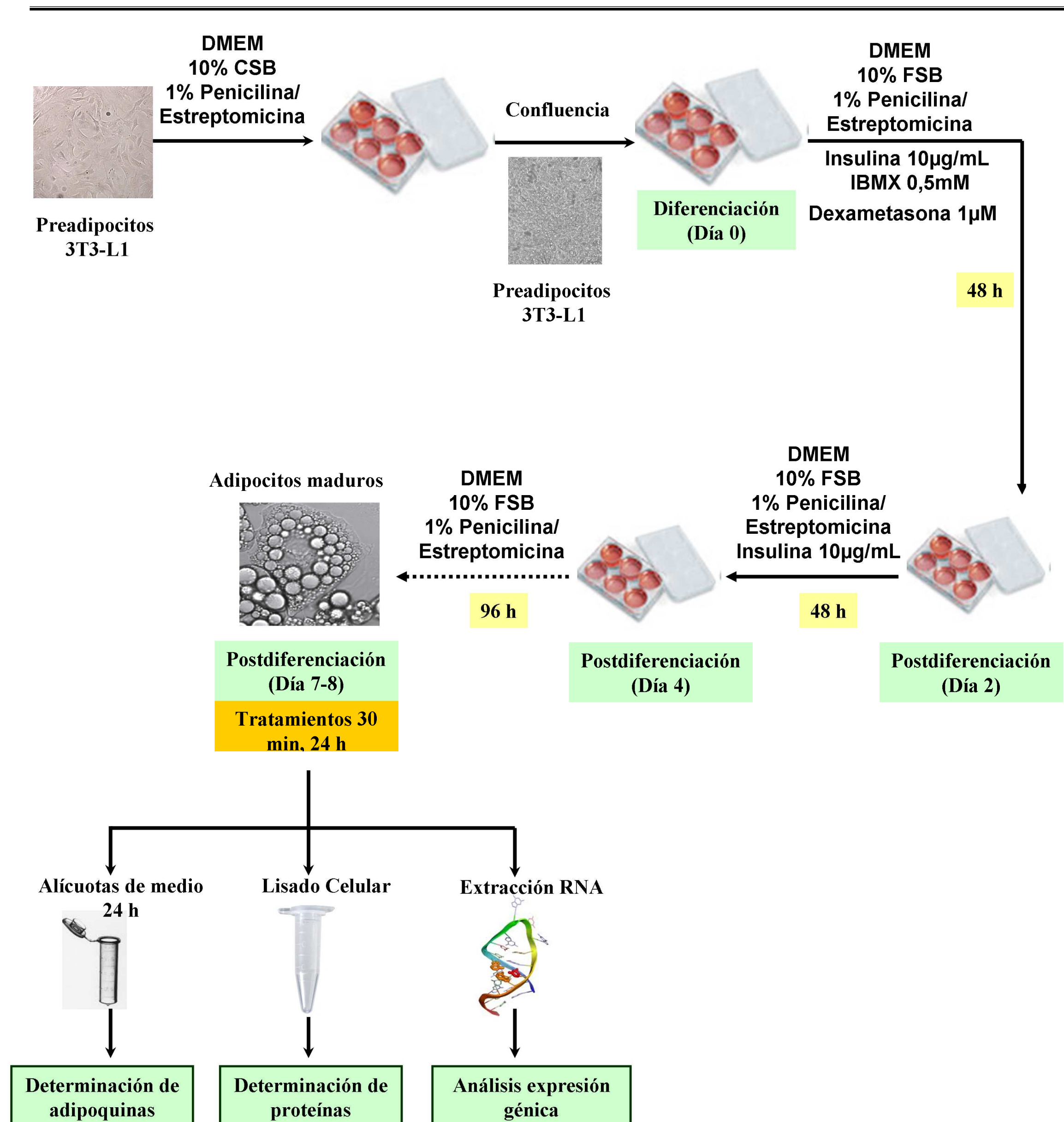


Figura 26.- Cultivo y proceso de diferenciación de 3T3-L1 y posteriores determinaciones.

3.2.1.4.- Tratamientos

La tarde previa al tratamiento de las células, el medio de cultivo de post-diferenciación se cambió a medio DMEM (4,5 g/L) suplementado al 1% ó 10% con SFB (dependiendo de las posteriores determinaciones) y estreptomicina/penicilina al 1%. Así, las células se mantuvieron en estas condiciones durante unas 14-15 horas previas a la realización del experimento. A continuación, se añadieron los medios con los tratamientos correspondientes: Control (Etanol, vehículo en el que el LA fue

disuelto) y LA (1-500 µM) en presencia o ausencia de distintos inhibidores específicos de las vías de señalización celular a estudiar y a su concentración adecuada. Se incubaron los adipocitos durante 24-48 horas a 37 ºC y 5 % CO_2 en condiciones de esterilidad. En el caso de utilizar inhibidores de diferentes vías de señalización, los adipocitos se preincubaron en presencia del inhibidor durante 1 hora y a continuación, se añadió el tratamiento correspondiente. Los adipocitos del grupo control fueron tratados con la misma cantidad del vehículo utilizado para disolver cada uno de los agentes en estudio (etanol y/o DMSO, según casos).

Tabla 6. Listado de tratamientos y sus correspondientes vías de señalización.

Tratamiento	Vía de señalización	Concentración
Ácido Lipoico (LA)		1-500 µM
SP600125	↓ JNK	20 µM
PD98059	↓ MAPK	50 µM
LY294002	↓ AKT	50 µM
Compound C	↓ AMPK	20 µM
AICAR	↑ AMPK	2 mM
Troglitazona	↑ PPARγ	10 µM
Insulina	↑ Akt	170 nM

Posteriormente, se tomó muestra del medio de cultivo para las determinaciones bioquímicas (glucosa y lactato) y hormonales (leptina, chemerina, adiponectina).

Seguidamente, y tras recoger todo el medio de cultivo, las placas se congelaron a – 80 ºC para su posterior extracción de RNA y análisis de la expresión génica.

3.2.2.- Cultivo primario de adipocitos de rata

3.2.2.1.- Animales de experimentación

Se utilizaron ratas Wistar macho de unos 250-280 g de peso procedentes del Centro de Investigación en Farmacobiología Aplicada (CIFA) de la Universidad de Navarra. Los animales se mantuvieron en unas condiciones de temperatura (22 ± 2ºC) y humedad constante alternando ciclos de luz/oscuridad cada 12 horas. Las ratas se alimentaron con una dieta referencia de laboratorio y agua *ad libitum.* La manipulación de estos animales se realizó de acuerdo con las Guías Nacionales e Institucionales de Cuidado y Uso de Animales y bajo la aprobación del Comité de Ética para Experimentación Animal (CEEA) de la Universidad de Navarra.

3.2.2.2.- Material

Medio de cultivo:

- *Dulbecco´s Modified Eagle Medium* (DMEM), bajo en glucosa (1 g/L) (Gibco-Invitrogen Corporation)
- Suero Fetal Bovino, *Heat Inactivated* (Gibco-Invitrogen Corporation)
- Penicilina/Estreptomicina (Gibco-Invitrogen Corporation)
- Nistatina (Gibco-Invitrogen Corporation)
- Aminoácidos no esenciales (Gibco-Invitrogen Corporation)

Buffer Hepes-Fosfato:

Para el cultivo primario de 4-6 ratas, se prepararon 500 mL del buffer HEPES-fosfato de la siguiente composición:

- 2 % Albúmina sérica bovina (BSA) (Sigma-Aldrich)
- 5 mM D-Glucosa
- 135 mM NaCl (Panreac)
- 2,2 mM $CaCl_2 \cdot 3H_2O$ (Panreac)
- 1,25 mM $MgSO_4 \cdot 7H_2O$ (Panreac)
- 0,45 mM KH_2PO_4 (Panreac)

- 2,17 mM Na_2HPO_4 (Panreac)
- 10 mM HEPES-1M (Sigma-Aldrich)
- 5 mM D-Glucosa (Panreac)

Se ajustó el pH entre 7,2 - 7,4 y se enrasó hasta el volumen adecuado con matraz aforado y se filtró con papel de filtro. Posteriormente, se esterilizó utilizando un filtro de 0,2 μm de diámetro y se conservó a 4 ºC.

3.2.2.3.- Preparación de los tratamientos

El medio de cultivo utilizado fue DMEM bajo en glucosa (1 g/L) suplementado con suero fetal bovino (1%), antibióticos (penicilina/estreptomicina) (1%), nistatina (1%) y aminoácidos no esenciales (1%). El LA (Sigma Aldrich), se disolvió en etanol y la insulina en DMEM. Con cierta antelación, se reconstituyeron los diversos productos en su disolvente adecuado (DMEM y etanol, respectivamente) y se prepararon las alícuotas stock (1000x) de cada agente en estudio. El día de la realización del cultivo, se prepararon los medios con los tratamientos necesarios a la concentración adecuada. Las células correspondientes al grupo control se trataron con la misma cantidad del vehículo utilizado para reconstituir los diferentes productos. El LA (250 μM) y la insulina (1,6 nM) se añadieron al medio al mismo tiempo. Las dosis de LA y de insulina han sido previamente utilizadas en nuestro Departamento o por otros grupos no observándose alteraciones en la viabilidad celular (Pérez-Matute *et al.* 2005; Cho *et al.* 2003; Mueller *et al.* 1998).

3.2.2.4.- Aislamiento y cultivo de los adipocitos

Las ratas fueron decapitadas e inmediatamente se procedió a la extirpación del tejido graso epididimal. El aislamiento de los adipocitos se realizó en condiciones de esterilidad, trabajando en campana de seguridad biológica de flujo laminar previamente esterilizada con luz ultravioleta.

El tejido adiposo extraído se colocó sobre una placa de Petri en buffer HEPES-fosfato atemperado a 37 ºC. El aislamiento de los adipocitos a partir del tejido se realizó según el protocolo de Rodbell (Rodbell, 1964) con ligeras

modificaciones (Peréz-Matute *et al.* 2005). De esta forma, tras pesar el tejido, se troceó con tijeras durante 90 segundos. Seguidamente, se incubó este tejido parcialmente digerido con una solución de colagenasa tipo I (Worthington Biochemical): 1,25 mg/mL por 0,5 g de tejido durante 30 min a 37 ºC y con agitación constante. Transcurrido este tiempo, se neutralizó la acción de la colagenasa mediante la adición de buffer HEPES-fosfato (24 mL) e inmediatamente se procedió a la filtración del tejido sin digerir para obtener los adipocitos aislados. Para esto último, se utilizaron membranas de nylon de 400 µM de tamaño de poro previamente esterilizadas.

A continuación, se realizaron dos lavados con buffer HEPES-fosfato con el fin de eliminar restos de colagenasa centrifugando la suspensión a 500 rpm durante 6 min. Tras la centrifugación, se obtiene en la parte superior la suspensión celular de adipocitos y en la fase inferior, los restos de buffer y otros tipos celulares como pre-adipocitos y células sanguíneas. Esta fase inferior se eliminaba utilizando cánulas metálicas revestidas de un aislamiento de goma (para evitar la lisis de los adipocitos en contacto con el metal) y acopladas a jeringas. Después, se realizó un último lavado utilizando DMEM (suplementado con FSB al 1%) y se procedió de nuevo a centrifugación en las mismas condiciones. Tras retirar de nuevo el infranadante, se volvió a añadir DMEM hasta 12-14 mL y los adipocitos aislados se incubaron a 37 ºC y 5 % de CO_2 durante 30- 40 min.

Durante este periodo se preparó la solución de colágeno (PureColTM, Inamed Biomaterials). Para ello, se enriqueció el colágeno comercial (PureColTM: 3 mg/mL; pH=2) con *Minimum Essential Medium* (MEM para 1 L, Gibco-Invitrogen Corporation) en una proporción 1:8 de MEM/colágeno y se ajustó el pH hasta 7,4-7,6. Esta solución se conservó a 4 ºC hasta su utilización.

Transcurridos los 30-40 min de incubación, se retiró la fase inferior hasta obtener una relación volumétrica 2:1 de células/medio, que se mezclaron hasta obtener una suspensión celular homogénea. Seguidamente, los adipocitos se cultivaron en placas de 6 pocillos anclados en una matriz de colágeno. Para ello, se añadieron 500 µL de la solución de colágeno preparada con anterioridad sobre cada pocillo y sobre ésta, 150 µL de la suspensión celular de adipocitos. La matriz de

colágeno con los adipocitos se extendió sobre la superficie del pocillo girando y agitando cuidadosamente hasta obtener una distribución homogénea de las células sobre la capa de colágeno. A continuación, las placas se incubaron durante 40-50 min (37 ºC y 5 % CO_2) para que se produjera la solidificación de la capa de colágeno en la que quedaban atrapados los adipocitos, favoreciendo así unas condiciones de cultivo más parecidas a la situación fisiológica de los adipocitos en el tejido adiposo. A continuación, se añadió a cada pocillo 2 mL de medio con los tratamientos correspondientes y posteriormente, se introdujeron las placas en el incubador durante 24-48 horas según la duración de los tratamientos. Cada 24 horas, se tomaron alícuotas del medio de cultivo y se repuso medio nuevo incluyendo los correspondientes tratamientos. Estas alícuotas se congelaron a -80 ºC para posteriores determinaciones de leptina, así como la cuantificación de los niveles de glucosa. Al final del período de incubación, se eliminó todo el medio de cultivo y las placas con los adipocitos se congelaron a -80 ºC para la posterior extracción de RNA y análisis de expresión génica.

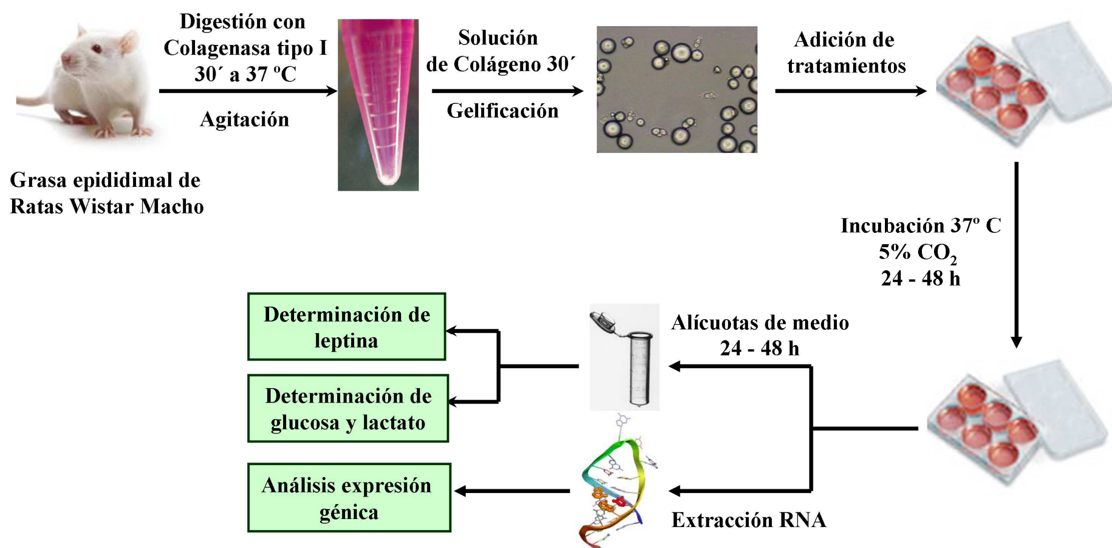


Figura 27.- Esquema del procedimiento utilizado para el aislamiento y cultivo de adipocitos y determinaciones posteriores.

3.2.3- Cultivo de adipocitos humanos

3.2.3.1.- Fundamento teórico

Los preadipocitos subcutáneos humanos se obtuvieron de Zen-Bio Inc (NC, USA). Los preadipocitos provienen de tejido subcutáneo adiposo humano, de 4-7 mujeres con sobrepeso – obesidad (IMC = 26,85 - 33,37 Kg/m^2), no diabéticas y con edades comprendidas entre 33-68 años. Los preadipocitos omentales (grasa visceral) humanos procedían de una mujer afroamericana, no diabética, con obesidad mórbida (IMC = 60,2 Kg/m^2) y con una edad de 34 años (Zen-Bio Inc., NC, USA). Ambos tipos celulares son capaces de transformarse en adipocitos en presencia de un medio de diferenciación comercial y han sido ampliamente utilizados tanto para el estudio de la adipogénesis como de la fisiología del adipocito maduro, incluyendo su metabolismo, y su función secretora (Tomlinson *et al.* 2010; Ortega *et al.* 2010; Ding *et al.* 2009).

3.2.3.2.- Material

- Medio de preadipocitos PM-1 (Zen-Bio Inc., NC, USA).
- Medio de diferenciación de adipocitos DM-2 (Zen-Bio Inc., NC, USA).
- Medio de mantenimiento de adipocitos AM-1 (Zen-Bio Inc., NC, USA).
- Medio de preadipocitos omentales OM-PM (Zen-Bio Inc., NC, USA).
- Medio de diferenciación de adipocitos omentales OM-DM (Zen-Bio Inc., NC, USA).
- Medio de adipocitos omentales OM-AM (Zen-Bio Inc., NC, USA).

3.2.3.3.- Procedimiento experimental

Los preadipocitos se cultivaron en medio específico, PM-1 (Zen-Bio Inc., NC, USA). Cuando estos preadipocitos alcanzaron el 100 % de confluencia, se indujo su diferenciación hasta adipocitos maduros. Para ello, se cultivaron en medio de diferenciación comercial DM-2, (Zen-Bio Inc., NC, USA). Después de 7 días se retiró parte de este medio de diferenciación (600 µl) y se añadieron 800 µl del medio de mantenimiento de adipocitos AM-1 (Zen-Bio Inc., NC, USA). Tras 2 días de incubación se retiraron 600 µl del medio y se sustituyeron por 600 µl de AM-1 y se mantuvieron hasta el día 14 de postdiferenciación en el cual aproximadamente el 100% de las células ya habían alcanzado la morfología y la funcionalidad típica de adipocitos maduros, con lo cual pudieron ser utilizadas como modelo *in vitro* de adipocitos humanos. Durante todo el proceso de crecimiento y diferenciación, las células fueron guardadas en un incubador a 37 ºC y 5 % de CO_2. Todos los medios contienen glucosa (3,15 g glucosa/L). El procedimiento para diferenciar los adipocitos provenientes de tejido omental es similar al anteriormente descrito pero usando los medios de cultivo específicos anteriormente mencionados para adipocitos omentales proporcionados también por la casa comercial.

3.2.3.4.- Tratamientos

La tarde previa al tratamiento de las células, se cambió el medio de cultivo de mantenimiento de los adipocitos AM-1. Así, las células se mantuvieron en estas condiciones durante unas 14-15 horas previas a la realización del experimento. A continuación, se añadieron los medios con los tratamientos correspondientes: Control (Etanol), LA (250 µM), TNF-α (100 ng/ml) y se incubaron los adipocitos durante 24 horas a 37 ºC y 5 % CO_2 en condiciones de esterilidad. Los adipocitos del grupo control fueron tratados con la misma cantidad del vehículo utilizado para disolver el LA (etanol).

Posteriormente, se tomaron muestra del medio de cultivo para las determinaciones hormonales (chemerina). Seguidamente, y tras recoger todo el medio de cultivo, las placas se congelaron a – 80 ºC para posterior extracción de RNA de los adipocitos y analizar los niveles de expresión génica.

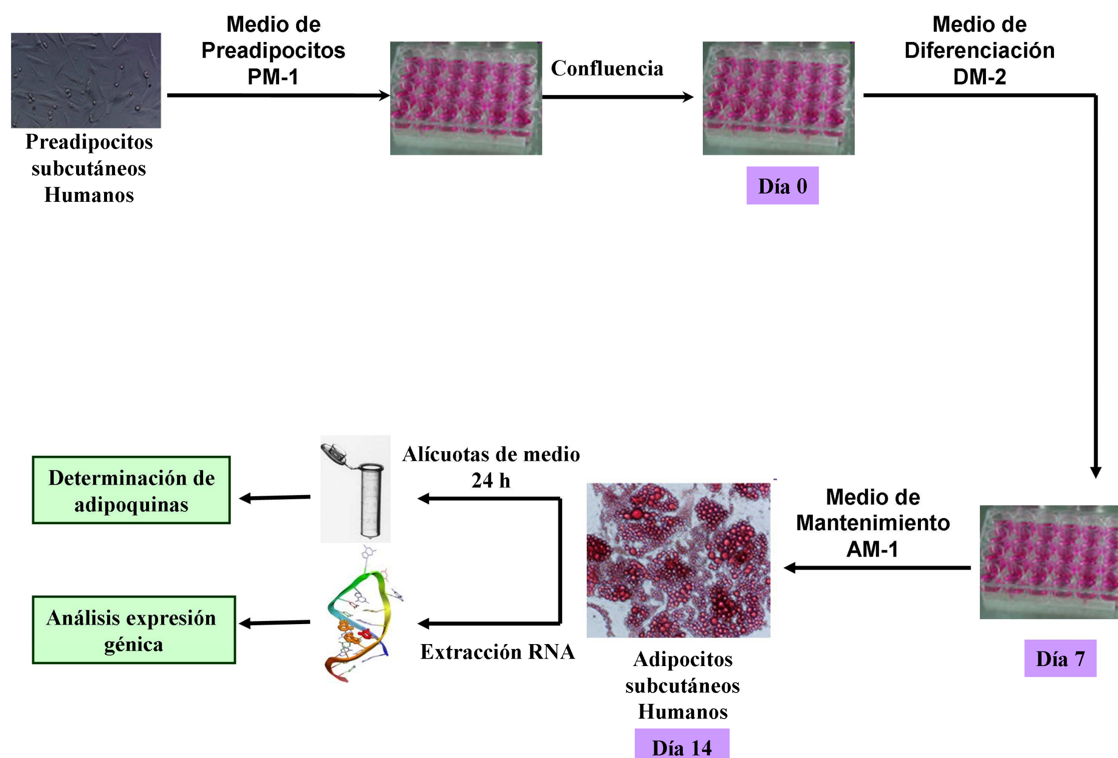


Figura 28.- Cultivo y proceso de diferenciación de adipocitos humanos y posteriores determinaciones.

3.2.4.- Determinaciones bioquímicas en medio de cultivo

Se determinaron los niveles de glucosa y lactato presentes en el medio de cultivo de 3T3-L1 y de adipocitos primarios de rata y de humanos tras 24 y/o 48 horas de incubación. Para ello se utilizó el autoanalizador Cobas - Mira (Roche Diagnostic, Basel, Suiza), según protocolos establecidos en el Departamento de Ciencias de la Alimentación, Fisiología y Toxicología de la Universidad de Navarra.

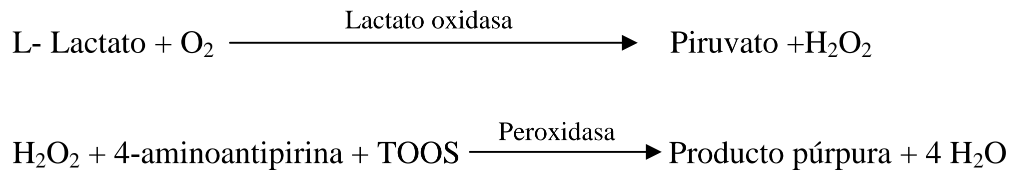
3.2.4.1.- Glucosa

La determinación de glucosa se realizó mediante un procedimiento enzimático (Glucose HK CP, ABX Pentra) tal y como ha sido expuesto anteriormente

La utilización de glucosa por los adipocitos se determinó restando el valor de glucosa inicial presente en el medio de cultivo de la concentración final obtenida tras la incubación con los distintos tratamientos. El resultado final se expresó como µmoles de glucosa captada.

3.2.4.2.- Lactato

Los niveles de lactato presentes en el medio de cultivo se determinaron mediante método colorimétrico (Lactate PAP, Randox Labratories S. L., Reino Unido) según la siguiente reacción:



De forma similar a la glucosa, la producción de lactato se calculó como el incremento de lactato en el medio de cultivo tras la incubación con los diversos agentes en estudio y se expresó como µmoles de lactato producido. La cantidad de glucosa transformada a lactato a lo largo del tiempo se expresó en forma de porcentaje ([µg de lactato producido]/[µg de glucosa captada]·100). El porcentaje de glucosa metabolizada a lactato es un buen indicador del metabolismo anaeróbico de la glucosa por los adipocitos.

3.2.5- Determinaciones hormonales mediante ELISA

Los niveles secretados de leptina, chemerina y adiponectina se analizaron mediante ELISA. Los kits empleados para ello fueron:

- Leptina: Mouse Leptin ELISA kit (Millipore, Estados Unidos).
- Chemerina: Mouse Chemerin ELISA kit (R&D Systems Inc., Estados Unidos).
- Chemerina: Human Chemerin ELISA kit (Biovendor R&D Inc., Estados Unidos).
- Adiponectina: Mouse Adiponectin ELISA kit (B-Bridge Internacional Inc., Estados Unidos

3.2.6- Determinacion de la actividad de Sp1

La actividad de unión al DNA del factor de transcripción Sp1 se analizó con el Sp1 *Transcription factor ELISA kit* (Panomics Inc., Estados Unidos).

3.2.6.1.- Obtención de extractos nucleares

Para el aislamiento de los extractos nucleares, se utilizaron adipocitos maduros (7-8 días post-diferenciación) procedentes de la línea celular 3T3-L1 cultivados según se ha descrito anteriormente. Los adipocitos se mantuvieron en medio DMEM (4,5 g/L) con 1% FSB durante 12-15 horas (overnight) previo al inicio del tratamiento. Entonces los adipocitos se trataron con LA (250 µM) durante 24 horas. Tras este periodo de incubación, se procedió a la obtención de los extractos nucleares según el método de Chu y colaboradores (2003) con ligeras modificaciones que se describen a continuación.

Tras eliminar el medio de cultivo, los adipocitos se lavaron con PBS. Posteriomente se añadió a las células 200 µl de buffer A (HEPES 10 mM pH 7,9; KCl 10 mM; EDTA 0,1 mM; EGTA 0,1 mM; DTT 1 mM; PMSF 0,5 mM con Nonidet P40 0.6 %) y se mantuvieron en hielo durante 15 min. Tras desprender las células por rascado, se centrifugaron a 800 x *g* durante 30 segundos a 4 ºC. Se

descartó el sobrenadante y el pellet obtenido se resuspendió en 1 ml de buffer B (HEPES 10 mM pH 7,9; KCl 10 mM; EDTA 0,1 mM; EGTA 0,1 mM; DTT 1 mM; PMSF 0,5 mM). Nuevamente, se centrifugó a 800 x *g* durante 30 segundos a 4 ºC. Seguidamente se retiró el sobrenadante y se resuspendió el pellet en 38 µl de buffer C (HEPES 10 mM pH 7,9; NaCl 0,4 mM; EDTA1 mM; EGTA 1 mM; DTT 1 mM; PMSF 1 mM). Esta suspensión nuclear se agitó a 1500 rpm durante 30 min a 4 ºC y se centrifugó a 10000 x *g* durante 10 min a 4 ºC con el fin de eliminar los restos insolubles. Los sobrenadantes obtenidos tras la centrifugación son los extractos nucleares y se almacenan a -80 ºC hasta su utilización. La concentración de proteína se determinó utilizando el método Bradford.

Con el fin de caracterizar si algunas de las acciones del LA eran revertidas al evitar la fosforilación de Sp1 algunos extractos nucleares fueron tratados con proteina fosfatasa 1 (1,25 U ó 5 U) (New England Biolabs, Reino Unido) durante 15 minutos a 30 ºC en baño seco (Chu *et al.* 2006).

3.2.6.1.1.- Procedimiento

Con el fin de cuantificar la actividad de unión al DNA del factor de transcripción Sp1 se utilizó el kit comercial *Transcription factor ELISA kit de Sp1* (Panomics, Estados Unidos). La técnica es altamente especifica y requiere de un mínimo de 5-10 µg de proteína en cada muestra, por ello previamente se procedió a la cuantificación de la cantidad de proteína por el método Bradford.

El fundamento de este kit está basado en la técnica *Enzyme-Linked ImmunoSorbent Assay* (ELISA) descrita anteriormente (Lequin, 2005; VanWeemen y Schuurs, 1971).

Se partió de un total de 6 µl de extracto nuclear al que se añadieron 6 µl de buffer de dilución del kit de *Transcription factor ELISA kit de Sp1* (Panomics, Estados Unidos) para la posterior determinación de la actividad de Sp1 mediante ELISA de acuerdo al protocolo descrito por el fabricante.

3.2.7.- Determinación de la fosforilación de proteínas mediante western blot

3.2.7.1.- Obtención de extractos proteicos

Para la preparación de los extractos proteicos, se utilizaron adipocitos maduros (7-8 días post-diferenciación) procedentes de la línea celular 3T3-L1 cultivados según se ha descrito anteriormente. La tarde anterior al experimento, se eliminó el suero fetal bovino del medio de cultivo y los adipocitos se mantuvieron en medio DMEM (4,5 g/L) con 1% FSB durante 12-15 horas (*overnight*). Tras este periodo se realizó una preincubación de 1 h con los distintos inhibidores: SP600125, PD98059, LY294002, y Compound C. A las células control se les añadió el vehículo correspondiente (DMSO). Posteriormente se añadieron los tratamientos (Control, LA 250 µM, AICAR 2 mM) durante 30 min ó 24 horas. Tras este periodo de incubación, se procedió a lisar las células y obtener los extractos proteicos.

Para la obtención de los extractos proteicos tras los distintos tratamientos se añadió a las células 250 µL del buffer de lisis que contenía: Tris HCl (pH 8) 2 mM; NaCl 137 mM; Glicerol 10%; Tritón X100 1%; Ortovanadato de sodio 1 mM; EDTA 2 mM; Cóctel inhibidor de proteasas 1 (Sigma-Aldrich) 1%; y PMSF 1 mM. Tras incubar a 4 ºC durante 30 min en presencia del buffer de lisis, se procedió a centrifugar 10 min a 200 *g*. Los sobrenadantes obtenidos, conteniendo el extracto protéico fueron congelados a -80 ºC.

Los niveles de proteína tanto total como fosforilada se realizaron mediante western blot, siguiendo un protocolo similar al descrito anteriormente (apartado 3.1.5). Los niveles de Akt y AMPK (total y fosforilado) se determinaron en extractos celulares según lo descrito anteriormente. Los niveles de Sp1 (total y fosforilado) se determinaron en extractos nucleares. La tabla 6 recoge el listado de anticuerpos utilizados en estas determinaciones.

Tabla 6. Listado de anticuerpos utilizados.

	Fabricante	Ref.	Especificidad
Anti-Sp1 fosfo T453	Abcam	59257	*Rabbit anti mouse*
Anti-Sp1 (H-225)	Santa Cruz biotechnoloy	14027	*Rabbit anti mouse*
Anti-AMPK	Cell signaling technology	2532	*Rabbit anti mouse*
Anti-AMPK fosfo Thr172	Cell signaling technology	2535	*Rabbit anti mouse*
Anti-Akt	Cell signaling technology	9272	*Rabbit anti mouse*
Anti-Akt fosfo Ser 473	Cell signaling technology	4058	*Rabbit anti mouse*
Anticuerpo Secundario	Bio Rad	170/6515	*Goat anti rabbit*

3.2.8.- Análisis de la expresión génica

La extracción de RNA total se realizó también aplicando el método del Trizol® (2 ml/pocillo). Tras la retrotranscripción el análisis de la expresión génica de las diferentes adipoquinas se realizó mediante PCR a tiempo real tal y como se ha detallado anteriormente.

Tabla 7. Listado de genes y las correspondientes sondas utilizadas para el análisis de la expresión génica

Nombre	Ref sonda Taqman	Gene symbol	Especie
Leptina	Mm00434759_m1	Lep1	*Mus musculus*
Adiponectina	Mm00456425_m1	Adipoq	*Mus musculus*
Chemerina	Mm00503579_m1	Rarres2	*Mus musculus*
Chemerina	Hs00161209_g1	Rarres2	*Homo sapiens*
Ciclofilina	Mm02342430_g1	Ppia	*Mus musculus*
Ciclofilina	Hs99999904_g1	Ppia	*Homo sapiens*
β-actina	Mm02619580_g1	Actb	*Mus musculus*
18s	Hs99999901_s1	18s	*Homo sapiens*

3.3.- ANÁLISIS ESTADÍSTICO

El análisis estadístico de todas las variables se realizó utilizando el programa informático GraphPad Prism 4.0 (GraphPad Software Inc, San Diego. CA, Estados Unidos). Se seleccionaron como estadísticos descriptivos de cada muestra la media como medida de tendencia central, y el error estándar de la media como medida de dispersión. El nivel de significación estadístico se situó en todos los casos en $P<0,05$.

La normalidad de todas las variables se verificó utilizando el programa estadístico SPSS para Windows versión 15.0 (SPSS Inc. Chicago, Estados Unidos) mediante los tests de Kolmogorov-Smirnof y Shapiro Wilk. Cuando las variables siguieron una distribución normal las comparaciones entre dos grupos se realizaron mediante un test de t-Student. Las comparaciones entre más de dos grupos fueron analizadas mediante ANOVA de un factor seguido de un test a posteriori de Bonferroni. En otros casos se utilizó el test estadístico ANOVA de dos factores, el cual permitió comparar el efecto tanto de la dieta como del tratamiento con LA en los animales del estudio, mostrando además si existía alguna interacción entre ambos factores. Cuando la interacción resultó ser significativa ($P<0,05$), se aplicó el test de t-Student para comparar los efectos de cada tratamiento. Cuando las muestras no siguieron una distribución normal se aplicaron los test de Kruskal Wallis o de U-Mann-Whitney.

Además, en algunos casos se estudió la posible asociación existente entre dos variables con el fin de detectar una posible correlación positiva o negativa y estadísticamente significativa entre ellas. El coeficiente de correlación empleado fue el de Pearson (r de Pearson), o Spearman en función de si las variables siguieron o no una distribución paramétrica.

3.4.- ESTUDIO BIBLIOGRÁFICO

Las búsquedas bibliográficas se realizaron mediante la consulta de la base informática PubMed (U.S. Nacional Library of Medicine) de acceso a través de Internet en la dirección URL www.ncbi.nlm.nih.gov/PubMed que accede a revistas de alto índice de impacto en el área en que se incluye esta tesis: nutrición, fisiología, endocrinología, etc.

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RESULTADOS

4.1.- Lipoic acid prevents body weight gain induced by a high fat diet in rats: Effect on intestinal sugar transport

Lipoic acid prevents body weight gain induced by a high fat diet in rats: Effects on intestinal sugar transport

Prieto-Hontoria PL, Pérez-Matute P, Fernández-Galilea M, Barber A, Martínez JA and Moreno-Aliaga MJ

J Physiol Biochem, 65 (1), 43-50, 2009.

Resumen

Varios estudios han sugerido que el estrés oxidativo podría actuar como desencadenante y agravante del estado inflamatorio asociado a la obesidad y podría ser un potencial nexo de unión entre la excesiva ganancia de peso y las co-morbilidades asociadas. Así, se ha propuesto el tratamiento con antioxidantes naturales como posible terapia contra el desarrollo de obesidad así como sus complicaciones asociadas. Por ello, el objeto del presente trabajo fue investigar en ratas Wistar macho los efectos de la suplementación de una dieta estándar o alta en grasa con un antioxidante, el ácido lipoico (LA) (0,25g/ 100g de comida) durante 56 días sobre la ganancia de peso corporal, la adiposidad, la eficiencia metabólica y la absorción intestinal de azúcares. La suplementación de la dieta con LA indujo una menor ganancia de peso corporal y redujo el tamaño del tejido adiposo blanco total, tanto en ratas alimentadas con dieta control como alta en grasa. Además, disminuyó la ingesta. La ganancia de peso en el grupo alimentado con dieta alta en grasa y LA fue menor que la de su correspondiente grupo *Pair-Fed* (P<0,05), el cual recibía la misma cantidad de comida que los animales tratados con LA pero sin este ácido. De hecho, la suplementación con ácido lipoico redujo la eficiencia metabólica y disminuyó significativamente la absorción intestinal de α-metilglucósido (α-MG) tanto en ratas control como obesas. Estos resultados sugieren que los efectos beneficiosos de la suplementación de la dieta con LA sobre la ganancia de peso corporal están mediados, al menos en parte, por la reducción observada en la ingesta de comida y en la eficiencia metabólica. Además, la acción inhibitoria del LA sobre el transporte intestinal de azúcares podría explicar, en parte, la menor eficiencia metabólica observada en los animales tratados con LA justificando, por consiguiente, los efectos beneficiosos del LA sobre la obesidad.

ABSTRACT

Several studies have suggested that oxidative stress might cause and aggravate the inflammatory state associated with obesity, being a potential link between excessive weight gain and its related disorders such as insulin resistance and cardiovascular diseases. Thus, antioxidant treatment has been proposed as a therapy to prevent and manage obesity and associated complications. Therefore, the aim of the present study was to investigate the effects of supplementation of a standard or high fat diet with the antioxidant lipoic acid (LA) during 56 days, on body weight gain, adiposity, feed efficiency and intestinal sugar absorption, in male Wistar rats. LA supplementation induced a lower body weight gain and adipose tissue size in both control or high fat fed rats accompanied by a reduction in food intake. The group fed on a high fat diet and treated with LA (OLIP group) showed a lower body weight gain than its corresponding Pair-Fed (PF) group ($P<0.05$), which received the same amount of food than LA-treated animals but with no LA. In fact, LA induced a reduction on feed efficiency and also, significantly decreased intestinal α-methylglucoside (α-MG) absorption both in lean and obese rats. These results suggest that the beneficial effects of dietary supplementation with LA on body weight gain are mediated, at least in part, by the reduction observed in food intake and feed efficiency. Furthemore, the inhibitory action of LA on intestinal sugar transport could explain in part the lower feed efficiency observed in LA-treated animals and therefore, highlighting the beneficial effects of LA on obesity.

INTRODUCTION

Obesity is defined as a multifactorial chronic disease characterized by an increase in fat mass, as a result of a positive energy balance maintained over the time (Barbany y Foz, 2002). In recent years, several studies have suggested that obesity is associated with a low degree chronic inflammation, which is accompanied by a high production of some cytokines and acute phase reactants as well as by the activation of the signaling pathways of inflammation (Hotamisligil, 2003). Furthermore, different studies in animal models and humans have shown that oxidative stress is related to the development of inflammation in obesity (García-Díaz *et al.* 2008; Vincent and Taylor, 2006). Indeed, oxidative stress might be the link between obesity and associated comorbidities, such as insulin resistance and cardiovascular disease (Vincent and Taylor, 2006; Keaney *et al.* 2003). Therefore, it is plausible to suggest that supplementation of the diet with ingredients with antioxidant properties (Chung *et al.* 2001) could prevent the development of obesity and associated comorbidities.

Lipoic acid (LA), a short chain fatty acid with eight atoms of carbon containing two sulfhydryl groups, is an important cofactor for mitochondrial bioenergetic enzymes (Smith *et al.* 2004). LA can be found in spinach and cabbage, liver and meat, wholewheat and yeast of beer, but it is also endogenously produced by the liver through the lipoic acid synthase (Padmalayam *et al.* 2009; Cakatay, 2006). Several studies have described the potential benefits of LA on obesity and its complications (Shen *et al.* 2005; Kim *et al.* 2004; Cho *et al.* 2003). Thus, it has been demostrated that LA reduces body weight and adiposity in rodents (Shen *et al.* 2005; Song *et al.* 2005; Kim *et al.* 2004).

In addition, LA has beneficial actions in both glucose and lipid metabolism, and it has been proposed as a potential therapy for type 2 diabetes (Sena *et al.* 2008; Song *et al.* 2005; Kim *et al.* 2004; Packer *et al.* 2001). In this context some antidiabetic agents such as metformin, sulfonylureas and thiazolidinediones, have shown to inhibit intestinal sugar absorption, and therefore to decrease postprandial glucose elevations (Levetan, 2007; Purnell and Hirsch, 1997). To our knowledge, there is not information available on the effects of LA on intestinal carbohydrate uptake.

Thus, the aim of this study was to investigate the effects of LA supplementation on food intake, feed efficiency and intestinal sugar transport both in lean and diet-induced obese Wistar rats.

RESULTS

Effects of LA on body weight gain, food intake and feed efficiency

As expected, the high fat diet induced a significant increase in final body weight compared with the control group (*P*<0.001). The dietary supplementation with LA induced a lower body weight gain in both control and high fat-fed animals, and was able to completely prevent the weight gain induced by the high fat diet (Figure 1).

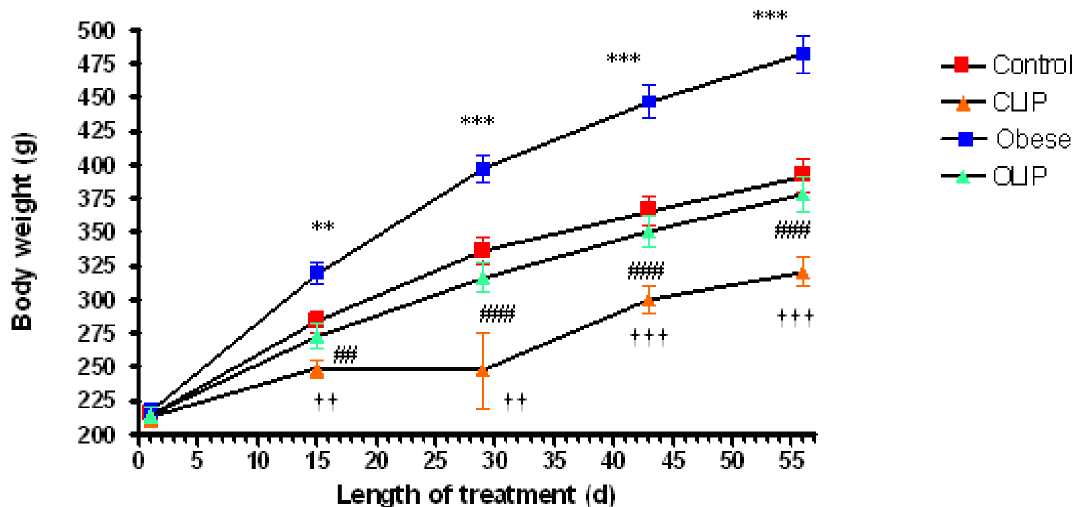

Figure 1. Effects of LA (56 days of treatment) on the growth curve in rats: control rats (*n* = 10), control rats treated with LA (CLIP *n* = 10), obese rats (*n* = 10), obese rats treated with LA (OLIP *n* = 12). Data are expressed as mean with standard errors represented by vertical bars. ***P*<0.01; *** *P*<0.001 Control *vs.* Obese. ##*P*<0.01; ###*P*<0.001 Obese *vs.* OLIP. ††*P*<0.01; †††*P*<0.001 Control *vs.* CLIP

LA decreased food intake both in lean (*P*<0.001) and obese (*P*<0.05) rats, which could explain, at least in part, the reduction of body weight gain observed in LA treated rats (Figure 2A).

In order to test if the inhibitory action of LA on food intake is the only mechanism involved in its effects on body weight reduction, we included two Pair-Fed groups, which received the same amount of food eaten by the groups CLIP and

OLIP respectively. Figure 2B shows that the groups treated with LA had lower body weight gain that their corresponding Pair-Fed groups, especially in the groups fed with a high fat diet (*P*<0.05).

Feed efficiency, the weight gain:energy consumed ratio, was significantly decreased (*P*<0.001) in those groups treated with lipoic acid in comparison with their respective Pair-Fed groups (Figure 3).

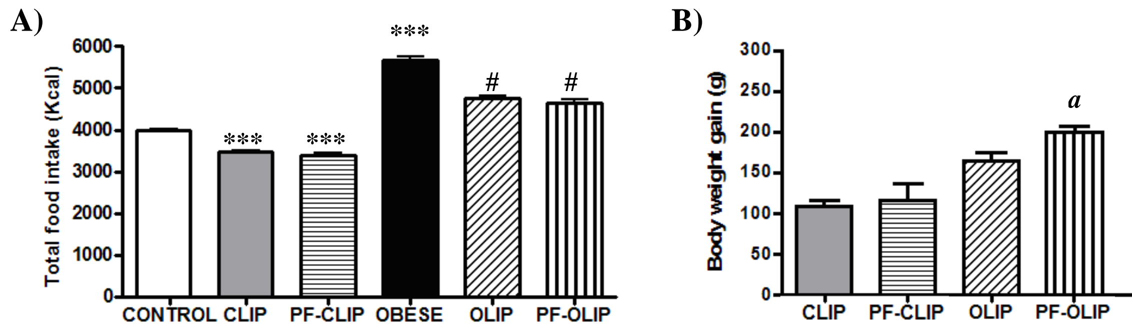

Figure 2. Effects of LA (56 days of treatment) on total food intake **(A)** Body weight gain in LA-treated animals (CLIP and OLIP) in comparision with their corresponding Pair-Fed groups (PF-CLIP and PF-OLIP) **(B)**. Data are expressed as mean with standard errors represented by vertical bars. ****P*<0.001 *vs*. Control. [#]*P*<0.05 *vs*. Obese. [a]*P*<0.05 *vs*. OLIP.

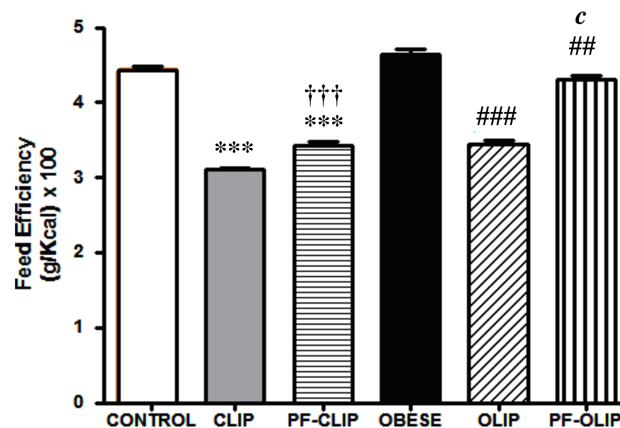

Figure 3. Effects of LA (56 days of treatment) on feed efficiency. Data are expressed as mean with standard errors represented by vertical bars. ****P*<0.001 *vs*. Control. †††*P*<0.001 *vs*. CLIP. [###]*P*<0.001 *vs*. Obese. [c]*P*<0.001 *vs*. OLIP.

Effects of LA on white adipose tissue weights

Treatment with LA decreased the weight of all white fat pads (Table 1). Our data also showed that in animals fed on a high fat diet, the decrease observed in the white fat depots is not only due to the reduction in energy intake induced by LA, since they showed smaller white fat depots than their corresponding Pair-Fed groups, being especially significant in the retroperitoneal depot (Table 1). However, the decrease in brown adipose tissue in LA-treated animal was similar to their corresponding Pair-Fed groups (Table 1).

Table 1. White adipose tissue weights in control, Pair-Fed groups and lipoic acid-treated lean and obese rats

	CONTROL (*n* = 10)	**CLIP (*n* = 10)**	**PF-CLIP (*n* = 6)**	**OBESE (*n* = 10)**	**OLIP (*n* = 12)**	**PF-OLIP (*n* = 6)**
Epididymal fat (g)	8.9± 0.6	4.7 ± 0.4 ***	5.2 ± 0.9	20.0 ± 1.1 ***	8.6 ± 0.7 ###	11.8 ± 1.2
Retroperitoneal fat (g)	11.2 ± 1.1	4.6 ± 0.5 ***	4.5 ± 1.2	24.6 ±1.4 ***	11.2 ± 1.1 ###	15.9 ± 0.5 *a*
Mesenteric fat (g)	2.8 ± 0.4	1.1 ± 0.1	1.1 ± 0.3	5.4 ± 0.9	2.2 ± 0.3	3.1 ± 0.2
Subcutaneous fat (g)	5.6 ± 0.5	3.4 ± 0.3 **	3.5 ± 0.8	14.7 ± 2.0 ***	6.7 ± 0.5 ###	8.2 ± 0.5
Brown adipose tissue(g)	0.41 ± 0.02	0.34 ± 0.02 *	0.33 ± 0.06	0.60 ± 0.07	0.53 ± 0.03	0.55 ± 0.04

Data (mean ± SE) were analysed by one-way ANOVA. ***P<0.001, **P<0.01, *P<0.05 *vs.* control group. $^{###}P$<0.001 *vs.* obese group. ^{a}P<0.05 *vs.* OLIP group.

Effects of LA on intestinal sugar absorption

In order to investigate the possible effects of LA on sugar intestinal absorption, α-MG was chosen since this sugar derivative is only transported in the intestine by the Na^+-dependent glucose transporter, SGLT1, and not by the facilitative glucose transporters. Figure 4 shows that LA induced a significant decrease in the absorption of α-MG after 10 min of incubation, both in lean and obese rats (P<0.01). Interestingly, the groups treated with LA showed lower intestinal sugar absorption that their corresponding Pair-Fed groups, especially in the group fed with a standard diet (0.40 ± 0.02 *vs.* 0.62 ± 0.03 µmol α-MG/g wet weight x 10 min, P<0.001, CLIP *vs.* PF-CLIP), suggesting a direct inhibitory effect of lipoic acid on intestinal sugar absorption.

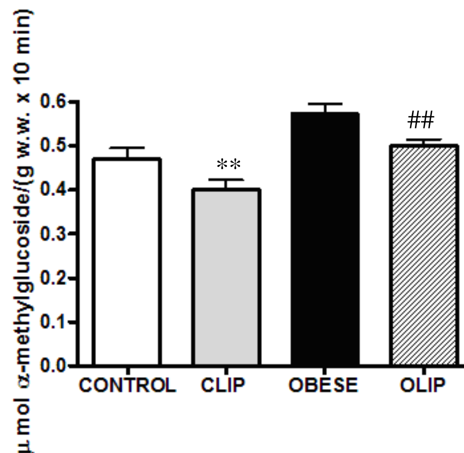

Figure 4. Effects of LA (56 days of treatment) on 1 mM α-methylglucoside uptake after 10 min of incubation by everted jejunum rings of rats. Data are expressed as mean with standard errors represented by vertical bars. $^{**}P<0.01$ *vs.* Control. $^{\#\#}P<0.01$ *vs.* Obese.

DISCUSSION

Animals fed with a high-energy-yielding diet during 56 days exhibited a significant increase in body weight gain and adiposity, as previously described (Jebelovszki *et al.* 2008; Mitra *et al.* 2008; Perez- Matute *et al.* 2007; Buettner *et al.* 2007). LA supplementation significantly decreased the body weight gain of the animals, both in those fed with the control diet as well as in those fed with a high fat diet, similarly as what was previously observed by Kim *et al.* (2004) This LA-induced reduction of body weight is mainly due to a significant reduction in the weight of total white adipose tissue along with a decrease in the size of every fat depot, corroborating previous studies (Yang *et al.* 2008; Shen *et al.* 2005; Kim *et al.* 2004).

Body weight gain in rodents is determined by the balance between food intake and energy expenditure. Our data showed that LA supplementation decreased food intake, which might explain the lowering of body weight gain. In this context, several authors had also observed this anorexigenic effect of LA (Yang *et al.* 2008; Shen *et al.* 2005; Song *et al.* 2005; Kim *et al.* 2004). However, LA-treated rats weighted significantly less than their corresponding Pair-Fed rats, suggesting that food intake inhibition is not the only mechanism underlying the body weight

lowering action of LA. In fact, we demonstrated in this study that feed efficiency, an index that relates body weight gain and energy consumed, was significantly decreased ($P<0.001$) in those animals treated with LA, suggesting an enhanced disruption of ingested energy. Thus, the decrease in feed efficiency induced by LA, together with the reduced food intake, could explain the lower body weight observed in LA-treated animals. These data are in concordance with the study of Kim *et al.* (2004), which showed that weight loss induced by LA is due, in part, to an enhancement of energy expenditure.

Thus, in order to test if the lower feed efficiency could be secondary to alterations in intestinal nutrient absorption, we analyzed intestinal α-MG uptake in jejunum everted rings, since this sugar derivative is only transported in the intestine by the Na^+-dependent glucose transporter, SGLT1, and not by the facilitative glucose transporters. Our data demonstrated for the first time that LA significantly decreased intestinal sugar absorption by inhibiting the sodium-dependent glucose transporter (SGLT1) both in lean and obese rats. Furthermore, this effect was not observed in the Pair-Fed groups, suggesting a direct inhibitory effect of LA on intestinal sugar absorption, which could contribute in some way to the lower feed efficiency observed in LA-treated animals. The precise contribution of the inhibitory action of LA on intestinal sugar absorption to the observed anti-obesity effects of LA remains to be determined. In this context, different studies have suggested that molecules with anti-obesity properties, such as green tea catechins could play a role in body weight reduction by controlling dietary glucose absorption by inhibiting intestinal glucose transporters (Wolfram *et al.* 2006; Shimizu *et al.* 2000).

Our data also suggest that the reduction induced by LA on adiposity is not only secondary to the lower energy intake. In fact, the OLIP group exhibited a more reduced adiposity, specially in the retroperitoneal depot than its corresponding Pair-Fed group. This observation suggested that other mechanisms are contributing to the anti-adiposity actions of LA. In this context, it can be hypothesized that the reduced size of the fat depots can be attributed to an increase in lipolysis (Garcia-Diaz *et al.* 2009) and/or a decrease in the number of adipocytes (by increasing apoptosis and/or decreasing adipogenesis). In this regard, the study of Cho *et al.* (2003) showed that LA in high concentrations (250-500 μM) inhibits insulin or troglitazone-induced adipogenesis in 3T3-L1 cells, indicating that co-treatment with LA would be beneficial for the prevention of obesity induced by several agonists of PPAR-γ.

However, necessary further studies are necessary to investigate whether an increase in apoptosis and/or lipolysis could also contribute to the reduction observed in the size of white adipose tissue after the treatment with LA.

In summary, these results suggest that the beneficial effects of dietary supplementation with lipoic acid on body weight gain are mediated, at least in part, by the reduction observed in food intake and feed efficiency. Furthemore, the inhibitory action of LA on intestinal sugar absorption could also contribute to the lower feed efficiency observed in LA-treated animals and therefore, to the beneficial effects of LA on obesity.

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4.2.- Effects of lipoic acid on AMPK and adiponectin in adipose tissue of high fat-fed rats and in 3T3-L1 adipocytes

Effects of lipoic acid on AMPK and adiponectin in adipose tissue of high fat-fed rats and in 3T3-L1 adipocytes

Prieto-Hontoria PL, Pérez-Matute P, Fernández-Galilea M, Martínez JA and Moreno-Aliaga MJ

Enviado

Resumen

El ácido lipoico (LA) es un antioxidante con propiedades antidiabéticas y antiobesidad en roedores y humanos. La adiponectina es una adipoquina con potentes propiedades anti-inflamatorias e insulino-sensibilizadoras. Se ha sugerido que la hipoadiponectinemia está involucrada en alteraciones del metabolismo vinculadas a la obesidad en tejidos periféricos como el hígado y el músculo. La proteína quinasa activada por AMP (AMPK) es una enzima clave que participa en la homeostasis de la energía celular. La activación de AMPK se ha considerado como una diana para revertir los desórdenes metabólicos asociados a la obesidad y a la diabetes tipo 2. El objetivo de este estudio fue determinar los efectos del LA en la fosforilación de AMPK y la producción de adiponectina en el tejido adiposo de ratas alimentadas con una dieta alta en grasa, así como en cultivos de adipocitos. La suplementación dietética con LA además de reducir el peso corporal y la adiposidad en ratas control y alimentadas con dieta alta en grasa, también mejoró la resistencia a la insulina, efecto que fue independiente, en parte, de la reducción de peso corporal. Por otra parte, la fosforilación de AMPK se vió incrementada en el TAB de ratas tratadas con LA en comparación con sus correspondientes *Pair-Fed*. La suplementación dietética con LA aumentó la expresión génica de adiponectina en TAB, mientras se encontró una correlación negativa entre los niveles de adiponectina corregidos por la adiposidad y el índice HOMA. En adipocitos 3T3-L1, el LA también activó AMPK, pero causó una inhibición dosis-dependiente en la expresión génica de adiponectina y en la secreción de la proteína. El co-tratamiento con el activador de AMPK, AICAR fue capaz de revertir la inhibición de adiponectina inducida por el LA. También el co-tratamiento con troglitazona también redujo moderadamente la acción inhibitoria del LA sobre la expresión génica de adiponectina. Nuestros datos sugieren que la

capacidad del LA para mejorar la sensibilidad a la insulina puede estar relacionada con el aumento de la fosforilación de AMPK y el incremento de adiponectina en TAB observados en ratas tratadas con LA. Sin embargo, el LA reduce la expresión génica y la secreción de adiponectina en adipocitos 3T3-L1, mientras que AMPK esta activada. Estos datos sugieren una compleja regulación e interacción entre la adiponectina y la activación de AMPK en adipocitos por el LA.

ABSTRACT

Lipoic acid (LA) is an antioxidant with antiobesity and antidiabetic properties in rodents and humans. Adiponectin is an adipokine with potent anti-inflammatory and insulin-sensitizing properties. It has been suggested that hypoadiponectinemia is involved in the metabolic alterations-linked to obesity on peripheral tissues such as liver and muscle. AMP-activated protein kinase (AMPK) is a key enzyme involved in cellular energy homeostasis. Activation of AMPK has been considered as a target to reverse the metabolic abnormalities associated to obesity and type 2 diabetes. The aim of this study was to determine the effects of LA on AMPK phosphorylation and adiponectin production in adipose tissue of high-fat fed rats as well as in cultured adipocytes. Dietary supplementation with LA reduced body weight and adiposity in control and high-fat fed rats. LA also ameliorated insulin resistance, which was in part independent of the body weight lowering actions. Furthermore, AMPK phosphorylation was increased in WAT from LA-treated rats as compared with pair-fed animals. Dietary supplementation with LA also upregulated adiponectin gene expression in WAT, while a negative correlation between adiposity-corrected adiponectin levels and HOMA index was found. In 3T3-L1 adipocytes, LA also activated AMPK, but caused a dose-dependent inhibition on adiponectin gene expression and protein secretion. Interestingly, the AMPK activator AICAR was able to reverse the LA-induced inhibition of adiponectin. Co-treatment with troglitazone also moderately prevented the LA inhibitory actions *in vitro* on adiponectin gene expression. Our present data suggest that the ability of LA to improve insulin-sensitivity could be related to the stimulation of AMPK phosphorylation and adiponectin in WAT observed in LA-treated rats. However, LA reduced adiponectin gene expression and protein secretion in 3T3-L1 adipocytes, while activated AMPK. These data suggest a complex regulation and cross-talk between adiponectin and AMPK activation in adipocytes by LA.

INTRODUCTION

Obesity is a complex disease affected by genetic and environmental factors (Marti *et al.* 2008). Obesity is currently reaching epidemic levels worldwide and is a major predisposing factor for a variety of life-threatening diseases including type 2 diabetes, and hypertension, which are major components of the metabolic syndrome (Ouchi *et al.* 2011; Poirier *et al.* 2006; Mokdad *et al.* 2003). Obesity involves a state of chronic low-grade inflammation and oxidative stress, which predisposes to some obesity-related comorbidities (Vincent *et al.* 2007; Hotamisligil, 2006; Shoelson *et al.* 2006).

LA is a naturally occurring short chain fatty acid, present in plants and animals, and synthesized by lipoic acid synthase (LASY) within the mitochondria (Shay *et al.* 2009; Packer *et al.* 2001). It has been shown that down-regulation of LASY reduced endogenous levels of LA as well as critical components of the antioxidant defence network. It also increased oxidative stress, which, in turn, leads to increased insulin resistance, mitochondrial dysfunction and inflammation. Moreover, LASY gene expression is down-regulated in animal models of diabetes and obesity both in skeletal muscle and adipose tissue (Padmalayam *et al.* 2009). Thus, LA has been proposed as a novel therapeutic approach for chronic inflammatory diseases such as diabetes and obesity. Several studies have demonstrated the potent anti-obesity properties of LA in rodents through the inhibition of hypothalamic adenosine monophosphate-activated protein kinase (AMPK) activity in hypothalamus, which leads to decreased food intake, and stimulated energy expenditure (Prieto-Hontoria *et al.* 2009; Shen *et al.* 2005; Kim *et al.* 2004). More importantly, two recent trials in humans have also supported that LA is a promising antioxidant candidate for the therapy of obesity related diseases (Koh *et al.* 2011; Carbonelli *et al.* 2010).

White adipose tissue (WAT) is an important endocrine organ that secretes several immunomodulators and bioactive peptides termed adipokines (Wang *et al.* 2010; Wozniak *et al.* 2009). Dysregulated adipokine secretion from the expanded WAT of obese individuals contributes to the development of systemic insulin resistance and metabolic diseases (Ouchi *et al.* 2011). Adiponectin is one of the adipocyte-derived adipokines with potent lipid-lowering, anti-inflammatory and insulin sensitizing properties. Circulating levels of adiponectin are decreased in obesity and increased after weight loss (Bruun *et al.* 2003). Thus,

hypoadiponectinemia then mediates the metabolic effects of obesity on the other peripheral tissues, such as liver and skeletal muscle (Funuhashi and Matsuzawa *et al.* 2006). Circulating adiponectin levels are increased by many commonly used insulin-sensitizing molecules such as thiazolidinediones (TZDs) and n-3 polyunsaturated fatty acids (Moreno-Aliaga *et al.* 2010; Kubota *et al.* 2006). Therefore stimulation of adiponectin could be a potential mechanism contributing to the anti-obesity and anti-diabetic properties of LA. However, this possibility remains unclear since there are few trials that have determined changes in adiponectin after LA supplementation and data obtained up to now are controversial reporting increase, no change or even decrease of adiponectin levels (Cheng *et al.* 2011; Cummings *et al.* 2010; Huong and Ide, 2008). Therefore, further studies are needed to get a better knowledge about the effects of adiponectin in LA-actions in animal models of obesity and type 2 diabetes.

AMP-activated protein kinase (AMPK) is a key enzyme involved in cellular energy homeostasis. AMPK stimulates pathways which increase energy production (glucose transport, fatty acid oxidation) and switches off pathways which consume energy (lipogenesis, gluconeogenesis) (Daval *et al.* 2006). Impairment of AMPK activity has been reported in obesity, type 2 diabetes, the metabolic syndrome and cardiovascular disease (Dzamko *et al.* 2009; Kola *et al.* 2008). AMPK has also a broader role in metabolism through the control of appetite. Regulation of AMPK activity at the whole-body level is coordinated by a growing number of hormones including adiponectin and leptin. Furthermore, activation of AMPK by bioactive food components or drugs has been considered as target to reverse the metabolic abnormalities associated to obesity and type 2 diabetes (Zhang *et al.* 2009).

Several investigations have analyzed the effects of LA on AMPK in different tissues including the hypothalamus, liver, muscle and pancreas (Park *et al.* 2008; Targonsky *et al.* 2006; Lee *et al.* 2005; Kim *et al.* 2004). The role of AMPK in mediating the metabolic actions of LA is complex and seems to be tissue-dependent. However, there is no information regarding the ability of LA to modulate AMPK in adipose tissue under an obesogenic environment (high fat diet).

The objective of this study was to determine the effects of dietary supplementation with LA on insulin sensitivity, AMPK activation in WAT and adiponectin production in control and high-fat fed rats. We also aimed to analyze the

direct effects of LA on AMPK and adiponectin in 3T3-L1 adipocytes as well as the potential signaling pathways involved.

RESULTS

Effects LA on body-weight gain, white adipose tissue weights, and serum glucose and insulin levels

Table 1 shows that consuming of a high fat diet for 56 days induced a significant increase ($P<0.001$) in body weight gain, which was prevented ($P<0.001$) by LA supplementation. Furthermore, LA also reduced ($P<0.001$) the body weight gain in control-fed rats. The body weight lowering actions of LA were accompanied by a significant reduction ($P<0.001$) of WAT weight in both control (CLIP group) and high fat-fed rats (OLIP group). Moreover, LA supplementation was able to counteract hyperinsulinemia ($P<0.001$) and the increase observed in the HOMA index ($P<0.001$) induced by the high-fat diet.

Because the body weight lowering actions of LA are due in part to reduced food-intake (Control: 71.16 ± 0.69, CLIP: 62.08 ± 0.40, Obese: 101.60 ± 1.40, OLIP: 84.84 ± 1.01 Kcal/day; $P<0.001$ for untreated vs-LA-treated groups), we compared the effects of LA supplementation with Pair-Fed (PF) animals. Our data showed that body weight gain (Figure 1A), WAT weights (Figure 1B), serum insulin levels (Figure 1C) and HOMA index (Figure 1D) were significantly lower in LA-supplemented groups than in their corresponding PF-groups.

Table 1. Effects of LA supplementation (0.25g/100g) during 56 days on body weight gain, white adipose tissue weight, and serum markers of glucose metabolism and insulin resistance in control and high fat-fed rats.

	CONTROL (*n* = 10)	CLIP (*n* = 10)	OBESE (*n* = 10)	OLIP (*n* = 12)	ANOVA 2X2		
					D	**LA**	**DXLA**
Body weight gain (g)	176.70 ± 8.31	108.00 ± 7.76	263.90 ± 11.67	163.70 ± 10.52	***	***	n.s.
WAT (g)	28.51 ± 2.18	13.86 ± 1.20 ***	64.65 ± 4.22 ***	28.82 ± 2.42 ###	-	-	***
Glucose (mg/dl)	99.74 ± 1.87	100.70 ± 2.13	122.70 ± 5.89	119.40 ± 3.87	***	n.s.	n.s.
Insulin (pM)	154.20 ± 22.55	93.86 ± 8.66 *	305.20 ± 24.39 ***	167.80 ± 14.28 ###	-	-	*
HOMA$^{\Phi}$	5.26 ± 0.97	3.32 ± 0.39	13.54 ± 1.47	6.31 ± 0.80	***	***	n.s.
Adiponectin (µg/ml)	22.47 ± 1.76	41.17 ± 2.13 ***	34.11 ± 2.52 ***	35.56 ± 2.39	-	-	***
Adiponectin/ WAT	0.83 ± 0.09	3.14 ± 0.25 ***	0.54 ± 0.04 *	1.33 ± 0.13 ###	-	-	***

Data are expressed as mean ± SE. Data were analyzed by two-way ANOVA: ***P<0.001; * P<0.05. When an interaction was found, comparison between groups were analyzed by a Student's *t*-test (***P<0.001; * P<0.05 when compared with control group; ###P<0.001 when compared with obese group) D: diet; LA: Lipoic Acid treatment; DxLA: interaction between diet and LA treatment

Φ HOMA = insulin (µUI/ml) x glucose (mmol/l)/22.5

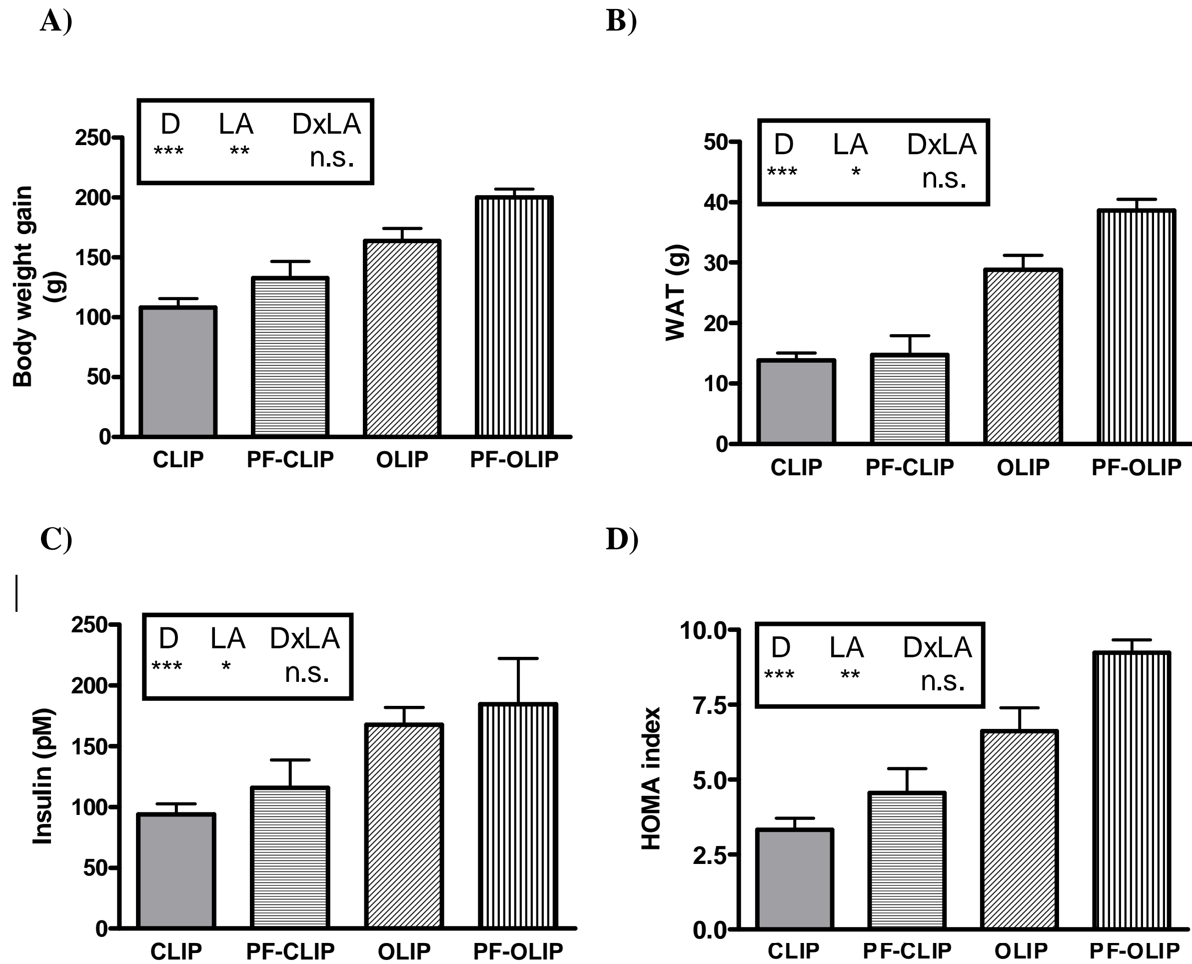


Figure 1. Effects of LA (0.25 g/100 g diet during 56 days) on (**A**) body weight gain, (**B**) total white adipose tissue, (**C**) serum insulin levels and (**D**) HOMA index. Data are expressed as mean ± SE. (n=6-12). Data were analyzed by two-way ANOVA: ***P<0.01; **P<0.01; *P<0.05 in comparison with Pair-Fed animals under control (PF-CLIP) or hight-fat (PF-OLIP) diet. D: diet; LA: Lipoic Acid treatment; DxLA: interaction between diet and LA treatment.

Effects of dietary supplementation with LA on adiponectin

The levels of the insulin-sensitizing adipokine adiponectin were significantly (P<0.001) elevated in the CLIP group (fed with control diet supplemented with LA). Surprinsingly, serum adiponectin levels were also higher in high fat-fed animals and no differences were induced by LA treatment (Table 1).

However, when adiponectin concentrations were corrected for adiposity, a marked reduction (P<0.05) in the amount of adiponectin per g WAT was observed in the high-fat fed obese animals, which was reversed (P<0.001) by dietary supplementation with LA (Table 1). A similar pattern was observed for adiponectin gene expression showing that LA supplementation was able to stimulate adiponectin

in control-fed rats and it was also able to override the inhibition in adiponectin gene expression induced by the high fat diet (Control: 1.00 ± 0.09, CLIP: 1.95 ± 0.94, Obese: 0.65 ± 0.16; OLIP: 1.31 ± 0.25, *P*<0.01 for untreated vs LA-treated groups). Interestingly, the ability of LA to stimulate adiponectin was not only secondary to its body lowering actions since statistically significant differences were observed between LA-treated groups (CLIP and OLIP) and their corresponding PF-groups (PF-CLIP and PF-OLIP) in both the amount of adiponectin/g WAT (*P*<0.01) and in adiponectin gene expression levels (*P*<0.05) in adipose tissue (Figures 2B and 2C). Furthermore, our data showed an inverse relationship (r= -0.598; *P*<0.001) between serum adiponectin levels corrected for adiposity and the HOMA index, a marker of insulin resistance (Figure 2D).

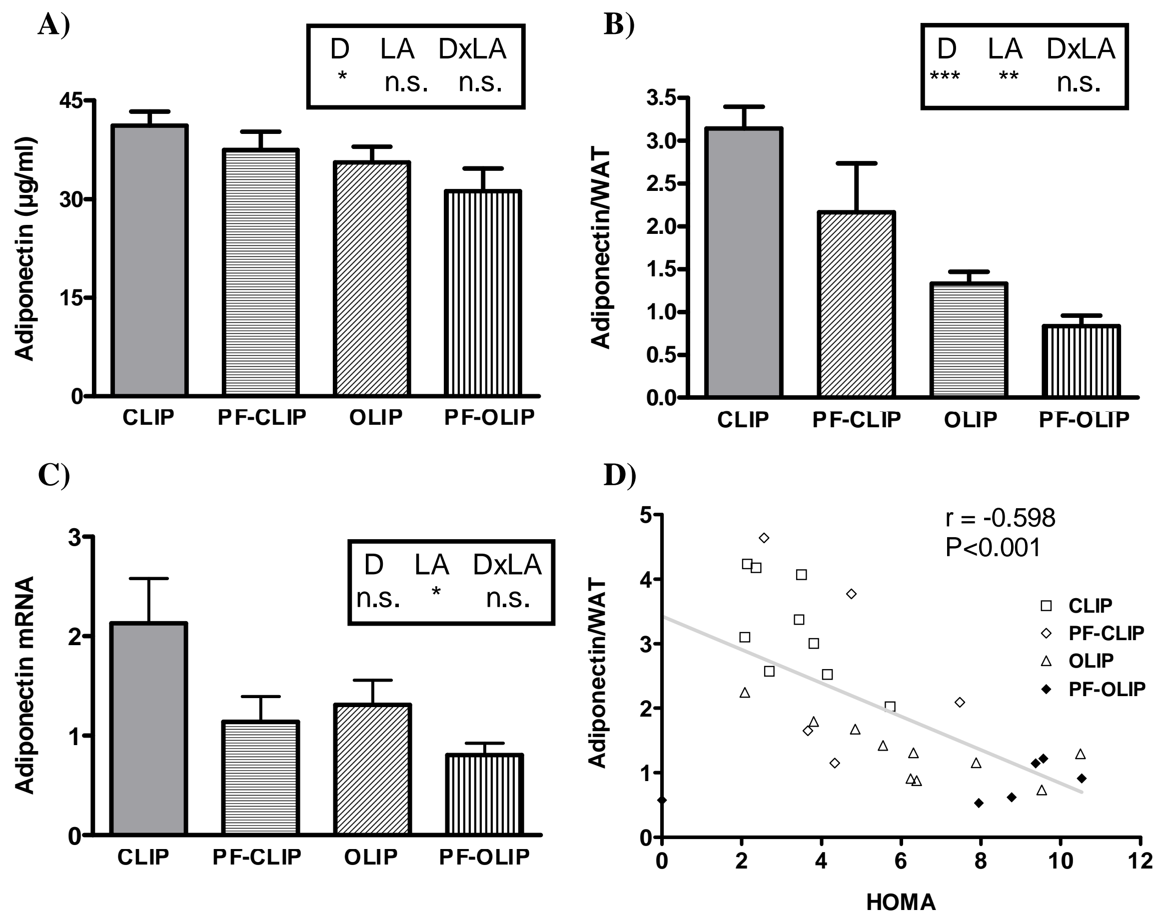


Figure 2. Effects of LA (0.25 g/100 g diet during 56 days) on (**A**) adiponectin circulating levels (**B**) adiponectin concentrations expressed per g of WAT and (**C**) adiponectin gene expression in epididymal fat (**D**) Pearson´s correlation between Adiponectin/WAT and HOMA index. Data are expressed as mean ± SE. (n=6-12). Data were analyzed by two-way ANOVA: ****P*<0.001; ***P*<0.01; **P*<0.05. D: diet; LA: Lipoic Acid treatment; DxLA: interaction between diet and LA treatment.

Effects of dietary supplementation with LA on AMPK in adipose tissue

Figure 3 shows that animals fed on a high fat diet exhibited a reduced phosphorylation levels of AMPK in WAT as compared to those under control diet ($P<0.05$). Interestingly, dietary supplementation with LA significantly increased ($P<0.05$) AMPK phosphorylation at Thr^{172} in both animals fed with control diet (CLIP group) and with high-fat diet (OLIP group) in comparison with the corresponding PF-groups.

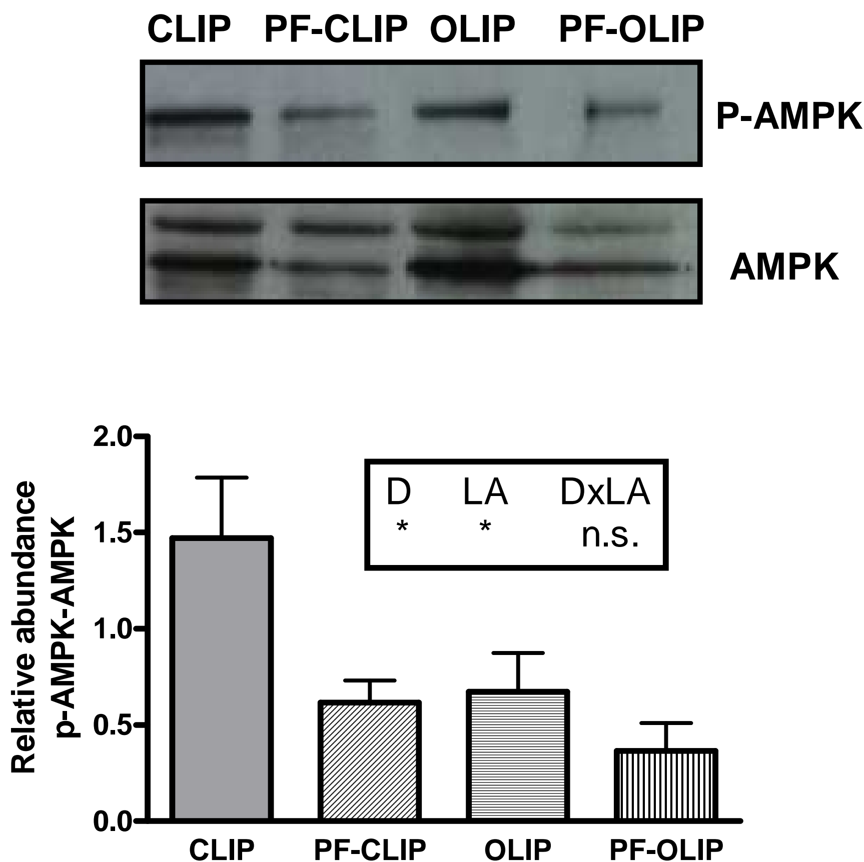


Figure 3. Effects of LA (0.25 g/100 g diet during 56 days) on AMPK phosphorylation in epididymal fat from control and high-fat fed rats. WAT lysates were subjected to gel electrophoresis and immunoblotted using specific antibodies for p-AMPKα (Thr-172) and AMPKα as described in material and methods. Representative blots and densitometric analysis of 5 independent experiments showing the effects of different treatments on AMPK phosphorylation. Data, expressed as mean ± SE, were analyzed by two-way ANOVA: *$P<0.05$. D: diet; LA: Lipoic Acid treatment; DxLA: interaction between diet and LA treatment.

Effects of LA on AMPK phosphorylation in 3T3-L1 adipocytes

In order to assess whether LA was able to directly stimulate AMPK phosphorylation, we treated 3T3-L1 adipocytes with LA during short (30 min) and long (24 h) periods. As expected, treatment for 30 min with AICAR (2 mmol/l) induced a strong increase on AMPK activation, and treatment with Compound C (20 µM) was able to block the phosphorylation of AMPK. Incubation of 3T3-L1 mature adipocytes for 30 min with LA (250 µM) tended to increase the phosphorylation of AMPK. Interestingly, LA was able to partially reverse the inhibitory effects of Compound C in AMPK phosphorylation (Figure 4A). Longer treatment (24 h) with LA (250 µM) caused a significant increase ($P<0.05$) in AMPK phosphorylation in 3T3-L1 adipocytes as compared with vehicle-treated cells (Figure 4B).

A)

B)

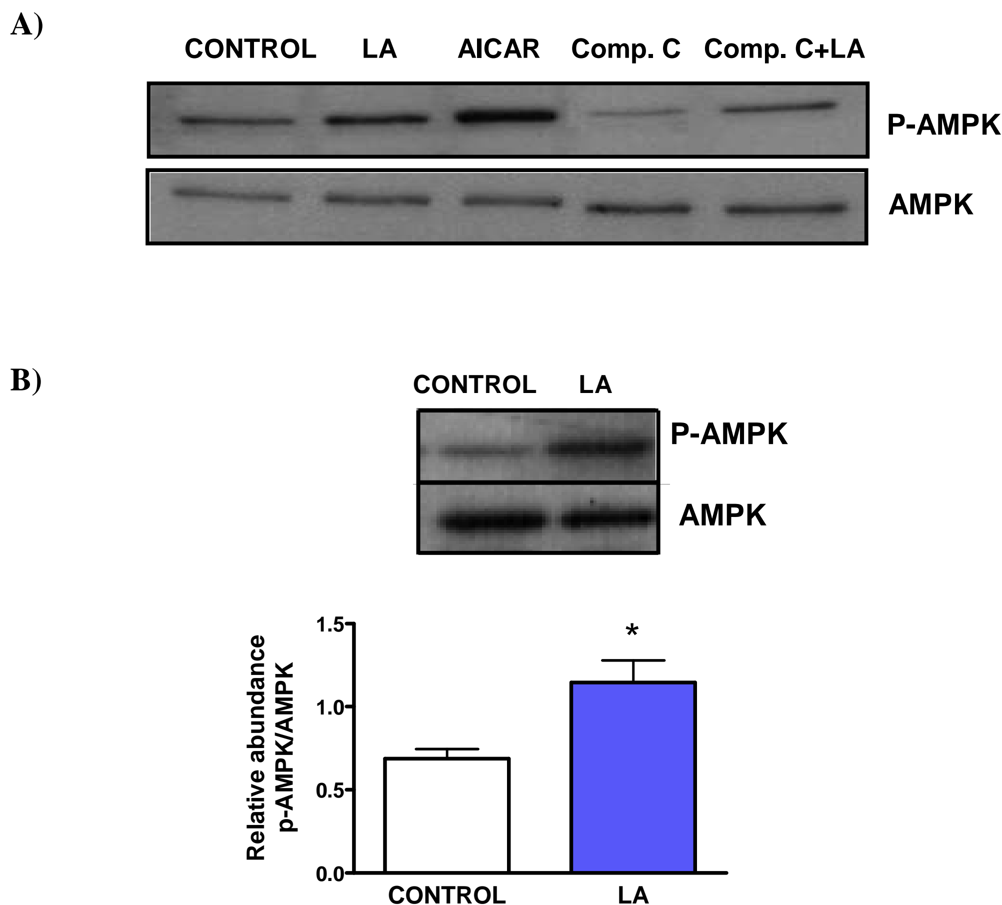


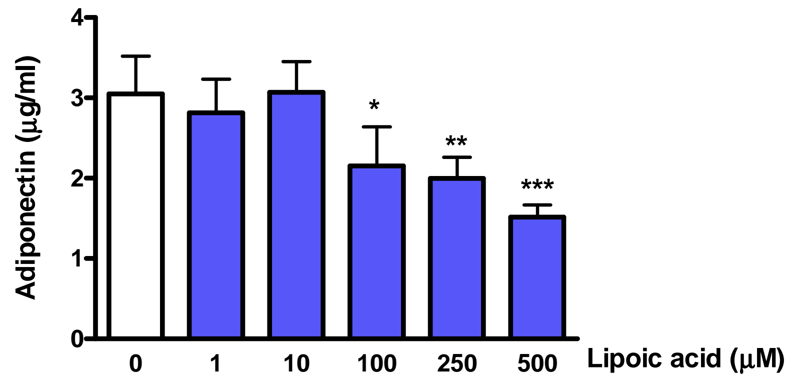
Figure 4. Analysis of AMPK phosphorylation in 3T3-L1 adipocytes. (**A**) p-AMPKα (Thr172) and total-AMPK in adipocytes treated with LA (250 µM) , AICAR (2 mmol/L), Compound C (20 µM) during 30 min. (**B**) Representative blots and densitometric analysis of p-AMPKα (Thr172) and total-AMPK in adipocytes treated during 24 hours with LA (250 µM). Whole cell lysates were subjected to gel electrophoresis and immunoblotted using specific antibodies for p-AMPKα (Thr-172) and AMPKα as described in material and methods. Data are expressed as mean ± SE of 6 independent experiments, *$P<0.05$ *vs* Control (vehicle-treated cells).

Effects of LA on adiponectin production and gene expression in 3T3-L1 adipocytes

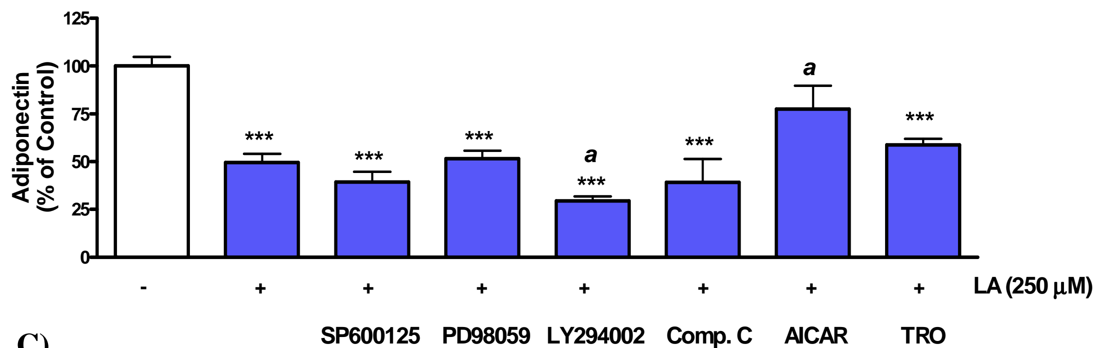
Treatment of 3T3-L1 adipocytes with LA for 24 h caused a concentration-dependent inhibition of adiponectin secretion (Figure 5A), which was only statistically significant at the highest concentrations tested (-30 to - 50%, $P<0.05$ - 0.001 for 100-500 μM). A similar dose-dependent inhibitory effect was observed on adiponectin gene expression in LA-treated adipocytes (data not shown).

Moreover, we tested the effects of several inhibitors and activators of different signaling pathways on adiponectin secretion and gene expression. Our data evidenced that treatment with the PI3K inhibitor LY294002 (50 μmol/l) significantly potentiated the inhibitory effect of LA ($P<0.05$) on adiponectin secretion (Figure 5B) without affecting adiponectin gene expression (Figure 5C). In contrast, co-treatment with the AMPK activator AICAR (2 mmol/l) was able to reverse the LA-induced inhibition of adiponectin secretion (Figure 5B, $P<0.05$) and gene expression (Figure 5C, $P<0.01$). Co-treatment with troglitazone (10 μM) also moderately prevented the LA inhibitory actions on adiponectin gene expression (Figure 5C, $P<0.05$) while no significant effects were observed on adiponectin secretion (Figure 5B). The JNK inhibitor SP600125, the MAPK inhibitor PD98059 and the AMPK inhibitor Compound C did not affect the inhibition of LA on adiponectin (Figure 5B and 5C).

A)



B)



C)

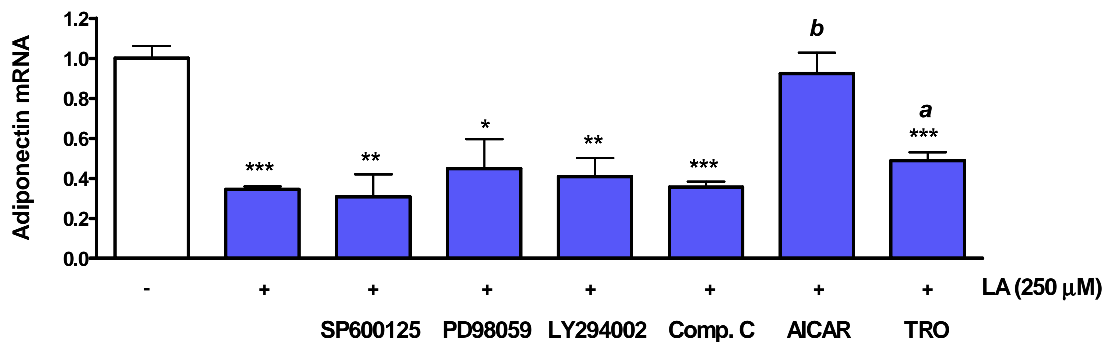


Figure 5. Effects of LA on adiponectin production in 3T3-L1 adipocytes. (**A**) Dose-dependent action of LA (1-500 µM) on basal adiponectin secretion. (**B-C**) Effects of the JNK inhibitor SP600125, the ERK1/2 inhibitor PD98059, the PI3K inhibitor LY294002, the AMPK inhibitor Compound C, the AMPK activator AICAR, and the PPARγ agonist Troglitazone (TRO) on LA-induced inhibition of (**B**) adiponectin secretion and (**C**) adiponectin gene expression over 24 hours of treatment in 3T3-L1 adipocytes. Data are expressed as mean ± SE of 3-4 independent experiments, ***P<0.001; **P<0.01; *P<0.05 *vs* Control (vehicle-treated cells); ^{b}P<0.01, ^{a}P<0.05 *vs* LA-treated cells.

DISCUSSION

Several studies have described the glucose-lowering and insulin-sensitizing action of LA both in rodents (Kandeil *et al.* 2011; Cummings *et al.* 2010; Wang *et al.* 2010) and in humans (Lee and Dogoua, 2011; Ziegler *et al.* 2004). Our current data also support the ability of dietary supplementation with LA to improve whole body insulin sensitivity even when a high-fat diet is consumed. These results are in agreement with those of Timmers *et al.* (2010) who found that fasting plasma insulin levels were lower if receiving LA in rats fed low and high-fat diets. Interestingly, our data demonstrated that the ability of LA to ameliorate insulin resistance was not only secondary to its body weight lowering actions since statistically significant differences were observed between LA-treated groups and their corresponding PF-groups. This outcome is in agreement with a recent study showing that LA (at doses that do not affect energy intake or body weight) ameliorates the onset of type 2 diabetes induced by fructose in UCD-T2DM rats (Cummings *et al.* 2010).

Several mechanisms have been proposed to mediate the insulin-sensitizing action of LA, including the improvement of glucose homeostasis by the preservation of beta-cell function (Cummings *et al.* 2010). In fact, similarly to metformin, LA inhibits insulin secretion in vitro from MIN-6 cells and isolated rat islets (Targonsky *et al.* 2006). It has been also suggested that LA prevents the development of diabetes in diabetes-prone obese rats by reducing triglyceride accumulation in non-adipose tissues such as muscle, pancreatic beta-cells and liver (Park *et al.* 2008; Song *et al.* 2005). Some of these positive effects on insulin sensitivity have been proposed to be mediated by the modulation of AMPK. Thus, it has been demonstrated that LA increases insulin sensitivity by activating AMPK in skeletal muscle (Lee *et al.* 2005) and beta-cells (Targonsky *et al.* 2006). Moreover, Park *et al.* (2008) described that LA decreases lipogenesis in liver through AMPK-dependent and AMPK-independent pathways. However, the study of Timmers *et al.* (2010) observed that prevention of high-fat diet-induced muscular lipid accumulation in rats by alpha-LA is not mediated by AMPK activation.

Regarding the role of AMPK activation in adipose tissue, it has been suggested to be beneficial in insulin-resistant states, particularly as AMPK activation also reduces cytokine secretion in adipocytes (Timmers *et al.* 2010; Daval *et al.*

2006). In the present trial, we have evaluated the role of LA in AMPK phosphorylation in epididymal fat depot of Wistar rats and 3T3-L1 adipocytes. To our knowledge the present study is the first to show the direct ability of LA to activate AMPK phosphorylation in WAT. A recent study also reported an increased phosphorylation of AMPK in WAT from ovariectomized rats treated with LA in parallel with the inhibition of food intake and adipose tissue size. However, the authors did not address if this stimulation of AMPK is merely the consequence of weight loss and reduced adiposity as pair-fed groups were not included in the study. The novelty of our study relies on the fact that we observed a significant stimulation of AMPK phosphorylation in LA-treated groups as compared with pair-fed animals, suggesting a direct ability of LA to activate AMPK. This finding is further supported by our observation that LA increases AMPK phosphorylation in cultured adipocytes. Previous studies have suggested that activation of AMPK in rodent adipocytes leads to a decreased lipogenic flux and triglyceride synthesis as well as increased fatty acid oxidation (Orci *et al.* 2004), which could contribute to the adipose-lowering effect of LA. Moreover, LA-induced activation of AMPK in adipose tissue may also contribute to its insulin-sensitizing properties since high levels of FFA have been suggested to cause insulin resistance in all major insulin target organs (Hardie, 2011; Daval *et al.* 2006).

Adiponectin is an adipokine with key insulin-sensiting properties (Ziemke *et al.* 2010). Stimulation of adiponectin production has been described for several insulin-sensitizing molecules including PPARγ activator Rosiglitazone, metformin, and omega-3 fatty acids (Tishinsky *et al.* 2011; Zulian *et al.* 2011; Moreno-Aliaga *et al.* 2010; Ziemke *et al.* 2010; Perez-Matute *et al.* 2007). However, few studies have addressed the effects of LA supplementation on adiponectin and controversial data have been obtained. Thus, the recent study of Cheng *et al.* (2011) suggests that LA treatment (200 mg/kg by gavage during 7 weeks) suppresses the elevation of adiponectin levels induced by ovariectomy. On the other hand, Cummings *et al.* (2010) did not observe any significant change in fasting plasma adiponectin levels in UCD-T2DM rats fed with fructose in the absence or presence of LA (80 mg/kg b.w. during 2 months). In contrast, Houng and Ide (2008) described an elevation in adiponectin circulating levels after dietary supplementation with LA (1-5 g/kg of diet) for 21 days. Our data indicate that dietary supplementation with LA is able to

upregulate adiponectin gene expression in WAT and suggest an increase in the amount of adiponectin produced per gram of adipose tissue. Interestingly, this ability of LA to stimulate adiponectin was not only secondary to its body weight lowering actions since statistically significant differences were observed between LA-treated groups and their corresponding PF-groups. These data suggest that the increase in adiponectin production by adipocytes could be involved in the insulin-sensitizing properties of LA. In fact, we found a negative correlation between adiposity-corrected adiponectin plasma levels and the HOMA index, a recognized marker of insulin-resistance. The different outcomes obtained in those trials could be related to differences in the animal models, the dose and the duration of treatments with LA.

In contrast, the stimulatory action of LA on adiponectin that we reported *in vivo* was not present in cultured adipocytes. This fact suggests that LA dietary supplementation stimulates adiponectin production by an indirect mechanism or that some in vivo metabolic processing is required to do so. Surprisingly, we found that in cultured adipocytes LA inhibits in a dose-dependent manner the secretion of adiponectin. The secretion of adiponectin from adipocytes is a complex process regulated by hormones and nutritional and metabolic changes (Wang *et al.* 2008). This action of LA seems to be independent of its antioxidant properties since the effects of different antioxidant on adiponectin secretion by cultured adipocytes widely differ. Thus, vitamin E but not NAC has been shown to induce adiponectin expression in 3T3-L1 adipocytes (Landrier *et al.* 2009). Moreover, Vitamin C does not modify adiponectin secretion in primary cultured rat adipocytes (Garcia-Diaz *et al.* 2010). Previous studies of our group have reported differential effects of same concentrations of LA on adipokine secretion. Thus, LA inhibits leptin but stimulates apelin secretion in 3T3-L1 adipocytes (Fernandez-Galilea *et al.* 2011; Prieto-Hontoria *et al.* 2011).

We also analyzed the potential signal transduction pathways mediating the inhibitory effects of LA on adiponectin secretion and gene expression. Several studies have related AMPK activation with the production of adiponectin, but the results are controversial. Thus, Lihn *et al.* (2004) described that the AMPK activator AICAR upregulates adiponectin gene expression in human adipose tissue explants. Recently, Wang *et al.* (2011) also described an increase in adiponectin protein levels in extracts from AICAR-treated 3T3-L1 adipocytes. In contrast, Huypens *et al.*

(2005) found that AICAR caused a decrease in adiponectin protein expression in 3T3-L1 adipocytes. Moreover, they described that the antidiabetic drug Metformin reduces adiponectin protein expression and release in 3T3-L1 adipocytes involving activation of AMPK. Similarly, we found that both AICAR (data not shown) and LA inhibit adiponectin mRNA levels and the amount of adiponectin secreted to the media, also supporting the idea that AMPK activation could explain the inhibitory effect of LA on adiponectin. However, our data showed that treatment with AICAR in combination with LA abolished the inhibitory effect of LA in adiponectin secretion and gene expression. On the other hand, evidence is accumulating that adiponectin, at least in part, acts by activating AMPK (Wu *et al.* 2004; Yamauchi *et al.* 2002) revealing a complex regulation and cross-talk between adiponectin and AMPK activation in adipocytes.

It is well known the ability of the PPARγ agonists thiazolidinedione family to increase the production of adiponectin (Lorente-Cebrian *et al.* 2006) in adipocytes, which has been related to their insulin-sensitizing effects (Yu *et al.* 2002). Our data revealed that Troglitazone partially prevented the LA inhibitory action on adiponectin gene expression, suggesting that PPARγ activation is able to override in part the transcriptional inhibition of adiponectin induced by LA.

In contrast, our results revealed that treatment with the PI3K inhibitor LY294002 mimicked LA actions (data not shown), and significantly potentiated the inhibitory effect of LA on adiponectin secretion. A recent study of our group has demonstrated that LA inhibits Akt phosphorylation in 3T3-L1 adipocytes (Prieto-Hontoria *et al.* 2011). Taking together these data suggest that the inhibition of adiponectin by LA is likely to be mediated by PI3K/Akt pathway. In this context, several studies have shown that inhibition of PI3K inhibits both basal and insulin-stimulated adiponectin secretion in 3T3-L1 (Blumer *et al.* 2008; Pereira *et al.* 2005). However, it should be mentioned that other study has found that insulin and PI3-kinase negatively regulate production of adiponectin (Fasshauer *et al.* 2002). The inhibitory action of LA on adiponectin seems to be independent of JNK and MAPK pathways.

In conclusion, our present data demonstrated the ability of LA to improve insulin-sensitivity, which could be related to the stimulation of AMPK phosphorylation and adiponectin in WAT observed in LA-treated rats. However, our

results also revealed that LA reduces adiponectin gene expression and protein secretion in 3T3-L1 adipocytes possibly through the activation of AMPK. These data suggest a complex regulation and cross-talk between adiponectin and AMPK activation in adipocytes by LA.

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4.3.- Lipoic acid inhibits leptin secretion and Sp1 activity in adipocytes

Lipoic Acid Inhibits Leptin Secretion and Sp1 Activity in Adipocytes

Prieto-Hontoria PL, Pérez-Matute P, Fernández-Galilea M, Martínez JA and Moreno-Aliaga MJ

Mol Nutr Food Res, doi: 10.1002/mnfr.201000534, 2011.

Resumen

El ácido lipoico (LA) es un antioxidante con potencial terapéutico en diversas enfermedades como la diabetes y la obesidad. La hiperleptinemia y el estrés oxidativo desempeñan un papel importante en el desarrollo de enfermedades relacionadas con la obesidad. El objetivo de este estudio fue examinar *in vivo* e *in vitro* los efectos del LA sobre la producción de leptina, así como dilucidar los mecanismos y vías de señalización implicadas en las acciones del LA. La suplementación dietética con LA disminuyó tanto la leptina circulante como los niveles de mRNA en el tejido adiposo de roedores. El tratamiento de los adipocitos 3T3-L1 con LA también provocó una inhibición dosis-dependiente de la expresión génica y de la secreción de leptina. Por otra parte, el LA estimuló la utilización anaeróbica de la glucosa a lactato, porcentaje que se relacionó negativamente con la secreción de leptina. Además, el LA aumentó la fosforilación de Sp1 e inhibió la actividad de Sp1 en los adipocitos 3T3-L1. Por otra parte, el LA inhibió la fosforilación de Akt, una diana de la cascada de señalización de PI3K. El tratamiento con el inhibidor de PI3K, LY294002, ejerció acciones similares a las del LA, y así inhibió dramáticamente la expresión génica y la secreción de leptina y estimuló la fosforilación de Sp1. Todos estos datos sugieren que la fosforilación de Sp1 y la correspondiente reducción de su unión al DNA es probable que estén involucrados en la inhibición de la leptina inducida por el LA, lo cual, a su vez, podría estar mediado, al menos en parte, por la supresión de la vía PI3K/Akt.

ABSTRACT

Lipoic acid (LA) is an antioxidant with therapeutic potential on several diseases such as diabetes and obesity. Hyperleptinemia and oxidative stress play a major role in the development of obesity-linked diseases. The aim of this study was to examine *in vivo* and *in vitro* the effects of LA on leptin production, as well as to elucidate the mechanisms and signaling pathways involved in LA actions. Dietary supplementation with LA decreased both circulating leptin, and adipose tissue leptin mRNA in rats. Treatment of 3T3-L1 adipocytes with LA caused a concentration-dependent inhibition of leptin secretion and gene expression. Moreover, LA stimulated the anaerobic utilization of glucose to lactate, which negatively correlated with leptin secretion. Furthermore, LA enhanced phosphorylation of Sp1 and inhibited Sp1 transcriptional activity in 3T3-L1 adipocytes. Moreover, LA inhibited Akt phosphorylation, a downstream target of PI3K. Treatment with the PI3K inhibitor LY294002 mimicked LA actions, dramatically inhibiting both leptin secretion and gene expression and stimulating Sp1 phosphorylation. All of these data suggest that the phosphorylation of Sp1 and the accompanying reduced DNA-binding activity are likely to be involved in the inhibition of leptin induced by LA, which could be mediated in part by the abrogation of the PI3K/Akt pathway.

INTRODUCTION

Obesity is a disease with serious public health implications, associated with insulin resistance, type 2 diabetes, hypertension, dyslipemia and atherosclerosis. A growing body of evidence supports that obesity is linked to a state of chronic oxidative stress, which may result from a combination of adipokine imbalance, and reduced antioxidant defenses (Vincent *et al.* 2007).

White adipose tissue (WAT) dysfunction plays a critical role in the development of obesity-associated disorders. Indeed, WAT is a complex and metabolically active organ, with an important relevance in regulating whole-body metabolism. In addition to its primary role as a fuel reservoir, WAT has been confirmed as a major endocrine organ, which synthesizes and secretes an array of sex steroids, and bioactive peptides termed 'adipokines', involved in the physiological regulation of fat storage, energy metabolism, food intake, insulin sensitivity, and immune function among others (Rasouli y Kern, 2008).

Leptin is an adipokine that plays an important role regulating food intake and energy balance. Although leptin deficiency leads to severe obesity in rodents and humans, it is clearly established that serum leptin concentrations are proportional to the amount of adipose tissue mass and therefore, most frequently obesity is accompanied by hyperleptinemia, associated with resistance to the actions of this adipokine (Havel, 2000). Hyperleptinemia itself has also been suggested to be involved in the pathogenesis of the co-morbidities associated to obesity such as type 2 diabetes mellitus, hypertension, cardiovascular disease, and cancer (Patel *et al.* 2008). Leptin has also been shown to induce perturbations in the anti-oxidative defence system and increase oxidative stress in both animals and humans (Bouloumie *et al.* 1999). In fact, hyperleptinemia is associated with oxidative stress in type 2 diabetes mellitus, and decreases insulin secretion from the pancreatic β cells (Fehmann *et al.* 1997). Therefore, antioxidant therapy has been proposed as an useful strategy for attenuation of oxidative stress and hyperleptinemia in obesity (Perez.Matute *et al.* 2009; Vincent *et al.* 2007).

Lipoic acid (LA; 1,2-dithiolane-3-pentanoic acid) is a naturally occurring short-chain fatty acid with sulphydryl groups, which is a necessary cofactor for mitochondrial enzymes (Kim *et al.* 2004; Packer *et al.* 2001). Aside from its enzymatic role, *in vitro* and *in vivo* studies suggest that LA also acts as a powerful

micronutrient with diverse pharmacological and antioxidant properties (Smith *et al.* 2004). A recent study has demonstrated that down-regulation of LA synthase (LASY), the enzyme involved in the endogenous synthesis of LA, reduced endogenous levels of LA as well as critical components of the antioxidant defence network, increasing oxidative stress. Moreover, this down-regulation of LA synthase induced a significant loss of mitochondrial membrane potential and decreased insulin-stimulated glucose uptake in skeletal muscle cells along with an increased inflammatory response which, in turn, leads to increased insulin resistance, mitochondrial dysfunction and inflammation (Padmalayam *et al.* 2009). Thus, LA can be considered as a promising therapeutic approach for chronic inflammatory diseases such as diabetes and obesity (Henriksen, 2006; Doggrell, 2004). Thus, it has been reported the ability of LA to reduced body weight gain in rodents by suppressing food intake and increasing energy expenditure (Timmers *et al.* 2010; Wang *et al.* 2010; Prieto-Hontoria *et al.* 2009; Kim *et al.* 2004). Studies of our group have also described that LA decreased feed efficiency and intestinal sugar transport, which could explain at least in part its reducing effects on body weight gain despite of high-fat diet feeding (Prieto-Hontoria *et al.* 2009). Moreover, a recent study in pre-obese and obese human subjects have evidenced that LA (800 mg/day for 4 months) induced significant reductions of body weight, BMI, blood pressure, and abdominal circumference, together with a decrease in inflammatory markers (Carbonelli *et al.* 2010).

Some of these studies have described that the reduction of body weight and adiposity induced by LA is accompanied by a decrease in plasma leptin levels (Huong and Ide, 2008; Song *et al.* 2005; Kim *et al.* 2004). However, it still remains to be elucidated if this reduction in circulating leptin levels is merely a consequence of reduced fat mass or if LA treatment can directly inhibit leptin production. Therefore, the first aim of the present study was to examine the effects of LA supplementation on leptin production in lean and highm fat-fed rats. Moreover, we investigate the *in vitro* effects of LA on leptin secretion and gene expression in cultured adipocytes. We also aimed to find out the potential molecular mechanisms involved. Because of the importance that glucose metabolism and Sp1 trancription factor play in the regulation of leptin secretion by the adipocytes (Moreno-Aliaga *et al.* 2007 and 2001; Mueller *et al.* 1998), we tested the effects of LA on the anaerobic utilization of glucose to lactate and on Sp1 activity and its regulation by

phosphorylation. Moreover, we also analyzed the potential signaling pathways underlying the effects of LA on leptin and Sp1.

RESULTS

Effects of LA on leptin circulating levels and gene expression in vivo

Table 1 summarizes that treatment with LA decreased food intake, body weight gain and adipose tissue size in both control or high fat-fed rats. Moreover, the group fed on a high-fat diet and treated with LA (OLIP group) had a lower body weight gain and reduced visceral fat weight than its corresponding PF-group ($P<0.05$), which received the same amount of food than LA-treated animals but without supplementation with LA. As expected, leptin circulating levels were significantly higher in the obese group in comparison with the control group ($P<0.001$). On the contrary, LA treatment significantly decreased leptin plasma levels ($P<0.001$ for CLIP and $P<0.01$ for OLIP) (Figure 1A). The patterns of LA effects on leptin gene expression were similar to those observed in plasma levels (Figure 1B). The decrease in leptin was also observed in the PF-groups, but it was less pronounced than in the LA-supplemented groups, and no statistically differences were found when comparing both groups (Figure 1A and 1B).

As expected, leptin levels were positively correlated with the size of total WAT ($P<0.001$) (Figure 1C).

Table 1. Body weight gain and white adipose tissue weights in control, Pair-Fed groups and lipoic acid-treated lean and obese rats**.**

	CONTROL (***n*** **= 10**)	**CLIP** (***n*** **= 10**)	**PF-CLIP** (***n*** **= 6**)	**OBESE** (***n*** **= 10**)	**OLIP** (***n*** **= 12**)	**PF-OLIP** (***n*** **= 6**)
Initial Body weight (g)	215.1 ± 6.4	212.3 ± 5.8	201.8 ± 7.6	218.1 ± 5.6	214.7 ± 6.0	209.8 ± 4.0
Final Body weight (g)	391.8 ± 12.2	320.3 ± 10.9 **	317.8 ± 24.3 *	482.0 ± 13.7 ***	378.4 ± 13.1 ###	409.8 ± 8.24 #
Body weight gain (g)	176.7 ± 8.3	108.0 ± 7.8 ***	116.0 ± 20.2 *	263.9 ± 11.7 ***	163.7 ± 10.5 ###	200.1 ± 6.86 #, *a*
Food Intake (g/day)	22.9 ± 0.2	20.0 ± 0.1 ***	Ψ	19.4 ± 0.3 ***	16.2 ± 0.2 ###	Ψ
Visceral fat (g)	22.9 ± 1.8	10.4 ± 0.9 ***	11.21 ± 2.36	49.9 ± 2.5 ***	22.0 ± 1.9 ###	30.3 ± 1.5 *a*
Subcutaneous fat (g)	5.6 ± 0.5	3.4 ± 0.3 **	3.5 ± 0.8	14.7 ± 2.0 ***	6.7 ± 0.5 ###	8.2 ± 0.5 *

Data (mean ± SE) were analysed by one-way ANOVA followed by Bonferroni *post-hoc* test.

***P<0.001, **P<0.01, *P<0.05 *vs.* Control group. [###]P<0.001, [#]P<0.05 *vs.* Obese group. [a]P<0.05 *vs.* OLIP group.

Ψ Same food intake that CLIP group. Ψ Same food intake that OLIP group.

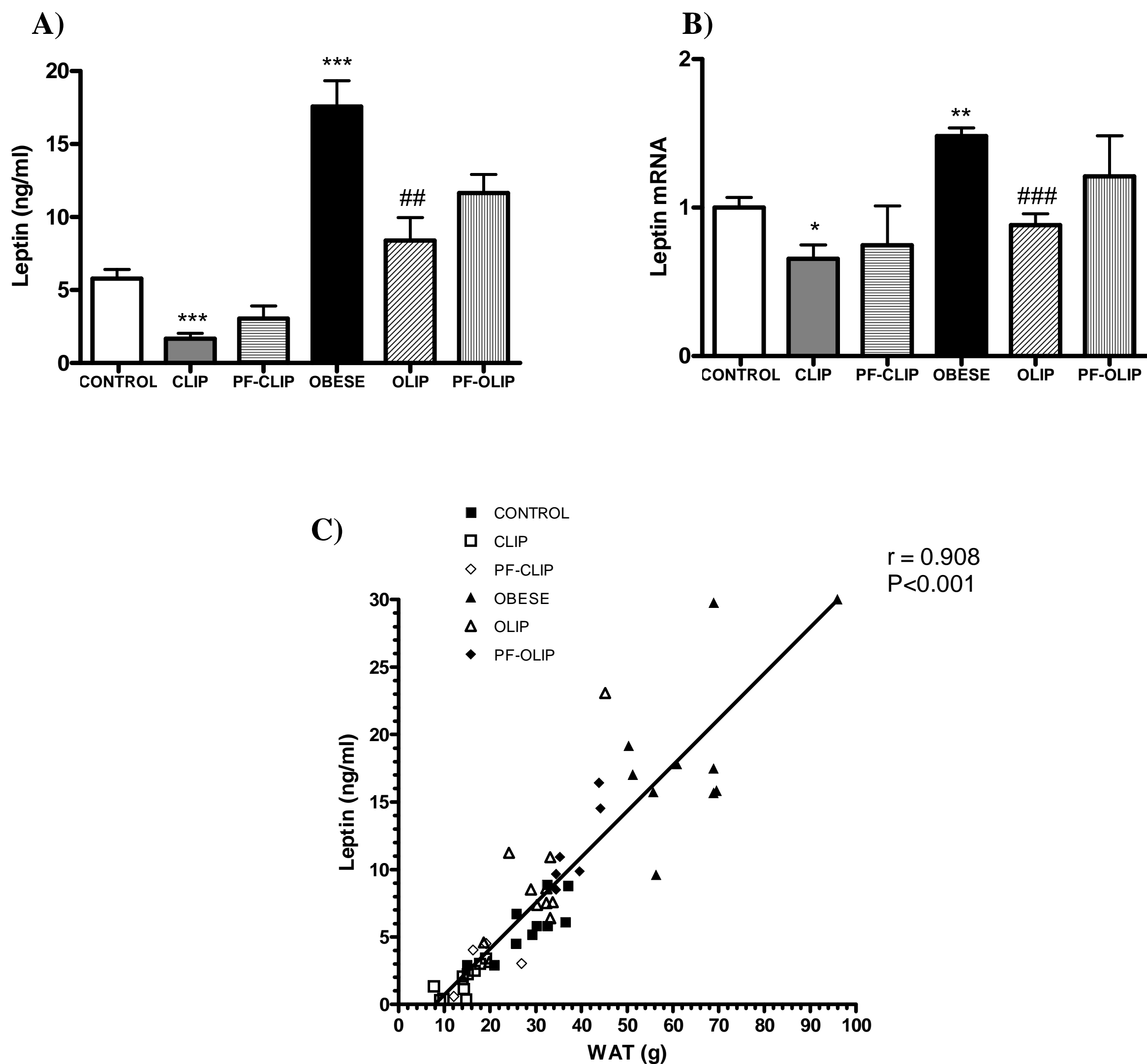

Figure 1. LA supplementation inhibits leptin in control and high-fat fed rats**.** (**A**) Effects of LA (0.25 g/100 g diet during 56 days) on leptin circulating levels and (**B**) leptin gene expression in epididymal fat. (**C**) Pearson´s correlation between leptin circulating levels and total WAT. Data are expressed as mean ± SE. (n=10-12 for Control, CLIP, Obese and OLIP; n=6 for PF-CLIP and PF-OLIP). ***P<0.001, *P<0.05 *vs.* Control; ###P<0.001, ##P<0.01 *vs.* Obese.

Effects of LA on leptin secretion and gene expression in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with LA caused a concentration-dependent inhibition of basal leptin secretion (Figure 2A), which was only statistically significant at the highest concentrations tested (-69 and - 91% for 250 and 500 μM respectively, P< 0.01). Basal leptin gene expression was also significantly inhibited by the presence of LA at concentrations of 100 and 250 μM (-81-91%, P<0.05-0.001, respectively) (Figure 2B). Moreover, we also tested the effects of LA

treatment on insulin-stimulated leptin secretion in a model of primary rat adipocytes anchored to a collagen matrix, which responds to more physiological concentrations of insulin than 3T3-L1 adipocytes. Figure 2C shows that LA was also able to partially inhibit the stimulatory effect of insulin on leptin secretion.

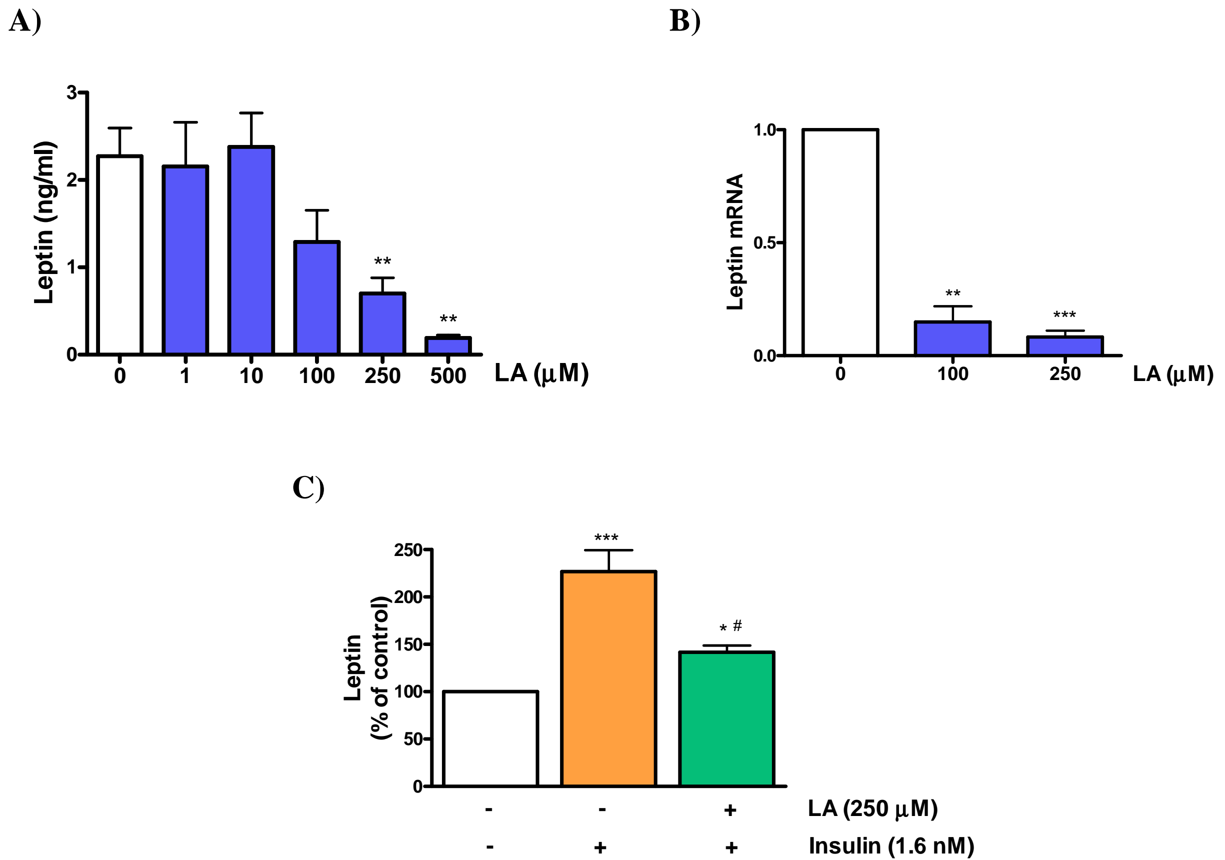

Figure 2. LA inhibits leptin production in 3T3-L1 adipocytes. Effects of different concentrations of LA (1-500 μM) on (**A**) basal leptin secretion and (**B**) leptin gene expression in 3T3-L1 adipocytes, and (**C**) on insulin-stimulated leptin secretion in primary cultured epididymal rat adipocytes treated over 48 h. Data are expressed as mean ± SE of 3-6 independent experiments, ****P*<0.001, ***P*<0.01, **P*<0.05 *vs* Control (vehicle-treated cells); #*P*<0.05 *vs.* insulin-treated cells.

Effects of LA on glucose uptake, lactate production and the percentage of glucose metabolized to lactate in 3T3-L1 adipocytes

Glucose uptake was not affected by LA-treatment (Figure 3A). However, LA increased lactate production by 44% at the highest concentration tested (500 μM, *P*<0.01), whereas lower concentrations did not show any effect (Figure 3B). Treatment of 3T3-L1 adipocytes with LA (0-500 μM) significantly increased in a concentration - dependent manner, the percentage of glucose that is metabolized to

lactate (*P*<0.01- *P*<0.001; 100-500 μM) (Figure 3C). Furthermore, the percentage of glucose metabolized to lactate was inversely correlated with leptin secretion, as shown in Figure 3D (r= -0.448; *P*<0.001).

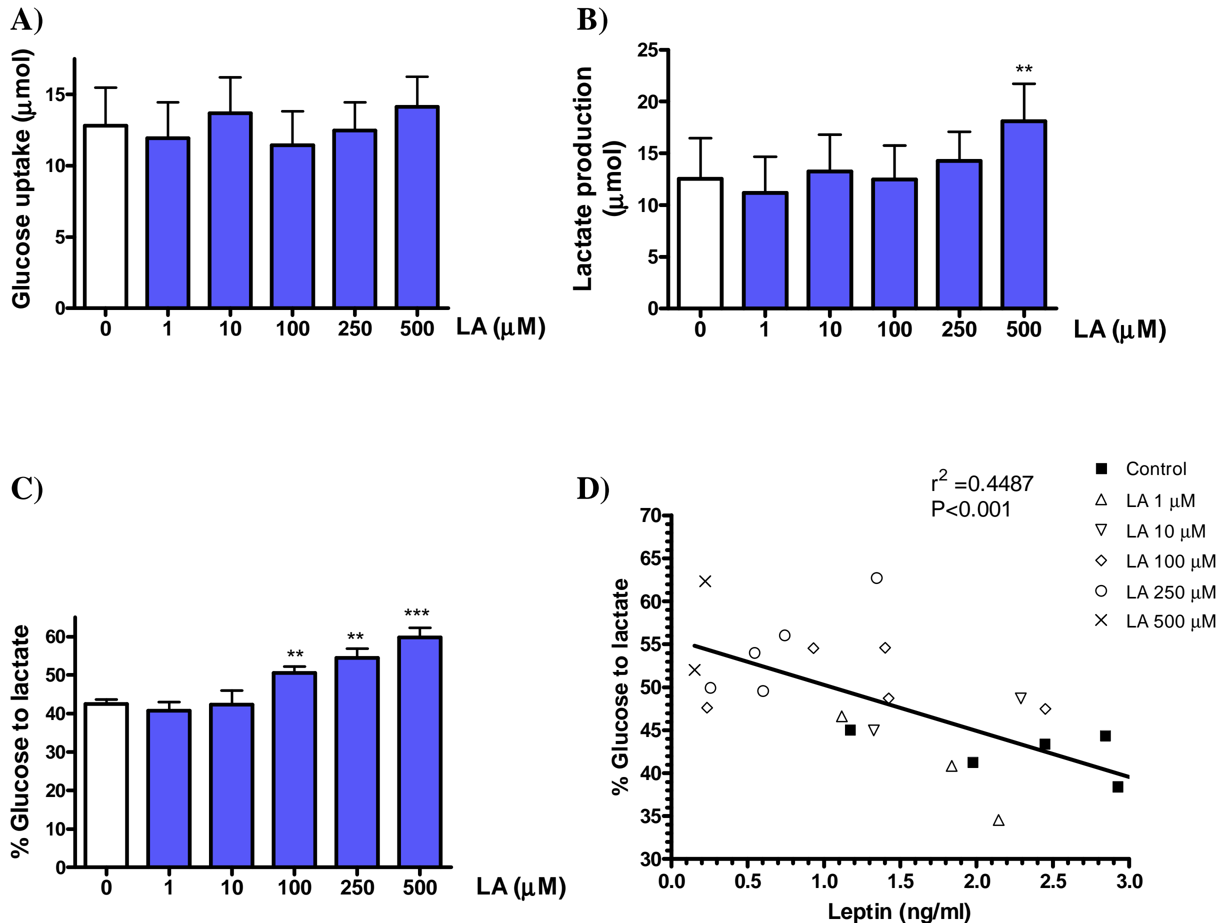

Figure 3. LA increases the anaerobical utilization of glucose in adipocytes. Effects of different concentrations of LA (1-500 µM) on (**A**) glucose uptake, (**B**) lactate productions and (**C**) the percentage of glucose metabolized to lactate in 3T3-L1 adipocytes treated over 24 h. Data are expressed as mean ± SE (n=6). (**D**) Relationship between leptin secretion levels and the percentage of glucose metabolized to lactate. ****P*<0.001, ***P*<0.01 *vs* Control.

Effects of LA on Sp1 -DNA binding activity

To define the mechanisms of LA inhibitory action on leptin, we study LA effects on the transcription factor Sp1, which has been shown to mediate the activation of leptin promoter in response to insulin-stimulated glucose metabolism (Moreno-Aliaga *et al.* 2001). As shown in Figure 4A, nuclear extracts from LA-treated adipocytes for 24 h

exhibited a decreased abundance of Sp1-DNA complexes ($P<0.01$), suggesting a significant inhibitory effect of LA on Sp1 transcription factor activity.

Several studies have demonstrated that changes in the phosphorylation status of Sp1 are controlling the ability of this transcription factor to bind to DNA (Tan y Khachigian *et al.* 2009). Our data showed that the phosphorylation of Sp1 in Thr453 was significantly increased ($P<0.05$) in LA-treated adipocytes (Figure 4B). Moreover, we found that treatment of nuclear extracts with protein phosphatase 1 (PP1) in order to dephosphorylate Sp1 abolished the inhibitory effect of LA on Sp1 activity (Figure. 4C).

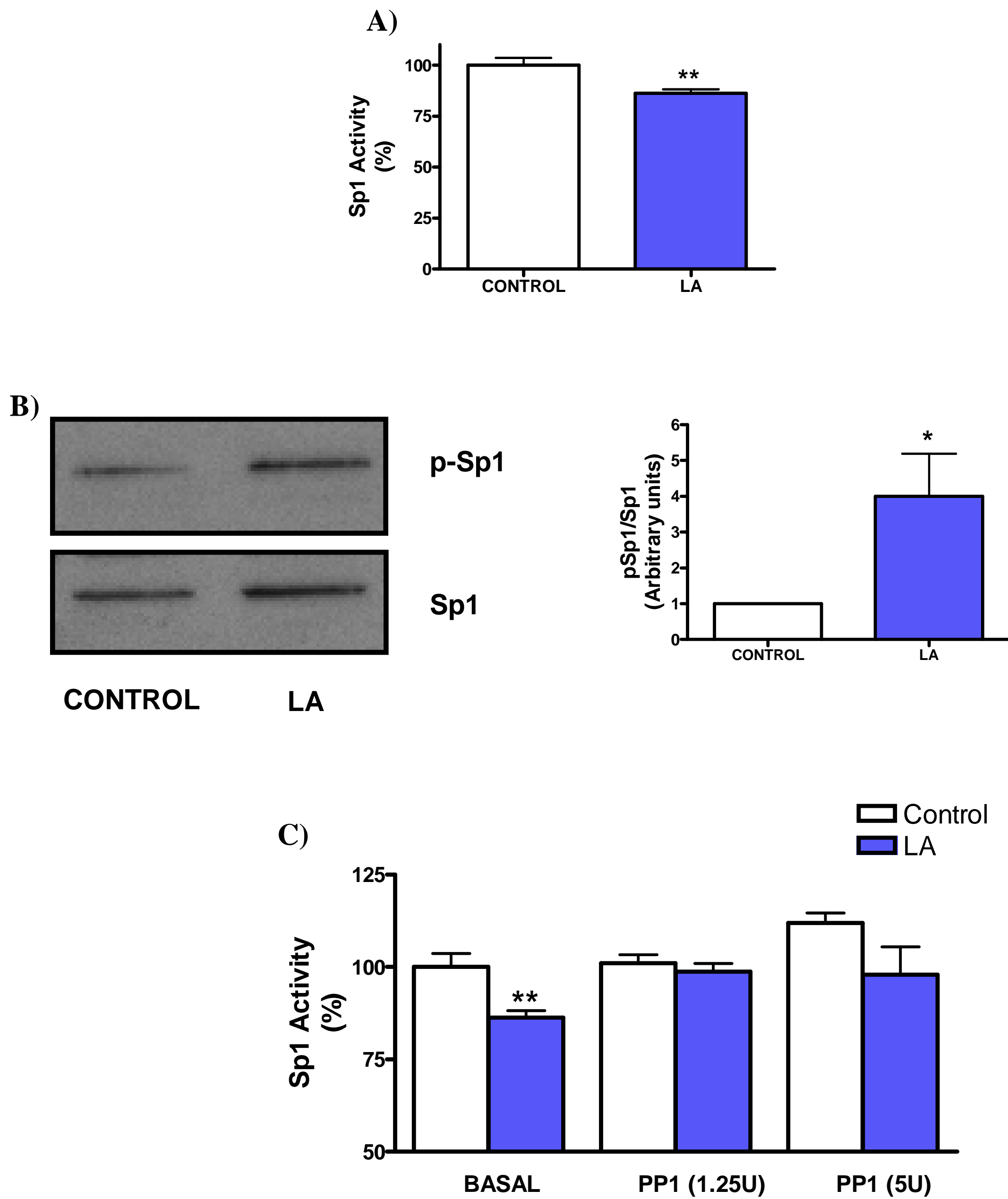

Figure 4. LA inhibits Sp1 activity through phosphorylation. (**A**) Sp1-DNA binding activity in nuclear extracts from LA (250 µM)-treated and untreated 3T3-L1 adipocytes during 24 h. (**B**) Effects of LA (250 µM) on Sp1 phosphorylation in 3T3-L1 adipocytes. (**C**) Protein Phosphatase 1 (PP1) abolishes the inhibitory effect of LA on Sp1 activity. Dephosphorylation reactions were performed by incubating with PP1 at 30ºC for 15 min the nuclear extracts obtained from control and LA-treated cells. Data are expressed as mean ± SE. (n=3). *P<0.05, **P<0.01 *vs.* Control.

Effects of PI3K, ERK1/2 and JNK inhibitors on LA inhibitory actions on leptin production and Sp1 phosphorylation in 3T3-L1 adipocytes

The effects of several inhibitors of different signaling pathways on both Sp1 phosphorylation and on leptin secretion and gene expression were tested.

Our data evidenced that treatment with the PI3K inhibitor LY294002 (50 μmol/l) mimicked LA actions, and dramatically reduced both leptin secretion (Figure 5A) and gene expression (Figure 5B), as well as increased Sp1 phosphorylation (Figure 5C). Moreover, we also found that LA treatment caused a significant ($P<0.05$) inhibition of Akt Ser437 phosphorylation in 3T3-L1 adipocytes (Figure 5D). The JNK inhibitor (SP600125) also stimulated Sp1 phosphorylation in adipocytes, but without affecting the LA actions on phosphorylation of Sp1 or leptin secretion. Moreover, the MAPK inhibitor (PD98059) was able to reverse the LA-induced phosphorylation of Sp1, without modifying the inhibitory action of LA on leptin secretion (Figures. 5A-5C).

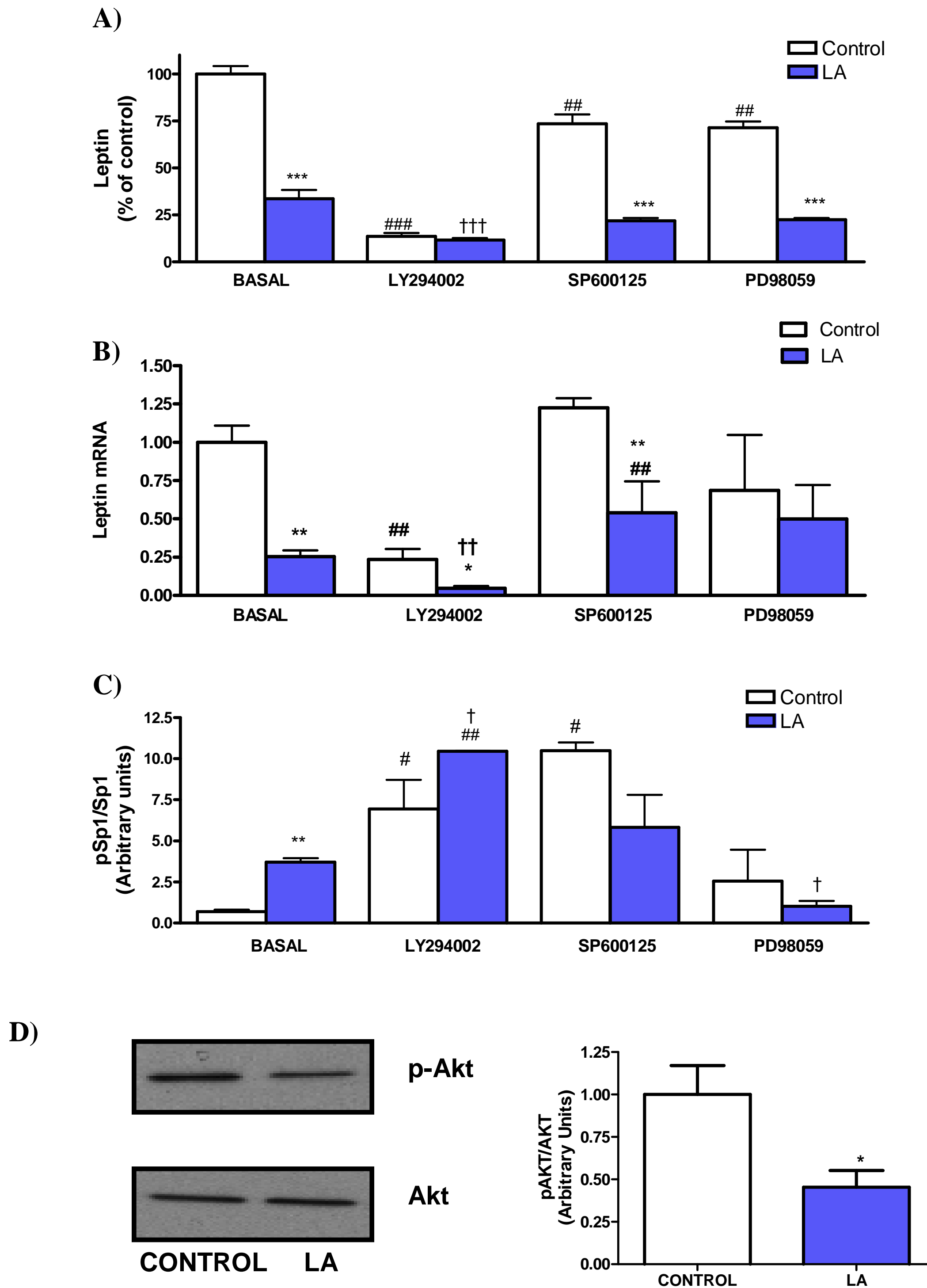

Figure 5. Analysis of the signaling pathways involved in LA actions on leptin and Sp1 phosphorylation. Effects of the PI3K inhibitor LY294002, the JNK inhibitor SP600125, and the ERK1/2 inhibitor PD98059 on LA-induced inhibition of (**A**) leptin secretion and (**B**) leptin gene expression, and (**C**) Sp1 phosphorylation in 3T3-L1 adipocytes. (**D**) Analysis of Akt (Ser-473) activation in mature 3T3-L1 adipocytes after treatment for 30 min with LA (250 µM). Data are expressed as mean ± SE. (n=3-8 independent experiments). $^{***}P<0.001$, $^{**}P<0.01$, $^{*}P<0.05$ *vs.* respective Control. $^{\#\#\#}P<0.001$, $^{\#\#}P<0.01$, $^{\#}P<0.05$ *vs.* Basal-Control (vehicle-alone treated adipocytes). †††$P<0.001$, ††$P<0.01$, †$P<0.05$ *vs.* Basal-LA-treated cells.

DISCUSSION

The previous studies of our group and others (using the same and higher doses than the used in the present study) have demonstrated that the antiobesity effects of dietary supplementation with LA are secondary, at least in part, to their inhibitory actions on food intake (Prieto-Hontoria *et al.* 2009; Kim *et al.* 2004). Moreover, it has been evidenced that leptin and its receptor are not essential for α-LA-induced anorexia since the reduction of food intake and body weight was also observed in leptin-deficient or leptin receptor–deficient mice (Kim *et al.* 2004).

In addition, it was reported LA given in food not only decreases food intake but also stimulates whole-body energy expenditure (Kim *et al.* 2004), and decreases intestinal α-methylglucoside (α-MG) absorption both in lean and obese rats (Prieto-Hontoria *et al.* 2009). Moreover, other studies showed that dietary supplementation with lower doses of LA that not affect food intake is able to improve glucose metabolism in diabetic rats (Cummings *et al.* 2010). All of these data suggest peripheral metabolic actions of LA in different tissues independently of their central actions on food intake.

In the present trial we have evaluated only the effects of LA on male rats, but it would be interesting to test if a similar or a differential response is observed in females. In this context, previous studies have described that LA stimulates glucose transport activity and insulin signaling in skeletal muscle of lean and obese female Zucker rats (Saengsirisuwan *et al.* 2004; Henriksen *et al.* 1997), as observed in other model of obese male rats (Gupte *et al.* 2009).

Our present data show that dietary supplementation with LA decreases adiposity as well as both circulating levels of leptin, and gene expression in WAT. Moreover, the observed changes in leptin levels were positively correlated with the changes in the size of adipose tissue. These results are in agreement with other studies (Kandeil *et al.* 2011; Huong and Ide, 2008; Song *et al.* 2005; Kim *et al.* 2004) also showing that the body and adipose weight reduction induced by LA treatment is accompanied by a parallel decrease in circulating leptin levels. Moreover, the fact that leptin levels were always slightly lower in LA-treated animals than in their corresponding pair-fed groups suggests a potential direct inhibitory effect of LA on the ability of adipocytes to secrete leptin. However, this possibility had not been yet addressed.

In the present study, we demonstrated for the first time a direct inhibitory effect of LA on both basal-and insulin-stimulated leptin secretions in adipocytes. This effect was concentration dependent, being significant at 250 and 500 µM. Moreover, similar effects of LA were observed on leptin gene expression (from 100 µM), suggesting that LA inhibition of leptin is mediated, at least in part, at transcriptional level. A previous study considered *in vitro* treatment with 50 µM LA as a physiological relevant dose (Shay *et al.* 2009). Moreover, similar concentrations of LA that the used in our present trial have been shown to be effective in regulating other biological and metabolic functions including adipocyte differentiation (Cho *et al.* 2003), mitochondrial biogenesis (Shen *et al.* 2010) and glucose uptake (Moini *et al.* 2002) in the same adipocyte line. We have not measured the circulating levels of LA reached after dietary supplementation, and therefore it is difficult to compare the correspondence between the doses used in the *in vivo* and *in vitro* approaches. However, our findings suggest that the observed decrease in plasma leptin levels after dietary supplementation with LA, is likely to be not only due to a decrease in the size of WAT depots induced by LA, but also a direct inhibitory effect of LA on the ability of adipocytes to secrete leptin could be contributing.

Several *in vitro* and *in vivo* studies have demonstrated that glucose metabolism is a major determinant of leptin production in adipocytes (Moreno-Aliaga *et al.* 2001; Wellhoener *et al.* 2000; Mueller *et al.* 1998). The ability of LA treatment (2.5 mM) for 2-60 min to stimulate glucose uptake in 3T3-L1 cells by inducing a redistribution of GLUT1 and GLUT4 transporters has been described previously (Yaworsky *et al.* 2000). Furthermore, another study in 3T3-L1 cells treated with 250 µM LA has also shown a stimulation of glucose uptake during the first 6 h of treatment. However, a longer pre-incubation period with LA (24-48 h) inhibited glucose uptake into adipocytes, suggesting that the time of treatment with LA is a key factor (Moini *et al.* 2002). Our data show that LA treatment (1-500 µM) during 24 h did not modify basal glucose uptake in 3T3-L1 adipocytes. Therefore, the different results between our present data and previous studies could be explained by the different concentrations tested and the period of treatment evaluated.

It has been suggested that uptake of glucose by itself is not the main determinant of the regulation of leptin production, but subsequent metabolic utilization of glucose is playing an important regulatory role of leptin levels (Moreno-Aliaga *et al.* 2001; Mueller *et al.* 1998). Indeed, leptin secretion has been

found to be inversely proportional to the amount of glucose anaerobically metabolized to lactate (Moreno-Aliaga *et al.* 2002; Perez-Matute *et al.* 2007 and 2005). In this context, our data demonstrate that LA significantly increased in a concentration-dependent manner, the percentage of glucose that is metabolized to lactate. Moreover, a negative correlation between the leptin secretion and the percentage of glucose anaerobically converted to lactate was observed, suggesting that this increase in the anaerobical utilization of glucose could explain, at least in part, the observed inhibitory effects of LA on leptin.

Sp1 is a ubiquitous transcription factor that may function as a cellular glucose sensor (Vaulont *et al.* 2000). Furthermore, Sp1 has been identified as an important transcription factor involved in the regulation of leptin gene. Thus, the site centered at -97 pb of the leptin promoter is conserved in evolution and binds Sp1 present in adipocyte nuclear extracts and contributes to promoter leptin activity (Mason *et al.* 1998). In fact, mutation of this Sp1 site in the proximal human and murine leptin promoters reduced promoter activity (Zhang *et al.* 2002; Mason *et al.* 1998). Moreover, previous studies of our group have demonstrated that Sp1 is involved in the induction of leptin by insulin-stimulated glucose metabolism (Moreno-Aliaga *et al.* 2007). Our results demonstrated that LA treatment (250 μM for 24 h) inhibited Sp1-DNA-binding activity in mature 3T3-L1 adipocytes. In support of these data, a previous study demonstrated that LA decreased the TNF-α and or IL-1β-induced Sp1-binding activity in human umbilical vein endothelial cells (Sung *et al.* 2005). Moreover, a recent trial has also observed that LA inhibited in a dose-dependent manner the activation of Sp1-binding was induced by TGF-β in HepG2 cells (Min *et al.* 2010). On the contrary, Cho *et al.* (2003) observed in adipocytes at the early stage of differentiation that LA (250 μM, 3 h of treatment) did not modify the transcriptional activity of Sp1 in the absence or presence of insulin. Taking together, the inhibitory action of LA on Sp1-binding activity and leptin production suggests that the inhibition of Sp1-mediated transcription is likely to be involved in the LA-induced reduction of leptin production. However, with our present data we cannot rule out the possibility that leptin production could be inhibited by LA through other mechanisms independently of Sp1 activity.

A growing body of evidence suggest that the DNA-binding and transcription activity of Sp1 may increase or decrease in response to the changes in

phosphorylation in many cell types (Chu and Ferro, 2005). Our present data clearly demonstrated the ability of LA to phosphorylate Sp1, and that dephosphorylation by PP1 abolished the inhibitory effect of LA on Sp1 transcriptional activity, strongly suggesting that phosphorylation of Sp1 is likely to be involved in the inhibitory action of LA on leptin gene expression and protein secretion. In this context, previous studies of our group showed that okadaic acid, a potent phosphatase inhibitor, inhibited the stimulation of leptin secretion and mRNA levels induced by insulin-stimulated glucose metabolism (Moreno-Aliaga *et al.* 2007), suggesting the involvement of Sp1 dephosphorylation. Moreover, dephosphorylation of Sp1 by PP1 has been involved in the glucose-mediated activation of several genes including acetyl-CoA carboxylase, aldolase and pyruvate kinase (Schafer *et al.* 1997; Daniel *et al.* 1996).

We also analyzed the potential signal transduction pathways mediating the inhibitory effect of LA on Sp1 phosphorylation and leptin secretion and gene expression. Several kinases including ERK1/2 and PI3K have been involved in the phosphorylation of Sp1 by different factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) (Reisinger *et al.* 2003; Zheng *et al.* 2001). Moreover, it has been shown that the insulin-induced up-regulation of leptin in breast cancer cells is regulated by PI3K and ERK1/2 and depends on Sp1 interaction with specific regions of the leptin promoter (Bartella *et al.* 2008). Our data show that treatment with the PI3K inhibitor LY294002 mimicked and potentiated LA actions on the phosphorylation of Sp1. Moreover, treatment with the PI3K inhibitor dramatically reduced both basal leptin secretion and gene expression, suggesting an important role of this pathway in controlling basal leptin secretion. This finding is in agreement with the observations of Maeda and Horiuchi (Maeda and Horiuchi, 2009). However, other studies using different cell types did not observe an inhibitory action of LY294002 on basal leptin secretion. Taking together, our data strongly suggest that the inhibition of the PI3K pathway by LA could be involved in the LA-induced phosphorylation of Sp1 as well as on the inhibitory effect of LA on basal leptin secretion. Indeed, we demonstrated here that LA treatment inhibited Akt phosphorylation, a downstream target of PI3K, in 3T3-L1 adipocytes. Shi *et al.* (2008) also described that LA (5 mM) causes Akt inhibition in hepatoma cell lines. On the contrary, other studies have reported that LA remediates the age-associated impairment of Akt phosphorylation in primary rat hepatocytes (Shay *et al.* 2009).

Moreover, it has been described that LA improves insulin-stimulated Akt phosphorylation in soleus muscle from high fat-fed rats (Gupte *et al.* 2009) and from the insulin resistant Goto-Kakizaki rats (Bitar *et al.* 2004). Therefore, the effect of LA on PI3K/Akt pathway is complex and seems to be dependent on the cell type, as well as on the characteristics of the treatment (dose/concentrations and duration) tested.

The previous studies have evidenced the ability of LA to interfere with the MAPK/ERK pathway. Some trials have shown that LA strongly activated ERK in adipocytes at early stages of differentiation (Cho *et al.* 2003), whereas others found that LA inhibited the TGF-β-induced phosphorylation of ERK in AML-12 cells (Min *et al.* 2010). Our present data show that inhibition of ERK1/2 was able to reverse the LA-induced phosphorylation of Sp1, but not the inhibition of leptin secretion, suggesting that this pathway is not involved in the inhibitory actions of LA on leptin production.

Here, we show the ability of antioxidant molecules like LA to inhibit leptin secretion. In the same way, other antioxidants such as Vitamin C and resveratrol also inhibit leptin secretion by adipocytes (Garcia-Diaz *et al.* 2010; Szkudelska *et al.* 2009). However, pro-oxidant agents (exposure to H_2O_2) have also been shown to alter adipokine gene expression in adipocytes, including the inhibition of leptin secretion (Kamigaki *et al.* 2006). JNKs are activated by reactive oxygen species, and are well known for regulating transcription factors through phosphorylation. Moreover, H_2O_2 has also been shown to induce JNK pathway and to phosphorylate Sp1 and reduce Sp1-binding to DNA, and JNK inhibitors are able to attenuate H_2O_2–induced Sp1 phosphorylation in human alveolar epithelial cells (Chu and Ferro, 2006). However, Min *et al.* (2010) described that LA inhibited the phosphorylation of JNK and also the TGF-β-stimulated Sp1-DNA-binding activity in HepG2 cells. In the same way, we have observed that in adipocytes LA is also able to induce Sp1 phosphorylation and decrease the Sp1-binding activity. Moreover, we also reported that inhibition of JNK pathway with SP600125 also stimulated Sp1 phosphorylation in adipocytes, without affecting the LA-induced phosphorylation of Sp1. In addition, the inhibition of JNK pathway was not able to reverse the LA actions on leptin secretion. Taking together all of these data, it can be hypothesized that the activation of JNK pathway is not likely to be involved either in the LA-induced phosphorylation of Sp1 or in the inhibitory action of LA on leptin secretion.

Taking together, our present results in cultured adipocytes and previous data of other groups in Hep G2 (Min *et al.* 2010; Park *et al.* 2008) and HUVEC (Sung *et al.* 2005) cells, suggest a role of Sp1 in mediating some of the actions observed for LA in *in vitro* models. However, it still remains to be addressed the potential physiological involvement of Sp1 in the effects of LA after dietary supplementation in animal models.

On the other hand, the fact that LA directly alters leptin secretion by adipocytes raises the possibility that LA could also regulate other bioactive adipokines (such as adiponectin, apelin and visfatin) that directly regulate nutrient metabolism and insulin sensitivity, opening future research perspectives to better understand the mechanisms of LA actions.

In summary, the present data clearly demonstrate that LA inhibits leptin secretion and gene expression, and suggest that increased anaerobic metabolism of glucose may be contributing to these effects. The phosphorylation of Sp1 and the reduced DNA-binding activity of this transcription factor are also likely to be involved in the inhibition of leptin induced by LA in adipocytes. Moreover, these effects of LA seem to be mediated in part by PI3K/Akt pathway. Furthermore, the findings provided in this study suggest that LA could be a potential therapeutic agent for the treatment of some of the metabolic complications associated to obesity in which hyperleptinemia is involved, including cancer. In fact, it has been suggested that insulin-stimulated leptin may promote breast cancer progression and that this process requires Sp1 and is partially regulated by the PI3K (Bartella *et al.* 2008).

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4.4.- Effects of lipoic acid on chemerin secretion in 3T3-L1 and human adipocytes

Effects of lipoic acid on chemerin secretion in 3T3-L1 and human adipocytes

Prieto-Hontoria PL, Pérez-Matute P, Fernández-Galilea M, Martínez JA and Moreno-Aliaga MJ

Enviado

Resumen

La chemerina es una nueva adipoquina asociada con la obesidad y la resistencia a la insulina. El ácido lipoico (LA) posee propiedades beneficiosas sobre la diabetes y la obesidad. Por ello, el objetivo de este estudio fue examinar los efectos del LA sobre la producción de chemerina en adipocitos en ausencia o presencia de TNF-α, insulina y AICAR. Se analizaron también las posibles vías de señalización implicadas en los efectos del LA sobre chemerina. Las acciones del LA (250 μM) sobre chemerina se estudiaron tanto en adipocitos maduros 3T3-L1 como en adipocitos humanos subcutáneos y omentales. Los niveles de ARNm de chemerina se midieron por RT-PCR y la cantidad de chemerina secretada al medio de cultivo se determinó por ELISA. El LA indujo a una inhibición significativa tanto de la secreción como de los niveles de ARNm de chemerina en los adipocitos 3T3-L1. El activador de AMPK, AICAR, así como el inhibidor de PI3K, LY294002, suprimieron drásticamente tanto la secreción como la expresión génica de chemerina y, además, potenciaron el efecto inhibidor del LA en la secreción de esta adipoquina. La insulina fue capaz de revertir la acción inhibitoria de LA en la secreción de chemerina. Además, el LA redujo la secreción basal de chemerina tanto en adipocitos subcutáneos como omentales de sujetos obesos, en los que también suprimió los efectos estimulantes de la citoquina pro-inflamatoria TNF-α sobre la secreción de chemerina. Nuestros datos demuestran por tanto, la capacidad del LA para inhibir la producción de chemerina (basal o estimulada por TNF-α), una adipoquina asociada al síndrome metabólico y la obesidad, y sugieren que la reducción de chemerina podría contribuir también a las propiedades antidiabéticas descritas para el LA.

ABSTRACT

Chemerin is a novel adipokine associated to obesity and insulin resistance. Lipoic acid (LA) has been shown to exert beneficial properties on diabetes and obesity. So, the aim of this study was to examine the effects of LA on chemerin production in adipocytes in absence or presence of TNF-α, insulin and AICAR. The potential signaling pathways involved in LA effects on chemerin were also analyzed. LA (250 μM) actions on chemerin were tested in differentiated 3T3-L1 adipocytes and in human subcutaneous and omental adipocytes. Chemerin mRNA levels were measured by RT-PCR and the amount of chemerin secreted to culture media was determined by ELISA. LA induced a significant inhibition on both chemerin secretion and mRNA levels in 3T3-L1 adipocytes. The AMPK activator AICAR and the PI3K inhibitor LY294002 dramatically abrogated both chemerin secretion and gene expression, and further potentiated the inhibitory effect of LA on chemerin secretion. Insulin was able to completely reverse the inhibitory action of LA on chemerin secretion. LA also reduced basal chemerin secretion in both subcutaneous and omental adipocytes from obese subjects. Moreover, LA was able to abolish the stimulatory effects of the pro-inflammatory cytokine TNF-α on chemerin secretion. Our data demonstrated the ability of LA to inhibit basal and TNF-α-stimulated production of chemerin, an adipokine associated to obesity and metabolic syndrome, suggesting that the reduction of chemerin could also contribute to the antidiabetic properties described for LA.

INTRODUCTION

Chemerin is a novel adipokine highly expressed in WAT in rodents and humans (Bozaoglu *et al.* 2007). Serum chemerin levels are elevated in obesity (Bozaoglu *et al.* 2009) and strongly associated with markers of inflammation and components of the metabolic syndrome (Lehrke *et al.* 2009). Moreover, it has been demonstrated that chemerin exacerbates glucose intolerance in mouse models of obesity and diabetes (Ernst *et al.* 2010). Recent studies have demonstrated that insulin and the proinflammatory cytokine TNF-α stimulate chemerin production *in vivo* and in cultured adipocytes (Parlee *et al.* 2010; Tan *et al.* 2009). In contrast, reduction of chemerin levels has been associated to improved insulin sensitivity (Tan *et al.* 2009).

Lipoic acid has demonstrated beneficial effects in chronic inflammatory diseases such as diabetes and obesity (Prieto-Hontoria *et al.* 2011; Konrad *et al.* 1999). On the other hand, AMPK has been recognized as an emerging drug target for diabetes and the metabolic syndrome, and seems to be mediating some of the LA actions on energy as well as on glucose and lipid homeostasis (Zhang *et al.* 2009).

Given the therapeutic potential of LA and AMPK for the treatment of type 2 diabetes and obesity, and the importance of chemerin in these pathologies, we examined the effect of LA and/or AICAR on chemerin production in adipocytes. We also analyzed other potential signaling pathways involved in LA effects on chemerin.

RESULTS

Effects of LA on chemerin secretion and gene expression in 3T3-L1 adipocytes

Treatment with LA (250 μM) during 24 h induced a significant inhibition on both chemerin secretion ($P<0.01$) and gene expression ($P<0.001$). The AMPK activator AICAR also caused a marked reduction ($P<0.001$) of chemerin secretion and gene expression. Moreover, AICAR potentitated inhibitory effect of LA on chemerin secretion but not on mRNA levels (Figure 1A and Figure 1B).

We also analyzed the potential signaling pathways mediating the inhibitory effects of LA treatment on chemerin secretion and gene expression. Our data showed that treatment with the JNK inhibitor (SP600125) and the MAPK inhibitor

(PD98059) did not significantly modify the inhibitory effect of LA on chemerin secretion and gene expression. However, we found that treatment with the PI3K inhibitor LY294002 dramatically abrogated both chemerin secretion (P<0.001) and gene expression (P<0.01). Moreover, the inhibitory effect of LA on chemerin secretion was further potentiated (P<0.01) by the presence of LY294002, without modifying LA actions on chemerin mRNA levels (Figure 1A and Figure 1B). We also found that co-treatment of adipocytes with insulin was able to completely reverse the inhibitory action of LA on chemerin secretion (Figure 1C).

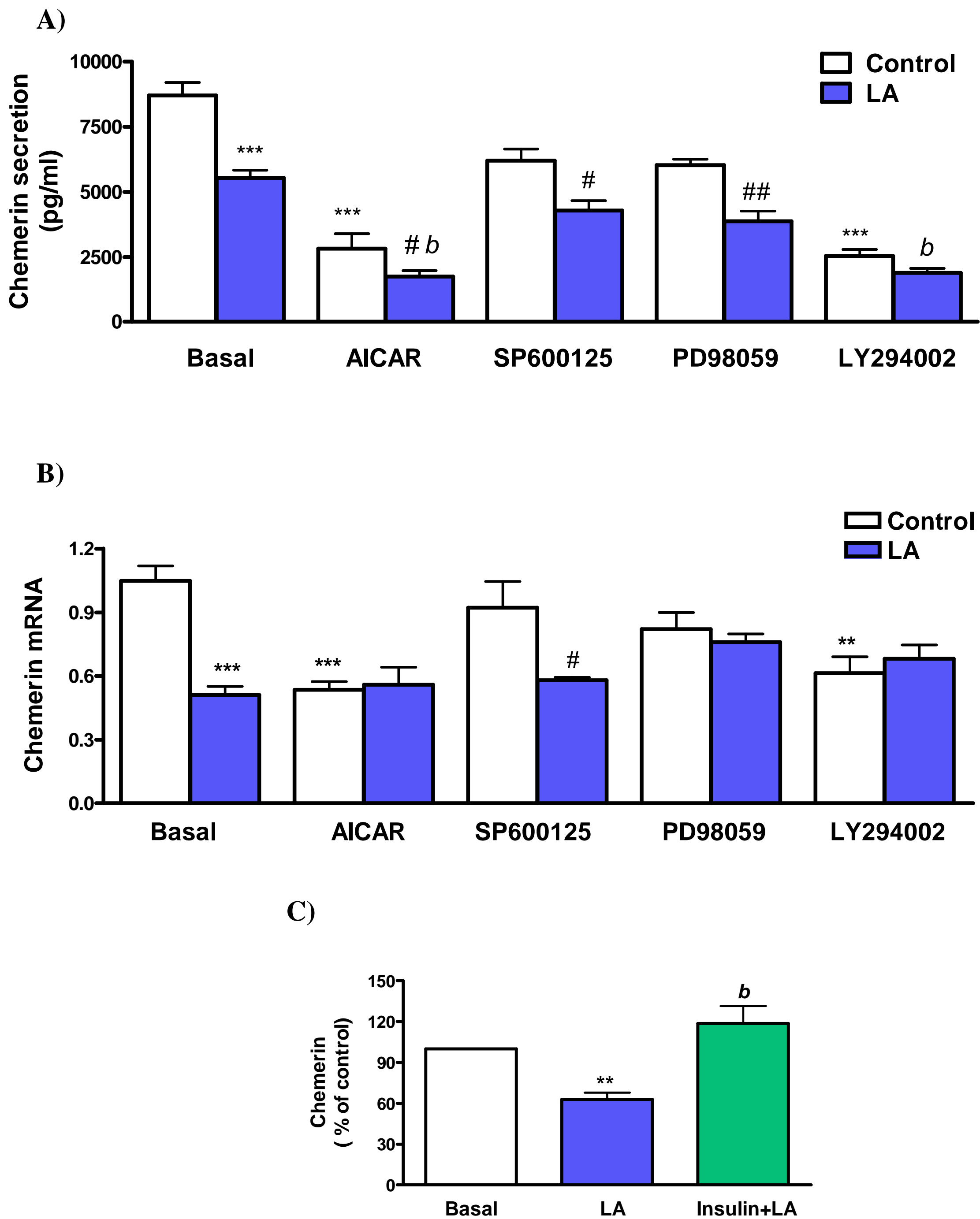

Figure 1. Effects of the AMPK activator AICAR (2 mM), the JNK inhibitor SP600125 (20 μM), the ERK1/2 inhibitor PD98059 (50 μM) and the PI3K inhibitor LY294002 (50 μM) on LA (250 μM)-induced inhibition of (A) chemerin secretion and (B) chemerin gene expression. (C) Effects of insulin (170 nM) on the inhibitory effect of LA on chemerin secretion. Fully differentiated 3T3-L1 adipocytes were treated with LA (250 μM) during 24 h in the absence or presence of the different treatments. Data are expressed as mean ± SE of 3-6 independent experiments. ***P<0.001, **P<0.01 *vs.* Basal-Control (vehicle-alone treated adipocytes). $^{\#\#}P$<0.01, $^{\#}P$<0.05 *vs.* respective Control. ^{b} P<0.01 *vs.* Basal-LA-treated cells.

Effects of LA on chemerin secretion and gene expression in human adipocytes

Similarly to what was observed in 3T3-L1 adipocytes, LA treatment (250 μM) reduced both basal chemerin secretion (P<0.05) and chemerin mRNA levels (P<0.05) in subcutaneous adipocytes from overweight-obese subjects. More importantly, LA was able to abolish the stimulatory effects of the pro-inflammatory cytokine TNF-α on chemerin secretion (P<0.001) and gene expression (P<0.05) (Figure 2A and Figure. 2B). The inhibitory effect of LA on basal chemerin was also observed in omental adipocytes from morbid obese subjects (2.30 ± 0.07 vs 1.99 ± 0.11 ng/ml, P<0.05).

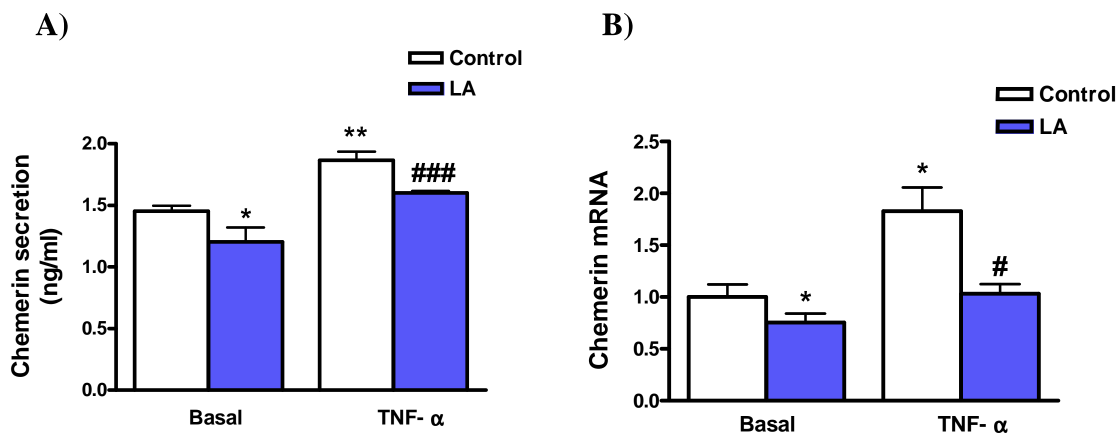

Figure 2. Effects of LA treatment on basal and TNF-α-induced chemerin secretion (A) and chemerin gene expression (B) in human subcutaneous adipocytes from overweight-obese individuals. Fully differentiated adipocytes were treated for 24 h with LA (250 μM) and/or TNF-α (100 ng/ml). Data are expressed as mean ± SE of 3-6 independent experiments, **P<0.01, *P<0.05 *vs.* Basal-Control (vehicle-alone treated adipocytes). ###P<0.001, #P<0.05 *vs.* TNF-α-treated cells.

DISCUSSION

Chemerin is a recently discovered adipokine associated with obesity and metabolic syndrome features (Bozaoglu *et al.* 2007). Thus, serum chemerin levels are elevated in obese compared with lean both in animals (Ernst *et al.* 2010; Parlee *et al.* 2010) and humans (Bozaoglu *et al.* 2009) and decreased after weight loss induced by bariatric surgery (Sell *et al.* 2010). Moreover, increased chemerin protein levels have been detected in adipose tissue from obese, diabetic mice (Ernst *et al.* 2010),

and chemerin secretion from human adipose tissue explants raised with obesity (Sell *et al.* 2009). Furthermore, it has been demonstrated that chemerin exacerbates glucose intolerance in mouse models of obesity and diabetes (Ernst *et al.* 2010). In fact, chemerin has been shown to induce insulin resistance in skeletal muscle *in vivo* by reducing insulin-stimulated Akt1 phosphorylation and activation of AMPK in mice (Becker *et al.* 2010). Moreover, chemerin also causes insulin resistance in primary human skeletal cells at the level of insulin receptor substrate 1, Akt and glycogen synthase kinase 3 phosphorylation, and glucose uptake (Sell *et al.* 2009).

Several studies have suggested that WAT acts as one of the primary source of chemerin in the whole organism (Bozaoglu *et al.* 2007) and that chemerin is involved in the cross talk between adipose tissue and other key metabolic organs such as liver and skeletal muscle (Becker *et al.* 2010). Therefore, controlling the increased secretion of chemerin from adipocytes especially in obesity could be a key target in the attenuation of obesity associated insulin-resistance. Our current data evidenced for the first time the ability of LA to decrease chemerin secretion and mRNA expression not only in 3T3-L1 adipocytes, but more importantly in human subcutaneous and omental adipocytes. It has been hypothesized that chronic low-grade inflammation that occurs in obesity could promote chemerin production by the adipocytes. In this way, Parlee *et al*. (2010) have recently shown that the proinflammatory cytokine TNF-α increased chemerin secretion in 3T3-L1 adipocytes and in primary mouse adipocytes. Here we also demonstrated that TNF-α increased chemerin production in human subcutaneous adipocytes, an effect that was completely reversed by treatment with LA.

In this study, we also aimed to elucidate the signaling pathways involved in the inhibitory action of LA on chemerin. Our data suggest that the inhibitory effect of LA on chemerin secretion is likely to be mediated by the inhibition of the PI3K/Akt pathway. In fact, a recent study of our group have demonstrated that the same dose of LA (250 μM) inhibits Akt phosphorylation in 3T3-L1 adipocytes (Prieto-Hontoria *et al.* 2011). In support of this idea, we found that the inhibitory action of LA on chemerin secretion was reversed by the presence of insulin, which activates Akt and stimulate chemerin secretion in adipocytes (Tan *et al.* 2009).

Another important novel finding of our study is that the AMPK activator AICAR also inhibited chemerin gene expression and secretion in adipocytes, suggesting that the inhibition of chemerin could be an additional mechanism

contributing to the well-known described potential antidiabetic properties of AMPK activator drugs (Zhang *et al.* 2009). Moreover, the inhibitory effect of the AMPK activator on chemerin was further potentiated by the presence of LA.

Therefore, the inhibition of chemerin by pharmacological agents represents a novel challenge for the treatment of diabetes and metabolic syndrome. In this context, a previous study has described that the antidiabetic drug metformin inhibits chemerin secretion in human omental adipose tissue explants, and also reported a reduction in serum chemerin levels after 6 months of metformin therapy with a concomitant decrease in insulin resistance in subjects with polycystic ovary syndrome (Tan *et al.* 2009). Taken together, those findings and our present data lead us to suggest that the ability of LA to reduce both basal and TNF-α stimulated chemerin production in adipocytes, and to potentiate the inhibitory effects of AMPK activation on chemerin could also contribute to the antidiabetic properties described for LA *in vivo* (Konrad *et al.* 1999). Further studies are need to elucidate if LA regulates chemerin *in vivo* and the relationship with the improvement in glucose and insulin metabolism.

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DISCUSIÓN

En el presente trabajo se han estudiado los potenciales efectos antiobesidad e insulino sensibilizadores del LA utilizando un modelo animal de obesidad inducida por una dieta alta en grasa. La administración de esta dieta hipercalórica durante 56 días indujo un aumento significativo en la ganancia de peso de las ratas, tal y como había sido descrito anteriormente (Lira *et al.* 2011; Jebelovski *et al.* 2008; Mitra *et al.* 2008; Buettner *et al.* 2007). La suplementación de la dieta con LA disminuyó de manera significativa la ganancia de peso de estos animales, apoyando las propiedades antiobesidad de este antioxidante. La dosis de LA utilizada es similar a la de otros ensayos en los que también se ha observado la capacidad del LA para prevenir o revertir la obesidad en modelos animales de obesidad tanto genéticos como dietéticos (Huong e Ide, 2008; Shen *et al.* 2005; Kim *et al.* 2004).

Respecto a los mecanismos implicados en la pérdida de peso inducida por el LA, nuestros datos demuestran que dicha pérdida se debe en parte a una disminución de la cantidad de alimento ingerida. Las propiedades anorexigénicas de dosis similares de LA en roedores han sido descritas en estudios previos (Shen *et al.* 2005; Song *et al.* 2005; Kim *et al.* 2004). La ganancia de peso fue menor en los grupos tratados con LA que en sus correspondientes grupos *Pair-Fed*, especialmente en los grupos alimentados con dieta alta en grasa, lo cual sugiere que el LA también podría incrementar el gasto energético. En este sentido, el estudio de Kim y colaboradores (2004) mostró un incremento del consumo de O_2, así como de la expresión de UCP1 especialmente en tejido adiposo pardo, pero también en el blanco, demostrando la capacidad del LA para estimular el gasto energético.

Los resultados del presente proyecto han mostrado que la eficiencia metabólica, un índice que relaciona la ganancia de peso respecto a las calorías ingeridas, está disminuida en los animales tratados con LA, lo que sugiere que a igual cantidad de calorías ingeridas, el LA induce un menor aprovechamiento de las mismas. En consonancia con este hallazgo, se ha observado una disminución en la absorción intestinal de α-metilglucósido en anillos intestinales de los animales tratados con LA, efecto que no fue observado en los grupos *Pair-Fed*. Todo ello sugiere una inhibición del transportador de glucosa dependiente de sodio (SGLT-1) por el LA, y avala la hipótesis de que la inhibición de la absorción intestinal de

azúcares podría contribuir a la menor eficiencia metabólica observada en los animales que recibieron la suplementación con LA.

La menor ganancia de peso corporal observada en las ratas tratadas con LA fue secundaria fundamentalmente a la reducción del tamaño de los diferentes depósitos grasos, en especial del tejido adiposo retroperitoneal, tal y como fue observado en el estudio de Shen y colaboradores (2005). Son varios los mecanismos que podrían contribuir a reducir el tamaño de los depósitos adiposos tales como el incremento en la lipólisis, la disminución de la adipogénesis y la lipogénesis, así como un aumento de la apoptosis adipocitaria. En este sentido el estudio de Cho y colaboradores (2003) demostró que el LA (250-500 µM) inhibe la adipogénesis en células 3T3-L1 inducida por insulina o troglitazona.

Varios estudios han descrito la acción hipoglucemiante e insulino-sensibilizadora del LA, tanto en roedores (Kandeil *et al.* 2011; Cummings *et al.* 2010; Wang *et al.* 2010) como en humanos (Lee y Dugoua, 2011; Ziegler *et al.* 2004). Nuestros datos demuestran que una suplementación dietética con LA mejora la sensibilidad a la insulina, incluso en el grupo alimentado con una dieta alta en grasa. Estos resultados están en consonancia con el estudio de Timmers y colaboradores (2010) quienes encontraron que los niveles plasmáticos de insulina en ayunas fueron más bajos tras la suplementación con LA de una dieta baja y/o alta en grasa administrada a roedores. Una aportación interesante del presente trabajo es que se demuestra que la capacidad del LA para mejorar sensibilidad la insulina no es sólo secundaria a la reducción de la ingesta inducida por el LA ya que existieron diferencias con sus correspondientes grupos *Pair-Fed*. Este resultado se corrobora con un estudio reciente que mostró que el LA (a dosis que no afectan a la ingesta de energía o el peso corporal) disminuye la aparición de diabetes tipo 2 inducida por la fructosa en ratas UCD-T2DM (Cummings *et al.* 2010).

Se han propuesto diversos mecanismos que podrían explicar las acciones insulino-sensibilizadoras del LA, incluyendo la mejora de la homeostasis de la glucosa por la conservación de la función de las células beta (Cummings *et al.* 2010). De hecho, de manera similar a la metformina, el LA inhibe la secreción de insulina *in*

vitro en células MIN-6 y en islotes pancreáticos de rata (Targonsky *et al.* 2006). También se ha sugerido que el LA impide el desarrollo de la diabetes en ratones propensos a obesidad mediante la reducción de la acumulación de triglicéridos en músculo, células beta pancreáticas e hígado (Park *et al.* 2008; Song *et al.* 2005). Algunos de estos efectos positivos sobre la sensibilidad a la insulina parecen ser mediados por la modulación de la AMPK. Así, se ha demostrado que el LA aumenta la sensibilidad a la insulina mediante la activación de la AMPK en músculo esquelético (Lee *et al.* 2005) y en células beta (Targonsky *et al.* 2006). Por otra parte, Park y colaboradores (2008) describieron que el LA disminuye la lipogénesis en el hígado a través de vías dependientes e independientes de la AMPK. Sin embargo, el estudio de Timmers y colaboradores (2010) observó que el efecto preventivo del LA sobre la acumulación de lípidos en el músculo tras una ingesta de una dieta alta en grasa no está mediada por la activación de la AMPK.

La activación de AMPK en el tejido adiposo también se ha relacionado con la mejora de la sensibilidad a la insulina, especialmente porque se ha asociado con la reducción de la secreción de adipoquinas inflamatorias en adipocitos (Timmers *et al.* 2010; Daval *et al.* 2006). En el presente trabajo, hemos evaluado el papel del LA en la activación de AMPK por fosforilación en la grasa epididimal de ratas Wistar y en adipocitos 3T3-L1. Los resultados obtenidos demustran la capacidad del LA para activar la fosforilación de la AMPK en adipocitos. Un estudio reciente ha observado un aumento de la fosforilación de la AMPK en el tejido adiposo blanco (TAB) de ratas ovariectomizadas tratadas con LA en paralelo con la inhibición de la ingesta de alimentos y el tamaño del tejido adiposo. Sin embargo, estos autores no analizaron si esta estimulación de AMPK es consecuencia de la pérdida de peso y/o adiposidad ya que no incluyeron en el estudio grupos *Pair-Fed*. La novedad de nuestro trabajo se basa en el hecho de que se observó una estimulación significativa de la fosforilación de AMPK en los grupos tratados con LA en comparación con sus correspondientes *Pair-Fed*, lo que sugiere una capacidad directa del LA para activar AMPK en adipocitos. Esta conclusión se ve apoyada por el aumento de la fosforilación de la AMPK observado en cultivos de adipocitos tratados con LA. Estudios previos han sugerido que la activación de la AMPK en adipocitos de roedores provoca una disminución del flujo de la lipogénesis y la síntesis de triglicéridos, así como un aumento en la oxidación de ácidos grasos (Orci *et al.* 2004) lo que podría contribuir a

la menor adiposidad mostrada en roedores tratados con LA. Por otra parte, la activación de AMPK inducida por LA en el tejido adiposo puede contribuir también a sus propiedades insulino-sensibilizadoras ya que se ha sugerido que niveles altos de ácidos grasos libres pueden para causar resistencia a la insulina (Hardie *et al.* 2011; Daval *et al.* 2006).

Asimismo, uno de los principales hallazgos del presente trabajo es que las acciones del LA sobre la reducción del peso corporal y la mejora de la sensibilidad a la insulina podrían estar mediadas por algunas adipoquinas relacionadas con la regulación del peso corporal y el metabolismo glucídico y lipídico, como la leptina, adiponectina y chemerina. Además, también se ha profundizado en el estudio de los posibles mecanismos intracelulares que podrían estar implicados en las acciones del LA sobre la producción de dichas adipoquinas por los adipocitos.

La adiponectina es una adipoquina con propiedades insulino-sensibilizadoras (Ziemke *et al.* 2010). La estimulación de la producción de adiponectina es un mecanismo común que presentan varias moléculas que promueven la sensibilidad a la insulina como el activador de PPARγ, rosiglitazona, metformina, y los ácidos grasos omega-3 (Tishinsky *et al.* 2011; Moreno-Aliaga *et al.* 2010; Zulian *et al.* 2010; Perez-Matute *et al.* 2007). Sin embargo, pocos estudios han abordado los efectos de la suplementación del LA sobre la adiponectina y los datos existentes son controvertidos. Así, el reciente estudio de Cheng y colaboradores (2011) sugiere que el tratamiento con LA (200 mg/kg/día mediante sonda intragástrica durante 7 semanas) suprime la elevación de los niveles de adiponectina observada en ratas ovariectomizadas. Por otra parte, Cummings y colaboradores (2010) no observaron cambios significativos en los niveles plasmáticos basales de adiponectina en ratas UCD-T2DM alimentadas con fructosa en ausencia o presencia de LA (80 mg/kg de peso corporal, durante 2 meses). Por el contrario, Houng e Ide (2008) han descrito una elevación en los niveles circulantes de adiponectina después de la suplementación dietética con LA (1-5 g/kg de dieta, durante 21 días). Nuestros datos indican que la suplementación dietética con LA es capaz de aumentar la expresión génica de adiponectina en TAB y sugieren un aumento en la cantidad de adiponectina producida por gramo de tejido adiposo. Es importante destacar que, esta capacidad del LA para estimular la adiponectina no es sólo secundaria a la pérdida de

peso ya que se observaron diferencias estadísticamente significativas entre los grupos tratados con LA y sus correspondientes grupos *Pair-Fed*. Estos datos sugieren que el aumento en la producción de adiponectina por los adipocitos podría estar involucrado en las propiedades sensibilizadoras a la insulina del LA. De hecho, se encontró una correlación negativa entre la adiposidad corregida por los niveles plasmáticos de adiponectina y el índice HOMA, un marcador de insulino-resistencia. Los resultados diversos obtenidos en los diferentes ensayos podrían estar relacionados con la diversidad de modelos animales, de dosis y de duración de los tratamientos empleados.

Sorprendentemente, los resultados obtenidos en cultivos de adipocitos revelan que el LA inhibe de forma dosis-dependiente la secreción de adiponectina. Este hecho sugiere que la estimulación de la producción de adiponectina observada tras la suplementación de la dieta con LA tiene lugar por un mecanismo indirecto o que se requieren de otros procesos metabólicos *in vivo* para llevar a cabo este efecto. Esta acción del LA parece ser independiente de sus propiedades antioxidantes ya que los efectos de diferentes antioxidantes sobre la secreción de adiponectina en adipocitos difieren ampliamente. Por ejemplo, la vitamina E, pero no la N-acetilcisteina, estimulan la expresión de adiponectina en las células 3T3-L1 (Landrier *et al.* 2009). Por otra parte, la vitamina C no modifica la secreción de adiponectina en cultivos primarios de adipocitos (García-Diaz *et al.* 2010).

Varios estudios han relacionado la activación de AMPK con la producción de adiponectina, pero los resultados son controvertidos. Lihn y colaboradores (2004) describieron que el AICAR, un activador de AMPK, aumenta la expresión génica de adiponectina en explantes de tejido adiposo humano. Recientemente, Wang y colaboradores (2011) también describen un aumento en los niveles de adiponectina en extractos de adipocitos 3T3-L1 que fueron tratados con AICAR. Por el contrario, Huypens y colaboradores (2005) encontraron que el AICAR causaba una disminución en la expresión proteica de adiponectina en adipocitos 3T3-L1. Además, describieron que la metformina reduce la secreción de adiponectina a través de la activación de AMPK. Del mismo modo, en el presente estudio se ha encontrado que tanto AICAR (datos no presentados) como el LA inhiben los niveles de ARNm de adiponectina y la cantidad de adiponectina secretada al medio de cultivo, apoyando

la idea de que la activación de AMPK podría explicar el efecto inhibitorio del LA sobre la adiponectina. Sin embargo, nuestros datos mostraron que el co-tratamiento con AICAR abolió el efecto inhibitorio del LA sobre la secreción de esta adipoquina. Por otro lado, está demostrado que la adiponectina, al menos en parte, activa AMPK (Wu *et al.* 2003; Yamauchi *et al.* 2002) lo que sugiere una compleja interacción entre la adiponectina y la activación de AMPK en los adipocitos.

Es bien conocida la capacidad de los agonistas del PPARγ, como las tiazolidinedionas para aumentar la producción de adiponectina en los adipocitos (Lorente-Cebrian *et al.* 2006) lo que se ha relacionado con sus efectos insulino-sensibilizadores (Yu *et al.* 2002). Nuestros datos revelaron que la troglitazona revierte parcialmente la acción inhibitoria del LA sobre la expresión génica de adiponectina, lo que sugiere que la activación del PPARγ es capaz de revertir, en parte, la inhibición de la expresión génica de adiponectina inducida por el LA.

La leptina es una de las principales adipoquinas implicada en la regulación de la ingesta y del gasto energético (Havel, 2004; Marti *et al.* 1999). Aunque un déficit de la misma provoca obesidad severa los niveles de leptina se encuentran elevados en la mayor parte de los obesos (Strobel *et al.* 1998; Montague *et al.* 1997), sugiriéndose una cierta resistencia a la acción de esta adipoquina en los individuos obesos (Meier y Gressner, 2004). La hiperleptinemia asociada a obesidad se ha relacionado con el desarrollo de varias de las complicaciones metabólicas y otras patologías asociadas a la obesidad, entre ellas la resistencia a la insulina, las complicaciones cardiovasculares y renales e incluso el cáncer (Kshatriya *et al.* 2010; Patel *et al.* 2008). Por tanto, el control de la hiperleptinemia asociada a obesidad podría ayudar a prevenir el desarrollo de las patologías asociadas a la misma.

Nuestros resultados demuestran la capacidad del LA disminuir los niveles plasmáticos así como la expresión de leptina en el tejido adiposo blanco, tras la suplementación de la dieta con dicho ácido. Estos resultados corroboran los de otros estudios (Kandeil *et al.* 2011; Huong e Ide, 2008; Song *et al.* 2005; Kim *et al.* 2004). Una contribución novedosa del presente estudio es la demostración del efecto inhibitorio directo del LA sobre la capacidad de los adipocitos de producir leptina. En cuanto a los mecanismos implicados, varios autores han demostrado la relación

entre el metabolismo de la glucosa y la producción de leptina por los adipocitos (Moreno-Aliaga *et al.* 2001; Wellhoener *et al.* 2000; Mueller *et al.* 1998). De hecho, la secreción de leptina es inversamente proporcional a la cantidad de glucosa utilizada anaeróbicamente y metabolizada a lactato (Perez-Matute *et al.* 2007 y 2005; Moreno-Aliaga *et al.* 2002). En este contexto, nuestros resultados demuestran que el LA aumentó significativamente y de una manera dosis-dependiente el porcentaje de glucosa que se metaboliza a lactato, y se observó una correlación negativa entre la secreción de leptina y el porcentaje de glucosa convertida a lactato, lo que sugiere que este incremento en la utilización anaeróbica de la glucosa podría explicar, al menos en parte, los efectos inhibitorios del LA sobre la leptina.

Varios estudios han puesto de manifiesto la importancia del factor de transcripción Sp1 en la regulación transcripcional del gen de la leptina (Mason *et al.* 1998). De hecho, la mutación del sitio de unión de Sp1 reduce la actividad del promotor de leptina tanto humano como de ratón (Zhang *et al.* 2002; Mason *et al.* 1998). Por otra parte, estudios previos de nuestro grupo han demostrado que Sp1 está involucrado en la estimulación de la producción de la leptina por el metabolismo de la glucosa mediado por la insulina (Moreno-Aliaga *et al.* 2007). Nuestros resultados demostraron que el tratamiento con LA inhibe la unión al DNA de Sp1, a través de la fosforilación del mismo, lo cual también podría contribuir a explicar las acciones inhibitorias del LA sobre la secreción de leptina en adipocitos maduros. Nuestros datos también sugieren que los efectos del LA sobre la leptina y Sp1 podrían estar mediados por la inhibición de la vía PI3K/Akt. Shi y colaboradores (2008) también describen que el LA provoca la inhibición de Akt en líneas celulares tumorales hepáticas. Por el contrario, otros estudios han descrito que el LA revierte la inhibición de la fosforilación de Akt asociada al envejecimiento en hepatocitos primarios de rata (Shay *et al.* 2009). Todo ello revela que el efecto de LA sobre la vía PI3K/Akt es compleja y parece depender del tipo celular, así como de las características del tratamiento (dosis, concentraciones y duración).

Finalmente, en el presente estudio se ha analizado también si los efectos beneficiosos del LA sobre la insulino-resistencia podrían estar mediados por sus acciones sobre la chemerina, una adipoquina de reciente descubrimiento relacionada con obesidad y síndrome metabólico (Bozaglou *et al.* 2007). Así, los niveles séricos

de chemerina están elevados en obesidad (Bozaglou *et al.* 2009) y fuertemente asociados con marcadores de inflamación y componentes del síndrome metabólico (Lehrke *et al.* 2009). Nuestros datos muestran que el tratamiento con LA, tanto en adipocitos maduros de la línea celular 3T3-L1 como en adipocitos subcutáneos y omentales de sujetos obesos, inhibe la secreción y expresión de chemerina. Se ha postulado que la inflamación crónica de bajo grado relacionada con la obesidad podría promover la producción de chemerina por los adipocitos. De hecho, Parlee y colaboradores (2010) han demostrado que la citoquina proinflamatoria TNF-α aumenta la secreción de chemerina en adipocitos de roedores. En nuestro estudio, se muestra que el LA es capaz de revertir los efectos estimulantes del TNF-α sobre la secreción y expresión de chemerina en adipocitos humanos. Al igual que lo descrito para la leptina, las acciones del LA sobre la chemerina parecen ser mediadas por la inhibición de la vía PI3K/Akt. En apoyo de esta idea, se encontró que la acción inhibitoria del LA sobre la secreción de chemerina fue revertida por el tratamiento con insulina, que activa Akt y estimula la secreción de chemerina en los adipocitos (Tan *et al.* 2009). Otro posible mecanismo por el que el LA pudiese inhibir la secreción de chemerina por los adipocitos es a través de la activación de la vía de señalización AMPK. Los resultados observados en este estudio sugieren que el AICAR (activador de AMPK) también inhibe la expresión y la secreción de chemerina en los adipocitos, y que los efectos inhibitorios del LA sobre chemerina se potencian en presencia de AICAR. Todos estos resultados sugieren que la reducción de la producción de chemerina por los adipocitos podría ser un mecanismo adicional que contribuya a las propiedades antidiabetes del LA y de los activadores de AMPK (Zhang *et al.* 2009), aunque son necesarios estudios *in vivo* que corroboren esta hipótesis.

En resumen, los datos del presente trabajo apoyan los potenciales efectos beneficiosos de la suplementación de la dieta con LA como posible nueva terapia para prevenir la obesidad y la resistencia a la insulina. Estos efectos del LA se deben en parte a sus propiedades anorexígenas a nivel central, pero también a importantes acciones periféricas sobre órganos clave para la homeostais de la energía y el metabolismo glucídico como el intestino y el tejido adiposo. Así, la reducción de la absorción intestinal de glucosa podría contribuir a la menor eficiencia metabólica observada en los animales tratados con LA. Por otra parte, la mejora de la

sensibilidad a la insulina observada tras el tratamiento con LA podría relacionarse con su capacidad de activar AMPK y de regular de manera directa o indirecta la producción de adipoquinas claves en el desarrollo/prevención de las complicaciones metabólicas asociadas a la obesidad tales como la leptina, adiponectina y chemerina.

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CONCLUSIONES

1.- La suplementación de la dieta con ácido lipoico durante 56 días previene la ganancia del peso corporal y de los depósitos grasos tanto en ratas alimentadas con una dieta control como en aquellas alimentadas con una dieta alta en grasa.

2.- Las propiedades antiobesidad del ácido lipoico se deben en parte a las acciones anorexígenas de esta molécula y también en parte a su capacidad para producir una menor eficiencia metabólica. De hecho, la ganancia de peso en el grupo alimentado con una dieta alta en grasa y ácido lipoico fue menor que la de su correspondiente grupo *Pair-Fed*.

3.- La suplementación con ácido lipoico provoca una disminución en la absorción intestinal de α-metilglucósido, sugiriendo una inhibición del transportador de glucosa dependiente de sodio (SGLT-1), lo que podría contribuir a la menor eficiencia metabólica observada en los animales tratados con este ácido.

4.- La suplementación de la dieta con ácido lipoico disminuyó la hiperinsulinemia y el índice de resistencia a la insulina -HOMA- apoyando las propiedades insulino-sensibilizadoras de esta molécula.

5. Las acciones antidiabéticas del ácido lipoico podrían ser secundarias a su capacidad para activar AMPK al estimular su fosforilación en el residuo Thr-172 en tejido adiposo.

6.- El ácido lipoico disminuye los niveles circulantes de leptina tanto en ratas alimentadas con dieta control como alta en grasa. Esta disminución no parece ser solo secundaria a la reducción de los depósitos grasos, ya que fue ligeramente superior en los grupos tratados con ácido lipoico que en sus correspondientes *Pair-Fed.*

7. El ácido lipoico provoca una inhibición dosis-dependiente tanto de la expresión del gen como de la secreción de leptina en adipocitos 3T3-L1. El incremento del metabolismo anaeróbico de la glucosa a lactato podría explicar, en parte, esa disminución en la producción de leptina. Además, la fosforilación de Sp1 y la consiguiente reducción de su capacidad para unirse al ADN podrían estar implicadas en los efectos inhibitorios del ácido lipoico sobre esta adipoquina, lo cual a su vez podría estar mediado por la inhibición de la cascada de señalización PI3K/Akt.

8.- La mejora de la sensibilidad a la insulina observada en los grupos suplementados con ácido lipoico podría ser secundaria también a la estimulación de la producción de adiponectina, una adipoquina insulino-sensibilizadora, tal y como pone de manifiesto la correlación negativa observada entre el HOMA y la expresión del gen de adiponectina en tejido adiposo y el cociente adiponectina circulante/ g tejido adiposo.

9.- En contraposición con lo observado *in vivo*, el ácido lipoico causa una inhibición dosis-dependiente de la secreción y expresión de adiponectina en adipocitos en cultivo, sugiriendo que la estimulación de adiponectina inducida por la suplementación dietética con ácido lipoico tiene lugar por un mecanismo indirecto o que se requieren de otros procesos metabólicos *in vivo* para llevar a cabo este efecto. La inhibición de adiponectina en adipocitos cultivados en presencia de ácido lipoico se produce en paralelo con la activación de AMPK; sin embargo, el activador de AMPK AICAR revierte los efectos inhibitorios del ácido lipoico sobre la adiponectina, revelando una compleja interacción entre la adiponectina y la AMPK en adipocitos.

10.- El ácido lipoico inhibe la secreción basal y la estimulada por TNF-α de chemerina, una adipoquina inflamatoria elevada en obesidad y síndrome metabólico. Además, el lipoico potencia los efectos inhibitorios de los activadores de AMPK sobre la chemerina, sugiriendo que la inhibición de la producción chemerina podría también contribuir a las propiedades antidiabéticas del ácido lipoico.

CONCLUSIÓN GENERAL

Los datos del presente trabajo apoyan los potenciales efectos beneficiosos de la suplementación de la dieta con ácido lipoico como posible nueva terapia para prevenir la obesidad y la resistencia a la insulina. Estos efectos del ácido lipoico se deben en parte a sus propiedades anorexígenicas a nivel central, pero también a importantes acciones periféricas sobre órganos clave para la homeostais energética y el metabolismo glucídico como el intestino y el tejido adiposo. Así, la reducción de la absorción intestinal de glucosa podría contribuir a la menor eficiencia metabólica observada en los animales tratados con lipoico. Por otra parte, la mejora de la sensibilidad a la insulina observada tras el tratamiento con ácido lipoico podría relacionarse con su capacidad de activar AMPK, así como su capacidad de regular de manera directa o indirecta la producción de adipoquinas claves en el desarrollo/prevención de las complicaciones metabólicas asociadas a la obesidad tales como la leptina, adiponectina y chemerina.

ANEXO I

Tabla 1. Efecto de la suplementación con LA (0,25g/100g de comida) durante 56 días sobre los diferentes órganos en ratas control y en ratas alimentadas con dieta alta en grasa.

	CONTROL (*n*=10)	**CLIP (*n*=10)**	**OBESO (*n*=10)**	**OLIP (*n*=12)**	**ANOVA 2X2**		
					D	**LA**	**DXLA**
M. Tibialis (g)	0,14±0,01	0,13± 0,01	0,17±0,01	0,13±0,01	n.s	*	n.s
M. gastrocnemio (g)	2,00±0,07	1,84±0,07	2,18±0,05	1,96±0,08	*	*	n.s
Riñón (g)	0,94±0,03	0,94±0,03	1,09 ±0,02	1,10±0,03	***	n.s	n.s
Hígado (g)	9,18±0,38	8,55±0,34	10,13±0,29	9,33±0,40	*	p=0.054	n.s
Corazón (g)	1,11±0,05	0,91±0,03	1,18±0,03	1,10±0,03	***	***	n.s
Páncreas (g)	0,63± 0,04	0,65±0,05	0,58±0,04	0,67±0,04	-	-	**
Bazo (g)	0,81±0,04	0,69±0,04	0,82±0,04	0,81±0,04	n.s	n.s	n.s

Los datos se expresan como media ± error estándar. El análisis estadístico se realizó mediante un ANOVA 2x2, *p< 0,05, **p<0,01 y ***p<0,001.

Tabla 2. Efecto de la suplementación con LA (0,25g/100g de comida) y de la restricción calórica (grupos *Pair-Fed*) durante 56 días sobre los diferentes órganos en ratas control y en ratas alimentadas con dieta alta en grasa.

	CLIP	PF-CLIP	OLIP	PF-OLIP	ANOVA 2X2		
					D	**LA**	**DXLA**
M. Tibialis (g)	0,13 ± 0,03	0,11 ± 0,06	0,13±0,01	0,17 ± 0,054	n.s	n.s	n.s
M. gastrocnemio (g)	1,84 ± 0,21	1,78 ± 0,30	1,96±0,08	1,98 ± 0,26	n.s	n.s	n.s
Riñón (g)	0,95 ± 0,10	0,82 ± 0,12	1,10±0,03	1,08 ± 0,12	***	n.s	n.s
Hígado (g)	8,56 ± 1,07	7,31 ± 1,53	9,33±0,40	9,01 ± 1,08	*	n.s	n.s
Corazón (g)	0,91 ± 0,10	0,96 ± 0,18	1,10±0,03	1,10 ± 0,11	***	n.s	n.s
Páncreas (g)	0,65 ± 0,15	0,65 ± 0,16	0,67±0,04	0,66 ± 0,12	n.s	n.s	n.s
Bazo (g)	0,69 ± 0,13	0,72 ± 0,20	0,81±0,04	0,83 ± 0,08	*	n.s	n.s

Los datos se expresan como media ± error estándar de al menos 6 animales por grupo. El análisis estadístico se realizó mediante un ANOVA 2x2, *p< 0,05, y ***p<0,001.

Tabla 3. Efecto de la suplementación con LA (0,25g/100g de comida) durante 56 días sobre diversos parámetros bioquímicos en ratas control y en ratas alimentadas con dieta alta en grasa.

	CONTROL	CLIP	OBESO	OLIP	ANOVA 2X2		
					D	LA	DXLA
TG(mg/dl)	69,54 ± 4,40	51,85 ± 2,51	63,67 ± 4,63	46,42 ± 1,97	n.s.	***	n.s.
Colesterol (mg/dl)	67,56 ± 2,59	60,20 ± 1,92†	69,26 ± 3,10	73,41 ± 3,16	-	-	**
LDL-colesterol (mg/dl)	48,52 ± 2,63	44,74 ± 2,19	54,32 ± 2,69	60,59 ± 3,40	***	n.s.	n.s.
HDL-colesterol (mg/dl)	23,14 ± 0,73	23,87 ± 0,61	21,16 ± 0,65	26,16 ± 0,84 †*c*	-	-	**
AG Libres (mmol/l)	0,84 ± 0,02	0,69 ± 0,04	0,72 ± 0,03	0,58 ± 0,02	***	***	n.s.
C. Cetónicos (mMol/l)	1,47 ± 0,14	1,28 ± 0,08	1,92 ± 0,19	1,28 ± 0,07	n.s.	**	n.s.

Los datos se expresan como media ± error estándar de al menos 7 animales por grupo. El análisis estadístico se realizó mediante un ANOVA 2x2, **p<0,01 y ***p<0,001.

La comparación entre grupos se realizó mediante una t-Student, †p<0,05 *vs*. grupo control y ^{c}p<0,001 *vs*. grupo obeso.

Tabla 4. Efecto de la suplementación con AL (0,25g/100g de comida) y de la restricción calórica (grupos *Pair-Fed*) durante 56 días sobre diversos parámetros bioquímicos en ratas control y en ratas alimentadas con dieta alta en grasa.

	CLIP	PF-CLIP	OLIP	PF-OLIP	ANOVA 2X2		
					D	**LA**	**DXLA**
TG(mg/dl)	51,85 ± 2,51	63,24 ± 5,55†	46,42 ± 1,97	39,48 ± 2,67	-	-	**
Colesterol (mg/dl)	60,20 ± 1,92	66,58 ± 3,40	73,41 ± 3,16	58,70 ± 2,81 *a*	-	-	**
LDL (mg/dl)	44,74 ± 2,19	47,40 ± 3,23	60,59 ± 3,40	48,02 ± 2,64 *a*	-	-	*
HDL(mg/dl)	23,87 ± 0,61	24,00 ± 1,00	26,16 ± 0,84	19,92 ± 0,81 *c*	-	-	***
AG Libres (mmol/l)	0,69 ± 0,04	0,83 ± 0,04	0,58 ± 0,02	0,66 ± 0,02	***	**	n.s
C. Cetónicos (mmol/l)	1,28 ± 0,08	2,42 ± 0,13 †††	1,28 ± 0,07	1,92 ± 0,09 *c*	-	-	*

Los datos se expresan como media ± error estándar de al menos 7 animales por grupo CLIP y OLIP, y al menos 5 animales por grupo *Pair-Fed*.
El análisis estadístico se realizo mediante un ANOVA 2x2, *p<0,05, **p<0,01 y ***p<0,001.
La comparación entre grupos se realizó mediante una t-Student, †p<0,05, †††p<0,001 *vs*. grupo CLIP y [a]p<0,05 [c]p<0,001 *vs*. grupo OLIP.

ANEXO II

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Lipoic acid prevents body weight gain induced by a high fat diet in rats: Effects on intestinal sugar transport

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Several studies have suggested that oxidative stress might cause and aggravate the inflammatory state associated with obesity and could be the link between excessive weight gain and its related disorders such as insulin resistance and cardiovascular diseases. Thus, antioxidant treatment has been proposed as a therapy to prevent and manage obesity and associated complications. Therefore, the aim of the present study was to investigate the effects of supplementation of a standard or high fat diet with the antioxidant lipoic acid (LA) during 56 days, on body weight gain, adiposity, feed efficiency and intestinal sugar absorption, in male Wistar rats. LA supplementation induced a lower body weight gain and adipose tissue size in both control or high fat fed rats accompanied by a reduction in food intake. The group fed on a high fat diet and treated with LA (OLIP group) showed a lower body weight gain than its corresponding Pair-Fed (PF) group ($P<0.05$), which received the same amount of food than LA-treated animals but with no LA. In fact, LA induced a reduction on feed efficiency and also significantly decreased intestinal α-methylglucoside (α-MG) absorption both in lean and obese rats. These results suggest that the beneficial effects of dietary supplementation with LA on body weight gain are mediated, at least in part, by the reduction observed in food intake and feed efficiency. Furthemore, the inhibitory action of LA on intestinal sugar transport could explain in part the lower feed efficiency observed in LA-treated animals and therefore, highlighting the beneficial effects of LA on obesity.

Key words: Lipoic acid, Obesity, Adiposity, Feed efficiency, Food intake, Intestinal sugar transport.

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Obesity is defined as a multifactorial chronic disease characterized by an increase in fat mass, as a result of a positive energy balance maintained over the time (1). In recent years, several studies have suggested that obesity is associated with a low degree chronic inflammation, which is accompanied by a high production of some cytokines and acute phase reactants as well as by the activation of the signaling pathways of inflammation (8). Furthermore, different studies in animal models and humans have shown that oxidative stress is related to the development of inflammation in obesity (6, 24). Indeed, oxidative stress might be the link between obesity and associated comorbidities, such as insulin resistance and cardiovascular disease (11, 24). Therefore, it is plausible to suggest that supplementation of the diet with ingredients with antioxidant properties (5) could prevent the development of obesity and associated comorbidities.

Lipoic acid (LA), a short chain fatty acid with eight atoms of carbon containing two sulfhydryl groups, is an important cofactor for mitochondrial bioenergetic enzymes (22). LA can be found in spinach and cabbage, liver and meat, wholewheat and yeast of beer, but it is also endogenously produced by the liver through the lipoic acid synthase (3, 16). Several studies have described the potential benefits of lipoic acid on obesity and its complications (4, 12, 20). Thus, it has been demostrated that lipoic acid reduces body weight and adiposity in rodents (12, 20, 23).

In addition, lipoic acid has beneficial actions in both glucose and lipid metabolism, and it has been proposed as a potential therapy for type 2 diabetes (12, 15, 19, 23). In this context some antidiabetic agents such as metformin, sulfonylureas and thiazolidinediones, have shown to inhibit intestinal sugar absorption, and therefore to decrease postprandial glucose elevations (13, 18). To our knowledge, there is not information available on the effects of lipoic acid on intestinal carbohydrate uptake.

Thus, the aim of this study was to investigate the effects of lipoic acid supplementation on food intake, feed efficiency and intestinal sugar transport both in lean and diet-induced obese Wistar rats.

Material and Methods

Animals and diets.– Male Wistar rats were obtained from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). Animals were housed in cages in temperature-controlled rooms (22 ± 2 °C) with a 12-hour light-dark cycle, fed a pelleted chow diet and given deionised water *ad libitum* for an adaptation period of 5 days. Rats were then assigned into 6 experimental groups for 8 weeks. The Control, CLIP and PF-CLIP groups, were fed on standard diet (Harlam Tekland Global Diets) containing 16.7 % of energy as protein, 78.6 % as carbohydrate and 4.6 % as lipid per dry weight. The Obese, OLIP, and PF-OLIP groups, were fed on a high fat diet (OpenSource diets Research Diets Inc) containing 60 % of energy as lipid, 20 % as carbohydrate and 20 % as protein per dry weight. The diet of the subgroups CLIP and OLIP was supplemented with lipoic acid in a proportion of 0.25 g LA/100 g of diet as previously described (12). The two Pair-Fed groups (PF-CLIP and PF-OLIP) received the same amount of food eaten by the groups CLIP or OLIP respectively, but without adding lipoic acid.

Body weight and food intake were recorded every 2-3 days. At the end of the

experimental period, rats were killed by decapitation and blood and tissue samples were collected, weighed and kept at –80 °C for subsequent analysis. All experimental procedures were approved and performed according to National and Institutional Guidelines for Animal Care and Use at the University of Navarra.

Sugar uptake measurements.– A segment (~ 15 cm) of the proximal jejunum was also obtained and washed in ice-cold Ringer´s solution (140 mM NaCl, 10 mM $KHCO_3$, 0.4 mM KH_2PO_4, 2.4 mM K_2HPO_4, 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$. pH 7.4). The jejunum segments were everted and cut into small pieces (0.3 – 0.5 cm) and the sugar uptake was measured as described (9). Briefly, groups of five intestinal rings were incubated for 10 min at 37 °C in Ringer´s solution containing 1 mM α-methylglucoside (MG) and 0.01 µCi/ml Methyl (U-^{14}C) glucopiranoside (PerkinElmer, Life Sciences Bostom, MA, specific activity 303 mCi/mmmol). At the end of the incubation period, rings were removed from the medium, weighed and the accumulated substrate was extracted from the rings for 15 h in 0.1 M HNO_3 at 4 °C. Finally, duplicate aliquot samples were taken for liquid scintillation counting (Wallac 1409, Pharmacia). MG uptake was estimated from the relationship between the counts per minute recorded for the incubation medium and the counts per minute obtained for the HNO_3 aliquots and expressed as micromoles of α-methylglucoside per gram of wet weight (w.w.) per 10 minutes (9).

Statistical analysis.– Data are expressed as means with standard errors. Differences were set up as statistically significant at *P<0.05*. Differences between the values for different variables were analysed by one-way ANOVA, Student´s *t* test or U-Mann Whitney after testing the normality with the Kolmogorov-Smirnoff and Shapiro-Wilk tests. The SPSS 15.0 version for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA) were used for the statistical analyses.

Results

Effects of lipoic acid on body weight gain, food intake and feed efficiency.– As expected, the high fat diet induced a significant increase in final body weight compared with the control group ($P<0.001$). The dietary supplementation with lipoic acid induced a lower body weight gain in both control and high fat-fed animals and was able to completely prevent the weight gain induced by high fat diet (Fig. 1).

Lipoic acid decreased food intake both in lean ($P<0.001$) and obese ($P<0.05$) rats, which could explain, at least in part, the

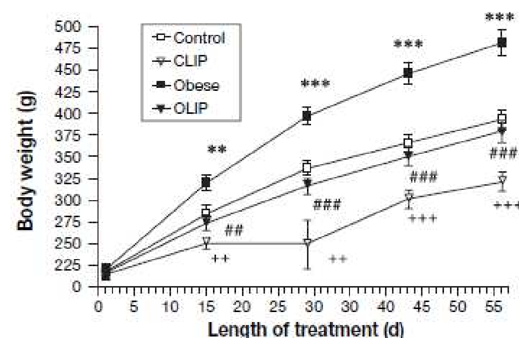

Fig. 1. *Effects of LA (56 days of treatment) on the growth curve in rats: control rats* (n=*10*)*, control rats treated with LA (CLIP* n=*10*)*, obese rats* (n=*10*)*, obese rats treated with LA (OLIP* n=*12*)*.*

Data are expressed as mean with standard errors represented by vertical bars. $^{**}p < 0.01$; $^{***}p < 0.001$ Control *vs.* Obese. $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ Obese *vs.* OLIP. $^{++}p < 0.01$; $^{+++}p < 0.001$ Control *vs.* CLIP.

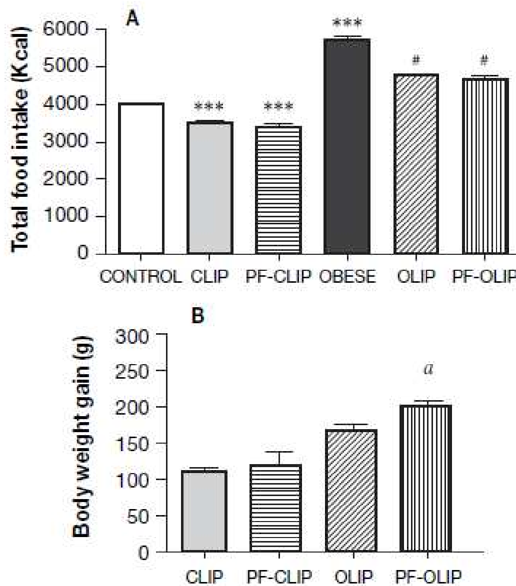

Fig. 2. *A. Effects of LA (56 days of treatment) on total food intake and B. Body weight gain in LA-treated animals (CLIP and OLIP) in comparison with their corresponding Pair-Fed groups (PF-CLIP and PF-OLIP).*

Data are expressed as mean with standard errors represented by vertical bars. ***p < 0.001 *vs.* Control. #p < 0.05 *vs.* Obese. ap < 0.05 *vs.* OLIP.

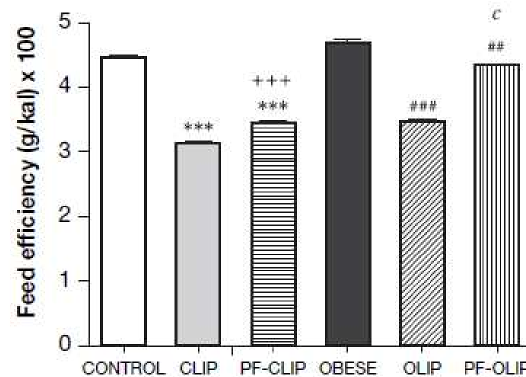

Fig. 3. *Effects of LA (56 days of treatment) on feed efficiency.*

Data are expressed as mean with standard errors represented by vertical bars. ***p < 0.001 *vs.* Control. +++p<0.001 *vs.* CLIP. ###p < 0.001 *vs.* Obese. cp<0.001 *vs.* OLIP.

reduction of body weight gain observed in lipoic acid treated rats (Fig. 2A). In order to test if the inhibitory action of lipoic acid on food intake is the only mechanism involved in its effects on body weight reduction, we included two Pair-Fed groups, which received the same amount of food eaten by the groups CLIP and OLIP respectively. Figure 2B shows that the groups treated with LA had lower body weight gain that their corresponding Pair-Fed groups, especially in the groups fed with a high fat diet (P<0.05).

Feed efficiency, the weight gain / energy consumed ratio, was significantly decreased (P<0.001) in those groups treated with lipoic acid in comparison with their respective Pair-Fed groups (Fig. 3).

Effects of lipoic acid on white adipose tissue weights.– Treatment with LA decreased the weight of all white fat pads (Table I). Our data also showed that in animals fed on a high fat diet, the decrease observed in the white fat depots is not only due to the reduction in energy intake induced by LA, since they showed smaller white fat depots than their corresponding Pair-Fed groups, being especially significant in the retroperitoneal depot (Table I). However, the decrease observed in brown adipose tissue in LA-treated animals was similar to their corresponding Pair-Fed groups (Table I).

Effects of lipoic acid on intestinal sugar absorption.– In order to investigate the possible effects of LA on sugar intestinal absorption, α-MG was chosen since this sugar derivative is only transported in the intestine by the Na^+-dependent glucose transporter, SGLT1, and not by the facilitative glucose transporters. Fig. 4 shows that lipoic acid induced a significant decrease in the absorption of α-MG after 10 min of incubation, both in lean and obese rats (P<0.01). Interestingly, the

Table I. *White adipose tissue weights (g) in control, Pair-Fed groups and lipoic acid-treated lean and overweight rats.*

Fat depot	CONTROL (10)	CLIP (10)	PF-CLIP (6)	OBESE (10)	OLIP (12)	PF-OLIP (6)
Epididymal	8.9±0.6	4.7±0.4***	5.2±0.9	20.0±1.1***	8.6±0.7###	11.8±1.2
Retroperitoneal	11.2±1.1	4.6±0.5***	4.5±1.2	24.6±1.4***	11.2±1.1###	15.9±0.5[a]
Mesenteric	2.8±0.4	1.1±0.1	1.1±0.3	5.4±0.9	2.2±0.3	3.1±0.2
Subcutaneous	5.6±0.5	3.4±0.3**	3.5±0.8	14.7±2.0***	6.7±0.5###	8.2 ±0.5
Brown adipose tissue	0.41±0.02	0.34±0.02*	0.33±0.06	0.60±0.07*	0.53±0.03	0.55±0.04

In parenthesis, number of animals. Data (mean ± SE) were analysed by one-way ANOVA. ***p<0.001, **p<0.01, *p<0.05 *vs.* control group. ###p<0.001 *vs.* obese group. [a]p<0.05 *vs.* OLIP group.

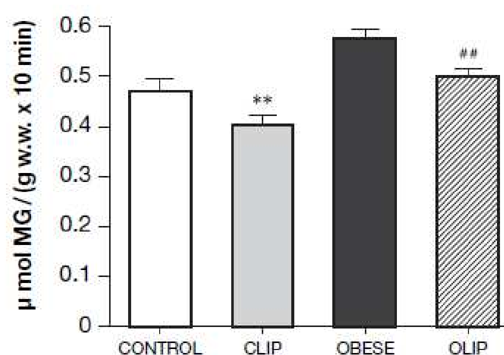

Fig 4. *Effects of LA (56 days of treatment) on 1 mM α-methylglucoside (MG) uptake after 10 min of incubation by everted jejunum rings of rats.*
Data are expressed as mean with standard errors represented by vertical bars. **p<0.01 *vs.* Control. ##p<0.01 *vs.* Obese.

groups treated with LA showed lower intestinal sugar absorption that their corresponding Pair-Fed groups, especially in the group fed with a standard diet (0.40 ± 0.02 *vs.* 0.62 ± 0.03 µmol α-MG/g wet weight x 10 min, *P*<0.001, CLIP *vs.* PF-CLIP), suggesting a direct inhibitory effect of lipoic acid on intestinal sugar absorption.

Discussion

Animals fed with a high-energy-yielding diet during 56 days exhibited a significant increase in body weight gain and adiposity, as previously described (2, 10, 14, 17). Lipoic acid supplementation significantly decreased the body weight gain of the animals, both in those fed with the control diet as well as in those fed with a high fat diet, similarly as what was previously observed by KIM *et al.* (12). This LA-induced reduction of body weight is mainly due to a significant reduction in the weight of total white adipose tissue along with a decrease in the size of every fat depot, corroborating previous studies (12, 20, 26).

Body weight gain in rodents is determined by the balance between food intake and energy expenditure. Our data showed that LA supplementation decreased food intake, which might explain the lowering of body weight gain. In this context, several authors had also observed this anorexigenic effect of LA (12, 20, 23). However, LA-treated rats weighted significantly less than their corresponding Pair-Fed rats, suggesting that food intake inhibition is not the only mechanism underlying the body weight lowering action of LA. In fact, we demonstrated in this study that feed efficiency, an index that relates body weight gain and energy consumed, was significantly decreased (*P*<0.001) in those animals treated with LA, suggesting an enhanced disruption of ingested energy. Thus, the decrease in feed efficiency induced by LA, together with

the reduced food intake, could explain the lower body weight observed in LA-treated animals. These data are in concordance with the study of KIM *et al.* (12), which showed that weight loss induced by LA is due, in part, to an enhancement of energy expenditure.

Thus, in order to test if the lower feed efficiency could be secondary to alterations in intestinal nutrient absorption, we analyzed intestinal α-MG uptake in jejunum everted rings, since this sugar derivative is only transported in the intestine by the Na^+-dependent glucose transporter, SGLT1, and not by the facilitative glucose transporters. Our data demonstrated for the first time that LA significantly decreased intestinal sugar absorption by inhibiting the sodium-dependent glucose transporter (SGLT1) both in lean and obese rats. Furthermore, this effect was not observed in the Pair-Fed groups, suggesting a direct inhibitory effect of LA on intestinal sugar absorption, which could contribute in some way to the lower feed efficiency observed in LA-treated animals. The precise contribution of the inhibitory action of LA on intestinal sugar absorption to the observed anti-obesity effects of LA remains to be determined. In this context, different studies have suggested that molecules with anti-obesity properties, such as green tea catechins could play a role in body weight reduction by controlling dietary glucose absorption by inhibiting intestinal glucose transporters (21, 25).

Our data also suggest that the reduction induced by LA on adiposity is not only secondary to the lower energy intake. In fact, the OLIP group exhibited a more reduced adiposity, specially in the retroperitoneal depot than its corresponding Pair-Fed group. This observation suggested that other mechanisms are contributing to the anti-adiposity actions of LA. In this context, it can be hypothesized that the reduced size of the fat depots can be attributed to an increase in lipolysis (7) and/or a decrease in the number of adipocytes (by increasing apoptosis and/or decreasing adipogenesis). In this regard, the study of CHO *et al.* (4) showed that LA in high concentrations (250-500 μM) inhibits insulin or troglitazone-induced adipogenesis in 3T3-L1 cells, indicating that co-treatment with LA would be beneficial for the prevention of obesity induced by several agonists of PPAR-γ. However, further studies are necessary to investigate whether an increase in apoptosis and/or lipolysis could also contribute to the reduction observed in the size of white adipose tissue after the treatment with LA.

In summary, these results suggest that the beneficial effects of dietary supplementation with lipoic acid on body weight gain are mediated, at least in part, by the reduction observed in food intake and feed efficiency. Furthemore, the inhibitory action of LA on intestinal sugar absorption could also contribute to the lower feed efficiency observed in LA-treated animals and therefore, to the beneficial effects of LA on obesity.

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P.L. PRIETO-HONTORIA, P. PÉREZ-MATUTE, M. FERNÁNDEZ-GALILEA, A. BARBER, J.A. MARTÍNEZ y M.J. MORENO-ALIAGA. *El ácido lipoico reduce la*

ganancia de peso inducida por dieta alta en grasa: efectos sobre el transporte intestinal de azúcares. J Physiol Biochem, **65** (1), 43-50, 2009.

Varios estudios han sugerido que el estrés oxidativo podría actuar como desencadenante y agravante del estado inflamatorio asociado a la obesidad y podría ser un potencial nexo de unión entre la excesiva ganancia de peso y las co-morbilidades asociadas. Así, se ha propuesto el tratamiento con antioxidantes naturales como posible terapia contra el desarrollo de obesidad así como sus complicaciones asociadas. Por ello, el objeto del presente trabajo fue investigar en ratas Wistar macho los efectos de la suplementación de una dieta estándar o alta en grasa con un antioxidante, el ácido lipoico (AL) (0,25g/ 100g de comida) durante 56 días sobre la ganancia de peso corporal, la adiposidad, la eficiencia metabólica y la absorción intestinal de azúcares. La suplementación de la dieta con AL indujo una menor ganancia de peso corporal y redujo el tamaño del tejido adiposo blanco total, tanto en ratas alimentadas con dieta control como alta en grasa. Además, disminuyó la ingesta. La ganancia de peso en el grupo alimentado con dieta alta en grasa y AL fue menor que la de su correspondiente grupo *Pair-Fed* (*P<0,05*), el cual recibía la misma cantidad de comida que los animales tratados con AL pero sin este ácido. De hecho, la suplementación con ácido lipoico redujo la eficiencia metabólica y disminuyó significativamente la absorción intestinal de α-metilglucósido (α-MG) tanto en ratas control como obesas. Estos resultados sugieren que los efectos beneficiosos de la suplementación de la dieta con AL sobre la ganancia de peso corporal están mediados, al menos en parte, por la reducción observada en la ingesta de comida y en la eficiencia metabólica. Además, la acción inhibitoria del AL sobre el transporte intestinal de azúcares podría explicar, en parte, la menor eficiencia metabólica observada en los animales tratados con AL justificando, por consiguiente, los efectos beneficiosos del AL sobre la obesidad.

Palabras clave: Ácido lipoico, Obesidad, Adiposidad, Eficiencia metabólica, Ingesta, Transporte intestinal de azúcares.

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RESEARCH ARTICLE

Lipoic acid inhibits leptin secretion and Sp1 activity in adipocytes

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Scope: Lipoic acid (LA) is an antioxidant with therapeutic potential on several diseases such as diabetes and obesity. Hyperleptinemia and oxidative stress play a major role in the development of obesity-linked diseases. The aim of this study was to examine in vivo and in vitro the effects of LA on leptin production, as well as to elucidate the mechanisms and signalling pathways involved in LA actions.

Methods and results: Dietary supplementation with LA decreased both circulating leptin, and adipose tissue leptin mRNA in rats. Treatment of 3T3-L1 adipocytes with LA caused a concentration-dependent inhibition of leptin secretion and gene expression. Moreover, LA stimulated the anaerobic utilization of glucose to lactate, which negatively correlated with leptin secretion. Furthermore, LA enhanced phosphorylation of Sp1 and inhibited Sp1 transcriptional activity in 3T3-L1 adipocytes. Moreover, LA inhibited Akt phosphorylation, a downstream target of phosphatidylinositol 3-kinase (PI3K). Treatment with the PI3K inhibitor LY294002 mimicked LA actions, dramatically inhibiting both leptin secretion and gene expression and stimulating Sp1 phosphorylation.

Conclusion: All of these data suggest that the phosphorylation of Sp1 and the accompanying reduced DNA-binding activity are likely to be involved in the inhibition of leptin induced by LA, which could be mediated in part by the abrogation of the PI3K/Akt pathway.

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Lipoic acid / Leptin / Obesity / PI3K / Sp1 transcription factor

1 Introduction

Obesity is a disease with serious public health implications, associated with insulin resistance, type 2 diabetes, hypertension, dyslipemia, and atherosclerosis. A growing body of evidence supports that obesity is linked to a state of chronic oxidative stress, which may result from a combination of adipokine imbalance, and reduced antioxidant defences [1].

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Abbreviations: **FBS**, fetal bovine serum; **LA**, lipoic acid; **PF**, pair-fed; **PI3K**, phosphatidylinositol 3-kinase; **PP1**, protein phosphatase 1; **SE**, standard error; **WAT**, white adipose tissue

White adipose tissue (WAT) dysfunction plays a critical role in the development of obesity-associated disorders. Indeed, WAT is a complex and metabolically active organ, with an important relevance in regulating whole-body metabolism. In addition to its primary role as a fuel reservoir, WAT has been confirmed as a major endocrine organ, which synthesizes and secretes an array of sex steroids, and bioactive peptides termed 'adipokines', involved in the physiological regulation of fat storage, energy metabolism, food intake, insulin sensitivity, and immune function among others [2].

Leptin is an adipokine that plays an important role regulating food intake and energy balance. Although leptin deficiency leads to severe obesity in rodents and humans, it is clearly established that serum leptin concentrations are proportional to the amount of adipose tissue mass and therefore, most frequently obesity is accompanied by

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hyperleptinemia, associated with resistance to the actions of this adipokine [3]. Hyperleptinemia itself has also been suggested to be involved in the pathogenesis of the co-morbidities associated to obesity such as type 2 diabetes mellitus, hypertension, cardiovascular disease, and cancer [4]. Leptin has also been shown to induce perturbations in the anti-oxidative defence system and increase oxidative stress in both animals and humans [5]. In fact, hyperleptinemia is associated with oxidative stress in type 2 diabetes mellitus, and decreases insulin secretion from the pancreatic β cells [6]. Therefore, antioxidant therapy has been proposed as an useful strategy for attenuation of oxidative stress and hyperleptinemia in obesity [1, 7].

Lipoic acid (LA; 1,2-dithiolane-3-pentanoic acid) is a naturally occurring short-chain fatty acid with sulphydryl groups, which is a necessary cofactor for mitochondrial enzymes [8, 9]. Aside from its enzymatic role, in vitro and in vivo studies suggest that LA also acts as a powerful micronutrient with diverse pharmacological and antioxidant properties [10]. A recent study has demonstrated that down-regulation of LA synthase (LASY), the enzyme involved in the endogenous synthesis of LA, reduced endogenous levels of LA as well as critical components of the antioxidant defence network, increasing oxidative stress. Moreover, this down-regulation of LA synthase induced a significant loss of mitochondrial membrane potential and decreased insulin-stimulated glucose uptake in skeletal muscle cells along with an increased inflammatory response which, in turn, leads to increased insulin resistance, mitochondrial dysfunction, and inflammation [11]. Thus, LA can be considered as a promising therapeutic approach for chronic inflammatory diseases such as diabetes and obesity [12, 13]. Thus, it has been reported that the ability of LA to reduce body weight gain in rodents by suppressing food intake and increasing energy expenditure [8, 14–16]. Studies of our group have also described that LA decreased feed efficiency and intestinal sugar transport, which could explain at least in part its reducing effects on body weight gain despite of high-fat diet feeding [14]. Moreover, a recent study in pre-obese and obese human subjects have evidenced that LA (800 mg/day for 4 months) induced significant reductions of body weight, BMI, blood pressure, and abdominal circumference, together with a decrease in inflammatory markers [17].

Some of these studies have described that the reduction of body weight and adiposity induced by LA is accompanied by a decrease in plasma leptin levels [8, 18, 19]. However, it still remains to be elucidated if this reduction in circulating leptin levels is merely a consequence of reduced fat mass or if LA treatment can directly inhibit leptin production. Therefore, the first aim of the present study was to examine the effects of LA supplementation on leptin production in lean and high fat-fed rats. Moreover, we investigate the in vitro effects of LA on leptin secretion and gene expression in cultured adipocytes. We also aimed to find out the potential molecular mechanisms involved. Because of the importance that glucose metabolism and Sp1 transcription factor play in the regulation of leptin secretion by the adipocytes [20–22], we tested the effects of LA on the anaerobic utilization of glucose to lactate and on Sp1 activity and its regulation by phosphorylation. Moreover, we also analysed the potential signalling pathways underlying the effects of LA on leptin and Sp1.

2 Materials and methods

2.1 Animal and diets

Six-wk-old growing male Wistar rats ($n = 54$) were obtained from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). Animals were housed in polycarbonate cages (three to four rats per cage) in temperature-controlled rooms (22 ± 2°C) with a 12-h light–dark cycle, fed a pelleted chow diet, and given deionized water ad libitum for an adaptation period of 5 days.

Rats were then assigned into six experimental groups for 8 wk. Control, CLIP, and pair-fed (PF)-CLIP groups were fed with a standard diet (Harlam Tekland Global Diets) containing 16.7% of energy as proteins, 78.6% as carbohydrates, and 4.6% as lipids per dry weight. The Obese, OLIP, and PF-OLIP groups were fed with a high-fat diet (Open-Source diets Research Diets) containing 60% of energy as lipids, 20% as carbohydrates, and 20% as proteins per dry weight, which has been widely used to induce obesity in rodents [23]. The diet of the subgroups CLIP and OLIP was supplemented with racemic α-LA (Sigma-Aldrich, St. Louis, MO, USA) in a proportion of 0.25 g LA/100 g of diet as described previously [8]. LA was thoroughly and homogeneously mixed with both diets (chow and high fat) using a blender. Food intake was measured three times per wk before the onset of the dark period, and the two pair-fed groups (PF-CLIP and PF-OLIP) were then received the same amount of food ingested by the groups CLIP or OLIP, respectively, but without LA supplementation. These two groups are necessary to distinguish what proportion of the LA actions is independent of LA effects on food intake.

Body weight and food intake were recorded every 2–3 days. At the end of the experimental period (56 days), rats were killed by decapitation and blood and tissue samples including WAT depots (epididymal, retroperitoneal, mesenteric, and subcutaneous) were collected as described previously [24]. Thus, epididymal WAT includes prominent bilateral intra-abdominal visceral depots in male rats attached to the epididymides; retroperitoneal WAT, bilateral depots in abdominal cavity behind the peritoneum on the dorsal side of the kidney; subcutaneous fat, bilateral superficial subcutaneous WAT between the skin and the muscle fascia posterior to the lower segment of the upper limbs; mesenteric fat is outlined by the two peritoneal leaflets holding the intestine against the posterior abdominal wall. All fat depots were weighed and kept at −80°C for the subsequent analysis. Visceral WAT depot was estimated by the sum of epididymal,

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retroperitoneal, and mesenteric depot weights. All experimental procedures were performed according to National and Institutional Guidelines for Animal Care and Use with the approval of the Ethical Committee for Animal Care and Use at the University of Navarra.

2.2 Cell culture and differentiation of 3T3-L1 cells

Mouse preadipose cell line 3T3-L1 was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were cultured in DMEM containing 4.5 g/L glucose, calf serum 10% v/v, and antibiotics 1% v/v, and were maintained in a water-jacketed incubator set to 37°C and 5% carbon dioxide. Confluent cells were induced to differentiate by incubating for 48 h with differentiation medium containing 1 μmol/L dexamethasone (Sigma-Aldrich), 0.5 μmol/L isobutylmethylxanthine (Sigma-Aldrich), 10 μg/mL insulin (Sigma-Aldrich), 10% v/v foetal bovine serum (FBS), and antibiotics (1%) in DMEM (Invitrogen, Grand Island, NY, USA). Then, cells were cultured with 10% v/v FBS and 10 μg/mL insulin and antibiotics (1%) in DMEM for 48 h. After 2 days, media were replaced with 10% FBS in DMEM and antibiotics, without insulin, and changed every 2 days. Different treatments were added to differentiated 3T3-L1 adipocytes (days 7 and 8 post-confluence) for a time period of 24 h [25].

2.3 Adipocyte isolation and primary culture

Adipocytes were isolated under sterile conditions from epididymal fat depots of 8-wk-old male Wistar rats (250–280 g) fed a pelleted chow diet and given deionized water ad libitum [25]. Briefly, adipose tissue fragments were digested with type I collagenase at 37°C with gentle shaking for 30 min. The resulting cell suspension was diluted in Hepes buffer (5 mmol/L D-glucose, 2% BSA, 135 mmol/L NaCl, 2.2 mmol/L $CaCl_2 \cdot 3H_2O$, 1.25 mmol/L $MgSO_4 \cdot 7H_2O$, 0.45 mmol/L KH_2PO_4, 2.17 mmol/L Na_2HPO_4, and 10 mmol/L Hepes, pH 7.4) and then filtered through a 400 μm nylon mesh. The isolated adipocytes were washed three times and resuspended in DMEM (5 mmol/L glucose) supplemented with 1% v/v FBS and incubated for 30–40 min at 37°C.

The isolated adipocytes were plated on 500 μL of a collagen matrix (Purecol; Inamed Biomaterials) in 6-well culture plates and, after 50 min incubation, culture medium containing the different treatments was added and the cells were cultured for up to 48 h. At the end of the incubation period, samples were collected to measure leptin.

2.4 Treatments

α-LA (Sigma-Aldrich) was dissolved in ethanol. LY294002 (Sigma-Aldrich), SP600125 (Biomol), PD98059 (Sigma-Aldrich), were dissolved in DMSO. Control cells were treated with the same proportion of the corresponding vehicle (ethanol and/or DMSO in a proportion of 0.01%). When the selective phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 (50 μM), JNK inhibitor SP600125 (20 μM), and MAPK inhibitor PD98059 (50 μM) were used, adipocytes were pre-incubated for 1 h with these inhibitors prior to the addition of LA (250 μM).

2.5 Biochemical measurements

Leptin concentrations in both serum and culture media were determined by a Rat/Mouse Leptin ELISA kit (Linco Research, MO, USA). The lowest level of leptin that can be detected by this assay is 0.05 ng/mL.

Glucose and lactate concentrations in the media were measured using an Autoanalyzer (Cobas Roche Diagnostic, Basel, Switzerland) as described previously [26]. The amount of carbon released as lactate per amount of carbon taken up as glucose was calculated as Δ[lactate]/Δ[glucose], where Δ is the difference, and expressed as a percentage.

2.6 Nuclear extracts and Sp1-binding activity

Nuclear extracts were prepared from fully differentiated 3T3-L1 adipocytes as described previously [27] with some modifications. The entire procedure was carried out at 4°C. Cells were collected and washed once with cold PBS and then with 200 μL buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF with 0.6% Nonidet P40) and kept on ice for 15 min. After centrifugation in a bench-top centrifuge at 800 × g for 30 s, the cell pellet was resuspended in 1 mL of buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF), and centrifuged at 800 × g for 30 s. Then, the cell pellet was resuspended in 38 μL of buffer C (10 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and shaked on 4°C at 1500 rpm for 30 min with occasional mixing. The suspension was then centrifuged at 10 000 × g for 10 min. Insoluble material was removed by centrifugation, and aliquots were kept at −80°C.

Nuclear extracts were left untreated or treated with Protein Phosphatase 1 (PP1) (1.25 or 5 U), and dephosphorylation reactions were performed at 30°C for 15 min according to the protocol recommend by the supplier (New England Biolabs, UK). Nuclear protein was then quantified using Bradford assay method. A total of 6 μL of nuclear protein and 6 μL nuclear extract dilution buffer from each treatment were analyzed for Sp1 activity using the Transcription Factor ELISA kit, which assesses Sp1 protein–DNA-binding activity (Panomics, Fremont, CA, USA).

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2.7 Western Blotting

3T3-L1 adipocytes were cultured and induced to differentiate as described previously [25]. Cells were serum-starved overnight and then incubated with the appropriate treatment. Nuclear extracts were prepared as reported previously. Briefly, equal amounts of protein samples were separated by 12% SDS-PAGE and transferred into PVDF membrane. Once blocked, the membrane was probed with primary antibodies against phospho-Sp1 (Thr 453) (Abcam plc, Cambridge, UK) and Sp1 (H-225) (Santa Cruz Biotechnology, USA). After further washings, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, CA, USA). The immunoreactive proteins were detected with enhanced chemiluminescence (Pierce Biotechnology, Rockford, IL, USA). Band intensities were quantified using a GS-800 calibrated densitometer (Bio-Rad Laboratories).

2.8 Real-time PCR

Total RNA was extracted from epididymal fat depots and from 3T3-L1 cells using TRIzol® reagent (Invitrogen, CA, USA) according to the manufacturer's instructions, and then the RNA was incubated with RNAse-free DNase kit (Ambion, Austin, TX, USA). Two microgram of RNA was reverse-transcribed to cDNA using MMLV (Moloney Murine Leukaemia Virus) reverse transcriptase (Invitrogen). Leptin mRNA levels were determined using pre-designed TaqMan® Assays-on-Demand (Rn00565158_m1* and Mm00434759_m1*, Applied Biosystems, Foster City, CA, USA). Quantitative real-time PCR was performed using the ABI PRISM 7000HT and ABI PRISM 7900HT Fast System Sequence Detection System (Applied Biosystems) Sequence as described previously [25]. Leptin mRNA levels were normalized by two different housekeeping genes Cyclophilin (Rn00690933_m1* and Mm02342430_g1) and/or Ubiquitin C (Rn01789812_g1, Applied Biosystems). Fold changes of gene expression were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.9 Data analysis

Data are expressed as mean with standard errors (SE). Differences were set up as statistically significant at $p<0.05$. Comparisons between the values for different variables were analysed by one-way ANOVA, followed by Bonferroni post-hoc test, or by Student's *t*-test, or by Mann–Whitney *U*-test after testing the normality with the Kolmogorov–Smirnov and Shapiro–Wilk tests. Furthermore, Pearson correlation analysis was performed to screen the potential association between two variables. SPSS 15.0 version for Windows (SPSS, Chicago, IL, USA) and GraphPad Prism 4.0 (GraphPad Software, San Diego, CA, USA) were used for the statistical analyses.

3 Results

3.1 Effects of LA on leptin circulating levels and gene expression in vivo

Table 1 summarizes that treatment with LA decreased food intake, body weight gain, and adipose tissue size in both control and high fat-fed rats. Moreover, the group fed on a high-fat diet and treated with LA (OLIP group) had a lower body weight gain and reduced visceral fat weight than its corresponding PF group ($p<0.05$), which received the same amount of food than LA-treated animals but without supplementation with LA. As expected, leptin circulating levels were significantly higher in the obese group in comparison with the control group ($p<0.001$). On the contrary, LA treatment significantly decreased leptin plasma levels ($p<0.001$ for CLIP and $p<0.01$ for OLIP) (Fig. 1A). The patterns of LA effects on leptin gene expression were similar to those observed in plasma levels (Fig. 1B). The decrease in leptin was also observed in the PF groups, but it was less pronounced than in the LA-supplemented groups, and no statistically differences were found when comparing both groups (Fig. 1A and 1B).

As expected, leptin levels were positively correlated with the size of total WAT ($p<0.001$) (Fig. 1C).

Table 1. Body weight gain and WAT weights in control, PF groups, and lipoic acid-treated lean and overweight rats

	Control ($n=10$)	CLIP ($n=10$)	PF-CLIP ($n=6$)	OBESE ($n=10$)	OLIP ($n=12$)	PF-OLIP ($n=6$)
Initial body weight (g)	215.1±6.4	212.3±5.8	201.8±7.6	218.1±5.6	214.7±6.0	209.8±4.0
Final body weight (g)	391.8±12.2	320.3±10.9**	317.8±24.3*	482.0±13.7***	378.4±13.1#	409.8±8.2#
Body weight gain (g)	176.7±8.3	108.0±7.8***	116.0±20.2*	263.9±11.7***	163.7±10.5#	200.1±6.8#,a
Food intake (g/day)	22.9±0.2	20.0±0.1***	Ψ	19.4±0.3***	16.2±0.2#	Ψ
Visceral fat (g)	22.9±1.8	10.4±0.9***	11.21±2.36	49.9±2.5***	22.0±1.9#	30.3±1.5a
Subcutaneous fat (g)	5.6±0.5	3.4±0.3**	3.5±0.8	14.7±2.0***	6.7±0.5#	8.2±0.5*

Data (mean±SE) were analysed by one-way ANOVA followed by Bonferroni post-hoc test. ***$p<0.001$, **$p<0.01$, *$p<0.05$ versus Control group. #$p<0.05$ versus Obese group. $p<0.05$ versus OLIP group. ΨSame food intake that of CLIP group. ΨSame food intake that of OLIP group.

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3.2 Effects of LA on leptin secretion and gene expression in 3T3-L1 adipocytes

Treatment of 3T3-L1 adipocytes with LA caused a concentration-dependent inhibition of basal leptin secretion (Fig. 2A), which was only statistically significant at the highest concentrations tested (−69 and −91% for 250 and 500 μM, respectively, $p<0.01$). Basal leptin gene expression was also significantly inhibited by the presence of LA at concentrations of 100 and 250 μM (−81 to −91%, $p<0.01$–0.001, respectively) (Fig. 2B). Moreover, we also tested the effects of LA treatment on insulin-stimulated

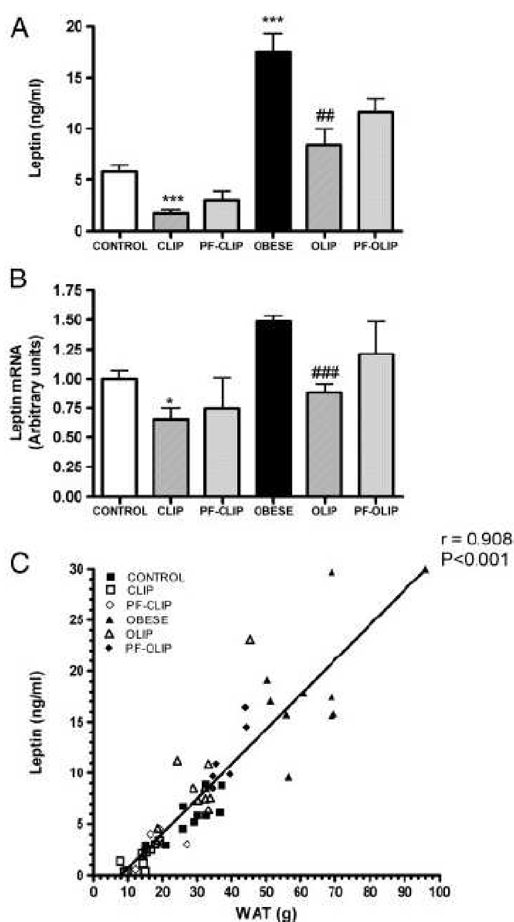

Figure 1. LA supplementation inhibits leptin in control and high fat-fed rats. (A) Effects of LA (0.25 g/100 g diet during 56 days) on leptin-circulating levels and (B) leptin gene expression in epididymal fat. (C) Pearson's correlation between leptin-circulating levels and total WAT. Data are expressed as mean±SE. (n = 10–12 for Control, CLIP, Obese, and OLIP; n = 6 for PF-CLIP and PF-OLIP). ***$p<0.001$, *$p<0.05$ versus Control; ###$p<0.001$, ##$p<0.01$ versus Obese.

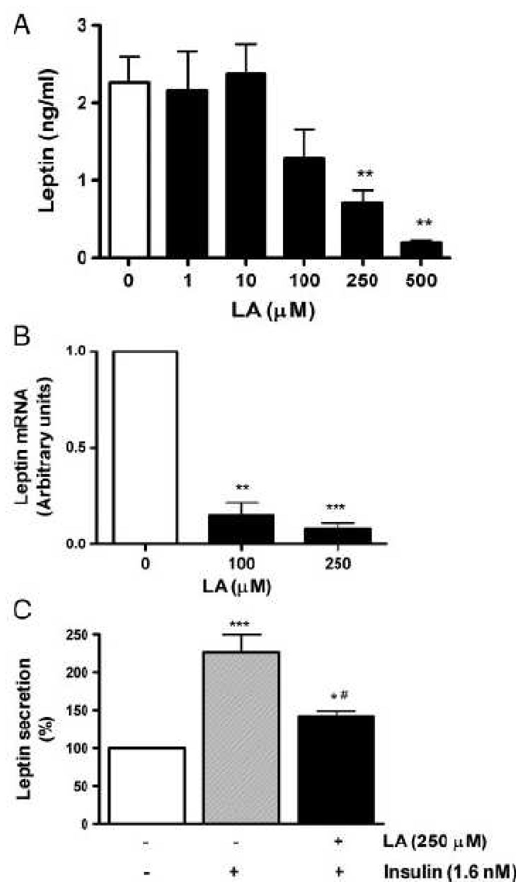

Figure 2. LA inhibits leptin production in 3T3-L1 adipocytes. Effects of different concentrations of LA (1–500 μM) on (A) basal leptin secretion and (B) leptin gene expression in 3T3-L1 adipocytes, and (C) on insulin-stimulated leptin secretion in primary-cultured epididymal rat adipocytes treated over 48h. Data are expressed as mean±SE of three to six independent experiments, ***$p<0.001$, **$p<0.01$, *$p<0.05$ versus Control (vehicle-treated cells); #$p<0.05$ versus insulin-treated cells.

leptin secretion in a model of primary rat adipocytes anchored to a collagen matrix, which responds to more physiological concentrations of insulin than 3T3-L1 adipocytes. Figure 2C shows that LA was also able to partially inhibit the stimulatory effect of insulin on leptin secretion.

3.3 Effects of LA on glucose uptake, lactate production and the percentage of glucose metabolized to lactate in 3T3-L1 adipocytes

Glucose uptake was not affected by LA treatment (Fig. 3A). However, LA increased lactate production by 44% at the

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highest concentration tested (500 μM, $p<0.01$), whereas lower concentrations did not show any effect (Fig. 3B). Treatment of 3T3-L1 adipocytes with LA (0–500 μM) significantly increased in a concentration-dependent manner, the percentage of glucose that is metabolized to lactate ($p<0.01$ to <0.001; 100–500 μM) (Fig. 3C). Furthermore, the percentage of glucose metabolized to lactate was inversely correlated with leptin secretion as shown in Fig. 3D ($r=-0.448$; $p<0.001$).

3.4 Effects of LA on Sp1–DNA-binding activity

To define the mechanisms of LA inhibitory action on leptin, we study LA effects on the transcription factor Sp1, which has been shown to mediate the activation of leptin promoter in response to insulin-stimulated glucose metabolism [21]. As shown in Fig. 4A, nuclear extracts from LA-treated adipocytes for 24 h exhibited a decreased abundance of Sp1–DNA complexes ($p<0.01$), suggesting a significant inhibitory effect of LA on Sp1 transcription factor activity.

Several studies have demonstrated that changes in the phosphorylation status of Sp1 are controlling the ability of this transcription factor to bind to DNA [28]. Our data showed that the phosphorylation of Sp1 in Thr453 was significantly increased ($p<0.05$) in LA-treated adipocytes (Fig. 4B). Moreover, we found that treatment of nuclear extracts with PP1 in order to dephosphorylate Sp1 abolished the inhibitory effect of LA on Sp1 activity (Fig. 4C).

3.5 Effects of PI3K, ERK1/2, and JNK inhibitors on LA inhibitory actions on leptin production and Sp1 phosphorylation in 3T3-L1 adipocytes

The effects of several inhibitors of different signalling pathways on both Sp1 phosphorylation and on leptin secretion and gene expression were tested.

Our data evidenced that treatment with the PI3K inhibitor LY294002 (50 μmol/L) mimicked LA actions, and dramatically reduced both leptin secretion (Fig. 5A) and gene expression (Fig. 5B), as well as increased Sp1 phosphorylation (Fig. 5C). Moreover, we also found that LA treatment caused a significant ($p<0.05$) inhibition of Akt Ser437 phosphorylation in 3T3-L1 adipocytes (Fig. 5D). The JNK inhibitor (SP600125) also stimulated Sp1 phosphorylation in adipocytes, but without affecting the LA actions on phosphorylation of Sp1 or leptin secretion. Moreover, the MAPK inhibitor (PD98059) was able to reverse the LA-induced phosphorylation of Sp1, without modifying the inhibitory action of LA on leptin secretion (Fig. 5A–C).

4 Discussion

The previous studies of our group and others (using the same and higher doses than the used in the present study) have demonstrated that the antiobesity effects of dietary supplementation with LA are secondary, at least in part, to their inhibitory actions on food intake [8, 14]. Moreover, it

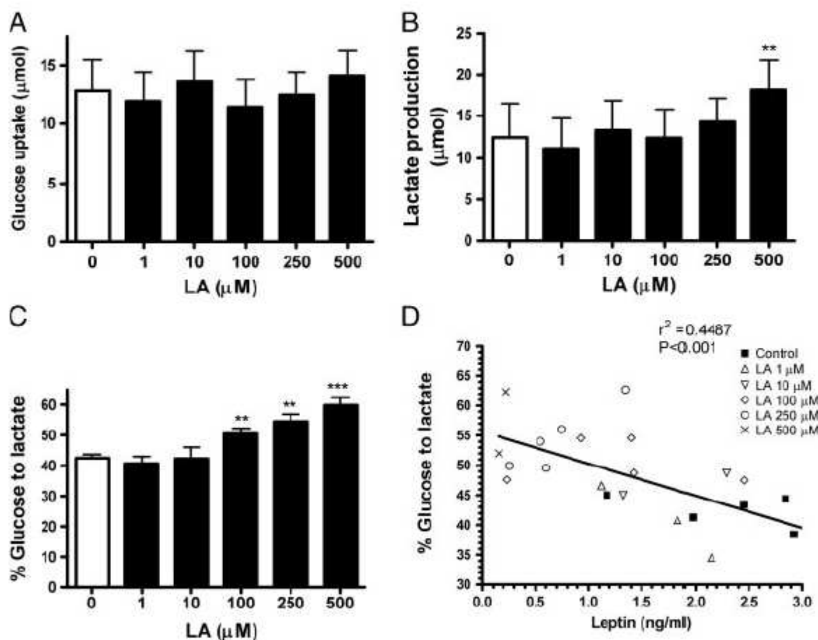

Figure 3. LA increases the anaerobical utilization of glucose in adipocytes. Effects of different concentrations of LA (1–500 μM) on (A) glucose uptake, (B) lactate production and (C) the percentage of glucose metabolized to lactate in 3T3-L1 adipocytes treated over 24 h. Data are expressed as mean ± SE ($n=6$). (D) Relationship between leptin secretion levels and the percentage of glucose metabolized to lactate. $^{***}p<0.001$, $^{**}p<0.01$ versus Control.

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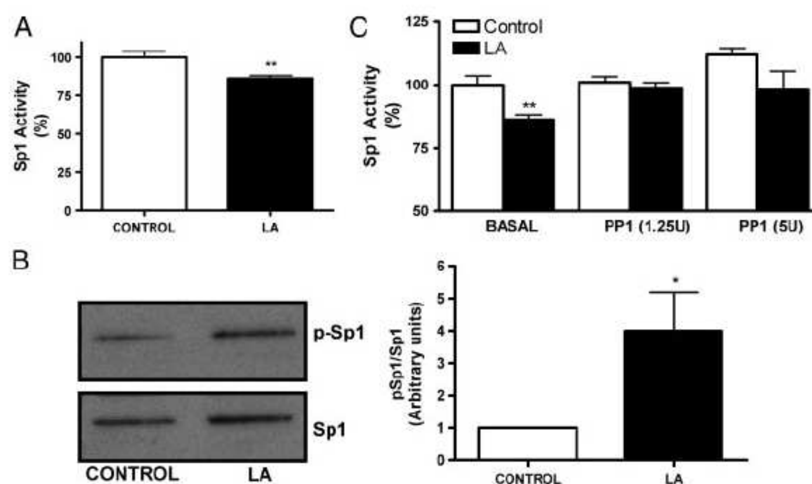

Figure 4. LA inhibits Sp1 activity through phosphorylation. (A) Sp1–DNA-binding activity in nuclear extracts from LA (250 μM)-treated and untreated 3T3-L1 adipocytes during 24 h. (B) Effects of LA (250 μM) on Sp1 phosphorylation in 3T3-L1 adipocytes. (C) PP1 abolishes the inhibitory effect of LA on Sp1 activity. Dephosphorylation reactions were performed at 30°C for 15 min by incubating with PP1 and the nuclear extracts were obtained from control and LA-treated cells. Data are expressed as mean ± SE. ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$ versus Control.

has been evidenced that leptin and its receptor are not essential for α-LA-induced anorexia since the reduction of food intake and body weight was also observed in leptin-deficient or leptin receptor-deficient mice [8].

In addition, it was reported that LA given in food not only decreases food intake but also stimulates whole-body energy expenditure [8], and decreases intestinal α-methylglucoside (α-MG) absorption both in lean and in obese rats [14]. Moreover, other studies showed that dietary supplementation with lower doses of LA that not affect food intake is able to improve glucose metabolism in diabetic rats [29]. All of these data suggest peripheral metabolic actions of LA in different tissues independently of their central actions on food intake.

In the present trial, we have evaluated only the effects of LA on male rats, but it would be interesting to test if a similar or a differential response is observed in females. In this context, the previous studies have described that LA stimulates glucose transport activity and insulin signalling in skeletal muscle of lean and obese female Zucker rats [30, 31], as observed in other models of obese male rats [32].

Our present data show that dietary supplementation with LA decreases adiposity as well as both circulating levels of leptin, and gene expression in WAT. Moreover, the observed changes in leptin levels were positively correlated with the changes in the size of adipose tissue. These results are in agreement with other studies [8, 18, 19, 33], also showing that the body and adipose weight reduction induced by LA treatment is accompanied by a parallel decrease in circulating leptin levels. Moreover, the fact that leptin levels were always slightly lower in LA-treated animals than in their corresponding PF groups suggests a potential direct inhibitory effect of LA on the ability of adipocytes to secrete leptin. However, this possibility had not been yet addressed.

In the present study, we demonstrated for the first time a direct inhibitory effect of LA on both basal- and insulin-stimulated leptin secretions in adipocytes. This effect was concentration dependent, being significant at 250 and 500 μM. Moreover, similar effects of LA were observed on leptin gene expression (from 100 μM), suggesting that LA inhibition of leptin is mediated, at least in part, at transcriptional level. A previous study considered in vitro treatment with 50 μM LA as a physiological relevant dose [34]. Moreover, similar concentrations of LA that are used in our present trial have been shown to be effective in regulating other biological and metabolic functions including adipocyte differentiation [35], mitochondrial biogenesis [36], and glucose uptake [37] in the same adipocyte line. We have not measured the circulating levels of LA reached after dietary supplementation, and therefore it is difficult to compare the correspondence between the doses used in the in vivo and in vitro approaches. However, our findings suggest that the observed decrease in plasma leptin levels after dietary supplementation with LA is likely to be not only due to a decrease in the size of WAT depots induced by LA but also due to a direct inhibitory effect of LA on the ability of adipocytes to secrete leptin could be contributing.

Several in vitro and in vivo studies have demonstrated that glucose metabolism is a major determinant of leptin production in adipocytes [20, 22, 38]. The ability of LA treatment (2.5 mM) for 2–60 min to stimulate glucose uptake in 3T3-L1 cells by inducing a redistribution of GLUT1 and GLUT4 transporters has been described previously [39]. Furthermore, another study in 3T3-L1 cells treated with 250 μM LA has also shown a stimulation of glucose uptake during the first 6 h of treatment. However, a longer pre-incubation period with LA (24–48 h) inhibited glucose uptake into adipocytes, suggesting that the time of treatment with LA is a key factor [37]. Our data show that LA treatment (1–500 μM) during 24 h did not modify basal glucose uptake in 3T3-L1 adipocytes. Therefore, the different results between our present data and the previous

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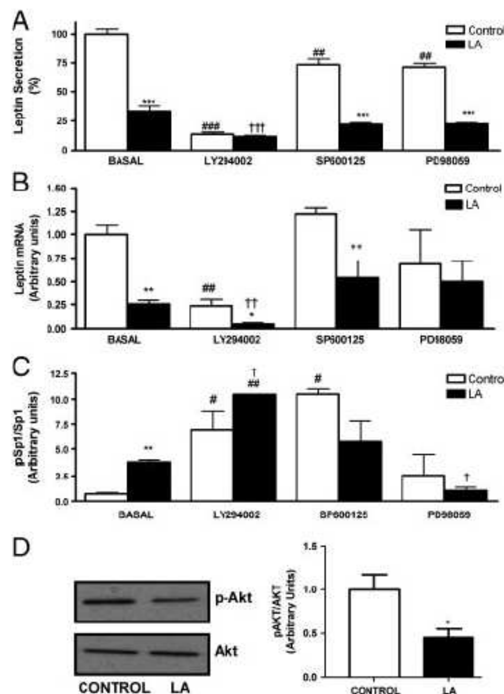

Figure 5. Analysis of the signalling pathways involved in LA actions on leptin and Sp1 phosphorylation. Effects of the PI3K inhibitor LY294002, the JNK inhibitor SP600125, and the ERK1/2 inhibitor PD98059 on LA-induced inhibition of (A) leptin secretion and (B) leptin gene expression, and (C) Sp1 phosphorylation in 3T3-L1 adipocytes. (D) Analysis of Akt (Ser-473) activation in mature 3T3-L1 adipocytes after treatment for 30 min with LA (250 μM). Data are expressed as mean ± SE. ($n = 3$–8 independent experiments). $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$ versus respective Control. $^{\#\#\#}p<0.001$, $^{\#\#}p<0.01$, $^{\#}p<0.05$ versus Basal-Control (vehicle-alone-treated adipocytes). $^{\dagger\dagger\dagger}p<0.001$, $^{\dagger\dagger}p<0.01$, $^{\dagger}p<0.05$ versus Basal-LA-treated cells.

studies could be explained by the different concentrations tested and the period of treatment evaluated.

It has been suggested that uptake of glucose by itself is not the main determinant of the regulation of leptin production, but subsequent metabolic utilization of glucose is playing an important regulatory role of leptin levels [20, 22]. Indeed, leptin secretion has been found to be inversely proportional to the amount of glucose anaerobically metabolized to lactate [40, 41, 26]. In this context, our data demonstrate that LA significantly increased in a concentration-dependent manner, the percentage of glucose that is metabolized to lactate. Moreover, a negative correlation between the leptin secretion and the percentage of glucose anaerobically converted to lactate was observed, suggesting that this increase in the anaerobical utilization of glucose could explain, at least in part, the observed inhibitory effects of LA on leptin.

Sp1 is a ubiquitous transcription factor that may function as a cellular glucose sensor [42]. Furthermore, Sp1 has been identified as an important transcription factor involved in the regulation of leptin gene. Thus, the site centered at −97 bp of the leptin promoter is conserved in evolution and binds Sp1 present in adipocyte nuclear extracts and contributes to promoter leptin activity [43]. In fact, mutation of this Sp1 site in the proximal human and murine leptin promoters reduced promoter activity [43, 44]. Moreover, the previous studies of our group have demonstrated that Sp1 is involved in the induction of leptin by insulin-stimulated glucose metabolism [21]. Our results demonstrated that LA treatment (250 μM for 24 h) inhibited Sp1–DNA-binding activity in mature 3T3-L1 adipocytes. In support of these data, a previous study demonstrated that LA decreased the TNF-α and or IL-1β-induced Sp1-binding activity in human umbilical vein endothelial cells [45]. Moreover, a recent trial has also observed that LA inhibited in a dose-dependent manner, and the activation of Sp1 binding was induced by TGF-β in HepG2 cells [46]. On the contrary, Cho et al. [35] observed in adipocytes at the early stage of differentiation that LA (250 μM, 3 h of treatment) did not modify the transcriptional activity of Sp1 in the absence or presence of insulin. Taking together, the inhibitory action of LA on Sp1-binding activity and leptin production suggests that the inhibition of Sp1-mediated transcription is likely to be involved in the LA-induced reduction of leptin production. However, with our present data we cannot rule out the possibility that leptin production could be inhibited by LA through other mechanisms independently of Sp1 activity.

A growing body of evidence suggests that the DNA-binding and transcription activity of Sp1 may increase or decrease in response to the changes in phosphorylation in many cell types [47]. Our present data clearly demonstrated the ability of LA to phosphorylate Sp1, and that dephosphorylation by PP1 abolished the inhibitory effect of LA on Sp1 transcriptional activity, strongly suggesting that phosphorylation of Sp1 is likely to be involved in the inhibitory action of LA on leptin gene expression and protein secretion. In this context, the previous studies of our group showed that okadaic acid, a potent phosphatase inhibitor, inhibited the stimulation of leptin secretion and mRNA levels induced by insulin-stimulated glucose metabolism [21], suggesting the involvement of Sp1 dephosphorylation. Moreover, dephosphorylation of Sp1 by PP1 has been involved in the glucose-mediated activation of several genes including acetyl-CoA carboxylase, aldolase, and pyruvate kinase [48, 49].

We also analysed the potential signal transduction pathways mediating the inhibitory effect of LA on Sp1 phosphorylation and leptin secretion and gene expression. Several kinases including ERK1/2 and PI3K have been involved in the phosphorylation of Sp1 by different factors such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) [50, 51]. Moreover, it has been shown

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that the insulin-induced up-regulation of leptin in breast cancer cells is regulated by PI3K and ERK1/2 and depends on Sp1 interaction with specific regions of the leptin promoter [52]. Our data show that treatment with the PI3K inhibitor LY294002 mimicked and potentiated LA actions on the phosphorylation of Sp1. Moreover, treatment with the PI3K inhibitor dramatically reduced both basal leptin secretion and gene expression, suggesting an important role of this pathway in controlling basal leptin secretion. This finding is in agreement with the observations of Maeda and Horiuchi [53]. However, other studies using different cell types did not observe an inhibitory action of LY294002 on basal leptin secretion. Taking together, our data strongly suggest that the inhibition of the PI3K pathway by LA could be involved in the LA-induced phosphorylation of Sp1 as well as on the inhibitory effect of LA on basal leptin secretion. Indeed, we demonstrated here that LA treatment inhibited Akt phosphorylation, a downstream target of PI3K, in 3T3-L1 adipocytes. Shi et al. [54] also described that LA (5 mM) causes Akt inhibition in hepatoma cell lines. On the contrary, other studies have reported that LA remediates the age-associated impairment of Akt phosphorylation in primary rat hepatocytes [34]. Moreover, it has been described that LA improves insulin-stimulated Akt phosphorylation in soleus muscle from the high fat-fed rats [32] and from the insulin-resistant Goto–Kakizaki rats [55]. Therefore, the effect of LA on PI3K/Akt pathway is complex and seems to be dependent on the cell type, as well as on the characteristics of the treatment (dose/concentrations and duration) tested.

The previous studies have evidenced the ability of LA to interfere with the MAPK/ERK pathway. Some trials have shown that LA strongly activated ERK in adipocytes at early stages of differentiation [35], whereas others found that LA inhibited the TGF-β-induced phosphorylation of ERK in AML-12 cells [46]. Our present data show that inhibition of ERK1/2 was able to reverse the LA-induced phosphorylation of Sp1, but not the inhibition of leptin secretion, suggesting that this pathway is not involved in the inhibitory actions of LA on leptin production.

Here, we show the ability of antioxidant molecules like LA to inhibit leptin secretion. In the same way, other antioxidants such as Vitamin C and resveratrol also inhibit leptin secretion by adipocytes [56, 57]. However, pro-oxidant agents (exposure to H_2O_2) have also been shown to alter adipokine gene expression in adipocytes, including the inhibition of leptin secretion [58]. JNKs are activated by reactive oxygen species, and are well known for regulating transcription factors through phosphorylation. Moreover, H_2O_2 has also been shown to induce JNK pathway and to phosphorylate Sp1 and reduce Sp1 binding to DNA, and JNK inhibitors are able to attenuate H_2O_2-induced Sp1 phosphorylation in human alveolar epithelial cells [59]. However, Min et al. [46] described that LA inhibited the phosphorylation of JNK and also the TGF-β-stimulated Sp1–DNA-binding activity in HepG2 cells. In the same way, we have observed that in adipocytes LA is also able to induce Sp1 phosphorylation and decrease the Sp1-binding activity. Moreover, we also reported that inhibition of JNK pathway with SP600125 also stimulated Sp1 phosphorylation in adipocytes, without affecting the LA-induced phosphorylation of Sp1. In addition, the inhibition of JNK pathway was not able to reverse the LA actions on leptin secretion. Taking together all of these data, it can be hypothesized that the activation of JNK pathway is not likely to be involved either in the LA-induced phosphorylation of Sp1 or in the inhibitory action of LA on leptin secretion.

Taking together, our present results in cultured adipocytes and previous data of other groups in Hep G2 [23, 46] and HUVEC [45], cells suggest a role of Sp1 in mediating some of the actions observed for LA in in vitro models. However, it still remains to be addressed the potential physiological involvement of Sp1 in the effects of LA after dietary supplementation in animal models.

On the other hand, the fact that LA directly alters leptin secretion by adipocytes raises the possibility that LA could also regulate other bioactive adipokines (such as adiponectin, apelin, and visfatin) that directly regulate nutrient metabolism and insulin sensitivity, opening future research perspectives to better understand the mechanisms of LA actions.

In summary, the present data clearly demonstrate that LA inhibits leptin secretion and gene expression, and suggest that increased anaerobic metabolism of glucose may be contributing to these effects. The phosphorylation of Sp1 and the reduced DNA-binding activity of this transcription factor are also likely to be involved in the inhibition of leptin induced by LA in adipocytes. Moreover, these effects of LA seem to be mediated in part by PI3K/Akt pathway. Furthermore, the findings provided in this study suggest that LA could be a potential therapeutic agent for the treatment of some of the metabolic complications associated to obesity in which hyperleptinemia is involved, including cancer. In fact, it has been suggested that insulin-stimulated leptin may promote breast cancer progression and that this process requires Sp1 and is partially regulated by the PI3K [52].

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The authors have declared no conflict of interest.

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Review

Role of obesity-associated dysfunctional adipose tissue in cancer: A molecular nutrition approach☆

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ABSTRACT

Obesity is a complex disease caused by the interaction of a myriad of genetic, dietary, lifestyle and environmental factors, which favors a chronic positive energy balance, leading to increased body fat mass. There is emerging evidence of a strong association between obesity and an increased risk of cancer. However, the mechanisms linking both diseases are not fully understood. Here, we analyze the current knowledge about the potential contribution that expanding adipose tissue in obesity could make to the development of cancer via dysregulated secretion of pro-inflammatory cytokines, chemokines and adipokines such as TNF-α, IL-6, leptin, adiponectin, visfatin and PAI-1. Dietary factors play an important role in the risk of suffering obesity and cancer. The identification of bioactive dietary factors or substances that affect some of the components of energy balance to prevent/reduce weight gain as well as cancer is a promising avenue of research. This article reviews the beneficial effects of some bioactive food molecules (*n*-3 PUFA, CLA, resveratrol and lipoic acid) in energy metabolism and cancer, focusing on the molecular mechanisms involved, which may provide new therapeutic targets in obesity and cancer. This article is part of a Special Issue entitled: Bioenergetics of Cancer.

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1. Energy balance and obesity

The concept of energy balance involves the exact equilibrium between caloric intake and energy utilization. Energy expenditure takes the form of physical activity, basal metabolism, and adaptive thermogenesis [1]. Physical activity refers to all voluntary movement, while basal metabolism includes the myriad biochemical processes necessary to sustain life. Adaptive thermogenesis refers to energy dissipated in the form of heat in response to environmental changes, such as diet or exposure to cold. In this context, it should be pointed out that the boundary between what is considered basal metabolism versus adaptive thermogenesis is not always clear-cut [1]. Obesity is defined as an abnormal or excessive fat accumulation that involves a risk to health. The fundamental cause of overweight or obesity is a positive energy balance, in which energy intake exceeds energy expenditure over a prolonged time leading to the increased body mass including the accumulation of subcutaneous and visceral fat [2]. However, obesity is a complex disease caused by different factors such as genetic, diet, lifestyle and environmental factors [3]. Some studies estimated that 40–70% of the variation in obesity-related phenotypes could be heritable [4]. In most of cases, obesity appears as a polygenic condition that is additionally affected by a myriad of environmental factors. In fact, the development of overweight and obesity is a consequence of the easy and cheap availability of high-calorie yielding foods, which is combined with sedentary lifestyle changes, occurring in modern societies and affecting genetically predisposed subjects (Fig. 1).

The prevalence of obesity among children, adolescents and adults has been dramatically increasing during the last decades [5,6]. The World Health Organization (WHO) estimates that there are currently more than 1.6 billion overweight adults and at least 400 million of these are obese. Moreover, they predict that by 2015 approximately 2.3 billion adults will be overweight and more than 700 million will be obese [7]. Thus, obesity is acquiring the characteristics of an authentic pandemia and it has been recognized as one of the major global health problems. Indeed, this health hazard is linked to several types of common diseases including cardiovascular disease [8], type 2 diabetes mellitus [9,10], hypertension, dyslipidemia, liver disease and also various types of cancer [9,11,12]. Therefore, the health consequences of obesity are huge and varied, ranging from an increased risk of premature death to several non-fatal but debilitating diseases that have adverse effects on the quality of life.

Furthermore, obesity typically leads to insulin and leptin resistance and a shift to dysfunctional adipose tissue. These conditions cause metabolic dysregulation with elevated circulating fatty acids and an increased secretion of pro-inflammatory adipokines. When left

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untreated, these conditions cause lipotoxicity, chronic inflammation, hypertension, atherosclerosis and cardiovascular disease [13,14]. The association between hypertension and obesity is well documented. Both systolic and diastolic blood pressure increase with BMI (Body Mass Index). Thus, obese people present higher risk to develop hypertension in comparison with lean people [15]. Obese individuals are frequently characterized by an impaired lipid profile, in which plasma triglycerides are raised, HDL-cholesterol concentrations are reduced and low-density lipoprotein apo B (LDL-apoB) levels are raised. This disturbed metabolic profile is more often seen in obese patients with a high accumulation of intra-abdominal fat and has consistently been related to an increased risk of cardiovascular diseases [16,17]. A positive association between obesity and the risk of developing type 2 diabetes mellitus has been also repeatedly reported in different studies. Intra-abdominal fat accumulation, has been associated with an increased risk of prediabetic conditions such as impaired glucose tolerance and insulin resistance [18].

Nonalcoholic fatty liver disease (NAFLD) is another of the consequences of the current obesity epidemic and the hepatic manifestation of the metabolic syndrome. This term encompasses a clinicopathologic spectrum of disease ranging from isolated hepatic steatosis to nonalcoholic steatohepatitis (NASH), the more aggressive form of fatty liver disease and characterized by steatosis, inflammation and progressive fibrosis, ultimately leading to cirrhosis and end-stage liver disease [19]. The most widely accepted theory that explains the pathogenesis of NASH is titled the 'Two Hit Theory' resulting from fatty infiltration of the liver due to obesity and insulin resistance, followed by inflammatory insults, potentially due to oxidative stress [20]. Recent studies estimate that NAFLD affects 30% of the general population and as high as 90% of the morbidly obese [21]. Furthermore, obese patients are at particularly high risk for NASH in view of the frequent coexistence of other features of the metabolic syndrome; thus, the prevalence of NASH in those patients ranges from 20%–30% against 5%–7% in the general population [22]. Although patients with isolated steatosis generally have a benign prognosis, some 26–37% of patients with NASH demonstrate progression of fibrosis over time period of up to 5.6 years, with up to 9% progressing to cirrhosis [23]. BMI and diabetes constitute independent risk factors associated with the progression of fibrosis [24]. Thus, it has been reported that about 40%–62% of patients with NASH-related cirrhosis develop a complication of cirrhosis after 5–7 years of follow-up [25]. The increase in the prevalence of childhood obesity results in a rising prevalence of metabolic syndrome and type 2 diabetes in populations. NASH was first observed in children in 1983 as a pattern of liver injury and it can even develop in obese children under 10 years of age [26]. The significant relation between fasting insulin, insulin resistance and NAFLD in obese children underlines the clinical dimension of these metabolic disturbances [27].

On the other hand, obesity is considered a major risk factor for the development and progression of sleep apnea [28]. Sleep apnea can be a problem with serious implications for anesthetic management, surgery, effect on pulmonary hypertension, stroke coronary artery disease and cardiac arrhythmias [29]. In addition, sleep apnea has a strong correlation with glucose metabolism [30]. Recently, the association between obesity and kidney disease onset has been accepted since several epidemiological and pathological studies support this relationship. A number of epidemiological studies have also provided sufficient evidence of a positive association between obesity and the incidence of cancer, particularly of hormone-dependent and gastrointestinal cancers. Modulation of energy balance, through increased physical activity, reduced the risk of many cancers, including cancers of the colon, breast and endometrium. In this context, it has been shown that weight loss by dietary and physical activity interventions partially reverse metabolic, endocrinal, inflammatory, and renal alterations associated with obesity [31].

2. Obesity and cancer

Energy imbalance is associated with obesity and different studies have observed a relationship between obesity and cancer [32–35].

The concept of a relationship between dysregulated metabolism and carcinogenesis was first enunciated by Otto Warburg [36]. In 2002, the International Agency for Research on Cancer (IARC) expert panel evaluated the link between weight and cancer [37] and concluded that some cancers could be prevented by avoiding weight gain. Since the IARC report, many observational and epidemiological studies have further investigated the association between adiposity and cancer, suggesting that obesity is associated with a significantly

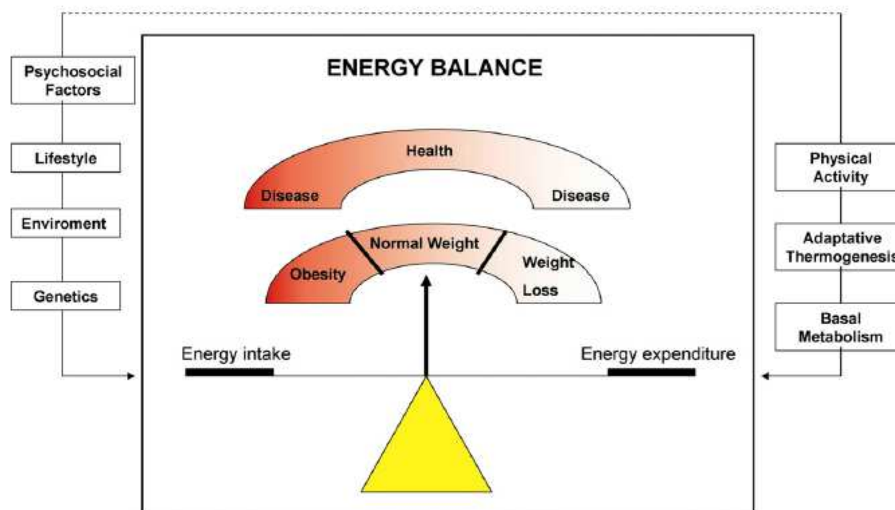

Fig. 1. Fundamental principles of energy balance. A positive energy balance occurs when energy intake is greater that energy expenditure and promotes weight gain/obesity. Conversely, a negative energy balance promotes weight loss.

increased risk of developing several cancer types including those of colon [38], esophagus, breast (in postmenopausal women) [39], endometrium, kidney, liver, gallbladder and pancreas [5,11,35,39,40].

Obesity management is an opportunity for cancer prevention [41], and adipose tissue has been suggested as a target organ in the treatment of hormone-dependent breast cancer and other types of cancer.

2.1. Obesity and breast cancer

Breast cancer is the second most common cancer in the world and the most common neoplasia among women. The association between indicators of body size and risk of breast cancer has been examined in numerous studies [42–44]. Obesity increases breast cancer risk in postmenopausal women by around 50%, probably by increasing serum concentrations of free estradiol [42,43,45]. Interestingly several studies established that the association between body size and the risk of breast cancer differed according to menopausal status [46,47]. In fact, BMI and body weight have been found to be positively related to the risk of breast cancer among postmenopausal women whereas some studies found inverse associations [11]. Furthermore, abdominal adiposity has been found to be positively associated with a higher risk of breast cancer in postmenopausal women, this relationship being stronger among nonhormone-replacement therapy users than among hormone replacement therapy users [48,49].

However, the mechanisms that underlie the association between obesity and breast cancer risk are not completely understood. Several hypotheses have been proposed, including alterations in sex hormones, growth factors and cytokines [11]. Another mechanism by which obesity may induce the development of breast cancer involves insulin and/or Insulin-like growth factors (IGFs) [50].

2.2. Obesity and endometrial cancer

There is convincing and consistent evidence from both case–control and cohort studies that overweight and obesity are strongly associated with endometrial cancer [51,52]. In fact, the risk of developing endometrial cancer is about 2 to 3-fold higher in obese women than in lean women [53] and about 40% of endometrial cancer incidence has been estimated to be attributable to excess body weight [54]. As with breast cancer, the potential mechanism for the increase risk of endometrial cancer associated with obesity is the increase in circulating estrogens [6].

2.3. Obesity and colorectal cancer

Colorectal cancer is the third most common cancer in the world. Incidence rates are approximately 10-fold higher in developed than in developing countries [53]. A possible association between an excess of body weight and risk of colon cancer has been examined in many epidemiological and cohort studies which have concluded that obesity is related with a higher risk of colorectal cancer [33,54,55]. Different studies have suggested that waist circumference and the waist/hip ratio are also strongly related to a higher risk of colorectal cancer and large adenomas in men, as supported by European Prospective Investigation into Cancer and Nutrition (EPIC), whereas body weight and BMI are associated with colon cancer risk in men but not in women [56,57]. The reasons for the gender difference are speculative. One hypothesis is that abdominal adiposity, more common in men than in women, is a stronger predictor of colon cancer risk than peripheral adiposity [58]. However, the mechanisms involved in the association between abdominal obesity and increased colon cancer risk remains still unclear. Another possible explanation is the protective role of exogenous estrogens on the risk of colorectal cancer [59].

2.4. Obesity and prostate cancer

Prostate cancer is the cancer most frequently diagnosed in men in Europe [60]. More than 40 studies, including prospective and case–control studies, examining the association between obesity and risk of prostate cancer have provided conflicting results [61]. However, a recent meta-analysis has suggested a weak significant positive association with an estimated increase in prostate cancer risk (5% excess risk per 5 unit increment of BMI) [62]. The association between waist circumference or waist hip-ratio and risk of prostate cancer has been examined in only a very few studies with most studies reporting no significant associations [62,63].

2.5. Obesity and esophageal cancer

Obesity is associated with a 3-fold increase in risk for adenocarcinoma of the esophagus [6,64]. The link between obesity and risk of esophageal cancer has recently been confirmed by quantitative meta-analysis that included twelve case–control studies and two cohort studies [65]. High BMI is associated with gastroesophageal reflux and frequent reflux is very strongly associated with esophageal adenocarcinoma [66,67]. Thus the increased occurrence in gastroesophageal reflux itself is considered to be a major risk factor for esophageal cancer.

2.6. Obesity and liver cancer

Primary liver cancer is one of the most common and deadly cancers worldwide. Incidence is increasing and hepatocellular carcinoma (HCC) has risen to become the fifth most common cancer and the third leading cause of cancer death [68,69]. Obesity has been established as a significant risk factor for liver diseases. A large prospective mortality study, demonstrated that high BMI was significantly associated with higher rates of liver cancer-related death. Compared to patients with normal BMI, the relative risk of mortality from liver cancer was 1.68 times higher in women and 4.52 times higher in men with BMI>35 kg/m^2 [51]. Similarly, data obtained from the United Network of Organ Sharing (UNOS) database on all liver transplantation from 1991 to 2000 carried out in the United States showed that the overall incidence of HCC in patients undergoing liver transplantation was 3.4% with a slightly higher prevalence among obese patients at 4.0%. Moreover, in this study obesity was confirmed to be an independent risk factor for HCC in patients with alcoholic cirrhosis (odds ratio [OR], 3.2) and cryptogenic cirrhosis (OR, 11.1) [70]. Obesity has definitively been established as a risk factor for the development of HCC. It is likely that this association represents the progression of underlying NAFLD to cirrhosis, but it remains unclear whether cirrhosis is a necessary prerequisite for the development of HCC [71]. Animal models of NAFLD support the hypothesis that obesity-related metabolic abnormalities, rather than cirrhosis, initiate the hepatic neoplastic process during obesity [72].

2.7. Other types of cancer

Obesity has also been linked to other types of cancer, although overall the amount of studies or data available is still limited. Several recent studies have suggested that high BMI may be associated with approximately a doubling of risk for pancreatic cancer in men and women [6]. Moreover, a recent meta-analysis supports a positive relationship between BMI and risk of pancreatic cancer [73]. However, in the study from the EPIC cohort, the BMI was not found to be significantly associated with pancreatic cancer. Two further studies have found some evidence for a positive association with waist circumference in men but not in women [74,75].

The role of obesity on ovarian cancer survival is unclear but it has been suggested that obesity may affect ovarian cancer survival by

having a negative impact on optimal surgical and cytotoxic treatment and increasing the likelihood of postoperative complications [76].

Few studies have investigated the association of BMI with cancers of gallbladder, stomach and uterine cervix but data are limited and inconsistent [32,77].

In summary, these studies have demonstrated that there is a clear association between obesity and different types of cancer (specially, breast, esophageal, colorectal…). However, the biological mechanisms that link overweight and obesity to different forms of cancer, other than those with an endocrine component, are poorly understood. Thus, further research to define the causal role of obesity in these types of cancers is needed.

3. Role of dysfunctional adipose tissue

Obesity is strongly associated with changes in the physiological function of adipose tissue, leading to insulin resistance, chronic inflammation, and altered secretion of adipokines [78]. White adipose tissue (WAT) is a complex and metabolically active organ, with a relevant important role in regulating whole-body metabolism. WAT is the largest energy storage organ, having an important lipid storing capacity in periods when energy input exceeds energy expenditure and with a lipolytic function (release of NEFA) during energy deprivation [1,79]. In addition to its primary role as a fuel reservoir, white adipose tissue has been confirmed as a major endocrine organ, since the tissue synthesizes and secretes an array of sex steroids, and bioactive peptides termed 'adipokines', involved in the physiological regulation of fat storage, energy metabolism, food intake, insulin sensitivity, and immune function among others [80]. In fact, adipose tissue dysfunction might play a crucial role in the different obesity-linked diseases including inflammation, insulin resistance and cancer. Several of these factors, such as insulin resistance, chronic inflammation, decreased levels of adiponectin, increased levels of plasminogen activator inhibitor-1 (PAI-1), endogenous sex steroids, visfatin and leptin, could be involved in carcinogenesis and cancer progression. In this section, we will review the pathophysiological mechanisms linking obesity to cancer, focusing on adipose tissue dysfunction as a potential unifying causal factor [78].

3.1. Sex steroids

WAT is an active organ that secretes different sex steroids. Obesity has an important impact on the synthesis and bioavailability of endogenous sex steroids. Indeed, obesity is associated with an increased serum concentration of estradiol and estrone and a decreased serum concentration of testosterone. Increased levels of estradiol are the result of the peripheral conversion of androgens to estradiol by an overall increased aromatase activity in WAT, secondary to the enhanced total adipose tissue mass [81]. In addition, obesity is associated with increased insulin and bioactive IGF-1 levels which downregulate the concentration of the circulating sex hormone-binding globulin, resulting in an increased fraction of bioavailable estradiol, but decreased testosterone production [82]. Prospective studies suggest this increased bioavailability of sex steroids, especially estrogen which is strongly associated with risk of endometrial and postmenopausal breast cancer [52]. The proliferative effect of estrogen on epithelial tissue of both breast and endometrium is believed to be the underlying mechanism.

3.2. Inflammation

It is well recognized that inflammation is involved in the promotion and progression of cancer [83–85]. Obesity is associated to systemic low-grade inflammation, which has been suggested to have an important role in the pathogenesis of some disorders such as insulin resistance, atherosclerosis and cancer [61,86]. In obesity, the expanding WAT makes a substantial contribution to the development of obesity-linked inflammation via dysregulated secretion (from both by adipocytes and the non-adipocyte fraction) of pro-inflammatory cytokines (Interleukin (IL)-6 and 1 and tumor necrosis factor alpha, TNF-α), chemokines (monocyte chemotactic protein 1, MCP-1) and adipokines (haptoglobin, PAI-1, leptin, visfatin, resistin and vascular endothelial growth factor, VEGF) and the reduction of anti-inflammatory adipokines (e.g. adiponectin, IL-10, antagonist IL-1) [87,88]. The precise role of these inflammatory components in carcinogenesis is not completely understood and therefore continues to be an appealing avenue of research.

TNF-α plays an important role in adaptive responses of the immune system and other organ systems. The anti-tumor effects of TNF-α have been related to activation of Caspase 3 and induction of apoptosis [89]. However, recent studies have suggested that TNF-α is involved in carcinogenesis because of its ability to activate NF-κB [90]. In almost all cell types, when exposed to TNF-α, NF-κB is activated and leads to the expression of a variety of inflammation-related genes. Also TNF-α appears to contribute to the development of the tissue architecture necessary for tumor growth and metastasis [91,92]. It also induces other cytokines, angiogenic factors and matrix metalloproteinases (MMPs) and thus drives to the increased growth and survival of tumor cells [93]. These tumor-promoting activities suggest that inhibition of TNF-α is an effective strategy for cancer therapy. Indeed, clinical trials with several TNF-α antagonists have shown promising effects. For example, D2E7 (a fully humanized anti-TNF-α monoclonal antibody), infliximab (a chimeric immunoglobulin G1 monoclonal antibody against TNF-α), pegylated recombinant humanized sTNF-R1, pegylated humanized anti-TNF-α fragment (CDP870) and TNF-α synthesis inhibitors (p38 kinase inhibitors) have been used to treat various tumors [94].

On the other hand, IL-6 is a pleiotropic inflammatory cytokine, involved in the maturation of B cells, with described cancer-stimulatory [95] and also cancer-inhibitory properties [96]. IL-6 is an important regulator of immune cell growth and differentiation. Recent studies have demonstrated that IL-6 regulates chronic inflammation, which can create a cellular microenvironment beneficial to cancer growth [95]. High circulating IL-6 concentrations in obesity correlated with overall cancer death and increased risk of cancer precursor lesions [78]. The activation of the IL-6 complex activates Janus kinases (JAK) and the signal transducer and activator of transcription 3 (STAT3) pathways, which regulate cell proliferation and apoptosis [97].

Obesity-induced inflammation involves other inflammatory components that could contribute to the development of cancer. These components include MMPs, which are associated with cancer-cell invasion and metastasis [78], suggesting that the strongly induced mRNA levels of several MMPs in obesity, as well as their role in adipocyte differentiation, might represent a potential molecular link between obesity and cancer.

3.3. Adipokines

3.3.1. Adiponectin

Adiponectin is a hormone mainly secreted by adipose tissue, and to a small degree is also produced by cardiac myocytes, muscle cells and endothelial cells [98]. The most important functions of adiponectin identified so far are anti-atherogenic, anti-inflammatory and insulin-sensitivity effects. In contrast to other adipokines, circulating levels of adiponectin are negatively associated with obesity, BMI, visceral fat accumulation and insulin resistance [99]. Several case–control studies have observed that serum adiponectin levels were significantly decreased in breast cancer patients [100]. One study described that adiponectin levels were significantly reduced in postmenopausal women with breast cancer, but not in premenopausal women and, most importantly, this inverse association with

adiponectin was independently associated with BMI [101]. However, another study observed reduced adiponectin levels in both premenopausal and postmenopausal women with breast cancer, and found that patients with serum adiponectin levels in the lowest tertile exhibited significantly larger tumors [102]. Moreover, *in vitro* studies have demonstrated that adiponectin treatment suppressed cell proliferation and caused cell growth arrest and apoptosis in breast cancer cells [103]. Moreover, Brakenhielm et al. [104] reported that adiponectin-induced antiangiogenesis and antitumor activity involves caspase-mediated endothelial cell apoptosis. The role of adiponectin in cancer etiology is not yet fully understood. Although it is possible that adiponectin provides indirect protection against carcinogenesis, by affecting insulin sensitivity and the inflammatory state, adiponectin may have direct anti-carcinogenic effects. The pathway that involves adiponectin and the adiponectin receptors, AdipoR1 and R2 mediates the activation of AMP-activated protein kinase (AMPK). Activated AMPK plays an important role in the regulation of growth arrest and apoptosis by stimulating p53 and p21 [105]. Independent of AMPK activation, adiponectin decreases the production of reactive oxygen species (ROS), which may result in decreased activation of mitogen-activated protein kinases (MAPK) and thereby inhibition of cell proliferation [78,100].

Moreover, the important anti-inflammatory and immunomodulatory properties of adiponectin could also contribute to its anti-carcinogenic effects. Thus, adiponectin has been shown to inhibit endothelial NF-κB signaling and to markedly reduce TNF-α production in cultured macrophages. Moreover, adiponectin also induces the production of anti-inflammatory cytokines IL-10 and IL-1RA in human leukocytes [106].

In conclusion, the decreased plasma levels of adiponectin in obesity may be associated with the increased risk of cancer in obesity. Thus, it has been proposed that upregulation of adiponectin levels might be of therapeutic use in the prevention or treatment of some cancers [100].

3.3.2. PAI-1

PAI-1 is a serine protease inhibitor produced by endothelial cells, stromal cells and adipocytes mainly in visceral adipose tissue. PAI-1 affects adipocyte differentiation and insulin signaling. Furthermore, increased PAI-1 levels have been associated with obesity, as the result of increased PAI-1 production from obese adipocytes [107].

Moreover, PAI-1 inhibits urokinase plasminogen activator (uPA), which acts as an inducer of fibrinolysis and extracellular matrix degradation, and is associated with tumor cell invasion and metastasis [108]. Paradoxically, PAI-1 is involved in tumor growth, invasion, metastasis, and angiogenesis by interacting with vitronectin, integrins, and other components of the uPA system and by affecting the extracellular matrix [78].

PAI-1 is a poor prognostic indicator for a number of cancers including breast cancer. However, there is no single mechanism to explain why an elevation in PAI-1 protein results in decreased patient survival [109]. In this context, there are a number of studies that suggest alternative roles for PAI-1 outside of the traditional protease inhibitor role. Specifically, several studies have indicated that PAI-1 promotes tumor growth through an inhibition of apoptosis [110], regulation of angiogenesis, as well as increased cell adhesion, and increased migration. In addition to the role of PAI-1 in breast cancer migration and invasion, it has been implicated in an inflammatory response. Taking together the current knowledge, it has been proposed that inhibition of PAI-1 might be a potential target in cancer therapy [109].

3.3.3. Visfatin/ NAMPT/PBEF

Visfatin was identified as an adipokine highly expressed in visceral fat of human subjects and rodents, whose plasma circulating levels were positively correlated with the size of visceral fat depots. Besides, this adipokine seemed not to be correlated with subcutaneous fat depots and consequently, it was termed visfatin (from visceral fat) [111]. This adipocytokine was initially discovered as pre-B cell colony-enhancer factor (PBEF), because it favored the development of lymphocyte B colonies [112]. Later on, it was described as nicotinamide phosphoribosyltranferase (NAMPT), a key enzyme that catalyses the first step in NAD biosynthesis from nicotinamide, which widens considerably its biological perspective [113].

The role of visfatin in obesity and linked metabolic disorders remains controversial [98]. Thus, several studies showed increased levels of visfatin in obesity, diabetes mellitus, and cardiovascular disease [114,115]. However, other studies reported lower levels of visfatin in these diseases [116–118]. The discrepancies in clinical studies may be attributed to the multifactorial regulation of visfatin as well as to the lack of concordance between commercially available visfatin assays [119]. Thus, more research is needed to better define the role of visfatin in metabolic diseases.

Moreover, several studies have shown an increased expression of Nampt/PBEF/visfatin in different types of cancers [120]. Thus, a recent study suggests that visfatin plays a role in prostate cancer progression, with particular relevance and emphasis in an obese population [121]. Furthermore, Wang et al. [122] described that NAMPT is prominently overexpressed in human prostate cancer cells and that elevation of NAMPT expression occurs early for the prostate neoplasia. Moreover, the inhibition of NAMPT significantly suppresses cell growth in culture and growth of xenografted prostate cancer cells in mice. Furthermore, they demonstrated that NAMPT knockdown sensitizes prostate cancer cells to chemotherapeutic treatment [122]. Nakajima et al. [123] observed that visfatin levels were gradually increased with stage progression in gastric cancer patients. Visfatin has also been related with breast cancer. In fact, visfatin has been shown to stimulate proliferation of MCF-7 human breast cancer cells [124]. Moreover, FK866/APO866 and CHS828/GMX1777 are two known inhibitors of visfatin and have been evaluated as anticancer agents in the clinic [120].

3.3.4. Leptin

Leptin, a 16 kDa adipokine produced by WAT, plays a critical role in the regulation of body weight and energy balance by inhibiting food intake and stimulating energy expenditure. Circulating leptin levels are actively transported through the blood-brain barrier and activates the hypothalamic anorexigenic neurons POMC/CART (pro-opiomelanocortin; cocaine and amphetamine regulated transcript) while inhibiting orexigenic NPY/AgRP (neuropeptide Y; agouti related peptide) neuropeptides leading to decreased appetite [125]. The key role of the leptin system in regulating body fat in animals and humans is demonstrated by the severe hyperphagia and obesity caused by leptin deficiency. However, leptin concentrations positively correlate with total body fat mass. Thus, leptin serum levels are high in obese subjects, suggesting that resistance to leptin action develops with chronic overfeeding and obesity. Leptin circulating levels rapidly decline under caloric restriction and weight loss [126].

Leptin has also shown to be involved in the inflammatory response, the regulation of insulin sensitivity as well as with carcinogenesis [127]. Leptin plays an important role in both adaptive and innate immunity. Accordingly, the leptin receptor is found to be expressed on a variety of immune cells. The most evident effects of leptin on the modulation of adaptive immune responses have been shown in leptin-deficient mice (*ob/ob*) and humans, which exhibited impaired immunity in parallel to metabolic disturbances [128]. These alterations are reversed by the administration of recombinant leptin [106]. With regard to innate immunity, leptin is a direct potent chemoattractant for monocytes and macrophages and also upregulates the phagocytic function of these leukocytes [127].

Leptin has also been shown to be a growth factor in cancer cell lines [129]. Indeed, leptin caused stimulation of normal and tumorous

cell growth as well as migration, invasion and enhancement of angiogenesis [130]. Moreover, elevated circulating leptin levels have been identified in patients with different types of cancer [128].

Numerous studies have investigated the complex mechanisms involved in the relationship between obesity, leptin and different cancers. Thus, Fig. 2 summarizes the potential pathways directly linking dysfunctional adipose tissue to obesity and cancer.

3.3.4.1. Leptin and breast cancer. It has been suggested that leptin induced proliferation of breast cancer cell lines, by activating JAK2–STAT3, PI3K–Akt–GSK3, ERK1/2, and AP-1 pathways, increasing the expression of proteolytic enzymes that are essential in metastatic process and stimulating angiogenesis, which is needed for tumor growth [131,132]. Specifically, in estrogen receptor-positive human breast cancer cell lines, leptin exerts its effects through activation of MAPK pathway [133,134]. Leptin itself can also enhance aromatase activity in MCF-7 cells and increase the production of estradiol or activate the telomerase which also promotes cell proliferation [135]. High levels of VEGF and leptin are strongly linked. Thus, leptin signaling upregulates VEGF in human and mouse mammary tumor cells (MT), which has been linked to worse prognosis of breast cancer, but the specific molecular mechanisms are largely unknown. A recent study demonstrated that leptin signaling regulates VEGF mainly through HIF-1alpha and NF-κB, and suggested that disruption of leptin signaling could be used as a novel way to treat breast cancer [136]. However, different studies exploring serum leptin levels in women with breast cancer showed inconsistent data. Thus, while some studies observed elevated plasma leptin levels and increased leptin gene expression and leptin receptor expression in breast cancer patients, other investigations do not support a relationship between systemic leptin levels and risk of breast cancer [87,131]. Further studies are needed in order to investigate the relationship between leptin and breast cancer as well as the underlying mechanisms.

3.3.4.2. Leptin and endometrial cancer. A close association between high leptin levels, as a consequence of obesity, and endometrial cancer has been described [137,138]. A recent study has shown that leptin may promote cell proliferation of endometrial cancer cells by the functional activation of cyclooxygenase-2 (COX-2) through JAK2/STAT3, MAPK/ERK, and PI3K/AKT-dependent pathways, suggesting that COX-2 may be a critical factor of endometrial carcinogenesis in obesity [139].

3.3.4.3. Leptin and colorectal cancer. There is accumulating evidence that leptin signaling might be involved in the development of colon cancer. Thus, data from a cohort study detected an almost 3-fold increased risk of colon cancer among people with high leptin levels [140]. Another case–control study in Japanese women also suggested that leptin substantially increases the risk of colorectal cancer [141]. Several *in vitro* experiments have also demonstrated a significant mitogenic activity of leptin in colonic epithelial cells [142,143]. Leptin can induce proliferation through the activation of the NF-κB and ERK1/2-dependent pathways [142,143], as well as by upregulating the c-fos expression in colon cancer cells [144].

Diverse nutritional trials demonstrated that diets rich in fats that increase circulating leptin promote carcinogenesis by stimulating colon cell proliferation [131,144]. Other studies carried out in animals supported the hypothesis that leptin is a growth factor in colonic epithelium and therefore that hyperleptinemia promotes epithelial dysplasia, which in turn promotes colorectal tumorigenesis. However, the relationship between leptin and colorectal cancer is not fully understood. Indeed, other research studies in rodents suggested that leptin treatment may have some protective effects against colon carcinogenesis [145]. Furthermore, controversial data were found regarding the association between serum leptin and colorectal cancer risk [128].

3.3.4.4. Leptin and prostate cancer. Several studies showed association between moderately elevated or high leptin levels with prostate cancer risk [146]. Leptin levels have also been significantly correlated with testosterone and specific prostatic antigen [147]. Leptin may interact with markers related to abdominal obesity such as sex hormones or IGF-1 increasing the risk of developing prostate cancer [131]. Several *in vitro* and *in vivo* studies reported that leptin increased cell proliferation, via JNK activation, and induced suppression of apoptosis, favoring tumor cell progression, invasion and metastasis [147], corroborating the role of this adipokine on prostate cancer.

3.3.4.5. Leptin and esophageal cancer. Several authors proposed a link between leptin and esophageal adenocarcinoma. *In vitro* studies reported that leptin stimulates cell proliferation on human esophageal cancer cells (OE-33, OE-19, KYSE-410) through activation of ERK and epidermal growth factor receptor system and through a reduction of apoptosis [128]. However, no studies have examined the association between serum leptin and esophageal cancer risk. In this sense, two recent case–control studies examined serum leptin concentrations in patients with Barrett's esophagus [148] describing a 3-fold increased risk of Barret's among men in the highest quartile of serum leptin. However, Francois et al. [149] did not find any association between plasma leptin with the risk of Barrett's.

In summary, all the previous studies support the important contribution of some key adipokines in the control of growth and proliferation of different types of tumors (see Table 1).

3.4. Insulin resistance, obesity and cancer

Dysfunctional adipose tissue in obese subjects makes a key contribution to the development of obesity-linked hyperinsulinemia and insulin resistance. Insulin upregulates hepatic production of IGF-1 and both act as growth factors able to promote cancer cell proliferation and decrease apoptosis [150]. These IGF-1 effects are mediated through several downstream signaling networks, including the phosphatidylinositol 3-kinase (PI3K)–AKT system and the Ras/Raf/MAPK systems, respectively [151]. Moreover, clinical studies have shown that patients with high levels of IGF-1 have an increased risk of several types of cancer, including colorectal, prostate, and postmenopausal breast cancer. Hyperinsulinemia is also an independent risk factor for breast cancer in postmenopausal women and increases the risk of colorectal and endometrial cancer; however, some controversial results have been found (reviewed by van Kruijsdijk et al. [78]).

3.5. Oxidative stress, obesity and cancer

Oxidative stress is generating much recent interest mainly because of its accepted role as a major contributor to the etiology of several pathologies with serious public health implications such as obesity and cancer [152,153]. Oxidative stress can result from an imbalance between reactive species production and body antioxidant defences. ROS primarily originate in mitochondria during oxidative phosphorylation. The study of Furukawa et al. [152] found that increased oxidative stress in accumulated fat is an early instigator of metabolic syndrome and that controlling the redox state in adipose tissue is a potentially useful therapeutic target for obesity-associated metabolic syndrome. In fact, this and other trials have observed that oxidative stress caused dysregulated production of adipokines, cytokines and chemokines including adiponectin, leptin, resistin, PAI-1, IL-6, and monocyte chemotactic protein-1 in adipocytes [154,155].

The role of oxidative stress in cancer is controversial. In fact, both pro- and anti-cancer effects have been attributed to ROS. Thus, increased ROS production control tumor cell proliferation and enhance metastatic potential of tumor cells [156]. However, several studies have suggested that a pharmacological regulation in favor of increasing intracellular ROS and/or depleting protective reducing

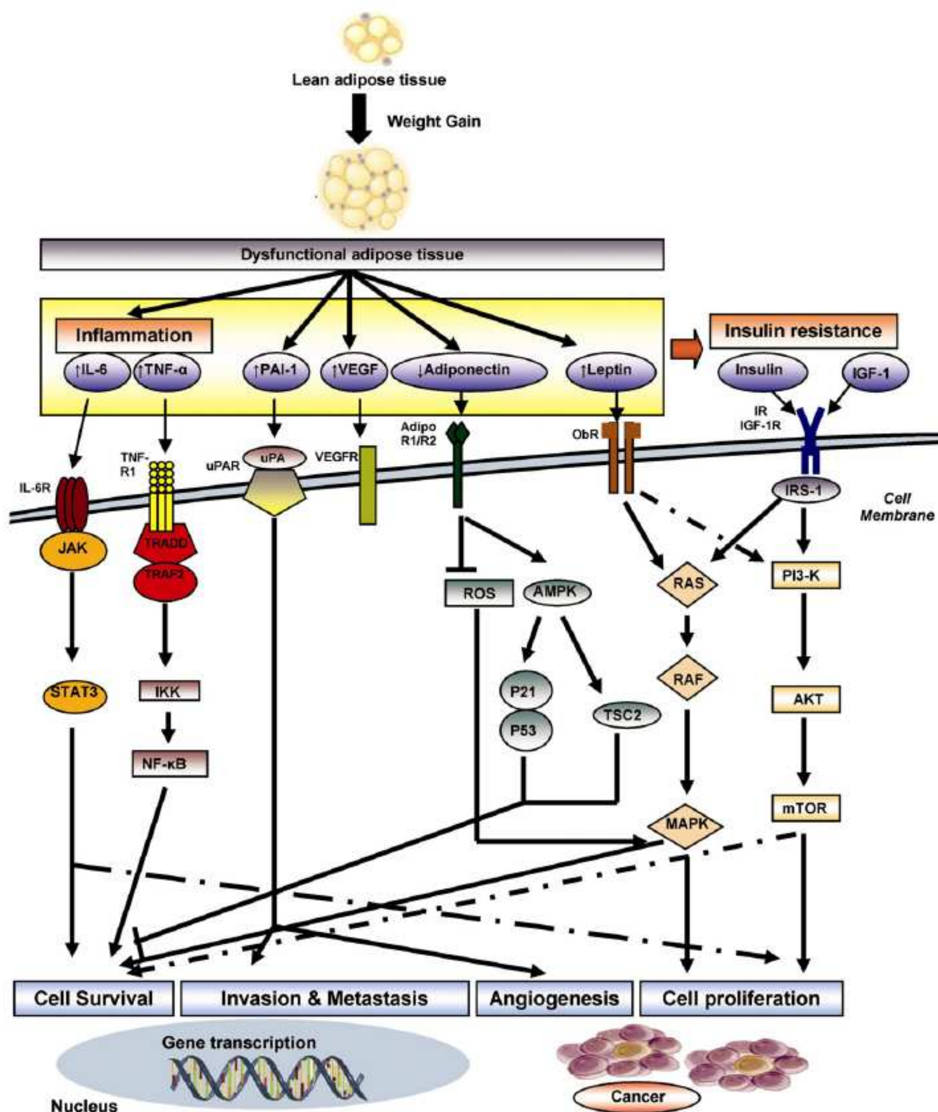

Fig. 2. Potential pathways directly linking obesity with cancer. AdipoR1/R2, adiponectin receptor 1/2; AMPK, 5′-AMPactivated protein kinase; IGF-1, insulin-like growth factor-1; IGF-1R, insulin-like growth factor-1 receptor; IKK, IκB kinase; IL-6, interleukin-6; IL-6R, interleukin-6 receptor; IR, insulin receptor; IRS-1, insulin receptor substrate-1; JAK, Janus kinase; MAPK, mitogen-activated-protein-kinase; mTOR, mammalian target of rapamycin; NF-κB, nuclear factor-κB; ObR, leptin receptor; PAI-1, plasminogen activator inhibitor-1; PI3K, phosphatidylinositol 3-kinase; ROS, Reactive oxygen species; STAT3, signal transducer and activator of transcription 3; TNF-α, tumor necrosis factor-α; TNF-R1, tumor necrosis factor-receptor 1; TRADD, TNFRSF1A-associated via death domain; TRAF2, TNF receptor-associated factor 2; TSC2, tuberous sclerosis complex 2; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.
Modified with permission from van Kruijsdijk et al. [78].

metabolites may lead to oxidative stress and resultant induction of apoptosis for the treatment of cancer [157].

Uncoupling protein-2 (UCP2) is expressed widely including in white adipose tissue, skeletal muscle, pancreatic islets, and central nervous system. Although the function of UCP2 is controversial, UCP2 may play a role in lipid metabolism as well as in energy expenditure Moreover, it has been suggested that UCP2 may function as a sensor of mitochondrial oxidative stress, being activated by ROS and providing protection against ROS production [158]. In fact, loss of UCP2 function may result in increased generation of ROS and reduced antioxidant capacity, whereas UCP2 overexpression conveys cytoprotection to various tissues by limiting oxidative injury [159,160]. These findings suggest a role for UCP2 in physiological states associated with oxidative stress including cancer [161]. In fact, cancer cell survival

depends on adaptive mechanisms that include modulation of oxidative stress. UCP2 expression has been found to be increased in different types of cancer, including human colon cancers, supporting the idea that UCP2 is part of a novel adaptive response by which oxidative stress is modulated in cancer [160]. However, it has been considered that the physiologically significant roles of UCP2 in the protection against reactive oxygen species remain still circumstantial [162].

3.6. Mitochondrial biogenesis, obesity and cancer

Mitochondrial dysfunction can lead to the development of several metabolic diseases such as obesity and cancer. In this sense, several studies have demonstrated that adipose mitochondrial biogenesis was overwhelmingly suppressed in both genetic and high-fat diet-induced rodent models of diabetes/obesity [163,164]. Peroxisome proliferator-activated receptor gamma coactivator (PGC-1α) and the NAD-dependent deacetylase SIRT1 have been characterized as master regulators of mitochondrial biogenesis [165]. Both, the expression of PGC-1α and SIRT1 are reduced in adipose tissue of obese subjects [166,167].

Therefore, developing therapeutics to improve mitochondrial function and/or biogenesis is an attractive strategy for preventing these disorders (reviewed by Pérez-Matute et al. [168]). In this sense, it has been recently demonstrated that the insulin sensitizer actions of pioglitazone could be due, at least in part, to its stimulatory effects on mitochondrial biogenesis in human subcutaneous adipose tissue [169]. Moreover, it has been observed that part of the mitochondrial dysfunction can be triggered by adverse nutrition conditions [170], and that bioactive food components may contribute to improve adipose tissue failure and mitochondria [171].

4. Molecular nutrition, energy metabolism and cancer

Cancer is known to be caused by a variety of factors including sedentary lifestyle, infections, radiation exposure and hormonal factors. Furthermore, breast, prostate, colorectal, esophageal and liver cancers seem to be also associated with dietary patterns. In fact, dietary factors account for approximately 30% of tumors in industrialized countries [172]. However, and despite these studies, there are some inconsistencies caused by the multi-factorial and complex nature of cancer as well as the different genetic background of individuals. In addition, not all macronutrients affect genes and oncogene expression in the same way [171]. Variation in cancer incidence among and within populations with similar dietary patterns suggests that an individual's response may reflect interactions with genetic factors, which may have ramifications in gene, protein and metabolite expression patterns (reviewed by Davis and Milner [173]).

Nutrigenomics considers the relationship between a specific nutrient or a diet and gene expression, whereas nutrigenetics determines how genetic variability affects the response to dietary pattern, functional food or supplement for a specific health outcome [174]. The specific fields of genome-health nutrigenomics and genome health nutrigenetics are proposed on the premise that a more useful approach to the prevention of diseases caused by genome damage is to take into consideration that (a) inappropriate nutrient supply can cause sizeable levels of genome mutation and alter expression of genes required for genome maintenance and (b) common genetic polymorphisms may alter the activity of genes that affect the bioavailability of micronutrients and/or the affinity for micronutrient cofactors in key enzymes involved in DNA metabolism or repair. Supplementation of the diet with appropriate minerals and vitamins could, in some cases, help to overcome inherited metabolic blocks in key DNA maintenance pathways [175,176].

In fact, during the last few years, research focused on the study of the mechanisms underlying the beneficial effects of bioactive food in energy metabolism and cancer. It is important to remember that an excess body weight is generally linked to enhanced cancer risk [32,33,35]. Thus, a large number of bioactive food components occurring in food may provide protection at several stages of the cancer process. Representative bioactive components found in food that are protective against cancer include essential nutrients (i.e., calcium, zinc, selenium, folate, Vitamins C, D and E) as well as non-essential food components (i.e., carotenoids, flavonoids, indoles, allyl sulfur compounds, conjugated linoleic acid, n-3 fatty acids, and lipoic acid) [177]. These bioactive food components may modify

Table 1
Effects of different adipokines on cancer cells.

Cancer type	Adipokines	Model of study	Effects and proposed mechanism of action	Reference
Breast cancer	Adiponectin	Breast cancer MDA-MB-231 cells	Induced growth arrest and apoptosis	[103]
	Visfatin	MCF-7 breast cancer cells	Stimulated cell proliferation by upregulating cyclin D1 and cdk2 and MMP-2, MMP-9, and VEGF	[124]
	PAI-1	MDA-MD-435 cell and human breast cancer cell	Promoted tumor growth by inhibition of apoptosis, regulation of angiogenesis and by increasing cell adhesion and migration	Reviewed by [109]
	Leptin	MCF-7 breast cancer cells	Proliferation of breast cancer cells by activation of JAK2–STAT3, PI3K–Akt–GSK3, ERK1/2, MAPK and AP-1	[131–133]
		MCF-7 breast cancer cells	Promoted cell proliferation by upregulating telomerase activity	[135]
		MCF-7 breast cancer cells	Enhanced aromatase expression via AP-1	[134]
Endometrial cancer	Leptin	Human endometrial cancer cells	Promoted cell proliferation by activation of COX-2, JAK2/STAT3, MAPK/ERK, and PI3K/AKT	[139]
Colorectal cancer	Leptin	Human colon cancer HT-29 cells, colonic epithelial cells	Stimulated proliferation of colon cancer cell lines by activating p42/44 MAPK	[142]
		Human colon cancer HT-29 cells treated with sodium butyrate	Induced proliferation by activation of NF-κB and ERK1/2	[143]
		Human colon cancer HT-29 cells, colonic epithelial cells	Stimulated growth and proliferation and upregulation of c-fos expression	[144]
Prostate cancer	Leptin	DU145 and PC-3 human prostate cancer cells	Increased cell proliferation through JNK, PI3K/Akt or ERK1/2 pathways, and suppression of apoptosis	Reviewed by [147]
	Visfatin	Human prostate cancer cells	Overexpression of NAMPT increased prostate cancer cell by increasing FOXO3 and oxidative stress resistance	[122]
		Human prostate tissue and Ln CaP or PC-3 cells	Increased PC-3 cell proliferation and the expression and activity of MMP-2 and -9 by activating MAPK, ERK 1/2 and p38.	[121]
	PAI-1	Human prostate cancer PC-3 cells	Promoted tumor growth through inhibition of apoptosis	[110]
Esophageal cancer	Leptin	Esophageal cancer cells (OE-33, OE-19, KYSE-410)	Stimulates cell proliferation by activation of ERK and epidermal growth factor receptor system. Suppression of apoptosis.	[128]

simultaneously more than one cancer development mechanism such as hormonal balance, cell signaling, cell-cycle control, apoptosis, and angiogenesis [178].

4.1. Dietary fatty acids, obesity and cancer

Dietary factors that potentially increase the risk of cancer include low fruit, vegetable, or fiber intake, high red meat or saturated fat consumption, and exposure to caffeine or alcohol [179]. Some case–control and cohort studies have also found positive associations between dietary glycemic index (a physiological assessment of a food's carbohydrate content through its effect on postprandial blood glucose concentrations), and risk of various cancers, including those of the colon, breast, and prostate [180]. Among the numerous dietary compounds that have been related to cancer, dietary lipids have been revealed as significative ones. Epidemiological and especially experimental studies have established a relationship between dietary fat and breast, colon and rectum tumors and, to a lesser extent, prostate [181,182]. A growing body of evidence suggests that not only the total amount of fat ingested, but also the type of fat included in the diet contribute to the development of obesity and insulin resistance [98]. Moreover, an expanding number of retrospective case–control investigations have also found an increase in cancer risk with increasing fat intake, especially with animal and saturated fat intake [183]. Thus, the association of high red meat or saturated fat consumption with increased colorectal cancer risk is supported by the preponderance of observational data [184]. In contrast to diets high in saturated fat, diets high in fish oil appear to prevent or attenuate the development of obesity and insulin resistance [185–187] and cancer [181].

4.1.1. n-3 Polyunsaturated fatty acids (n-3 PUFAs)

The *n*-3 PUFAs present in fish oil, are known to have numerous beneficial effects on health. These include improvement of endothelial function, anti-arrhythmic effects, reductions in platelet aggregation and serum triglyceride concentrations, and amelioration of pathological conditions such as inflammatory diseases, hypertension and cancer. Diets containing high levels of *n*-3 PUFAs have been shown to reduce WAT and adipocyte size and to prevent non-insulin-dependent diabetes in rats. Indeed, eicosapentaenoic acid (EPA) [20:5 (*n*-3)], one of the prominent *n*-3 PUFAs contained in fish oil, has been reported to be useful in preventing the onset of insulin resistance and diabetes in animal models of obesity and diabetes [188].

Regarding the mechanisms underlying the beneficial effects of marine *n*-3 PUFA consumption, it was demonstrated that *n*-3 PUFA are important regulators of key metabolic transcription factors including the peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding protein (SREBP) [98]. *n*-3 PUFAs have anti-inflammatory effects in a range of chronic inflammatory conditions, because their ability to decrease the production of classic inflammatory mediators such as arachidonic acid-derived eicosanoids and inflammatory cytokines. Moreover, it was recently described that *n*-3 PUFAs serve as substrates for the conversion to a novel series of lipid mediators designated resolvins and protectins, with more potent anti-inflammatory properties than their *n*-3 precursors [189].

AMPK activation could be also involved in *n*-3 PUFA-induced improvements on energy metabolism and insulin sensitivity. A recent study of our group has demonstrated that EPA strongly stimulates AMPK phosphorylation in 3T3-L1 adipocytes [190]. Moreover, two recent trials have described the ability of *n*-3 PUFAs to *in vivo* activate AMPK [191,192]. *n*-3 PUFAs have also been shown to upregulate mitochondrial biogenesis and induce beta-oxidation in white fat in mice, associated with a three-fold stimulation of the expression of genes encoding regulatory factors for mitochondrial biogenesis and oxidative metabolism such as PGC-1α and nuclear respiratory factor-1 (Nrf-1) [193].

Furthermore, the beneficial properties of *n*-3 PUFAs may also partially result from the modulation of WAT metabolism and the secretion of bioactive adipokines that directly regulate energy homeostasis, insulin sensitivity and carcinogenesis. Thus, different studies of our group and others have shown that *n*-3 PUFAs are important regulators both *in vitro* and *in vivo* of the secretion and gene expression of leptin [188,194], adiponectin [194–196], visfatin [118,190] and apelin [118,197], among other adipokines.

The proposed mechanisms for the anticancer actions of *n*-3 PUFAs include suppression of neoplastic transformation, inhibition of cell proliferation, enhancement of apoptosis, and antiangiogenic. A recent study suggests that *n*-3 PUFAs inhibit hepatocellular carcinoma cell growth through blocking beta-catenin and cyclooxygenase-2 [153]. Another investigation suggests that DHA induce apoptosis in human MCF-7 breast cancer cells both *in vitro* and *in vivo*. The induction of apoptosis in these cells is selectively mediated via caspase 8 activation [198]. Recently, it has been suggested that resolvins biosynthesized from *n*-3 PUFAs may play a role as anti-inflammatory and proresolving mediators in colon cancer [199].

4.1.2. Conjugated linoleic acid (CLA)

CLA was initially discovered in 1987 by Ha et al. [200] and it was first identified as an anticarcinogen molecule. Several studies have indicated that CLA exert anti-obesity effects in rodents, although its effects in humans are controversial. The potential mechanisms responsible for the antiobesity properties of 10,12-CLA isomer in rodent models include decreased energy intake by suppressing appetite, increased energy expenditure, decreased lipogenesis and adipogenesis, increased lipolysis and apoptosis [201,202]. Several studies have also shown that CLA regulates both leptin and adiponectin *in vivo* [203] and *in vitro* [194]. Furthermore, it has been suggested that this inhibition of leptin and adiponectin induced by CLA may contribute to the insulin resistance observed in some CLA-treated animals and humans [204].

On the other hand, a number of assays have demonstrated that CLA exerts chemopreventive and therapeutic activities in a number of rodent and human tumor models. Thus, the 10t, 12c isomer of CLA inhibits tumorigenesis and tumor growth in human breast (MCF-7), colon (HT-29) and prostate (LNCaP) cancer cell lines. This inhibitory effect of CLA on tumor growth is mediated in part by its pro-apoptotic activity [205,206].

4.2. Antioxidants, obesity and cancer

The attenuation or complete suppression of oxidative stress as a way to improve several diseases has flourished as one of the main challenges of research in the last years. A number of trials have examined the effects of supplementation with different antioxidants on oxidative stress associated to obesity and/or cancer [205–213]. However, and although several positive effects have been found [207–209] there are some controversial results, specially in the field of antioxidant supplementation and cancer. Thus, a trial found a statistically significant reduction in total and specific cancer incidence and mortality after supplementation with antioxidants [210]. However, other studies observed a lack of effect of supplementation with antioxidants on cancer [211,212]. Furthermore, the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study (ATBC) and the β-Carotene and Retinol Efficacy Trial, especially on lung cancers did not observe reduction in the incidence of lung cancer among male smokers after five to eight years of dietary supplementation with alpha-tocopherol or beta carotene. In fact, these trials raise the possibility that these supplements may actually have harmful as well as beneficial effects [213,214]. Vitamin C also seems to have a controversial role in cancer [215]. Possible reasons for these discrepancies in relation to the efficacy of antioxidant to counteract oxidative stress and improve health relate to (1) the type of antioxidant used (some of the

antioxidants examined were ineffective and nonspecific and dosage regimen or duration of therapy were inefficient), (2) patient cohort included in trials, (3) the trial design itself and (4) inappropriate or insensitive methodologies to evaluate oxidative state (reviewed by Pérez-Matute et al., 2009 [168]).

4.3. Resveratrol, obesity and cancer

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a well-known polyphenolic compound of red wine with numerous beneficial activities, including cardioprotective actions [216], anti-cancer effects [217] and anti-inflammatory and antioxidant properties [218]. Recently, this broad spectrum of effects is enlarged by new data demonstrating a great potency of this compound in relation to obesity and diabetes. It is well established that resveratrol exerts beneficial effects in rodents fed with a high fat diet, substantially reducing visceral fat and body weight gain [219,220]. The mechanisms underlying these resveratrol effects include: induction of genes for oxidative phosphorylation and mitochondrial biogenesis (reviewed by Szkudelska and Szkudelski [221]), inhibition of preadipocyte proliferation and adipogenic differentiation, stimulation of basal and insulin-stimulated glucose uptake, and inhibition of *de novo* lipogenesis [222]. Resveratrol may also influence the secretion and plasma concentrations of some adipokines such as adiponectin and TNF-α and inhibits leptin secretion from rat adipocytes [223,224]. However, data regarding the effects of resveratrol on adipokines are still insufficient to be conclusive.

Several studies have suggested that activation of SIRT1 and AMPK plays a key role in the metabolic effects of resveratrol [225,226]. A recent research has also shown that resveratrol modulates tumor cell proliferation and protein translation via SIRT1-dependent AMPK activation [227]. In this context, resveratrol has been proposed as a potential dietary compound against various cancers including breast and colon tumors. Resveratrol may affect all three discrete stages of carcinogenesis (initiation, promotion, and progression) by modulating signal transduction pathways that control cell division and growth, apoptosis, inflammation, angiogenesis, and metastasis [228]. Recently, it has been shown that resveratrol suppresses IGF-1 induced cell proliferation and elevates apoptosis in human colon cancer cells, via suppression of IGF-1R/Wnt and activation of p53 signaling pathways [217].

4.4. Lipoic acid, obesity and cancer

α-Lipoic acid (LA) or 1,2-dithiolane-3-pentanoic acid is a promising dietary bioactive molecule because of its recognized therapeutic potential on several diseases such as diabetes, vascular disease, hypertension and inflammation [229]. Both LA and its reduced form dihydrolipoic acid (DHLA) exert powerful antioxidant properties although DHLA seems to be a more effective [230]. Their antioxidants functions involve: quenching ROS (reactive oxygen species), regeneration of endogenous and exogenous antioxidants involving vitamin C, vitamin E and glutathione, chelation of redox metal including Cu (II) and Fe (II) and repair of oxidized proteins. LA can be found in different foods such as spinach and cabbage, liver and meat, whole wheat and yeast of beer, but it is also endogenously produced by the liver through the lipoic acid synthase (LASY) machinery. Deficiency of LASY results in an overall disturbance in the antioxidant defence network, leading to increased inflammation, insulin resistance and mitochondrial dysfunction [231].

LA is an important cofactor for mitochondrial bioenergetic enzymes, and therefore, plays a critical role in mitochondrial energy metabolism [232,233]. In addition, there are increasing scientific and medical interests in the potential therapeutic uses of LA. In this sense, several studies have described the putative benefits of LA on obesity and associated complications. Thus, LA reduces body weight and adiposity in rodents [234,235] and humans [236]. Several mechanisms may contribute to the anti-obesity effects of LA including the suppression of hypothalamic AMPK activity [237], which in turn leads to a reduction on food intake, and the stimulation of energy expenditure by increasing Ucp-1 mRNA levels in brown adipose tissue [234] and by enhancing adenosine monophosphate-activated protein kinase (AMPK)-peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α) signaling in the skeletal muscle [238]. Furthermore, the inhibitory action of LA on intestinal sugar transport could also contribute to a lower feed efficiency observed in LA-treated animals [235].

In addition, LA has also beneficial actions in both glucose and lipid metabolism, and it has been proposed as a potential therapy for insulin resistance and type 2 diabetes [239–241]. LA positively interacts with the insulin pathway and glucose handling in muscle and adipocytes, by modulating the IR/PI3K/Akt pathway and GLUT4 translocation [229]. LA also promotes mitochondrial biogenesis in adipocytes and muscle through a stimulation of PGC-1α, contributing to improve the defective mitochondrial function associated to diabetes/obesity [242,243].

Several trials have also suggested the potential use of LA in cancer therapy [244] due to its ability to induce apoptosis in cancer cells [245,246]. However, the molecular mechanisms underlying the apoptotic effect of LA are not well understood. Shi et al. [246] suggested that the inhibition of ROS generation mediated LA-induced apoptosis in hepatoma cells. Moreover, LA, through scavenging ROS, inhibits PI3K signaling and induces mitochondrial pathway mediated apoptosis [246]. However, Moungjaroen et al. [247] demonstrated that LA induced-ROS generation mediates caspase activation and apoptosis in human lung epithelial cancer cells through Bcl-2 downregulation.

A recent study have suggested that LA exerts an inhibitory effect on cell proliferation via EGFRs and Akt signal transduction and induces cancer cell apoptosis in MDA-MB-231 human breast cancer cells [248]. Further studies are needed to better characterize the mechanisms involved in the anti-carcinogenic action of LA.

5. Perspectives

There is emerging evidence of strong associations between obesity and the incidence of cancer. In obesity, the expanding adipose tissue could make a clinically relevant contribution to the onset and development of cancer via dysregulated secretion of pro-inflammatory cytokines, chemokines and adipokines such as TNF-α, IL-6, leptin, adiponectin and PAI-1. More investigation in order to better understand the molecular mechanisms that link dysregulated adipose tissue function and cancer is worth pursuing, as it may provide new therapeutic targets to prevent or treatment in cancer. Nevertheless, tackling obesity is a priority for reducing the incidence in addition to mortality of certain cancers. The identification of bioactive dietary factors or substances that affects some of the components of energy balance to prevent/reduce weight gain is a promising avenue of research. However, the mechanisms by which dietary components modulate obesity and cancer are not fully understood, partly because of the lack of appropriate research tools to identify the complex mechanisms involved. With the emergence of Nutrigenomics, it is now possible to exploit genome-wide changes in gene expression profiles related to molecular nutrition in obesity and cancer. Evolution of '-omics' epigenomics, transcriptomics, proteomics, and metabolomics will allow a better understanding of how dietary factors may affect both energy metabolism and carcinogenesis, leading to healthier foods and, in turn, healthier people and lifestyles.

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ORIGINAL PAPER

Effects of lipoic acid on apelin in 3T3-L1 adipocytes and in high-fat fed rats

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Abstract Lipoic acid (LA) is an antioxidant with therapeutic properties on several diseases like diabetes and obesity. Apelin is a novel adipokine with potential beneficial actions on glucose metabolism and insulin resistance. The aim of this study was to examine in 3T3-L1 adipocytes the effects of LA on apelin gene expression and secretion, as well as elucidate the signaling pathways involved. We also tested the regulation of adipose apelin gene expression by LA supplementation in a model of high-fat diet-induced obesity. LA increased apelin secretion but not apelin gene expression in 3T3-L1 adipocytes. The AMPK inhibitor Compound C induced an increase in LA-stimulated apelin production, and, on the contrary, the AMPK activator AICAR completely reversed the LA stimulatory effects on apelin secretion, also inducing a significant reduction in apelin mRNA levels in this in vitro model. Apelin mRNA levels were increased in those animals fed with the high-fat diet, while the caloric restriction decreased apelin mRNA to control levels. However, apelin gene expression was not significantly modified in rats treated with LA compared with the obese group. The current data suggest the ability of LA to modulate apelin secretion by adipocytes. However the insulin-sensitizing effect of LA in vivo is not related to changes in apelin gene expression in our model of diet-induced obesity.

Keywords Lipoic acid · Apelin · Insulin resistance · AMPK · Adipocytes · Obesity

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Introduction

Apelin is a bioactive peptide identified as the endogenous ligand of the orphan G protein-coupled receptor, APJ. It is secreted by adipocytes as well as by several tissues including heart, brain, lungs, and pancreatic islet cells among others [14]. Apelin has been shown to be involved in the regulation of cardiovascular functions, fluid homeostasis, vessel formation, and cell proliferation [5, 31]. More recently, apelin has been described as an adipocyte-secreted factor (adipokine) with an emerging role in energy metabolism. In fact, several studies have also described that apelin is up-regulated in obesity and insulin resistance [2, 32]. Also, insulin and TNFα have been found to be important up-regulators of

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apelin gene expression in adipose tissue [8]. Recent studies have demonstrated that apelin, administered in a physiological range, improves in vivo glucose metabolism in normal and insulin-resistant high-fat-fed mice by increasing glucose utilization in insulin-sensitive tissues [10]. A very recent study has also demonstrated that apelin stimulates glucose uptake in human adipose tissue ex vivo [1]. Because of this and other beneficial actions of apelin, it has been suggested that over-production of apelin could be one of the last protective defense before the emergence of obesity-related disorders such as insulin resistance and type 2 diabetes [5, 6, 9, 38].

Lipoic acid (LA) is a short chain fatty acid, described as an important cofactor of several mitochondrial bioenergetic enzymes [36] and with important antioxidant properties due to its ability to chelate free radicals and regenerate other intracellular antioxidants [39]. Several studies have demonstrated the beneficial properties of LA on obesity and its related comorbidities. In this context, previous studies from our group described that ingestion of a high-fat diet supplemented with LA decreased body weight gain by a reduction of food intake, feed efficiency, and inhibition of intestinal sugar absorption [27]. Moreover, Butler et al. [3] showed that LA improves hypertriglyceridemia in rats by down-regulating hepatic GPAT-1 and DGAT-2 gene expression, by inhibiting liver TG secretion as VLDL, and by stimulating clearance of TG-rich lipoproteins. Recent studies in humans also reported that LA has beneficial effects in the treatment of pre-obese and obese subjects by decreasing body weight, blood pressure and abdominal circumference [4]. In addition, LA has also beneficial effects on glucose metabolism and it has been proposed as a potential therapy for type 2 diabetes [15, 23, 30, 34, 37]. Thus, LA treatment improved glucose tolerance and insulin sensitivity of skeletal muscle in obese insulin-resistant Zucker rats by reducing oxidative stress [16, 35]. Furthermore, studies carried out in 3T3-L1 adipocytes described that LA stimulates basal and insulin-stimulated glucose transport [33]. Moreover, LA also provides partial protection against the impaired insulin-stimulated glucose transporter 4 translocation and protein kinase B/Akt activation mediated by oxidative stress [11].

In the present study, we investigated the direct effects of LA on apelin production by 3T3-L1 adipocytes and the signaling pathways involved. Furthermore, we also aimed to investigate the effects of dietary supplementation with LA on apelin mRNA levels in white adipose tissue (WAT) in rats fed a high-fat diet in order to find out if LA effects on apelin could explain, at least in part, its protective properties against the development of insulin resistance.

Materials and methods

Culture and differentiation of 3T3-L1 cells

Mouse 3T3-L1 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 10% (*v/v*) calf bovine serum (Invitrogen, CA, USA), and 1% (*v/v*) antibiotics (penicillin/streptomycin) (Gibco, Invitrogen Corporation, CA, USA) and mantained in an incubator set to 37°C and 5% of carbon dioxide [20, 22]. Two days post-confluence pre-adipocytes were induced to differentiate into adipocytes by culturing them for 48 h in DMEM containing 4.5 g/L glucose, 10% fetal bovine serum (FBS) (Invitrogen), antibiotics, and supplemented with dexamethasone (1 mM; Sigma, St. Louis, MO, USA), isobutylmethylxantine (0.5 mM; Sigma) and insulin (10 mg/mL; Sigma). Then, cells were cultured with 10% FBS and insulin for another 48 h. After that, media were replaced with 10% FBS and antibiotics, without insulin and changed every 2 days up to day 8 post-confluence, when cells were considered to be differentiated into mature adipocytes.

Different treatments were added to differentiated 3T3-L1 adipocytes day 8 post-confluence for a period time of 24 h.

Treatments

α-Lipoic acid (Sigma-Aldrich) was dissolved in ethanol. The inhibitors SP600125 (Calbiochem, San Diego, CA, USA), PD98059 (Sigma), LY294002 (Sigma), Compound C (Calbiochem), H89 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA), and KT5823 (Calbiochem) were dissolved in DMSO. The AMPK activator AICAR (Sigma) was dissolved in deonized sterile water. All compounds were prepared as 1,000× stock solutions and added to the culture medium. Control cells were treated with the same proportion of the corresponding vehicle.

Prior to the addition of the appropriate treatments, fully differentiated 3T3-L1 adipocytes were serum starved overnight. Then, cells were treated with or without LA (250 μM) during 24 h. For the identification of the signaling pathways involved in LA action on apelin, 3T3-L1 adipocytes were preincubated for 1 h with selective inhibitors or activators, the JNK inhibitor, SP600125 (20 μM), the MAPK inhibitor PD98059 (50 μM), the PI3K inhibitor, LY 294002 (50 μM), the AMPK inhibitor, Compound C (20 μM), and the AMPK activator AICAR (2 mmol/l).

Animals and diets

Six-week-old male Wistar rats were obtained from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). Animals were housed in polycarbonate cages (3–4 rats per cage) in temperature-controlled rooms (22±2°C) with a 12-h light–dark cycle, fed a pelleted chow diet, and given deionized water ad libitum for an adaptation period of 5 days.

Rats were then assigned into four experimental groups for 8 weeks. The control group ($n=10$) was fed with a standard diet (Harlam Tekland Global Diets, Madison, WI, USA) containing 16.7% of energy as protein, 78.6% as carbohydrate, and 4.6% as lipid per dry weight. The other three experimental groups (obese, OLIP, and PF) were fed with a high-fat diet (OpenSource diets Research Diets Inc. New Brunswick, NJ, USA) containing 60% of energy as lipid, 20% as carbohydrate, and 20% as protein per dry weight. The obese group was fed ad libitum with the high-fat diet ($n=10$), the OLIP group was fed ad libitum with the high-fat diet supplemented with LA in a proportion of 0.25 g LA/100 g of diet ($n=12$), and the pair-fed (PF) group received the same amount of high-fat diet eaten by the group OLIP, but without adding LA ($n=6$). At the end of the experimental period, rats were euthanized, and blood and tissue samples were collected, including WAT depots (epididymal, retroperitoneal, mesenteric, and subcutaneous) as previously described [7]. All fat depots were weighed and kept at −80°C for subsequent analysis. Visceral WAT depot was estimated by the sum of epididymal, retroperitoneal, and mesenteric depot weights.

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

Assays

Apelin concentration in the media was determined after 24 h of culture by ELISA for mouse/rat apelin from Phoenix Peptide (Burlingame, CA, USA).

Serum insulin levels were measured by ELISA for rat/mouse Insulin ELISA kit (Linco, St. Charles, MI, USA).

Serum glucose levels were assayed using a Cobas Mira Autoanalyzer (Roche Diagnostic, Basel, Swiss), as previously described [26].

Analysis of mRNA levels

Total RNA was extracted from 3T3-L1 cells and epididymal fat depots using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA concentrations and quality were measured by Nanodrop Spectrophotometer ND1000 (Thermo Scientific, Wilmington, DE, USA). RNA was then incubated with an RNAse-free kit DNase (Ambion, Austin, TX, USA) for 30 min at 37°C. RNA (2 μg) was reverse-transcribed to cDNA using MMLV (Moloney Murine Leukaemia Virus) reverse transcriptase (Invitrogen). For the real-time quantitative polymerase chain reaction analyses, 4.5 μl of 1/50 dilution of cDNA per reaction were used in a final reaction volume of 10 μl. Apelin mRNA levels were determined using predesigned TaqMan® Assays-on-Demand (Mm00443562_m1 and Rn00581093_m1) (Applied Biosystems, Foster City, CA, USA). Taqman Universal Master Mix was also provided by Applied Biosystems. The reaction conditions were followed according to manufacturer's instructions. Amplification and detection of specific products were performed using the ABI PRISM 7900HT Fast System Sequence Detection System (Applied Biosystems).

Apelin mRNA levels were normalized by the housekeeping gene beta actin also obtained from Applied Biosytems (Mm02619580 and Rn00667869_m1). All samples were analyzed in duplicate. Ct values (the cycle where the emitted fluorescence signal is significantly above background levels and is inversely proportional to the initial template copy number) were generated by the ABI software. Finally, the relative expression level of each gene was calculated as $2^{-\Delta\Delta Ct}$.

Data analysis

Data are expressed as mean with standard errors (SE). Differences were set up as statistically significant at

$p<0.05$. Comparisons between the values for different variables were analyzed by one-way ANOVA, followed by Bonferroni post hoc test or by Student's *t* test or Mann–Whitney *U* test after testing the normality with the Kolmogorov–Smirnoff and Shapiro–Wilk tests. GraphPad Prism 4.0 (Graph-Pad Software Inc., San Diego, CA, USA) was used for the statistical analyses.

Results

Effects of LA on apelin secretion and gene expression in 3T3-L1 adipocytes

Treatment with LA (250 μM) during 24 h significantly increased basal apelin secretion ($p<0.01$) in 3T3-L1 adipocytes (Fig. 1a). A similar pattern of expression was observed in apelin mRNA levels after LA treatment, although it did not reach statistical significance (Fig. 1b).

Signaling pathways involved in the LA stimulatory effects on apelin secretion

To further characterize the stimulatory effects of LA on apelin secretion, 3T3-L1 adipocytes were preincubated with several kinase inhibitors or activators as described in the "Materials and methods" section. Interestingly, the AMPK inhibitor Compound C stimulated ($p<0.05$) apelin secretion and induced an additional and significant ($p<0.05$) increase in LA-stimulated apelin production (Fig. 2a), but without significantly affecting apelin gene expression (Fig. 2b). On the contrary, the AMPK activator AICAR completely reversed ($p<0.05$) the LA stimulatory effects on apelin secretion (Fig. 2a), suggesting that AMPK inhibition could be involved in the regulation of apelin secretion by LA. However, treatment with AICAR alone did not modify basal apelin secretion, while it induced a significant reduction in apelin mRNA levels, which was also observed in the presence of LA (Fig. 2b).

Treatment with the PI3K inhibitor LY294002 increased ($p<0.05$) basal apelin secretion, while a significant down-regulation was observed on apelin mRNA levels (Fig. 2b).

Finally, treatment with the JNK inhibitor SP600125 or the MAPK inhibitor PD98059 did not modify either apelin gene expression (Fig. 2b) or protein secretion (Fig. 2a).

Effects of LA supplementation on body and white adipose tissue weights

As expected, the high-fat diet intake significantly increased ($p<0.001$) both body weight (Fig. 3a) and fat mass (Fig. 3b) compared with the control group, which was completely prevented by the dietary supplementation with LA. This anti-obesity effect of LA was secondary in part to reduced food intake (19.39±0.27 and 16.19±0.19 g/day, $p<0.001$ for obese vs. OLIP groups). However, the fat mass lowering actions observed in LA-treated animals were higher than those observed in the PF group ($p<0.05$) (Fig. 3b).

Effects of LA supplementation on glucose metabolism

As expected, high-fat feeding caused hyperglycemia ($p<0.05$) (Fig. 3c) and hyperinsulinemia ($p<0.01$) (Fig. 3d) as well as increased HOMA index ($p<0.001$) (Fig. 3e), suggesting the development of insulin resistance in these animals.

Interestingly, dietary supplementation with LA dramatically decreased insulin levels and HOMA

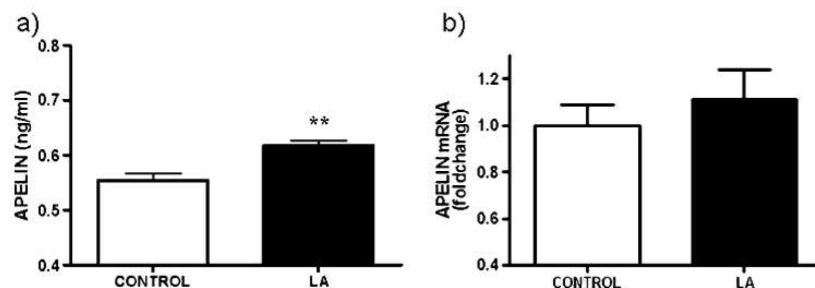

Fig. 1 Effects of LA (250 μM) treatment on **a** apelin secretion and **b** mRNA levels in 3T3-L1 adipocytes. **$p<0.01$ vs. control group

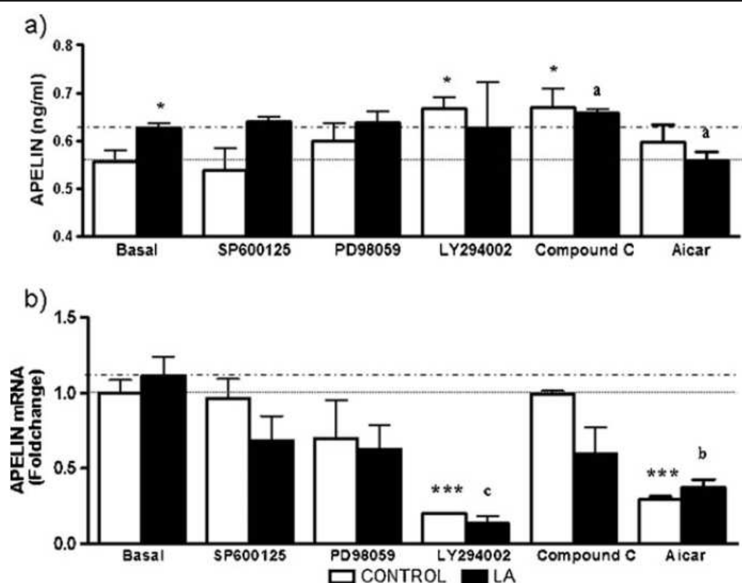

Fig. 2 Signaling pathways involved in the regulation of **a** apelin secretion and **b** gene expression. 3T3-L1 adipocytes were treated with LA (250 μM) in the presence or absence of JNK inhibitor SP600125, the MAPK inhibitor PD98059, the PI3K inhibitor LY294002, the AMPK inhibitor Compound C, and the AMPK activator AICAR. $^{*}p<0.05$ and $^{***}p<0.001$ vs. basal control group; *a* $p<0.05$, *b* $p<0.01$, and *c* $p<0.001$ vs. basal LA-treated group

index ($p<0.001$ and $p<0.01$, respectively), indicating that LA supplementation improves the insulin resistance caused by the high-fat diet. Moreover, the reduction in insulin levels observed in the OLIP animals was higher than the one observed in the PF group, suggesting a direct effect of LA on insulin homeostasis and not secondary to calorie restriction (Fig. 3d).

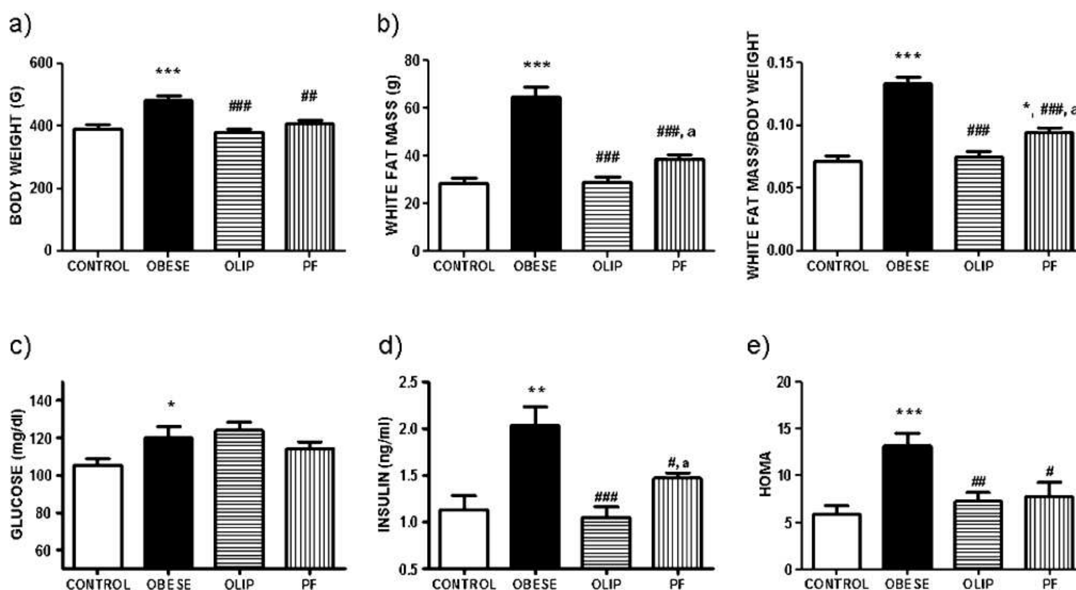

Fig. 3 Effects of dietary supplementation with LA (0.25% *w/w*) during 56 days on **a** body weight, **b** white fat mass, **c** glucose, and **d** insulin, as well as **e** HOMA index. $^{*}p<0.05$; $^{**}p<0.01$, and $^{***}p<0.001$ vs. control group. #$p<0.05$, ##$p<0.01$, and ###$p<0.001$ vs. obese group. *a* $p<0.05$ vs. OLIP group

Effects of LA on apelin gene expression in high-fat-fed rats

We next evaluated the effects of a high-fat diet and LA supplementation on apelin gene expression. As shown in Fig. 4, apelin mRNA levels were increased in those animals fed with the high-fat diet ($p<0.05$), whereas calorie restriction (PF group) decreased apelin mRNA to control levels. However, apelin gene expression was not modified in rats treated with LA in comparison with the obese group while a tendency to increase apelin mRNA levels in comparison with the PF group was observed.

Discussion

Previous studies have demonstrated the ability of LA to regulate the production of several adipokines involved in energy metabolism such as leptin [13, 28, 29].

However, this is the first study where the direct effects of LA on apelin production in adipocytes have been analyzed. Thus, we have demonstrated the ability of LA treatment to increase apelin secretion in 3T3-L1 adipocytes. Previous studies have also reported that other bioactive molecules such as the omega-3 eicosapentaenoic acid (EPA) are able to stimulate apelin production in 3T3-L1 adipocytes [21] as well as in adipose tissue [25].

The lack of significant effects of LA on apelin gene expression suggests that LA stimulates apelin production via post-transcriptional mechanisms. Moreover, our data also suggest that these mechanisms could involve inhibition of AMPK in adipocytes. The role of AMPK pathway in mediating LA actions is complex and seems to be tissue-dependent. In this context, the anti-obesity effects of LA are mediated by suppression of hypothalamic AMPK [15]. However, other studies have reported that AMPK activation is mediating some of the beneficial effects of LA in different disease models. Thus, α-LA acid increases insulin sensitivity by activating AMPK in skeletal muscle in Otsuka Long Evans Tokushima Fatty (OLEFT) rats [18]. Moreover, it has been found that LA decreases hepatic lipogenesis through both AMPK-dependent and independent mechanisms [24]. LA has been also shown to prevent endothelial dysfunction in obese rats via activation of AMPK [17].

Furthermore, we also found that the AMPK activator AICAR caused a down-regulation of basal apelin gene expression, which suggests that this pathway could be also involved in the transcriptional regulation of apelin. To our knowledge, this is the first study reporting the potential role of AMPK in regulating apelin production, although the mechanisms seem to be complex and require further investigation.

Our data also showed that treatment with the PI3K inhibitor LY294002 dramatically inhibited apelin gene expression, as it has been previously reported [2]. However, we found that basal apelin secretion was increased after treatment with this inhibitor, suggesting a complex post-transcriptional regulation of this adipokine, as we have previously described [21].

Many studies have reported an upregulation of apelin production in obesity associated to hyperinsulinemia both in humans and in rodents [2, 6, 19]. However, it has been suggested that the overproduction of apelin in obesity could be one of the last protections before the emergence of the obesity-related disorders such as type 2 diabetes [25]. In this context, a previous study from our group has demonstrated that oral supplementation with EPA ethyl ester induced a significant increase in apelin gene expression in adipose tissue of rats fed a high-fat

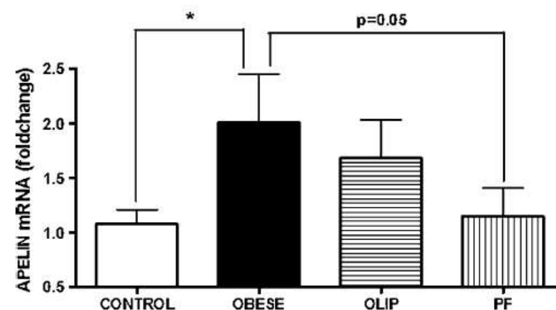

Fig. 4 Effects of dietary supplementation with LA (0.25% *w/w*) during 56 days on apelin gene expression in epididymal fat from high-fat-fed rats. *$p<0.05$ vs. control group

diet. Moreover, HOMA, an index of insulin resistance, was negatively correlated with apelin, suggesting that, to some extent, the insulin-sensitizing effects of EPA could be also related to its stimulatory action on apelin gene expression in visceral fat [25]. Other studies found that dietary supplementation of high-fat diet with vitamin C counteracts the upregulation of apelin mRNA expression induced by high-fat diet in subcutaneous fat, probably as a consequence of the reduction observed on the size of this fat depot [12]. In the present study, we found an over-expression of apelin mRNA levels in epididymal fat from obese and hyperinsulinemic rats, as previously reported [2, 25]. This upregulation of apelin gene expression was reversed by caloric restriction (PF group) in parallel with the reduction of adiposity and hyperinsulinemia. In contrast, rats treated with LA, which also exhibited lower adiposity and insulin levels, did not show a significant decrease in apelin mRNA levels in comparison with the obese group and even a certain stimulatory effect of LA on this adipokine was observed when compared with the PF group. This fact suggests a certain stimulatory action of LA on apelin in vivo in agreement with our in vitro data. However, no significant relationships were observed between these effects of LA on apelin and the HOMA index or insulin levels, suggesting that the previously described insulin-sensitizing effects of LA are not mediated by apelin, at least under our experimental conditions.

In conclusion, our data suggest that LA increased apelin secretion in 3T3-L1 adipocytes. However, we cannot conclude that changes in apelin mRNA levels are involved in the insulin-sensitizing effects of LA observed in an in vivo model of obesity induced by a high-fat diet.

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