PHENOLIC COMPOUNDS AS MODULATORS OF LEPTIN SIGNALLING PATHWAY IN PERIPHERAL TISSUES

Andrea Ardid Ruiz

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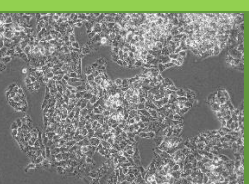
Phenolic compounds as modulators of leptin signalling pathway in peripheral tissues

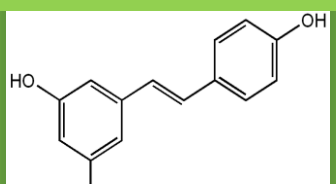
Andrea Ardid Ruiz

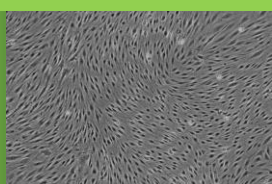
Doctoral Thesis

Universitat Rovira i Virgili

2018







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Phenolic compounds as modulators of leptin signalling pathway in peripheral tissues

Doctoral Thesis

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WE STATE that the present thesis, entitled **Phenolic compounds as modulators of leptin signalling pathway in peripheral tissues**, presented by **Andrea Ardid Ruiz** to obtain the award of Doctor, has been carried out under our supervision in the Nutrigenomics Research Group at the Department of Biochemistry and Biotechnology of this university and fulfils the demanded requirements to get the European Mention.

FEM CONSTAR que la present tesi, titulada **Phenolic compounds as modulators of leptin signalling pathway in peripheral tissues**, presentada per l'**Andrea Ardid Ruiz** per a l'obtenció del títol de Doctora, ha estat realitzada sota la nostra direcció al Grup de Recerca de Nutrigenòmica al Departament de Bioquímica i Biotecnologia d'aquesta universitat, i que acompleix els requeriments per a optar a la Menció Europea.

Tarragona, 3rd of September of 2018

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Dr. Gerard Aragonès Bargalló Dr. Manuel Suárez Recio Prof. Lluís Arola Ferrer

“Si vols, pots”.

AGRADECIMIENTOS – AGRAÏMENTS - ACKNOWLEDGMENTS

La vida es una sucesión de elecciones, tanto buenas cómo malas. Constantes elecciones que nos llevan a lo que realmente somos. Nunca me había planteado hacer un doctorado y mucho menos en Nutrición y Metabolismo. Pero es una elección que tomé en su debido momento puesto que, cuando acabé de realizar las prácticas de final de Grado tuve claro que me encantaba el mundo de la investigación. Ha habido momentos buenos y malos pero, echando la mirada atrás, no me arrepiento de esta elección en concreto. Soy cómo soy a día de hoy por esta elección y estoy más que orgullosa de hasta donde he llegado y mi crecimiento personal y profesional.

Cuando estaba llevando a cabo mi estancia en Szeged, empecé a ver una serie de televisión y recuerdo que me sorprendió gratamente des del primer episodio. Especialmente por una frase que no dejaban de repetir los personajes: “la familia es poder”, decían. Y pienso que es completamente cierto. No puedo estar más orgullosa de la familia que tengo. Estoy muy agradecida a todos ellos por el apoyo que me han blindado durante estos años de estudio, especialmente en los cuatro años de doctorado y, con más hincapié, cuando tuve que estar fuera, en Hungría.

Empezaré con mi yaya María. Ella dio a luz a mi fantástico padre y a mi tía y me gustaría agradecerle el esfuerzo de educar a sus hijos sin la ayuda de su marido. Esto demuestra lo fuerte que ha sido a lo largo de su vida. Gracias, yaya, por ayudarlos y

luchar por ellos para que tuviesen una buena vida y, por supuesto, por compartir tantos momentos especiales a lo largo de mi vida.

Ahora es el turno para mis abuelos que no están aquí pero que tienen la misma importancia. Primero de todo, quiero mencionar a mi abuelo Adolfo. Él nos dejó hace mucho tiempo (incluso mucho antes de que yo naciera) pero todo el mundo dice que era una persona extraordinaria y lo comparan siempre con mi padre. Si mi padre es cómo lo eras tú… Estoy completamente segura que eras una fantástica persona. Me hubiese encantado conocerte y, aunque no ha sido posible, sé que estás conmigo mediante mi padre y espero que estés orgulloso de mí.

Les toca a los padres de mi madre. No tuve la oportunidad de conocer a mi otra abuela María (sólo la vi un par de veces cuando era muy pequeña) pero mi madre siempre nos explica que era muy limpia, pulcra y educada. Y esto no me sorprende en absoluto porque todas estas cosas las puedo ver reflejadas en mi madre. Por otro lado, está mi abuelo Francisco. Tuve la oportunidad de conocerlo en el 2009 y fue realmente un placer. Su espontaneidad, educación y fuerza de espíritu, a pesar de su edad, me sorprendieron des del minuto cero. Pienso que mi madre tiene esa alegría por la vida gracias a él.

Estoy muy orgullosa de tener estos 4 maravillosos abuelos en mi vida. Me han influenciado muchísimo, directa e indirectamente, y estoy aquí escribiendo esta tesis por ellos.

Pienso que ahora estaría bien seguir con la familia de mi padre. Me gustaría dar las gracias a todos ellos: tía Marta, tío José, Beatriz, Pol, Alèxia, Pau, Irene, Cesc, tío Raimundo, tía Tere, Alberto, Carlos, tía María Pilar, tío Monchu, tía Pili y tío Macario.

Y, por supuesto, está la familia de mi madre: tío Paco, tía María Ángeles y Héctor. Gracias por estar siempre allí y por ayudarme a mí y a mi madre en algunas situaciones complicadas. Habéis demostrado lo grandes personas que sois.

Voy a volver a mi familia un poco más tarde pero me gustaría hablar de una persona que ha entrado recientemente en mi vida y a la que tengo que agradecerle mucho. Ella es Gina. Gracias, guapi, por iluminar el camino de mi hermana. Ahora es muy muy feliz y eso es porque te ha conocido a ti. Si mi hermana está bien, yo lo estoy más y, por lo tanto, tanto cómo la cuido a ella te cuidaré y querré a ti.

Ahora quiero centrarme en mi estada en Hungría. Era la primera vez que dejaba a mi familia y mi ciudad y, por supuesto, tenía miedo y estaba preocupada. Sin embargo, después de pasar esta experiencia puedo decir que fue fantástico y muy necesario para mí. No sólo porque me ha hecho ser más fuerte y he crecido muchísimo cómo persona en todos los sentidos sino también porque tuve la oportunidad de aprender muchas cosas nuevas e interesantes al lado de fantásticos profesionales y, lo más importante para mí, increíbles personas. Köszönöm szépen: Dr Mária Deli, Dr Zsófia Hoyk, Dr Szilvia Veszelka, Raquel, Ilona, András, Marika, Lilla, Bandi, Dori, Judit, Vego and Gergő. All of you are so nice and I am so happy of meeting all of you, the amazing Biological Barriers Research Group.

Ara és el torn de la Universitat. He estat pràcticament la meitat de la meva vida allà i, durant aquest període, he pogut conèixer moltes persones increïbles. Crec que tot el món té dubtes quan comença la seva carrera i jo no vaig ser l'excepció. Em preguntava: serà la Bioquímica la tria correcta? La resposta és clara, sí. M'encanta aquest tema i totes les coses que pot explicar de la vida. M'agradaria donar les gràcies a tots els professors perquè m'han fet arribar els seus coneixements per ser on sóc: el /la Cinta, Lluís, Pepa, Anna Arola, Paqui, Begoña, Miquel, Santi, Gerard Pujadas, Ximena, Mayte, Anna Ardèvol, Montse, Raúl i Juan. A més a més, he pogut conèixer meravellosos joves investigadors amb moltíssima motivació: el/la Kathe, Albert, Lisard, Àngela, Susu, Anna, Iris, Carlos Alberto, Carol, Paula, Maria Ibars, Aïda, Cris, Roger, Maria Guirro, Miguel, Héctor, Andreu, Mª José, Sarah, Aleix, Adrià, Carme i tots els investigadors que van estar prèviament. Tots vosaltres sou uns fantàstics científics i, sobretot, gent molt bona. No em vull oblidar de tres joves investigadors que m'han ajudar durant aquests anys tan durs i que sé que, sense el seu suport, la meva tesis probablement no estaria acabada a dia d'avui: David, Érika i Imane. Espero que en el seu moment aprenguéssiu alguna cosa de mi com jo vaig aprendre de vosaltres quan vam treballar junts. Gràcies a la Niurka i la Rosa, per ajudar-me en els moments de crisis i sempre estar allà animant-me.

Finalment, vull fer menció especial als meus supervisors directes: el Gerard i el Manuel. Volia donar-vos les gràcies per tot el que heu fet per mi durant aquest llarg període. Només tinc paraules d'agraïment per tot el vostre suport i paciència, per donar-me tants coneixements, les vostres opinions,… En definitiva, per estar sempre

allà per a mi i fer-me créixer com a investigadora i, sobretot, com a persona. Us desitjo lo millor perquè heu sobreviscut a mi durant aquests quatre anys i no tothom ho pot dir.

Des de que tenía ocho años tuve la oportunidad de disfrutar del más fantástico deporte que he conocido nunca: el baloncesto. Durante este período (17 años), he tenido momentos buenos y malos que me han influenciado mucho y que han hecho que sea la persona que veis hoy en día. Sólo el deporte de por sí me ha cambiado muchísimo y creo que me ha hecho ser más paciente, amigable, respetuosa… Definitivamente, una mejor persona. Pero no únicamente me ha influido el deporte, por supuesto, sino también todas las personas que he ido conociendo durante el proceso y que me han dado la oportunidad de disfrutar de este maravilloso *hobbie*. Des de mi período del CBT, pasando por Torredembarra y, finalmente, mi tiempo en el Manyanet de Reus. Me encantaría centrarme en estos dos últimos debido a los buenos recuerdos que tengo de ellos. Quiero acordarme de todos ellos: Gemma, Érika, Saray, Silvia, Tere, Noe, Farah, Scott y Pep (del Torredembarra) y Anaïs, Júlia, Patri, Sara, Maria, Lopes, Adri, Bultó, Marta, Sandra, Cris, Mireia, Marina Ribas, Marina Giné, las Jurado (Marta y Cris), Alba, Laia, Damián, Joan Marqui y Xavi (del Pare Manyanet). Finalmente, quiero mencionar especialmente a una persona que el baloncesto puso en mi vida: Núria Samperi.

Pero durante este largo período no todo ha sido perfecto. Sufrí una importante lesión en mi rodilla en Setiembre del 2015 que me sacó de las pistas durante seis meses. Sin

embargo, este momento malo me llevó a la que es una de mis pasiones favoritas. Después de realizar una larga recuperación, decidí empezar a ir al gimnasio para mejorar muscularmente. Lo que nunca hubiese imaginado es que el gym se convertiría en mi pasión y mi estilo de vida. Y, des de mi punto de vista, esto es debido a las personas maravillosas que he tenido oportunidad de conocer allí y a las que considero parte de mi familia: Tomás, Ana, Xavi, señora Carmen y muchos más. Sempre seré la teva princesa, superBenjamí. Ets un gran suport per a mi tots els dies i m'encanta parlar amb tu de les nostres coses (potser Galímedes?). No puc evitar pensar i riure de les teves persecucions pel gym.

Finalment, em vull centrar en una persona molt especial: el Dani. No sé ni per on començar per fer-te entendre com has canviat la meva vida des de que t'he conegut. Ets un fantàstic professional i tot el que sé d'aquest món és degut a tu. La teva passió per aquest esport, aquest món, em dóna moltíssima motivació per intentar millorar cada dia. Però la teva persona és molt millor: sempre escoltant-me, ajudant-me, recolzant-me, donant-me la teva mà en els meus pitjors i tristos moments,… Em fas ser una persona més forta, una persona que veritablement confia en ella mateixa i, ara, sé que puc aconseguir tot el que em proposo. Estàs canviant constantment moltes coses en la meva vida: des de coses més concretes relacionades amb el gym fins a la meva manera de veure i entendre el món, el meu món. És per això que mai seré capaç d'agrair-te tot el que fas per mi. Ets fantàstic.

Tornant a la Universitat, ella em va donar una de les persones més importants en la meva vida, la meva millor amiga: la Sheila. Com expressar en paraules el que sento per tu, xurry? Bé, ja coneixem la meva opinió sobre ella però tinc la necessitat de explicar com d'excepcional arriba a ser. Han hagut de passar molts anys per conèixer veritablement la meva ànima bessona però aquí la tenim! Ens coneixem i entenem com si haguéssim coincidit des del minut zero d'haver nascut. Sempre està per mi, per a tot, escoltant-me, recolzant-me, fent-me sentir millor i feliç. Mai havia conegut una persona tan meravellosa abans de tu i espero (i sé) que sempre estarem juntes. Perquè sé que si tu estàs en la meva vida, sóc molt més forta, feliç, una millor persona. T'estimo molt floreta i et desitjo lo millor perquè t'ho mereixes.

Finalmente, había dicho que volvería a mi familia. Y aquí estamos… Hay tres personas especiales en mi vida. Mis padres, Daniel y Angelines, y mi hermana pequeña, Claudia. Quiero decir tantas cosas buenas sobre vosotros… Primero de todo, gracias por estar siempre aquí, des de que nací, creando mi persona. Durante todos estos años hemos tenido buenos y malos momentos pero siempre nos las hemos arreglado para salir adelante y esto es realmente ser familia… Estoy orgullosísima de vosotros y no puedo imaginar mi vida sin vosotros y sin vuestras cualidades fantásticas y especiales que me influencian tan positivamente. Mi padre tiene un fuerte carácter pero un gran corazón y muchísima paciencia. Mi madre es muy nerviosa pero apasionada y muy educada. Mi hermana está un poco loca pero siempre hace lo mejor para el resto y siempre dice lo que está pensando. Por supuesto, tienen imperfecciones pero son estas imperfecciones las que los hacen especiales. He heredado algunas de

ellas pero siempre me ayudan a intentar superarlas y a ser mejor persona. Vuestro apoyo durante todos mis estudios (y también durante mi vida) es incondicional y sé que no hubiese acabado esto sin vosotros, estoy completamente segura. Hacéis que mi vida tenga sentido y deseo con todo mi corazón que nunca nos separemos porque con vosotros, con mi familia, tengo el poder suficiente para conseguir y hacer todo lo que me proponga.

Mi familia es el poder que realmente necesito.

Quiero acabar mis agradecimientos con la palabra de origen hawaiano ‘Ohana, que significa familia, y la familia significa que nadie te deja de lado o te abandona. Durante mi tesis mucha gente me ha apoyado. Pero como siempre digo, tengo mi pequeña ‘Ohana formada por cinco personas que han convertido este difícil camino en un bonito final.

GRACIAS, GRÀCIES, THANK YOU.

A mi ‘Ohana:

Mi preciosa familia: mis padres, Daniel y Angelines, y mi hermana, Claudia.

La meva amiga de l’ànima: la Sheila.

El meu model a seguir: el Dani.

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SUMMARY

Obesity is a current and worldwide extended problem and one of the main factors related with other chronic pathologies. Conventional therapies, normally based on increasing the exercise and reducing the consumption of energy-dense food used to prevent or palliate obesity, are ineffective. In this sense, the use of bioactive compounds as polyphenols, a group of plant secondary metabolites with a wide range of beneficial healthy effects, arises as a novel strategy to combat obesity and its related pathologies.

Leptin is a key hormone secreted proportionally by the amount of adipocytes that acts primarily in the central nervous system controlling the energy balance. In this process, leptin transport across the blood-brain barrier is especially important. In addition, leptin is implicated in the regulation of peripheral homeostasis, mainly modulating the lipid and carbohydrate metabolism, in organs such as liver, muscle and white adipose tissue. However, obesity is related with an impaired action of leptin, namely leptin resistance, causing hyperleptinemia and an increase in the energy intake.

In this context, the aim of this thesis is to identify phenolic compounds with the capacity to restore the obesogenic-leptin resistance condition caused in peripheral tissues (liver, skeletal muscle and epididymal white adipose tissue) and to increase the leptin transport across the blood-brain barrier.

Our results demonstrate the effects of resveratrol and its metabolites acting in the peripheral leptin signalling pathway on reducing body fat accumulation in an

obesogenic rat model. Moreover, resveratrol restores the leptin sensitivity in a palmitate-induced model of steatotic human hepatocellular carcinoma cell line by increasing the leptin receptor content. Finally, the capacity of different phenolic compounds to increase the leptin receptor content and to protect against pro-inflammatory cytokine-induced damage in rat brain endothelial cells is described.

This research provides novel information that can be useful for the functional food industry identifying bioactive compounds that can be used to potentially treat obesity and its related pathologies.

RESUM

L'obesitat és una patologia que, avui en dia, està àmpliament estesa per tot el món i és un dels principals factors relacionats amb altres malalties cròniques. Les teràpies convencionals utilitzades per prevenir o pal·liar l'obesitat, normalment basades en l'exercici i en la reducció del consum d'aliments molt energètics, són inefectives. En aquest sentit, l'ús de compostos bioactius com són els polifenols, un grup de metabòlits secundaris de les plantes amb un ampli espectre d'efectes beneficiosos per la salut, es presenta com a una estratègia innovadora per combatre l'obesitat i les seves patologies associades.

La leptina és una hormona clau que es secreta proporcionalment a la quantitat d'adipòcits i que actua principalment en el sistema nerviós central controlant el balanç energètic. En aquest procés, el transport de la leptina a través de la barrera hematoencefàlica és especialment important. Addicionalment, la leptina està implicada en la regulació de l'homeòstasi perifèrica, principalment modulant el metabolisme lipídic i dels carbohidrats, en òrgans com el fetge, el múscul i el teixit adipós blanc. Però, l'obesitat està relacionada amb un deteriorament de l'acció de la leptina, procés anomenat resistència a la leptina, causant hiperleptinèmia i un increment en la ingesta energètica.

En aquest context, l'objectiu de la present tesi és identificar compostos fenòlics amb la capacitat de restaurar la condició obesitat-resistència a la leptina causada en teixits

perifèrics (fetge, múscul esquelètic i teixit adipós blanc de l'epidídim) i que puguin incrementar el transport de la leptina a través de la barrera hematoencefàlica.

Els nostres resultats demostren els efectes del resveratrol i els seus metabòlits actuant en la via perifèrica de senyalització de la leptina, reduint l'acumulació de greix corporal en un model de rata obesa. A més a més, el resveratrol restaura la sensibilitat a la leptina en un model esteatòtic de cèl·lules cancerígenes hepàtiques humanes tractades amb palmitat, incrementant el contingut del receptor de la leptina. Finalment, s'ha descrit la capacitat de diferents compostos fenòlics en incrementar el contingut del receptor de la leptina i en protegir contra un dany induït per citocines pro-inflamatòries en cèl·lules endotelials de cervell de rata.

Aquesta investigació proporciona una informació innovadora que pot ser útil per a la indústria d'aliments funcionals, identificant compostos bioactius que poden ser utilitzats per a tractar potencialment l'obesitat i les seves patologies associades.

ABBREVIATIONS

AA Amino acid

AGRP Agouti-related peptide

alpha-MSH Alpha-melanocyte-stimulating hormone

AMPK AMP-activated protein kinase

ANS Autonomic nervous system

APOJ Apolipoprotein J or Clusterin

ARH Arcuate nucleus

ASP Agouti signalling protein

ATF Activating transcription factor

BA Benzoic acid

BAT Brown adipose tissue

BBB Blood-brain barrier

BMI Body mass index

B-AR B-adrenoceptor

BW Body weight

CA Cinnamic acid

CAF Cafeteria

cAMP Cyclic adenosine monophosphate

CART Cocaine and amphetamine regulated transcript

CBG Cytosolic B-glucosidase

CBR Cannabinoid receptor

CCK Cholecystokinin

CD38 Cyclic adenosine diphosphate (ADP)-ribose hydrolase

CEBP CCAAT/enhancer-binding protein

cGMP Cyclic guanosine monophosphate

CHOP DNA damage inducible transcript

CIS Cytokine-inducible sequence

CK Cytokines

CLA Conjugated linoleic acid

CNS Central nervous system

COMT Cathecol-*O*-methyltransferase

COX Cyclooxygenases

CPE Carboxypeptidase E

CPT Carnitine palmitoyltransferase

CR Caloric restriction

CREB cAMP-responsive element-binding protein

CRH Corticotropin-releasing hormone

CVO Circumventricular organs

DB Leptin receptor gene

DGK Diacylglycerol kinase

DHA Docosahexanoic acid

DIO Diet induced obese model

DMH Dorsomedial hypothalamus

DPP Dipeptidyl peptidase

EC (-)-Epicatechin

EDHF Endothelium-derived hyperpolarizing factor

EGCG Epigallocatechin-3-gallate

EPA Eicosapentaenoic acid

ER Endoplasmatic reticulum

ERK Extracellular regulated kinase

FA Fatty acid

FAS Fatty acid synthase

FFA Free-fatty acid

FOX Forkhead box protein

FRUC Fructose

FXR Farnesoid X receptor

GA Gallic acid

GADD45 Growth arrest and DNA damage

GAL Galanine

GH Growth hormone

GHRH Growth hormone releasing hormone

GLC Glucose

GLP Glucagon-like peptide

GLUT Glucose transporter

GSPE Grape seed proanthocyanidin extract

G-6-P Glucose-6-phosphate

G6PDH Glucose-6-phosphate dehydrogenase

HEPG2 Immortalized human hepatocellular carcinoma cell line

HFD High fat diet

HTR2C 5-hydroxytryptamine receptor 2C

IGF Insulin-like growth factor

IKKB Inhibitor of nuclear factor kappa B kinase-beta

IL Interleukin

IRS Insulin receptor substrate

JAK Janus kinase

JNK c-Jun N-terminal kinase

LD Long day

LHA Lateral hypothalamic area

LOX Lipoxygenase

LPH Phloridzin hydrolase

LPR2 Low-density lipoprotein (LDL) receptor-related protein-2 or Megalin

LR Leptin resistance

LSR Leptin sensitivity ratio

MAPK Mitogen-activated protein kinase

MCH Melanin-concentrating hormone

MRP Multidrug resistance protein

MS Mass spectrometry

mTOR Mechanistic target of rapamycin

NA Nicotinic acid

NAAD Nicotinic acid adenine dinucleotide

NAD^+ Nicotinamide adenine dinucleotide

NADPH Nicotinamide adenine dinucleotide phosphate

NADSYN NAD^+ synthetase

NAM Nicotinamide

NAMN Nicotinic acid mononucleotide

NAMPT Nicotinamide phosphoribosyltransferase

NF Neurofibromin

NF-kB Nuclear factor-kappa B

NMN Nicotinamide mononucleotide

NMNAT Nicotinamide mononucleotide adenylyltransferase

NMR Nuclear magnetic resonance

NO Nitric oxide

NOS Nitric oxide synthase

NPY Neuropeptide Y

NR Nicotinamide riboside

NT Neurotransmisser

NTS Nucleus of tractus solitarius

OB Leptin gene

OBR or LEPR Leptin receptor

OXM Oxyntomodulin

PAC Proanthocyanidins

PALM Palmitate

PARP Poly(ADP-ribose) polymerase

PCR Protein C reactive

PDE Phosphodiesterase

PFA Perifornical area

PG Prostaglandin

PGC Peroxisome proliferator-activated receptor gamma coactivator

PHE Phenylananine

PI Phosphatidylinositol

PKA Protein kinase A

PKB or Akt Protein kinase B

PKC Protein kinase C

PL Phospholipase

POMC Proopiomelanocortin

PP Pancreatic polypeptide

PPAR Peroxisome proliferator-activated receptor

PPIA Peptidylprolyl isomerase A

PPP Pentose phosphate pathway

PTEN Phosphatase tensin homolog deleted on chromosome 10

PTP Protein tyrosine phosphatase

PUFA Polyunsaturated fatty acid

PVN Paraventricular nucleus

PYY Peptide YY

QPRT Quinolinate phosphoribosyltransferase

RBEC Rat brain endothelial cells

RNS Reactive nitrogen species

ROS Reactive oxygen species

RPS6 Ribosomal protein S6

RSV Resveratrol

R-5-P Ribulose-5-phosphate

SCD Stearoyl-CoA desaturase

SD Short day

SF Steroidogenic factor

SGLT Sodium-dependent glucose transporter

SIRT Sirtuin

SNS Sympathetic nervous system

SOCS Supressor of cytokine signalling

SOD Superoxide dismutase

SREBP Sterol regulatory element-binding protein

STAT Signal transducer and activator of transcription family

STD Standard

SULT Sulfotransferase

TAG Triacylglycerides

TC Cholesterol

TDO2 Tryptophan 2,3-dioxygenase

TEER Transepithelial/transendothelial electrical resistance

TFAP2B Transcription factor AP-2B

TLR Toll-like receptor

TNF Tumor necrosis factor

TRH Thyrotropin-releasing hormone

TRP Tryptophan

TYR Tyrosine

UCP Uncoupling protein

UGT Uridine 5'-diphospho (UDP)-glucuronyl transferase

UPR Unfolded protein response

VH Vehicle

VMN Ventromedial hypothalamic nucleus

WAT White adipose tissue

WT Weight

sXBP Spliced x-box binding protein

I. INTRODUCTION

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1. Obesity and body weight regulation

1.1. Obesity

The origins of the obesity go back to 30,000 years ago, in the Prehistory. The individuals who stored energy more efficiently had more probabilities to survive fasting and famine. However, many centuries later, this natural selection became an inconvenient for humans when a more sedentary lifestyle and higher fat and sugar-processed diets spread in the population.

For millennia, obesity was something desired. In fact, some cultures prized obese people because it was a status of wealth. The ancient Greeks were the first to remark the problems caused by obesity and its association with the disease. Similarly, Egyptians described the diet as the main factor to preserve the health, remarking the importance of the quantity and the quality of the food. In this sense, Hippocrates stated that diet and exercise could help to reduce fat accumulation in the body. In this line, the first texts about diet and exercise wanted to preserve the health rather than cure the pathology. However, in the 17th century, the number of obese people increased and the term obesity started to be used in a medical context. Moreover, several researchers observed that obesity was the combination of different conditions such as skin disorders, circulatiory problems, depression, etc. It was not until in the 18th century when fats gained relevance and started to be considered important in the onset of the pathology. Then, the first diet programme was written [1].

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Nowadays, obesity is considered one of the most important epidemic worldwide and in the last years it has spread from richer regions to lower-income countries due to several factors but mainly changes in dietary patterns [2]. In 2016, 39 % of adult women and 39 % of adult men were overweight and 18 % of global population under 18 years were considered overweight or obese [3].

Conceptually, overweight and obesity are defined as abnormal or excessive fat accumulation that may disrupt health. Body mass index (BMI), which is defined as a person's weight (wt) divided by the square of his height (Kg/m^2) is used to roughly classify obesity in adults. People who have a BMI between 25 and 30 are classified as overweight whereas a 30 or higher index corresponds to obesity. The main cause of obesity is the imbalance between the energy intake and its expenditure, caused by (**1**) an increased intake of energy-dense foods higher rich in fat and carbohydrates, and (**2**) a decrease in physical activity.

Higher levels of BMI are correlated with obesity but also with other non-associated diseases such as cardiovascular diseases (heart disease and stroke), diabetes, musculoskeletal disorders (osteoarthritis), cancer (endometrial, breast, ovarian, prostate, liver, among others), hypertension, insulin resistance and psychological effects, among others [4].

1.2. Body weight regulation

The daily energy intake is very variable among individuals and it is not always correlated with the daily energy expenditure [5]. In general, the body weight (bw) and

its composition are stable during long periods, being energy intake equal to energy expenditure [6]. Despite short-term imbalances, usually, most of the people match the energy intake with its expenditure when measured in a long-term period that includes different daily meals [5]. This phenomenon is called energy homoeostasis and is based on promoting the equilibrium in the amount of energy stored in the body as fat [7].

The hyperphagia (increase of food intake) induced by a period of fasting is an example of this food intake regulation. In 1953, Kennedy explained that the fat reservoirs of the organism generate inhibitory signals proportionally to their amounts, acting in the brain and thus reducing food intake. When there is a situation of caloric restriction (CR), these fat reservoirs are reduced, there is also a reduction of these inhibitory signals. To counteract this effect, the organism reacts increasing the food intake, thus correcting the energy deficit. However, the mechanism by which energy intake is accurately corrected during each meal is not completely known [8]. In this sense, Gibbs and Smith proposed that certain signals when eating, the so-called satiety factors, provide information to the brain that leads to stop to eat [9].

It is important to highlight that the mechanisms implicated in bw regulation are altered by several factors like mutations in some genes, an increased food availability, non-active life-style and a decrease in physical activity, thus promoting the obesity [10]. In addition, emotions, social factors, circadian rhythms and convenience of costs are some non-biological variables that also affect the energy intake [7].

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Focusing on the process of regulation itself, it is known that protein and carbohydrate stores in adults are in equilibrium. Hence, the bw regulation is basically foundamented in the adipose tissue mass. When there is a chronic deregulation of the equilibrium between incoming and outcoming energy, the quantity of adipose tissue changes. In this sense, adipose tissue self-controls by means of different signals its own regulation acting on central nervous system (CNS) receptors and, activating effectors in the autonomic nervous system (ANS). Therefore, three important sites in the control of the bw are described: (**1**) one sensor that monitors the quantity of energy (hormones secreted in proportion to the fat depots); (**2**) the hypothalamic centres that receive and proceed the signal and its intensity (the targets are hormone-responsive neurons that produce several neuropeptides); and (**3**) the effectors that change the energy intake and/or waste (tissues that contain neurons with receptors for those neuropeptides) [6].

Within this context, appetite is defined as a series of complex interactions that combines the signals between peripheral and central systems. Thus, the gastrointestinal tract has mechano- and chemoreceptors that inform the brain, mainly through the vagus nerve, about the nutrient content [11]. When the nutrients of the last meal have been absorbed, hunger, which is defined as the sensation that promotes to eat, is activated [12]. The amount of energy that enters to our body depends on the size and the frequency of each ingested meal, being regulated by different mechanisms. When the requirement of nutrients are covered there is a suppression of hunger which leads to stop eating. This process is called satiation and its mechanisms of action determines the meal size. Therefore, satiety and hunger are key elements in

the cycle of food intake [13,14]. The mechanisms that promote satiation differ from those that determine the duration of the satiety. Thus, the size and the frequency of the meals are differently controlled. In this sense, the composition, size, caloric density and the organoleptic properties of the macro-nutrients play a key role in the satiation [15]. Moreover, the body size and composition and the social activities play a essential role in the control of food intake [6,16] (**Figure 1**).

1.2.1. Hypothalamic regulation of body weight

Hypothalamic and brain stem centres control food intake and energy expenditure [6]. Thus, nuclei within the lower brain stem integrate and process the information from peripheral autonomic and endocrine organs and other forebrain structures. On the other hand, nuclei in the pars-midbrain and thalamus interpret the information of sensory properties of food. The hypothalamic nuclei respond to neural inputs, circulating hormones and substrates while forebrain nuclei (amygdala and frontal cortex) are implicated in the aversive or the good aspect of the food. Many inputs, such as neural inputs from the vagus, hormones and changes in the concentration of substrates inform all these centres about the metabolic status of the body [17].

The signals that control food intake can be divided into long- or short-term signals. These two categories are linked since long signals, such as insulin and leptin, modulate responses to short-term nutritional inputs [18].

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Insulin, the pancreatic hormone that reduces the food intake entering to the brain from circulation, was the first hormone descried as implicated in the control of bw in the CNS [19]. The hyperphagia and obesity in *ob/ob* mice provided the study of a second adiposity signal implicated in the regulation of food intake, leptin [20]. Both hormones are secreted proportionally to the fat depots [21,22] and enter to the CNS according to the amount of plasma levels [23,24]. Receptors for both hormones are expressed in brain neurons which are involved in energy intake [25,26]. The administration of these peptides directly into de brain reduces food intake [19,27] whereas their deficiency causes the opposite effect [23,28].

When bw is increased, the insulin secretion rises both in basal conditions and after a meal in order to compensate the insulin resistance and to maintain the glucose (Glc) homoeostasis [29]. This increase in the insulin production can cause a failure in the pancreatic beta-cells originating hyperglycaemia and type 2 diabetes mellitus [7]. The ratio of insulin - Glc used in the adipocytes is an important factor in the leptin secretion via body fat mass [30]. Hence, the adipose Glc metabolism is affected by acute changes in the energy balance leading to the dissociation of the leptin secretion and the total amount of fat. As a consequence, food deprivation causes a higher decrease of plasma leptin concentration than of the body fat content.

It is know that leptin is more implicated than insulin in the CNS control of the energy homoeostasis. Thus, the leptin deficiency causes obesity although even in case where the insulin levels are correct whereas a deficit of insulin does not directly produce

obesity. However, there is a complicated link between these two hormones since insulin is responsible of the fat storage and leptin synthesis and secretion is done by the adipose tissue [7].

Several neuropeptides involved in the regulation of food intake had been described. These neuropeptides can be classified as orexigenic (stimulators of food intake) or anorexigenic (inhibitors of food intake) [7]. Within the group of the orexigenic peptides it is remarkable the Neuropeptide Y (NPY) [7,31], which is released from the arcuate nucleus (ARH) when there is feeding (fasting or hypoglycemia) and its inhibition is promoted when nutrients are absorbed [32]. Another one is the Agouti-related peptide (AgRP), which acts as antagonist of melanocortin-4 receptor (MC4R) in hypothalamic cells and increases feeding behaviour [7]. Others are melanin-concentrating hormone (MCH) [7], hypocretin 1 and 2 or orexin A and B [7], galanin [7,33], catecholamines (adrenalin, noradrenalin and dopamin) [7,34]. Glucocorticoids and growth hormone releasing hormone (GHRH) also increase the food intake [35].

As anorexigenic neuropeptides, the most important is alpha-melanocyte-stimulating hormone (alpha-MSH), produced by proopiomelanocortin (POMC) neurons in the ARH, which exerts a tonic inhibition of food intake [7,36,37]. The corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), cocaine and amphetamine regulated transcript (CART), interleukin 1 beta (IL-1B), urocortin [7], oxytocin, neurotensin, serotonin [7] and opioid peptides [6] are others that belong to this group.

1.2.2. Gut regulation of body weight

In addition to the hypothalamus, the gut is also implicated in the energy balance as it synthetizes numerous peptides that can modify the appetite. These peptides have different targets such as gastrointestinal exocrine glands, muscles, afferent nerve terminals and the brain. Afferent signals from the gut to the brain are transported via vagal and splanchnic nerve. Vagal afferent is activated when there is a luminal chemical stimuli, physiological levels of distension or nutrients in the portal circulation whereas splanchnic afferents transmit information about noxious stimuli. The main parts of the gut implicated in the secretion of these peptides are the stomach, the proximal small intestine, the distal ileum and the colon. A lot of peptides can be produced by a specific cell type and each peptide has different effects. Prohormone convertases and post-translational modifications (acylation and sulfation) increase the amount of signalling peptides. In addition, there are different types of receptors types which are implicated in different several physiological effects according to the cell type in which they are located [18].

Cholecystokinin (CCK) is a satiety hormone and interferes in the early-phase of satiety, when proteins and lipids are eaten. It is produced by mucosal enteroendocrine cells from the duodenum and the jejunum and it is secreted when food is still present in the gut lumen. Therefore, CCK is implicated in the reduction of the meal size via CCK1 receptors which are activated in the piloric region of the stomach and the signal is transported via vagus to the nucleus of the tractus solitarius [18,38].

On the other hand, preproglucagon yields to important peptides implicated in satiety, glucagon-like peptide-1 (GLP-1) and oxyntomodulin (OXM) [18]. They are released from L cells in response of to some nutrients, specially fatty acids (FA) and carbohydrates [18,39,40]. GLP-1(7-36) inhibits the secretion and emptying of acid gastric and it also stimulates the release of postprandial insulin, inhibits the secretion of glucagon and B-cell neogenensis, acting in the endocrine pancreas [41]. Dipeptidyl peptidase IV (DPP-IV) is a key enzyme with a specific function of inactivating circulating GLP-1, having a half-life in circulation of 2 minutes (min) [42]. Agonists of GLP-1 that are resistant to DPP-IV reduce food intake and induce wt loss in rats [43]. It is important to highlight that the brain contain receptors for GLP-1 and it is known that human treated with this hormone presented a small reduction in the food intake [44]. Glc-dependent insulinotropic polypeptide (GIP) is another incretin hormone that is quickly inactivated by DPP-IV, as GLP-1. Its release by K cells from duodenum is stimulated by the ingested fat. This peptide acts in adipocytes having multiple effects: enhancement of insulin-stimulated Glc transport and stimulation of FA synthesis incorporating them into triacylglycerides (TAG) [18].

OXM inhibits food intake and suppresses the appetite in rodents [45] and humans [46], respectively, linking with GLP-1-like receptors. However, OXM and GLP-1 stimulate different areas of the brain: GLP-1 acts in the brainstem [47] and other autonomic sites [48] whereas OXM works in the ARH [49]. Preproglucagon can also be transformed into GLP-2 and glucagon. It has been seen that the administration of

GLP-2 in the brain of rats reduces their feeding although it seems that is not implicated in appetite or food intake in humans [50].

The ileum and colon contain enteroendocrine cells implicated in the production of peptide YY (PYY) after a meal. PYY acts delaying gastric emptying. In the brain, PYY binds to presynaptic Y2 receptors in the hypothalamus inhibiting NPY neurons, stimulating POMC neurons and reducing feeding [18]. Furthermore, pancreatic polypeptide (PP) is secreted from the pancreatic islet cells, under nutrient conditions [51]. It acts on Y4 and Y5 receptors in the brain and also in the periphery modifying exocrine pancreatic and biliary function, gastric acid secretion and gut motility [18].

Moreover, PP reduces appetite and food intake in humans [52]. Both PYY and PP cause a reduction in food intake when they are administered peripherally but there is an increase in the food intake when they are administered centrally. It is thought that this double effect is produced due to their action in Y4 receptors, which are in the area postrema area and reduce food intake, and Y5 receptors, which are present in all the brain and increase the food intake [18].

Ghrelin, an acylated hormone formed by 28 amino acids (aas), is implicated in the stimulation of the appetite and the release of growth hormone (GH) [53]. Some cells from the gastrointestinal tract can secreted it, concretely those which are in the fundus of stomach. Its plasma levels are higher during fasting and after meals and decrease after one hour of food intake, having a role in the initiation of the meal [54]. It activates NPY-expressing neurons in the ARH of the hypothalamus [53]. Moreover, ghrelin

can also exert its functions via stimulation of the gastric vagal afferents [55] and acting directly on the dorsal vagal complex [56]. The role of ghrelin has not been yet established since its levels are low in obese subjects but increase after dietary wt loss [57]. In addition, there are not changes in the food intake or in bw when either hormone or its receptor are deleted [58,59].

Islet amyloid polypeptide (amylin) is a 37-aas polypeptide secreted simultaneously to insulin by B-cells [60]. It delays gastric emptying and decreases food intake acting in the area postrema in rats [61]. Enteroestatin, a pentapeptide produced from pancreatic procolipase, which is released from exocrine pancreas, reduces selectively intake of a high-fat diet (HFD) [62]. However, it has not been seen any effect on food intake, appetite, energy expenditure and bw when it is administered orally to humans [63].

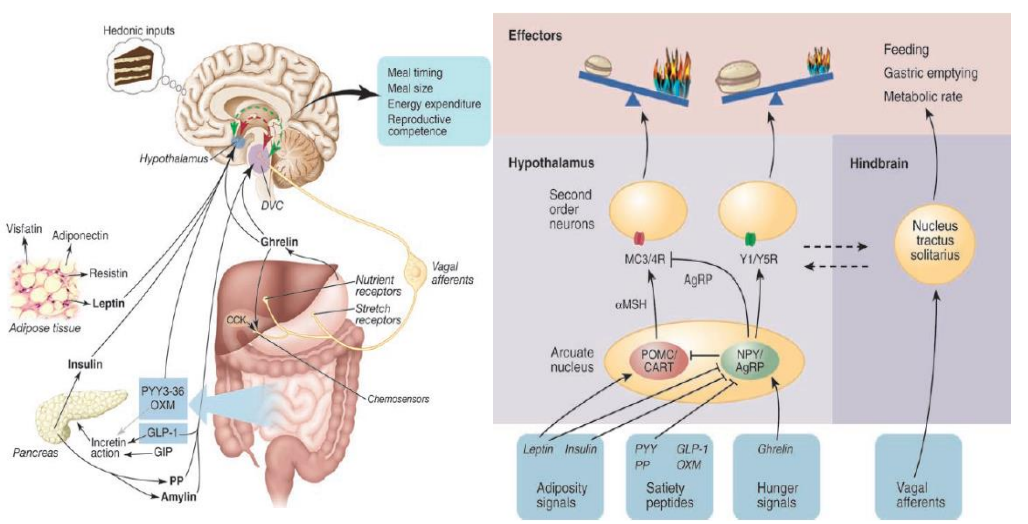

Figure 1. Mechanisms implicated in the regulation of body weight. Differents signals (adiposity signals, satiety peptides, hunger signals and vagal afferents) from different regions of the body are involved in the control of this sophisticated process mainly acting in the central nervous system,

controlling different types of neurons which, in turn, trigger different responses according to the appetite. The figure was adapted from [18].

1.2.3. Metabolic signals controlling body weight

In addition, there are signals from metabolic origin that also contribute to the satiety of mammals. It is known the degree of oxidative metabolism of Glc and free-fatty acids (FFA) in the liver [64]. Some peripheral satiety signals from adipose tissue can act as a feedback signals: satietin [65], adipsin [66] and oleoyl-estrone [67]. A deficit in one of the macronutrients activates a compensatory ingestion of the same calories from the diet rather than an increase in the ingestion of the same macronutrient [68,69]. However, when there is an excessive intake, in general, the following food intake decreases. Nutrients exerts acute and chronic effects on food intake. In fact, macronutrients can be classified according to the capacity to create satiety and to increase the period to the next meal [6].

1.2.4. Genes controlling body weight

It is also known that some genes are implicated on the wt gain increase and in the probabilities of an individual to develop obesity in environmental conditions that positively favours the energy balance. Hence, some studies performed in twins raised in different environtments confirm that genes influence obesity [70]. The level of heritability varies for BMI between 25-40 % [71]. Age-related changes in body fatness and total body fat in adult life are also heritable [72,73]. Genetic also influences both body fat mass and the partitioning of central and peripheral fat depots [74]. In the past,

the capacity to store fat in depots conferred survival and reproductive capacities. Thus, mutations of genes implicated in energy storage and metabolic efficiency promoted the survival of individuals when it was not enough food. However, the fact that nowaydays there is more probabilities to access to energy-dense foods and the physical activity have decreased make these genes a problem [75].

Apart of the monogenic mutations which inhibits the expression of the *leptin* (*ob/ob*) and its receptor (*db/db*) (explained in Section 2) other mutations lead to obesity. Firstly, a mutation in the agouti locus, which encodes for a 131-aa peptide with the name agouti signalling protein (ASP). This causes an overexpression of *Asp* in the brain, interfering with the action of alpha-MSH on MC4R, implicated in the inhibition of food intake [76,77]. The mutation of the gene coding for carboxypeptidase E (CPE) is another one implicated in obesity [78]. CPE function is to cleavage pro-hormones like proinsulin or POMC, which function is the suppression of food intake. Moreover, a reduction of the brain levels of alpha-MSH, GLP-1, CRH or MCH can also be implicated in [79]. Homozygous mutation of the prohormone convertase 1 gene causes obesity, hypoglycaemia, hypogonadism and hypocortisolism [80]. Other mutation is that produced in 5-hydroxytryptamine receptor 2C (HTR2C) which causes a loss-of-function of the brain serotonin receptor [81]. The mutation produced in MC4R causes a defective brain receptor for alpha-MSH and ASP [77]. There is a loss-of-function of the transcription factor required for hypothalamic neuronal development when a mutation in nescient basic helix-loop-helix 2 (NHLH2) is produced [82]. POMC can also suffers a mutation and, as a consequence, neither

alpha-MSH nor even B-endorphin are produced [83]. Finally, a mutation in tubby protein causes a loss-of-function in this transcription factor present in hypothalamus and retina [52].

2. Leptin

The word leptin comes from greek *leptos* which means *thin* [84]. Leptin was discovered in 1994 [20] and its gene (*ob*) is located in human chromosome 7q31.3 [85]. The transcription of the *ob* gene (mRNA of 3.5 kb) leads to a 167 aa protein with a N-terminal end (21 aas) that, once it is translocated into the microsomes, is removed originating the 146 aa final leptin product (16 kDa) [20].

There is a correlation between the plasma leptin levels and BMI, as well as the body fat mass [22]. In fact, both are considered good predictors of the levels of circulating leptin. The main role of leptin is to regulate food intake and energy expenditure, not only via hypothalamus but also in peripheral tissues. This way it acts in different processes: the maturation of reproductive axis, regulation of bw, neuroendocrine adaptations to fasting conditions [86,87], Glc homeostasis [88], angiogenesis, haematopoiesis, lipid and carbohydrate metabolism and even exerting effects on cardiovascular and immune systems [89].

Leptin deficiency mice (*ob/ob*), and leptin resistant (*db/db*) mice and (*fa/fa*) rats show hyperphagia, insulin resistance and decreased energy expenditure, causing obesity. In

addition, these animals present diabetes mellitus independent of insulin, insulin resistance, hypothermia, infertility and lower muscle body indexes [90].

As previously explained, leptin is a hormone produced and secreted mainly by adipocytes proportionally to their amount [84] (**Figure 2**). However, site-specific variations in its expression of depending on the type of adipose tissue have been identified [91,92]. For example, in rodents, it is basically secreted by visceral white adipocytes whereas in humans is the subcutaneous adipose tissue which exerts this function [92]. In the case of the rodents, males have higher amounts of *leptin* expression in the epidydimal white adipose tissue (WAT) and both sex also present important levels in perirenal adipose tissue [92]. Moreover, leptin is more expressed when the adipocytes are larger [91]. It is important to remark that depending onto the age of the rodents some differences in the place where leptin is most expressed are observed. Especially important in adults are the gonadal and perirenal tissues [91] whereas in suckling rats it is predominantly expressed in subcutaneous fat [93]. In humans, the differences in the leptin production due to the gender are due to the stimulating effect of oestrogens and the suppressor role of androgens [94].

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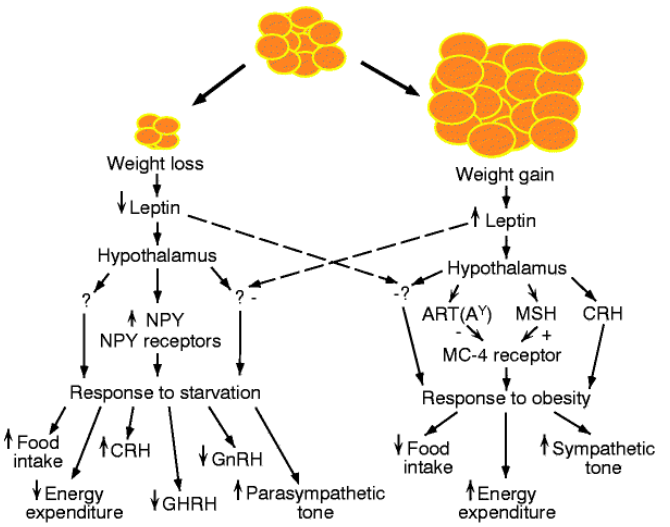

Figure 2. Leptin secretion and action according to the amount of white adipose tissue. Low circulating leptin levels activate orexygenic peptides, promoting the mechanisms to start to eat whereas when there is an increase of leptin in blood, the hyphotalamus initiates the response to obesity, activating the anorexygenic neurons. The figure was adapted from [88].

Brown adipose tissue (BAT) also produces leptin [95,96]. There are two hypothesis about the role of the leptin expressed in BAT. The first one considers that it is added to the total amount of circulating leptin [84]. The other says that this expression could be a reflection of infiltration of WAT to the BAT [91].

Other tissues which also express leptin are placenta and ovaries [97-99]. Two hypothesis are postulated about the function of the leptin produced in the placenta. The first one is that this leptin is a growth factor and the second one is that it can act as a signal of energy state between the mother and the foetus [84]. Moreover, it is known that placenta expresses leptin receptors (ObR), being a target tissue for leptin, which acts as an autocrine factor [97]. The foetus also expresses leptin in a several tissues including brain, lung, kidney, heart, liver and cells of the hair follicle [100].

Leptin disappears from circulation in two manners: free or linked with other proteins. Hence, leptin can be found linked to tissue receptors, to non-specific sites in the tissues and to carrier molecules in the plasma [84,101]. For example, in rats, free leptin is removed from plasma very quickly, having a half-life of 3.4 min, whereas the bound form remains in plasma about 71 min [101]. Van Heek *et al.* (1996) observed that, after the intraperitoneal injection of ^{125}I-leptin in *ob/ob* and *db/db* mice, it rapidly appeared in serum and it was stored in fat, intestines, liver and kidneys. Authors concluded that the distribution of leptin is affected by its hydrophobic character (link to non-specific binding sites) independent of its bind to the ObR [102]. In humans, the clearance of leptin was 1.5 mL/kg/min, being biologically active during bigger periods than in rodents [103].

2.1. Factors influencing leptin secretion

Leptin is not considered a short-term signal of satiety although it is necessary to control food intake. This is because the changes in leptin plasma levels due to the consumption of food occur some hours later after the ending of the meal [104].

The expression and secretion of leptin are regulated by many factors. For example, in both obese human and animals, the mRNA levels of *leptin* are increased in the WAT and also in the circulation [22,105]. It has been reported that the practise of exercise increases the energy expenditure and the metabolic rate [106] but still remains unknown the mechanism of regulation between training and the circulating levels of leptin. In this sense, there is high controversy in the bibliography about the effects of

the exercise in the leptin production. In addition, this production can be affected by many other factors such as gender, time and intensity of the exercise, habit practising sport, and, of course, the BMI among others [107]. Other factors that increase its expression are re-feeding [108], over-feeding [109] and surgical stress [110]. Insulin [111], glucocorticoids [112], serotonin [113], oestrogen [114], testosterone, Glc [95] and tumor necrosis factor-alpha (TNF-alpha) [86] also stimulate leptin secretion.

It has been observed that plasma leptin levels decrease in response to several types of stress such as fasting [115], cold exposure [91] and CR [116], confirming that the regulation of leptin reflects the body energy demand. The regulation by these stressors is probably dependent of the **s**ympathetic nervous system (SNS) [117]. For example, some studies have pointed out that the inhibitory action of low temperatures in the leptin synthesis may be due to an increase in the adrenergic function, activating the B_3-adrenoceptor (B_3-AR) in the adipose tissue both in rats [96] and mice [91]. Hence, animals living in large seasonal temperature variations show a decrease in plasma leptin levels suggesting an adaptive response in order to increase the number of fat depots when temperatures decrease. Thus, it is possible that there is a feedback between the BAT and the hypothalamus (the *leptin* expression is inhibited when the SNS is activated) [91,96]. Decreased leptin levels due to fasted conditions have been observed in rodents [105] and humans [102] and it seems that it is also modulated by the SNS and B_3-AR. In this sense, the so-called uncoupling proteins (UCPs) play an essential role in the response of the organisms to stress. For example, leptin increases

the use of energy stimulating thermogenesis mediated by UCPs while UCP-1 is over-expressed during cold conditions and it decreased during fasting [118].

Some studies have revealed that leptin has the capacity to regulate its own expression acting on the SNS. Hence, leptin feedback to the SNS induces a reduction in the *leptin* expression independently of the adipose levels via the activation of B_3-AR [91]. This way, in rodents and humans it has been seen that B-AR agonists (noradrenaline and isoprenaline) decrease the expression of *leptin* in WAT and BAT and, consequently, decrease the leptin circulating levels [119]. In addition, in humans, it has been seen that leptin controls lipolysis in the subcutaneous adipose tissue [120]. Leptin also preserves TAG from mobilization for use by muscle and promotes its use for thermogenesis, increasing the turnover of noradrenaline in BAT and the sympathetic flow to other sites of the body [121,122]. Leptin increases energy expenditure and causes higher core temperatures and metabolic rates [123] via its interaction with insulin (mobilizing fuels) and increasing SNS.

Some transcription factors related with the SNS are implicated in the regulation of the leptin transcription. Thus, the transcription factor AP-2B (TFAP2B) [124] and the peroxisome proliferator-activated receptor gamma (PPARgamma) [112] inhibit its expression while neurofibromin (NF1) and forkhead box protein L2 (FOXL2) have positive effects [125,126]. Moreover, leptin can be controlled by CCAAT/enhancer-binding proteins (CEBPs) [127], transcription factor SP1, glucocorticoid receptor (GR) [128], cyclic adenosine monophosphate (cAMP)-responsive element-binding

protein 1C (CREB) and sterol regulatory element-binding protein 1C (SREBP1C) [129]. The last ones seem that are not implicated neither in the SNS-mediated *leptin* expression nor even in the fasting and feeding regulation according to the leptin levels [130].

Finally, it is important to take into account that a diverse range of diseases also affect the levels of leptin in the organisms. Hence, obesity, overfeeding, impaired renal function, insulin, Glc, glucocorticoids, TNF-alpha, oestrogen, endotoxin, IL-1, alcohol, androgens, fasting, B_3-adrenoceptors agonists, GH, cold exposure, long-term exercise, somatostatine, cAMP, thiazolidinedions, smoking, insulin-like growth factor-1 (IGF-1) and FFA have an effect on the levels of this hormone [84].

2.2. Leptin receptors

It was in 1995 when Tartaglia *et al.* (1995), identified the ObR as a product of the *db* gene [127]. ObR belongs to gp130 family of cytokine (CK) receptors [131,132] and it has six alternative spliced forms (ObRa-ObRf) [133]. All isoforms have identical extracellular (ligand-binding domain) and transmembrane domain. There are multiple domains in the extracellular region of these receptors [134] (**Figure 3**).

INTRODUCTION

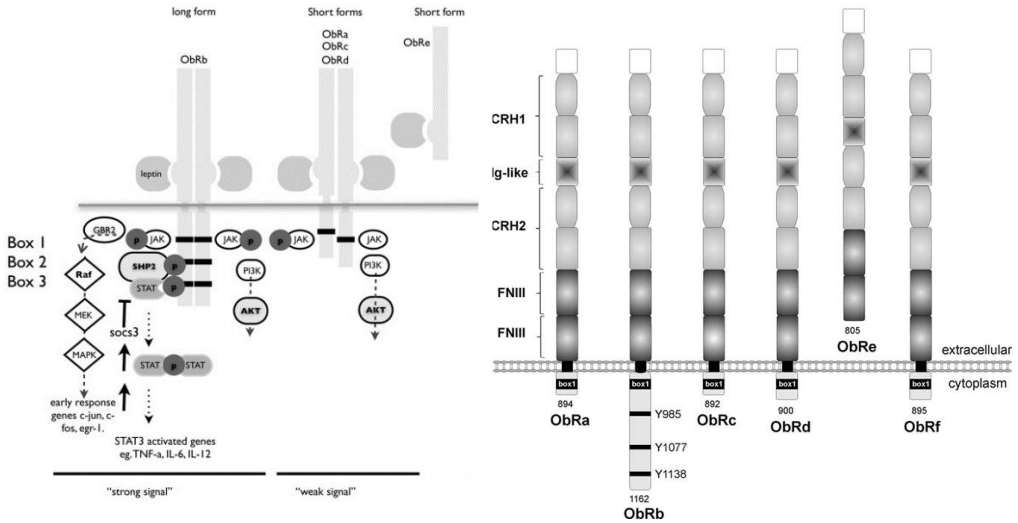

Figure 3. The different leptin receptor isoforms. All of them contain the same domains in the extracellular region whereas they differ in the intracellular estructure conferring them different functions and properties.

The extracellular region contains 816 aas (counting the N-terminal region) and has two motifs CK-like binding (Trp-Ser-X-Ser-Trp, CRH1 and 2) and a fibronectin type III domain (FNIII) [131]. Remarkably, only the second leptin binding domain (CRH2 domain) is implicated in the linkage of leptin and in the activation of receptor [135]. On the other hand, the transmembrane domain is formed by 23 aas. This domain appears in all the isoforms except in ObRe, which changes before the membrane-spanning domain (has different sequence after His796) and it is secreted [133]. Finally, the intracellular domain located at C-terminal end, differs among the isoforms. ObRb is the longest isoform as it has a long cytoplasmatic domain of 320 aas [133] whereas the short forms have 32-40 aas [131]. Mostly of ObR transcripts are found in the short isoform [136]. Regarding their functionality, ObRe, also known as the soluble isoform, is important in the regulation of the circulating leptin levels

because it acts as a binding protein via buffering the free circulating leptin [137,138]. Thus, the proportion of leptin circulating free or bound to a protein is important to determine its action [139].

On the other hand, ObRa, also known as the short form, plays a key role in transporting leptin from blood to the cerebrospinal fluid, where it is pumped by diffusion into different brain centres to control bw. It was has been observed that rats without ObRa show a decrease in leptin transport across the blood-brain barrier (BBB). Remarkably, ObRa has a particular signal pathway, different from that performed by ObRb. This fact was confirmed by the addition of leptin to cells which expressed ObRb and ObRa. The two types of cells showed an increased expression of mRNA levels of *c-fos*, *c-jun* and *jun-B*, demonstrating that both ObRs have the capacity to activate the signal transduction. It is known that the ObRa has low potency but its abundance in peripheral tissues can mediate some effects of leptin [132].

ObRd is only described in mouse [133] whereas ObRf is present in rats [139]. ObRc and ObRd isoforms play a role in the leptin clearance from the circulation [133]. Murine foetal liver express another new splicing variant of the ObR called B219 and it is associated with hematopoietic stem cells, indicating that leptin plays a role in haematopoiesis [140,141].

ObRs are expressed in the brain and in peripheral tissues such as liver, heart, kidneys, lungs, small intestine, pituitary cells, testes, ovaries, spleen, pancreas, adrenal gland and adipose tissue [97,106,102,133,136,141-146]. However, it is known that the most

affinity binding sites for leptin are the CNS [147-149] and the kidneys [150]. In the hypothalamus, ObRb is found in many nuclei: ARH, ventromedial (VMN), dorsomedial (DMH) and lateral hypothalamic (LHA) nuclei and the paraventricular nucleus (PVN) [151,152].

ObRa is found in spleen, testes, intestine, heart, lung, liver, skeletal muscle, adrenal gland [112] and adrenal medulla [153]. Most of the organs that express ObRa also express ObRb in the same cells, although in some of them it is weaker [152]. Therefore, it seems that the long isoform is present in higher levels in the hypothalamus [152] and in lower levels in different peripheral tissues [154] while ObRa is expressed ubiquitously and is the most prevalent in the periphery [152]. The other short forms are expressed less than these two [152].

It is important to highlight that leptin is widely distributed in peripheral tissues compared to the CNS because the mRNA expression of *ObR* is higher in the periphery for rodents and humans [133,136,142,155].

Once leptin binds to its receptor, all the complex is internalized via clathrin-coated vesicles into endosomes and the ObR is processed for degradation or recycled and returned back to the cell surface. Thus, it this is an important step for the leptin signalling regulation. Remarkably, in conditions in which leptin is not acting, only 5-25 % of the cellular ObRs are in the cell surface being most of them in pools inside the cell [156]. Both ObRa and ObRb are implicated in this clathin-mediated leptin internalization and the next step, the lisosomes degradation [157]. It seems that in this

degradation, the longest isoform is more efficient [156] whereas the shorter is faster recycling the leptin to the cell surface [158]. Therefore, the preferential action of leptin for ObRb could play a role in the human obesity [159].

Apart from ObRs, another important molecule related with their functionality is clusterin. Clusterin, also named apolipoprotein J (ApoJ), is an anorexigenic neuropeptide which potentiates the anorexigenic effect of leptin [160]. Its action is based on an increase of the leptin-binding to its receptor [160]. This effect is mediated through LDL receptor-related protein-2 (LPR2, megalin), which mutually acts with ObRb [160,161]. It has been seen that the central administration of clusterin induces anorexia and wt loss [161]. And, oppositely, the inhibition of hypothalamic clusterin increases food intake and bw [160,161]. It is observed that ObRb deficiency or LPR2 suppression causes an impairment of clusterin signalling and actions in hypothalamus [161].

2.3. Leptin signalling pathway

In the organism, leptin is modulated by means of a complex signalling pathway in which several interrelated molecules participate. In brief, ObRs act via a pathway that involves cytoplasmatic tyrosine (Tyr) kinases from the Janus kinase family (JAKs) and the signal transducers and activators of transcription (STATs). This said, all the isoforms of the receptor include a transmembrane domain that contains the Box 1 JAK binding domain. In addition, ObRb also has the Box 2 and the STAT binding sites. This fact is essential as these two Boxs are needed for the activation of the JAK/STAT

signalling pathway [132] (**Figures 3** and **4**). Therefore, the short forms of ObR (such as ObRa, ObRc, ObRd, ObRe and ObRf) cannot activate the JAK/STAT signalling pathway [162,163] because their intercellular domain only contains the motif Box1 [131,133]. However, in some cases, only the motif Box 1 is enough to create a signal, as in the case of ObRa, which can modulate the insulin receptor substrate (IRS)-1 / mitogen-activated protein kinase (MAPK) cascade [164].

Human ObRb has 5 Tyr residues in the cytoplasmatic domain and each one is related with a different signalling pathway [132]. Long and short isoforms have the capacity of forming homodimers but dimerization, essential to activate the pathway [165], is not playing a role in the activation of the receptor [166]. As each receptor binds one leptin molecule the result is the linkage of four receptors with four leptins (tetramer) [167], crucial to activate the cascade [135].

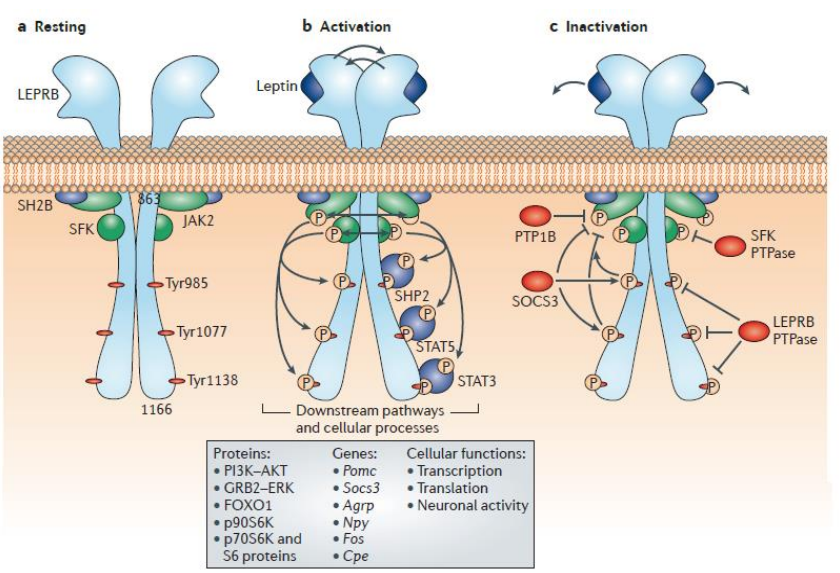

Figure 4. Leptin receptor action in the presence or absence of leptin and the consequences in the leptin signalling pathway. (A) When ObRb is resting, which means that leptin is not interacting with it, the leptin cascade is not activated. (B) Once leptin is linked to its receptor, the downstream signals

cause the secretion of different protein and an increase of many genes resulting in the start of different cellular functions in which leptin is involved in. (C) Moreover, there are some inactivators of the pathway, being SOCS3 and PTP1B the most importants. ObRb or LEPRb: Leptin receptor; PTP1B: Protein tyrosine phosphatase 1B; SOCS3: Supressor of cytokine signalling 3. The figure was adapted from [82].

ObR has not Tyr kinase activity *per se* and some signalling events depend on kinases like JAK2. Once leptin binds to the ObRb and the oligomerization is produced, JAK2 is recruited to by the receptor and it is phosphorilated [164]. JAK2 is a receptor-associated protein Tyr kinase used by ObRb to phosphorilate itself (Tyr985, Tyr1077 and Tyr1138) and many targets, as STAT proteins. These transcription factors are recruited by the ObRb/JAK2 complex through Src Homology (SH2 and SH3) domains and are activated upon Tyr phosphorilation. Thus, it forms homo- or hetero-dimers that are translocated into the nucleus and interact in the promoters of key genes regulating their expression [168]. Concretely, STAT3 binds to POMC and AgRP promoters, stimulating and inhibiting them, respectively [169]. The sequence which comprises Tyr1138 (YXXQ) in ObRb is a consensus motif for STAT3 binding [170]. Thus, Tyr1138 is phosphorilated when leptin is bind to the ObRb and STAT3 is subsequent activated [171]. Short isoforms can interact with JAK2 but they cannot activate STAT3 pathway [136,163].

Supressor of cytokine signalling (SOCS) proteins negatively regulates signalling induced by CK in temporal and quantitative manner [172]. CKs like interleukine-6 (IL-6), GH and erythropoietin activate the expression of many *Socs* isoforms, creating a negative feedback loop and the potential for crosstalk with signalling started by other

ligands [172,173]. They contain a central SH2 domain which leads to bind and inhibit the phosphorilated JAK proteins or they can also act via their direct interaction with phosphorilated receptors [172]. SOCS participate in the ubiquitinization and direct proteasome digestion of many signalling molecules [174]. Concretely, SOCS3 has the capacity to inhibit leptin-stimulated phosphorilation of JAK2 and ERK, acting as a negative feedback in leptin signalling [173]. SOCS3 mediates its action by binding to JAK2 or Tyr985 or Tyr1077 of the ObRb [175,176]. SOCS3 is increased in obese, leptin resistant, acquired or diet-induced obesity (DIO) rats and *ob/ob* mice [175,177]. Moreover, when there are changes in *Socs3* expression, it can cause leptin resistance (LR) in obese human. This molecule also binds to the insulin receptor at Tyr960 and inhibits the STAT5B link [178], having different roles apart of inhibiting leptin signalling. Another important molecule is protein tyrosine phosphatase 1B (PTP1B), which acts as an inhibitor of the leptin cascade binding to Tyr985 and, consequently, inhibits the JAK2/STAT3 pathway [179]. CK-inducible sequence (CIS) also acts as an intracellular negative-feedback loop, inhibiting JAK [175,180]. In contrast, Src-homology 2 domain 1 (SH2B1) increases JAK2 activity, acting as a positive regulator [181].

Extracellular regulated kinases (ERKs) members of the mitogen-activated protein kinases (MAPK) family are serine/threonine kinases (42 or 44 KDa) and belong to the Ras/MAPK signalling pathway [182]. The phosphorilation of Tyr985 in ObRb controls the activation of ERK involving the recruitment of SHP-2 and its phosphorilation [171]. The activation of ERK causes the expression of many genes

(*c-fos*). It is thought that p38, another member of the MAPK family, increases its phosphorilation when leptin interacts with its receptor [183].

Phosphatidylinositol 3 (PI) 3-kinase is regulated by lots of ligands, particularly growth factors like insulin. PI 3-kinase stimulates protein kinases such as protein kinase B (Akt or PKB) and protein kinase C (PKC) [184]. Leptin stimulates PI 3-kinase and it regulates phosphodiesterase (PDE) 3B in pancreatic B cells [185], the pump Na^+-K^+ in fibroblasts [186], invasiveness in colon epithelial cells [187], hormone-sensitive lipase activity in macrophages [188], Glc uptake in muscle cells [189] and actin reorganization and activation of K_{ATP} channels in insulinoma cells [190,191]. Leptin may stimulates different isoforms of PI 3-kinase generating different products and this ability can be tissue-specific [159]. The activation of PI 3-kinase by leptin implies phosphorilation of IRS. In some cells [164], leptin can phosphorilates IRS via activation of JAK (JAK2 activates IRS1, IRS2 and p85alpha) [159]. PI 3-kinase affects AgRP and POMC neurons [192]. Moreover, this pathway has the capacity to inhibit PTP1B and forkhead box protein O1 (FoxO1), which enhances the expression of *Npy* and *Agrp*, down-regulates *Pomc* and reduces the STAT3 action in these cells [193]. Due to most of the insulin actions are dependent of the PI 3-kinase activation [194], there is an interaction between leptin and insulin, although leptin does not influence on the capacity of the insulin to activate this pathway and this stimulation is lower in leptin compared with insulin [159].

The serine/threonine kinase Akt are involved in several of molecular events such as cell survival and carbohydrate metabolism [195]. Leptin increases the phosphorilation of Akt but the effect is small and less compared with insulin [196]. The activation of Akt produced by leptin is dependent of high intracellular cAMP levels [197] although the effect depends on the experimental conditions and the cell type.

Nitric oxide (NO) is implicated in many physiological processes, interacting with guanylate cyclase [198]. Moreover, it interacts with oxygen and superoxide radicals producing reactive oxygen and nitrogen species (ROS and RNS), thus, modifying nucleic acids, lipids and proteins [199]. Leptin increases non-ubiquitously NO production but its action is not excessively relevant [200]. In addition, leptin stimulates SNS to peripheral organs, increasing blood pressure [201]. It seems that this effect of leptin is attributed to an increase of endothelium-derived hyperpolarizing factor (EDHF) [200]. Leptin alters partly NO production via regulation of nitric oxide synthase (NOS) levels. Neuronal nitric oxide synthase (nNOS) is important in the role of leptin and it increases the *nNOS* expression [202,203]. Moreover, leptin partially induces expression of entothelial (*eNOS*) and inducible (*iNOS*) nitric oxide synthase [204].

Leptin increases the production of ROS [183,205]. It also stimulates the production of prostaglandin E2 (PGE2) and F2alpha [206]. Diacylglycerol kinase-ζ (DGK-ζ) interacts in the cytoplasmatic domain of ObRb (not ObRa) [207]. p90 ribosomal protein S6 (RPS6) and ribosomal protein S6 kinase beta-1 (p70S6K) are activated by

leptin [208]. In addition, leptin activates phospholipase C (PLC) and L-type and N-type Ca^+ channels which depend of voltage, inducing catecholamines secretion [209].

2.3.1. Central effects of leptin

Leptin in the CNS, concretely in hypothalamus, exerts a neuroendocrine function, modulating food intake and energy expenditure, participating in many metabolic processes and regulating the expression and release of signal molecules such as *Npy*, galanine (*Gal*), *Mch*, neurotransmisser (*Nt*), *Pomc* (symphatetic tone), *Igf-1* and *Gh* [84]. Hences, many pathways that involve neuropeptides in the hypothalamus can be modulated by leptin in the CNS.

The most important circuit modulating the anabolic pathway involves NPY, which potentially stimulates food intake [210] and down-regulates energy waste via the induction of lipogenesis in liver and WAT [211]. Therefore, a continuous administration of NPY in the hypothalamus induces obesity [210,212]. The reduction of body fat stores [213,214] and the leptin signalling in the brain [215] increase the gene expression and secretion of *Npy* in the hypothalamus. Moreover, the expression of *Npy* in the ARH is inhibited by leptin [216] although mice that have NPY blocked (but they are not genetically altered) do not present changes in the feeding behaviour when leptin levels are normal [217]. AgRP, orexin and MCH are signalling molecules which also have anabolic action.

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Melanocortins such as alpha-MSH, CRH, TRH, CART and IL-1B are neuropeptides involved in the down-regulation of energy balance. When there is an increased signalling of adiposity in the brain, these molecules start to be expressed. Melanocortins are peptides that result from the cleavage of POMC and play their actions linking with MC3R and MC4R [218]. Both in mice [77] and humans [219], the lack of MC4R leads to hyperphagia and wt gain. One of the antagonist of MC3R and MC4R is AgRP [220]. *Agrp* expression is located in the ARH [221] and it is up-regulated by fasting [222] and low circulating leptin levels [221], indicating that antagonism of MCRs is markedly important in the bw control. Higher AgRP levels in brain cause hyperphagia and an increase in food intake. NPY is the most potent orexigenic peptide but it exerts a short-term action comparing to AgRP, being the second one more robust in therms of cumulative increment of energy intake [223].

As mentioned previously, hypothalamus is the main region of the body that controls food intake and bw. The VMN is called the “satiety centre” whereas the LHA is the “hunger centre” [7]. The ARH is located adjacent to the floor of the third ventricle. It is elongated and it is formed by a group of neuronal cell bodies that comprises one-half of the hypothalamus. NPY and AgRP are in the ARH neurons [222], informing that a group of neurons can co-express different neuropeptides. POMC and CART are co-localized in a different, but adjacent, group of neurons in the ARH [224], suggesting that this area of the brain contain circuits specialized on controlling energy metabolism.

Most of the NPY/AgRP and POMC/CART neurons express ObR [26,225] and leptin oppositely regulates the two circuits. Leptin inhibits NPY/AgRP (activated when leptin levels in plasma are low) [216,222]. When there is a reduction in leptin, POMC [226] and CART [227] are inhibited (**Figure 5**). Thus, this information is useful to understand that ARH is a key part of the brain implicated in transducing input signals from leptin and to create output neuronal responses. There are other areas of the brain that are innervated from neurons, which origin is the ARH, and they contain second-order neurons with a role in the energy homeostasis. The most enriched areas of the brain that contain axons from the ARH POMC/CART and NPY/AgRP neurons are the PVN, zona incerta, perifornical area (PFA) and LHA [228]. The anorexigenic signalling molecules come from PVN whereas those which belong to orexigenic response are from LHA [7]. Thus, neuropeptides generated from PVN neurons reduce bw and food intake. An example is CRH, implicated in anorexia, activates the SNS and acts as a regulator of the hypothalamic-pituitary-adrenal axis [35]. TRH belongs to the thyroid axis and reduces food intake. Oxytocin regulates the function of the uterus and reduces food intake [229]. Because these second-order catabolic neurons located in the PVN are the downstream components of the ARH, they should be activated by the melanocortin system and/or CART and inhibited by NPY [7].

As previously mentioned, the meal size is determined by the satiation. Hence, hypothalamic pathways involved in the control of energy interact with others implicated in the response of these satiety signals [230]. Changes in adiposity signalling and energy storages influences the meal size. For example, animals that

received a treatment with leptin eat the same number of meals but they are smaller [231]. Therefore, signals from energy homoeostasis regulate the size of individual meals to control food intake [7]. The satiety information originated during a meal is formed in afferent fibres of vagus nerve and of upper gastrointestinal tract, passing through the spinal cord and arriving to the hindbrain [232]. All this information arrives to an area of caudal brainstem called nucleus of tractus solitarius (NTS) that incorporates signals from the gastrointestinal duct, the oral cavity and the abdominal viscera [233]. Mechanical or chemical stimulation of the stomach and small intestine as well as inputs from the metabolism in the liver and humoral signals, like CCK, initiate signals to induce the satiety in NTS [234,235]. Thus, the process of finishing the meal is not associated with the hypothalamus.

Regarding the interaction of adiposity signals that arrive to forebrain and the changes in the amounts of food consumed during each meal, evidence suggests that leptin potentiates the effect of CCK to activate NTS neurons to inform about the satiety [236]. It is known that neurons from NTS integrate information of satiety from neurons of forebrain involved in energy homoeostasis. Some forebrain areas, such as PVN, are interconnected with NTS [237], being multiple brain areas linked about the satiety and energy homoeostasis. Moreover, the substrates originated to respond the central effector peptides involved in energy homoeostasis are in the NTS and in the hypothalamus (MC4R) [238]. In addition, ObRs [151] and POMC neurons [239] are in the NTS, concluding that both hindbrain and forebrain regulate energy homoeostasis. Therefore, NTS and ARH have neurons that receive information from

leptin, via ascending projections to forebrain, and both contribute to the adaptation of the feeding according to the body fat depots [7].

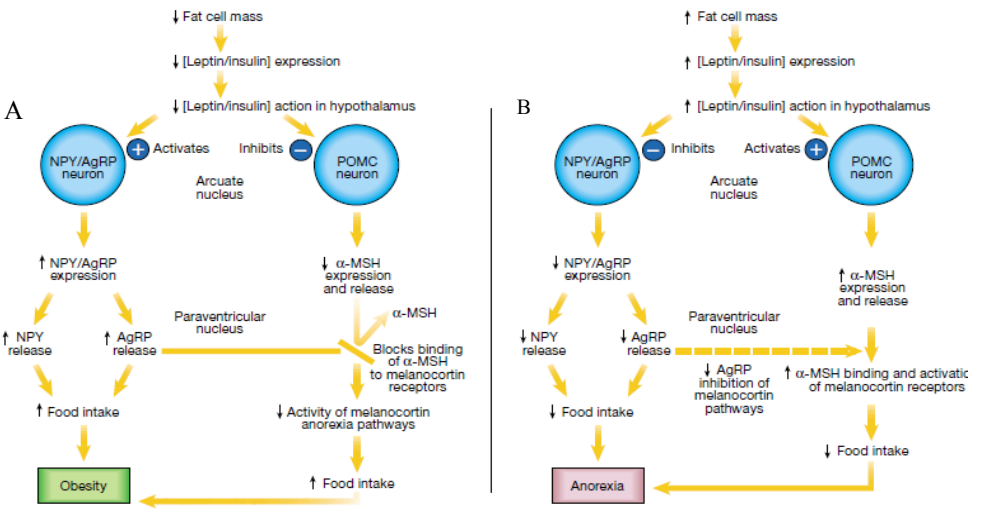

Figure 5. The central effects of leptin. (A) When leptin production is descreased due to the fat depots are reduced, there is an activation of NPY/AgRP neurons (orexygenic response) and an inhibition of POMC/CART neurons (anorexygenic response), thus, causing a serie of events that increase food intake. (B) Contrarily to this situation, when leptin is higher present in blood, is the anorexygenic peptide which is activated and, consequently, there is a decrease of food intake. AgRP: Agouti-related peptide; CART: Cocaine and amphetamine regulated transcript; NPY: Neuropeptide Y; POMC: Proopiomelanocortin. The figure was adapted from [7].

2.3.2. Peripheral effects of leptin

2.3.2.1. Liver

Leptin is implicated in the insulinic action in the liver [84]. In hepatic cell models, through its shorter isoform, it stimulates PI 3-kinase binding to its receptors, causing the activation of PI 3-kinase and PKB and, consequently, the activation of cyclic nucleotide PDE 3B, which is an enzyme implicated in the cAMP degradation. It also

inhibits glucagon-induced cAMP increase in a PI 3-kinase-dependent manner causing an inhibition of insulin secretion [240,241].

Moreover, it is postulated that leptin plays a role in the acute regulation of lipoproteins metabolism in liver. It decreases the TAG levels and secretion by the liver and increases hepatic FA oxidation and ketogenesis, mainly due to an inhibition of the ACC activity [242].

According to the hepatic Glc metabolism, leptin mimics the insulin effect on glycogenolisis and glucagon-like effect on gluconeogenesis. It is involved in activating hepatic substrate oxidation from carbohydrates to lipids and in the control of liver glycogen stores in obesity. In obese patients, there is not an increase in the production of endogenous Glc although the gluconeogenesis is increased, suggesting a suppression of glycogenolysis. Therefore, higher leptin plasma levels help to suppress hepatic glycogen turnover and maintain glycemia levels in obesity [243] (**Figure 6**).

2.3.2.2. Muscle

Muscle is an important tissue implicated in insulin-stimulated Glc uptake and lipid oxidation and it is the most important in the resting metabolic rate. Leptin is expressed in skeletal muscle [225] and both ObRa and ObRb are present in this tissue [131]. Although only the 8 % is the ObRb isoform, both ObRa and ObRb mediate the signal transduction [136]. Leptin up-regulates FA oxidation activating the AMP-activated

protein kinase (AMPK) which phosphorilates and inactivates the enzyme ACC [226,242]. Moreover, it participates in the clearance of serum FFA and TAG [84]. Therefore, leptin plays a key role in inhibiting glycogen synthesis, Glc transport and lipid partitioning [84].

In the muscle, leptin acts synergistically with insulin increasing the uptake of FFA and the uptake of Glc via a mechanism mediated by CNS. Furthermore, they antagonize in the inhibition of insulin-mediated suppression of FA oxidation [246]. As insulin is anabolic, it has the capacity to increase the storage of fuels and aas whereas leptin mobilize TAG, stimulates the use of FA and blocks the insulin-mediated mechanisms of long-term Glc storage and oxidation [84] (**Figure 6**).

2.3.2.3. White adipose tissue

Leptin can regulate the sympathetic tone to WAT. It has been seen that leptin administered centrally causes a reduction in the gonadal fat wt without changes in food intake [247]. Moreover, it decreases lipogenesis in gonadal fat [248]. In inguinal WAT, it increases the sympathetic tone [249], the lipolysis through SNS and insulin regulation [84,250,251], and it stimulates UCP2 [84].

Leptin has the capacity to inhibit insulin binding in rat adipocytes, impairing insulin-mediated stimulation of Glc transport, glycogen synthase activity, lipogenesis (inhibition of ACC), inhibition of isoprenaline-induced lipolysis and protein kinase A (PKA) activation and synthesis [252,253].

Insulin is involved directly in the gene expression and production of leptin by this tissue. Higher insulin plasma levels causes an increase in the circulating leptin and its expression in WAT, both in humans and rodents [111,254], via an increase in Glc transport and metabolism [255] (**Figure 6**).

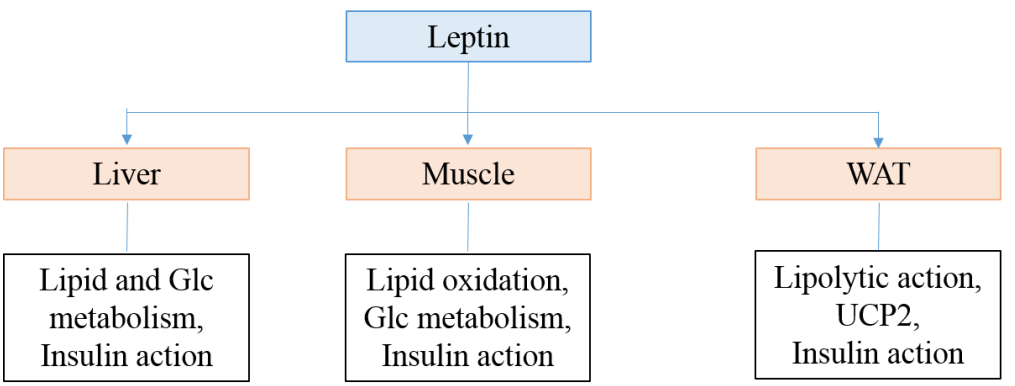

Figure 6. Pheripheral actions of leptin. In liver, the main action of leptin is related with insulin, lipid and Glc metabolism. In muscle, leptin is implicated in glycogen synthesis, Glc transport and lipid metabolism. In WAT, it is responsible of thermogenesis and insulin and lipolytic action. Glc: Glucose; UCP2: Uncoupling protein 2; WAT: White adipose tissue.

2.4. Leptin resistance

LR is defined as the failure of endogenous or exogenous leptin to promote anticipated salutary metabolic outcomes in states of over-nutrition or obesity, although the hormone's inability to promote desired responses in specific situations results from multiple molecular, neural, behavioral, and environmental mechanisms [256].

LR was first documented in mice (*db*/*db*) [257] and in rats (*fa*/*fa*) [258]. Both models are mutant of ObR and develop obesity. Moreover, there are other mice models that

develop obesity for different reasons: genetic ablation of thermogenic BAT [259], lack of MC4R [260], agouti (A^y/a) [261] and those which are fed with HFD [257].

LR is the consequence of the contribution of different mechanisms. One of them is the inability of the circulating leptin to enter in the brain due to a disruption in the uptake through endothelial cells of the BBB. In this sense, ObRs expressed by endothelial cells in the BBB are implicated in the leptin transport into the brain, acting as leptin transporters [262]. LR can appear when a failure of one or more neuronal circuits is produced [7]. Inflammation, endoplasmatic reticulum (ER) stress and a low Sirtuin 1 (SIRT1) activity also promote LR [263] (**Figure 7**). Following, some of the main factors that contribut to the development of LR are reviewed.

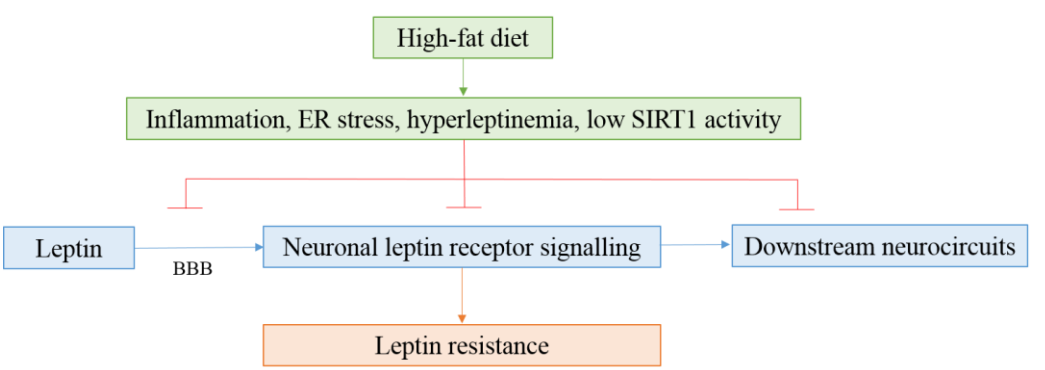

Figure 7. The main causes of leptin resistance. Obesity, which can be caused due to HFD, is related with inflammation, ER stress, hyperleptinemia and low SIRT1 activity. All these conditions influence on LR acting on different processes. The LR can appear do to an impairment of leptin transport from blood into the brain via BBB. Other cause is the disruption of the leptin signalling pathway. In addition, it can be a dysregulation in the downstream neuronal circuits that also can lead to LR. ER: Endoplasmatic reticulum; HFD: High-fat diet; LR: Leptin resistance; SIRT1: Sirtuin 1.

2.4.1. Disrupted leptin transport across de BBB

Because leptin is produced in the periphery and most of the functions are produced in the CNS it needs to be transported via a specific and saturable transport across the BBB, concretely in the endothelium of cerebral microvessels and epithelium of choroid plexus [264]. ObRa and ObRe are expressed in these microvessels and help leptin to pass through the BBB [265]. Another leptin transporter protein found in the choroid plexus epithelium is LPR2 [266].

The association between LR and the defect in the leptin pass via BBB is observed in obese human and rodents with reduced leptin levels in the cerebrospinal fluid compared with controls [267]. There are different hypothesis that explain this fact. Polyunsaturated FA influences in peripheral LR increasing the expression of the thigh junctions, called *occludins*, in hypothalamus, thus, reducing the transport of leptin paracellulary in the brain [268]. In addition, a deregulation in *ObRa* and *ObRe* expression can alter the leptin transport, being the major cause of this defective leptin transport [269]. When the TAG and/or protein C reactive (PCR) are abnormally high there is a reduction in the leptin transport across the BBB [268].

2.4.2. A down-regulation of leptin signalling pathway in the hypothalamus

An interruption of the ObRb signalling pathway in the brain is also a cause of LR. In fact, this is considered the primary factor that produces central LR and it is mainly produced, basically, due to two molecules, SOCS3 and PTP1B.

It is observed that SOCS3 specific deletion in brain of rats caused a lower incidence of the DIO and, as a consequence, LR decreases [270]. The consumption of a fat-rich diet and inflammation increase the expression of *Ptp1b*, which plays an important role in LR [271]. Thus, the PTP1B deletion in brain protects from obesity and increases leptin sensitivity [272].

2.4.3. Inflammation in hypothalamus

In obesity, LR can appear due to the prevalence of inflammation in both periphery and the hypothalamus. Rodents with a HFD show an up-regulation of inflammation, having activated the inhibitor of nuclear factor kappa B kinase-beta/Nuclear factor-kappa B (IKKB/NF-kB) pathway [273]. However, an increase in leptin sensitivity and reduced-*Socs3* expression is observed when this pathway is inhibited in the ARH [274] and also when there is a deletion of IKKB in AgRP neurons [275].

Toll-like receptor 4 (TLR4) is a receptor implicated in immune system and activates the IKKB/NF-kB pathway in hypothalamus when saturated FA interact with it [276].

c-Jun N-terminal kinase (JNK) also induces inflammation and it is highly expressed in the ARH of rodents fed with HFD [273] although the connection between this molecule and the LR is not well established yet.

It is important to highlight that inflammation not only causes LR but also produces changes in the structure of hypothalamus, altering the neuronal connexions in areas of the brain implicated in the regulation of food intake in obese human and rodents [277].

2.4.4. ER stress

ER is the organelle involved in the synthesis and folding of proteins, creating active proteins in the ER lumen. The proteasome complex avoids the accumulation of miss-folded or un-folded proteins. If this complex is unbalanced, defective proteins are accumulated in ER lumen, originating ER stress and the activation of un-folded protein response (UPR). When there is an acute ER stress, the UPR response restores the homoeostasis of the ER by reducing the protein production, increasing the folding of the proteins and removing the damaged ones [278].

Chronic ER stress causes LR increasing the leptin levels in plasma and originating obesity [275]. Therefore, the inhibition of ER stress increases leptin sensitivity and reduces food intake and bw [279]. Rats with a HFD show decreased conversion of POMC to alpha-MSH caused by hypothalamic ER stress [280]. It is known that the inflammatory IKKB-NF-kB pathway and ER stress interact each other causing LR [273].

2.4.5. Low SIRT1 activity

SIRT1 is a nicotinamide adenine dinucleotide (NAD^+)-dependent protein deacetylase and it is implicated in LR when its activity is reduced [281,282]. In contrast, there is an increase in energy waste and a reduction in food intake when it is activated in hypothalamus [281]. This is due to the fact that SIRT1 reduces PTP1B [281], SOCS3 [281] and FoxO1 [283] levels. It also reduces inflammation [284] and ER stress [285].

2.5. Leptin and photoperiod

Rhythmic events, like daily and seasonal cycles, affect animals, thus, developing endocrine rhythms to try to adapt to the nature changes. The autonomic rhythmicity of a cell in cycles of 24 h has the name of circadian rhythm, and is involved in controlling physiological functions and endocrine rhythms. Most animals which are living in temperate climate use photoperiod to sense seasonal modifications in their environment. In fact, the regulation of photoperiod in energy metabolism is a key strategy used by animals to survive. These photoperiod changes in energy metabolism are found both in domestic and experimental animals such as different species of hamsters, voles and sheep [286]. Until the date, the most used animal for this purpose is the Siberian Hamster (*Phodopus sungorus*) [287]. Moreover, there is one rat strain, the inbred Fischer 344 (F344) rat, which is sensitive to the length of daylight exposure by modifying its physiological phenotype and reproductive condition depending on the season [288]. The bw and food intake in these hamsters and rats are lower exposing them to short day (SD) photoperiod [289].

In seasonal mammals, the annual cycles of food intake and adiposity are related with changes in the levels of circulating leptin and with modifications in its expression in WAT [287]. This is because the photoperiodic regulation in bw affects the sensitivity of the hypothalamic energy centre against leptin [286]. Concretely, in Siberian hamster, it has been observed that long day (LD) photoperiods caused LR. In addition, an increase in bw [290,291], abdominal fat depots [291,292], food intake [291], serum

leptin concentration [291], mRNA *leptin* levels in WAT [292], mRNA *ObRb* and mRNA *Pomc*. In contrast, the levels of mRNA *Npy*, *Agrp* and *Cart* [293] are not different compared with those submitted to a SD photoperiod, which are leptin sensible. Moreover, *Socs3* expression increases during long photoperiod independently of circulating leptin levels and fat depots [294].

Similar patterns in leptin and adiposity changes due to seasons are also observed in domestic sheep [295] and woodchucks [296]. Sheep, deer and in general large seasonal mammals show high amplitude seasonal cycles of food intake with low seasonal changes in metabolic rate [297]. However, in rodents, changes in the energy waste regulate changes in adiposity via sympathetic influence in WATs [298].

In young Fisher rats the dietary preferences are modulated by photoperiod as a consequence of modified energetic requirements from specific macronutrients. Carbohydrates are the major determinants of these preferences while fat is balanced according to photoperiod. When rats are forced to eat low-fat high-carbohydrate or high-fat low-carbohydrate diets, energy intake, *Socs3* expression in the hypothalamus and feed efficiency are regulated by the interaction between dietary composition and photoperiod, implying that seasonal mammals can choose specific nutrition in accordance to changes in the environment [286], being in relation with seasonal cycle of nutrition in humans [299].

In humans, leptin circulating levels are pulsatile and they have circadian patterns, having a pick during the night and decreasing at its lower amounts at morning

[91,300,301]. Insulin levels or food intake did not influence the circadian rhythm of leptin levels [302] while the leptin levels are altered according to the meal timing [301]. In rats, the pick is reached during their active period (late-dark) and there is a down-regulation during the inactive period, produced in the late-night [303].

However, these variations in the circadian rhythm of leptin are 1.5 times lower compared with the modifications which can suffers from metabolic changes (fasting or cold) that can quickly alter the expression of *leptin* without acting in fat depots [91,228].

3. Phenolic compounds

Phenolic compounds, the major sub-class of phytochemicals [304,305], are plant secondary metabolites characterized by the presence of at least one phenolic ring in their structure. This family of compounds is involved in several aspects of the plants such as morphological development, physiological functions (colouring for camouflage, defence against herbivores, antibacterial, antimicrobial and antifungal activities, UV protectants) and reproduction (incorporating attractive substances to accelerate pollination and seed-dispersing animals) [306-308]. In addition, they give bitterness, astringency, colour, flavour, odour and oxidative stability to plants and foods that contain them [305].

Nowadays, there are approximately 8,000 phenolic compounds described [306,308,309]. Fruits, vegetables, cocoa products and beverages (tea and wine) are

the main sources of phenolic compounds in the human Western diet [304,307]. Concretely, raspberries, cranberries, strawberries, apples, grapes, pears and jams are foods that contain some of the highest concentrations of polyphenols [307]. Phenolic compounds are distributed in different parts of the plants such as roots, leaves, fruits and vegetables. Leaves and stems contain higher amounts of these compounds, primarily in their simple form, having variations between species. Complex polyphenols are present in vacuoles, leaf, epidermis, flowers and fruits. Barks, wood and fruit pods contain complex structures such as tannins and flowers are rich in flavonoids [309]. Taking into consideration all this, it can be considered that phenolic compounds are the most essential non-nutrient in our diet [305,309].

The content of phenolic compounds varies according to several factors such as the genotype, type of culture, climatic factors and agricultural factors. Moreover, the daily intake of polyphenols fluctuates due to environmental conditions, storage and the processing of the foods [305].

Phenolic compounds are synthesized through the pentose phosphate and shikimic acid by phenylpropanoid metabolization [306,307] (**Figure 8**). In the shikimate pathway, phenylananine (Phe) and/or Tyr are the precursors for the synthesis of phenolic acids [309]. The first step of the synthesis of phenolic compounds is the entrance of Glc to the pentose phosphate pathway (PPP) and the conversion of Glc-6-phosphate (G-6-P) to ribulose-5-phosphate (R-5-P), via the enzyme Glc-6-phosphate dehydrogenase (G6PDH). This conversion causes the reduction of nicotinamide adenine dinucleotide

phosphate (NADPH) for anabolic processes. Moreover, PPP originates erythrose-4-phosphate that is combined with phosphoenolpyruvate from glycolysis and it is used by the phenylpropanoid pathway in order to create phenolic compounds [307].

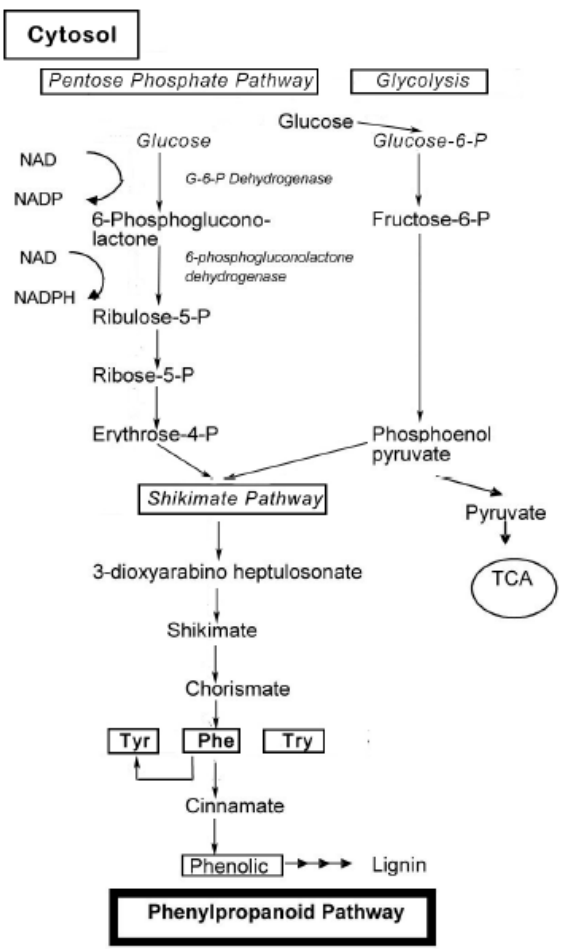

Figure 8. The route of synthesis of phenolic compounds. Phenolic compounds can be formed from pentose phosphate pathway and glycolysis. These pathways originate erythrose-4-phosphate and phosphoenol pyruvate, which enter to shikimate pathway and start different metabolic transformation until phenolic compounds are formed. This figure was adapted from [307].

The main core of the phenolic compounds is formed by at least one phenol ring, ranging from simple molecules (one phenol ring) to highly polymerized compounds

(more than one ring, also known as polyphenols) [306,307,309]. The hydrogen is usually replaced by other more active residues like hydroxyl, methyl or acetyl groups. The aromatic ring helps to form strong hydrogen bounds, increasing the water solubility and raising the boiling and melting points. Phenolic compounds present a wide range of colours, from colourless to intense colours like red or violet [306]. The diverse biological properties of each phenolic compound varies according to the structures, substitutes and cellular targets [304,306]. Furthermore, they can link with other molecules like amines, carboxylic and organic molecules, lipids or other phenols [305].

3.1 Structure and classification

According to their complexity, phenolic compounds can be classified as simple or complex phenolics (**Figure 9**). Benzoic acids (BA) and cinnamic acids (CA) are the most common simple phenolic compounds found in nature having 6- and 9-C in their skeleton, respectively. Both have a carboxylic group linked to the benzene ring and one or more hydroxyl or methyl groups associated with it. Complex phenols have a high molecular wt and are found in cell vacuoles. As examples we found tannins and flavonoids, commonly present in fruits and vegetables. Flavonoids are formed by 2 or more phenolic rings being attached with one oxygenated heterocyclic pyran ring. According to the oxygenated levels of the pyran rings, flavonoids are classified in different groups such as anthocyanins, flavones and flavanols [309].

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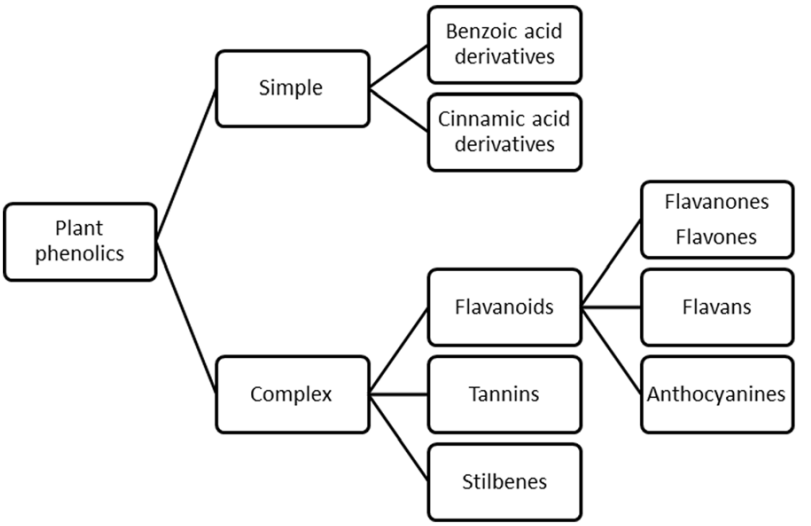

Figure 9. One classification of phenolic compounds. Plant phenolics can be classified according to their complexity in simple, including benzoic and cinnamic acid derivates, or complex. The last one is divided in flavonoids (i.e., flavanones and flavones, flavans and anthocyanines), tannins and stilbenes. This figure was adapted from [309].

Due to the importance of flavonoids, another classification that can be found is the division of phenolic compounds into flavonoids and non-flavonoids [304,306,308] (**Figure 10**).

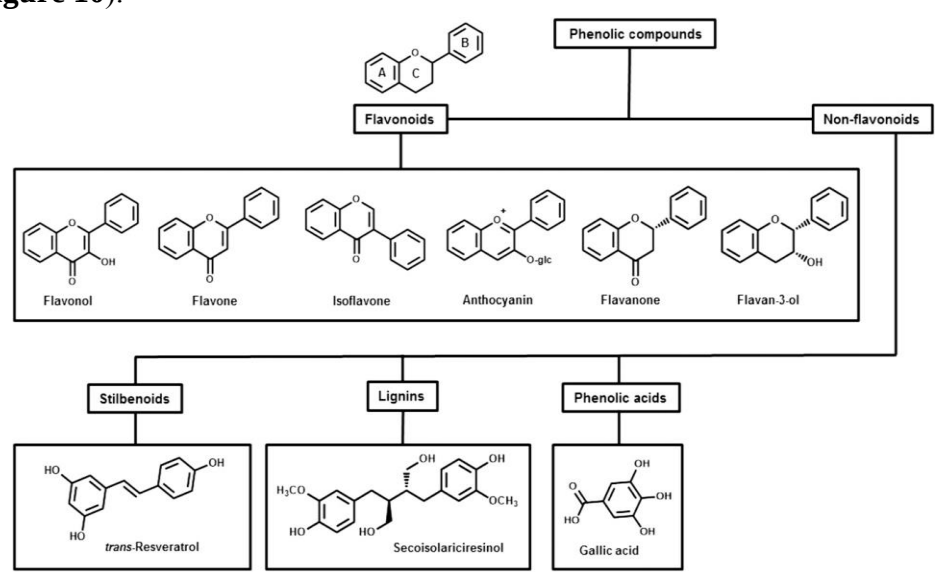

Figure 10. The other classification of phenolic compounds is according if they are flavonoids or non-flavonoids. The first group is composed of flavonols, flavones, isoflavones, anthocyanins,

flavanones and flavan-3-ols whereas in the second one stilbenoids, lignins and phenolic acids are included in. This figure was adapted from [304].

3.1.1. Flavonoids

This is the most largest and diverse group of phenolic compounds. Flavonoids contain two aromatic rings (15 C) connected by a bridge of three carbons (C_6-C_3-C_6) and are divided in six subclasses: flavonols, flavones, flavanones, flavan-3-ols, isoflavones and anthocyanidins (**Figure 11**). They are usually associated with sugar. Other minnor groups are chalcones, dihydrochalcones, dihrydroflavonols, flavan-3,4-diols, coumarins and aurones.

They are usually found in the vacuolar juices and in the epidermis. The main sources of flavonoids are fruits and vegetables but they also appear in some grains, seeds, spices, wine, tea, coffee, cocoa and herbal essences [306,308].

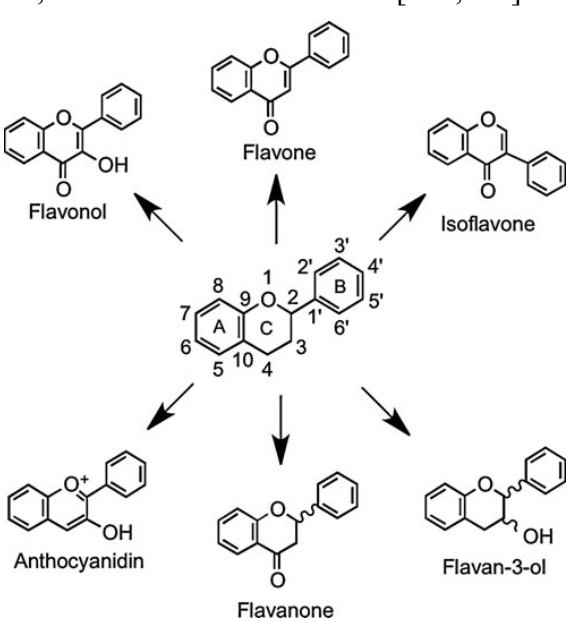

Figure 11. Schematic representation of the structure of the different sub-families of flavonoids. In the center of the image, there is the basic skeleton of a flavonoid that, after some changes in its

structure, it can be transformed to the different members: flavonols, flavones, isoflavones, anthocyanidins, flavanones and flavan-3-ols. This figure was adapted from [308].

3.1.1.1. Flavonols

The most known flavonols are kaempferol, quercetin, isorhamnetin and myricetin. They are usually found as glycosides having the conjugation in the positions 5, 7, 3', 4' and 5'. Commonly, flavonols are found in the most consumed fruits, vegetables and beverages depending their amounts due to local agronomical conditions, climatic factors and the variety, among others [308].

3.1.1.2. Flavones

Apigenin, luteolin, wogonin and baicalein have similar structure to flavonols but they do not have an oxygenation at C-3. They present several substitutions such as hydroxylationa, methylationa, *O*- and *C*-glycosilationa and alkyationa. They are only found in celery, parley and some herbs. Many of them occur as 7-*O*-glycosides [308].

3.1.1.3. Flavanones

Naringenin and hesperetin are the most common flavanones and they are characterized by the absence of $\Delta^{2,3}$ double bound and by the presence of a chiral centre at C-2. They are predominantly found as *S*- or (-)-enantiomer with the C-ring linked with the B-ring at C-2 in the alpha-configuration. They appear as hydroxyl, glycosylated and *O*-methylated derivates and basically they are present in citrus fruits. The most important flavanone glycoside is hesperetin-7-*O*-rutinoside (hesperidin) [308].

3.1.1.4. Flavan-3-ols

Flavan-3-ols are the most complex group of flavonoids ranging from monomers to polymeric proanthocyanidins (PAC) (condensed tannins). The chiral centres at C-2 and C-3 of the monomer generate four isomers for each level of B-ring hydroxylation. (+)-catechin and (-)-epicatechin (EC) are the most prevalent flavan-3-ols found in nature. PAC have another quiral centre in C-4 and they can appear as polymers of more than 50 units. Those polymers formed by (epi)catechin units are called procyanidins and are the most abundant in plants. A less common group of PAC are those formed by (epi)afzelechin and (epi)gallocatechin units namely as propelargonidins and prodelphinidins, respectively. Green tea contains higher amounts of these monomers [308].

3.1.1.5. Isoflavones

Isoflavones have the B-ring linked at C-3 rather than C-2 and they are found basically in leguminous. The most importants are daidzein and genistein in soybean and principally as a 7-*O*-(6”-*O*-malonyl)glucosides with low levels of 7-*O*-(6”-*O*-acetyl)glucosides, 7-*O*-glucosides and aglycones. Due to their structure similarity to of oestrogen, they are classified as phytoestrogens (non-flavonoid lignans) being a diverse group found in cereal grains [308].

3.1.1.6. Anthocyanidins

The commonly found aglycones are pelargonidin, cyanidin, delphimidin, peonidin, petunidin and malvidin, forming conjugates with sugars and organic acids to generate

multiple anthocyanins with lots of colours (from orange/red to blue/purple). They are present in fruits and flowers [308].

3.1.2. Non-flavonoids

Non-flavonoids are formed by: phenolic acids (hydroxybenzoates C_6-C_1 and hydroxycinnamates C_6-C_3), lignans (C_6-C_3)$_2$, stilbenes C_6-C_2-C_6, tannins and lignins (both are biopolymers) [306,308].

3.1.2.1. Phenolic acids

The most common phenolic acids in plant tissues are hydroxycinnamic acids which includes caffeic acid, chlorogenic acid (C_6-C_3 hydroxycinnamates which are conjugated with tartaric acid and quinic acid), *o*-, *m*- and *p*-coumaric acids, ferulic acid and sinapic acid. These acids are associated with esters or forming glycosides. Concretely, hydrocycinnamic acids are in the ester form linked with carboxyl acids or Glc. Caffeic acid, the most prevalent hydroxycinnamic acid, is found in coffee, apples, potatoes, spinach, cabbage, olive oil, wine and tobacco leaves. The second group of phenolic acids are those which belong to hydroxybenzoic acids such as gallic acid (GA), *p*-hydroxybenzoic acid, protocatechuic acid, vanillic and syringic acids. This group is found mainly as glycoside form. In plants, phenolic acids can be bound to different compounds like flavonoids, FA, sterols and other polymers [306].

GA can appear as a complex sugar esters generating gallotanins. The related ellagic acid-based ellagitannins are found in a wide range of foods such as raspberries,

strawberries, blackberries and others like pomegranate, persimmon, walnuts, hazelnuts and oak-aged wines [308].

3.1.2.2. Lignans

The principal lignan compounds are called secoisolariciresinol, pinoresinol and matairesinol which are phenylpropanoid dimers. The main presence of these compounds are in seed of flax and sunflower and they are found in small amounts in grains, vegetables, fruits, nuts, tea and coffee [306].

3.1.2.3. Tannins

Tannins are a group of polyphenols divided into hydrolyzable (with sugar polymerization) and condensed (the combination of different flavonoids) [306].

3.1.2.4. Lignins

The exact structure of lignins is not known because of its complex polymeric structure and the random couplings. The composition, molecular wt and their amount vary between plants decreasing in the following order: softwoods > hardwoods > grasses. The components are linked with B-*O*-4, 5-5, B-5, 4-*O*-5, B-1, dibenzodioxocin and B-B, being the B-*O*-4 the most common form [310].

3.1.2.5. Stilbenes: Resveratrol

This group of compounds has a C_6-C_2-C_6 structure and are phytoalexins produced by plants to revert diseases, injuries and stress [308]. The most representative stilbene is resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RSV) which is present in a lot of plant

species (grapes, peanuts and berries) [306,311]. It is synthesized in plants as defence mechanism to combat mechanical injuries, infection from pathogens and UV radiation. Its structure is formed by two aromatic rings connected by a styrene double bound, allowing two orientations, *trans*- and *cis*-isomers. The first one, *trans*-RSV, has a potent biological activity against high pH and UV radiation [306]. In humans, bioavailability studies wih *trans*-RSV have showed rapid absorption and metabolism and relative low excretion in urine and feces. The peak of free RSV in the plasma reach its maximum 30 min after ingestion. The Cmax of free RSV is low (< 37 nM) after oral ingestion at physiological doses (1-110 μmol). RSV-*O*-glucuronides and sulfates are the major plasma and urine metabolites, being the sulfates forms the predominants. Thus, RSV-3-*O*-glucuronide (R3G), RSV-4'-*O*-glucuronide (R4G), RSV-3-*O*-sulfate (R3S), RSV-4'-*O*-sulfate (R4S), RSV-*O*-disulfate (RDS), RSV-*O*-glucuronide-*O*-sulfate (RGS) and RSV-C/O-conjugated diglucuronide (RDG) are the major RSV metabolites detected in plasma and urine (**Figure 12**). In contrast, the most prevalent metabolites of *trans*-RSV found in plasma coming from microbiota are dihydroRSV-*O*-glucuronide (DRG), dihydroRSV-*O*-sulfate (DRS) and dihydroRSV-*O*-sulfate-*O*-glucuronide (DRSG) [312].

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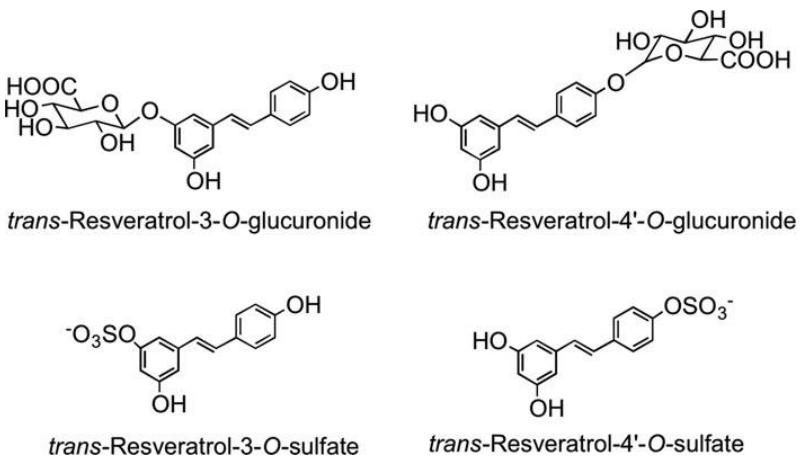

Figure 12. Different metabolites of resveratrol formed by phase II enzyme transformations. Phase II enzymes are responsible of adding the glucuronide or sulfate group to RSV creating different metabolites. From them, sulfates are prevalent in plasma and urine whereas glucuronides are found in minor amounts. RSV: Resveratrol. This figure was adapted from [308].

Remarkably, RSV has been associated with several beneficial effects, being useful in the treatment of cardiovascular diseases, cancers and degenerative disorders [306,311]. On the one hand, RSV is one of the possible candidates about related with the benefits observed in the "French Paradox". According to the "French Paradox", it was observed that French population showed a low incidence of cardiovascular diseases in spite of the fact they consumed high amounts of saturated fats. These beneficial effects are attributed, in part, to the consumption of the RSV contained in red wine [313].

On the other hand, CR is described as a moderate reduction in the calories consumed (between 20-40 %) without having malnutrition or deficits in vitamins or minerals, thus, conferring an increase in lifespan and protection to the loss of biological functions and pathological conditions due to the ageing process [314]. It seems that

RSV mimics the action of CR, exerting antitumoral and cardio-protective actions, protecting animals against atherogenic plaque development, increasinge endothelial NO production (vasodilatation), reducing inflammation in the wall of arteries, decreasing oxidative stress and improving myocardial perfusion, as well as ameliorating plasma lipid profiles and inflammatory markers, among others [311].

RSV is a SIRT1 allosteric activator and extends lifespan in lower eukaryotes in a SIRT1-dependent manner [315] mimicking one of the most important effects of CR: lifespan increase. Hence, mice with HFD are protected against bw gain, reduce body Glc and improve lipid metabolism as well as increase the lifespan when they are supplemented with RSV [316,317]. Moreover, they present expression patterns in peripheral tissues that are closely similar to younger animals. They reduced osteoporosis, cataracts, vascular dysfunction and increase motor coordination [318].

In addition to these findings, it has been seen that RSV enhances energy expenditure by affecting mitochondrial respiration, lipid oxidation and improving some associated pathologies, including insulin resistance, sarcopenia, cognitive decline and cancer [316]. Peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1alpha) is one of the SIRT1 targets which is involved in mitochondrial biogenesis [319]. Once SIRT1 desacetylates PGC-1alpha, it increases its activity as co-activator of many transcriptional factors and nuclear receptors which control mitochondrial gene expression [320]. RSV treatment increases mitochondria in skeletal muscle and BAT [316].

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It has also been seen that RSV has the capacity to activate AMPK (a key nutrient sensor with the capacity to control body metabolism) in liver, skeletal muscle and WAT, faster than SIRT1 [321]. Its mechanism of action is based on the RSV capacity to bind and inhibit mitochondrial F1F0-ATPase/ATP synthase (Complex V), impairing ATP production [322]. Metabolic effects of RSV depend on AMPK activation: decreases lipid accumulation in liver [323], increases Glc transport in skeletal muscle [324] and improves insulin sensitivity, Glc tolerance, physical endurance and mitochondrial biogenesis [325] (**Figure 13**). Other effectors described for RSV are cyclooxygenases (COX), PDE, NF-kB, PI 3-Kinase/Akt signalling, mechanistic target of rapamycin (mTOR) signalling, oestrogen receptors and MAPK signalling [311].

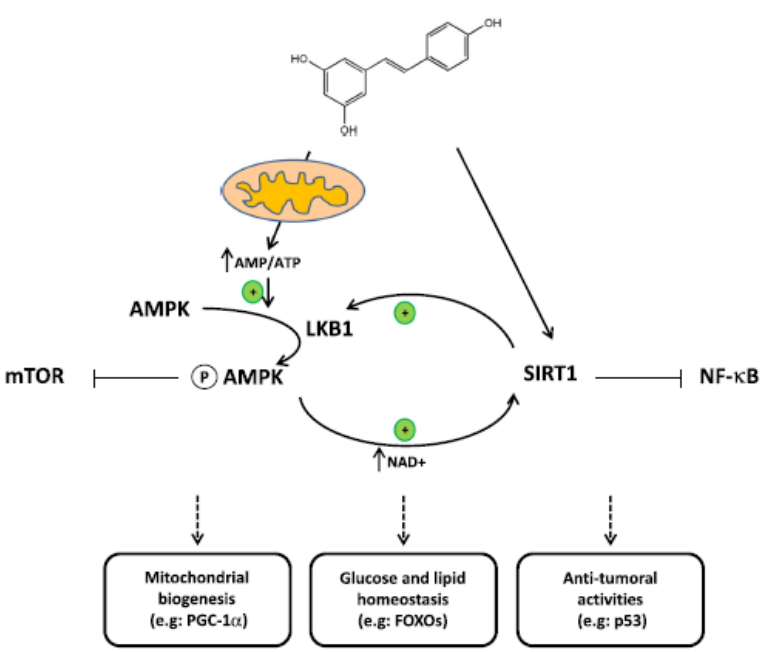

Figure 13. The beneficial effects of resveratrol due to its action in sirtuin 1 and AMP-activated protein kinase pathways. RSV is an activator of SIRT1 and it also increase the phosphorylation of AMPK. Both molecules have beneficial effects on human health acting through different mechanisms

and pathways. For example, they are involved in mitochondrial biogenesis, in the mantainance of Glc and lipid metabolism and they have anti-tumoral activities, among others. They can act directly on indirectly via activation or inhibition of other signalling molecules. AMPK: AMP-activated protein kinase; Glc: Glucose; RSV: Resveratrol; SIRT1: Sirtuin 1. This figure was adapted from [311].

3.2. Intake, bioavailability and metabolism

The efficacy of the dietary polyphenols depends on the ability of these compounds to be absorbed by the organism and then transported to target tissues where they can exert their biological action. Therefore, the bioavailability of these compounds is a critical point that has to be considered when evaluating their activity [304]. In this sense, there are multiple factors that can influence the potential therapeutic effects of polyphenols including absorption, metabolism and transport in the upper and lower gastrointestinal tract [304] (**Figure 14**). Polyphenols in nature appear in the form of esters, glycosides and also as a part of complex strcutures. This diversity of structures, dramatically affects their bioavailability [305,309]. It has to be highlighted that, usually, the forms present in blood or tissues are different from those contained in food, due to a complex process of metabolization [305]. In addition, nowadays toxicity of phenolic compounds is one of the current topics of research, with different publications focused on studying the effect of a wide range of concentrations of polyphenols [305].

In the absorptive process, the first important step is the release of phenolic compounds from foods matrixs to accessible and soluble forms in the gut lumen. The soluble forms cross the lumen, via active or passive transport, to the enterocyte [304]. On the

other hand, some structures require the activity of enzymes to be absorbed. For example, the absorption of flavonoid glycosides needs the action of lactase phloridzin hydrolase (LPH) to cleavage and release the aglycone in the brush border of the small intestine epithelial cells. This enzyme has high specificity for flavonoid-*O*-B-D-glucosides. The resultant aglycone can enter epithelial cells via passive diffusion. Another enzyme involved in absorption is the cytosolic B-glucosidase (CBG) from the epithelial cells. Polar glucosides need to be transported into epithelial cells, by means of the sodium-dependent Glc transporter 1 (SGLT1). There are two mechanisms by which glycoside conjugates are hydrolyzed and the aglycones are transported into epithelial cells: LPH/diffusion or transport/CBG [308]. Furthermore, phenolic compounds can be returned from enterocytes into the lumen using ATP-binding cassette (ABC) transporters, including multidrug resistance protein (MRP) and P-glycoprotein [304,308]. Phenolic compounds have the capacity to highly specifically or broadly inhibit or decrease the activity of these transporters [304]. MRP-3 and Glc transporter 2 (GLUT2) are implicated in the efflux of metabolites from the basolateral site of enterocytes [308].

Inside the enterocyte, phenolic compounds are metabolized and further transported to the portal vein for the circulation. They arrive to the liver where they continue metabolization before entering to the systemic circulation (phase II metabolism), excretion into the bile (enterohepatic recirculation) or urine or distribution to other tissues.

Those compounds which are not absorbed, basically polyphenol conjugates with sugar that are resistant to the action of LPH or CBG, arrive to the lower gut being substrate for microbiota. In there, microbiota transforms them into compounds with low molecular wt, including phenolic acids and hydroxycinnamates. Then, these compounds can be absorbed and metabolized in the liver before being excreted by the urine [304,308].

The xenobiotic metabolism, present mainly in the intestine and the liver, but also in other tissues such as the BBB, is in charge of transforming and transporting phenolic compounds. Hence, phenolic compounds suffer phase II conjugation producing metabolites, which are more easily transported and excreted via bile, faeces or urine [304]. Phase II enzymes comprises cathecol-*O*-methyltransferase (COMT), sulfotransferases (SULTs) and UDP-glucoronyl transferase (UGT). COMT is the enzyme implicated in the methylation of phenolic compounds giving more hydrophobic and less chemically active compounds. The change of their structure causes alterations in their transport through the enterocyte and other cells, causing an accumulation of these methylated forms in some specific tissues like brain [304]. SULTs are another class of enzymes that conjugate anionic sulfate groups producing more hydrophilic compounds which can confer less or more bioactivity depending of the phenolic compound and the target tissue [304]. The process called glucoronidation consist on conjugating glucoronic acid to phenolic compounds via the enzyme UGT, resulting in hydrophilic species which can be easily eliminated from the body [304].

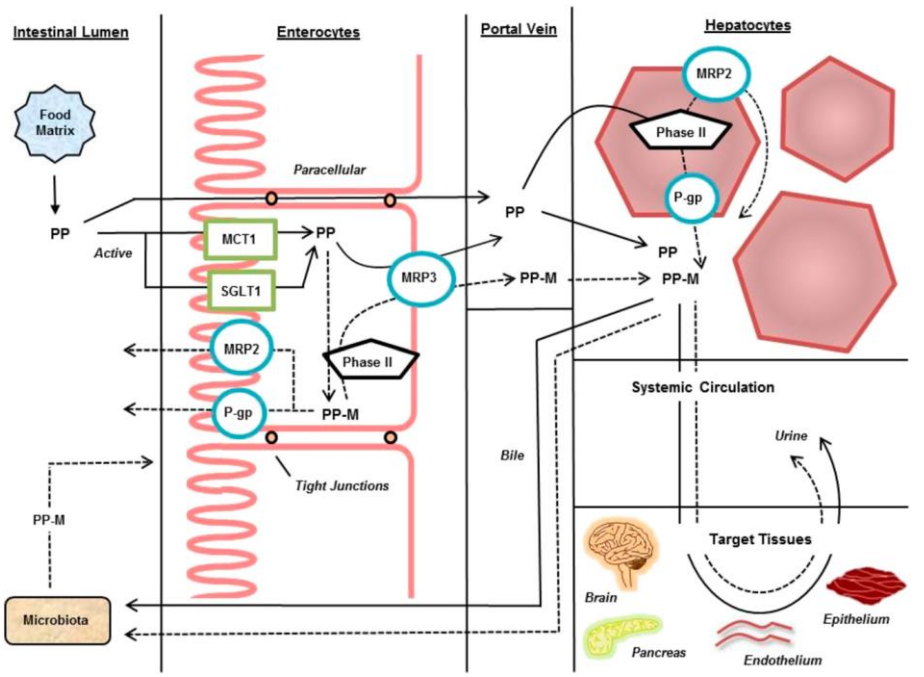

Figure 14. Absorption and biodisponibility of phenolic compounds once they are ingested. In the intestinal lumen, phenolic compounds can pass to portal circulation via parecellular transport or through active transport entering to enterocytes. Once they are inside the enterocyte cells, many phase II reactions can occur forming different metabolites which, in turn, can return to intestinal lumen or enter to portal vein. When they arrive to hepatocytes, they continue being transformed by enzymes of phase II and, finally, they enter to systemic circulation being distributed to target tissues such as brain, pancreas, among others. Moreover, when they are in the hepatocytes they can be expulsed by bile or urine. Futhermore, phenolic compounds that arribe to lower gut are used by microbiota and can be metabolized and absorved by enterocytes. This figure was adapted from [304].

3.3. Main functions

The concentrations used of phenolic compounds in the different experiments range from nM to mM. However, after ingestion, dietary polyphenols usually appear in the circulation as phase II and microbiota metabolites and their concentrations are limited at nM [308].

The biological activity of phenolic compounds results on a wide range of healthy properties [304,308] acting in different fields such as anti-ageing, anti-inflammatory, antioxidant and anti-proliferative agents [307,309] (**Figure 15**). These beneficial effects are basically due to the fact that they can modulate carbohydrate and lipid metabolisms, attenuate hyperglycemia, dyslipidaemia and insulin resistance, improve B-cell dysfunction, increase insulin secretion, improve adipose tissue metabolism and reduce oxidative stress and inflammatory processes. Thus, these properties confer phenolic compounds the ability to prevent the complications of cancer, neurodegenerative disorders, long-term type 2 diabetes and the metabolic syndrome, including cardiovascular problems, neurophathy, nephropathy and retinopathy [307-309]. Because of the wide range of beneficial effects, the introduction of phenolic compounds in functional foods and dietary supplements is currently under study [304,309]. Next, some of the main functions of phenolic compounds are briefly summarized.

3.3.1. Anti-oxidative properties

The most studied property of phenolic compounds is their antioxidant activity [306,307]. It is well described that ROS (superoxide, singlet oxygen, hydrogen peroxide and hydroxyl radical) are toxic and mutagenic. An excess of ROS leads to oxidative stress (characterized by an imbalance between ROS and the antioxidant defence system), causing cell damage and affecting lipids, proteins and DNA. Thus, it can originate disorders such as cancer, inflammation, cataract, hypertension, diabetes, cardiovascular disease and neurodegenerative diseases, including Alzheimer

and Parkinson. ROS are also implicated in immunological processes and ageing also influencing in skin inflammatory diseases [306].

There is a tight relation between the antioxidant properties of phenolic compounds and their structure being important the central annular structure, the presence of conjugated double bonds and the existence of functional groups in the ring. Several mechanisms of action have been described: inhibition of ROS formation and trapping and the suppression of singlet oxygen (scavenging free radicals), reduction of chelated metal ions, interruption of the cascade of free radical reactions in lipid peroxidation and reduction the hydroperoxide formation and with their antioxidant properties [305,306].

3.3.2. Anti-inflammatory properties

External factors cause in the organism different damages, irritation and allergies and the mechanism of action of our body to combat these negative effects is the inflammation, causing the release of free radicals. The formation of ROS and RNS activates biological responses such as the release of the transcription factor activator protein-1 (AP-1) and NF-kB which are involved in the regulation of secretion of signalling molecules such as pro-inflammatory CK and IL. Polyphenols inhibit these pro-inflammatory agents, block free radicals, ROS and RNS and, consequently, lipid peroxidation. Moreover, during the inflammatory process, the cell membranes release arachidonic acid via the enzyme PLA2, activated in oxidative stress conditions. Arachidonic acid can be transformed in the COX or lipoxygenase (LOX) pathways.

Phenolic compounds can block these reactions, interrupting the substrate-enzyme interaction, disrupting the hydrogen bonding system or chelating ions in the active site of the enzyme [306].

3.3.3. Beneficial effects on skin

Phenolic compounds not only affect internal organs but also the skin using them both in topical and surface treatments (cosmeceuticals). They have showed properties to maintain homogeneity and healthy of the skin. Different mechanisms are involved in these effects: acting in cell renewal, elastin and collagen stimulation, inhibiting enzymes involved in collagen degradation and reducing the excess of melanin synthesis [306]. Therefore, phenolic compounds are currently being used in the formulation of antiageing and skin-protective creams.

3.3.4. Cardioprotection properties

Foods rich in flavonoids significantly reduce cardiovascular risks factors, including hypertension, endothelial dysfunction, lipid metabolism and platelet activation [308]. It is also described the effects of polyphenols reducing postprandial hyperlipidaemic and oxidative stress, both contributors of atherosclerosis. Atherosclerosis is a silent pathology that can produce acute myocardial infarction, angina and sudden cardiac death. Polyphenols can reduce the risk of suffering thrombosis, one of the main causes of myocardial infarction. Moreover, epidemiologic studies confirm that

Mediterranean diet, enriched with vegetables, fruits, fish and wine, confers protection in the coronary heart disease compared to other diets [305].

3.3.5. Anti-cancer properties

Cancer is one of the major causes of death around the world and polyphenols have beneficial effects described in cancers present in mouth, stomach, duodenum, colon, liver, lung, mammary gland and skin. PAC, flavonoids, RSV, tannins, epigallocatechin-3-gallate (EGCG), GA and anthocyanins have shown protective effects in many models although their mechanism of action is different in each case and not fully understood [305]. Thus, polyphenols may influence in carcinogenesis and tumour development, DNA oxidation/damage and protect against lymphocyte DNA damage [308].

3.3.6. Anti-neurodegenerative action

The mechanisms of action of polyphenols to improve neurological health include their capacity to interact with intracellular neuronal and glial signalling, influencing peripheral and cerebrovascular blood flow, and to reduce the neuronal impairment due to neurotoxins or neuroinflammation. Their consumption improves cognitive performance, increases the positive effects on cognitive outcome measures and affects several aspects of memory and learning. Some of them are beneficial in reversing the

course of neuronal and behavioural ageing and others have shown the capacity to change positively the psychomotor activity in older animals [308].

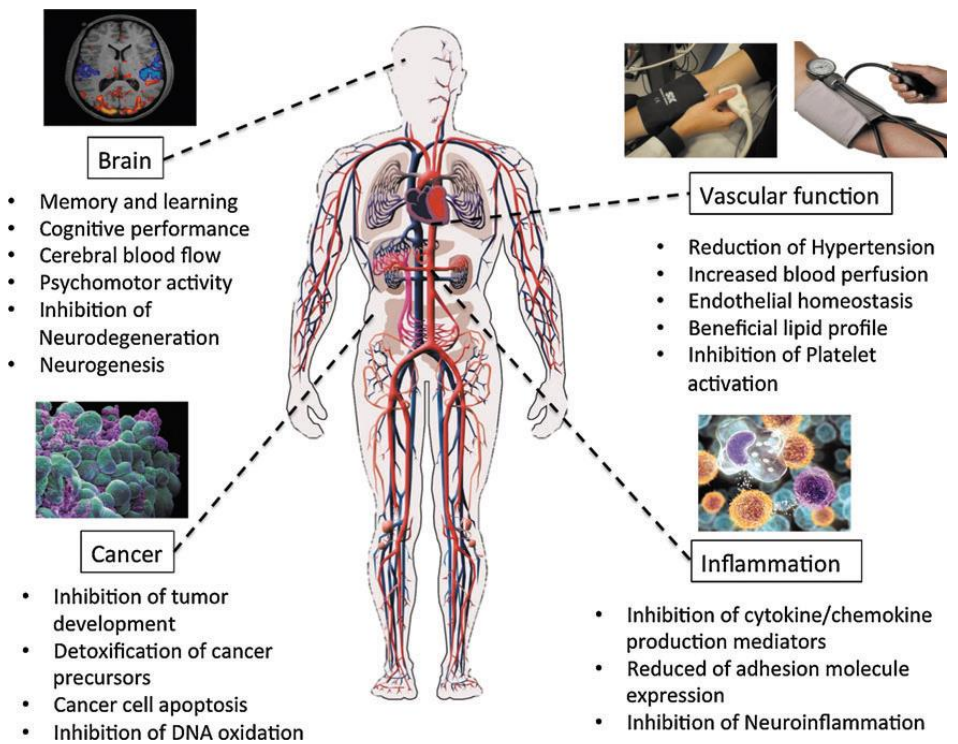

Figure 15. Schematic representation of the main actions of phenolic compounds. Phenolic compounds have a lot of described beneficial effects on human health at different levels. The mainly actions are anti-oxidant, anti-cancer, anti-inflammatory, cardioprotection, neuroprotection, among others. This figure was adapted from [308].

4. Effects of polyphenols on leptin signalling pathway

Manuscript 1

Modulation of leptin resistance by food compounds

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Manuel Suárez, Cinta Bladé

Mol Nutr Food Res. 2016, 60(8), 1789-1803

INTRODUCTION

PREFACE

The aim of this manuscript is to review the current avalaibale information about leptin, its signalling pathway and the causes and consequences of leptin resistance. Moreover, a compilation of the experimental data published until the date regarding the natural compounds that can restore leptin sensitivity in different animals and cellular models was done. Furthermore, when known, their mechanism of action is also reviewed. The objective was to introduce the topic of the thesis and to justify, partially, our hypothesis and objectives.

INTRODUCTION

Modulation of leptin resistance by food compounds

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Abbreviations: blood-brain barrier (BBB), white adipose tissue (WAT), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), agouti-related peptide (AgRP), neuropeptide Y (NPY)

Keywords: AgRP, megalin, STAT3, POMC, PTP1B.

INTRODUCTION

Abstract

Leptin is mainly secreted by white adipose tissue and regulates energy homeostasis by inhibiting food intake and stimulating energy expenditure through its action in neuronal circuits in the brain, particularly in the hypothalamus. However, hyperleptinemia coexists with the loss of responsiveness to leptin in common obese conditions. This phenomenon has been defined as leptin resistance and the restoration of leptin sensitivity is considered to be a useful strategy to treat obesity. This review summarizes the existing literature on potentially valuable nutrients and food components to reverse leptin resistance. Notably, several food compounds, such as teasaponins, resveratrol, celastrol, caffeine and taurine among others, are able to restore the leptin signalling in neurons by overexpressing anorexigenic peptides (POMC) and/or repressing orexigenic peptides (NPY/AgRP), thus decreasing food intake. Additionally, some nutrients, such as vitamins A and D, can improve leptin transport through the blood brain barrier. Therefore, food components can improve leptin resistance by acting at different levels of the leptin pathway; moreover, some compounds are able to target more than one feature of leptin resistance. However, systematic studies are necessary to define the actual effectiveness of each compound.

INTRODUCTION

1. Introduction

Overweight and obesity, the epidemic of the 21st century according to World Health Organization (WHO) information, are defined by chronic disease with an elevated accumulation of adipose tissue due to an imbalance between energy intake and energy expenditure, which affects both physical and psychosocial health. Currently, obesity is considered a health problem in both developed and developing countries, significantly increasing in prevalence in many nations worldwide with the expectation that more than 300 million people will be obese in 2035. However, presently, there is no successful long-term treatment for obesity, with the exception of bariatric surgery, which is expensive and risky. Thus, society needs innovative anti-obesity strategies that cause a significant reduction in food intake and/or an increase in energy expenditure with higher efficacy, safety, and selectivity. In this sense, the discovery of leptin in 1994 [1] opened a new field within the therapeutic strategies driven to combat obesity, and fortunately, leptin therapy has been found to be relevant for patients with very low leptin or leptin deficiency [2]. However, the administration of leptin is absolutely inefficient in decreasing the body weight of obese humans who are not leptin-deficient but instead have high levels of circulating leptin associated with loss of responsiveness to leptin [3]. This hyposensitivity to leptin is, currently, identified as leptin resistance, and its prevention and treatment could represent a major challenge in obesity research for the next decade.

Understanding the biological function of leptin and its receptors is an important step to identify potential dietary food compounds that could provide new strategies for restoring leptin sensitivity. Accordingly, dietary food compounds, such as amino acids, terpenoids, and flavonoids, act through a variety of mechanisms to improve leptin sensitivity. Herein, we review the roles of dietary food compounds that have demonstrated a clear improvement in leptin signalling, as well as briefly summarize the latest advances in the molecular mechanisms involved in leptin resistance, to supply new ideas for the management of obesity.

2. Biology of leptin

Leptin, which is a 16-KDa circulating protein with 167 amino acids synthesized from the LEP gene, is mainly secreted by white adipose tissue (WAT) and acts in the brain to regulate energy homeostasis [4]. The quantity of leptin released into circulation is directly proportional to the amount of body fat in the organism, reflecting the status of long-term energy stores [5]. Apart from WAT, there are other tissues with the capacity to secrete leptin, such as the placenta, mammary glands, ovaries, skeletal muscle, stomach, pituitary gland, lymphoid tissue and brown adipose tissue. Notably, leptin is secreted in a pulsatile fashion and displays a circadian rhythm. Its levels fluctuate according to changes in calorie intake, decreasing during starvation and increasing in overfed and obese states [6]. Additionally, women have higher levels of circulating leptin than men because of their higher estrogens levels, which increase the leptin serum concentration; meanwhile, male androgens suppress the leptin serum levels [7]. In addition to sex steroids, circulating leptin levels are also modulated by

other hormones, including catecholamine, insulin, glucocorticoids and cytokines [8-10].

To date, six isoforms of the leptin receptor are identified, including five short isoforms (namely LEPRa, LEPRc, LEPRd, LPERe and LEPRf) and one long isoform (LEPRb) [11]. All of the isoforms have an extracellular domain to link leptin, but only LEPRb has the complete intracellular domain required to activate the cellular signalling cascade of leptin. LEPRb belongs to the gp130 class I cytokine receptor family and is the main receptor implicated in leptin signalling in neurons. The highest expression of *LEPRb* in the brain is located in the hypothalamus, particularly in the arcuate nucleus (ARH) and ventromedial hypothalamus. However, LEPRb is also expressed in many extra-hypothalamic brain regions, such as the ventral tegmental area, hippocampus and brainstem [12], as well as in peripheral tissues [13].

After crossing the blood-brain barrier (BBB) through a receptor-mediated process, leptin directly targets two different neuronal populations in the arcuate nucleus, one co-expressing the proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) and the other co-expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY) [14]. Leptin stimulates *Pomc/Cart* expression and inhibits *Agrp/Npy* expression, reducing food intake and increasing energy expenditure, which consequently decrease body weight. In addition, leptin also inhibits feeding by reducing the expression of the melanin-concentrating hormone (*Mch*) and orexins in the lateral hypothalamic area (LHA), as well as by enhancing

the expression of brain-derived neurotrophic factor and steroidogenic factor-1 (*Sf-1*) in the ventromedial hypothalamus (VMH) [15].

Leptin is also implicated in the regulation of other physiological functions, such as glucose and lipid metabolism, reproduction and sexual maturation, thermogenesis, heart rate and blood pressure, the hypothalamic-pituitary-adrenal system, neuroendocrine and neuroprotection functions, thyroid and growth hormones, angiogenesis and platelet-aggregation, hematopoiesis, immune and pro-inflammatory responses and bone remodeling.

3. Molecular leptin signalling

When leptin interacts with LEPRb, the conformational change and dimerization induced in the receptor promote the activation of Janus kinase 2 (JAK2) and its auto-phosphorylation. Moreover, JAK2 phosphorylates three tyrosine (Tyr) residues, which include Tyr985, Tyr1077 and Tyr1138, in the cytoplasmic domain of the receptor, activating it and initiating different intracellular signalling pathways.

Phosphorylated Tyr1138 (pY1138) recruits the signal transducer and activator of transcription 3 (a transcription factor, STAT3), which also becomes phosphorylated [16]. Subsequently, STAT3 dimerizes and translocates from the cytoplasm into the nucleus, where it binds to POMC and AgRP promoters, stimulating *Pomc* expression and inhibiting *Agrp* [17]. This is what is known as the JAK2/STAT3 signalling pathway, which is regulated by both positive and negative regulators. The suppressor of cytokine signalling protein 3 (SOCS3) and protein tyrosine phosphatase 1B

(PTP1B) act as feedback inhibitors of leptin signalling by binding to pY985 and preventing the activation of the JAK2/STAT3 pathway [18,19]. However, the Src-homology 2 domain 1 (SH2B1) markedly enhances JAK2 activity, which is conducive to the activation of this signalling pathway [20].

In addition to the JAK2/STAT3 signalling pathway, the activation of LEPRb also activates the extracellular signal-regulated kinase (ERK) and phosphoinositide-3-kinase (PI3K) pathways (**Figure 1**). The ERK pathway is activated through the recruitment of the protein tyrosine phosphatase non-receptor type 11 (PTP11 or also called SHP2) to pY985 of LEPRb [21], whereas the activation of the PI3K pathway is mediated by the phosphorylation of insulin receptor substrate 2 (IRS2) [22]. The PI3K pathway also affects the neuronal activity and neuropeptide release of AgRP and POMC neurons [22]. Notably, there is evidence that this pathway inhibits PTP1B and forkhead box protein O1 (FOXO1). FOXO1 stimulates the expression of *Npy* and *Agrp*, inhibits the expression of *Pomc*, and blocks STAT3 action in these neurons; therefore, the inactivation of FOXO1 via PI3K allows STAT3 to bind to POMC and AgRP promoters [23].

INTRODUCTION

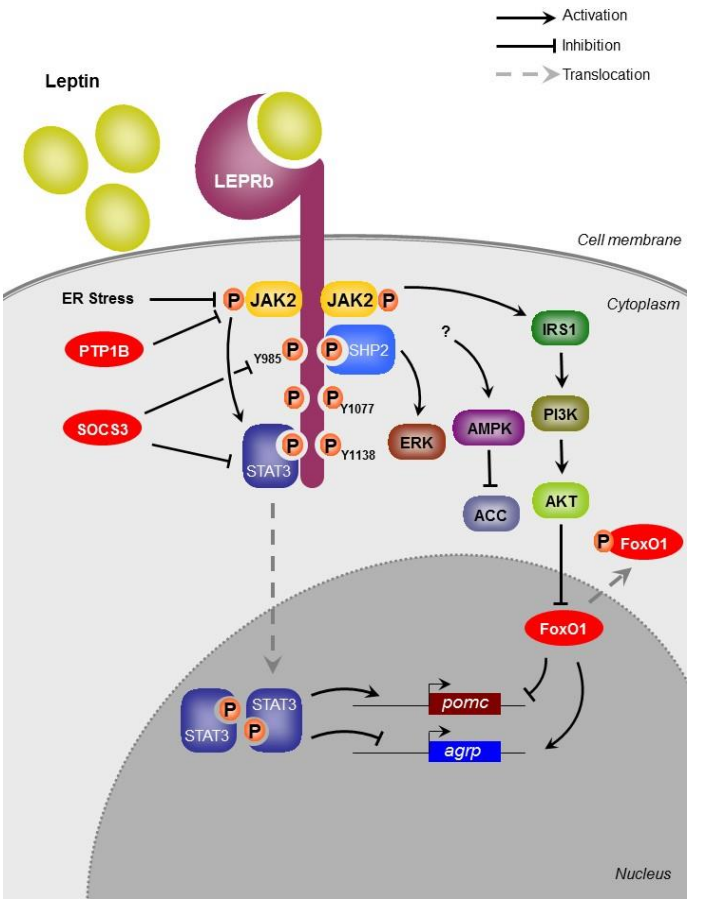

Figure 1. Schematic representation of the main components and regulators of leptin pathway targeted by food compounds. Leptin signalling pathway is modulated by the effect of several food compounds on targets from different upstream and downstream levels.

Finally, the AMP-activated protein kinase (AMPK) and mechanistic target of rapamycin (mTOR) pathways are also involved in the regulation of leptin signals. Specifically, leptin inhibits AMPK in several hypothalamic regions stimulating hypothalamic acetyl-CoA carboxylase (ACC) action, consequently decreasing food intake and body weight [24].

4. Mechanisms of leptin resistance in obesity

As previously explained, the term leptin resistance is commonly used to define states of obesity in which hyperleptinemia coexists with a decreased responsiveness to leptin administration. Although the exact mechanisms that lead to leptin resistance are still unclear, some have been proposed (**Figure 2**), including impaired leptin transport across the BBB and the disruption of the leptin signalling cascade within neurons from specific brain areas. Besides, hypothalamic inflammation, endoplasmic reticulum (ER) stress and loss of sirtuin 1 (SIRT1) activity also promote leptin resistance.

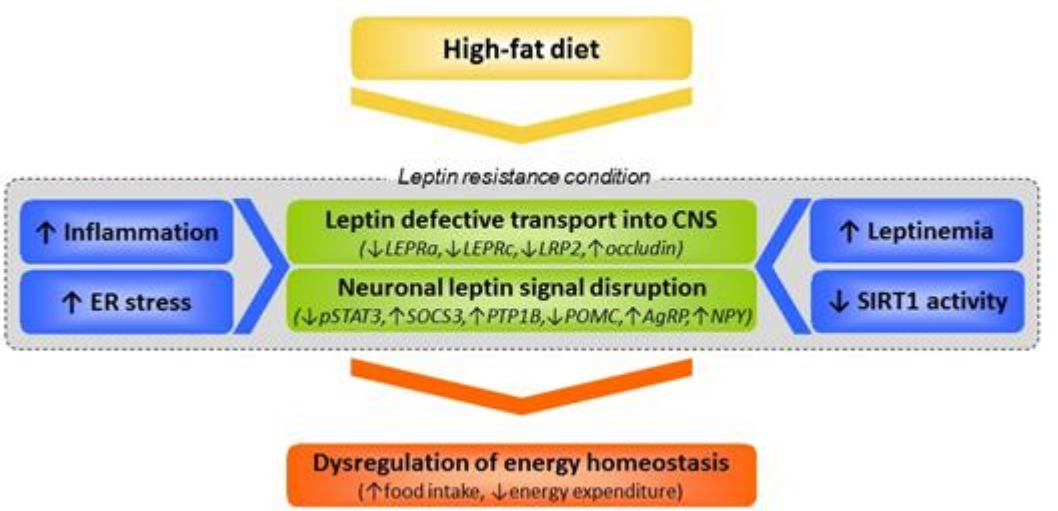

Figure 2. Schematic diagram for molecular mechanisms of leptin resistance. The molecular basis for leptin resistance is not yet completely understood but it have been mainly attributed to several mechanisms. These include reduced transport across the blood-brain barrier and the enhancement of intracellular processes that attenuate cellular signalling such as inflammation, endoplasmic reticulum stress, hyperlepinemia and sirtuin 1 dysfunctionality. CNS: central nervous system; ER: endoplasmic reticulum; SIRT1: sirtuin 1.

4.1 Defective leptin transport across the blood-brain barrier (BBB)

Most known functions of leptin within the central nervous system (CNS) are presumably mediated by leptin produced in the periphery. Therefore, leptin must be transported across the BBB. Several studies have shown that this is most likely conducted using a specific and saturable transport system that is located at both the endothelium of the cerebral microvessels and the epithelium of the choroid plexus [25]. LEPRa and LEPRc short form leptin receptor isoforms have both been shown to be highly expressed in the microvessels of the brain and are suggested to facilitate the BBB transport of leptin [26]. Furthermore, low density lipoprotein-related protein 2 (LRP2), also known as megalin, has been identified as potential novel leptin transporter protein at the choroid plexus epithelium. It functions by binding circulating leptin and transporting the hormone into the CNS [27].

Several reports in obese humans and rodents, in which the leptin levels in the cerebrospinal fluid are significantly decreased compared with control group, have suggested that leptin resistance is associated with a defect in the transport of leptin through the BBB [28]. From these studies some hypotheses have been proposed as causes of this defective transport of leptin. For example, polyunsaturated fatty acids have been reported to induce peripheral leptin resistance via an increase in the expression of hypothalamic *occludin*, one of the main proteins of the tight junctions, reducing paracellular transport of leptin into the brain [29]. Alternatively, an impaired expression of the transporters *LEPRa* and *LEPRc* at the BBB has also been suggested,

but this aspect is quite controversial because some studies have suggested that a decreased capacity of the transporter to bind and transport leptin into the brain is the major cause of the defective leptin transport [30,31]. Additionally, some studies indicate that high levels of both triglycerides and protein C reactive also reduce leptin transport across the BBB [29]; therefore, in states of hypertriglyceridemia and/or inflammation, such as obesity or starvation, decreased leptin transport into the CNS is expected.

4.2 Attenuation of the leptin signalling cascade in the hypothalamus

Another mechanism involved in the development of leptin resistance is the disruption of LEPRb signalling in the hypothalamus. In fact, this mechanism has been considered to be one of the leading factors and the primary defect that induces central leptin resistance. This loss of leptin signalling is mainly due to two parallel molecular mechanisms, including the up-regulation of SOCS3 and PTP1B.

As explained before, SOCS3 is a key protein that inhibits the signal transduction process of various cytokines in the body, including leptin. By binding to Tyr985 of LEPRb and JAK2, SOCS3 inhibits the leptin-induced phosphorylation of STAT3 through a negative feedback mechanism [18]. In this sense, the incidence of diet-induced obesity (DIO) and leptin resistance is significantly decreased in rats with brain-specific deletion of SOCS3 [32]. Moreover, PTP1B is a non-receptor protein tyrosine phosphatase located in the cytoplasmic face of the endoplasmic reticulum in the hypothalamic regions enriched with leptin-responsive neurons [19]. PTP1B

dephosphorylates JAK2, thereby inhibiting leptin signalling. The expression of *Ptp1b* is increased by high-fat feeding and inflammation, suggesting that PTP1B may play a crucial role in the etiology of leptin resistance [33]. Additionally, brain-specific deletions of PTP1B improve leptin sensitivity and offer protection from obesity [34]. Thus, as important targets to increase leptin sensitivity and improve obesity, SOCS3 and PTP1B have emerged as their inhibition may facilitate this process.

In addition to the JAK2/STAT3 signalling cascade, other signalling pathways such as the PI3K/Akt and AMPK pathways, as mentioned above, jointly participate in the LEPRb signal transduction. Consequently, the study of regulators in these secondary pathways, including the phosphatase tensin homolog deleted on chromosome 10 (PTEN) and ACC, among others, can potentially also provide new targets for the management of leptin resistance.

4.3 Hypothalamic inflammation

Most evidence indicates that inflammation, both in peripheral tissues and the hypothalamus, is a cause of the development of leptin resistance in obesity. Specifically, the inflammatory pathway IκB kinase-β/nuclear factor-κB (IKKβ/NF-κB) is activated in the hypothalamus of rodents fed a high-fat diet (HFD) [35]. Notably, genetic inhibition of this pathway in the neurons of the ARH [36] or AgRP neuron-specific deletion of IKKβ [37] protects from HFD-induced obesity, enhances leptin signalling and reduces *Socs3* expression. Remarkably, the promoter of SOCS3

has two putative motifs for binding NF-κB [37], thus connecting hypothalamic inflammation with leptin resistance.

Toll-like receptor 4 (TLR4), a membrane receptor that functions in the innate immune system, is activated by saturated fatty acids in the hypothalamus, triggering the IKKβ/NF-κB pathway [38]. Moreover, genetic and pharmacological inhibition of TLR4 restored leptin signalling in rodents fed a HFD [38]. Therefore, TLR4 is proposed to act upstream from IKKβ/NF-κB in the inflammatory process induced by a HFD in the hypothalamus.

Besides IKKβ/NF-κB pathway, c-Jun N-terminal kinase (JNK) is another pro-inflammatory signalling component up-regulated in the ARH of murine fed a HFD [35]. However, the actual implication of JNK on inducing leptin resistance and obesity is controversial.

Recently, 15-deoxy-D12,14-prostaglandin J2 (15d-PGJ2), which regulates key aspects of immunity, has been also involved in the development of leptin resistance [39]. Specifically, it has been described that 15d-PGJ2 inhibits the leptin-induced phosphorilation of STAT3 *in vitro* and the leptin-induced anorexia *in vivo* [39].

In addition to leptin signalling dysfunction, inflammatory processes are also responsible for structural changes in the hypothalamus, which alter the hypothalamic circuits. Interestingly, neuronal injury has been observed in specific brain areas that regulate food intake in obese humans and rodents [40].

4.4 Endoplasmic reticulum stress

The transmembrane proteins are synthesized and folded by ER in order to form active proteins in the ER lumen. Miss-folded and un-folded proteins are eliminated via proteasome complex. An imbalance in this process causes an accumulation of defective proteins in ER lumen, forming ER stress and, consequently, the induction of unfolded protein response (UPR) [41]. If there is a short-term ER stress, the UPR restores the ER homeostasis reducing the protein synthesis, increasing their folding capacity and degrading the miss- and un-folded proteins [41].

Notably, ER stress has also been suggested to be an inducer of leptin resistance. In this sense, long-term ER stress inhibits the leptin signalling pathway, leading to hyperleptinemia and obesity [37], and several studies have shown that obese animals display significant ER stress in multiple tissues (i.e., liver, adipose, and brain tissues) [42,43]. Therefore, the inhibition of ER stress increases leptin sensitivity and reduces food intake and body weight [44]. At molecular level, hypothalamic ER stress results in decreased post-translational conversion of POMC to α-melanocyte-stimulating hormone (α-MSH) in HFD fed rats [45], thus connecting ER stress and leptin resistance.

The pro-inflammatory IKKβ/NF-κB pathway may act both upstream and downstream from ER stress and experimental evidences show that hypothalamic IKKβ/NF-κB pathway and ER stress positively feedback each other under conditions of overnutrition [35] further worsening leptin resistance.

4.5 Sirtuin 1 activity

Recently, a reduced activity of SIRT1, which is a NAD^+-dependent protein deacetylase, has been implicated in the appearance of leptin resistance [46,47]. On the contrary, the activation of hypothalamic SIRT1 increases energy expenditure and reduces food intake [46]. SIRT1 can improve leptin sensitivity by decreasing the levels of PTP1B [46], SOCS3 [46] and FOXO1 [48]. Moreover, SIRT1 activation reduces inflammation [49] and ER stress [50], two dysfunctions that also induce leptin resistance. Therefore, SIRT1 appears as a new promising target to improve leptin resistance.

5. Food compounds useful for counteracting leptin resistance

Several nutrients and food components with the ability to reverse leptin resistance have been described. This review compiles food compounds that are able to reduce hyperleptinemia, promote leptin transport across the BBB or modulate leptin cascade in the hypothalamus. However, food compounds able to reduce inflammation and ER stress or increase SIRT1 activity, but without experimental evidence of promoting hypothalamic leptin sensitivity, are not included in this review.

5.1 Food compounds controlling circulating leptin levels

Hyperleptinemia is one of the characteristics of leptin resistance, and numerous studies have been conducted to identify compounds with anti-hyperleptinemic activity. It should be noted that most of these studies are performed in animal models,

using basically rats and mice. **Table 1** summarizes a list of food compounds and extracts that have shown the ability to decrease the levels of circulating leptin in *in vivo* studies.

Among the molecules listed in **Table 1** numerous phenolic compounds can be found. This group of compounds, which is widely distributed in fruits and vegetables, has been shown to reduce the level of circulating plasmatic leptin in a large range of *in vivo* studies, using different types of models and treatments. This capacity has been observed by both using pure phenolic compounds, such as resveratrol [51] and myricetin [52], as well as some precursors or derivatives of phenols, such as polydatin [53] and KMU-3 [54] (a synthetic derivative obtained from gallic acid). In addition, some polyphenolic rich-extracts, obtained from natural sources such pecans [55], brown algae [56] and peach and plum juices [57], have shown the same behavior. Among all of these studies carried out with phenolic compounds, it is worthwhile to highlight that some of them have been conducted in humans. For example, fraxin, a glucoside of an *o*-methylated coumarin, and curcumin have been confirmed to reduce hyperleptinemia in overweight and obese humans [58], thus emphasizing their suitability for use in the formulation of functional foods directed towards weight reduction. Taking into consideration all of these studies, it seems clear that phenolic compounds are a very interesting family of molecules for finding new molecules from natural sources that could be used to improve hyperleptinemia and leptin resistance.

In addition to phenolic compounds, other plant secondary metabolites, such isothiocyanates [59] and some terpenoids including thymol [60], saponins [61] and lycopenes [62] among others, have also been proven to be effective in reducing circulating leptin levels when administered to rodents. Furthermore, other compounds different from plant secondary metabolites have shown effects reversing leptin resistance. For example, some peptides [63] and protein hydrolysates [64], as well as polyunsaturated fatty acids (PUFAs), such as docosahexanoic acid (DHA) [65], eicosapentaenoic acid (EPA) [65] and conjugated linoleic acid (CLA) [66], also exhibit anti-hyperleptinemic action. Interestingly, some of them have been proven in humans. For example, this is the case for the CLA isomer t10c12-CLA, thus highlighting its suitability and potential to be used in treatments directed towards the reversal of leptin resistance [66].

Although there are several studies describing a reduction in the leptin level due to the effect of natural compounds, only some of them describe the mechanisms by which this reduction is produced. One of these mechanisms is through the repression of the leptin gene. Notably, cranberries [67] and KMU-3 [54] have the ability to repress *leptin* gene expression in the adipocyte cell line 3T3 L1 (**Table 1**). However, to confirm the contribution of leptin gene repression on the anti-hyperleptinemic effect of a food component, it would be necessary to determine whether the expression of the *leptin* gene in WAT correlates with the reduction in the leptin level induced by a specific food compound in *in vivo* models. The CB1 cannabinoid receptor (CB1R) is the main CBR found in the brain, and it is present in endocrine cells and other

peripheral tissues [68], such as pancreas, fat, liver and skeletal muscle tissues [69]. It is known that the use of brain-penetrating CB1R antagonists can cause neuropsychiatric side effects, but a selective targeting of peripheral CB1R results in an improved hormonal-metabolic profile without the observation of the brain secondary consequences [69]. The antagonistic action of CB1R causes an increase in appetite, insulin resistance and an increase in the hepatic lipogenesis, suggesting its implication in obesity [69]. Therefore, there are synthetic CB1R antagonists that increase the leptin sensitivity [69] or CB1R inverse agonists that reverse the leptin resistance, decreasing *leptin* expression and secretion by adipocytes and increasing leptin clearance via the kidney [70]. Although, to date, no natural compounds have been described as CB1R antagonists, the search and use of molecules from natural sources that antagonize this target could be one mechanism to reverse leptin resistance.

As indicated above, many food compounds have been described as anti-hyperleptinemic. However, food compounds could improve leptin sensitivity by a direct action in the brain, targeting hypothalamic leptin signalling and leptin transport across the BBB, or either secondary to body weight reduction, as a result of targeting peripheral tissues such as liver and adipose tissue. In this sense, serum leptin levels strongly correlates with the reduction of body weight induced by several polyphenols in rats fed a HFD [71]. Therefore, this review focus on food compounds that target hypothalamic leptin signalling and leptin transport across the BBB.

INTRODUCTION

Table 1. Food compounds that reduce hyperleptinemia.

Class	Compound/s	Dietary source	Experimental model	Reference
Phenolic compounds	Resveratrol	Grapes, red wine	Wistar rats	[51]
	O-coumaric acid	Vinegar	Male Wistar rats	[106]
	7-*O*-galloyl-D-sedoheptulose	*Cornifructus*	*db/db* mice	[107]
	Neohesperidin	Citrus	Male KKAyand C57BL/6 mice	[108]
	Polydatin	*Polygonum cuspidatum*	Male Sprague Dawley rats	[53]
	Myricetin	Vegetables, fruits, nuts, berries, tea, red wine	Male C57BL/6J mice	[52]
	Fraxin	*Fraxinus sp*	Elderly overweight/obese human	[58]
	Gingerol	Ginger	Male Wistar rats	[109]
	Polyphenol-rich extracts	Pecan nut	Male Wistar rats	[55]
		Brown algae	High-fat diet induced obese mice	[56]
		Peach and plum juice	Zucker rats	[57]
		Zygophyllum album	Female Wistar rats	[110]

Terpenes	Thymol	Thyme	High-fat diet-induced obese mice	[60]
	Lycopene	Tomatoes, watermelon, papaya, orange	Male Wistar rats	[62]
	Teasaponin	Tea	Male C57Bl/6J mice	[61]
	Ginsenoside Rb1	Ginseng	Male C57Bl/6 mice	[87]
PUFAs	DHA, EPA	Fish oils, golden algae oil	Male C57BL/6J mice	[55]
	CLA	Beef, lamb, dairy foods	Human and mice	[66]
Soluble fiber	Pectin	Fruits	Male Wistar rats	[95]
Other	Protein lysates	Rice bran	A high carbohydrate diet-induced obese rats	[64]
	Isothiocyanates	*Moringa oleifera*	Male C57BL/6J mice	[59]
	Mate aqueous solution	*Ilex paraguariensis*	Male Wistar rats weaned prematurely	[101]

Abbreviations: CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFAs: polyunsaturated fatty acids.

5.2 Food compounds that modulate leptin transport across the brain blood barrier

As stated in previous sections, leptin resistance can be the consequence of the impairment of its transport across the BBB, reducing leptin accessible to the CNS. Thus, increasing leptin transport across the BBB could be a good strategy to increase central leptin sensitivity. Despite that several transporters have been described, most studies have focused on the capacity of food compounds to increase the expression of the transporter *megalin*. Some of these compounds are listed in **Table 2**. Notably, increased bile acid production due to a lithogenic diet [72] produce an overexpression of *megalin* in mice, whereas vitamin A and vitamin D [73] are effective in producing the overexpression of *megalin* in several cell lines. Interestingly, synthetic peroxisome proliferator-activated receptor (PPARα and PPARγ) agonists induce the expression of *megalin* [74]. Therefore, it can be hypothesized that food components that could act as PPAR agonists, such as PUFAs [75], coumarins [76], flavonoids [77] or even polyphenol rich extracts from fruit juices [57], could be good candidates to improve leptin transport across the BBB.

In addition to megalin, other proteins can modulate the transport of leptin across the BBB. In this sense, clusterin (also called ApoJ) is a plasma leptin-binding protein for which megalin has been identified as an endocytic receptor [78]. Additionally, clusterin modulates leptin signalling in cell lines expressing LEPRb [78]. Therefore, increasing the expression and/or the activity of clusterin could be a good way to

increase leptin interactions with LEPRb and megalin. Notably, the coumarin fraxin upregulates *clusterin* gene expression in cell lines [79]. As an overexpression of *clusterin* induces the overexpression of *megalin* [80], fraxin is considered a potential stimulator of leptin transport across the BBB.

It is also important to highlight the findings obtained by Indra *et al*. [81], in which the administration of quercetin, a flavonol found in many fruits, produced an overexpression of *LEPRa* in HUVEC cells. This finding is very important due to the involvement of this receptor in the transport of leptin through the BBB. However, as quercetin is actively sulfated and glucoronidated upon intestinal uptake, further experiments should be carried out using these quercetin metabolites in this cell line to obtain stronger conclusions.

Table 2. Food compounds that modulate leptin transport across the brain blood barrier.

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Reference
Phenolic compounds	Fraxin	*Fraxinus sp*	Upregulates *clusterin* gene expression	HUVEC cells	[79]
	Quercetin	Onion, broccoli	Increases LEPRa protein levels	HUVEC cells	[81]
Vitamins	Retinoic acid	Sweet potatoes, carrot	Upregulates *Lpr2* gene expression	Male C57BL/6J mice	[73]
	Cholecalciferol	Fish liver oils, fatty fish species, beef liver			
Bile acids	Cholic acid	Lithogenic diets	Upregulates *Lpr2* gene expression	RPT, JEG-3 and EC-F9 cells	[72]
	Chenocholic acid				

Abbreviations: LEPRa, leptin receptor isoform a; LPR2, low density lipoprotein-related protein 2 also known as megalin.

5.3 Food compounds enhancing leptin signalling in the hypothalamus

Several food compounds, most of them from vegetal sources, have the capacity of reaching the hypothalamus, where they regulate leptin signalling pathways. These food compounds can either cross the BBB or pass through fenestrated capillaries of circumventricular organs (CVO) and medial eminence (ME) and target ARH neurons, which express LEPR [82], i.e., POMC/CART and AgRP/NPY neurons, where they can act at different levels. Some compounds improve central leptin sensitivity by increasing the expression of *LEPRb*, the phosphorylation of STAT3 and the expression of downstream components, mainly neuropeptides [83]. Additionally, some food compounds target the negative regulators of the leptin cascade, such as SOCS3 and PTP1B, reduce ER stress and/or modulate other leptin cascades, such as PI3K/Akt [84]. Food components that modulate leptin signalling activity are summarized in **Table 3**.

Table 3. Food compounds involved in the modulation of the leptin signalling cascade.

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Tissue	Reference
Phenolic compounds	Curcumin	Turmeric	Reduces LEPRb phosphorylation Reduces STAT3 phosphorylation	Male Sprague Dawley rats	Hepatic stellate cells	[111]
	Resveratrol	Grapes, red wine	Increases STAT3 phosphorylation	Male Wistar rats	Hypothalamus	[83]
			Increases AMPK phosphorylation	Male Wistar rats	Liver, muscle	[94]
	Fucoxanthin	Brown algae	Increases AMPK phosphorylation	Male C57BL/6J mice	eWAT	[96]
Terpenes	Teasaponin	Tea	Increases STAT3 phosphorylation Inhibits SOCS3 activity	Male C57BL/6J mice	Hypothalamus	[61]
	Ginsenoside Rb1	Ginseng	Increases STAT3 phosphorylation Inhibits SOCS3 activity	Males C57Bl/6 mice	Hypothalamus	[87]
			Increases Akt phosphorylation	Male Long-Evans rats	Hypothalamus	[93]
	Celastrol	*Tripterygium wilfordii*	Increases STAT3 phosphorylation	Male C57BL/6J mice	Hypothalamus	[88]

		Celastrus regelii	Upregulates *Socs3* mRNA levels Reduces ER stress			
Soluble fibre	Pectin	Fruits	Increases STAT3 phosphorylation Increases AMPK phosphorylation	Male Wistar rats	Liver, eWAT	[95]
Plant extracts	Triterpenoids	*Schisandra chinensis*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[112]
	Phenolic compounds	*Cyclocarya paliurus*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[113]
	Xanthones and fllavonoids	*Cudrania tricuspidata*	Inhibits PTPB1 activity	Cell-free bioactivity assay	-	[114]
Others	Caffeine	Coffee beans, tea bush, kola nuts	Increases STAT3 phosphorylation Reduces ER stress	SH-SY5Y-Ob-Rb cells	Liver, eWAT	[89]
	Leucine	Soybeans, beef	Increases LEPRb phosphorylation Increases STAT3 phosphorylation Inhibits SOCS3 activity	Male Sprague-Dawley rats	Hypothalamus prWAT	[115]

Taurine	Shellfish, turkey dark meat	Increases STAT3 phosphorylation Reduces ER stress	Male C57Bl/6 mice	Hypothalamus	[90]
Safranal	Saffron	Inhibits PTPB1 activity	Male C57BL/6J mice	C2C12 myoblast	[116]

Abbreviations: AMPK, 5' adenosine monophosphate-activated protein kinase; Akt, protein kinase B; ER, endoplasmic reticulum; eWAT, epididymal white adipose tissue; LEPRb, leptin receptor isoform b; prWAT, perirenal white adipose tissue; PTP1B, protein-tyrosine phosphatase 1B; SOCS3, suppressor of cytokine signalling 3; STAT3, signal transducer and activator of transcription 3.

To the best of our knowledge, only leucine has been described as a food component that increases the expression of *LEPRb* [85]. Currently, the best marker of leptin signalling activity is pSTAT3 which is the transcription factor that mediates leptin anorexigenic actions [86]. Notably, several food compounds increase the level of pSTAT3 in the hypothalamus of rodents fed a HFD, such as teasaponin [61] and ginsenoside Rb1 (the main bioactive compound of ginseng) [87], which also inhibit SOCS3, thus increasing leptin sensitivity. Interestingly, resveratrol increases pSTAT3 levels in the hypothalamus concomitantly with a decreased adiposity [51]. Other compounds, such as celastrol (a pentacyclic triterpene extracted from the roots of thunder god vine) [88], caffeine [89] and taurine [90] are also STAT3 activators, and their actions seem to be mediated through the decline of ER stress in the hypothalamus.

Other studies have focused instead on negative factors that attenuate leptin receptor signalling, such as PTP1B. However, only *in vitro* studies had found potential natural compounds that would inhibit this target [91]. Therefore, the capacities of these compounds to increase leptin sensitivity in the hypothalamus are speculative and more research is needed to confirm these findings.

Current studies have shown that the activation of PIK3/Akt and AMPK pathways is essential to maintain energy homeostasis, as they are involved in the anorexigenic effect of leptin [92]. Notably, ginsenoside Rb1 activates Akt in both the hypothalamus of obese rats and in hypothalamic cell lines [93]. Moreover, it has been hypothesized

that SIRT1 activation would inhibit proteins involved in leptin resistance, thus reducing ER stress [47]. Therefore, compounds that either directly or indirectly activate SIRT1, such as resveratrol, could increase central leptin sensitivity [94].

Other studies have focused on the capacity of several food compounds, which lower body weight and visceral fat mass, to modulate leptin signalling in peripheral tissue. For example, it has been confirmed that pectin [95] and fuxocanthin [96] increase pSTAT3 levels and AMPK activity in adipocytes, thus relating it with the reversal of leptin resistance.

First order neurons POMC and AgRP, will project to second order neurons in the paraventricular nucleus (PVN), VMH, dorsomedial hypothalamus (DMH) and LHA, where they produce the anorexigenic and orexigenic effects, respectively [97]. Leptin anorectic effects are mediated by CART and POMC neurons. The later produces α-MSH peptide, which binds to the melanocortin-3/4 receptor (MC3/4R) in second order neurons and will inhibit food intake [98,99]. Many natural compounds are able to increase either POMC or CART levels. In this sense, apigenin [100], ginsenoside Rb1 [87], teasaponin [61], taurine [90], leucine [85] and yerba mate extracts [101] have been shown to increase the expression of *Pomc* in the hypothalamus and to reduce food intake and body weight (**Table 4**). Moreover, some of these compounds, including resveratrol [102], ginsenoside Rb1 [87] and taurine [90], are also able to inhibit the orexigenic neuropeptides AgRP and NPY, suggesting an increased effectiveness to modulate food intake and energy expenditure. However, celastrol,

which reduces body weight in DIO mice, increases *Agrp* mRNA levels [88]. Therefore, more studies are necessary to thoroughly understand this mechanism.

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Table 4. Food compounds targeting neuropeptides that regulate energy homeostasis.

Class	Compound/s	Dietary source	Molecular mechanism/s and targets	Experimental model	Reference
Phenolic compounds	Apigenin	Fruits, vegetables	Upregulates *Pomc* mRNA levels Upregulates *Cart* mRNA levels	N29-2 and SH-SY-5Y neuronal cells	[100]
	Resveratrol	Grapes, red wine	Downregulates *Agrp* mRNA levels Increases NPY protein expression	N29-4hypothalamic cells	[51]
Terpenes	Teasaponin	Tea	Upregulates *Pomc* mRNA levels	Male C57Bl/6 mice	[61]
	Ginsenoside Rb1	Ginseng	Downregulates *Agrp* mRNA levels Upregulates *Pomc* mRNA levels	Male C57Bl/6 mice	[87]
			Increases NPY protein expression Upregulates *Pomc* mRNA levels	Male Long-Evans male rats	[93]
	Celastrol	*Tripterygium wilfordii* *Celastrus regelii*	Upregulates *Agrp* mRNA levels	Male C57Bl/6 mice	[88]

	Retinoic acid	Sweet potatoes, carrot	Increases methylation levels in the POMC gene	Leukocytes of obese men	[117]
Others	Taurine	Shellfish, turkey dark meat	Increases NPY protein expression Upregulates *Pomc* mRNA levels Upregulates *Cart* mRNA levels	Male C57Bl/6 mice	[90]

Abbreviations: AgRP, Agouti-related protein; CART, cocaine-and amphetamine-regulate transcript; NPY, neuropeptide Y; POMC, proopiomelanocortin.

5.4 Food compounds against leptin resistance: a holistic overview

In the previous sections, food compounds have been listed according to the level where they improve leptin resistance. However, it is important to take into account that some of these compounds act at several levels of the leptin pathway. Thus, some food compounds stand out from the others. For example, resveratrol, a phenolic compound obtained from the skin of grapes, is able to reduce the circulating levels of leptin not only by reducing its secretion but also by promoting the activation of STAT3 in the hypothalamus, thereby increasing leptin sensitivity and down-regulating *Agrp* and NPY. Other compounds that can be highlighted are teasaponin and ginsensoside, which reduce hyperleptinemia, activate hypothalamic STAT3, increase *Pomc* mRNA levels, and inhibit SOCS3. In addition, ginsensoside increases pFOXO1 and inhibits PTP1B.

It is also worthwile to take into account that some food compounds also modulate proinflammatory cytokines. These cytokines contribute to the development of leptin resistance, and some food compounds reduce proinflammatory cytokines, such as teasaponin and ginsensoside.

Therefore, the selection of a compound taking into consideration its influence within the entire set of mechanisms involved in the onset of leptin resistance is the best way to assure its functionality. **Figure 1** shows the leptin pathway components and regulators targeted by food compounds.

6. Methods for the identification of new natural compounds with anti-leptin resistance activities.

To deeply study the mechanisms involved in the development of leptin resistance and its reversal using natural compounds, several strategies have been followed by researchers in the last two decades. These strategies involve a broad variety of techniques, ranging from the more theoretical ones, which are based in the use of bioinformatics-aided tools, to biological studies that evaluate the *in vivo* response in different models.

Traditionally, both *in vitro* cellular models and *in vivo* studies have been used to study leptin resistance. Focusing on cell models, different points of view can be followed. For example, it is very important to evaluate if the compounds being studied are able to cross the BBB and reach the selected targets. To do this, a specific cellular model using endothelial cells should be used. Until now, several endothelial cell lines are described in the bibliography for the simulation of the BBB, such as RBEC1, GP8/3.9, GPNT and RBE4 [103]. Other studies are focused on the evaluation of the signalling cascade pathway. These studies are carried out using astrocyte cultures and neuronal cell lines.

Regarding the *in vivo* studies aimed to evaluate leptin resistance, several models of metabolic syndrome using animals with a non-functional leptin pathway have been used. In general, these animals can be classified into two groups: genetically altered or diet-induced altered animals. In the first group, all of the animals (in general mice

and rats) that present a modification in any of the genes involved in the onset of leptin resistance can be included. These modifications can be either from natural origin or generated in the laboratory, including knock-out animals that had a deficiency in the leptin receptor or animals that do not produce leptin (*ob/ob* mice, *db/db* mice, etc.). In the other group, animals in which leptin resistance originates by means of the nutritional composition of the diet are included. For example, a HFD produces the onset of leptin resistance in animals. This last group of animal models is very interesting to simulate the most typical situation that originates leptin resistance in humans.

The most novel approach to study leptin resistance is the so-called *in silico* strategy, which is based in the use of bioinformatics. This set of techniques is focused on the search through the virtual screening of new potent molecules from natural products with the capacity to directly act in one of the mechanisms that is implicated in leptin resistance, including molecules that have the capacity to directly bind to therapeutic targets and consequently either inhibit or activate them. To carry out these *in silico* studies and successfully do the virtual screening, some prerequisites have to be accomplished. For example, the existence of crystallized structures within databases is necessary to know their 3-D conformations, which is essential in this process. The existence of ligands described for the selected targets is also required to complete the design of the pharmacophore, which contains the information of the biological conformation and electrostatic features that the ligands should have to interact with the binding site of the target proteins. To date, some bioinformatics results focusing

on the reversal of leptin resistance have been carried out. For example some papers have reported molecules with the ability to inhibit PTP1B [104,105], whereas no results against SOCS3 and SH2B1 have been found. Following this virtual screening, the selected set of molecules that fulfil the prerequisites should be used in an *in vitro* assay to confirm the theoretical activity described.

Taking into consideration this wide spectrum of strategies, the most logical sequence of action in the search for compounds that could revert leptin resistance is as follows: start with *in silico* studies to select potentially active food compounds, test the molecules selected in the virtual screening *in vitro* by using several cellular models, and finally test the most actives ones in *in vivo* conditions, first in animals models and then in humans, to determine their efficacies.

7. Concluding remarks

Leptin resistance is commonly used to define states of obesity in which hyperleptinemia coexists with a decreased responsiveness to leptin administration. Notably, numerous food components, mainly polyphenols, are able to reduce hyperleptinemia, suggesting that these compounds could improve leptin resistance. However, only a few studies have focused on the mechanism by which these food components could primarily restore leptin sensitivity. The results of these studies indicate that food components can reverse leptin resistance by increasing leptin access to the brain and/or activating the intracellular signalling cascade of leptin in the hypothalamus. Nevertheless, the conclusions that could be extracted from these

studies are limited because they focus only on one of the levels of leptin resistance. Thus, new studies considering the activity of a particular food compound at all levels of leptin resistance and using validated markers of leptin sensitivity, such as pSTAT3, are indispensable to clearly ascribe the property of leptin sensitizer.

8. Acknowledgments

This work was supported by grant number AGL2013-40707-R from the Spanish Government.

The authors declare no conflict of interest.

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II. HYPOTHESIS AND OBJECTIVES

II. HYPOTHESIS AND OBJECTIVES

This PhD thesis has been performed in the Nutrigenomics Research Group of the Universitat Rovira i Virgili, within the frame of one research project carried out in this group: "Development of an integrated food to maintain body weight and to prevent the risk of obesity related pathologies (AGL2013-40707-R)".

Obesity is one of the most dominant diseases in our present society [2]. Although leptin is a key hormone that plays an essential role in food intake and energy metabolism, it is well stablished that high circulating levels appear in obesity [22,106]. This is partially explained by an imbalance in the energy homeostasis in overweight people due to the impaired action of this adypokine [4].

This is the phenomenon called leptin resistance (LR) and it can appear due to many factors. The main events that can promote LR are: (**1**) the disruption leptin transport across the blood-brain barrier (BBB), (**2**) a down-regulation of the leptin signalling pathway in the hypothalamus, (**3**) inflammation in the hypothalamus, which is closely related with obesity, (**4**) endoplasmatic reticulum (ER) stress and (**5**) a low sirtuin 1 (SIRT1) activity [263].

It is known that some food components are able to modulate the leptin signalling pathway, thus reverting LR. Amongst them, phenolic compounds, a group of secondary plant metabolites, stand out due to their wide range of beneficial effects. In this sense, the Nutrigenomics Research Group has a long history working with phenolic compounds, some of them directly related with obesity prevention and

HYPOTHESIS AND OBJECTIVES

modulation of the leptin signalling pathway. Especially the group has deep experience working with a grape seed proanthocyanidin extract (GSPE).

This thesis was raised from two previous investigations using GSPE that reached the following conclusions: (**1**) GSPE increases the hypothalamic leptin signalling pathway and proopiomelanocortin (*Pomc*) gene expression in rats fed with high fat diet (HFD) [326] and (**2**) GSPE boosts hepatic nicotinamide adenine dinucleotide (NAD^+) metabolism and SIRT1 expression and activity in a dose-dependent manner in healthy rats. In addition, GSPE has the capacity to revert lipid accumulation in human hepatocarcinoma cell line (HepG2 cells) in a SIRT1-dependent manner (**Manuscript 5**) [327].

These results lead us to think that maybe some polyphenols could help to revert the LR acting in the central nervous system (CNS) but also in peripheral tissues and that they could exert this effect modulating SIRT1 activity. Therefore, **the hypothesis of the present doctoral thesis is that phenolic compounds, including resveratrol (RSV), could act reverting the LR caused by obesity in specific peripheral tissues (liver, skeletal muscle and epididymal white adipose tissue (eWAT)) in a SIRT1-dependent manner**. In addition, as mentioned previously, the impaired leptin transport through the BBB is another cause of LR. Hence, **we postulate that polyphenols could also exert beneficial effects reverting LR due to they increase the leptin transport into the brain via their ability to potentiate the expression of leptin receptors (*ObR*s) in the BBB.**

HYPOTHESIS AND OBJECTIVES

This hypothesis is reinforced by the fact that there are a lot of polyphenols described by the bibliography that can help to modulate and counteract the different factors that can promote the LR. Most of these phenolic compounds are reviewed in the **Manuscript 1**, Modulation of leptin resistance by food compounds [263]. RSV is described as a reductive of hyperleptinemia [328] and it has also been seen that it increases the phosphorylation of signal transducer and activator of transcription 3 (STAT3) in the hypothalamus [329] and the levels of AMP-activated protein kinase (AMPK) in the liver and muscle [330]. Both effects were obtained in Wistar rats. In addition, RSV is described as a modulator of the neuropeptides involved in the regulation of energy homeostasis. Specifically, it down-regulates agouti-related peptide (*Agrp*) and increases neuropeptide Y (*Npy*) gene expression in N29-4 hypothalamic cells [328]. Additionally, other phenolic compounds such as fraxin [331] and quercetin [332] are described as a modulators of the leptin transport in human umbilical vein endothelial cells (HUVEC).

In order to confirm the postulated hypothesis three main objectives were defined:

1. To evaluate the general effect of RSV in reverting a condition of LR in cafeteria (CAF)-fed male Wistar rats and to determine the metabolites that have a major contribution in this beneficial effect (**Manuscript 2**).

For this purpose, we stablished different partial objectives:

HYPOTHESIS AND OBJECTIVES

• To assess how RSV, at different doses, reverts the LR caused by the CAF diet in peripheral organs (liver, skeletal muscle and eWAT) by modulating some molecules involved in the leptin signalling pathway.

• To stablish which metabolites of RSV are prevalent in the serum of these rats according to the dose and which ones are the cause of the improvement in those animals that are LR.

2. To study the mechanisms by which RSV modulates the LR in HepG2 cells (**Manuscript 3**).

For this purpose, we stablished different partial objectives:

• To stablish a LR-*in vitro* model in HepG2 cells evaluating if the ObRb/STAT3 cascade is activated by leptin.

• To determine if RSV can activate the leptin signalling pathway and revert the LR caused in this *in vitro* model.

• To determine the molecular mechanisms by which RSV can revert the LR in HepG2 cells.

3. To study the mechanisms by which different polyphenols could increase the leptin transport across the BBB and their capacity to revert the inflammation caused by pro-inflammatory cytokines in rat brain endothelial cells (RBEC) (**Manuscript 4**).

For this purpose, we defined the following partial objectives:

HYPOTHESIS AND OBJECTIVES

• To determine if the selected natural compounds improve the leptin transport through the BBB due to their capacity to increase the gene expression of leptin transporters in these cells.

• To create a model of obese-damaged BBB inducing inflammation by cytokines and to evaluate if phenolic compounds are able to revert this pathological condition.

The research work performed in this PhD has been supported by the grant AGL2013-40707-R from the Spanish government. This thesis (**Manuscripts 1, 2, 3** and **5**) was carried out in the Nutrigenomics Research Group laboratories of the Universitat Rovira i Virgili under Universitat Rovira i Virgili and FPU predoctoral fellowships (2015PMF-PIPF-60 and FPU14/01202, respectively) from the Spanish government. An international phase has been completed in the Biological Barriers Research Group in the Biological Research Centre, Hungarian Academy of Sciences (Szeged, Hungary) under the supervision of Dr. Mária Deli (**Manuscript 4**) to obtain the International Doctorate Mention. This phase was supported by the fellowship "Programa de movilidad internacional Erasmus+".

II. HIPÒTESI I OBJECTIUS

La present tesi doctoral s'ha realitzat en el Grup de Recerca en Nutrigenòmica de la Universitat Rovira i Virgili, en el marc d'un projecte d'investigació realitzat en aquest grup: "Desenvolupament d'un aliment integrat per mantenir el pes corporal i per prevenir el risc de patologies relacionades amb l'obesitat (AGL2013-40707-R)".

L'obesitat és una de les patologies predominants en la societat actual [2]. Tot i que la leptina és una hormona clau que juga un paper essencial en el consum d'aliments i el metabolisme energètic, es sap que, durant l'obesitat, hi ha elevats nivells d'aquesta en sang [22,106]. Aquest fet pot ésser explicat parcialment degut al desajust en l'homeòstasi energètica produïda en la gent amb sobrepès, el que comporta una alteració de la seva acció [4].

Aquest fenomen s'anomena resistència a la leptina (LR) i pot aparèixer com a conseqüència de diferents factors. Els principals promotors de la LR són: (**1**) una interrupció del transport de la leptina a través de la barrera hematoencefàlica (BBB), (**2**) una baixa regulació de la via de senyalització de la leptina en l'hipotàlem, (**3**) una inflamació en l'hipotàlem, estretament relacionada amb l'obesitat, (**4**) l'estrès de reticle endoplasmàtic (ER) i (**5**) una baixa activitat de l'enzim sirtuina 1 (SIRT1) [263].

Es sap que alguns components dels aliments són capaços de modular la via de senyalització de la leptina, revertint la LR. Entre ells trobem els compostos fenòlics,

un grup de metabòlits secundaris de les plantes, que destaquen degut al seu ampli espectre d'efectes beneficiosos. En aquest sentit, el Grup de Recerca en Nutrigenòmica disposa d'una llarga trajectòria treballant amb els compostos fenòlics, alguns d'ells directament relacionats amb la prevenció de l'obesitat i la modulació de la via de senyalització de la leptina. El grup té especial experiència en l'estudi de l'extracte de proantocianidines de la llavor del raïm (GSPE).

Aquesta tesis sorgeix a partir dels resultats obtinguts en dues investigacions prèvies en les que es feia servir el GSPE i a partir de les quals es va arribar a les següents conclusions: (**1**) el GSPE incrementa la via de senyalització de la leptina en l'hipotàlem i l'expressió gènica de proopiomelanocortina (*Pomc*) en rates alimentades amb una dieta rica en greixos (HFD) [326] i (**2**) el GSPE estimula el metabolisme hepàtic de la nicotinamida adenina dinucleòtid (NAD^+) i l'expressió gènica i l'activitat de la SIRT1 d'una manera dosi-depenent en rates sanes. Addicionalment, el GSPE té la capacitat de revertir l'acumulació lipídica en la línia cel·lular cancerígena humana d'hepatòcits (cèl·lules HepG2) d'una manera depenent de SIRT1 (**Manuscrit 5**) [327].

Aquests resultats ens van portar a pensar que potser alguns polifenols podrien ajudar a revertir la LR actuant a nivell de sistema nerviós central (CNS) però també en teixits perifèrics i que podrien realitzar-ho mitjançant una modulació de l'activitat SIRT1. Llavors, **la hipòtesi de la present tesi doctoral és que els compostos fenòlics, incloent el resveratrol (RSV), podrien revertir la LR causada per l'obesitat en**

teixits perifèrics específics (fetge, múscul esquelètic i teixit adipós blanc de l'epidídim (eWAT) de forma depenent de SIRT1). A més a més, com s'ha comentat anteriorment, el deteriorament en el transport de la leptina a través de la BBB és una altra causa de la LR. Per tant, **postulem que els polifenols podrien exercir efectes beneficiosos revertint la LR degut a la seva capacitat per incrementar el possible transport de la leptina cap al cervell a través de la seva habilitat per potenciar l'expressió dels receptors de la leptina (*ObR*s) en la BBB.**

Aquesta hipòtesi es veu reforçada per la informació trobada en la bibliografia que ens indica que hi ha molts polifenols que poden ajudar a modular i contrarestar els diferents factors que poden originar la LR. Molts d'aquests compostos fenòlics es troben en la revisió del **Manuscrit 1**, Modulació de la resistència a la leptina per compostos alimentaris [263]. El RSV està descrit com a reductor de la hiperleptinèmia [328] i també s'ha vist que incrementa la fosforilació del transductor de senyals i activador de transcripció 3 (STAT3) en l'hipotàlem [329] i els nivells de proteïna quinasa activada per AMP (AMPK) en el fetge i en el múscul [330]. Ambdós efectes es van obtenir en rates Wistar. Addicionalment, el RSV està descrit com a modulador dels neuropèptids implicats en la regulació de l'homeòstasi energètica. Específicament, regula a la baixa el pèptid relacionat amb agouti (*Agrp*) i incrementa l'expressió gènica del neuropèptid Y (*Npy*) en cèl·lules hipotalàmiques N29-4 [328]. A més a més, altres compostos fenòlics, com la fraxina [331] i la quercetina [332] estan descrits com a moduladors del transport de la leptina en cèl·lules endotelials humanes de cordó umbilical (HUVEC).

HIPÒTESI I OBJECTIUS

Per tal de confirmar la hipòtesi postulada, es van definir tres objectius principals:

1. Avaluar l'efecte general del RSV en la reversió d'una condició de LR en rates Wistar mascles alimentades amb una dieta de cafeteria (CAF) i determinar els metabòlits que tenen una major contribució al seu efecte beneficiós (**Manuscrit 2**).

Per a aquest propòsit, es van establir diferents objectius parcials:

• Avaluar com el RSV, a diferents dosis, reverteix la LR causada per una dieta de CAF en els òrgans perifèrics (fetge, múscul esquelètic i eWAT) a través de la modulació d'algunes de les molècules implicades en la via de senyalització de la leptina.

• Establir quins metabòlits del RSV són els majoritaris en el sèrum d'aquestes rates, depenent de la dosi, i quins són els causants de la millora en aquest animals amb LR.

2. Estudiar el mecanisme pel qual el RSV modula la LR en cèl·lules HepG2 (**Manuscrit 3**).

Per a aquest propòsit, es van establir diferents objectius parcials:

• Establir un model *in vitro* de LR en les cèl·lules HepG2 avaluant si la cascada ObRb/STAT3 està activada per la leptina.

• Determinar si el RSV pot activar la via de senyalització de la leptina i revertir la LR creada en aquest model *in vitro*.

HIPÒTESI I OBJECTIUS

• Determinar el mecanisme molecular a través del qual el RSV pot revertir la LR en les cèl·lules HepG2.

3. Estudiar els mecanismes a partir dels quals diferents polifenols podrien incrementar el transport de la leptina a través de la BBB i la seva capacitat per revertir la inflamació causada per citocines pro-inflamatòries en cèl·lules endotelials de cervell de rata (RBEC) (**Manuscrit 4**).

Per a aquest propòsit, es van establir diferents objectius parcials:

• Determinar si els compostos naturals seleccionats milloren el transport de la leptina a través de la BBB degut a la seva capacitat per incrementar l'expressió gènica dels transportadors de leptina en aquestes cèl·lules.

• Crear un model de BBB danyada en condicions d'obesitat induint inflamació amb citocines i avaluar si els compostos fenòlics són capaços de revertir aquesta condició patològica.

El treball d'investigació dut a terme en aquesta tesi doctoral ha estat finançat per la beca AGL2013-40707-R del govern d'Espanya. Aquesta tesi (**Manuscrits 1**, **2**, **3** i **5**) es va realitzar en els laboratoris del Grup de Recerca en Nutrigenòmica de la Universitat Rovira i Virgili sota la subvenció de les beques pre-doctorals de la Universitat Rovira i Virgili i FPU (2015PMF-PIPF-60 i FPU14/01202, respectivament) del govern d'Espanya. Es va dur a terme una estada internacional en el Grup de Recerca en Barreres Biològiques en el Centre d'Investigació Biològic,

HIPÒTESI I OBJECTIUS

Acadèmia Hongaresa de les Ciències (Szeged, Hongria) sota la supervisió de la Dra. Mária Deli (**Manuscrit 4**) per a obtenir el Doctorat amb la Menció Internacional. Aquesta estada va ser recolzada per la beca "Programa de mobilitat internacional Erasmus+".

III. WORK PLAN

III. WORK PLAN

In order to carry out all the defined objectives, we stablished the following work plan:

First year.

First of all, a bibliographic search was done, using scientific books and publications, to review the information related with the topic of the Doctoral Thesis and keep updated. From this research, **Manuscript 1** was written. Simultaneously, postgraduate courses were performed, obtaining new knowledges that were later used during the thesis, particularly in topics such as how to publish and statistical analysis, among others. Moreover, part of that year was focused on helping in the experimental part and the preparation of the **Manuscript 5**.

Second and third year.

The experimental work during the second and third years included a combination of *in vivo* and *in vitro* studies and was organised in order to fulfil two of the three proposed objectives (**Manuscript 2** and **3**):

To study, using an *in vivo* model of cafeteria (CAF)-fed Wistar rats, how resveratrol (RSV) acts to restore leptin resistance (LR) and to determine the metabolites implicated in this process **(Manuscript 2)**.

On the other hand, the mechanisms by which RSV acts reverting LR wanted to be elucidated. For this purpose, an *in vitro* LR-model using human hepatocarcinoma cell line (HepG2 cells) was created **(Manuscript 3)**.

WORK PLAN

Fourth year.

The last year was used to focus on the last objective using the stay abroad in a well-recognized international group, expertize in cell barriers, to learn about the issue and to obtain the results. The idea was to determine whether different phenolic compounds had the capacity to increase the leptin transport across the blood-brain barrier (BBB) and if they had the capacity to revert an inflammatory/obese state produced by pro-inflammatory cytokines in an *in vitro* model of rat brain endothelial cells (RBEC) which simulated BBB model. In addition, the statistical analysis and the redaction of the **Manuscripts 2**, **3** and **4** and the thesis was done.

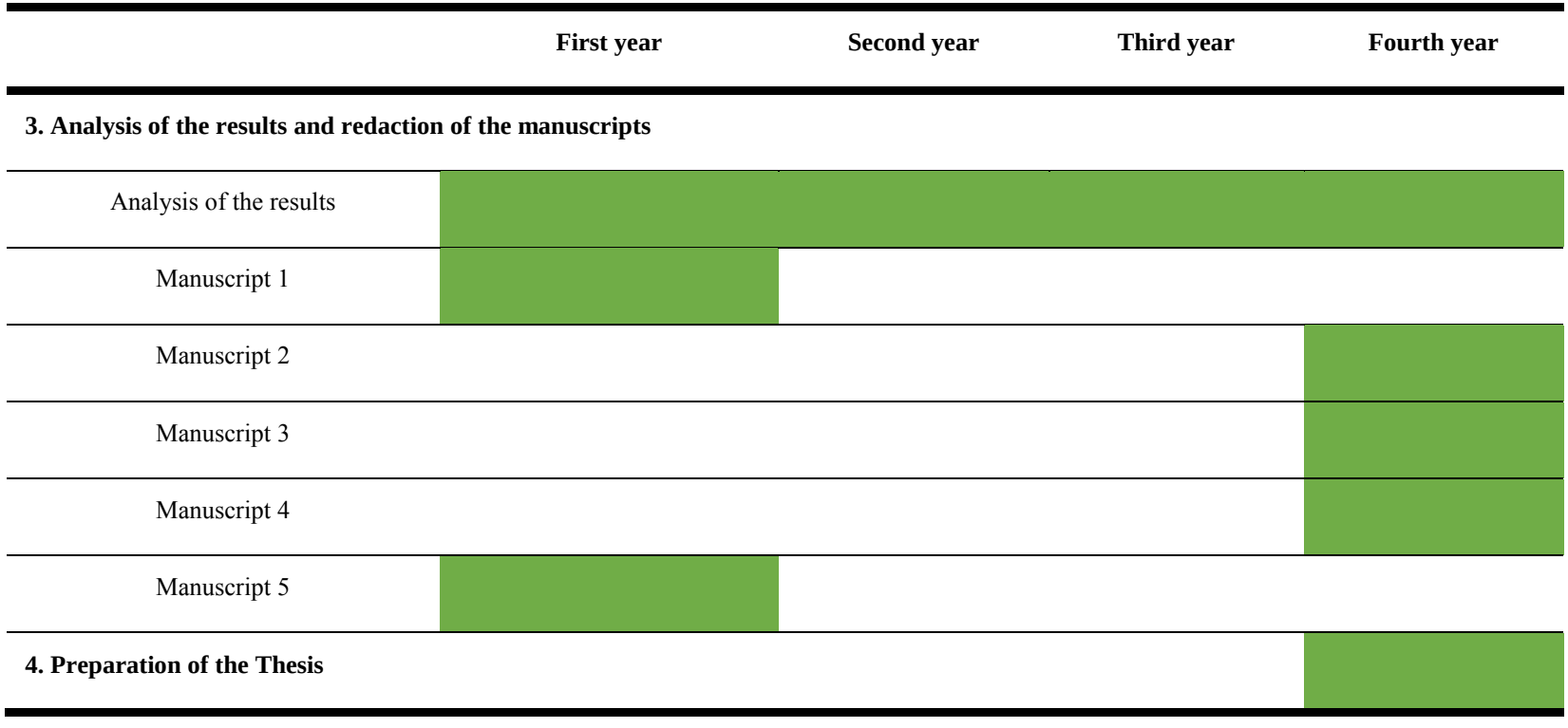
WORK PLAN

Table 1. Distribution of the activities during the four years of the thesis.

	First year	Second year	Third year	Fourth year
1. Bibliographic review and courses				
Bibliographic research				
Doctoral courses				
Conferences and congresses				
2. Experimental part				
***2.1. In vitro* studies**				
HepG2 studies				
RBEC studies				
***2.2. In vivo* studies**				
Wistar rats studies				

WORK PLAN

Table 1. Distribution of the activities during the four years of the thesis.



V. RESULTS

RESULTS

Manuscript 2

Potential involvement of peripheral leptin/STAT3 signalling in the effects of resveratrol and its metabolites on reducing body fat accumulation

Andrea Ardid-Ruiz, Maria Ibars, Pedro Mena, Daniele del Rio, Begoña Muguerza, Cinta Bladé, Lluís Arola, Manuel Suárez, Gerard Aragonès

Mol Nutr Food Res (Submitted)

RESULTS

PREFACE

This paper is focused in evaluating the capacity of resveratrol to re-establish the alterations caused by a cafeteria diet in male Wistar rats, a model that generates obesity with the development of all the associated pathologies. The idea, according to the **hypothesis** of the present doctoral thesis**,** is that resveratrol can revert the altered biometric parameters and the lipid profile as well as the leptin resistance produced in specific peripheral tissues (i.e., liver, skeletal muscle and epididymal white adipose tissue) in this obesogenic animal model. Thus, this article studies the general effects of resveratrol in the modulation of the last commented items. In addition, we decided to determine the metabolites of resveratrol present in the serum of the animals in order to know which ones are the main influencers in the observed beneficial effects of resveratrol.

RESULTS

Potential involvement of peripheral leptin/STAT3 signalling in the effects of resveratrol and its metabolites on reducing body fat accumulation

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Keywords: Cafeteria diet, leptin resistance, metabolites, microbiota, obesity, sirtuin.

RESULTS

Abstract

Background: Obesity is characterized by an imbalance in energetic homeostasis that causes ectopic fat accumulation in the peripheral organs. Current therapies, which are mainly based on energy restriction and exercise, are not sufficient. Bioactive compounds such as polyphenols have increasing in importance in recent years, and among them, resveratrol (3,5,4'-trihydroxy-*trans*-stilbene) has generated great interest as an anti-obesity agent. Recent investigations have highlighted the importance of leptin signalling in lipid metabolism in peripheral organs.

Objective: In this context, the aims of this study were (**1**) to investigate whether resveratrol can reduce fat accumulation in peripheral tissues including the liver, skeletal muscle and adipose tissue by increasing their leptin sensitivity and (**2**) to identify which resveratrol-derived circulating metabolites are potentially involved in these peripheral metabolic effects.

Methods: Serum leptin levels and the leptin signalling pathway were assessed by immunoassay, Western blot and quantitative PCR in diet-induced obese rats to elucidate the molecular mechanisms by which resveratrol exerts its anti-obesity effects. Moreover, serum metabolites of resveratrol were studied by UHPLC-MS^n.

Results: The daily consumption of 200 mg/kg of resveratrol but not doses of 50 and 100 mg/kg reduced body weight and fat accumulation in obese rats and restored leptin sensitivity in the periphery. These effects were due to increases in sirtuin 1 activity in the liver and leptin receptors in muscle and protection against endoplasmic reticulum

stress in adipose tissue. In general, the resveratrol metabolites associated with these beneficial effects were derived from both phase II and microbiota metabolism, although only those derived from microbiota metabolism increased proportionally with the administered dose of resveratrol.

Conclusions: Resveratrol reversed leptin resistance caused by diet-induced obesity in peripheral organs using tissue-specific mechanisms to restore leptin action. Resveratrol metabolites derived from the gut microbiota explained, at least partially, the contribution of the dose of 200 mg/kg but not lower doses reducing the metabolic disorders associated with an obesogenic diet.

RESULTS

1. Introduction

Obesity, defined by the World Health Organization (WHO) as excessive fat accumulation, has been increasing in recent decades and is now reaching epidemic proportions [1]. The increased consumption of energy-dense foods and the significant reduction of physical activity in our daily lives have led to dysregulation of the homeostatic control of energy balance and, consequently, body weight [2]. The current options for body weight management are energy restriction and physical activity [3]. However, compliance with these treatments is frequently poor, especially in the long term, and thus they are less successful than expected [4]. In this context, the scientific community is interested in naturally occurring bioactive compounds such as polyphenols that may be useful in body weight management [5]. Among these molecules, resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, RSV), a non-flavonoid polyphenol, has been found to be beneficial for many metabolic diseases [6], and in most studies in rodent models of diet-induced obesity (DIO), RSV alleviated the effects of this dietary pattern [7-9]. In addition, RSV is a well-described activator of NAD^+-dependent deacetylase sirtuin 1 (SIRT1) [10] and AMP-activated protein kinase (AMPK) [11], both of which are considered metabolic sensors that act on gene expression according to the metabolic state of the cell and are closely linked with benefits of caloric restriction [12].

RESULTS

Leptin, a hormone secreted mainly from white adipose tissue (WAT), is the main messenger that carries information about peripheral energy stores to the hypothalamus [13]. The interaction of leptin with its longest receptor isoform (ObRb) promotes the phosphorylation of signal transducer and activator of transcription 3 (STAT3). Subsequently, STAT3 dimerizes and translocates from the cytoplasm into the nucleus, stimulating anorexigenic factors and reducing body weight [14]. For this reason, the role of leptin in controlling energy homeostasis has thus far focused on hypothalamic receptors and neuroendocrine signalling pathways [15,16]. However, accumulating evidence indicates that leptin's effects on energy balance are also mediated by direct peripheral actions on key metabolic organs such as the liver, skeletal muscle, and adipose tissue [17]. In fact, several studies have recently indicated that peripheral leptin signalling regulates cellular lipid balance to stimulate lipolysis and fatty acid oxidation in WATs [17-19] and skeletal muscle [17,20], decrease triglyceride (TAG) content and secretion rates in liver [17,21], and even suppress insulin expression and secretion in pancreatic β-cells [17]. However, leptin is unable to exert its effect during DIO, and several molecular alterations have been associated with attenuated leptin/STAT3 signalling. These include enhanced endoplasmic reticulum (ER) stress and inflammation, impaired SIRT1 activity and the overexpression of inhibitory factors such as suppressor of cytokine signalling 3 (*Socs3*) and protein-tyrosine phosphatase (*Ptp1b*) [14].

In this context, we previously showed that a polyphenol-rich extract from grape seeds could improve peripheral and central leptin signalling by increasing SIRT1 functionality and protecting against neuroinflammation [22]. However, to the best of our knowledge, RSV has not been previously studied for its impact on leptin signalling in these organs.

Therefore, the aim of the present study was to examine whether RSV exerts part of its anti-obesity effect by modulating leptin sensitivity in the liver, skeletal muscle and adipose tissue. Thus, both serum leptin concentrations and the STAT3/leptin signalling pathway were evaluated in DIO animals to investigate the effects of this compound in hyperleptinemic animals with impaired leptin signalling. In addition, as RSV is quickly metabolized by both phase II enzymes and gut microbiota, it was necessary to simultaneously analyze its derived circulating metabolites to obtain a better understanding of the mechanism of action of this compound.

2. Materials and Methods

2.1 Animal handling

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili (reference number 4249 by Generalitat de Catalunya).

Male Wistar rats (n=30; 200 ± 50 g body weight) were purchased from Charles River Laboratories (Barcelona, Spain). The animals were housed in pairs under a 12 h light-

dark cycle at 22 ºC, fed a standard chow diet (Panlab A04, Barcelona, Spain) *ad libitum*, and were provided access to tap water during the adaptation week. Then, the animals were distributed into equal groups composed of 6 rats. One group was fed a standard chow diet (STD group) with a calorie breakdown of 14 % protein, 8 % fat and 73 % carbohydrates, while the others were fed an STD plus cafeteria diet (CAF group). The CAF diet was composed of 14 % protein, 35 % fat and 51 % carbohydrates and consisted of bacon, carrots, cookies, foie-gras, cupcakes, cheese and sugary milk. Nine weeks later, an oral treatment with RSV (Fagron Iberica, Barcelona, Spain) was administered together with the CAF diet for 22 days. The treatment groups were supplemented daily with 50, 100 or 200 mg/kg body weight of RSV dissolved in low-fat sugary milk diluted 1:1 in water. The STD and CAF diet groups were supplemented with the same quantity of vehicle (VH) (750 µL) (**Figure 1S**). Before supplementation, all rats were trained to voluntarily lick the milk to avoid oral gavage. On the day of sacrifice, the rats received VH or RSV and then were fasted for 3 h before sacrifice by decapitation. Blood was collected, and the serum was obtained by centrifugation (1,500 x *g*, 4 °C and 15 min) and stored at -80 ºC. Metabolic tissues such as the liver, calf skeletal muscle and epididymal and retroperitoneal WATs (eWAT and rWAT, respectively) were excised, weighed, immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.2 Body weight and composition analysis

Body weight was weekly monitored until the end of the experiment. In addition, the day before sacrifice, total body composition in live animals was assessed by nuclear magnetic resonance (NMR) using an EchoMRI-700 system (Echo Medical Systems, Houston, TX, USA). Direct measurements of fat mass were obtained in triplicate for each animal, and the results were expressed as a percentage of total body weight.

2.3 Hormonal and metabolic serum parameters

Serum glucose, total cholesterol (TC) and triacylglycerol (TAG) were measured by enzymatic colorimetric kits (QCA, Barcelona, Spain). Serum leptin and insulin concentrations were measured using ELISA kits (Millipore, Madrid, Spain) according to the manufacturer's instructions.

2.4 Tissue lipid analysis

The total lipid content in liver, calf skeletal muscle and eWAT was extracted using the Folch method [23]. Briefly, 0.5 g of either liver or eWAT or 0.1 g of calf skeletal muscle was homogenized with 0.45 % NaCl in chloroform:methanol (2:1) in an orbital shaker at 4 ºC overnight. Then, the homogenate was filtered and washed with 0.45 % NaCl solution and 0.9 % NaCl solution. An aliquot of each extract was subjected to gravimetric analysis to measure the total lipid concentration. The remainder was allowed to evaporate under nitrogen flow, dissolved in isopropanol and stored at -80 ºC until further analysis. The TAG and TC concentrations from the extracts were also measured using QCA enzymatic colorimetric kits (QCA).

2.5 Leptin signalling analysis

Leptin signalling in the liver, calf skeletal muscle and eWAT was assessed by calculating the activation of STAT3 using an ELISA kit with a phospho-specific antibody for STAT3 phosphorylation (pSTAT3) at tyrosine 705 (Abcam, Cambridge, UK). Briefly, 100 µL of positive control or sample homogenate was added to wells in duplicate and incubated at room temperature for 2.5 h on an orbital microtiter plate shaker. After washing, 100 µL of the anti-pSTAT3 antibody was applied, and the plate was sealed and incubated for 1 h with shaking. After washing, 100 µL of HRP-conjugated anti-rabbit IgG against rabbit anti-pSTAT3 was applied, and the plate was sealed and incubated for 1 h with shaking. Then, the wells were washed, and the TMB One-Step Substrate Reagent was incubated for 30 min in the dark. Finally, 50 µL of the Stop Solution was added, and the plates were immediately read at 450 nm on an EON microplate automatic plate reader (BioTek, Vermont, USA).

2.6 Leptin sensitivity index

As cellular pSTAT3 levels are mainly attributable to leptin action in peripheral tissues, leptin sensitivity in the liver, calf skeletal muscle and eWAT was objectively estimated as the ratio of pSTAT3 levels in each tissue to the leptin concentration in serum.

2.7 qRT-PCR analysis

Total RNA was extracted from the liver, calf skeletal muscle and eWAT using TRIzol LS Reagent (Thermo Fisher, Madrid, Spain) and RNeasy Mini Kit (Qiagen, Madrid, Spain) according to the manufacturers' protocols. The quantity and purity of RNA were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Madrid, Spain). Only samples with an adequate RNA concentration (A260/A280 ≥ 1.8) and purity (A230/A260 ≥ 2.0) were selected for reverse transcription. Complementary DNA (cDNA) was generated using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher), and 10 ng was subjected to quantitative PCR (qPCR) with iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain) using the 7900HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal profile settings were 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min. The forward (FW) and reverse (RV) primers used in this study were obtained from Biomers.net (Ulm, Germany) and can be found in **Table 1S**. A cycle threshold (Ct) value was generated by setting the threshold during the geometric phase of the cDNA sample amplification. The relative expression of each gene was calculated by referring to cyclophilin peptidylprolyl isomerase A (*Ppia*) mRNA levels and normalized to the STD group. The ΔΔCt method was used and corrected for primer efficiency [24]. Only samples with a quantification cycle lower than 35 were used for fold change calculation.

2.8 Western blot analysis

Protein levels of the ObRb leptin receptor isoform in the liver, calf skeletal muscle and eWAT were determined by western blot analysis. Tissues were homogenized at 4 ºC in 800-1,000 µL of Radio-Immunoprecipitation Assay (RIPA) lysis buffer (100 mM Tris-HCl and 300 mM NaCl pH 7.4, 10 % Tween, 10 % Na-Deox) containing protease and phosphatase inhibitor cocktails using a TissueLyser LT (Qiagen). The homogenate was incubated for 30 min at 4 ºC and then centrifuged at 12,000 x *g* for 20 min at 4 ºC. The supernatant was placed in fresh tubes and used to determine total protein and for immunoblotting analyses. The total protein content of the supernatant was measured using the Pierce BCA protein assay kit (Thermo Scientific). Samples were denatured by mixing with loading buffer solution (Tris-HCl 0.5 M pH 6.8, glycerol, SDS, β-mercaptoethanol and Bromophenol Blue) and then heated at 99 ºC for 5 min in a thermocycler (Multigen Labnet, Barcelona, Spain). Acrylamide gels were prepared using TGX Fast Cast Acrylamide Kit, 10 % (Bio-Rad), and 25 µg of protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) in electrophoresis buffer (glycine 192 mM, Tris base 25 mM and 1 % SDS). Proteins were electrotransferred onto supported PVDF membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad). After blocking with 5 % non-fat dried milk, the membranes were incubated with gentle agitation overnight at 4 °C with a specific antibody for ObRb (ab177469, Abcam) diluted 1:1,000. For β-actin analysis as a loading control, membranes were incubated with a rabbit anti-actin primary antibody (A2066, Sigma-Aldrich, Madrid, Spain), diluted 1:1,000. Finally, membranes were

incubated with anti-rabbit horseradish peroxidase secondary antibody (NA9344, GE Healthcare, Barcelona, Spain), diluted 1:10,000. Protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare) and GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). The protein bands were quantitated by densitometry using ImageJ software (W.S Rasband, Bethesda, MD, USA) and each band was normalized by the corresponding β-actin band, and finally, the treatment groups were normalized by the STD group.

2.9 SIRT1 activity assay

The SIRT1 activity in liver, calf skeletal muscle and eWAT was determined using a SIRT1 direct fluorescent screening assay kit (Cayman, Ann Arbor, MI) as previously described [25]. Briefly, a total of 25 μL of assay buffer (50 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$), 5 μL of tissue extract (1.5 mg/mL), and 15 μL of substrate (Arg-His-Lys-Lys(ε-acetyl)-)-7-amino-4-methylcoumarin) solution were added to all wells. The fluorescence intensity was monitored every 2 min for 1 h using a BertholdTech TriStar2S fluorescence plate reader (Berthold Technologies, Germany) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results were expressed as the rate of reaction for the first 30 min, when there was a linear relationship between fluorescence and time.

2.10 Resveratrol metabolite extraction from serum samples

Serum samples were extracted as previously reported by Savi, *et al*. (2017) [26] with minor modifications. Briefly, 300 µL of serum was diluted with 1 mL of acidified acetonitrile (2 % formic acid, Sigma-Aldrich). The samples were vortexed vigorously, ultrasonicated for 10 min, and centrifuged at 12,000 rpm for 5 min. Then, the supernatant was dried under vacuum by rotary evaporation, and the pellet was suspended in 100 µL of methanol 50 % (v/v) acidified with formic acid 1 % (v/v) and centrifuged at 12,000 rpm for 5 min prior to analysis by ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS/MS).

2.11 UHPLC-MS^n analysis

Samples were analyzed by an Accela UHPLC 1250 coupled to a linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). The chromatographic and ionization parameters for the analysis of the samples were set as previously described [27]. Metabolite identification was performed by comparing the retention time with authentic standards and/or MS^n fragmentation patterns in negative ionization mode (**Table 2S**). The glucuronide forms of RSV and dihydroresveratrol (DR) were fragmented using a collision-induced dissociation (CID) value of 16 (arbitrary units), whereas aglycones and sulfate conjugates required CID values of 34 and 23, respectively. Pure helium gas was used for CID. Data processing was performed using Xcalibur software from Thermo Scientific. Quantification was

performed using specific MS^2 full scans and calibration curves of pure standards in the case of RSV, resveratrol-3-*O*-sulfate (R3S), resveratrol-4'-*O*-sulfate (R4S), resveratrol-3-*O*-glucuronide (R3G) and DR. When a standard was not available, the conjugated metabolites were quantified based on the most structurally similar compound and expressed as their equivalents.

2.12 Statistical analysis

The data are expressed as the means ± standard errors of the means (SEM). Groups were compared by Student's t-test or two-way ANOVA and Bonferroni's test.

Outliers were determined by Grubbs' test. MetaboAnalyst (Xia Lab, McGill University, Quebec, Canada) was used to perform multivariate statistical analyses. Correlation analysis was performed using the nonparametric Spearman test. Statistical analyses were performed using XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel (Addinsoft, Paris, France (2017)). Graphics were prepared using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant, and $p < 0.1$ was considered to indicate tendency.

3. Results

3.1 RSV attenuates diet-induced body fat increase, hypertriglyceridemia and hyperleptinemia

The CAF diet for 12 weeks consistently resulted in obesity as indicated by significantly higher body weight gain (50.1 % higher) and total body fat mass (124.3

% higher, assessed by NMR scanning) compared to the STD group. Notably, body weight gain was 17 % lower in animals supplemented with RSV at 200 mg/kg daily compared to the CAF group (**Figure 1A**), and this reduction was associated with a significant decrease in total body fat mass (**Figure 1B**). Importantly, the consumption of 200 mg/kg of RSV partially reversed the hyperleptinemia induced by CAF diet (40.1 % lower) (**Figure 1C**), reinforcing the robust metabolic correlation between leptin levels and total body fat mass in our experimental model ($\rho = 0.93$, $p < 0.05$). In addition, at this dose, RSV was also effective in normalizing serum concentrations of TAG, glucose and insulin in a fasting state (**Figure 1D-F**), indicating that RSV has an insulin-sensitizing effect in DIO model. By contrast, the daily consumption of 50 and 100 mg/kg of RSV for 22 days did not exert any beneficial effects with respect to body weight, total body fat accumulation and hormonal and metabolic serum parameters.

3.2 A multivariate analysis shows that RSV partially reverses the metabolic alterations induced by the cafeteria diet

To further evaluate the effect of RSV from a multivariate point of view, principal component analysis (PCA) was performed to analyze globally the distribution of animals among all anthropometric, metabolic and biochemical variables. Accordingly, the PCA score plot for the STD and CAF groups accounted for 91.6 % of the variance of the original matrix, and each animal was clearly clustered according to their diet (**Figure 1G)**. In addition, when the multivariate analysis was used to

evaluate the effect of RSV consumption on DIO, we observed that animals treated at doses of 50 and 100 mg/kg could not be clustered separately with respect to CAF animals, and only animals daily supplemented at a dose of 200 mg/kg were clustered in an intermediate position between the CAF and STD groups, indicating that RSV at this dose could exert a tendency to reverse the metabolic alterations induced by an obesogenic diet and a return to the basal situation (**Figure 1H**).

RESULTS

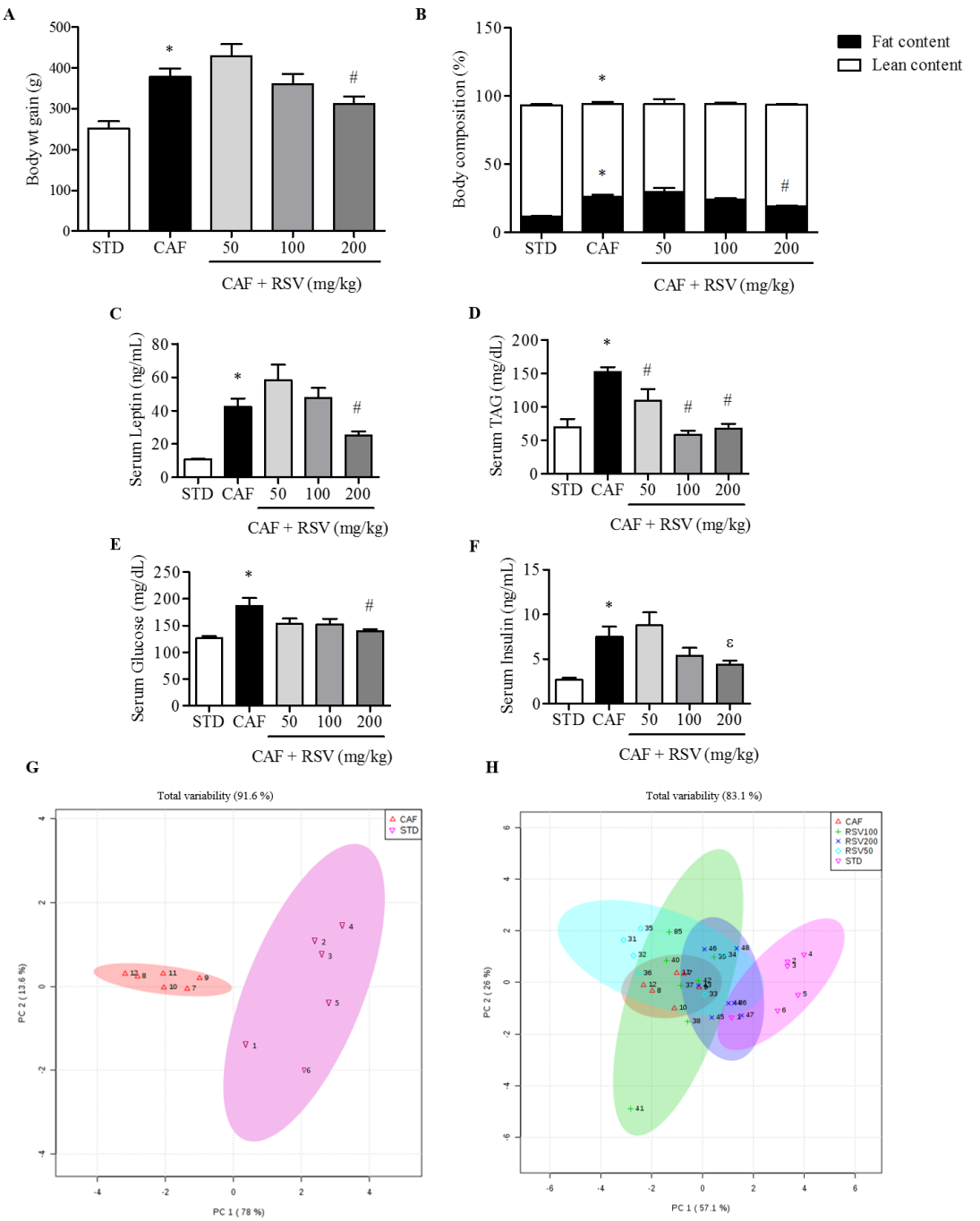

Figure 1. Metabolic parameters of rats fed the STD or CAF diet and treated with three concentrations of RSV (50, 100 and 200 mg/kg). The rats were fed the STD or CAF diet for 9 weeks. Then, the STD rats were treated orally with vehicle (VH) for 3 weeks, whereas the CAF rats were treated orally with VH or RSV (50, 100, or 200 mg per kg of body wt) for 3 weeks. (A) Body wt gain

(g) from the first day of the experiment until the last day. (B) Body composition (%) as assessed by NMR, including fat and lean content. The panels in (C) to (F) show serological levels of leptin, TAG, glucose and insulin, respectively. (G) and (H) are PCAs representing the clusters between the different groups according to the studied biometric parameters.

Data are expressed as the mean ± SEM, n=6. * $p < 0.05$ and $^{\phi}$ $p < 0.1$, Student's t-test comparing the CAF group to the STD group. $^{\#}$ $p < 0.05$ and $^{\varepsilon}$ $p < 0.1$, Student's t-test comparing the RSV group to the CAF group.
CAF: cafeteria diet; NMR: nuclear magnetic resonance; RSV: resveratrol; STD: standard chow diet; TAG: triacylglycerol; VH: vehicle; wt: weight.

3.3 RSV decreases diet-induced lipid content in liver, skeletal muscle and adipose tissues

To assess the contribution of visceral fat accumulation to the decrease in total body fat mass, we next evaluated the effect of RSV on fat deposition in three important metabolic peripheral tissues: visceral WAT, liver and calf skeletal muscle. Again, the CAF diet for 12 weeks resulted in a significant increase in two different visceral WAT depots compared to the STD group, including eWAT (18.6 ± 1.4 vs 8.9 ± 0.9 g, respectively) and retroperitoneal WAT (rWAT, 8.4 ± 0.9 vs 3.6 ± 0.1 g, respectively). Notably, at a dose of 200 mg/kg, RSV elicited a significant decrease in the weights of these depots compared with CAF animals, and this effect was more evident in eWAT (14.6 ± 1.4 g, 21 % lower) than in rWAT (7.1 ± 0.7 g, 16 % lower). In addition, RSV also tended to reduce the total fat content in eWAT (**Figure 2A**) and significantly in the liver (**Figure 2B**) but above all in the calf skeletal muscle, although not in a dose-dependent manner (**Figure 2C**). Interestingly, this decrease in fat depots in peripheral organs was directly associated with significant reductions of both TC and TAG

content (**Table 3S**), and in turn, it was positively and significantly related to serum leptin levels in the liver ($\rho = 0.54$, $p < 0.05$) and eWAT ($\rho = 0.43$, $p < 0.05$), implicating leptin in the regulation of lipid metabolism in peripheral tissues.

3.4 RSV directly down-regulates *leptin* transcription in adipose tissues

To further examine the mechanism by which RSV regulates lipid accumulation in peripheral tissues, we assessed the gene expression of lipid-regulating enzymes by RT-qPCR in these liver, calf skeletal muscle and eWAT. Completely contrary to our expectations, we found that the expression levels of genes involved in lipogenesis but not fatty acid oxidation, such as *Acc*, *Scd1* and *Fas*, were significantly increased in the liver of animals supplemented with 50 and 100 mg/kg of RSV (**Figure 2D**). By contrast, no significant changes were observed in the expression of genes encoding enzymes for lipogenesis, whereas RSV down-regulated fatty acid oxidation in eWAT (**Figure 2E**). Moreover, we did not observe any significant changes in thermogenesis, mitochondrial biogenesis and fatty acid oxidation in calf skeletal muscle (**Figure 2F**). Importantly, in eWAT and rWAT, at a dose of 200 mg/kg, RSV consumption tended to down-regulate *leptin* mRNA levels compared with CAF animals (**Figure 2G-H**), indicating that animals undergoing RSV treatment more efficiently regulated leptin production and secretion in these tissues than those in the CAF group.

RESULTS

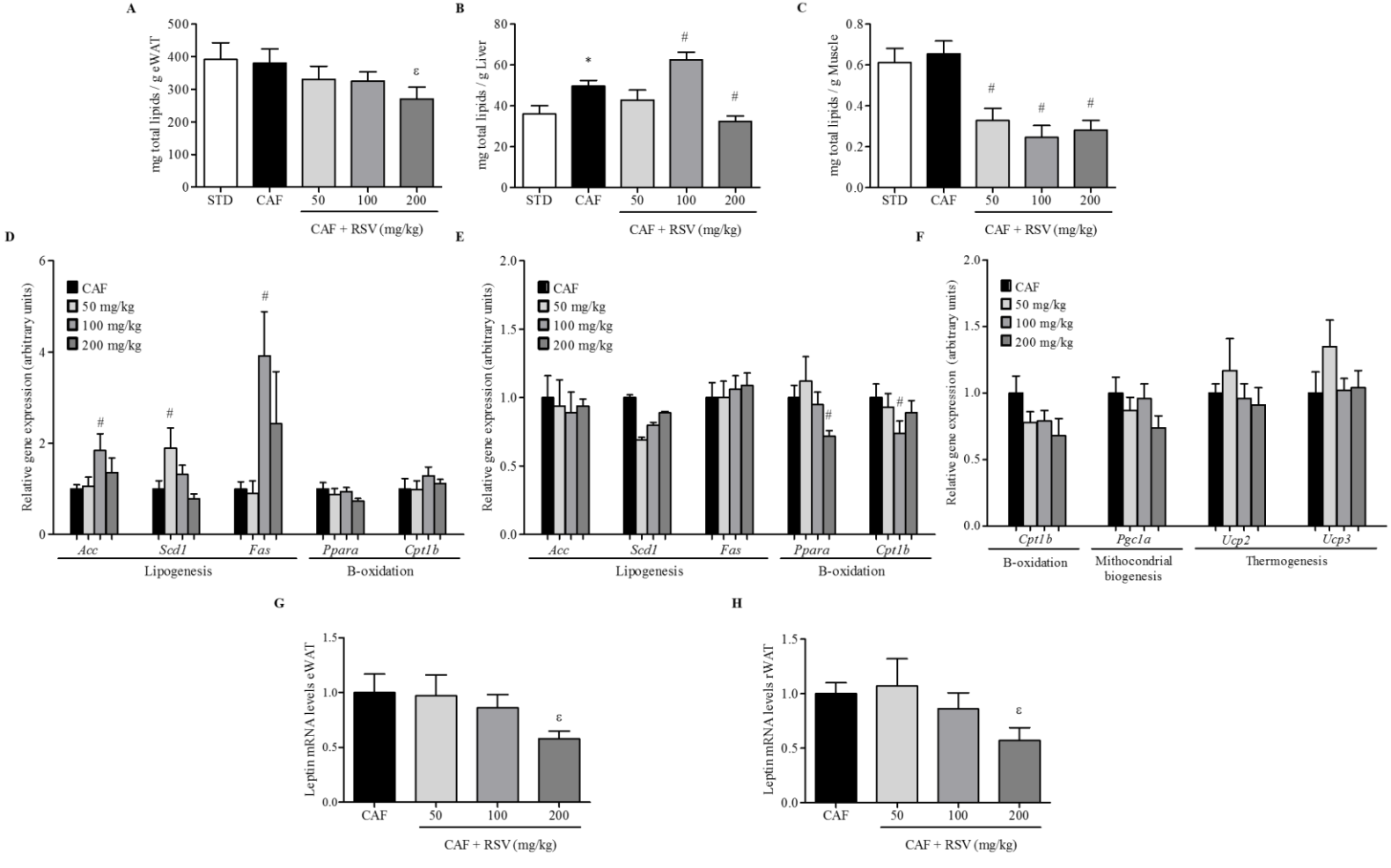
A
mg total lipids / g eWAT
STD
CAF
50
100
200
CAF + RSV (mg/kg)
B
mg total lipids / g Liver
C
mg total lipids / g Muscle
D
Relative gene expression (arbitrary units)
CAF
50 mg/kg
100 mg/kg
200 mg/kg
Acc
Scd1
Fas
Ppara
Cpt1b
Lipogenesis
B-oxidation
E
F
Pgc1a
Ucp2
Ucp3
Mithocondrial biogenesis
Thermogenesis
G
Leptin mRNA levels eWAT
H
Leptin mRNA levels rWAT

Figure 2. Lipid profile of rats fed the STD or CAF diet and treated with three concentrations of RSV (50, 100 and 200 mg/kg). The rats were fed the STD or CAF diet for 9 weeks. Then, the STD rats were treated orally with vehicle (VH) for 3 weeks, whereas the CAF rats were treated orally with VH or RSV (50, 100, or 200 mg per kg of body wt) for 3 weeks. (A), (B) and (C) Total lipids in eWAT, liver and calf skeletal muscle in mg for each g of tissue. (D) Expression in the liver of genes related to lipogenesis (*Acc*, *Scd1* and *Fas*) and β-oxidation (*Ppara* and *Cpt1b*). (E) Expression in eWAT of some genes related to lipogenesis (*Acc*, *Scd1* and *Fas*) and β-oxidation (*Ppara* and *Cpt1b*). (F) Expression in calf skeletal muscle of genes related to β-oxidation (*Cpt1b*), mitochondrial biogenesis (*Pgc1a*) and thermogenesis (*Ucp2* and *Ucp3*). (G) and (H) Leptin expression for total eWAT and rWAT content, respectively.

Data are expressed as the mean ± SEM, n=6. * $p < 0.05$ and $^{\phi}$ $p < 0.1$, Student's t-test comparing the CAF group to the STD group. $^{\#}$ $p < 0.05$ and $^{\varepsilon}$ $p < 0.1$, Student's t-test comparing the RSV group to the CAF group. CAF: cafeteria diet; eWAT: epididymal white adipose tissue; RSV: resveratrol; rWAT: retroperitoneal white adipose tissue; STD: standard chow diet; VH: vehicle; wt: weight.
Acc (acetyl-CoA carboxylase); *Cpt1b* (carnitine palmitoyltransferase 1b); *Fas* (fatty acid synthase); *Pgc1a* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), *Ppara* (peroxisome proliferator activated receptor alpha); *Scd1* (stearoyl-CoA desaturase 1); *Ucp2* (mitochondrial uncoupling protein 2); Ucp3 (mitochondrial uncoupling protein 3).

3.5 RSV potentiates leptin sensitivity in liver, calf skeletal muscle and adipose tissue

To determine if the observed decreases in leptin production and circulating levels could indicate that RSV directly affects leptin signalling in peripheral tissues, we assessed leptin sensitivity in liver, calf skeletal muscle and eWAT by detecting STAT3 activation (pSTAT3). Because pSTAT3 levels are mainly attributable to leptin action in theses tissues, we assessed the ratio of tissue-specific levels of pSTAT3 to the circulating leptin concentration to estimate the degree of sensitivity of each tissue to this hormone. In this context, the leptin sensitivity of CAF animals was significantly reduced compared to the STD group in all three studied tissues, and importantly, when

RSV was administered at dose of 200 mg/kg, the leptin sensitivity significantly increased to basal levels, indicating partial reversion of the situation observed in CAF animals (**Figure 3A**). By contrast, no significant effects on leptin sensitivity were observed at lower doses in any of the tissues assessed.

Next, we studied the gene expression levels of *Socs3* and *Ptp1b*, negative feedback regulatory molecules involved in leptin signalling by qRT-PCR. However, *Socs3* and *Ptp1b* mRNA levels were not significantly altered by RSV consumption (**Figure 2S**). Finally, we also investigated the impact of RSV on two metabolic processes closely associated with leptin signalling disruption: local inflammation and ER stress. However, *iNos* mRNA expression levels were not significantly regulated in any tissue (**Figure 3S)**. In a similar manner, in liver and muscle, transcripts related to ER stress were not modulated in any group of animals undergoing RSV supplementation (**Figure 4S)**. Interestingly, a significant decrease in ER stress markers was observed in eWAT in animals under the highest dose of RSV (**Figure 3B**).

3.6 RSV distinctively modulates sirtuin-1 (SIRT1) activity and leptin receptor (ObRb) protein expression in peripheral tissues

To elucidate the molecular mechanisms by which RSV potentially rescues leptin sensitivity in these tissues, we next evaluated whether RSV consumption could result in enhanced SIRT1 functionality, which could be an additional mechanism involved in the regulation of leptin signal transduction in the periphery. Thus, we analyzed the deacetylase activity of SIRT1 in liver, calf skeletal muscle and eWAT (**Figure 3C)**.

Notably, robust activation of SIRT1 was observed in the liver of animals supplemented with 50 and 200 mg/kg of RSV, indicating that a relatively low dose of RSV (50 mg/kg) is sufficient to efficiently activate this enzyme in the liver. By contrast, at these same doses, SIRT1 activity was notably decreased in calf skeletal muscle and was not significantly affected in eWAT, suggesting that if RSV is a true leptin sensitizer, this activity is not mediated by an increase in SIRT1 functionality in calf skeletal muscle and eWAT. Therefore, we also investigated by immunoblotting whether the modulation of leptin sensitivity in these tissues could also be directly mediated by increasing the cell content of the long leptin receptor isoform ObRb (**Figure 3D**). Interestingly, the consumption of RSV resulted in a dose-dependent significant increase in ObRb protein levels in calf skeletal muscle, although statistically significant differences were only observed at a dose of 200 mg/kg. Importantly, in contrast to calf skeletal muscle, ObRb protein levels in liver were decreased significantly in animals under RSV at doses of 50 and 200 mg/kg, whereas in eWAT, ObRb protein levels were not significantly affected at any dose. These results indicate that RSV can modulate different cellular processes in a tissue-specific manner.

RESULTS

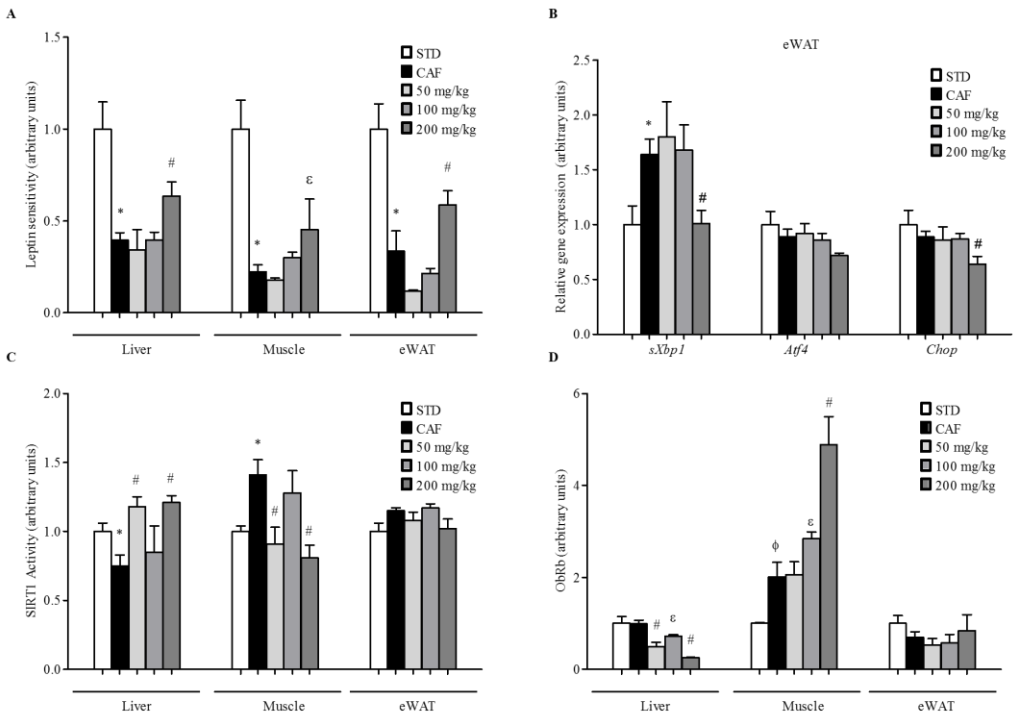

Figure 3. Analyzed parameters to explain the effects of the restoration by RSV of leptin sensitivity in rats fed the STD or CAF diet and treated with three concentrations of RSV (50, 100 and 200 mg/kg) or vehicle (VH). The rats were fed the STD or CAF diet for 9 weeks. Then, the STD rats were treated orally with vehicle (VH) for 3 weeks, whereas the CAF rats were treated orally with VH or RSV (50, 100, or 200 mg per kg of body wt) for 3 weeks. (A) The leptin sensitivity index (LSI). (B) The expression of some ER stress markers in eWAT. (C) SIRT1 activity. (D) The WB results for ObRb. The three graphics show the results for the three studied tissues: liver, calf skeletal muscle and eWAT.

Data are expressed as the mean ± SEM, n=6. * $p < 0.05$ and $^{\phi}$ $p < 0.1$, Student's t-test comparing the CAF group with the STD group. $^{\#}$ $p < 0.05$ and $^{\varepsilon}$ $p < 0.1$, Student's t-test comparing the RSV group with the CAF group.

CAF: cafeteria diet; ER: endoplasmic reticulum; eWAT: epididymal white adipose tissue; LSI: leptin sensitivity index; ObRb: leptin receptor isoform b; RSV: resveratrol; SIRT1: NAD^{+}-dependent deacetylase sirtuin-1; STD: standard chow diet; VH: vehicle; WB: western blot; wt: weight.

Atf4 (activating transcription factor 4), *Chop* (DNA damage inducible transcript 3), s*Xbp1* (spliced x-box binding protein 1).

RESULTS

3.7 Different RSV metabolites, including microbial and phase II conjugates, could explain the body fat-lowering effects of RSV consumption

Since the efficacy of orally administered RSV depends on its absorption and metabolism, next we investigated whether RSV and its metabolites found in the bloodstream can account for the observed anti-obesity effects after the daily consumption of 200 mg/kg of RSV for 22 days. **Table 1** details the serum concentrations of each metabolite of RSV. The administration of RSV at 50, 100 and 200 mg/kg led to high serum concentrations of some metabolites, in the range of μM. Interestingly, 10 different RSV-derived metabolites, but not the parent compound, were detected in serum 3 h after the last RSV treatment. These metabolites included seven phase II metabolites of RSV (R3G, R4G, RDG, R3S, R4S, RDS and RSG), and three for gut-microbial metabolites, including the glucuronide and sulfate conjugates of DR (DRG, DRS and DRGS).

Table 1. The results obtained for each metabolite (μM) present in serum for each group of rats treated with RSV.

RSV Metabolites (μM)		50 mg/kg	100 mg/kg	200 mg/kg
Phase II	**R4G**	18.52 ± 4.54	15.45 ± 1.69	20.53 ± 7.73
	R3G	12.44 ± 4.85	8.43 ± 3.79	1.84 ± 0.61*
	RDG	0.19 ± 0.04	0.29 ± 0.09	0.23 ± 0.04
	R4S	0.12 ± 0.02	0.16 ± 0.05	0.27 ± 0.09
	R3S	7.06 ± 2.66	5.69 ± 1.21	5.59 ± 2.04

RESULTS

	RDS	0.80 ± 0.31	1.39 ± 0.31	0.66 ± 0.09
	RSG	0.83 ± 0.15	1.00 ± 0.14	0.83 ± 0.21
	DRG	3.95 ± 0.60	2.72 ± 0.66	9.43 ± 2.28
	DRS	1.15 ± 0.46	1.24 ± 0.63	3.92 ± 1.30
Microbiota	**DRSG**	0.11 ± 0.02	0.15 ± 0.04	0.21 ± 0.06

The rats were fed the CAF diet for 9 weeks. Then, the CAF rats were treated orally with RSV (50, 100 or 200 mg per kg of body wt) for 3 weeks.

Data are expressed as the mean ± SEM, n=6. * $p < 0.05$, two-way ANOVA and Bonferroni's test comparing to 50 mg/kg group.

Abbreviations: R4G: resveratrol-4'-*O*-glucuronide; R3G: resveratrol-3-*O*-glucuronide; R3S: resveratrol-3-*O*-sulfate; R4S: resveratrol-4'-*O*-sulfate; RDS: resveratrol disulfate; RDG: resveratrol diglucuronide; RSG: resveratrol sufate glucuronide; DRG: dihydroresveratrol glucuronide; DRS: dihydroresveratrol sulfate; DRSG: dihydroresveratrol sufate glucuronide.

When the serum distribution of these two types of metabolites was analyzed, phase II RSV metabolites were found to be predominant over DR metabolites derived from microbiota by more than twofold (**Figure 4A**). Interestingly, the concentration of microbial DR metabolites significantly increased at a dose of 200 mg/kg, whereas the opposite occurred for phase II RSV metabolites. Thus, the largest circulating levels of microbial metabolites were found at the highest dose of 200 mg/kg. In addition, total glucuronide metabolites (R3G, R4G, RDG and DRG) were also detected in higher levels than total sulfate conjugates (R3S, R4S, RDS and DRS) at all doses (**Figure 4B**). However, the concentration of glucuronide conjugates tended to decrease when

the RSV dosage was increased, whereas sulfate metabolites significantly increased at doses of 100 and 200 mg/kg.

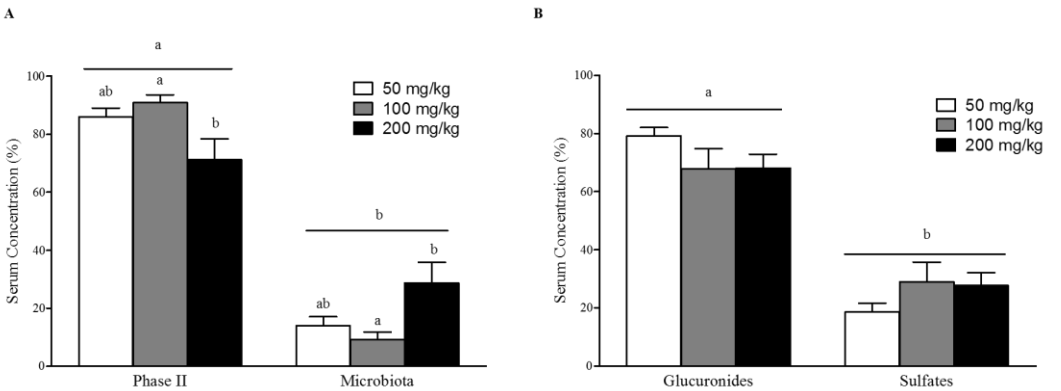

Figure 4. Percentage of serum RSV metabolites in rats fed the CAF diet and treated with three concentrations of RSV (50, 100 and 200 mg/kg). The rats were fed the CAF diet for 9 weeks. Then, the CAF rats were treated orally with RSV (50,100 or 200 mg per kg of body wt) for 3 weeks. The metabolites present in serum were classified as phase II RSV metabolites or microbial metabolites (A) and as glucuronide or sulfate metabolites (B).

Data are expressed as the mean ± SEM, n=6. ^{a,b,c} $p < 0.05$, two-way ANOVA and Bonferroni's test comparing groups.
CAF: cafeteria diet; RSV: resveratrol; STD: standard chow diet; wt: weight.

Finally, to determine which blood RSV metabolites could potentially be involved in the anti-obesity effects of RSV, we used Spearman's correlation test to evaluate the relationship of RSV metabolites with body and fat mass as well as with leptin sensitivity in each peripheral tissue (**Table 4S**). R4G and R3S were the only phase II RSV metabolites that showed significant and negative correlations with total body fat mass (ρ = -0.67 and -0.76, p = 0.033 and 0.011, respectively) and circulating leptin levels (ρ = -0.66 and -0.60, p = 0.038 and 0.067, respectively). In addition, R4G was also positively associated with leptin sensitivity in liver (ρ = 0.72, p = 0.03), calf

skeletal muscle (ρ = 0.69, p = 0.05) and eWAT (ρ = 0.79, p = 0.021), whereas R4S was positively associated with leptin sensitivity in calf skeletal muscle (ρ = 0.81, p = 0.015) and eWAT (ρ = 0.79, p = 0.021). When the correlation coefficients were analyzed for the microbial DR metabolites, only DRSG presented a negative and significant correlation with diet-induced body weight increase (ρ = 0.66, p = 0.038). In addition, DRSG was related to leptin sensitivity in calf skeletal muscle (ρ = 0.81, p = 0.015) and eWAT (ρ = 0.71, p = 0.047), whereas DRS was related to leptin sensitivity in calf skeletal muscle (ρ = 0.69, p = 0.05).

4. Discussion

Previous studies of by group indicated that chronic consumption of grape-seed proanthocyanidins (GSPE) for three weeks by DIO rats significantly decreased both hepatic fat content and circulating plasmatic leptin levels, presumably by restoring SIRT1 functionality and leptin signalling in both the hypothalamus and liver [22,25]. Nonetheless, studies of other compounds with complementary or more powerful effects are necessary to combat metabolic diseases associated with leptin dysfunction, such as obesity. Accordingly, in the present study, we demonstrated that RSV, a dietary non-flavonoid polyphenol found in grapes and red wine, decreased body fat mass and leptinemia by restoring leptin sensitivity in the liver, calf skeletal muscle and adipose tissue (eWAT).

RESULTS

Leptin is a pleiotropic hormone with a variety of functions within the organism and activity in different tissues. Liver and skeletal muscle are the tissues with greatest metabolic activity and, together with adipose tissue, constitute important targets for leptin regulation of lipid metabolism [28,29]. However, pathological states such as obesity have been related to peripheral leptin resistance (LR) development, and dietary components have been proposed to modulate leptin actions in these peripheral tissues, suggesting that LR may also result from specific nutrient intake [30,31]. In this sense, our CAF-induced obesity rat model exhibited body weight/fat increase, hyperleptinemia and peripheral LR as indicated by the impairment of leptin-induced STAT3 phosphorylation in these tissues. pSTAT3 levels are widely studied to evaluate leptin sensitivity as STAT3 is proportionally activated by leptin concentrations in these tissues [32].

Remarkably, our results showed an ability of RSV at 200 mg/kg to normalize tissue fat content and leptin expression and secretion as well as to enhance peripheral leptin sensitivity, highlighting the overall beneficial effect of RSV in the modulation of DIO at this dose. Conversely, RSV did not regulate the gene expression of enzymes directly involved in peripheral lipid metabolism, such as *Acc*, *Fas*, *Cpt1b* and *Scd1*. In addition, RSV did not down-regulate the gene expression of relevant enzymes involved in leptin signalling, such as *Socs3* and *Ptp1b*. Contradictory results have been published about the effect of RSV on these markers of leptin signalling in different tissues [33-35], indicating that the duration of the treatment and the grade of obesity achieved can directly influence the effect of RSV in these tissues.

RESULTS

The daily consumption of 50 and 100 mg/kg of RSV for 22 days in combination with the CAF diet did not change any metabolic parameter or leptin sensitivity in our experimental model. Some contradictory results have been published about the effectiveness of RSV on metabolic alterations in rodents. Andrade, *et al*. (2014) reported that the consumption of 30 mg/kg of RSV by FVN/N mice for 60 days in combination with a CAF-rich diet exerted beneficial effects on body fat and weight [36]. By contrast, supplementation of DIO C57BL/6J mice with 22.5 and 45 mg/kg of RSV for 12 weeks [37] or 200 mg/kg for 20 weeks [38] did not cause any significant change in body weight, indicating that the effect of RSV in rodents might depend on the treatment length, RSV dosage and the percentage of fat present in the diet. In our study, the impact of the CAF diet was too robust, as the lowest doses of RSV administered could not counteract the diet-induced dysregulation of lipid metabolism and leptin signalling.

The induction of peripheral LR in diet-induced models has been primarily attributed to the induction of pro-inflammatory signalling and ER stress. However, in this study, we did not find significant differences in inflammatory status in any of the three tissues studied. In contrast to our results, Kimbrough, *et al*. (2015) observed down-regulation of *iNos* by RSV in hepatocytes in an inflammatory experimental model [39], as did Centeno-Baez, *et al*. (2011) in muscle and WAT in LPS-treated C57BL6 mice [40]. Conversely, our results showed a significant reduction of *sXBP1* gene expression in eWAT, suggesting that this local decrease in ER stress in adipocytes is one of the mechanisms by which RSV re-establishes appropiate leptin sensitivity in

this tissue. In addition, SIRT1 functionality and ObRb levels have been highlighted as mediators of leptin action in peripheral organs. Thus, both the overexpression of SIRT1 in the liver and the enhanced ObRb protein content in calf skeletal muscle induced by RSV could be mechanisms by which this compound increases leptin signalling in these tissues. In fact, this beneficial effect of RSV on SIRT1 activity in the liver is in accordance with a previous report [41]. However, the different responses of the liver, calf skeletal muscle and eWAT to RSV suggest different functions of SIRT1 and ObRb in peripheral tissues, and thus further studies are required to clarify the molecular mechanism by which RSV regulates leptin signalling in each tissue under obesogenic conditions.

The efficacy of orally administered RSV depends on its absorption and metabolism. RSV is quickly absorbed in the intestine via simple intestinal transepithelial transport and by ATP-dependent binding cassette transporters, but most RSV undergoes rapid and extensive phase II metabolism in enterocytes before entering the blood and further into the liver [42,43]. According this metabolism, RSV is mainly converted into glucuronide and sulfate metabolites. Interestingly, in the present study, we detected RSV metabolites but not RSV. Our results are in line with some previously published finding of RSV metabolites but not free RSV in different peripheral tissues when rats were supplemented with 300 [44] or 60 mg/kg [45]. In addition, total glucuronide RSV metabolites (R3G, R4G and RDG) were also detected in higher levels than total sulfate RSV conjugates (R3S, R4S and RDS) at all doses. Similarly, in a previous study using male Sprague-Dawley rats, Marier, *et al*. (2002) observed 46 times more

glucuronidated forms in plasma than other metabolites 4 h after oral administration of 50 mg/kg of RSV [46]. However, other researchers have reported that the sulfate forms were prevalent over glucuronides in male Wistar rats orally supplemented with 300 mg/kg of RSV during 8 weeks, whereas no RSV conjugates were detected in the group with a dose of 50 mg/kg [44].

Notably, in our study, the concentration of glucuronide RSV conjugates tended to decrease as the RSV dosage increased, whereas the sulfate metabolites increased at the highest doses. Similarly, Andres-Lacueva, *et al.* (2012) observed that as the dose of RSV in rats increased (6, 30 and 60 mg/kg/day during 6 weeks), there was an increase in the sulfate forms compared with the glucuronides [45]. These results may suggest that glucuronidation but not sulfation is a saturable metabolic pathway, at least in the range of doses used in the present study. Nevertheless, the potential degradation of glucuronide metabolites, as well as more rapid elimination, cannot be discarded. Consequently, further studies are needed to better elucidate this issue.

Only a few studies have considered the determination of RSV-derived microbial metabolites after RSV consumption. Consequently, we also assessed DR concentrations in serum in free form or as glucuronide and sulfate conjugates. Notably, in our study, the largest circulating levels of microbial metabolites were found at the highest dose of 200 mg/kg. These data could provide a clue to explain the protective effects on body fat accumulation and leptin sensitivity observed only at this dose of RSV. In fact, our results showed negative correlations of levels of DRSG

metabolites from microbiota and with body fat mass, circulating leptin levels and body weight gain. However, the levels of most of these DR metabolites detected in serum were low in comparison to RSV metabolites and thus it is difficult to understand how they contributed to the effects observed. In addition, some studies also showed that, in 3T3-L1 cells, R3S, R3G and R4G decreased both mRNA and leptin secretion [47], increased the expression of *Atgl*, *Cpt1*, *Sirt1* and *Pgc1a*, and decreased the expression of *Fas* [48]. Consequently, more studies are needed to explain the *in vivo* effects induced by this polyphenol after long-term treatment.

In summary, we can conclude that RSV can reverse the disruption of metabolic parameters and the lipid profile in DIO rat model. This beneficial effect could be explained by the restoration of leptin sensitivity in the three peripheral organs described as more metabolically active. In the liver, RSV could act via a SIRT1-dependent manner, whereas in calf skeletal muscle and eWAT, its action was mediated by increasing ObRb content and protecting against ER stress, respectively. However, further studies are required to clarify the molecular mechanisms by which RSV regulates leptin signalling in obesity. Finally, the metabolites derived from the gut microbiota may partially explain the contribution of the highest dose of RSV reducing the metabolic alteration caused by obesity.

5. Acknowledgments

This work was supported by the Ministerio de Economía, Industria y Competitividad (AGL2013-49500-EXP, AGL2016-77105-R and FPU14/01202).

We gratefully acknowledge the aid of Niurka Dariela Llópiz and Rosa Pastor, the laboratory technicians. Authors also thank Dr. Luca Calani for his valuable support in metabolite identification.

6. Supplementary information

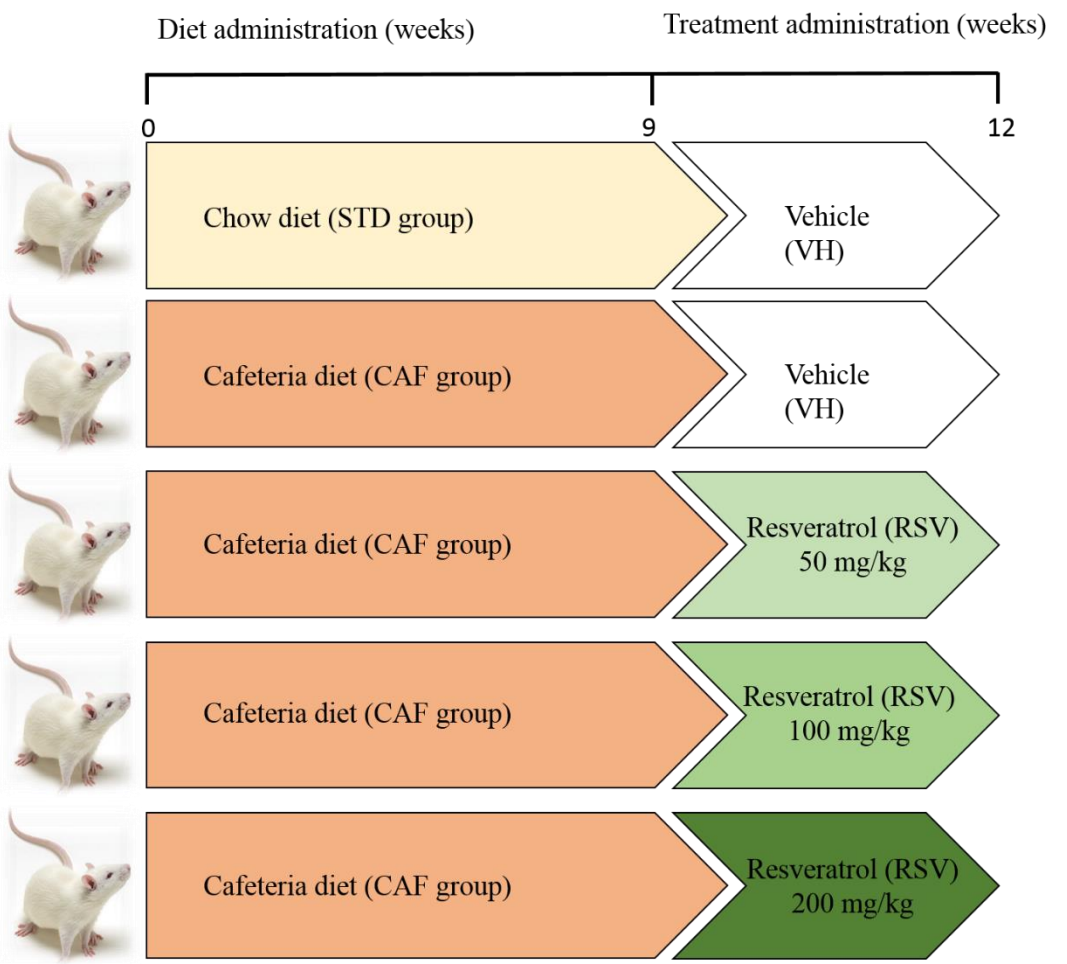

Figure 1S. A scheme of the distribution of animals in the study. Each group was composed of 6 rats. During the first nine weeks, one group was fed the standard chow diet (STD group), whereas the other group was fed the cafeteria diet (CAF group). After nine weeks, the animals were orally administered either vehicle (VH) or resveratrol (RSV) + VH at three doses (50, 100 and 200 mg/kg). On week twelve, the animals were sacrificed.

CAF: cafeteria diet; RSV: resveratrol; STD: standard chow diet; VH: vehicle.

RESULTS

Table 1S. A summary of the rat-specific primer sequences used for qRT-PCR analysis.

Primer name	Direction	Primer sequences (5'-3')	Primer length (nucleotides)	Tm (ºC)	Amplicon lenght (nucleotides)
Acc	FW	gcggctctggaggtatatgt	20	51	156
	RV	tctgtttagcgtggggatgt	20	52	
Atf4	FW	attcttgcagcctcttccct	20	52	213
	RV	aggtaggactcagggctcat	20	49	
Chop	FW	tactcttgaccctgcatccc	20	51	170
	RV	actgaccactctgtttccgt	20	48	
Cpt1b	FW	tcatgtatcgccgcaaactg	20	55	199
	RV	agccaaaccttgaagaagcg	20	54	
Fas	FW	tggtgatagccggtatgtcc	20	52	153
	RV	tcagctttccagaccgctta	20	53	
iNos	FW	gtaccctcagttctgtgcct	20	48	220
	RV	tgttgcgttggaagtgtagc	20	51	

Pgc1a	FW	gtggatgaagacggattgcc	20	54	219
	RV	ggtgtggtttgcatggttct	20	52	
Ppara	FW	aacggcgttgaaaacaagga	20	55	191
	RV	aaggaggacagcatcgtgaa	20	52	
Ppia	FW	cttcgagctgtttgcagacaa	21	53	138
	RV	aagtcaccaccctggcacatg	21	57	
Ptp1b	FW	ccctttgaccacagtcgga	20	55	119
	RV	ttggtaaagggccctgggtg	20	58	
Scd1	FW	tgggttgccagtttctttcg	20	55	192
	RV	accacaagaagccacgtttc	20	52	
Socs3	FW	ctggacccattcgggagttc	20	56	148
	RV	ctgggagctaccgaccattg	20	54	
Ucp2	FW	agaccattgcacgagagaa	20	52	156
	RV	aagggaggtcgtcgttcatg	20	51	

Ucp3	FW	acgccattgtcaattgtgct	20	53	179
	RV	agcgttcatgtatcgggtct	20	51	
sXbp1	FW	ttaaggacacgcttggggat	20	54	193
	RV	gcaacagcgtcagaatccat	20	52	

Abbreviations: *Acc* (acetyl-CoA carboxylase), *Atf4* (activating transcription factor 4), *Chop* (DNA damage inducible transcript 3), *Cpt1b* (carnitine palmitoyltransferase 1b), *Fas* (fatty acid synthase), *iNos* (inducible nitric oxide synthase), *Pgc1a* (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), *Ppara* (peroxisome proliferator activated receptor alpha), *Ppia* (peptidylprolyl isomerase a), *Ptp1b* (protein-tyrosine phosphatase 1b), *Scd1* (stearoyl-CoA desaturase 1), *Socs3* (suppressor of cytokine signalling 3), *Ucp2* (mitochondrial uncoupling protein 2), *Ucp3* (mitochondrial uncoupling protein 3), s*Xbp1* (spliced x-box binding protein 1).

RESULTS

Table 2S. Chromatographic and fragmentation characteristics of RSV metabolites quantified by UHPLC-MSn in serum samples.

Metabolite	Abbreviation	RT (min)	$[M-H]^-$ (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)	MS^4 ions (*m/z*)
trans-resveratrol	RSV	2.89	**227**	185, 183, 159, 157, 143, 141		
resveratrol-4'-*O*-sulfate	R4S	2.30	**307**	*227*, 261	185, 183, 159, 157	
resveratrol-3-sulfate [a]	R3S	3.45	**307**	*227*, 243	185, 183, 159, 157	
resveratrol-4'-*O*-glucuronide [b]	R4G	2.57	**403**	*175, 227*	175: 113; 227: 185, 183, 159, 157	
resveratrol-3-*O*-glucuronide	R3G	2.75	**403**	*175, 227*	175: 113; 227: 185, 183, 159, 157	
resveratrol-sulfate-sulfate [a]	RDS	3.89	**387**	*307*, 369	*227*	185, 183, 159, 157
resveratrol-glucuronide-sulfate [b]	RSG	2.25	**483**	*307*, 403, 227	*227*	185, 183, 159, 157
resveratrol- glucuronide-glucuronide [b]	RDG	1.31	**579**	403		
dihydroresveratrol	DR	3.29	**229**	123		
dihydroresveratrol-sulfate [c]	DRS	2.84	**309**	*229*	123	

dihydroresveratrol-glucuronide [c]	DRG	2.24	**405**	175, *229*	123	
dihydroresveratrol-glucuronide -sulfate [c]	DRSG	2.25	**485**	*309*, 405	*229*	123, 187

MS^2 and MS^3 ions in italic were those subjected to MS^3 MS^4 fragmentation for unambiguous identification.

[a] Quantified as R4S equivalents

[b] Quantified as R3G equivalents

[c] Quantified as DR equivalents

Abbreviations: R4G: resveratrol-4'-*O*-glucuronide; R3G: resveratrol-3-*O*-glucuronide; R3S: resveratrol-3-*O*-sulfate; R4S: resveratrol-4'-*O*-sulfate; RDS: resveratrol disulfate; RDG: resveratrol diglucuronide; RSG: resveratrol sufate glucuronide; DRG: dihydroresveratrol glucuronide; DRS: dihydroresveratrol sulfate; DRSG: dihydroresveratrol sufate glucuronide.

RESULTS

Table 3S. Different biometric parameters of liver, calf skeletal muscle and eWAT. wt was represented as the percentage of the total body wt, whereas mg of TAG and TC content were calculated for each g of the respective tissue.

	STD	CAF	CAF + RSV 50 mg/kg	CAF + RSV 100 mg/kg	CAF + RSV 200 mg/kg
Liver					
Wt (%)	2.92 ± 0.12	2.94 ± 0.07	3.16 ± 0.04	3.11 ± 0.13	3.18 ± 0.07
TAG (mg/g)	6.81 ± 1.20	6.80 ± 0.59	5.31 ± 0.33	10.92 ± 0.72 $^{\#}$	5.25 ± 0.67
TC (mg/g)	1.85 ± 0.04	3.28 ± 0.33 *	2.38 ± 0.26 $^{\varepsilon}$	3.76 ± 0.25 $^{\#}$	2.66 ± 0.29 $^{\varepsilon}$
Calf skeletal muscle					
Wt (%)	0.60 ± 0.01	0.45 ± 0.01 *	0.44 ± 0.03	0.47 ± 0.01	0.51 ± 0.02
TAG (mg/g)	0.12 ± 0.01	0.12 ± 0.01	0.08 ± 0.02 $^{\varepsilon}$	0.03 ± 0.01 $^{\#}$	0.04 ± 0.01 $^{\#}$
TC (mg/g)	0.49 ± 0.06	0.54 ± 0.05 $^{\phi}$	0.25 ± 0.05 $^{\#}$	0.22 ± 0.06 $^{\#}$	0.24 ± 0.04 $^{\#}$
eWAT					

Wt (%)	2.04 ± 0.16	3.97 ± 0.27 *	4.07 ± 0.35	3.42 ± 0.16	2.91 ± 0.21 #
TAG (mg/g)	2.38 ± 0.09	2.56 ± 0.10	2.57 ± 0.14	3.04 ± 0.15 #	2.57 ± 0.11
TC (mg/g)	0.47 ± 0.05	0.67 ± 0.07 ϕ	0.82 ± 0.12	0.89 ± 0.15	0.50 ± 0.07

Data are expressed as the mean ± SEM, n=6. * $p < 0.05$ and ϕ $p < 0.1$, Student's t-test comparing the CAF group to the STD group. # $p < 0.05$ and ε $p < 0.1$, Student's t-test comparing the RSV group respect to the CAF group.

Abbreviations: CAF: cafeteria diet; eWAT: epididymal white adipose tissue; RSV: resveratrol; STD: standard chow diet; TAG: triacylglycerol; TC: total cholesterol; wt: weight.

RESULTS

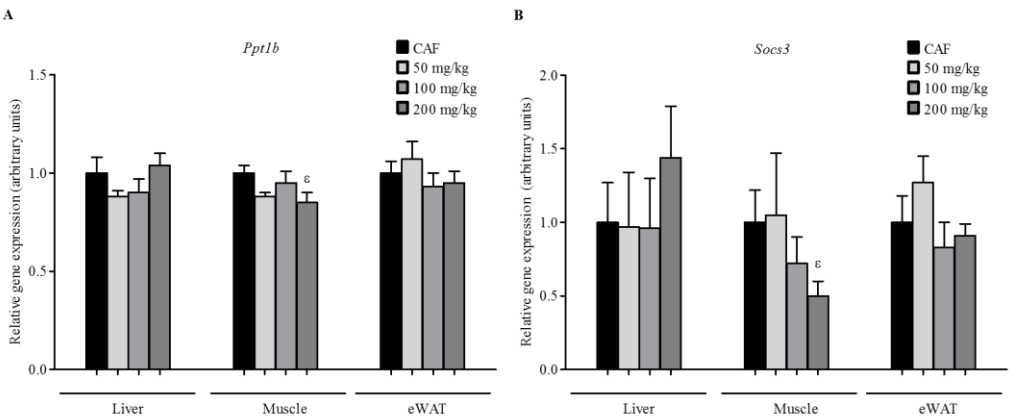

Figure 2S. Inhibitors of the leptin signalling cascade analyzed in rats fed the CAF diet and treated with three concentrations of RSV (50, 100 and 200 mg/kg). The rats were fed the CAF diet for 9 weeks. Then, the CAF rats were treated orally with VH or RSV (50, 100, or 200 mg per kg of body wt) for 3 weeks. (A) *Ptp1b* gene expression; (B) *Socs3* gene expression. The two graphics show the results for the three studied tissues: liver, calf skeletal muscle and eWAT.

Data are expressed as the mean ± SEM, n=6. $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$, Student's t-test comparing the RSV group with the CAF group.

CAF: cafeteria diet; eWAT: epididymal white adipose tissue; RSV: resveratrol; VH: vehicle; wt: weight.

Ptp1b (protein-tyrosine phosphatase 1b); *Socs3* (suppressor of cytokine signalling 3).

RESULTS

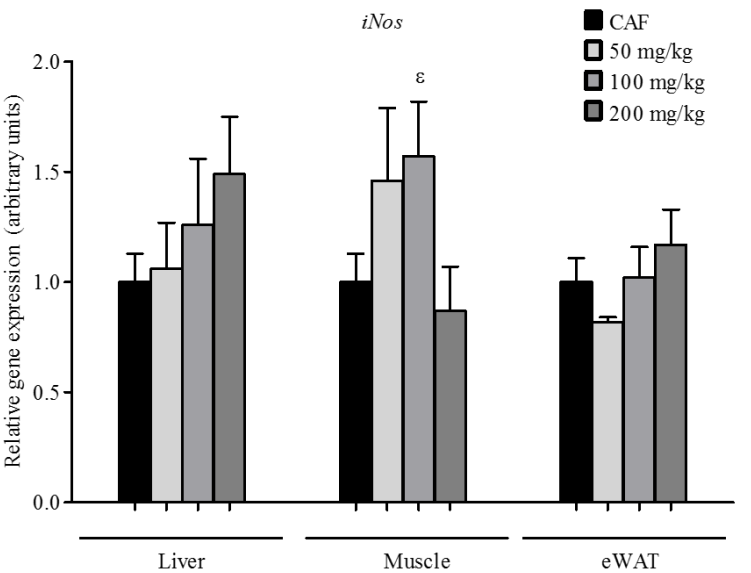

Figure 3S. Inflammatory marker expression analyzed in rats fed the CAF diet and treated with three concentrations of RSV (50, 100 and 200 mg/kg). The rats were fed the CAF diet for 9 weeks. Then, the CAF rats were treated orally with VH or RSV (50, 100, or 200 mg per kg of body wt) for 3 weeks. *iNos* gene expression. In the graphic we can observe the results for the three studied tissues: liver, calf skeletal muscle and eWAT.

Data are expressed as the mean ± SEM, n=6. $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$, Student's t-test comparing the RSV group with the CAF group.

CAF: cafeteria diet; eWAT: epididymal white adipose tissue; RSV: resveratrol; VH: vehicle; wt: weight.

iNos (inducible nitric oxide synthase).

RESULTS

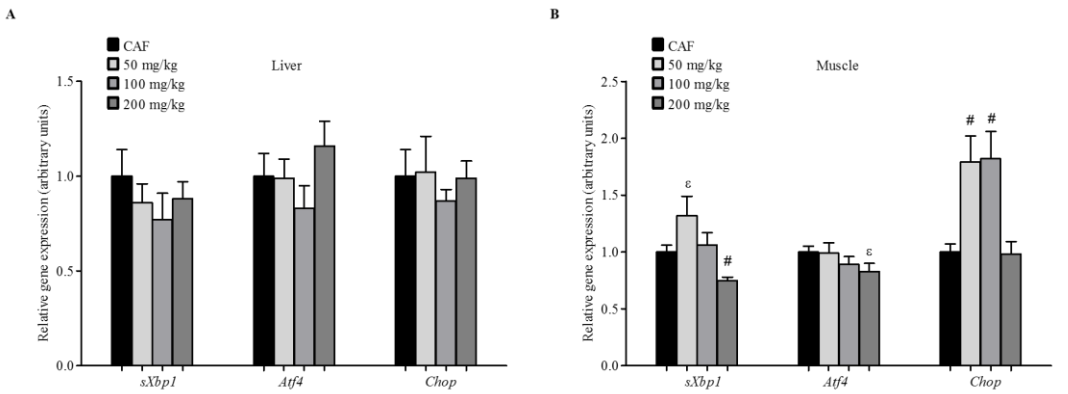

Figure 4S. ER stress marker expression analyzed in rats fed the CAF diet and treated with three concentrations of RSV (50, 100 and 200 mg/kg). The rats were fed the CAF diet for 9 weeks. Then, the CAF rats were treated orally with VH or RSV (50, 100, or 200 mg per kg of body wt) for 3 weeks. (A) The results for gene expression in the liver; (B) the results for gene expression in calf skeletal muscle. The two graphics show the results for the three studied genes: s*Xbp1*, *Atf4* and *Chop*.

Data are expressed as the mean ± SEM, n=6. $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$, Student's t-test comparing the RSV group with the CAF group.

CAF: cafeteria diet; RSV: resveratrol; VH: vehicle; wt: weight.

Atf4 (activating transcription factor 4), *Chop* (DNA damage inducible transcript 3), s*Xbp1* (spliced x-box binding protein 1).

RESULTS

Table 4S. Correlation analysis of the most relevant biometric parameters or the leptin sensitivity index (LSI) of liver, calf skeletal muscle and eWAT and the metabolites of RSV present in the serum of rats treated with 50, 100 or 200 mg/kg RSV.

RSV Metabolites (μM)		Body wt gain (g)	Total body fat content (%)	Serum leptin (ng/mL)	LSI Liver	LSI Muscle	LSI eWAT
Phase II	**R4G**	ns	-0.67 *	-0.66 *	0.72 *	0.69 *	0.79 *
	R3G	ns	ns	ns	ns	ns	ns
	RDG	ns	ns	ns	0.60 #	ns	ns
	R4S	ns	-0.60 #	ns	ns	0.81 *	0.79 *
	R3S	ns	-0.76 *	-0.60 #	ns	ns	ns
	RDS	ns	ns	ns	ns	ns	0.67 #
	RSG	ns	ns	ns	ns	0.67 #	ns
Microbiota	**DRG**	ns	ns	ns	0.60 #	ns	ns
	DRS	ns	ns	ns	ns	0.69 *	ns
	DRSG	-0.66 *	ns	ns	ns	0.81 *	0.71 *

Data are expressed as a mean ± SEM, n=6. Non-parametric Spearman test. * $p > 0.05$ and # $p > 0.1$.

Abbreviations: eWAT: epididymal white adipose tissue; ns: non-significant; RSV: resveratrol; wt: weight.

R4G: resveratrol-4'-*O*-glucuronide; R3G: resveratrol-3-*O*-glucuronide; R3S: resveratrol-3-*O*-sulfate; R4S: resveratrol-4'-*O*-sulfate; RDS: resveratrol disulfate; RDG: resveratrol diglucuronide; RSG: resveratrol sufate glucuronide; DRG: dihydroresveratrol glucuronide; DRS: dihydroresveratrol sulfate; DRSG: dihydroresveratrol sufate glucuronide.

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RESULTS

Manuscript 3

Resveratrol enhances the cellular response to leptin by increasing the cell surface expression of ObRb in palmitate-induced steatotic HepG2 cells

Andrea Ardid-Ruiz, Maria Ibars, Pedro Mena, Daniele del Rio, Cinta Bladé, Lluís Arola, Begoña Muguerza, Gerard Aragonès, Manuel Suárez

Nutrients (Submitted)

RESULTS

PREFACE

This paper continues studying our proposed **hypothesis**, that phenolic compounds such as resveratrol can revert leptin resistance. In this case, we focused on the elucidation of the exact mechanism by which resveratrol acts reverting the leptin resistance induced in a specific type of cells. Concretely, the research is focused in human hepatocarcinoma cell line, HepG2 cells, and the objective is to found the best dose of resveratrol to try to counteract the altered parameters in leptin resistant liver cells. After finding the best dose and incubation time in which leptin acts activating its pathway, we developed an *in vitro* model of leptin resistance. Moreover, we wanted to know if the longest isoform leptin receptor/signal transducer and activator of transcription 3 cascade is only activated when leptin is added to these cells. Finally, the changes caused by resveratrol in the altered lipid profile and gene expression of key enzymes implicated in this metabolism are analysed and the most important molecules involved in the leptin signalling pathway are studied.

RESULTS

Resveratrol enhances the cellular response to leptin by increasing the leptin receptor (ObRb) content in palmitate-induced steatotic HepG2 cells

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Keywords: palmitate, leptin resistance, liver, STAT3, ObRb, SIRT1.

RESULTS

Abstract

Background: When leptin interacts with its hepatic longest receptor isoform (ObRb) promotes the phosphorylation of STAT3 and, consequently, protects the liver from lipid accumulation. However, leptin cannot carry out an effective cell signalling during hepatic steatosis, causing leptin resistance in this tissue. One of the current strategies to combat it, is the use of bioactive compounds including polyphenols.

Objective: Since Resveratrol (RSV) is described as a potent modulator of many metabolic alterations in the liver, we aimed to investigate whether RSV could also re-establish appropriate leptin action and fat accumulation in palmitate-induced HepG2 cells.

Methods: An *in vitro* model of steatosis-leptin resistance was created incubating HepG2 cells with 0.5 mM Palmitate + 30 mM Glucose during 48 h and different doses and incubation times of RSV were tested to try to restore the leptin cascade. The mRNA expression and protein content of molecules implicated in the leptin sensitivity were analysed.

Results: Accordingly, RSV 10 μM counteracted the leptin resistance produced in our *in vitro* model of hepatocellular steatosis by increasing the protein levels of ObRb, without changing SIRT1 activity. Moreover, RSV down-regulated expression of relevant genes involved in lipogenic pathways. No changes in the expression of ER stress and inflammation markers were observed.

Conclusions: Our results demonstrates that RSV restored leptin sensitivity in a cellular model of hepatic steatosis and, therefore, it could be a valid nutritional strategy to complement the existing ones for treating hepatic fat accumulation.

RESULTS

1. Introduction

Liver steatosis, a condition characterized primarily by excessive lipid accumulation in the form of triglycerides and cholesterol in this organ, is increasing among both adults and children in proportion to the rapid rise in obesity epidemic [1]. In fact, liver steatosis is considered the hepatic component of the metabolic syndrome associated with obesity and leptin resistance (LR) [1,2]. In this sense, it has been reported that exogenous leptin administration regulates the hepatic lipid and glucose homeostasis in rodent models of dysfunctional leptin signalling such *ob/ob* and *db/db* mice, suggesting that hepatic leptin action is essential for the maintenance of a correct fat and carbohydrate metabolism in this tissue [3,4].

At molecular level, when leptin interacts with its hepatic longest receptor isoform (ObRb) promotes the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) through the activation of Jak2 tyrosine kinase (JAK2). Subsequently, STAT3 dimerizes and translocates into the nucleus [5], down-regulating hepatic lipogenesis and up-regulating fatty acid oxidation [6]. Thus, defects in hepatic leptin action, as occur in states of diet-induced obesity (DIO), impair the function of liver leading to hyperlipidemia and hepatic steatosis [1,4]. The molecular basis for this lack of leptin response in the steatotic liver is not yet completely known but it has been mainly attributed to several mechanisms. These include ObRb insensitivity, enhanced endoplasmic reticulum (ER) stress and inflammation, impaired nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase sirtuin 1

(SIRT1) activity and the overexpression of inhibitory factors such as suppressor of cytokine signalling 3 (SOCS3) and protein-tyrosine phosphatase (PTP1B) [4,5,7].

As pharmacologic approaches to restoring leptin sensitivity in liver have not yet been found, one strategy would be using dietary bioactive food compounds to complement the existing therapeutic strategies to combat fatty liver [8-10]. In this sense, we previously reported that a polyphenol-rich extract from grape seeds protected against hepatic fat accumulation by increasing cellular NAD^+ availability and SIRT1 functionality [11], but the role of SIRT1 in the regulation of leptin signalling in the liver is still unclear and further studies are needed [12,13]. In addition, resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, RSV), a dietary non-flavonoid polyphenol found in grapes and red wine, has been demonstrated to exert protective effects on the liver against lipid metabolic disorders in various rodent models of hepatic steatosis [14]. However, the exact mechanisms by which RSV realize its beneficial effect are still unclear, although it has been shown to modulate SIRT1 activity and ER stress [15]. Given that little is currently known about how RSV impact on leptin sensitivity in the liver, the effects of this compound on the cellular response to leptin in an experimental model of hepatic fat accumulation will be of interest. Therefore, in this study, we aimed to investigate the effects of RSV on leptin signalling in palmitate-induced steatotic HepG2 cells and determined the role of leptin action in the beneficial effects of RSV on liver steatosis.

2. Materials and Methods

RESULTS

2.1 Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), penicillin/streptomycin (P/S), L-glutamine (L-Glut) and fetal bovine serum (FBS) were purchased from Lonza (Barcelona, Spain). Non-essential amino acids (NEAA), amphotericin B, Hepes, palmitate (Palm), non-essential fatty acid (NEFA), free-bovine serum albumin (BSA), 2-mercaptoethanol and $CaCl_2$ obtained from Sigma-Aldrich (Madrid, Spain). Glucose (Glc), fructose (Fruc), neutral red dye and glacial acetic acid were purchased in Panreac AppliChem (Barcelona, Spain). Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, RSV) was purchased from Fagron Iberica (Barcelona, Spain). Formaldehyde and ethanol were obtained from Millipore (Madrid, Spain). Recombinant human leptin was obtained from BioVision (San Francisco, USA).

2.2 Cell culture and general experimental treatment

The human hepatoma cells (HepG2, HB-865; ATCC, Virginia, USA) were cultured in DMEM, supplemented with 100 X NEAA, 10,000 U/mL P/S, 250 mg/L amphotericin B, 200 mM L-Glut, FBS and 1.25 M Hepes at 37 °C in a humidified atmosphere containing 5 % CO_2, with medium changes three times a week. Cells were incubated in 12-well plates at a density of $5x10^5$ cells/well for 48 h or until they were 70-80 % confluent before starting the experimental treatments. Hepatic steatosis was induced by treating cells with Palm (0.5 mM), Glc (30 mM), Palm + Glc (0.5 mM and 30 mM, respectively) or Glc + Fruc (30 mM and 5.5 mM, respectively) for 48 h using albumin as fatty acid carrier (7:1, Palm-to-albumin ratio). After successfully

producing hepatic steatosis model, cell were treated with or without RSV (1, 5, 10, 50 μM) for different times intervals (20 min, 6 h and 24 h). Afterwards, cells were starved during 16 h with serum-free media and, then, cells were exposed to 10, 100 or 1,000 ng/mL recombinant human leptin during 20 min.

2.3 Palmitate-BSA solution preparation

The Palm-BSA complexes were prepared as previously described (Rojas, *et al.* (2014)) with minor modifications [17]. Briefly, 13.9 mg sodium Palm was dissolved in 0.5 mL sterile water (100 mM Palm stock solution) by heating (70 °C) and mixing (250 rpm) for 10 min in a thermomixer (Grant-Bio). A portion of the Palm stock solution (50 μL) was added to 950 μL serum-free DMEM containing 5 % NEFA-free BSA (the 5 mM Palm working solution). The Palm working solution was also heated (40 °C) and shaken (250 rpm) for 1 h. Finally, the working solution was filtered (20 nm diameter filter) and immediately used to treat the cells. Serum-free DMEM containing 5 % NEFA-free BSA was used as the control vehicle.

2.4 Determination of cell viability by Neutral Red assay

Cells were incubated with Neutral Red dye to assess toxicity as previously described [17,18]. Briefly, 1 mL of freshly prepared neutral red solution (0.05 mg/mL) was pre-warmed to 37 °C and added to each well (12-well plate). The cells were then incubated for 3 h at 37 °C. Then, the dye was removed and cells were exposed to 1 mL/well of fixative solution ($CaCl_2$ and 37 % formaldehyde). After washing, cells were incubated with 1 mL of decolorizing solution (1 % glacial acetic acid and 50 % absolute ethanol).

Following 10 min of shaking at room temperature to release all of the dye from the cells, absorbance was measured at 540 nm using an automatic plate reader (EON Microplate, BioTek, Vermont, USA).

2.5 Quantification of triglycerides content

The triacylglycerides (TAG) concentration in cell lysates were measured using the GPO Method enzymatic colorimetric assay (QCA, Barcelona, Spain) according to the manufacturer's instructions. All results were normalized to total protein content measured using the BCA method and all data were calculated respect to the control group.

2.6 SIRT1 activity assay

The SIRT1 activity was determined using a SIRT1 direct fluorescent screening assay kit (Cayman, Ann Arbor, MI) as previously described [11]. Briefly, a total of 25 µL of assay buffer (50 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$), 5 µL of tissue extract (1.5 mg/mL), and 15 µL of substrate (Arg-His-Lys-Lys(ε-acetyl)-)-7-amino-4-methylcoumarin) solution were added to all wells. The fluorescence intensity was monitored every 2 min for 1 h using the fluorescence plate reader BertholdTech TriStar2S (Berthold Technologies, Germany), applying an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results are expressed as the rate of reaction for the first 30 min, when there was a linear correlation between the fluorescence and this period of time.

2.7 Total RNA isolation and gene expression analysis

The total RNA was obtained from the cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). The NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure the quantity and purity of RNA. Samples with an adequate RNA concentration (A260/A280 ≥ 1.8) and purity (A230/A260 ≥ 2.0) were selected for reverse transcription. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Madrid, Spain) was used to generate the cDNA. 15 ng of cDNA were subjected to quantitative PCR (qPCR) with iTaq Universal SYBR Green Supermix (Bio-Rad), using the 7900HT Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The thermal profile settings were 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min. The forward (FW) and reverse (RV) primers used in this study were obtained from Biomers.net (Ulm, Germany) and can be found in **Table 1S**. A cycle threshold (Ct) value was generated by setting the threshold during the geometric phase of the cDNA sample amplification. Relative expression of each gene was calculated referring to the cyclophilin peptidylprolyl isomerase A (*Ppia*) mRNA levels and normalized to the control group. ΔΔCt method was used and corrected for primer efficiency [19]. For that, only samples with a quantification cycle lower than 30 were used for fold change calculation.

2.8 Western blotting analysis

The HepG2 cells were harvested and homogenized in 300 μL RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % NP40, and 0.25 % Na-deoxycholate,

containing protease and phosphatase inhibitors). The total protein content was measured using the Pierce BCA protein assay kit (Thermo Scientific Madrid, Spain). Samples were denatured by mixing with loading buffer solution (0.5 M Tris-HCl pH 6.8, glycerol, SDS, 2-mercaptoethanol and Bromophenol Blue) and then heated at 99 ºC during 5 min using a thermocycler (Multigen Labnet, Barcelona, Spain). Acrylamide gels were prepared using TGX Fast Cast Acrylamide Kit, 10 % (Bio-Rad, Barcelona, Spain) and 25 µg of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) using electrophoresis buffer (glycine 192 mM, Tris base 25 mM and 1 % SDS). Proteins were electrotransfered onto supported PVDF membranes (Trans-Blot Turbo Mini PVDF Transfer Packs, Bio-Rad). After blocking with 5 % of non-fat dried milk, membranes were incubated with gentle agitation overnight at 4 °C with specific antibody for pSTAT3 (ab68153; Abcam, Cambridge, UK) and ObRb (ab177469; Abcam) diluted 1:1,000. For β-actin analysis as a loading control, membranes were incubated with a rabbit anti-actin primary antibody (A2066; Sigma-Aldrich), diluted 1:1,000. Finally, membranes were incubated with anti-rabbit horseradish peroxidase secondary antibody (NA9344, GE Healthcare, Barcelona, Spain), diluted 1:10,000. Protein levels were detected with the chemiluminescent detection reagent ECL Select (GE Healthcare) and using GeneSys image acquisition software (G:Box series, Syngene, Barcelona, Spain). Lastly, protein bands were quantitated by densitometry using ImageJ software (W.S Rasband, Bethesda, MD, USA) and each band was normalized by the corresponding β-actin band and finally the treatment groups were normalized by the control group.

2.9 Immunofluorescence analysis

ObRb content in steatotic HepG2 cells treated with resveratrol at 10 and 50 μM for 24 h were visualized by immunofluorescence detection. After fixation, the cells were blocked with 1 % BSA in PBST for 1 h at room temperature and incubated with primary anti-leptin receptor antibody (5 μg/mL, ab60042; Abcam) in a humidified chamber overnight at 4 °C. After washing with PBS, the cells were incubated with the secondary antibody (1/250, ab96899; Abcam) for 1 h at room temperature in the dark. Subsequently, the cells were exposed to 1 μg/mL 4,6-diamidino-2-phenylindole (DAPI, Thermo Scientific) for 1 min at room temperature, mounted with Vectashield (Vector Laboratories) and sealed with nail polish to prevent drying and movement under microscope. Images were taken by using a Nikon Eclipse TE2000-E laser scanning confocal microscope at 60 X magnification.

2.10 siRNA knockdown of ObRb in HepG2 cells

RNA interference to reduce ObRb expression was performed with a set of three siRNA oligonucleotides (Trilencer-27 human siRNA) obtained from OriGene Technologies Inc. (SR302671, Rockville, MD, USA). The trilencer-27 universal scrambled negative control siRNA duplex (SR30004, OriGene Technologies Inc.) was used as the scrambled siRNA control. The HepG2 cells were transfected for 36 h with 10 nM of each of three siRNA using the siTRAN 1.0 transfection reagent (TT300002, OriGene Technologies Inc.) following the manufacturer's protocol. After that, cells were starved during 16 h with serum-free media and were treated with 10

ng/mL recombinant human leptin during 20 min. The total protein extract from these transfected cells were obtained and analyzed for ObRb knockdown and pSTAT3 levels by Western blotting as previously described.

2.11 UHPLC-MS^n analysis of cell media

Cell media supernatants were collected and analyzed by UHPLC-MS^n to determine the stability and intracellular metabolism of RSV in cell culture. Cell media was extracted according to Sala, *et al.* (2015) [20] and analyzed according to Bresciani, *et al.* (2014) [21]. Briefly, a mixture of 150 µL of cell media and 150 µL of cold methanol acidified with formic acid 1 % (v/v) was vortexed for 1 min. The mixture was then centrifuged at 12,000 rpm for 5 min at room temperature. The supernatant was directly injected into the UHPLC-MS system. Samples were analyzed by Accela UHPLC 1250 equipped with linear ion trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). Metabolite identification was carried out by comparing the retention time with authentic standards and/or MS^2 fragmentation patterns. Specifically, the glucuronide forms of RSV, with a m/z at 403 were fragmented using a collision induced dissociation (CID) equal to 16 (arbitrary units), whereas sulfate conjugates (m/z 307) required a CID of 23 and then free RSV was further fragmented with a CID of 34. Data processing was performed using Xcalibur software from Thermo Scientific. Quantification was performed with

calibration curves of pure standards in the case of resveratrol (RSV), resveratrol-3-*O*-sulfate (R3S), resveratrol-4'-*O*-sulfate (R4S) and resveratrol-3-*O*-glucuronide (R3G).

2.12 Statistical analysis

The data are expressed as the means ± SD of at least three independent assays for each experiment. The data were evaluated by Student's T-test to identify significant differences between the controls and the treatments. Outliers were determined by Grubbs' test. The statistical analyses were performed using XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel (Addinsoft, Paris, France, 2017). Graphics were done using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). The differences were considered significant when the *p* values were less than 0.05 or considered as a tendency when they were less than 0.1.

3. Results

3.1 ObRb/STAT3 signalling pathway was directly activated by physiological concentrations of leptin in HepG2 cells

In order to establish the experimental conditions needed to activate the hepatic leptin signalling pathway, HepG2 cells were incubated at physiological (10 ng/mL) and pharmacological concentrations of leptin (100 and 1,000 ng/mL) during different incubation times (5, 10, 15, 20 and 30 min). Notably, only the incubation of cells with 10 and 100 ng/mL of leptin during 20 min resulted in a significant increase of the phosphorylation levels of STAT3 (pSTAT3) (**Figure 1A**). Thus, leptin at 10 ng/mL

for 20 min was used in subsequent experiments to achieve maximal activation of STAT3 at physiological conditions. In addition, to further determine the role of leptin receptor ObRb in STAT3 activation, HepG2 cells were pre-treated for 36 h with a specific siRNA against ObRb. Accordingly, pSTAT3 levels were mainly attenuated when ObRb was silenced (**Figure 1B**), indicating that the phosphorylation of STAT3 in our experimental model was a direct consequence of the activation of ObRb with leptin.

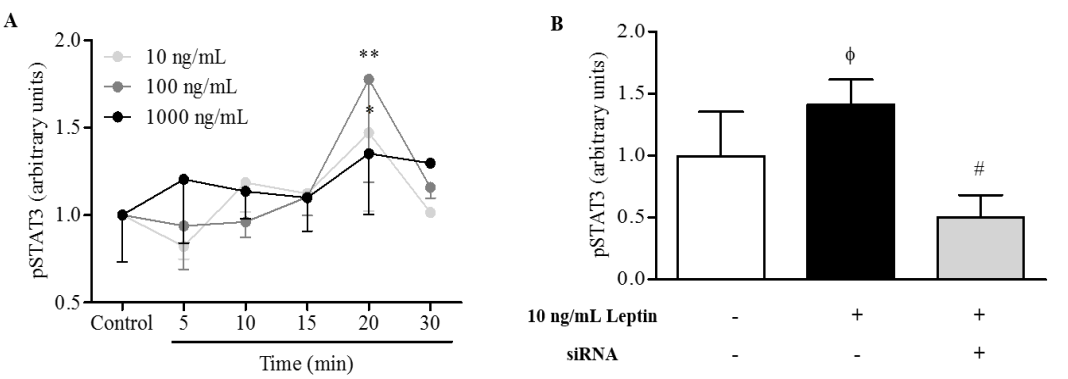

Figure 1. Activation of the leptin signalling pathway in HepG2 cells. (A) pSTAT3 protein levels of different leptin concentrations (10, 100 and 1,000 ng/mL) and incubation times (5, 10, 15, 20 and 30 min) used to activate the leptin signalling pathway in HepG2 cells. $^{*}p < 0.05$ and $^{\phi}p < 0.1$ comparing leptin conditions respect to control group making a T-student test. Data are expressed as a mean ± SD of 3 replicates. (B) pSTAT3 protein levels incubating HepG2 cells during 36 h with ObRb siRNA, to observe if pSTAT3 amounts were reduced due to the ObRb silencing. Moreover, 10 ng/mL of leptin were added, or not, the last 20 min of the treatment. $^{*}p < 0.05$ and $^{\phi}p < 0.1$ comparing the absence and presence of leptin and $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$ comparing the absence and presence of ObRb siRNA making a T-student test. Data are expressed as a mean ± SD of 4 replicates.

RESULTS

3.2 A mixture of palmitate and glucose for 48 hours induced leptin resistance and fat-overloading in HepG2 cells

Initially, HepG2 cells were incubated with different treatments to produce LR and cellular steatosis. In this sense, the supplementation of the cell media with 0.5 mM palmitate (Palm) or with 0.5 mM Palm + 30 mM of glucose (0.5 mM Palm + 30 mM Glc) during 48 h were the two most effective treatments to abolish STAT3 phosphorylation despite the presence of 10 ng/mL of leptin in the cell media (**Figure 2A**). In addition, to further confirm the loss of leptin signalling induced by these supplements, the effect on lipid accumulation was also monitored after each treatment. Accordingly, the incubation for 48 h with 0.5 mM Palm + 30 mM Glc resulted in the highest significant increase in cellular TAG content as compared to the control condition (**Figure 2B)**, and this supplementation (0.5 mM Palm + 30 mM Glc) was therefore used in subsequent experiments to successfully induce LR and fat accumulation in HepG2 cells.

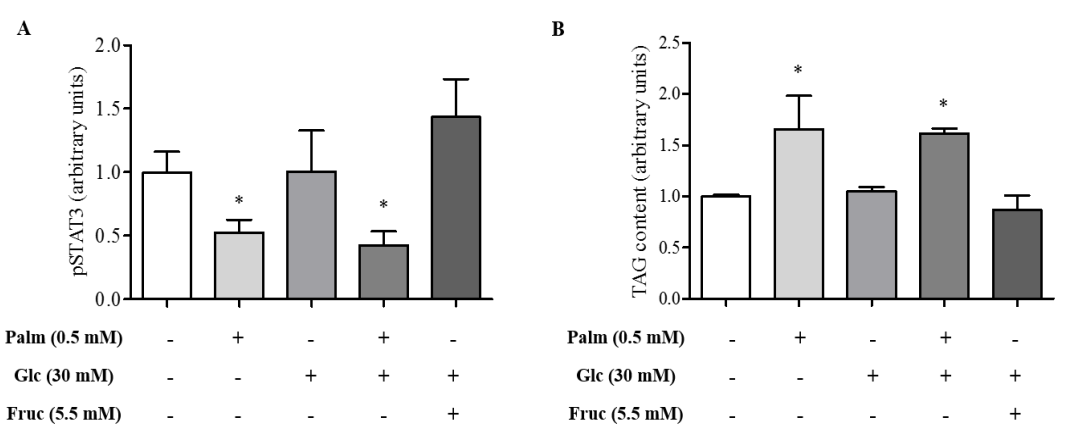

Figure 2. The creation of the leptin resistance (LR) model in HepG2 cells. pSTAT3 protein levels (A) and TAG content (B) of the different experimentally-induced LR model in HepG2. Cells were treated with 0.5 mM Palm, 30 mM Glc, 0.5 mM Palm + 30 mM Glc and 30 mM Glc + 5.5 mM Fruc during 48 h and the leptin signalling pathway was activated after adding 10 ng/mL of leptin the last 20 min. * $p < 0.05$ and $^{\phi}$ $p < 0.1$ comparing treatment conditions respect to control group making a T-student test. Data are expressed as a mean ± SD of 3 replicates.

3.3 Resveratrol improved leptin signalling and lipid content in steatotic HepG2 cells by modulating lipogenic gene expression but not fatty acid oxidation

Thereafter, in order to evaluate whether RSV could sensitize our experimental cell model of hepatocellular steatosis to leptin, cells were treated with or without RSV (1, 5, 10, 50 μM) for different times intervals (20 min, 6 h and 24 h) and then were exposed to 10 ng/mL of leptin the last 20 min. Notably, the treatment of HepG2 cells with 1, 5, 10 and 50 μM of RSV during 20 min had no effect on pSTAT3 levels (**Figure 3A**) and TAG content (**Figure 3B**), and after 6 h of treatment, only the dose of 50 μM of RSV was capable to revert the effects on STAT3 phosphorylation (**Figure 3C**), although no significant changes were observed in fat accumulation (**Figure 3D**). In contrast, after 24 h of treatment, both 10 and 50 μM of RSV significantly restored pSTAT3 levels in steatotic cells to values similar to those observed in non-steatotic cells (**Figure 3E**), indicating that RSV could be a valid tool to rescue leptin sensitivity in our cellular model. In addition, after 24 h of treatment with RSV, the intracellular concentrations of TAG decreased significantly to basal levels in all doses tested, reversing the fat accumulation observed in the untreated cells, although the dose of 50 μM did not reach statistical significance (**Figure 3F**).

RESULTS

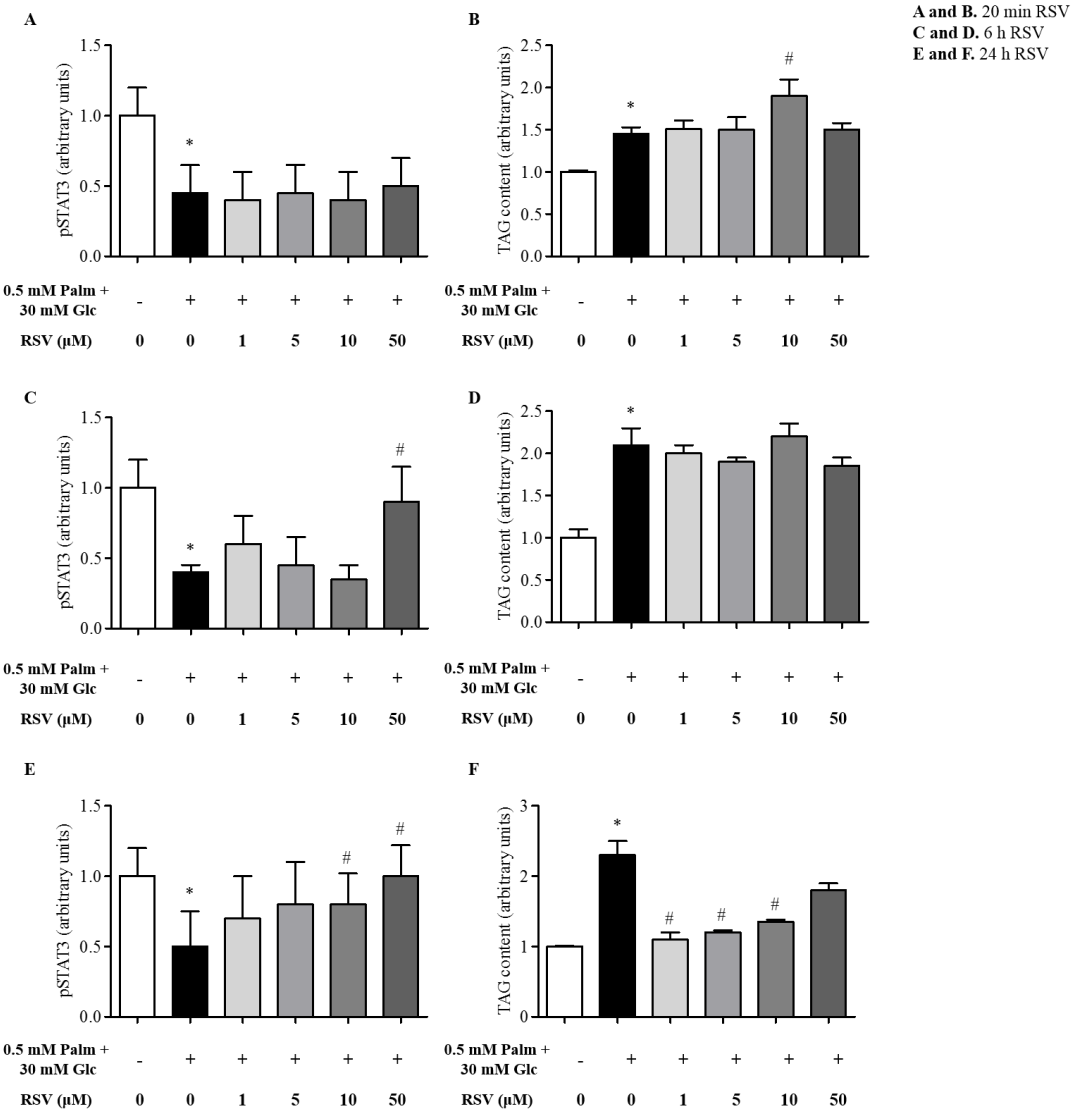

Figure 3. The leptin signalling pathway activation using RSV. pSTAT3 (A, C and E) and TAG content (B, D and F) were analysed after inducing the leptin resistance (LR) incubating HepG2 with 0.5 mM Palm + 30 mM Glc during 48 h. Additionally, cells were treated with RSV at different doses the last 20 min (A and B), 6 h (C and D) and 24 h (E and F) and, finally, the leptin signalling pathway was activated using 10 ng/mL leptin the last 20 min. $^{*}p < 0.05$ and $^{\phi}p < 0.1$ comparing 0.5 mM Palm + 30 mM Glc group respect to control group and $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$ comparing RSV groups respect to 0.5 mM Palm + 30 mM Glc group making an T-student test. Data are expressed as a mean ± SD of 6 replicates.

RESULTS

Accordingly, when the mRNA levels of the key genes involved in lipid metabolism were assessed by RT-qPCR, treatment with 10 μM of RSV for 24 h resulted in decreased *fatty acid synthase* (*Fas*) and *stearoyl-CoA desaturase-1* (*Scd1*) mRNA levels compared to the untreated cells, indicating that the delipidating effect of RSV may be mediated by limiting the capacity for *de novo* lipogenesis (**Figure 4A**). In contrast, and contrary to our expectations, no significant changes were observed in the expression of genes that encode enzymes for fatty acid oxidation (**Figure 4B**).

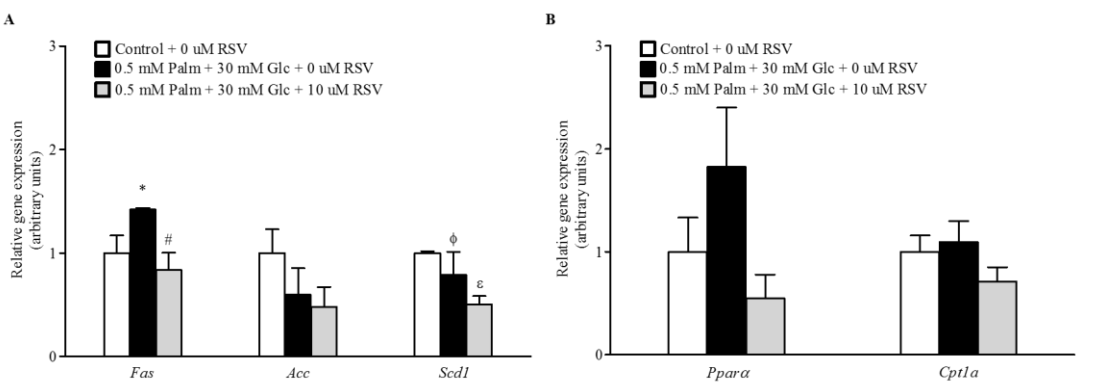

Figure 4. The state of genes implicated in lipid metabolism. The mRNA expression of lipogenic (A) and lipolytic (B) genes in HepG2 cells after 48 h incubation with 0.5 mM Palm + 30 mM Glc and, additionally, the last 24 h supplemented with 10 μM RSV. 10 ng/mL of leptin were added the last 20 min to activate the leptin signalling pathway. *Fas* (fatty acid synthase), *Acc* (acetyl-CoA carboxylase) and *Scd1* (stearoyl-CoA desaturase-1) were evaluated to study the lipids formation. *Pparα* (peroxisome proliferator-activated receptor α) and *Cpt1a* (carnitine palmitoyltransferase 1a) were analyzed to study the β-oxidation pathway. $^{*}p < 0.05$ and $^{\phi}p < 0.1$ comparing 0.5 mM Palm + 30 mM Glc group respect to control group and $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$ comparing RSV groups respect to 0.5 mM Palm + 30 mM Glc group making an T-student test. Data are expressed as a mean ± SD of 3 replicates.

Finally, it should be noted that the exposure of steatotic HepG2 cells to the highest concentration of RSV tested for 24 h did not decrease cell viability compared to untreated cells as assessed using the neutral red assay (**Figure 1S**).

3.4 Resveratrol did not modulate the expression of inflammation and ER stress related genes in steatotic HepG2 cells

In our attempt to determine the molecular mechanism by which RSV enhances the cellular response to leptin, we next assessed by qRT-PCR the expression levels of selected genes involved in two cellular processes well associated with leptin signalling disruption such as ER stress and inflammation. Although *activating transcription factor 4 (Atf4)* expression was down-regulated after 24 h of RSV treatment, no significant differences were found in *DNA damage inducible transcript 3 (Chop)*, *spliced x-box binding protein 1 (sXBP1)* and *inducible nitric oxide synthase (iNos)* mRNA levels, indicating that the supplementation of cell media with 0.5 mM Palm + 30 mM Gluc for 48 h did not clearly induce ER stress or inflammation in HepG2 cells and, in turn, that these transcripts were also not significantly affected by RSV after 24 h of treatment (**Figure 5A**). In addition, to further investigate the effects of RSV treatment on the leptin signalling pathway, the mRNA expression of negative regulatory molecules involved in STAT3 activation such as *suppressor of cytokine signalling 3* (*Socs3*) and *protein-tyrosine phosphatase 1b* (*Ptp1b*) were also assessed by qRT-PCR in steatotic HepG2 cells treated with 10 μM of RSV for 24 h. However, RSV could not revert the increased expression levels of *Socs3* observed in steatotic cells, and *Ptp1b* mRNA levels were not statistically altered in any experimental condition (**Figure 5B**).

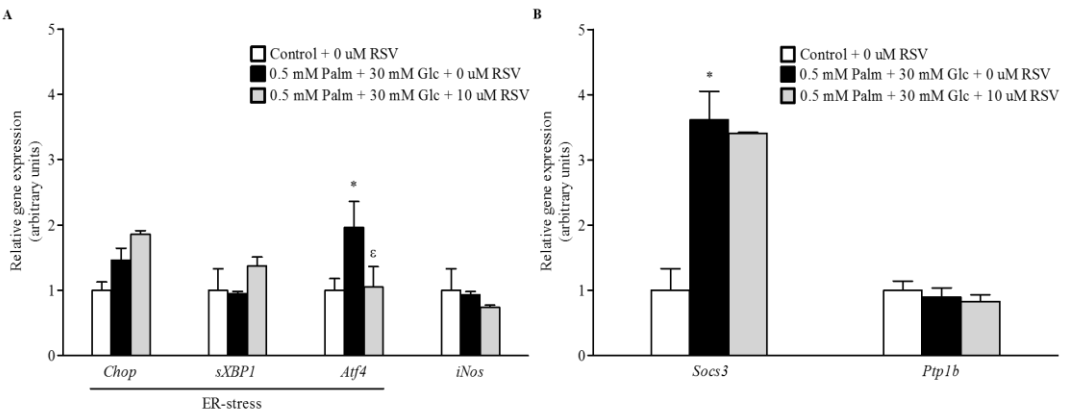

Figure 5. Mechanisms of action of RSV. HepG2 cells were incubated during 48 h using 0.5 mM Palm + 30 mM Glc and, additionally, the last 24 h with 10 μM RSV. 10 ng/mL of leptin were added the last 20 min to activate the leptin signalling pathway. mRNA expression of (A) ER-stress markers: *Chop* (DNA damage inducible transcript 3), *sXbp1* (spliced x-box binding protein 1) and *Atf4* (activating transcription factor 4) and inflammation: *iNos* (inducible nitric oxide synthase), and (B) inhibitors of leptin signalling pathway: *Socs3* (suppressor of cytokine signalling 3) and *Ptp1b* (tyrosine-protein phosphatase non-receptor type 1). $^{*}p < 0.05$ and $^{\phi}p < 0.1$ comparing 0.5 mM Palm + 30 mM Glc group respect to control group and $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$ comparing RSV groups respect to 0.5 mM Palm + 30 mM Glc group making an T-student test. Data are expressed as a mean ± SD of 3 replicates.

3.5 Resveratrol increased the protein levels of leptin receptor ObRb but not SIRT1 activity in steatotic HepG2 cells

We next evaluated whether RSV consumption could result in enhancing SIRT1 activity which could be an additional mechanism involved in the regulation of leptin signalling in hepatic cells. However, the deacetylase activity of SIRT1 was not significantly affected after 24 h of treatment with RSV at any dose (**Figure 6A**), indicating that if RSV had the ability to enhance the cellular response to leptin, it is not mediated by an increase of SIRT1 activity in our experimental model. So, next we determined by immunoblotting whether the modulation of pSTAT3 was directly

mediated by increasing the cell content of the long leptin receptor isoform ObRb. Interestingly, RSV treatment during 24 h, resulted in a significant increase in the protein levels of ObRb in all doses, although only statistically significant differences were observed at 5 and 10 μM (**Figure 6B**). These results were further confirmed by immunocytochemistry using a specific anti-ObRb antibody. Remarkably, ObRb expression was more highly detected in steatotic HepG2 cells treated with 10 and 50 μM of RSV than in untreated cells (**Figure 6C**).

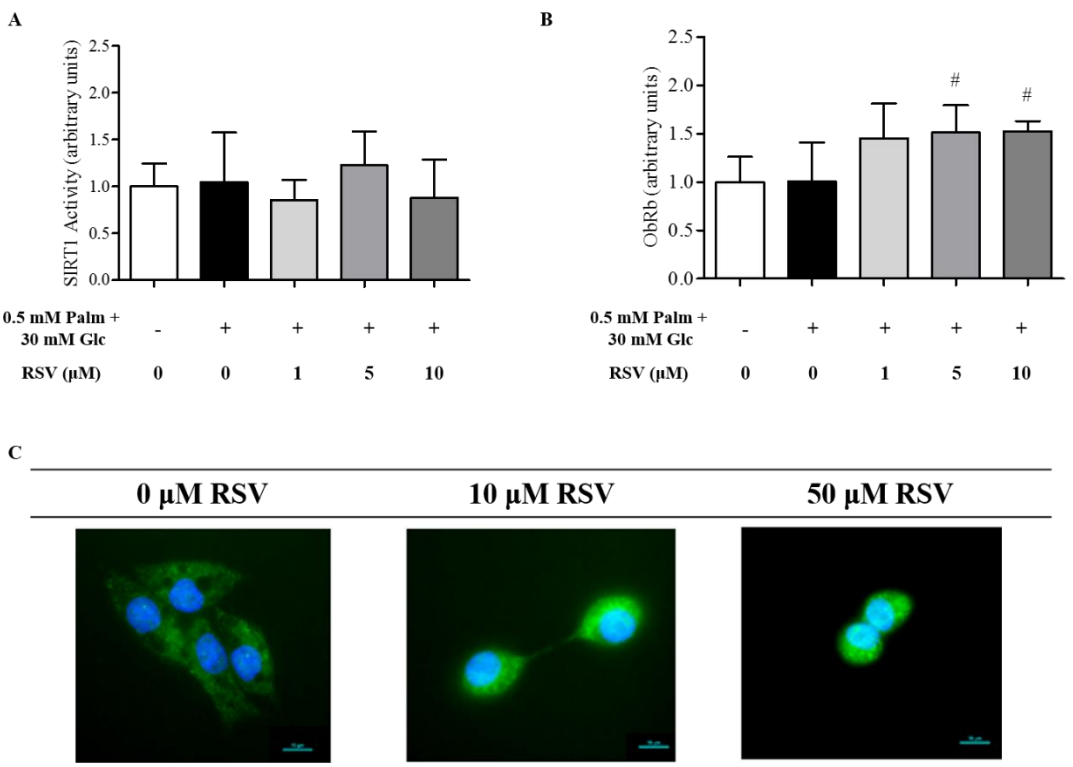

Figure 6. Mechanisms of action of RSV. SIRT1 enzyme activity (A) and the ObRb protein content using WB (B) were measured. $^{*}p < 0.05$ and $^{\phi}p < 0.1$ comparing 0.5 mM Palm + 30 mM Glc group respect to control group and $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$ comparing RSV groups respect to 0.5 mM Palm + 30 mM Glc group making an T-student test. Data are expressed as a mean ± SD of 6 replicates for SIRT1 activity whereas for WB results, data are expressed as a mean ± SD of 6 replicates. (C) It shows

a fluorescent representative image of ObRb content in HepG2 cells treated with 0.5 mM Palm + 30 mM Glc during 48 h and the last 24 h with 0, 10 and 50 μM RSV.

3.6 Resveratrol was rapidly metabolized in HepG2 cells into resveratrol-*O*-3-sulfate

Finally, in order to verify the metabolism of RSV in steatotic HepG2 cells and to get insights into the real molecules behind the effects observed on ObRb expression after 24 h of treatment, cell media was analysed by UHPLC-MS^n at the beginning and end of each incubation time (20 min, 6 h and 24 h) in HepG2 cells. In addition, non-incubated cell media was used to test RSV stability in the absence of cells. Notably, RSV was entirely conjugated to resveratrol-*O*-3-sulfate (R3S) at the concentration of 10 μM after 24 h of incubation. As shown in **Figure 7A**, the proportion of R3S in steatotic HepG2 cells was 5 % after 20 min of incubation, increased to 45 % at 6 h, and reached almost 100 % at 24 h. It should be noted that the incubation of RSV in non-steatotic HepG2 cells did not alter the metabolic activity observed in our experimental model of steatosis, since the percentage of metabolic transformations of RSV to R3S was almost equally than those observed in steatotic HepG2 cells (**Figure 7B**).

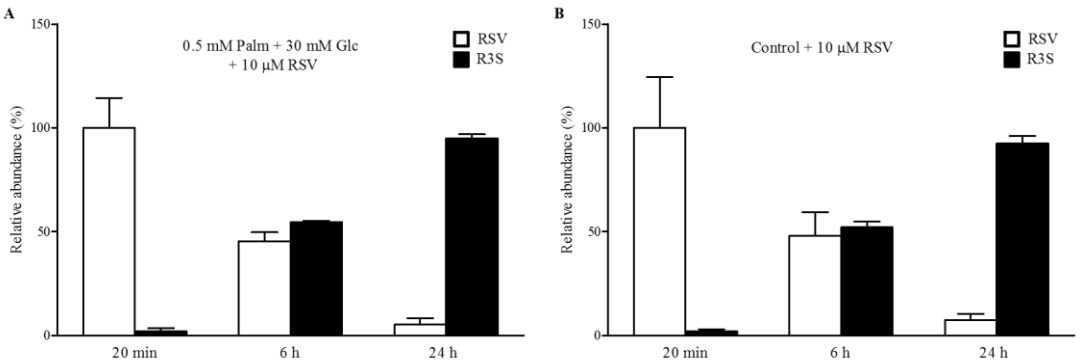

Figure 7. Metabolization of RSV in HepG2 cells. Graphic shows the conversion of 10 µM RSV to R3S in cells treated at different hours (20 min, 6 h and 24 h) with the phenolic compound in normal (A) and 48 h-treated 0.5 mM Palm + 30 mM Glc conditions (B).

4. Discussion

In order to evaluate *in vitro* the impact of RSV into the leptin signalling pathway, firstly, it was necessary to develop a suitable experimental model. Hence, based on some bibliographic references [22-26], we tested different doses (10, 100 and 1,000 ng/mL) and incubation times (5, 10, 20 and 30 min) of leptin in our cell line (HepG2) to determine the conditions in which the leptin signalling pathway is activated. As a result, based on pSTAT3 levels, we determined that the leptin pathway was activated at its maximum when cells were incubated 20 min using 10 and 100 ng/mL of leptin. We selected the lowest dose (10 ng/mL) because no differences were observed compared to 100 ng/mL.

To confirm that STAT3 was being phosphorylated by the activation of the leptin signalling pathway [27] through its receptor ObRb we decided to perform an experiment in which this receptor was silenced by siRNA. A significant reduction in

the levels of pSTAT3 was observed between silenced and non-silenced cells, highlighting the role of ObRb in the activation of the leptin signalling pathway. Therefore, these results confirmed that pSTAT3 is a useful candidate to monitor the leptin signalling pathway in our cellular model.

Then it was necessary to generate a condition of LR in the HepG2 cells. For this purpose, we evaluated 0.5 mM Palm, 30 mM Glc, the combination, and 30 mM Glc + 5.5 mM Fruc as they were previously studied in our group to generate a NAFLD model [19]. We could observe that HepG2 cells incubated with Palm at a concentration of 0.5 mM and the combination of 0.5 mM Palm + 30 mM Glc during 48 h were the most damaged in terms of showing a reduction in the pSTAT3 levels and, consequently, causing a higher LR. Both Palm and Glc are well-known molecules implicated in the damage of the liver since Palm is an inductor of accumulation and synthesis of fatty acids (FA) [16,19], produces ER stress and apoptosis [16], insulin resistance [28], induces LR and impairs hepatic Glc and lipid metabolism in mice [29]. On the other hand, it was described that the incubation of HepG2 cell using higher amounts of Glc induced apoptosis due to an increase of the oxidative stress [30] and inhibited leptin-induced phosphorilation of STAT3 [31].

Since in liver disease there is an increased synthesis of FA [1] and the leptin signalling pathway can partially revert this damage increasing the oxidation of lipids [2], we postulated that a reduction in the leptin signalling pathway in liver may increase TAG accumulation. This fact was confirmed with our results. Thus, we also selected the

TAG content as a way to confirm the presence of LR in our cellular model. Again, the 0.5 mM Palm and 0.5 mM Palm + 30 mM Glc was the treatment whose cells accumulated more TAG. Similarly, Kuo, *et al*. (2012) observed higher amounts of accumulated lipids in these cells compared with the control [32].

These results lead us to choose 48 h with 0.5 mM Palm + 30 mM Glc as the inductor of the LR. To evaluate its effect in the viability of the cells, HepG2 were incubated with 0.5 mM Palm + 30 mM Glc during 48 h and we confirmed that there was not significant changes in the mortality compared to the control (data not shown). Rojas, *et al*. (2014) observed significant results according to the mortality of the cells when they treated them with 0.7 and 1 mM Palm during 24 h [16].

Once established the conditions of the LR, we evaluated the effect of different doses and incubation time of RSV: adding either the last 20 min, 6 h or 24 h RSV at different doses (0, 1, 5, 10 and 50 μM). The supplementation with RSV the last 24 h at the different doses did not altered the viability of the cells previously incubated during 24 h with the 0.5 mM Palm + 30 mM Glc. Hence, as this was the longest incubation time, we could state that RSV treatments, at the conditions evaluated did not alter the viability of HepG2. These results differ from those obtained by Rojas, *et al*. (2014) who observed that the co-treatment of HepG2 during 24 h with Palm (0.7 and 1mM) and RSV (25 and 50 μM) were toxic [16]. This discrepancy in the result may be explained by the differences in the procedures followed, for instance, the combination of Palm and Glc may cause a different reaction of the cells to the treatment.

RESULTS

To determine if RSV was able to act directly (20 min), in a middle time (6 h) or if it had a long-time action (24 h) in the leptin signalling pathway, we analysed the protein levels of pSTAT3 and the TAG content was measured. We obtained that the best time of incubating cells using RSV to revert the LR was 24 h, concluding that RSV exerts its function as a leptin sensitizer in a long-time manner. Focusing at 24 h, RSV at 10 and 50 μM had the capacity to restore the pSTAT3 protein content to basal levels. Moreover, the TAG levels were reduced using all the concentrations unless the highest one. Because 50 μM RSV is considered a higher dose and due to its effect increasing the TAG content in the cells, we decided to perform the next experiments using 10 μM RSV. Rojas, *et al*. (2014), did different experiments and they measured the oil red presented in HepG2 cells. Using 0.5 mM Palm and 25 μM RSV (20 h pre-treatment with RSV + 8 h co-treatment with Palm), they observed a significant reduction in the lipid content of the cells. In addition, they used the same times for doing another experiment using 0.25 mM Palm and different concentrations of RSV (0, 5, 10, 25, 50 and 100 μM). Only 50 and 100 μM RSV reduced the lipids whereas the lowest did not caused any reduction [16]. We postulated that maybe due to we are working at different times and doses of both compounds (0.5 mM Palm + 30 mM Glc and RSV) we obtained different results in the RSV action according to the concentration used. Moreover, returning to the discussion made by Rojas, *et al*. (2014), they suggested that RSV has beneficial effects decreasing TAG content but it has a threshold that can activate ER stress and apoptosis. Thus, when the inhibition of RSV is strong and continuous (24 h and 50 μM), the remaining of Palm that is inside cells promotes the

damage effects caused by this saturated FA [16].

The next step was to know the mechanism of how RSV reduced the lipid content increase in HepG2 cells caused by the addition of 0.5 mM Palm + 30 mM Glc. For this purpose, we analyzed the expression of genes implicated in lipogenesis (i.e., *Fas*, *Acc* and *Scd1*) and in β-oxidation (*Pparα* and *Cpt1a*). According to the genes involved in the lipid synthesis, only *Fas* expression was increased when cells were treated with 0.5 mM Palm + 30 mM Glc and this effect was counteracted using 10 μM RSV. No changes were observed in *Acc* and, finally, there was a significant reduction in the *Scd1* expression when cells were supplemented with RSV although we could not observe an increase in the *Scd1* expression when only treated them with the inductor of the LR. Similarly to our results, Cheng, *et al*. (2015), obtained that C57BL/6 male mice which were icv administered with Palm increased the expression of target lipogenic genes (both *Fas* and *Scd1*) [28]. Rojas, *et al.* (2014) studied the *Scd1* expression in cells treated 28 h with 25 μM RSV and the last 8 h they were co-treated with 0.5 mM Palm. They did not found any significant change [16]. Liu, *et al*. (2010), obtained an increase in the expression of *Fas* when treated HepG2 with Palm at a dose of 0.4 mM. Theoretically, Acc is the responsible of converting acetyl-CoA to malonyl-CoA. When it is combined with another acetyl-CoA, Fas converts them in Palm. Scd1 transforms it into Palmitoleate. As we are working with the combination of Palm and Glc, we hypothesize that maybe cells have a lot of TAG accumulated because of the conversion of the added Palm into complex lipids. Therefore, from the added Glc they increase the expression of *Fas* to obtain Palm but cells stop the process in this step

(down-regulating *Scd1*).

There was a tendency of the 0.5 mM Palm + 30 mM Glc to increase *Pparα* and RSV partially reverted this effect. In the case of *Cpt1a*, we could not observe any significant change between the groups. Liu, *et al*. (2010), obtained an increased expression of *Cpt1* when HepG2 cells were treated with 0.4 mM Palm during 24 h [33]. In one study did by Cui, *et al*. (2010), they obtained a reduction of the Pparα protein content in HepG2 treated during 24 h with oleic acid at 2 mM [34]. Theoretically, Pparα is a nuclear receptor implicated in β-oxidation. One of its target gene is Cpt1. We believe that because the higher amounts of accumulated lipids in the cells, HepG2 try to increase the expression of *Pparα* (tendency) to start the β-oxidation and, consequently, the lipolysis although the expression of *Cpt1a* was not altered yet.

Other studies did in HepG2 by Gnoni, *et al*. (2009), Zang, *et al*. (2006), Shang, *et al*. (2008) and Wang, *et al*. (2009), used higher amounts of Glc or Palm to increase the fat accumulation in the liver and the incubation of these cells with RSV from 10 to 50 μM RSV during 24 h prevented lipid accumulation and reduced the TAG content via reducing Acc activity or increasing its phosphorilation (inhibition), increasing the phosphorilation of AMPK (activation), reducing the expression of *Srebp1c* and *Fas* and increasing the *Sirt1* [35-37].

The last step was to determine how exactly the RSV acts to counteract the LR caused in HepG2 cells. For this purpose, we analyzed the expression of some genes implicated in the inhibition of the leptin signalling pathway (*Socs3* and *Ptp1b)*, in the

ER stress (*Chop*, s*Xbp1* and *Atf4*) and inflammation (*iNos*). Moreover, the activity of SIRT1 and the protein content of ObRb was studied.

After treating HepG2 cells with 25 μM RSV during 28 h being co-treated with 0.5 mM Palm the last 8 h, Rojas, *et al*. (2014) found that all the markers of ER stress were increased in the presence of RSV (the *Atf4* and *Chop* expression and the Xbp1 splicing) [16]. It seems that in our case, both *Chop* and s*Xbp1* tended to increase their expression in the presence of RSV although no significant results were found. Surprisingly, the expression of *Atf4* was increased when cells were damaged and the supplementation with RSV tended to return it to the basal levels. It is well-known that the activation of Atf4 induces the expression of *Chop* [38]. Maybe it is due to in these cells there was a higher presence of ER stress than those which experimented Rojas, *et al*. (2014) and RSV tried to revert this problem reducing the expression of this key enzyme (*Atf4*) and this effect did not arrive to the *Chop* expression yet. A possible explanation could be the TRB3 pseudokinase, which its expression is promoted by ER stress (concretely by Atf4/Chop). An excess of its expression causes a negative feedback in the Atf4/Chop, down-regulating its own expression [39,40].

No changes were obtained in the expression of the *iNos*, the inflammatory marker. Jiang, *et al*. (2015), obtained a high protein content of iNos when HepG2 cells were treated during 4 h with Palm [41]. However, another study where HepG2 cells were treated with oleic and palmitic acids (2:1) for 24 h, there were not differences in the iNos protein content between the control and the treated cells [42].

According to our results, the expression of *Socs3* was increased when cells were treated with 0.5 mM Palm + 30 mM Glc and the RSV could not counteract this effect. Only observing the leptin signalling pathway, it not make sense that damaged cells presented higher amounts of *Socs3* when the leptin pathway is decreased because one of the Socs3 functions is to act as a negative feedback to compensate the continuous activation of the pathway. To explain this, we should know that the expression of *Socs3* is induced by both STAT3 and NF-kB. In our case, cells presented ER stress and we believe that NF-kB was over-activated. A mechanism to counteract this problem could be the up-regulation of *Socs3* to attenuate the activation of NF-kB [43]. In this case, RSV had not the capacity to counteract this possible mechanism of protection activated by the damaged cells. No significant results were obtained for *Ptp1b*. Contrarily to our results, a study made in patients with nonalcoholic steatohepatitis (NASH), authors obtained an increased expression of *Ptp1b* in liver [44,45].

No changes in SIRT1 activity were obtained. Wang, *et al*. (2009), incubated HepG2 cells during 24 h with 0.2 mM Palm and 40 μM RSV. SIRT1 expression and protein content was higher in Palm-treated cells and this effect was higher when the RSV was added [36]. It is known that SIRT1 is implicated in the down-regulation of lipogenesis and with an increase in the lipids oxidation [13]. In our case, the fact that there was not changes neither in its expression nor in its activity informs us that the mechanisms implicated in the restoration of the leptin sensitivity by the RSV is via a non SIRT1-dependent pathway.

The protein content of ObRb did not change between cells that were resistant to leptin and control cells. Remarkably, when we added RSV to the treated cells the expression of the leptin receptor increased. We believe that the behavior of RSV increasing the protein content of the leptin receptor in cells damaged by the treatment of 0.5 mM Palm + 30 mM Glc is to increase the leptin transport across the membrane of the cell in order to re-establish the truncated leptin signalling pathway. The ObRb results obtained in the WB study were confirmed using fluorescence in order to detect all the ObRb content present in treated cells.

To sum up, in this study we could create a LR model in human hepatocarcinoma cell line and to determine that RSV had the capacity to restore the leptin sensitivity in this damaged *in vitro* model, increasing the pSTAT3 levels. Its main mechanism of action was increasing protein content of ObRb without changing SIRT1 levels. Moreover it modulated the expression of target lipolytic and lipogenic genes. Therefore, we postulate RSV as a useful natural agent to create food bioactives in order to combat the onset of obesity.

5. Acknowledgements: This work was supported by the Ministerio de Economía, Industria y Competitividad (AGL2013-49500-EXP, AGL2016-77105-R and FPU14/01202).

RESULTS

6. Supplementary information

Table 1S. A summary of the human-specific primer sequences used for qRT-PCR analysis.

Primer name	Direction	Primer sequences (5'-3')	Primer length (nucleotides)	Tm (ºC)
Acc	FW	gctgaaccagcactctgat	19	47
	RV	ccttgcacataaggtccagc	20	52
Atf4	FW	gggttctccagcgacaaggctaag	24	61
	RV	aacagggcatccaagtcgaactc	23	58
Chop	FW	agggagaaccaggaaacggaaaca	24	61
	RV	tcctgcttgagccgttcattctct	24	60
Cpt1a	FW	Cgg ´ñgggaatgtcaagaggtt	20	55
	RV	caagaaatgtcgcacgagcc	20	56
Fas	FW	tctactacaagctgcgtgcc	20	51
	RV	aacctctcccaggtatgcga	20	54
iNos	FW	caagcagcagaatgagtccc	20	52
	RV	ctgggtcctctggtcaaact	20	50
ObRb	FW	aattgttcctgggcacaagg	20	54
	RV	gagagaccacagttgttggc	20	49
Pparα	FW	aggctgcaagggcttctttc	20	55
	RV	tcgggatgtcacacaacgc	19	55
Ppia	FW	ctcctttgagctgtttgcag	20	50
	RV	caccacatgcttgccatcc	19	54
Ptp1b	FW	tgggaaatgcagggagttct	20	53
	RV	ccacgacccgacttctaact	20	51
Scd1	FW	acttggagctgtgggtgagg	20	54
	RV	cgctggcacatcaacttcac	20	54

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Sirt1	FW	tggcacagatcctcgaacaa	20	54
	RV	catgaaacagacaccccagc	20	53
Socs3	FW	caggaatgtagcagcgatggaa	22	57
	RV	cctgtccagcccaatacctga	21	56
sXbp1	FW	gagttaagacagcgcttggg	20	52
	RV	gatgttctggaggggtgaca	20	52

Abbreviations: *Acc* (acetyl-CoA carboxylase), *Atf4* (activating transcription factor 4), *Chop* (DNA damage inducible transcript 3), *Cpt1a* (carnitine palmitoyltransferase 1a), *Fas* (fatty acid synthase), *iNos* (inducible nitric oxide synthase), *ObRb* (leptin receptor isoform b), *Pparα* (peroxisome proliferator activated receptor α), *Ppia* (peptidylprolyl isomerase a), *Ptp1b* (protein-tyrosine phosphatase 1b), *Scd1* (stearoyl-CoA desaturase 1), *Sirt1* (NAD^+-dependent deacetylase sirtuin-1), *Socs3* (suppressor of cytokine signalling 3) and *sXbp1* (spliced x-box binding protein 1).

RESULTS

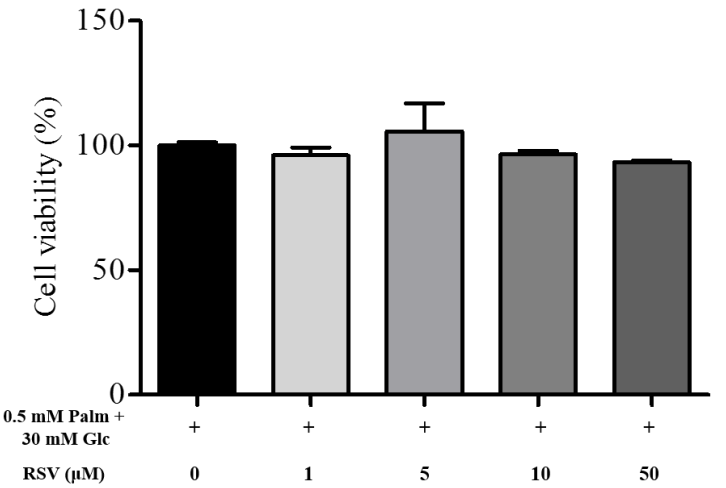

Figure 1S. Neutral red viability results in the leptin resistance (LR) model and supplementing HepG2 cells with different doses of RSV. Cells were incubated 48 h with 0.5 mM Palm + 30 mM Glc and the last 24 h were supplemented with 0, 1, 5, 10 and 50 µM RSV. After that, the viability of the cells was measured using the neutral red method. $^{\#}p < 0.05$ and $^{\varepsilon}p < 0.1$ comparing RSV groups respect to the 0.5 mM Palm + 30 mM Glc group making an T-student test. Data are expressed as a mean ± SD of 3 replicates.

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Manuscript 4

Effects of resveratrol, epicatechin, gallic acid and a grape seed proanthocyanidin-rich extract on primary rat brain endothelial cells: expression of leptin receptors and protection against cytokine-induced damage

Andrea Ardid-Ruiz, András Harazin, Lilla Barna, Cinta Bladé, Lluís Arola, Maria A. Deli, Manuel Suárez, Gerard Aragonès

(Manuscript under preparation)

RESULTS

PREFACE

This paper is framed in answering the part of the **hypothesis** about the role of phenolic compounds in the increase of the leptin transport across the blood-brain barrier. Because one of the leading causes of leptin resistance is the impairment of the leptin to arrive to the brain, we decided to test some selected phenolic compounds to evaluate their effect in this transport. For this, we tested different doses and incubation times of these 3 pure phenolic compounds, resveratrol, (-)-epicatechin and gallic acid, and also evaluated the effect of a complex extract of phenolic compounds, the grape seed proanthocyanidin extract, in rat brain endothelial cells (RBEC). Once we identified the doses and incubation time of the selected compounds more adecuate in order to avoid an impairment in the barrier, we analysed the gene expression of leptin receptors (*leptin receptor isoform b*, *leptin receptor isoform a* and *LDL receptor-related protein-2*) as well as some molecules implicated in the leptin signalling pathway. As the onset of obesity is closely linked with inflammation we induced inflammation using pro-inflammatory cytokines in this cellular model to try to simulate an obese condition and to test if polyphenols have a beneficial effect reverting the damaged caused in the barrier generated in the RBEC model.

Effects of resveratrol, epicatechin, gallic acid and a grape seed proanthocyanidin-rich extract on primary rat brain endothelial cells: expression of leptin receptors and protection against cytokine-induced damage

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Keywords: blood-brain barrier; brain endothelial cell; cytokines; epicatechin; gallic acid; grape-seed proanthocyanidin-rich extract; leptin receptors; ObRa; resveratrol.

RESULTS

Abstract

Background: Dietary obesity is generally associated with central leptin resistance. Although the molecular basis for this metabolic disturbance is not yet completely understood, it has been mainly attributed to defective leptin transport across the blood-brain barrier (BBB) and systemic chronic and hypothalamic inflammation.

Objective: Within this context, the aim of this study was to investigate the effects of resveratrol (RSV), epicatechin (EC), gallic acid (GA) and a grape-seed proanthocyanidin-rich extract (GSPE) on the expression of leptin receptors and cytokine-induced endothelial damage using an *in vitro* BBB model.

Methods: Primary cultures of rat brain endothelial cells (RBEC) were treated with different concentrations of RSV, EC, GA and GSPE. The expression of leptin receptors was assessed by qPCR and western blot. In order to determine the ability of these compounds to attenuate the cytokine-induced barrier disruption, RBEC were incubated with tumor necrosis factor-α and interleukin-1 β (TNFα + IL-1β; 10 + 10 ng/mL) for 24 h. RBEC cell viability was evaluated by the measurement of impedance.

Results: Incubation with the selected doses of the compounds (10 μM RSV, 10 μM EC, 1 μM GA or 10 μg/mL GSPE) for 24 h did not affect the cell viability measured by impedance. Remarkably, the gene expression of the short leptin receptor isoform, *ObRa,* was significantly up-regulated in this cellular model. The mRNA levels of *LPR2* and *Clusterin,* other leptin receptors also known as megalin and apolipoprotein

J, respectively, were not affected. No significant changes were observed in the direct modulation of the cell surface content of the long leptin receptor isoform, ObRb, by these compounds. Importantly, the treatment with the compounds at the selected doses partially protected against the cytokine-induced RBEC damage.

Conclusions: RSV, EC and GSPE by upregulating the short leptin receptor isoform at the BBB may modify leptin transport into the brain and leptin-resistance associated with diet-induced obesity (DIO). Our results also suggest that natural food compounds may protect against cellular inflammation associated with obesity.

RESULTS

1. Introduction

Obesity is considered one of the most important epidemics worldwide which is related with other pathologies such as cardiovascular disease, diabetes, cancer, hypertension, among others [1,2]. Obese subjects present an excessive adipose tissue mass due to hypertrophy and hyperplasia. In this sense, the adipose tissue is a crucial endocrine organ implicated in the energy storage and with an active metabolism. Thus, its correct function, secreting balanced amounts of pro- and anti-inflammatory adipokines, is crucial to maintain the energy balance and the metabolism. An excessive production of pro-inflammatory adipokines induces systemic inflammation, insulin resistance and other related pathologies [3]. Therefore, there is an association between obesity and a systemic chronic low-grade inflammation [4,5].

It has been reported that obesity causes an increase in the levels of circulating leptin, a hormone secreted proportionally to adipocytes that regulates food intake acting on the central nervous system (CNS). However, this excess of leptin is not functional, resulting in a state known as leptin resistance (LR) [6,7]. LR appears as a consequence of the imbalance of many mechanisms. One of them is the prevalence of inflammation in both periphery and hypothalamus during obesity [8]. Another is the inability of leptin to cross the blood-brain barrier (BBB) due to the disruption of its uptake by the endothelial cells [9]. Because leptin is produced in the periphery and its main function is exerted in the CNS, it needs to be transported across the BBB [10]. The association

between LR and the deficient pass of leptin across the BBB has been observed in both obese patients and rodent models of obesity [11].

In this line, leptin receptors play an essential role in the proper functioning of this signalling pathway. The group of leptin receptors (ObR) belongs to the gp130 family of cytokine receptors [12,13] and is composed by six spliced isoforms (ObRa-ObRf). ObR are basically expressed in the brain although they also appear in some peripheral tissues such as the liver [12,14]. The longest isoform is ObRb, which contains a cytoplasmatic domain involved in the activation of the leptin signalling pathway [12,14] through janus kinase 2 (JAK2) and the signal transducer and activator of transcription 3 (STAT3) [12]. Suppressor of cytokine signalling 3 (SOCS3) and the protein tyrosine phosphatase 1B (PTP1B) are known inhibitors of the leptin cascade [15,16].

Other isoforms of ObR are ObRa and ObRe that lack intracellular signalling domains and which main action is to transport leptin from the blood to the cerebrospinal fluid. Moreover, clusterin, also known as apolipoprotein J (ApoJ), was described recently as a peptide which stimulates the anorexigenic effect of leptin, increasing the interaction of leptin with its receptor [17]. This effect is regulated via LDL receptor-related protein-2 (LPR2, megalin) that simultaneously acts against ObRb [17,18]. Thus, ObRa and LPR2 are implicated in the transport of leptin across the BBB. ObRa and LPR2 are expressed in both the endothelium of cerebral microvessels and choroid plexus [19-22].

RESULTS

Recent studies demonstrate that many phenolic compounds have the capacity to revert the hyperleptinemia present in obesity although the mechanisms in which they are involved still remain unknown [12]. Phenolic compounds are plant secondary metabolites [23-25] found in fruits, vegetables and beverages [26] such as cocoa, olive and grapes, among others. The basic structure of phenolic compound is one phenol ring and they range from simple molecules to polymerized compounds (called polyphenols) [23,26]. The biological activities of phenolic compounds include anti-ageing, anti-inflammatory, antioxidant and anti-proliferative, among others [26,27]. According to the anti-inflammatory action, it is known that polyphenols have the capacity to inhibit the production of pro-inflammatory adipokines [5] and exert a protective effect on the BBB [28].

In this sense, previous studies from our research group have shown that the ingestion of grape seed proanthocyanidin extract (GSPE) enhanced the activation of STAT3 and increased the proopiomelanocortin (*Pomc*) mRNA levels in the hypothalamus of obese rats. In the skeletal muscle, it restored the expression of *Socs3* and *Ptp1b* [29]. Hence, it seems clear that the ingestion of phenolic compounds can, to some extent, modulate the leptin signalling pathway.

Considering all this information, the aim of this study was to determine whether a selection of phenolic compounds have the capacity to modify the expression of leptin receptors or the molecules implicated in the leptin transport and signalling pathway in primary cultures of rat brain endothelial cells (RBEC), an *in vitro* model of the BBB. Furthermore, since inflammation is involved in both obesity and LR we evaluated if

they could also counteract the alterations caused by pro-inflammatory cytokines in brain endothelial cells.

2. Materials and methods

2.1 Reagents and compounds

GSPE was obtained from white grape seeds and was provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to previous analysis performed in our group using HPLC-MS/MS [30], this proanthocyanidin (PA)-rich extract contained EC (33.6 ± 12.7 mg/g), GA (15.1 ± 3.5 mg/g), but not RSV (0.0 ± 0.0 mg/g) (**Table 1S**). GA (G7384) and EC (E4018) were purchased from Sigma-Aldrich (Madrid, Spain). RSV was purchased from Fagron Iberica (Barcelona, Spain).

2.2 Cell culture

Primary rat brain endothelial cells (RBEC) were isolated from 3 week-old outbred Wistar rats (Harlan Laboratories, Indianapolis, IN, USA) as described in our previous paper [31]. Forebrains were collected and meninges were removed, then the tissue was cut into 1 mm^3 pieces by scalpel and digested with enzymes (1 mg/mL collagenase type II, and 15 µg/ml deoxyribonuclease type I (Roche, Basel, Switzerland) in Dulbecco's modified Eagle medium (DMEM/F12, Gibco, Life Technologies, Carlsbad, CA, USA) at 37 °C for 55 min. Microvessels were separated from the myelin rich brain tissue fraction by centrifugation in 20 % BSA-DMEM gradient (1,000 × *g*, 20 min, 3 times). The collected vessels were further digested with enzymes (1 mg/mL collagenase-dispase, and 15 µg/ml deoxyribonuclease type I;

Roche, Basel Switzerland) in DMEM/F12 at 37 °C for 35 min. Brain microvascular endothelial cell clusters were separated on a 33 % continuous Percoll gradient (1,000 × *g*, 10 min), collected, and washed twice in DMEM/F12. Cells were seeded onto Petri dishes (100 mm; Orange Scientific, Braine-l'Alleud, Belgium) coated with collagen type IV and fibronectin (100 µg/ml each). Cultures were maintained in DMEM/F12 supplemented with 15 % plasma-derived bovine serum (First Link, Wolverhampton, UK), 5 µg/mL insulin-transferrin-sodium selenite (Pan Biotech, Aidenbach, Germany), 1 ng/mL basic fibroblast growth factor (Roche, Basel, Switzerland) 100 µg/mL heparin and 5 µg/mL gentamycin. For the first 3 days of culture cells were grown in medium with 3 µg/mL puromycin to eliminate P-glycoprotein negative contaminating cell types [32]. When endothelial cells reached 90 % confluency, they were subcultivated for different experiments.

2.3 Treatments

RBEC were treated at increasing concentrations of RSV, EC and GA ranging from of 0.03 to 100 µM. GSPE was tested in the range from 1 to 50 µg/mL. These concentration ranges were selected based on our previous studies [30,33,34]. Stock solutions were prepared in ethanol. Treatment solutions were diluted in culture medium. The final concentration of ethanol was below 0.1 % in the treatment solutions.

2.4 Real-time cell microelectronic sensing

Real-time cell electronic sensing is an impedance-based, label-free technique for dynamic monitoring of living adherent brain endothelial cells [35]. The RTCA-SP instrument (ACEA Biosciences, San Diego, CA, USA) registers the impedance of cells in every 10 min and for each time point cell index is calculated as (Rn - Rb)/15, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the medium alone. E-plates, special 96-well plates with built in gold electrodes, were coated with collagen type IV and fibronectin for RBECs at room temperature and dried for 20 min under UV and air-flow. For background measurements culture medium (60 μL) was added to each well, then 50 μL of RBEC suspension was distributed at a cell density of 5×10^3 cells/well. After cells reached a steady growth phase they were treated with different phenolic compounds and cytokines.

2.5 Total RNA isolation and gene expression analysis

The expression of key genes from the leptin signalling pathway was evaluated. For RNA isolation and to obtain the cDNA, TriFast (PeqLab, VWR International, Vienna, Austria) and Maxima First Strand cDNA Synthesis kit with dsDNase (Thermo Scientific, Massachusetts, USA), were respectively used according to the manufacturer instructions. Finally, qPCR with iTaq Universal SYBR Green Supermix (Bio-Rad, Barcelona, Spain) was performed to analyse the gene expression of Peptidylprolyl Isomerase A (*Ppia*), leptin receptor isoform b (*ObRb*), leptin receptor

isoform a (*ObRa*), low density lipoprotein-related protein 2 (*Lpr2* or megalin), clusterin (*Clusterin* or ApoJ), suppressor of cytokine signalling 3 (*Socs3*) and protein-tyrosine phosphatase 1B (*Ptp1b*). **Table 1** shows the forward and reverse primers for the various genes used in this study. They were obtained from Biomers.net (Ulm, Germany). A cycle threshold (Ct) value was generated by setting the threshold during the geometric phase of the cDNA sample amplification. The fold change in expression of each mRNA was calculated with respect to the Control group using the ΔΔCt method corrected for the primer efficiency and converted to relative expression ratio with peptidylprolyl isomerase A (*Ppia)* as the reference gene [36].

RESULTS

Table 1. A summary of the rat-specific primer sequences used for qRT-PCR analysis.

Primer name	Direction	Primer sequences (5'-3')	Primer length (nucleotides)	Tm (ºC)	Amplicon lenght (nucleotides)
Clusterin	FW	gctctcagcaatcgggtgaa	20	55	153
	RV	atgccattgtcccaggtcag	20	54	
Lpr2	FW	ggagccagtcagtagccaag	20	51	136
	RV	cctgggaggacagccaattt	20	55	
ObRa	FW	cactgttaatttcacaccagag	22	55	235
	RV	gtcattcaaaccatagtttagg	22	53	
ObRb	FW	ccagtacccagagccaaagt	20	59	122
	RV	ggatcgggcttcacaacaagc	21	62	
Ppia	FW	cttcgagctgtttgcagacaa	21	53	138
	RV	aagtcaccaccctggcacatg	21	57	
Ptp1b	FW	ccctttgaccacagtcgga	20	55	119
	RV	ttggtaaagggccctgggtg	20	58	
Socs3	FW	ctggacccattcgggagttc	20	56	148

RV	ctgggagctaccgaccattg	20	54

Abbreviations: *Lpr2* (low density lipoprotein-related protein 2), *ObRa* (leptin receptor isoform A), *ObRb* (leptin receptor isoform B), *Ppia* (peptidylprolyl isomerase A), *Ptp1b* (protein-tyrosine phosphatase 1B), *Socs3* (suppressor of cytokine signalling 3).

2.6 Protein determination and Western blot

Radioimmunoprecipitation assay buffer (RIPA) supplemented with protease and phosphatase inhibitor cocktail was used to extract the protein content from RBEC previously treated with phenolic compounds during 24 h. It was quantified by Pierce BCA Protein Assay Kit (Thermo Scientific, Madrid, Spain). To perform the WB, a total of 25 μg of protein was solubilized and boiled for 5 min in a loading buffer solution containing 0.5 M Tris HCl pH 6.8, glycerol, SDS, 2-Mercaptoethanol and 1 % bromophenol blue. The total protein extracts were separated using SDS-polyacrylamide gel electrophoresis of the TGX FastCast acrylamide kit, 10 % (Bio-Rad, Barcelona, Spain) and electrotransferred onto supported PVDF membranes using TransBlot Turbo RTA Transfer kit, PVDF (Bio-Rad). After blocking, the membranes were incubated overnight in agitation at 4 ºC with an antibody specific for detecting ObRb (ab177469, Abcam, Cambridge, UK), The protein levels were detected by the chemiluminescent detection reagent ECL Select Western blotting detection reagent (GE Healthcare, Barcelona, Spain) using GeneSys image acquisition software (G-Box series; Syngene, Barcelona, Spain). Finally, protein band quantification was performed using ImageJ (W.S Rasband, NIH, MD, USA). The studied protein levels were normalized respect to β-actin.

2.7 Evaluation of anti-inflammatory properties of the studied phenolic compounds

To determine the capacity of the selected phenolic compounds to revert the inflammation caused by cytokines in RBEC cells an *in vitro* assay was carried out. RBECs were pretreated with 10 μM RSV or EC, 1 μM GA or 10 μg/mL GSPE for 30 min, then 10 ng/mL TNFα + 10 ng/mL IL-1β were added to the cells. The co-treatment with phenolic compounds was maintained during 24 h during which cell viability was continuously monitored by impedance measurement. The treatment protocol is shown **Figure 1S**.

2.8 Statistical analysis

The data are expressed as the means ± SEM. Multiple independent groups were compared by one-way analysis of variance (ANOVA) followed by Tukey or Dunnett post hoc test when it necessary. Outliers were determined by Grubbs' test. The statistical analyses were performed using XLSTAT 2017: Data Analysis and Statistical Solution for Microsoft Excel. Addinsoft, Paris, France (2017). Graphics were done using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

3. Results

RESULTS

3.1 Effect of phenolic compounds on brain endothelial cell viability

RBECs were treated with the selected phenolic compounds at different doses for 48 h (**Figure 1).** GSPE was tested at 1, 5, 10 and 50 µg/mL concentrations, and only the highest one (50 µg/mL) had a negative impact (26 % decrease) on cell viability at 48-hour incubation time-point (**Figure 1A**). EC and GA, components of GSPE, were not toxic to RBECs (**Figure 1B** and **1C**) at any concentration tested through the period of time evaluated. RSV, a well examined polyphenol, which is not present in GSPE, showed a concentration and time-dependent effect on RBECs cells viability (**Figure 1D**). The highest concentrations of RSV, 50 and 100 µM, caused a marked reduction (25 % and 75 % decrease) in the cell index when time increased, thus reducing the integrity of the cell layer.

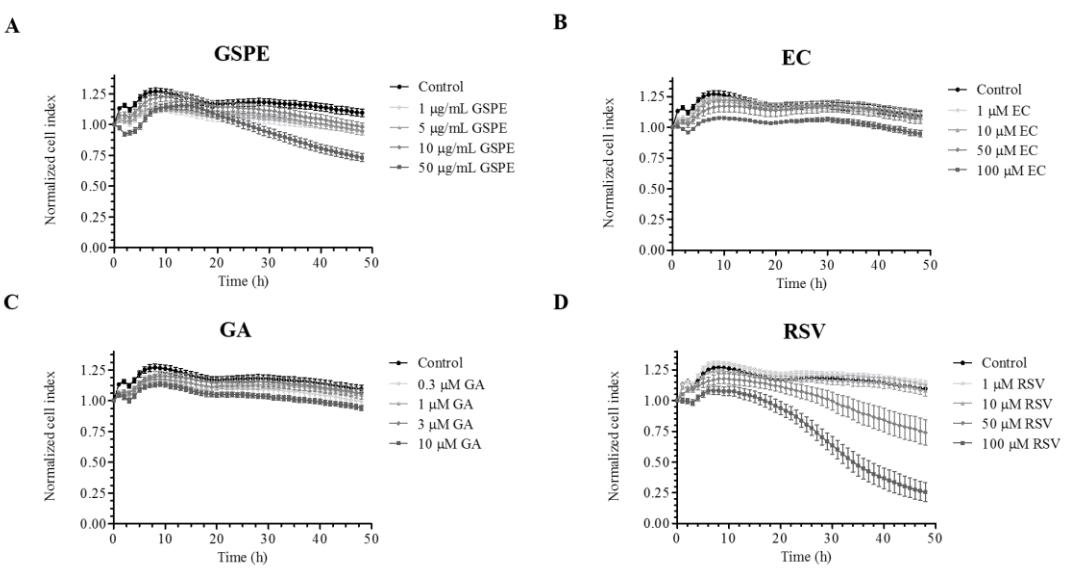

Figure 1. Impedance monitoring of the viability of rat brain endothelial cells treated with different phenolic compounds. Cells were treated during 48 h with 1, 5, 10 and 50 µg/mL grape seed proanthocyanidin-rich extract (A); 1, 10, 50 and 100 µM epicathecin (B); 0.3, 1, 3 and 10 µM gallic

acid (C); and 1, 10, 50 and 100 μM resveratrol (D). Data are expressed as a mean ± SEM, n=6. EC, epicathecin; GA, gallic acid; GSPE, grape seed proanthocyanidin-rich extract; RSV, resveratrol.

At 24-hour time point, the selected length for all further experiments, GSPE at 50 μg/mL presented a statistically significant decrease (13.5 %) in the cell viability compared to the control (**Figure 2A**). This reduction of cell index at the highest dose led us to select the 10 μg/mL concentration of GSPE for the next assays. EC also caused a small, but significant decrease (11 %) in the cell viability of cells treated with the highest concentration with respect to the control (**Figure 2B**). On the other hand, GA did not change the cell index at any evaluated concentration (**Figure 2C**). The biggest decrease in the brain endothelial cell impedance (29 %) was seen at the highest concentration of RSV (100 μg/mL) compared to the other conditions (**Figure 2D**).

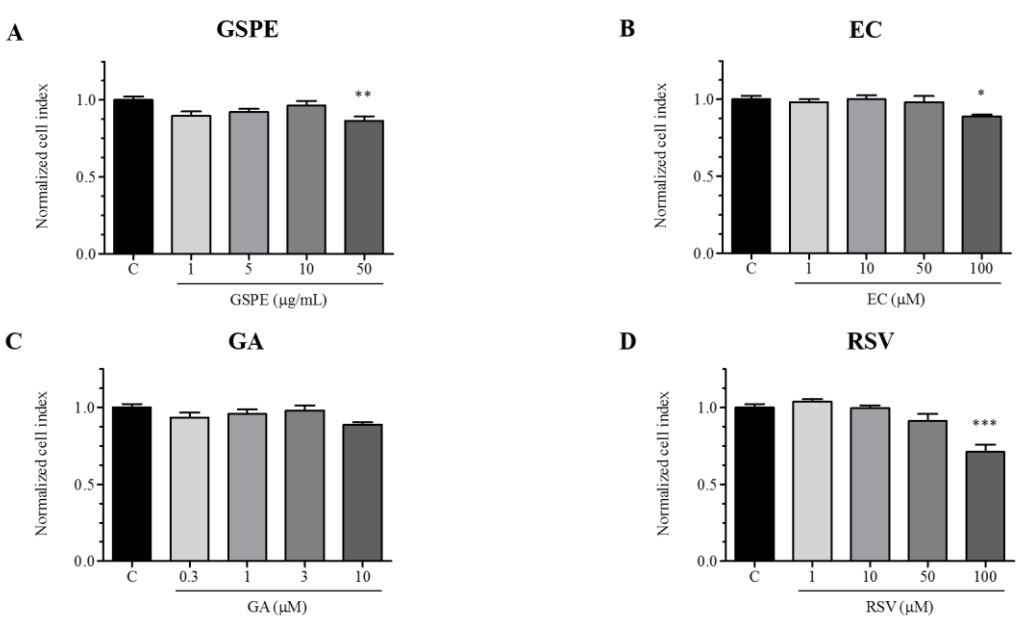

Figure 2. Impedance monitoring of the viability of rat brain endothelial cells treated with different polyphenols at 24 h. Cells were treated with 1, 5, 10 and 50 μg/mL grape seed proanthocyanidin-rich extract (A); 1, 10, 50 and 100 μM epicathecin (B); 0.3, 1, 3 and 10 μM gallic

acid (C); and 1, 10, 50 and 100 μM resveratrol (D). Data are expressed as a mean ± SEM, n=6. *$p <$ 0.05, ANOVA and Tukey test. EC, epicathecin; GA, gallic acid; GSPE, grape seed proanthocyanidin-rich extract; RSV, resveratrol.

Therefore, after analyzing the cell index of RBECs incubated with several concentrations of the phenolic compounds we selected 10 μg/mL GSPE, 10 μM RSV or EC, and 1 μM GA to treat cells for 24 h in order to evaluate the gene and protein expression.

3.2 The effect of polyphenols on receptors implicated in the leptin signalling pathway in rat brain endothelial cells

After selecting the best time and concentrations of phenolic compounds to work with cells without causing toxicity, their gene expression was evaluated to determine the effect of the treatments on some key components of the leptin signalling pathways.

Figure 3A informs about the *ObRa* expression in RBEC cells after the 24-hour treatments. In this case, GSPE, EC and RSV markedly increased the expression of *ObRa* (15-fold, 8-fold, 12-fold, respectively) as compared to the control. **Figure 3B** represents the relative gene expression of *ObRb* when cells were incubated with polyphenols. For this receptor, no significant changes were observed as compared to the control. In the case of *LPR2* expression, we could not observe any significant difference between the treatment groups (**Figure 3C**). In addition, the gene expression of other important components of the leptin pathway, namely *Clusterin*, *Socs3* and *Ptp1b*, was also evaluated. In all the cases no significant changes were observed between the treated and the control cells (**Figure 3D-F**).

RESULTS

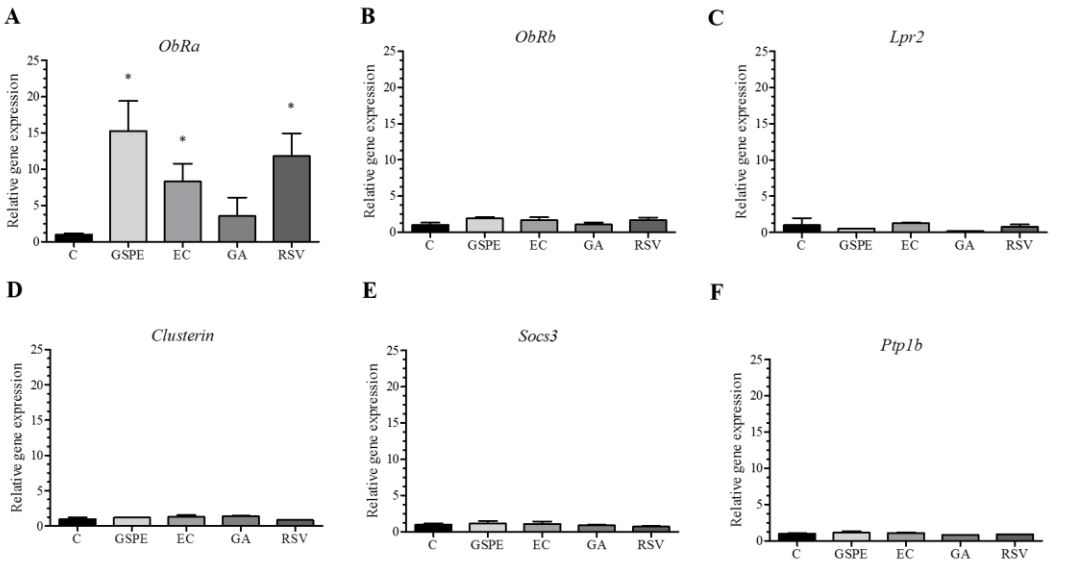

Figure 3. Relative gene expression of leptin receptors and other components of the leptin cascade in rat brain endothelial cells. Cells were treated with 10 μM resveratrol , 10 μM epicathecin , 1 μM gallic acid and 10 μg/mL grape seed proanthocyanidin-rich extract for 24 h. (A) Represents the expression of leptin receptor isoform b (*ObRa*); (B) Shows the expression of leptin receptor isoform a (*ObRb*); and (C) Graphics the expression of low density lipoprotein-related protein 2 (*Lpr2*). (D) Represents the expression of *Clusterin*; (E) Shows the expression of suppressor of cytokine signalling 3 (*Socs3*); and (F) Graphics the expression of protein-tyrosine phosphatase 1b (*Ptp1b*). Data are expressed as a mean ± SEM, n=2. $*p < 0.05$, ANOVA and Dunnett test. EC, epicathecin; GA, gallic acid; GSPE, grape seed proanthocyanidin-rich extract; RSV, resveratrol.

3.3 The effect of polyphenols on Obrb protein content in cells treated with the selected phenolic compounds for 24 hours

After analysing the gene expression, we decided to study if phenolic compounds could increase the protein expression of ObRb in RBECs. ObRb is the longest isoform of leptin receptors and, consequently, activates the leptin cascade. **Figure 4** shows how RBE cells cultured with 10 μg/mL GSPE, 10 μM EC, 1 μM GA and 10 μM RSV for 24 h did not present any significant change compared to control cells in the case of the protein content of ObRb.

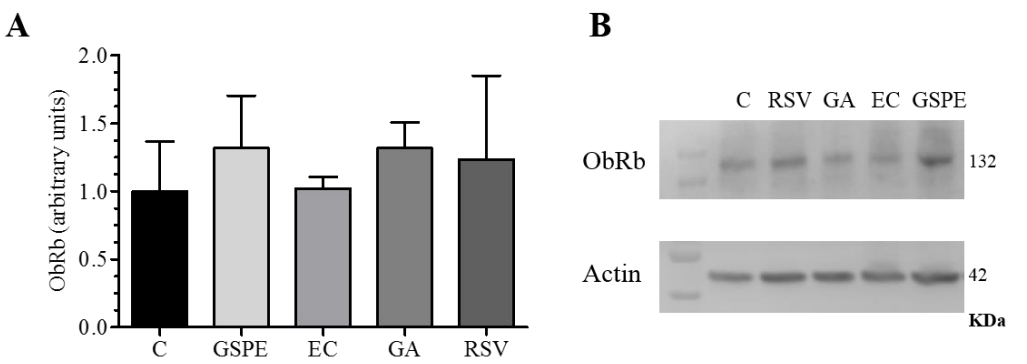

Figure 4. Expression of leptin receptor isoform b (ObRb) protein in rat brain endothelial cells. Cells were treated for 24 h with 10 μM resveratrol, 10 μM epicathecin, 1 μM gallic acid and 10 μg/mL grape seed proanthocyanidin-rich extract. (A) Represents the graphical results. (B) Shows a representative image of the western blot. Data are expressed as a mean ± SEM, n=2. EC, epicathecin; GA, gallic acid; GSPE, grape seed proanthocyanidin-rich extract; RSV, resveratrol.

3.4 The effect of the selected phenolic compounds on damage induced by cytokines in rat brain endothelial cells

Figure 5A represents cells treated with phenolic compounds and cytokines. As shown on the bar graph, cells incubated with cytokines presented a very low cell index compared with the control and RBEC co-treated with cytokines and phenolic compounds partially reverted the damage induced by TNFα + IL-1β treatment for 24 h, which was reflected in cell index values higher than the control + CK group at all the time points. Due to the fact that the normalized cell index of RBEC treated with cytokines already dropped to very low level (23 % of the control) at 6 h, the effects of co-treatment with phenolic compounds was evaluated at this time point (**Figure 5B).** As compared to the control group, the cytokine treatment resulted in a drastic drop of cell viability. This toxic effect was partially reverted by the phenolic compounds,

although there was no statistically different change as compared to the cytokine-treated group. The cell index of the phenolic compound co-treated groups did not significantly differ from the control group, thus it may reflect a protective effect on brain endothelial cells against damages exerted by the cytokines.

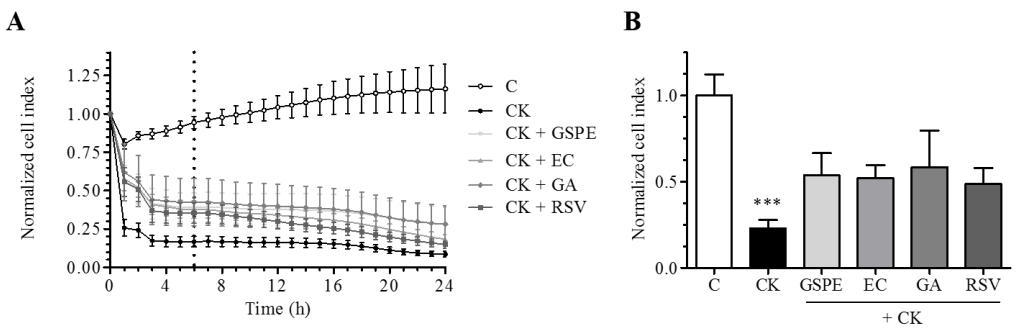

Figure 5. Impedance monitoring of the viability of rat brain endothelial cells co-treated with cytokines and different polyphenols. Cells were pre-treated with 10 μg/mL grape seed proanthocyanidin-rich extract, 10 μM epicathecin, 1 μM gallic acid or 10 μM resveratrol for 30 min, then co-treated with pro-inflammatory cytokines TNFα + IL-1β (10 + 10 ng/mL) for 24 h (A). Panel (B) represents the results of the co-treatment at the 6-hour time-point. Data are expressed as a mean ± SEM, n=8. $^{***}p < 0.001$, ANOVA and Tukey test. EC, epicathecin; GA, gallic acid; GSPE, grape seed proanthocyanidin-rich extract; RSV, resveratrol.

4. Discussion

As explained above, the aim of this work was to study the effect of phenolic compounds on the leptin signalling pathway and their potential protective effects on brain endothelial cells. Hence, RSV, EC, GA and GSPE were selected. EC belongs to the flavonoid family whereas RSV and GA are non-flavonoids, thus, giving information about the behaviour of the two main families in which phenolic compounds are divided. In addition, GSPE is a complex mixture of different phenolic

compounds, mainly but not all of them, from the flavonoids family. Therefore, its study provides useful information about the beneficial effects of these compounds.

To select the concentrations and incubation time of each one of the treatments with the selected phenolic compound we based on previous literature data [30,33,34]. As explained before, **Figure 1** shows the results of the impact of each one of the studied phenolic compounds on the viability of brain endothelial cells and it can be seen that, overall, only GSPE and RSV at the highest concentrations had a significant negative impact on this parameter. In this sense, recent experiments performed using another type of endothelial cells, specifically human umbilical endothelial cells (HUVEC), showed that treatment with RSV at concentrations ranging from 10 nM to 100 μM did not affect cell viability whereas higher concentrations caused a significant reduction on their survival [37-41]. The differences observed compared with our results, in which the concentrations of 50 and 100 μM produced a significant reduction in the cell index, could be explained by the specific characteristics of RBEC. Although both cell types are endothelial, thus, sharing certain properties, they have essential differences regarding the expression of the thigh junctions and their permeability that may explain the differences observed in our study [42].

On the other hand, regarding GSPE, previous studies performed in HUVEC demonstrated that at concentrations between 5 and 25 μg/mL it reduced pro-oxidative parameters [43] while at 1-5 μg/mL it showed anti-inflammatory properties [44]. In addition, in a human hepatocarcinoma cell line (HepG2 cells) GSPE had beneficial

effects reducing lipid content at concentrations of 25-100 μg/mL [32]. Comparing with our results, we observed a significant reduction of the cell index after 20 hours of incubation at the concentration of 50 μg/mL pointing that, although this extract has showed beneficial effects at higher concentrations in other cell models, it has a negative impact on the RBEC barrier model. This fact may be explained by the complexity of the extract, composed of a diverse range of molecules at different levels, mainly all of them of high molecular weight. In this sense, we hypothesised that at high concentrations these complex mixture of compounds can be affecting the integrity of the barrier. Therefore, taking in consideration all the results, we decided to select 10 μg/mL of GSPE, 10 μM of RSV and EC, and 1 μM of GA during 24 h to carry out the treatments.

After deciding the time and the concentration of each treatment that did not cause significant cellular toxicity, the next step was to test if these phenolic compounds had the capacity to influence the leptin transport pathway at the BBB. For this purpose, we studied if they could increase the expression of leptin transporters in RBECs. Therefore, we evaluated the relative gene expression of *ObRa*, *ObRb* and *Lpr2*. As mentioned before, ObRb is the longest isoform, being the main ObR implicated in the leptin signalling pathway [14]. In this case, we could not observe any significant change compared to the control when RBEC were treated with the selected four compounds (**Figure 3B**). Moreover, the ObRb protein content did not change (**Figure 4**). However, the results obtained on *ObRa,* described as the most important receptor isoform implicated in the leptin transfer across brain endothelial cells [45], were

especially remarkable. Surprisingly, three of the four phenolic compounds significantly and highly increased *ObRa* levels compared to the control (**Figure 3A**). Only in the case of GA we could not observe any significant change. Based on this gene expression result GSPE, EC and RSV may potentially help the leptin entry into the brain. Our results are novelty because recent investigations are focused on designing leptin analogs or ObR agonists to selectively improve leptin transport across the BBB without disturbing the integrity of the barrier [46]. In this sense, the GSPE had a more important impact on the overexpression of *ObRa* pointing to a synergistic effect of the phenolic compounds when tested as a complex mixture. The exact content of each phenolic compound in 10 μg/mL GSPE was 1.16 μM for EC, 0.89 μM for GA and 0 μM for RSV [30] indicating a synergistic action of EC in low amount with other compounds present in the extract. The third receptor implicated in the leptin action in the brain is the LPR2 [17,18]. The results were similar to those obtained for *ObRb*, demonstrating that the compounds do not modulate the *Lpr2* expression in RBEC (**Figure 3C**).

There are three points of entry for leptin in the brain: (i) the endothelial cells of the BBB [10], (ii) the epithelial cells of the choroid plexus that form the blood-CSF barrier [47] and (iii) the mediobasal hypothalamus surrounded by tanycytes which form a barrier between the median eminence and CSF by thigh junctions [48]. ObRb is expressed in epithelial and brain endothelial cells as well as in tanycytes [9,48-51] whereas LPR1 and LPR2 are alternative transporters as observed in *in vitro* studies [52,53]. However, brain endothelial and choroid plexus epithelial cells highly express

ObRa compared with ObRb [9]. In addition, the Km of the leptin transport at the BBB is about 1 nM [54], due to its good affinity to both ObRa and ObRb [55]. However, in the case of LPR2, leptin presents a lower affinity and a dissociation constant of 200 nM [56], suggesting that ObR are most important binding sites for leptin. In the case of the expression of additional molecules which are involved in the leptin signalling pathway, *clusterin* [17], *Socs3* [56,57] and *Ptp1b* [16], we could not observe any change when cells were treated with polyphenols as compared to the control (**Figure 3**). Our results that brain endothelial cells express higher *ObRa* than *ObRb* and *Lpr2* levels are in agreement with literature data [9].

The relationship between obesity, inflammation and higher plasma leptin levels is well known [58]. Data supports that brain permeability to leptin is impaired during obesity and the plasma levels of this hormone are elevated compared to non-obese patients [11], thus, contributing to LR in obesity. The two most prominent pro-inflammatory cytokines are TNFα and IL-1β which directly damage brain endothelial cells [59]. Therefore, our other aim was to induce in RBECs a pro-inflammatory state and to test if the phenolic compounds could counteract this effect. RBECs which were incubated with cytokines for 24 h presented low cell index compared to the control and all the four compounds tested partially reversed it (**Figure 5**). This model of pro-inflammatory damage in RBEC was previously described by our group and similar results on brain endothelial toxicity were obtained [60].

As a conclusion, three of the four phenolic compounds, namely GSPE, EC and RSV, increased the gene expression of *ObRa*, the main leptin transporter from blood to the brain across the BBB, in cultured brain endothelial cells. We also observed that natural compounds partially reverted the cytokine-induced inflammation process, which is associated with obesity. These effects of phenolic natural compounds at the level of BBB may be beneficial in the leptin resistance present in obese conditions. Therefore, our experiments shed light about the mechanisms by which phenolic compounds are involved in interacting with leptin signalling pathway and, consequently, highlight their potential to be used as possible ingredients in functional food.

5. Acknowledgements

This work was supported by the Ministerio de Economía, Industria y Competitividad (AGL2013-49500-EXP, AGL2016-77105-R and FPU14/01202).

6. Supplementary information

Table 1S. Identified phenolic compounds and derivatives determined by reversed-phase HPLC-MS/MS [30].

Phenolic compound	mg compound/g GSPE extract
GA	15.1 ± 3.5
Vanillic acid	0.2 ± 0.05
Procyanidin dimer	82.2 ± 13.1
Dimer B2	30.8 ± 8.3
Catechin	51.8 ± 14.9
EC	33.6 ± 12.7
Gallate dimer	23.9 ± 7.4
Epigallocatechin gallate	0.1 ± 0.0
Trimer C1	4.4 ± 1.0
EC gallate	10.3 ± 5.5
Quercetin	0.2 ± 0.0
RSV	0.0 ± 0.0

Abbreviations: EC (Epicatechin), GA (Gallic acid), RSV (Resveratrol).

RESULTS

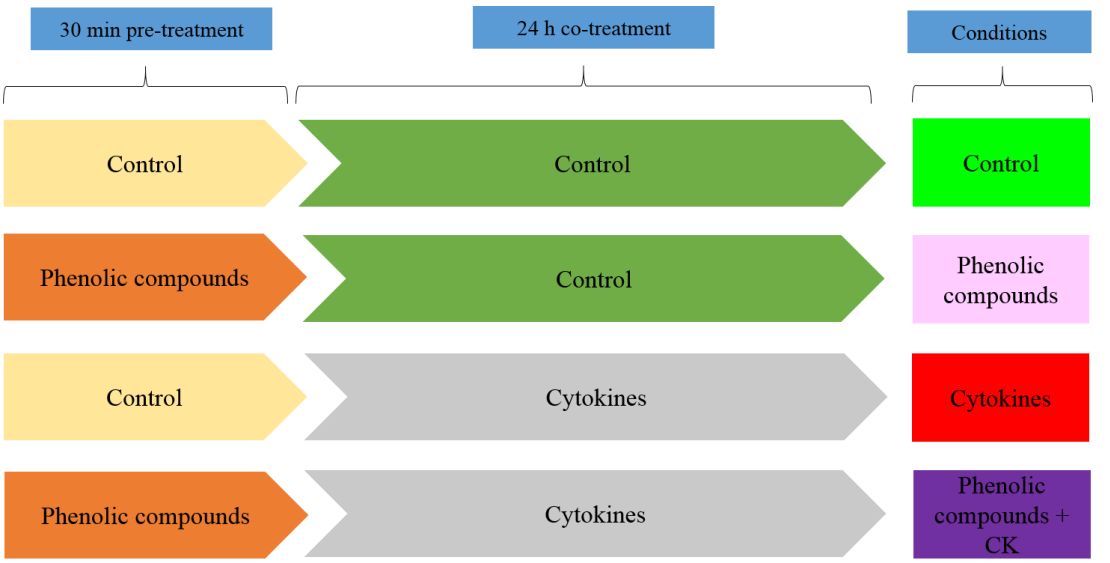

Figure 1S. Schematic representation about the experiment to study if phenolic compounds had the capacity to revert the inflammation caused by cytokines in rat brain endothelial cells. Firstly, cells were pre-treated 30 min with phenolic compounds and, after that, they were simultaneously co-treated with cytokines during 24 h and monitored with the real-time cell electronic sensing.

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V. GENERAL DISCUSSION

V. GENERAL DISCUSSION

Obesity is a current worldwide pathology originated by a prevalent sedentary lifestyle and an incorrect feeding behaviour leading to an excessive fat accumulation resulting from the prolonged imbalance between energy intake and its expenditure. Moreover, it is closely related with other pathologies such as type II diabetes mellitus, hypertension, non-alcoholic fatty acid liver disease and inflammation, among others [1]. Within this context, leptin, a hormone secreted by the adipocytes proportionally to their amounts, plays a crucial role mainly in the CNS controlling food intake and energy expenditure [2]. Basically, it activates anorexigenic neurones (POMC/CART) and down-regulates those neurons implicated in food intake, called orexigenics neurons (NPY/AgRP) [3]. In addition, leptin interacts with specific receptors in many peripheral tissues (i.e., liver, skeletal muscle and WAT) influencing in key metabolic pathways [4].

In obese subjects there are higher amounts of leptin in circulation, a phenomenon called hyperleptinemia. However, it is known that this leptin cannot act normally in these patients originating leptin resistance (LR) [5]. LR appears due to different causes that can be summarized in: (**1**) the impaired leptin pass across the blood-brain barrier (BBB), (**2**) a disruption of the leptin signalling pathway in the hypothalamus, (**3**) inflammation in the hypothalamus, which is closely related with obesity, (**4**) ER stress and (**5**) a low SIRT1 activity [6].

GENERAL DISCUSSION

Current strategies to combat obesity are primarily focused in (**1**) treating radically the pathology via pharmacotherapies [7] or surgery [8], which can cause different problems to the patient, and (**2**) using therapies based on different diets and exercise, although it is extensively demonstrated that this last strategy is difficult to be maintained in prolonged periods of time [9]. Because of that, recent investigations centre the research on the identification of new bioactive compounds from natural origin that can aid in the prevention and development of this problem [10].

In this sense, phenolic compounds are excellent candidates to be used as functional ingredients in obese patients. Phenolic compounds are plant secondary metabolites with a wide described range of healthy beneficial effects, including anti-oxidative, anti-inflammatory, cardio-protective, anti-carcinogenic and anti-neurodegenerative diseases, among others. Regarding the beneficial effects of phenolic compounds it has been described that GSPE: (**1**) increases the leptin signalling pathway and *Pomc* expression in the hypothalamus of HFD-fed rats [11], (**2**) boosts NAD^+ metabolism and SIRT1 expression and activity in a dose-dependent manner in the liver of healthy rats and (**3**) reverts the lipid accumulation in HepG2 cell line in a SIRT1-dependent manner (**Manuscript 5**) [12].

Additionally, as previously mentioned, another cause of LR is the impaired leptin transport into the brain. In the bibliography, it can be found that some phenolic compounds also have the capacity to increase the crossing of leptin through other barriers which are closely similar to the BBB (**Manuscript 1**) [6].

GENERAL DISCUSSION

In this sense, this thesis studies whether resveratrol (**RSV) can act reverting the LR caused by obesity in specific peripheral tissues (liver, muscle and eWAT) in a SIRT1-dependent manner. Furthermore, the capacity of RSV to increase the leptin transport across the BBB, using an *in vitro* model has also been evaluated.**

Hence, the **first objective (Manuscript 2)** has been to evaluate the general effect of RSV restoring the leptin sensitivity in peripheral tissues of CAF-fed male Wistar rats and to determine which of its metabolites the major contributors to this beneficial effect are. For this, we designed an experimental study using Wistar rats that were divided into five groups of six rats. During the first nine weeks, one group was fed with STD diet whereas the others were fed with CAF diet. After nine weeks, either VH or RSV + VH at three doses (50, 100 and 200 mg/kg) were orally administered to the animals. On the twelfth week, the animals were sacrificed and serum, liver, calf skeletal muscle and eWAT were obtained.

The selection of the animal model was done in order to simulate the characteristics present in a situation of human obesity. Experimental animal models can be divided into two groups: genetically engineered mutants and diet-induced models (DIO). Because obesity is caused by a combination of environmental and behavioural factors, the last ones are extensively used for this purpose. Our model is classified into the DIO models, which includes CAF and HFD-induced obesity. The advantage of CAF respect to HFD diet is that this kind of feeding simulates what humans consume in their diet, including high-salt and fat products, low-fiber, processed food with fried

products and energy dense food. It is well-described how a CAF diet causes an increase in body weight, adiposity, hyperinsulinemia, hyperglycemia, glucose intolerance, insulin resistance, inflammation, and hepatoesteatosis, being a perfect model to study obesity and metabolic syndrome [13]. In addition, Myers, *et al.* (2010) concluded that the high-caloric diet and the associated increased adiposity in the DIO animals promote LR, preventing the leptin signalling cascade although the leptin levels in blood are increased. Therefore, it makes the DIO animal a perfect model to study LR in a general context of obesity [14].

We used three different doses of RSV: 50, 100 and 200 mg/kg bw/day, being all of them in the range considered as non-toxic. In rodents, the range of doses of RSV used in the studies is large (1-450 mg/kg bw/day). These doses are safe attending at the values obtained in the transformation using the Reagan-Shaw's formula [15]. Additionally, the duration of the treatments used in the different experiments is quite variable (from 4 to 10 weeks) [16].

A general consensus that RSV reduces body fat accumulation in a rat model of obesity has emerged [17,18] which is confirmed by our results. We obtained a significant reduction in the body fat gain, in hypertriglyceridemia and in the lipid content in liver, calf skeletal muscle and eWAT when rats were supplemented with the highest dose of RSV (200 mg/kg). In this sense, bibliographic references summarize the beneficial effects of RSV in the reduction of the lipid profile due to its capacity to increase adipogenesis in WAT, increase of *de novo* lipogenesis in WAT and liver and decrease

in lipid oxidation in skeletal muscle [19-23]. This information confirms that some of the most important peripheral tissues in which RSV acts are WAT, liver and skeletal muscle, the same used in our study. It is well described that many of the peripheral effects observed by leptin are prevalent in these three tissues due to the fact that leptin receptor is expressed in all of them [24], having different actions according to the tissue. In liver, it modulates the insulin action [25] and it also controls lipid [26] and glucose (Glc) metabolism [27]. According to the skeletal muscle, leptin influences glycogen synthesis, Glc transport and lipid partitioning. Finally, in WAT, leptin is described as a modulator of thermogenesis and insulin and to have lipolytic action [4].

Due to the fact that both leptin and RSV modulate the lipid metabolism in the three peripheral studied tissues, we postulate that if RSV can increase leptin sensitivity in this obesogenic model, maybe its effect is mediated by changes in the lipid profile. However, we could not observe a clear action of RSV modulating the expression of genes implicated in the regulation of the lipid metabolism.

In WAT, RSV acts primarily in the pre-adipocyte differentiation, in *de novo* lipogenesis and the assembly of TAG. Furthermore it is also involved in lipolysis, fatty acid (FA) oxidation and mitochondriogenesis [28] (**Figure 1**). Among all of this pathways, we decided to focus on *de novo* lipogenesis (*Fas* and *Acc* gene expression), the assembly of TAG (*Scd1* gene expression) and on the FA oxidation (*Ppara* and *Cpt1b* expression). We could observe a reduction in the expression of genes involved in the lipid oxidation. However, literature shows that RSV can reduce the expression

and activity of *Fas*, *Acc* and *Scd1* [20] and increases *Ppara* and *Cpt1a* protein content [22].

In liver and skeletal muscle, RSV modulates FA oxidation and mitochondriogenesis and only in liver it also has influence in *de novo* lipogenesis (**Figure 1**). From our results, only in liver RSV markedly increased the expression of genes involved in lipogenesis. In contrast, other authors observed that RSV increased in liver the gene expression and activity of *Cpt1a* [29], *Ppara* [30] and *Acc* and decreased *Fas* [22] whereas in muscle, they found an increase in *Ucp3* and *Pgc1a* [31].

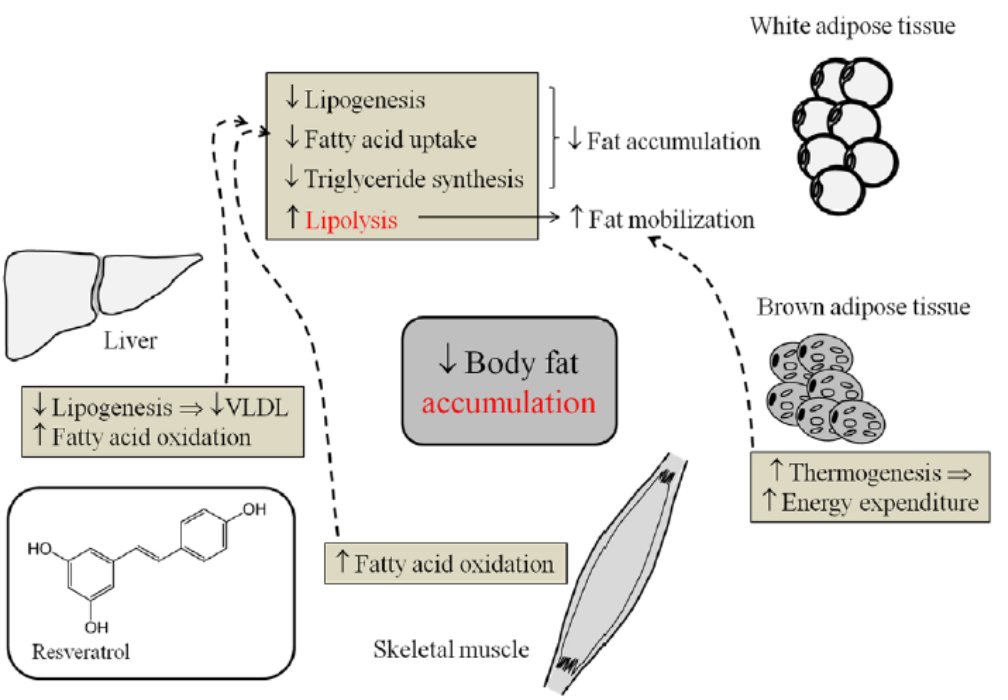

Figure 1. Lipid metabolism crosstalk caused by resveratrol (RSV) in the peripheral tissues: liver, skeletal muscle, brown adipose tissue and white adipose tissue. RSV has many and different actions according to the type of tissue and most of them are interconnected causing a reduction in body fat accumulation. This figure was adapted from [15].

GENERAL DISCUSSION

In light of the results we postulate that the animal model, the RSV doses and the time of the treatment really influence in the differences obtained in our experiment compared with other investigations.

Because we could not find an exact mechanism by which RSV restores the body fat levels caused by the CAF diet, we decided to analyse the *leptin* expression in WATs, concretely in rWAT and eWAT and, surprisingly, we observed a tendency of RSV to reduce its expression. This result points that the ingestion of RSV may activate a compensatory pathway in order to restore homeostasis in the organism. Therefore, we postulate that RSV maybe had these beneficial effects on biometrical parameters because of its capacity to restore the leptin sensitivity.

To confirm this, firstly we decided to study the main molecules involved in the leptin cascade: (**1**) pSTAT3 because it is the most common molecule used in research to evaluate the activation of the leptin signalling pathway [32], (**2**) SOCS3 due to the fact that it can be linked to leptin receptor attenuating the leptin pathway (negative feedback), (**3**) and PTP1B, which is another independent inhibitor of the cascade and dephosphorilates JAK2 [6]. We could not find any significant change for none of them.

Considering that pSTAT3 levels are mainly attributable to the action of leptin in these tissues, we decided to perform a ratio between tissue-specific levels of pSTAT3 and circulating leptin concentration in order to estimate the degree of sensitivity of each

tissue to this hormone. In all the cases evaluated the ingestion of RSV could, at least, partially revert the loss of leptin sensitivity caused by the CAF diet.

Once we determined that RSV increases the leptin sensitivity, we decided to investigate the mechanisms by which it could exert this action. As previously mentioned, LR appears due to many factors, including ER stress (reflected in the increase of *sXbp1, Atf4 and Chop* gene expression), inflammation (increased *iNOS* expression), impaired leptin signalling pathway (altered ObRb protein content) and low SIRT1 activity. In liver, RSV up-regulated SIRT1 activity, suggesting that the mechanism in which RSV restores leptin sensitivity in the liver is via SIRT1-dependent manner. In muscle, an increased ObRb protein content was the possible cause of leptin sensitivity restoration when animals were treated with RSV. In the case of eWAT, we could observe a reduction in the expression of ER stress markers.

Finally, we decided to identify which metabolites of RSV were the agents that cause the beneficial effects in the obese rats. For this purpose, we quantified the presence of metabolites derived from RSV in the serum of the animals treated with the three doses of RSV. It was demonstrated that the profile of metabolites obtained after the ingestion of the highest dose is quite different comparing with the other doses. In general, phase II metabolites are prevalent comparing with those which belong to the microbiota although the dose of 200 mg/kg RSV produced a significant increase in these metabolites from the microbiota respect to the rest. According to the structure, glucuronides were the most prevalent followed by sulfates. The higher presence of

phase II [33] and glucuronide forms obtained in our results agrees with other results that can be found in the bibliography [34]. The dose of administration does not affect absorption but its metabolic conversion can be saturated [35]. The orally administration of RSV at the level of mg/kg leads to tissue concentrations of nmol/g tissue but the differences found among tissues depending on the bibliographic source could be due to changes in the methodological or experimental design of the procedures. In a closely related study in which rats were chronically administered with 6, 30 and 60 mg/kg RSV during 6 weeks (lowest doses but higher time), they analysed RSV metabolites in the three tissues and obtained higher concentrations in liver (ranging from 5 to 100 nmol/g tissue depending on the dose of RSV administered), followed by WAT (ranging from 0.1 to 3 nmol/g tissue depending on the dose of RSV administered) and skeletal muscle (ranging from 0.4 to 0.7 nmol/g tissue depending on the dose of RSV administered) [36]. However, our results are in accordance with the distribution of metabolites of RSV that we obtained in the serum analysis. Hence, from phase II metabolism, the most important metabolites according to the correlation results were R4G, R3S and R4S whereas for those ones which belongs to microbiota family, the DRSG was the prevalent on restoring the damaged parameters caused by the CAF diet.

After finishing the first objective, we focused on a **second objective** (**Manuscript 3**) in which we wanted to describe the exact mechanisms by which RSV was involved in modulating the LR. For this, we decided to work with human liver cancer cells, HepG2. There were two reasons because we decided to work with this kind of cells.

GENERAL DISCUSSION

First of all, it was demonstrated that ObRb is highly expressed in liver cells [24], thus, being active the leptin signalling pathway in this tissue. Secondly, the group has other experiences with this kind of cells. Moreover, in a previous study, HepG2 were used to study the capacity of the GSPE to revert the lipid accumulation and we observed that it was possible and occurred via SIRT1-dependent manner (**Manuscript 5**). That result confirmed other reports, where a restoration of lipid metabolism, concretely a reduction in TAG content in liver, was observed [16].

Because we created different cellular models of fat accumulation using different inductors and, as mentioned previously, there is a crosstalk between lipid metabolism and leptin pathway, we hypothesised that this model of lipid altered metabolism could be a good model for studying LR in these cells. Once we confirmed this, the next step was to verify if ObRb/pSTAT3 pathway was only activated when leptin was added to our cells and it was confirmed using small interfering RNA (siRNA) for ObRb.

The next step was to treat the cells using different doses of RSV and incubation times to try to increase the leptin sensitivity lost in these cells. Thus, the range used was comprised from 1 to 50 μM and the times of incubation were 20 min, 6 h and 24 h. As *in vivo* studies, *in vitro* studies from bibliographic references comprised a wide spectrum of doses used in different cells lines [37]. Concerning HepG2 cells, the incubation time extent from 30 min to 48 h and the doses comprises from 1 μM to 100 μM [16]. However, it is extensively recommended to work using a maximum dose of 10 μM in cells. In this case, we studied pSTAT3 as a main factor of the leptin cascade

and, to confirm the results, we also evaluated the capacity of RSV to reduce the TAG content. Moreover, we performed the same experiments using non-LR cells incubated with RSV at the different conditions and we did not obtain any significant change between the groups.

Once we obtained the best dose and incubation time (10 μM and 24 h, respectively) to work with RSV in this LR model induced in HepG2, the last step was to elucidate the mechanism through RSV acted. For this, we studied both the expression of genes related with lipid metabolism and the different factors that are related with LR, namely the inhibitors of the leptin cascade, ER stress, inflammation, SIRT1 activity as well as the protein content of its receptor. In the case of the studied genes related with lipogenesis, *Fas*, *Acc* and *Scd1*, we observed a tendency to reduce their expression caused by RSV which was significant in *Fas*. These results are in accordance with those obtained by other authors both *in vivo* [22] and *in vitro* [16]. Although we did not observe any significant change between the groups in the case of genes that belong to the lipid oxidation pathway, we could see a reduction in the *Ppara* expression caused by RSV compared with control and inducted cells. The expression of this marker changes according to the dose, time and type of polyphenol used [16].

In the case of the different factors that can influence in the LR, we observed that RSV cannot counteract the increased expression of *Socs3* caused by our used LR inductor and, it was observed a tendency on reducing one of the ER stress markers (*Atf4*). Surprisingly, we could not observe any change when we studied SIRT1 activity, as

we obtained in the case of the *in vivo* study or other reports [38,39]. Our most markedly result was that it seemed that RSV increased the leptin sensitivity of these damaged cells via increasing the protein content of ObRb, results confirmed by western blot and immunofluorescence.

Additionally, we evaluated the metabolites derived from RSV in normal and LR cells at 20 min, 6 h and 24 h and, in both cases, we observed that RSV was converted to R3S when the time passed. Thus, the treatment did not cause an alteration in the enzymes involved in the metabolization of RSV to R3S in these cells. Moreover, we have two hypothesis to explain how RSV restores the leptin sensitivity in this cell model: (**1**) RSV, *per se,* modifies the expression of some molecules that counteract the LR in a long period of time (24 h) and R3S is the result of this process, or (**2**) it needs to be firstly converted to R3S and, after that, this metabolite acts modifying the expression of these molecules.

Taking into consideration all these results, we hypothesised that maybe the discordance between the *in vitro* and *in vivo* results about the mechanism of action of RSV in liver (ObRb- and SIRT1-dependent manner, respectively) was due to the fact that we were working with liver cells with a heavy LR-induced condition than those that were obtained in the liver of CAF-fed animals. For this, different *in vitro* experiments using other less-potent inductors or maybe using shorter times of incubation with these inductors could be performed to try to confirm the *in vivo* results.

Moreover, rats had a chronicle treatment of RSV during three weeks whereas HepG2 cells received an acute dose of this stilbene. Another point is the dose used in the two conditions. In spite of the fact that we knew that for both experiments the doses used were not toxic, they were different, thus, conditioning the final results. The study performed by Andrés-Lacueva, *et al*. (2012), obtained from 5 to 100 nmol/g RSV metabolites in liver when rats were treated during six weeks with 6, 30 and 60 mg/kg RSV [36]. For our dose used in the *in vitro* study, 10 μM, the amount of RSV was 2.28 mg/L. In addition, it should be noted that we worked with a human and immortalized cell line which means that the behaviour can be different if we compare respect to non-cancerous cells from the liver of the rat.

The last important point is the RSV *per se*. RSV is quickly absorbed in the intestine, through a simple transepithelial transport and ATP-binding cassette transporters [40] and most of it undergoes phase II metabolism in enterocytes before passing to the blood (circulating as glucuronides, sulfates or free, linked to albumin or lipoproteins) and arrive into the liver [33,41]. Therefore, RSV arrives to the liver as its metabolites and in the original form and, again, phase II metabolism occurred there [42].

This is an important point because in our *in vitro* study, we worked with trans-RSV and not the metabolites. We have to remark that, in the liver, the direct addition of RSV causes its transformation basically into R3S. Although the metabolites that we obtained in the *in vivo* study were from the serum and not from this specific tissue, we proposed working in HepG2 cells with R4G and R3S, those ones which seemed that restored better the leptin sensitivity in CAF-fed rats.

GENERAL DISCUSSION

RSV presents lipophilic properties leading to a high absorption [43] but the distribution in the tissues is low. Consequently, the interpretation of the *in vitro* results should be done with caution when we try to extrapolate to *in vivo*. Despite its low bioavailability, it is confirmed its efficacy *in vivo*. This can be explained by the conversion of sulfates and glucuronides into RSV in liver [33], by the enterohepatic recirculation of RSV metabolites, which are deconjugated in the intestine and reabsorbed [44] or by the activity of its metabolites *per se*.

Once studied how RSV modulates the leptin signalling pathway in the periphery, the last step was to evaluate its action in the leptin transport across the BBB and if it had the capacity to revert an inflammatory/obese condition caused by pro-inflammatory cytokines (**Manuscript 4**). Moreover, we decided to study other members of the phenolic compounds namely EC (flavonoid), GA (non-flavonoid) and the full grape seed extract, GSPE. Until the date, the only phenolic compounds described as modulators of the leptin transport studied in an *in vitro* model of HUVEC cells were fraxin [45] and quercetin [46]. Recent reports are focused, basically, on creating leptin analogues or ObR agonists improving selectively the leptin transport and BBB permeability without disturbing the integrity of the barrier [47]. Therefore, the confirmation that phenolic compounds can modulate the leptin transport in the brain endothelial cells and the elucidation of their mechanism of action is an unexplored topic which can lead to obtain beneficial effects in terms of health.

For this purpose, we used primary cultures of RBEC. This type of cell cultures is bibliographically described as the best model to simulate BBB because it shows better

thigh junctions pattern, paracellular barrier tightness, influx transporters and drug permeability and a correct expression of metabolic enzymes and the best efflux transporters than other cellular models [48] (**Figure 2**).

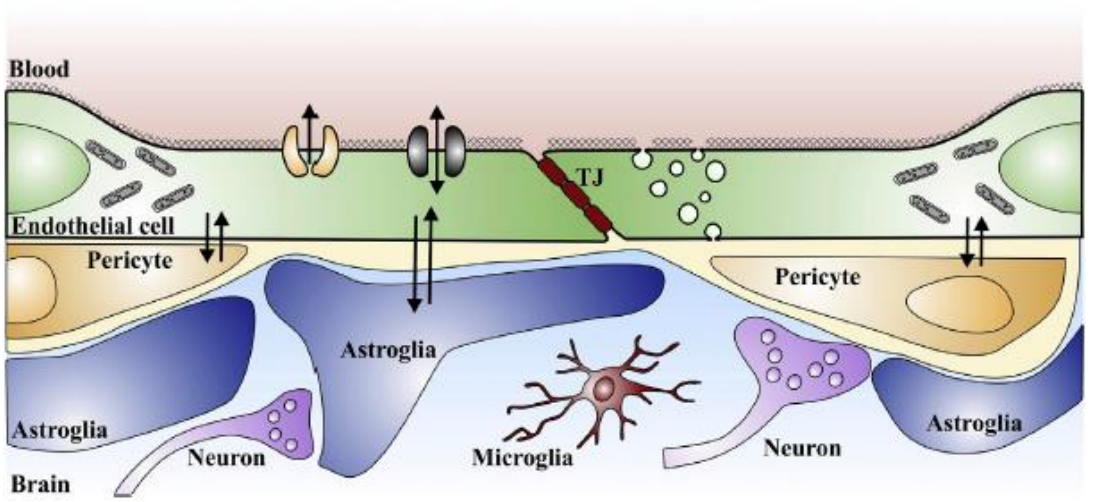

Figure 2. Cells that form blood-brain barrier (BBB) and the interconnections and transport mechanisms implicated in. The BBB is a highly selective semipermeable membrane that separates the circulating blood from the brain and extracellular fluid in the central nervous system (CNS). It is formed by endothelial cells of the capillary wall, astrocyte end-feet ensheathing the capillary, and pericytes embedded in the capillary basement membrane. This figure was adapted from [50].

Hence, firstly we decided to determine the best doses and time of incubation in which cells maintained their barrier integrity. To do this, we performed a real-time cell microelectronic sensing measuring the TEER values, which is a properly BBB validation marker [49]. After performing the experiment, we decided to work with 10 μM RSV and EC, 1 μM GA and 10 μg/mL GSPE during 24 h.

The next step was to evaluate whether the natural compounds had de capacity to increase the expression of leptin receptors (i.e., *ObRb*, *ObRa* and *Lpr2*) as it is

described that leptin only enters to the brain via receptor-mediated transport [50]. In RBEC, the major leptin receptor is ObRa [51] although the presence of ObRb and LPR2 is also reported [52,53]. Surprisingly, all the compounds evaluated, unless GA, remarkably increased the gene expression of *ObRa*.

Furthermore, we postulated that phenolic compounds could modulate the expression of molecules involved in the regulation of the leptin cascade such as *clusterin*, *Socs3* and *Ptp1b*. However, we could not observe significant changes between the groups. Clusterin was described as a molecule with the property to help the leptin transport via LPR2-mediated endocytosis [54].

There is an association between obesity-LR states and a systemic chronic low-grade inflammation [55,56] due to the fact that in obesity there is an over-production of pro-inflammatory adipokines [57]. Thus, we considered adequate to induce a pro-inflammatory state in the BBB model to study a condition of obesity-LR. For this, we pre-treated RBEC during 30 min with phenolic compounds and, after that, cells were co-treated with 10 ng/mL TNFα + 10 ng/mL IL-1β during 24 h. All the phenolic compounds partially reduced the loss of the barrier integrity caused by the pro-inflammatory cytokines. This model was previously described by Harazin, *et al.*, 2018 [58].

Taking into consideration the results obtained in the present thesis, we confirm the potential of phenolic compounds, particularly RSV, on preventing or treating the LR caused by obesity.

GENERAL DISCUSSION

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VI. CONCLUSIONS

VI. CONCLUSIONS

The main conclusions obtained in the present thesis are:

1. Resveratrol (RSV) restores the leptin sensitivity in the three peripheral tissues (liver, skeletal muscle and epididymal white adipose tissue (eWAT)) studied in cafeteria (CAF)-fed male Wistar rats. Moreover, any circulating RSV metabolite, either from phase II or from microbiota, stands out as major responsible of the observed beneficial effects of this phenolic compound in this animal model **(Manuscript 2)**.

- Only the dose of 200 mg/kg RSV, the highest used in this experiment, has the capacity to revert the altered biometrical parameters and lipid profile caused by the CAF diet.

- In addition, 200 mg/kg RSV reverts the leptin resistance (LR) caused by the obesogenic diet in the three studied tissues. However, the mechanism of action differs depending on the tissue evaluated.

- RSV metabolites correlate positively with the leptin sensitivity and negatively with the biometrical parameters (body weight gain, total body fat content and serum leptin levels).

- The intake of higher doses of RSV (200 mg/kg) increases only the levels of metabolites derived from the microbiota pointing to the crucial role of these metabolites in the beneficial effects observed with this dose.

CONCLUSIONS

2. RSV has the capacity to counteract the LR induced in human hepatocarcinoma cell line (HepG2 cells) (**Manuscript 3**).

- 10 ng/mL leptin during 20 min are required to activate the leptin cascade in HepG2 cells and 48 h with 0.5 mM Palmitate + 30 mM Glucose to induce LR.

- RSV, at doses of 10 and 50 μM, can activate the leptin signalling cascade reverting the down-regulated levels of phosphorylated signal transducer and activator of transcription 3 (pSTAT3) caused by the LR inductor.

- The mechanism by which RSV reverts LR in HepG2 cells is via an increase of the protein levels of the leptin receptor isoform b (ObRb).

3. The phenolic compounds evaluated in this study, namely RSV, (-)-epicatechin (EC), gallic acid (GA), and the grape seed proanthocyanidin extract (GSPE), may promote leptin transport across the blood-brain barrier (BBB) modulating the expression of potential leptin transporters. Moreover, they have the capacity to revert the barrier disruption caused by inflammation in rat brain endothelial cells (RBEC) (**Manuscript 4**).

- Selected doses of RSV, EC and GSPE increase the expression of *ObRa* at 24 h in RBECs.

- RBEC co-treated with pro-inflammatory cytokines and selected doses of phenolic compounds can revert, partially, the pathological conditions caused in this *in vitro* model of BBB.

VI. CONCLUSIONS

Les principals conclusions obtingudes en la present tesis són:

1. El Resveratrol (RSV) restaura la sensibilitat a la leptina en els tres teixits perifèrics (fetge, múscul esquèlètic i teixit adipós blanc de l'epidídim (eWAT)) que es van estudiar de les rates Wistar mascles alimentades amb una dieta de cafeteria (CAF). A més a més, cap dels metabòlits circulants del RSV, tant de fase II com de microbiota, destaca majoritàriament com a responsable dels efectes beneficiosos d'aquest compost fenòlic en aquest model animal (**Manuscrit 2**).

• Únicament la dosi de 200 mg/kg RSV, la més alta utilitzada en l'experiment, té la capacitat de revertir l'alteració en els paràmetres biomètrics i en el perfil lipídic causada per la dieta de CAF.

• Addicionalment, 200 mg/kg RSV reverteixen la resistència a la leptina (LR) causada per la dieta obesogènica en els tres teixits estudiats. Però, el mecanisme d'acció difereix depenent del teixit avaluat.

• Els metabòlits del RSV correlacionen positivament amb la sensibilitat a la leptina i negativament amb els paràmetres biomètrics (guany del pes corporal, el contingut total de greix en l'organisme i els nivells de leptina en sèrum).

• La ingesta de dosis elevades de RSV (200 mg/kg) incrementa només els nivells de metabòlits provinents de la microbiota, suggerint un rol crucial d'aquests en els efectes beneficiosos observats amb aquesta dosi.

CONCLUSIONS

2. El RSV té la capacitat de contrarestar la LR induïda en la línea cel·lular cancerígena humana d'hepatòcits (cèl·lules HepG2) (**Manuscrit 3**).

- Es requereixen 10 ng/mL de leptina durant 20 min per activar la seva via de senyalització en les cèl·lules HepG2 i incubar-les durant 48 h amb 0.5 mM Palmitat + 30 mM Glucosa per induir la LR.

- El RSV, a les dosis de 10 i 50 μM, pot activar la via de senyalització de la leptina revertint els baixos nivells de fosforilació del transductor de senyals i activador de transcripció 3 (pSTAT3) causats per l'inductor de la LR.

- El mecanisme pel qual el RSV reverteix la LR en les cèl·lules HepG2 és a través de l'increment dels nivells proteics de la isoforma b del receptor de la leptina (ObRb).

3. Els compostos fenòlics avaluats en aquest estudi (RSV, (-)-epicatequina (EC), àcid gàl·lic (GA)) i l'extracte de proantocianidines de la llavor del raïm (GSPE) podrien promoure el transport de la leptina a través de la barrera hematoencefàlica (BBB) modulant l'expressió de possibles transportadors de la leptina. A més a més, tenen la capacitat de revertir l'alteració de la barrera causada per un procés inflamatori en les cèl·lules endotelials de cervell de rata (RBEC) (**Manuscrit 4**).

- Dosis seleccionades de RSV, EC i GSPE incrementen l'expressió d'*ObRa* a les 24 h en les cèl·lules RBEC.

CONCLUSIONS

• El co-tractament de les RBEC amb citocines pro-inflamatòries i les dosis seleccionades dels compostos fenòlics poden revertir, parcialment, les condicions patològiques causades en aquest model *in vitro* de BBB.

VII. APPENDICES

VII. APPENDICES

1. List of publications

• Aragonès G, Ardid-Ruiz A, Ibars M, Suárez M, et al. Modulation of leptin resistance by food compounds. Mol Nutr Food Res. 2016, 60(8), 1789-1803.

• Aragonès G, Suárez M, Ardid-Ruiz A, Vinaixa M, et al. Dietary proanthocyanidins boost hepatic NAD(+) metabolism and SIRT1 expression and activity in a dose-dependent manner in healthy rats. Sci Rep. 2016, 6, 24977.

• Ibars M, Ardid-Ruiz A, Suárez M, Muguerza B, et al. Proanthocyanidins potentiate hypothalamic leptin/STAT3 signalling and Pomc gene expression in rats with diet-induced obesity. Int J Obes (Lond). 2017, 41(1), 129-136.

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• Ibars M, Aragonès G, Ardid-Ruiz A, Gibert-Ramos A, et al. Seasonal consumption of polyphenol-rich fruits affects the hypothalamic leptin signalling system in a photoperiod-dependent mode. Sci Rep. 2018, 8(1), 13572.

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• Ardid-Ruiz A, Ibars M, Mena P, del Rio D, et al. Potential involvement of peripheral leptin/STAT3 signalling in the effects of resveratrol and its metabolites on reducing body fat accumulation. Mol Nutr Food Res (Submitted).

• Ardid-Ruiz A, Ibars M, Mena P, del Rio D, et al. Resveratrol enhances the cellular response to leptin by increasing the leptin receptor (ObRb) content in palmitate-induced steatotic HepG2 cells. Nutrients (Submitted).

• Ardid-Ruiz A, Harazin A, Barna L, Bladé C, et al. Effects of resveratrol, epicatechin, gallic acid and a grape seed proanthocyanidin-rich extract on primary rat brain endothelial cells: expression of leptin receptors and protection against cytokine-induced damage. Manuscript under preparation.

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2. List of posters

• Ardid-Ruiz A, Vizárraga D, Ibars M, Bladé C, Suárez M, Aragonès G. Grape-Seed Proanthocyanidins decrease the triglycerides content in HepG2 cells by a sirtuin-dependent mechanism. NuGOweek 2015, 12th edition: Mechanisms of a long-life health.

• Ibars M, Ardid-Ruiz A, Suárez M, Arola L, Bladé C, Aragonès G. Resveratrol increases the expression of leptin receptor in the hypothalamus of healthy mice after short-term treatment. ICPH 2015.

• Ibars M, Ardid-Ruiz A, Suárez M, Arola L, Bladé C, Aragonès G. Proanthocyanidins overexpress POMC and reduce food intake in leptin resistant DIO rats. NuGOweek 2015, 12th edition: Mechanisms of a long-life health.

• Ardid-Ruiz A, Ibars M, Bladé C, Aragonès G and Suárez M. Resveratrol rescues hepatic leptin signal transduction via stat3 pathway in a cellular model of fat accumulation induced by palmitic acid. XXVIIIth International Conference on Polyphenols 2016.

• Ardid-Ruiz A, Djemmah I, Ibars M, Bladé C, Aragonès G, Bravo FI, Suárez M. Chronic consumption of resveratrol reduces adiposity and circulating leptin in diet-induced obese rats. IX seminario sobre alimentación y estilos de vida saludables, 2017.

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• Suárez M, Ardid-Ruiz A, Ibars M, Mena P, del Rio D, Bladé C, Aragonès G. Understanding the anti-obesity effects of resveratrol: phase II conjugates vs gut microbiota metabolites. NuGOweek 2017, 14th edition: Molecular nutrition - understanding how food influences health.

• Aragonès G, Suárez M, Ardid-Ruiz A, Ibars M, Mena P, del Rio D, Arola L, Bladé C. Sulfate-conjugated resveratrol metabolites are associated with increased hepatic leptin/STAT3 signalling in obese rats treated with a high-dose of trans-resveratrol. Wine health 2017.

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• Martin M, Mas-Capdevila A, Gibert A, Ardid-Ruiz A, Aragonès G, Muguerza B. Reduction of hypertriglyceridemia and improvement of triglycerides homeostasis in cafeteria diet-fed rats by a chronic administration of natural extract. SEBBM 2017.

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• Ardid-Ruiz A, Harazin A, Barna L, Deli M, Bladé C, Suárez M and Aragonès G. Beneficial effects of resveratrol and epicatechin on leptin transport across the blood-brain barrier in primary cultures of rat brain capillary endothelial cells. FBHC 2018.

VIII. ANNEXES

ANNEXES

Manuscript 5

Dietary proanthocyanidins boost hepatic NAD^+ metabolism and SIRT1 expression and activity in a dose-dependent manner in healthy rats

Gerard Aragonès, Manuel Suárez, Andrea Ardid-Ruiz, Maria Vinaixa, Miguel A Rodríguez, Xavier Correig, Lluís Arola, Cinta Bladé

Sci Rep. 2016, 6, 24977

PREFACE

This paper studies the role of grape seed proanthocyanidin extract in the modulation of nicotinamides. Specifically, it is seen that it modulates the hepatic concentrations of the major nicotinamide adenine dinucleotide precursors as well as the gene expression of enzymes involved in the cellular metabolism of nicotinamide adenine dinucleotide. Notably, *sirtuin 1* gene expression and activity is also significantly up-regulated in a dose-dependent manner in healthy rats. In addition, grape seed proanthocyanidin extract has the capacity to revert lipid accumulation in human hepatocarcinmoma cell line, HepG2 cells, in a sirtuin 1-dependent manner. It is known that one of the causes of leptin resistance is the reduction of the sirtuin 1 activity. Therefore, this lead us to postulate that if resveratrol is a sirtuin 1-modulator, maybe it can restore leptin sensitivity in peripheral organs.

ANNEXES

Dietary proanthocyanidins boost hepatic NAD^+ metabolism and SIRT1 expression and activity in a dose-dependent manner in healthy rats

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ANNEXES

Abstract

Proanthocyanidins (PACs) have been reported to modulate multiple targets by simultaneously controlling many pivotal metabolic pathways in the liver. However, the precise mechanism of PAC action on the regulation of the genes that control hepatic metabolism remains to be clarified. Accordingly, we used a metabolomic approach combining both nuclear magnetic resonance and mass spectrometry analysis to evaluate the changes induced by different doses of grape-seed PACs in the liver of healthy rats. Here, we report that PACs significantly increased the hepatic nicotinamide adenine dinucleotide (NAD^+) content in a dose-dependent manner by specifically modulating the hepatic concentrations of the major NAD^+ precursors as well as the mRNA levels of the genes that encode the enzymes involved in the cellular metabolism of NAD^+. Notably, *Sirtuin 1* (*Sirt1*) gene expression was also significantly up-regulated in a dose-response pattern. The increase in both the NAD^+ availability and *Sirt1* mRNA levels, in turn, resulted in the hepatic activation of SIRT1, which was significantly associated with improved protection against hepatic triglyceride accumulation. Our data clearly indicates that PAC consumption could be a valid tool to enhance hepatic SIRT1 activity through the modulation of NAD^+ levels.

ANNEXES

1. Introduction

Natural dietary polyphenols and specifically proanthocyanidins (PACs), the most structurally complex subclass of flavonoids, are bioactive food compounds that are primarily present in fruits and vegetables and exhibit many protective effects against cardiovascular disease [1]. In this context, our group and others have reported many healthy and beneficial effects of PACs on different metabolic syndrome-related pathologies, such as insulin resistance, dyslipidemia, obesity, hypertension and inflammation [2-7]. Furthermore, other studies performed at the molecular level have demonstrated that PACs could play an important role in the regulation of the transcriptional networks that control various critical metabolic processes in the liver. Specifically, PAC consumption was shown to protect the liver from lipid accumulation by reducing the expression of target lipogenic genes and up-regulating fatty acid oxidation [8-10].

Several mechanisms by which PACs reduce these hepatic metabolic disturbances have been described, such as the direct interaction with intracellular signalling pathways [11] and modulation of epigenetic factors, including both microRNAs [12,13] and components of the DNA methylation machinery [14]. However, the actual molecular mechanisms involved in the health benefits of PAC consumption in the liver remain mostly speculative and the global mechanism of action is still largely unknown. This might be because previous studies have primarily focused on genomic or proteomic changes instead of at the direct changes in the hepatic metabolites. Certainly,

metabolomics, one of the *most rapidly* growing fields of contemporary science [15], might be a good alternative to characterize the hepatic metabolites that are modified as a result of exogenous challenges, such as PAC consumption, and provide new evidence linking the cellular pathways to the biological mechanisms.

In this context, two different technologies have the potential to discover the metabolic alterations in liver samples: nuclear magnetic resonance (NMR) and mass spectrometry (MS) [16-18]. However, to the best of our knowledge, there are no previous metabolomic studies in the literature aimed at evaluating the effect of PAC consumption on liver metabolites. Therefore, here, we performed a multiplatform approach combining both NMR and MS metabolomic analysis of the liver of healthy rats that were chronically supplemented with different doses of PACs. Because homeostasis is very robust, challenging homeostasis is more informative than static homeostatic studies [19]. Therefore, we aimed to quantitatively identify the alterations in hepatic metabolites in response to a fat overload challenge to further elucidate the mechanism by which PAC consumption can modulate lipid metabolism in liver. Our results revealed that PAC consumption could be relevant to improve the hepatic lipid metabolism in an *in vivo* model by regulating the liver's response through a metabolic increase in both cellular nicotinamide adenine dinucleotide (NAD^+) availability and Sirt activity.

2. Materials and Methods

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2.1 Grape seed PAC extract composition

The grape seed PAC extract was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, the extract is mainly composed of phenolic compounds (total content higher that 96 %) being this phenolic content composed of monomers or flavan-3-ols (21.3 %), dimers (17.4 %), trimers (16.3 %), tetramers (13.3 %) and oligomers (5-13 units; 31.7 %) of procyanidins. The phenolic composition of this extract was deeper analyzed by Quiñones, *et al.* (2013) [45]. Briefly, from their manuscript it can be seen that the most important phenolic compounds contained in the extract were: catechin (58 μmol/g), epicatechin (52 μmol/g), epigallocatechin (5.50 μmol/g), epicatechin gallate (89 μmol/g), epigallocatechin gallate (1.40 μmol/g), dimeric procyanidins (250 μmol/g), trimeric procyanidins (1568 μmol/g), tetrameric procyanidins (8.8 μmol/g), pentameric procyanidins (0.73 μmol/g), and hexameric procyanidins (0.38 μmol/g).

2.2 Animal handling

The investigation has been conducted in accordance with the ethical standards and according to the Declaration of Helsinki and has been approved by the Ethics Review Committee for Animal Experimentation of the Universitat Rovira i Virgili. Forty six-week-old male Wistar rats were purchased from Charles River Laboratories (Barcelona, Spain). The animals were singly housed in a 12 h light-dark-cycle at 22 °C and were provided a standard chow diet (Panlab 04, Barcelona, Spain) and tap water *ad libitum*. After one week of adaptation, the animals were randomly divided

into four groups (n = 10) and supplemented with 0 (control group), 5, 25 or 50 mg of PACs/kg body weight (bw) for 21 days. The grape seed PAC extract was dissolved in 750 µL of commercial sweetened skim condensed milk (Nestle; 100 g: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates, 1175 kJ) at the appropriate concentrations. Before supplementation, all of the rats were trained to voluntarily lick the milk, and all groups were administered with the same volume of condensed milk for 21 days. The treatment was administered every day at 9:00 am. After 21 days of supplementation, the rats were fasted overnight. At 9:00 am, the rats were orally gavaged with lard oil (2.5 mL/kg bw) with the appropriate dose of the extract (0, 5, 25 or 50 mg/kg bw). Three hours later, the rats were sedated using a combination of ketamine (70 mg/kg bw, Parke-Davis, Grupo Pfizer, Madrid, Spain) and xylazine (5 mg/kg bw, Bayer, Barcelona, Spain). After anesthetization, the rats were exsanguinated from the abdominal aorta. The liver was excised and frozen immediately in liquid nitrogen and stored at -80 ºC until the RNA and metabolites were extracted.

2.3 ^{1}H-NMR analysis

The liver metabolite extraction was performed according to the procedure described by Vinaixa, *et al.* (2010) [16]. Briefly, 50 mg of hepatic tissue was removed, flash-frozen, and mechanically homogenized in 1 mL of H_2O/CH_3CN (1/1). The homogenates were centrifuged at 5,000 × *g* for 15 min at 4 °C. The supernatants (hydrophilic metabolites) were separately lyophilized overnight and stored at -80 °C until further analysis. For the NMR measurements, the hydrophilic dried extracts were

reconstituted in 600 μL of D_2O containing 0.67 mM trimethylsilyl propionic acid (TSP). The samples were then vortexed, homogenized for 20 min, and centrifuged for 15 min at 6,000 × *g* at 4 °C. Finally, the redissolved samples were placed into 5 mm NMR tubes. The ^{1}H-NMR spectra were recorded at 300 K on an Avance III 600 spectrometer (Bruker, Germany) operating at a proton frequency of 600.20 MHz using a 5 mm CPTCI triple resonance (^{1}H, ^{13}C, ^{31}P) gradient cryoprobe. The one-dimensional ^{1}H pulse experiments were performed using the nuclear Overhauser effect spectroscopy (NOESY) presaturation sequence (RD-90°-t1-90°-tm-90° ACQ) to suppress the residual water peak. Solvent presaturation with an irradiation power of 75 Hz was applied during the recycling delay (RD = 5 seconds) and mixing time (tm = 100 ms). The 90° pulse length was calibrated for each sample and varied from 6.57 to 6.99 ms. The spectral width was 12 kHz (20 ppm), and a total of 256 transients were collected into 64 k data points for each ^{1}H spectrum. The exponential line broadening applied before Fourier transformation was 0.3 Hz. The frequency domain spectra were phased and baseline-corrected using TopSpin software (version 2.1, Bruker).

2.4 LC-QqQ-MS/MS analysis

NAD^+ and its related metabolites and end-products, Nam, NMN, Na, Nr, NaMN, NaAD, nicotinamide *n*-oxide, nicotinuric acid, *n*-methylnicotinamide, and Trp, were measured in the liver samples using an LC-QqQ-MS/MS system consisting of an Agilent HPLC 1200 Series (Agilent Technologies, Palo Alto, U.S.A.). Briefly, 60 mg

of the lyophilized liver samples were vigorously vortexed in 0.5 mL of physiological saline for 30 s, followed by 30 s of ultrasonication in a Vibra Cell (Sonics, Newton, USA). Then, 0.5 mL of acetone was added and the samples were centrifuged at 10,000 x *g* for 15 min at 20 ºC. This procedure was repeated twice; the upper phases were mixed and evaporated under nitrogen flow to dryness. Finally, the dried samples were dissolved in 100 µL of the mobile phase at the initial conditions and injected into the liquid chromatograph (LC). A Scherzo SM-C18 (3 mm; 150 mm x 2 mm i.d.; Imtakt, Japan) was used to perform the analysis. The LC was coupled to a triple quadrupole (QqQ) mass spectrometer (MS) 6410 (Agilent Technologies, Palo Alto, USA).

2.5 Cell culture and treatments

The HepG2 human hepatoma cells obtained from the American Type Culture Collection (ECACC code 85011430) were cultured in DMEM containing 25 mM Hepes, 10 % (vol/vol) FBS, 2 mM L-glutamine, 0.1 mM NEAA, 100 U/mL penicillin, and 100 mg/mL streptomycin, 2.5 mg/L Amphotericin B solution at 37 °C in a humidified atmosphere containing 5 % CO_2, with medium changes three times a week. The cells were incubated in 12-well plates at a density of $5x10^6$ cells/well for 48 h or until the cells were 70 % confluent before starting the experimental treatments. To establish a hepatocellular model of fat-overloading, the HepG2 cells were incubated with a mixture of 0.5 mM palmitate (P9767, Sigma-Aldrich, Madrid, Spain) combined with 0.5 % fatty acid free-BSA (A3803, Sigma-Aldrich) and 30 mM glucose (131341.1211, Panreac, Barcelona, Spain) for 48 h. For the last 24 h of incubation,

the cells were treated with 100 mg/L PACs in the presence or absence of 10 or 100 μM sirtinol (10523, Cayman Chemical, Tallin, Estonia), a SIRT1 inhibitor. The treatments were carried out in triplicate in three independent experiments. Finally, the cell lysates were collected and stored at -80 °C for the triglyceride and RT-qPCR analyses.

2.6 qRT-PCR analysis

The total RNA was extracted from the liver and cultured cells using TRIzol reagent and an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. The quantity and purity of RNA was measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). Samples with the RNA concentration (A260/A280 ≥ 1.8 ng/μL) and purity (A230/A260 ≥ 2.0 ng/μL) were selected. Additionally, RNA integrity was assessed by denaturing gel electrophoresis stained with SYBR Green dye (BioRad). Complementary DNAs were generated using the High-Capacity complementary DNA Reverse Transcription Kit from Life Technologies (Uppsala, Sweden). The relative mRNA expression levels of the selected genes were assessed in the liver and normalized to the cyclophilin peptidylprolyl isomerase A (*Ppia*) mRNA levels. The forward (F) and reverse (R) primers used in this study can be found as Supplementary **Table 1S**. Briefly, a total of 10 ng of the cDNAs was subjected to quantitative RT-PCR amplification using the SYBR Green PCR Master Mix from Bio-Rad (Barcelona, Spain). The reactions were run on a CFX96 real-time system-C1000 Touch Thermal Cycler (Bio-Rad); the

thermal profile settings were 50 °C for 2 min, 95 °C for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min. Finally, the fold change in the mRNA levels was calculated and normalized to the linear form by the $2^{-\Delta\Delta Ct}$ calculation [46]. For that, only samples with a quantification cycle lower than 30 were used for fold change calculation.

2.7 SIRT1 activity assay

Hepatic SIRT1 activity was determined according to the method described by Becatti, *et al*. (2012) [47] with some modifications. Liver extracts were obtained using a mild lysis buffer (50 mM Tris-HCl pH 8, 125 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 10 % glycerol, and 0.1 % NP40). SIRT1 activity was measured using a SIRT1 direct fluorescent screening assay kit (Cayman, Ann Arbor, MI), following the manufacturer's protocol. Briefly, a total of 25 µL of assay buffer (50 mM Tris-HCl, pH 8.0, containing 137 mM NaCl, 2.7 mM KCl, and 1 mM $MgCl_2$), 5 µL of extract, and 15 µL of substrate solution were added to all wells. The fluorescence intensity was monitored every 2 min for 1 h using the fluorescence plate reader Bio-Tek FLx800, applying an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The results are expressed as the rate of reaction for the first 30 min, when there was a linear correlation between the fluorescence and this period of time.

2.8 Lipid analysis

The liver lipids (0.5 g) were extracted using the Folch method [48]. An aliquot of the extract was subjected to gravimetric analysis to measure the total lipid concentrations.

The remaining extract was allowed to evaporate under a nitrogen flow and dissolved in 1 mL of lipoprotein lipase buffer, containing 1,4-piperazinediethanesulfonic acid disodium salt (P3768, Sigma-Aldrich), $MgCl_2 \cdot 6H_2O$ (M9272, Sigma-Aldrich), albumin-free fatty acids (A8806, Sigma-Aldrich) and 0.1 % sodium dodecyl sulfate (L3771, Sigma-Aldrich). The triglycerides and cholesterol concentrations in the dissolved extract and in the cultured cell lysates were measured using QCA enzymatic colorimetric kits (QCA, Barcelona, Spain) according to the manufacturer's protocols.

2.9 Data analysis and statistical methods

The pure compound references in BBioref AMIX (Bruker), HMDB and Chenomx databases were used for the ^{1}H NMR metabolite identification. In addition, we assigned the metabolites by ^{1}H - ^{1}H homonuclear correlation (COSY and TOCSY) and ^{1}H - ^{13}C heteronuclear (HSQC) 2D NMR experiments and by correlation with the pure compounds run in-house. After baseline correction, the specific ^{1}H NMR regions identified in the spectra were integrated using the AMIX 3.9 software package (Bruker, GmBH) and the resulting dataset was explored through principal component analysis (PCA). LC-QqQ-MS/MS data acquisition was performed using Masshunter software. The analyses were performed in positive electrospray ionization (ESI+) mode. The selected reaction monitoring (SRM) transitions and the individual fragmentor voltage and collision energy for each compound were evaluated using commercial standards to obtain the best instrumental conditions. Two transitions were acquired for each compound: one for quantification and a second for confirmation

purposes (data not shown). The quality parameters of the analytical method (linearity, recovery, accuracy, reproducibility, limit of detection and limit of quantification) were evaluated to confirm the reliability of the method. These parameters were determined by spiking the basal liver extract with known concentrations of the standards. All of the reported values are expressed as the means ± SEM (standard error) and were analyzed using the IBM SPSS for Windows statistical package (v.21.0). The differences between groups were initially calculated using ANOVA (single-factor or two factors) and the Mann-Whitney *U*-test; Student's unpaired *t* test and Fisher's exact test were used as necessary. Spearman's correlation coefficients were also used to evaluate the degree of association between variables. Finally, a multiple linear regression model was fitted to evaluate which gene expression and metabolomic variables were independently associated with the hepatic fat content. The P-values for the gene expression analyses were calculated using the $2^{-\Delta\Delta Ct}$ method. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1 Metabolomics revealed that PAC consumption robustly increased the hepatic NAD^+ levels in a dose-response manner

The liver metabolic profile changes associated with PAC consumption were initially assessed only in the liver samples from animals receiving 0, 5 and 25 mg of PAC/kg bw using an untargeted ^{1}H-NMR-based metabolomics approach. A total of 46 spectral regions were identified and quantified from the ^{1}H-NMR spectra acquired from liver

extracts. Then, a multivariate principal components analysis (PCA) was performed on the resulting data for exploratory purposes. The PCA score plot accounted for a 57 % variance of the original matrix and did not show any clear clustering trend of the data according to the PAC consumption groups (**Figure 1A**). Subsequently, we used a one variable at a time 1-way ANOVA to compare the PAC consumption groups. The concentrations of fumaric acid (singlet, 2×CH, δ = 6.5 ppm) were slightly decreased as a consequence of PAC consumption (P = 0.04 by Tukey's HSD post-hoc test), whereas the hepatic nicotinamide (Nam) levels (singlet, H-2, δ=8.9 ppm & doublet, H-6, δ = 8.7 ppm) were robustly increased in those animals supplemented with 25 mg of PAC/kg bw (**Figure 1B**).

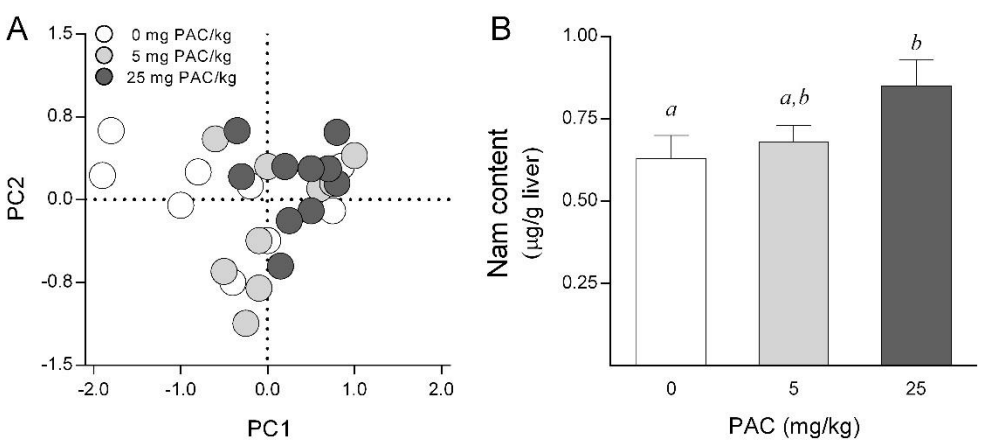

Figure 1. Metabolic profile assessed by untargeted H^1NMR in the liver of healthy rats supplemented with different doses of PACs. (A) The PC analysis did not show statistically significant changes in liver metabolic profile following PAC consumption. (B) The animals supplemented with 25 mg of PACs/kg bw displayed higher hepatic Nam levels compared to the control group. The rats were fed a standard chow diet supplemented with 0 (control group), 5 or 25 mg of PACs/kg of body weight for 21 days. The values shown are the means ± SEM of 10 animals per group. The letters denote a significant difference between groups (P < 0.05; one-way ANOVA and Tukey's HSD post-hoc test comparison). Nam: nicotinamide; PACs: proanthocyanidins; PC: principal component.

Next, as Nam is one of the main liver precursors of NAD^+, these results were validated and complemented by quantifying the NAD^+ levels with targeted analyses of the liver samples using triple-quadrupole mass spectrometry (QqQ-MS/MS). Notably, in order to further investigate the linear dose-dependent related effects of PAC consumption, additional liver samples of another group of animals receiving a higher dose of PAC (50 mg/kg bw) were also evaluated in targeted analyses. Accordingly, we confirmed that PAC consumption significantly increased the hepatic NAD^+ levels in a dose-dependent manner (**Figure 2A**), indicating that PAC administration could be a valid tool to boost the cellular NAD^+ content in the liver. In addition, the hepatic Nam levels were clearly higher in the PAC-supplemented animals compared to the control group (**Figure 2B**), as previously observed by global NMR. Then, we tested whether the increase in the NAD^+ and Nam content would be paralleled by changes in the other major liver metabolites involved in the NAD^+ cycle. Accordingly, the tryptophan (Trp), nicotinic acid (Na) and nicotinamide mononucleotide (NMN) concentrations were also significantly increased in animals supplemented with 50 mg of PAC/kg bw compared to the control rats (**Figure 2C**). Additionally, a positive and significant relationship between the hepatic NAD^+ concentrations and the levels of their major immediate precursors in the liver, Trp ($rho = 0.622$, $P < 0.001$) and Nam ($rho = 0.604$, $P < 0.001$), was also observed, suggesting that the increase in the hepatic NAD^+ content is due to changes of these two NAD^+ precursors. Finally, other liver intermediates and the end-products of NAD^+ catabolism were also measured. However, the nicotinamide riboside (Nr), nicotinic acid mononucleotide (NaMN),

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nicotinic acid adenine dinucleotide (NaAD), nicotinamide *n*-oxide, nicotinuric acid and *n*-methylnicotinamide levels did not display significant differences among the groups (data not shown) or were below the limit of detection in all groups of animals.

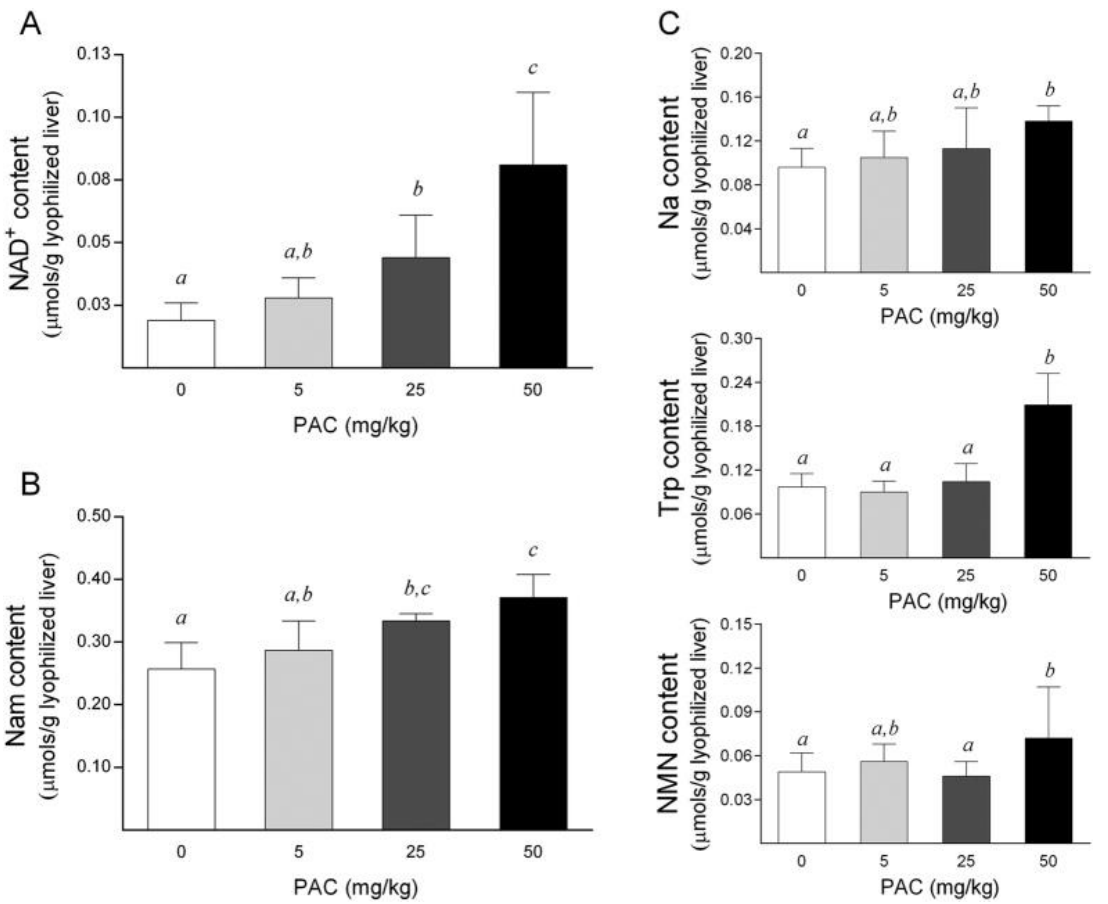

Figure 2. Hepatic levels of NAD$^+$ and the main NAD$^+$ precursors in healthy rats supplemented with different doses of PACs. (A-B) PAC consumption increased the hepatic NAD$^+$ (A) and Nam (B) levels in a dose-dependent manner. (C) Similarly, the Trp, Na and NMN concentrations were also significantly increased in animals supplemented with 50 mg of PACs/kg bw compared to the control rats. The animals were fed a standard chow diet supplemented with 0 (control group), 5, 25 or 50 mg of PACs/kg bw for 21 days. The values shown are the means ± SEM of 10 animals per group. The letters denote a significant difference between groups ($P < 0.05$; one-way ANOVA and LSD post-hoc test comparison). Na: nicotinic acid; NAD$^+$: nicotinamide adenosine dinucleotide; Nam: nicotinamide; NMN: nicotinamide mononucleotide; PACs: proanthocyanidins; Trp: tryptophan.

3.2 PAC consumption induced an overexpression of the genes that encode the enzymes involved in the de novo NAD^+ biosynthesis pathway

To analyze these changes further, we next evaluated whether the enhanced NAD^+ levels upon PAC consumption could be derived from increased NAD^+ biosynthesis. Thus, we determined the mRNA levels of the major enzymes of the NAD^+ biosynthetic pathways. The first step of the *de novo* NAD^+ biosynthesis pathway is the conversion of Trp into N-formylkynurenine through an enzymatic reaction catalyzed by tryptophan 2,3-dioxygenase (Tdo2). N-formylkynurenine is then directed to spontaneous cyclization to quinolinic acid, which is converted to NaMN through quinolinate phosphoribosyltransferase (Qprt) activity. NaMN is then transformed to NaAD by the nicotinamide mononucleotide adenylyltransferase (Nmnat) enzymes, and NaAD is finally amidated to NAD^+ by NAD^+ synthetase 1 (Nadsyn1). Thus, we examined the effect of PAC consumption on the levels of the *Tdo2*, *Qprt* and *Nadsyn1* mRNAs. Although we could not detect differences in the *Tdo2* mRNA levels between the animal groups, PAC consumption significantly up-regulated the *Qprt* and *Nadsyn1* mRNA levels in a dose-dependent manner (**Figure 3A**). Notably, the increased levels of these transcripts were already significant at a dose of 5 mg of PAC/kg bw, indicating that this dose was sufficient to efficiently increase the mRNA levels of the major *de novo* NAD^+-biosynthetic enzymes.

Alternatively, NAD^+ is also synthesized through the NAD^+ *salvage* pathway from its precursors Na, Nam and Nr. Beginning with Na, the first step in NAD^+ synthesis is

catalyzed by nicotinic acid phosphoribosyltransferase 1 (Naprt1) and leads to the formation of NaMN. Similarly, Nam is converted to NMN by Nampt; NMN is also the product of phosphorylation of Nr by nicotinamide riboside kinase 1 (Nrk1). Both NaMN and NMN are then converted to NaAD by Nmnat, after which the NaMN-derived NaAD requires final amidation through Nadsyn1. Importantly, our results showed that PAC consumption did not modify the levels of the *Nampt*, *Naprt1* or *Nrk1* mRNAs (**Figure 3B**). In fact, we could not even detect a statistically significant difference at a dose of 50 mg of PAC/kg bw compared to the control group, indicating that the higher NAD^+ levels observed in the livers of the PAC-supplemented animals were not due to an increase in direct NAD^+ synthesis from Nam, Na or Nr. Together, these results indicated that PAC consumption increases the cellular NAD^+ content by a direct effect on *de novo* NAD^+ biosynthesis rather than by indirectly affecting the major NAD^+ *salvage* pathway.

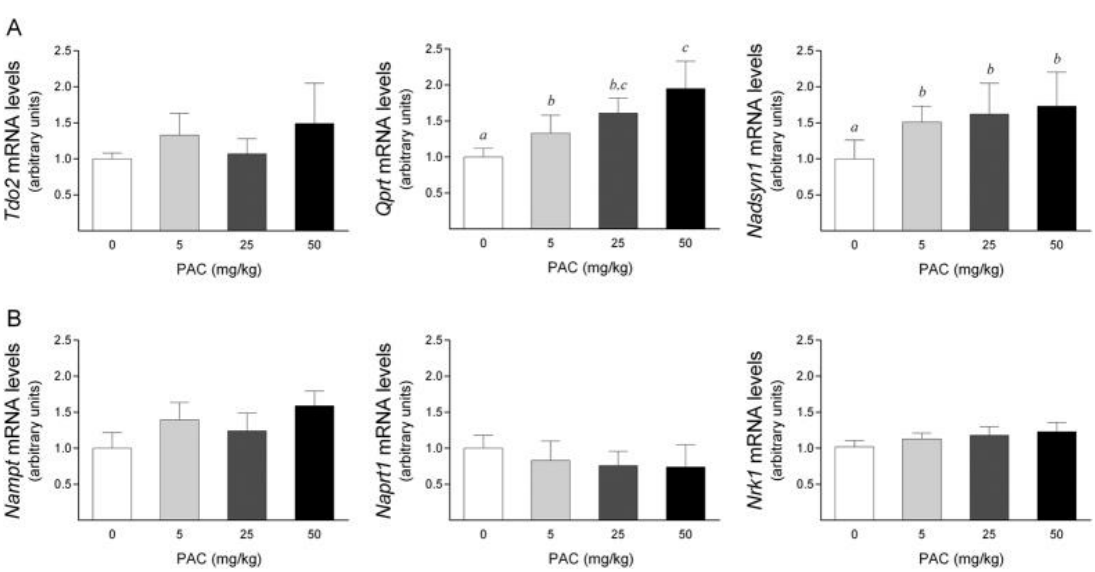

Figure 3. Hepatic mRNA levels of selected genes of NAD^+ biosynthesis in healthy rats supplemented with different doses of PACs. (A-B) PAC consumption up-regulated the mRNA levels

of specific genes involved in *de novo* (A), but not the *salvage* (B) NAD^+ biosynthetic pathways. The mRNA levels of the selected genes were normalized to the *Ppia* mRNA levels. The animals were with a standard chow diet supplemented with 0 (control group), 5, 25 or 50 mg of PACs/kg bw for 21 days. The values shown are the means ± SEM of 10 animals per group. The letters denote a significant difference between groups ($P < 0.05$; one-way ANOVA). NAD^+: nicotinamide adenosine dinucleotide; Nadsyn1: NAD^+ synthetase; Nampt: nicotinamide phosphoribosyltransferase; Naprt1: nicotinate phosphoribosyltransferase 1; Nrk1: nicotinamide riboside kinase1; PACs: proanthocyanidins; Qprt: quinolinate phosphoribosyltransferase; Tdo2: tryptophan 2,3-dioxygenase.

3.3 PAC consumption modified the activity of the major NAD^+-consuming enzymes by simultaneously down-regulating the hepatic levels of the *Parp1* and *Cd38* mRNAs

As ADP-ribosylation reactions consume NAD^+, we also analyzed whether the mRNA levels of the major genes involved in this process, *Poly(ADP-ribose) polymerase 1* (*Parp1*) and *Cyclic ADP-ribose hydrolases* (*Cd38*), could also contribute to the increase in the NAD^+ levels. Notably, at dose of 50 mg/kg bw, PAC consumption significantly down-regulated the *Parp1* and *Cd38* mRNA levels compared to the control animals ($P = 0.04$ and $P = 0.014$, respectively; Mann-Whitney test), without any effect at lower doses (**Figure 4**). PARP1 and CD38 are the major NAD^+-consuming enzymes, thus the decrease in their activities may have also contributed to the increase in the hepatic NAD^+ content. Indeed, there was a negative and significant correlation between the hepatic NAD^+ concentrations and the *Parp1* mRNA levels (*rho* = -0.661, $P = 0.004$) that was not as evident for the *Cd38* mRNA values (*rho* = -0.457, $P = 0.075$).

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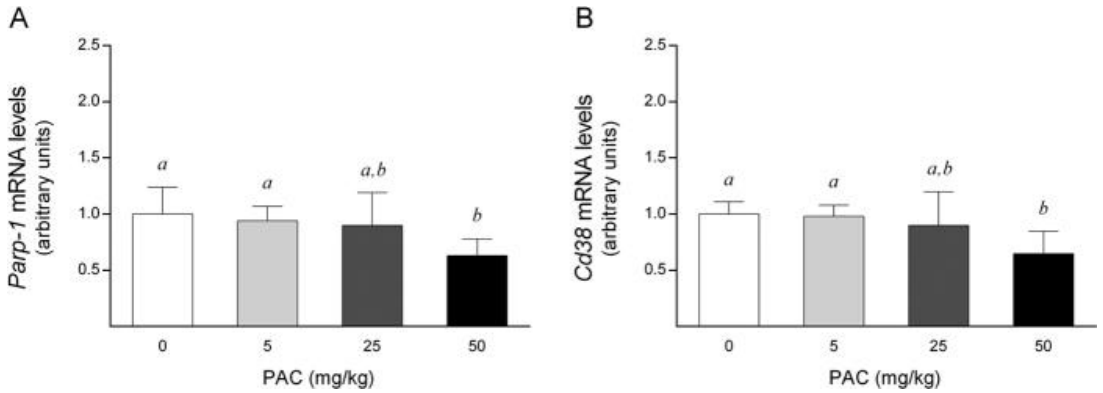

Figure 4. Hepatic mRNA levels of selected genes of NAD^+ consumption in healthy rats supplemented with different doses of PACs. (A-B) PAC consumption down-regulated the mRNA levels of two major enzymes, *Parp1* (A) and *Cd38* (B), implicated in NAD^+ consumption. The mRNA levels of the selected genes were normalized to the *Ppia* mRNA levels. The animals were fed a standard chow diet supplemented with 0 (control group), 5, 25 or 50 mg of PACs/kg bw for 21 days. The values shown are the means ± SEM of 10 animals per group. The letters denote a significant difference between groups ($P < 0.05$; one-way ANOVA). Cd38: cluster of differentiation 38, also known as cyclic ADP ribose hydrolase; NAD^+: nicotinamide adenosine dinucleotide; PACs: proanthocyanidins; Parp1: poly(ADP-ribose) polymerase 1.

3.4 PAC consumption robustly enhanced both the hepatic *Sirt1* mRNA levels and SIRT1 activity in a dose-dependent manner

The ability of PAC to increase the intracellular NAD^+ levels *in vivo* prompted us to test whether it could also modulate the SIRT1 levels. PAC consumption resulted in a significant up-regulation of the *Sirt1* mRNA levels in a dose-dependent manner (**Figure 5A**). Moreover, we observed a positive and significant relationship between the *Sirt1* mRNA levels and the hepatic NAD^+ content (*rho* = 0.493, $P = 0.03$). The combination of higher NAD^+ concentrations with higher *Sirt1* mRNA levels provides an excellent *scenario* for increased SIRT1 activity. Accordingly, SIRT1 activity was

increased in a dose-response manner, with a significant activation in the liver of rats treated with 25 or 50 mg PACs/kg bw (**Figure 5B**).

Moreover, indirect measurements of SIRT1 activity also reinforced the actual activation of SIRT1 in liver by PACs. Considering that the deacetylation of forkhead O-box protein 1 (FOXO1) is mediated by SIRT1, we evaluated the transcriptional activation of FOXO1 by determining the mRNA levels of its target genes *Superoxide dismutase 2* (*Sod2*) and *Growth Arrest and DNA Damage* (*Gadd45*) to confirm this hypothesis [20]. Accordingly, their mRNA levels were significantly up-regulated in the livers of the PAC-supplemented animals in a dose-dependent manner (**Figure 5C**). Consistent with SIRT1 being a negative regulator of *Mitochondrial uncoupling protein 2* (*Ucp2*) expression [21], PAC consumption significantly decreased the *Ucp2* mRNA levels in a similar dose-dependent pattern (**Figure 5C**). Finally, as the altered NAD^+ levels could also potentially impact other SIRT proteins, we also tested the levels of *Sirt2* and *Sirt3* transcripts, which act as cytosolic and mitochondrial SIRTs, respectively. However, no changes were observed in the mRNA levels of these genes with respect to control group (**Figure 5D**).

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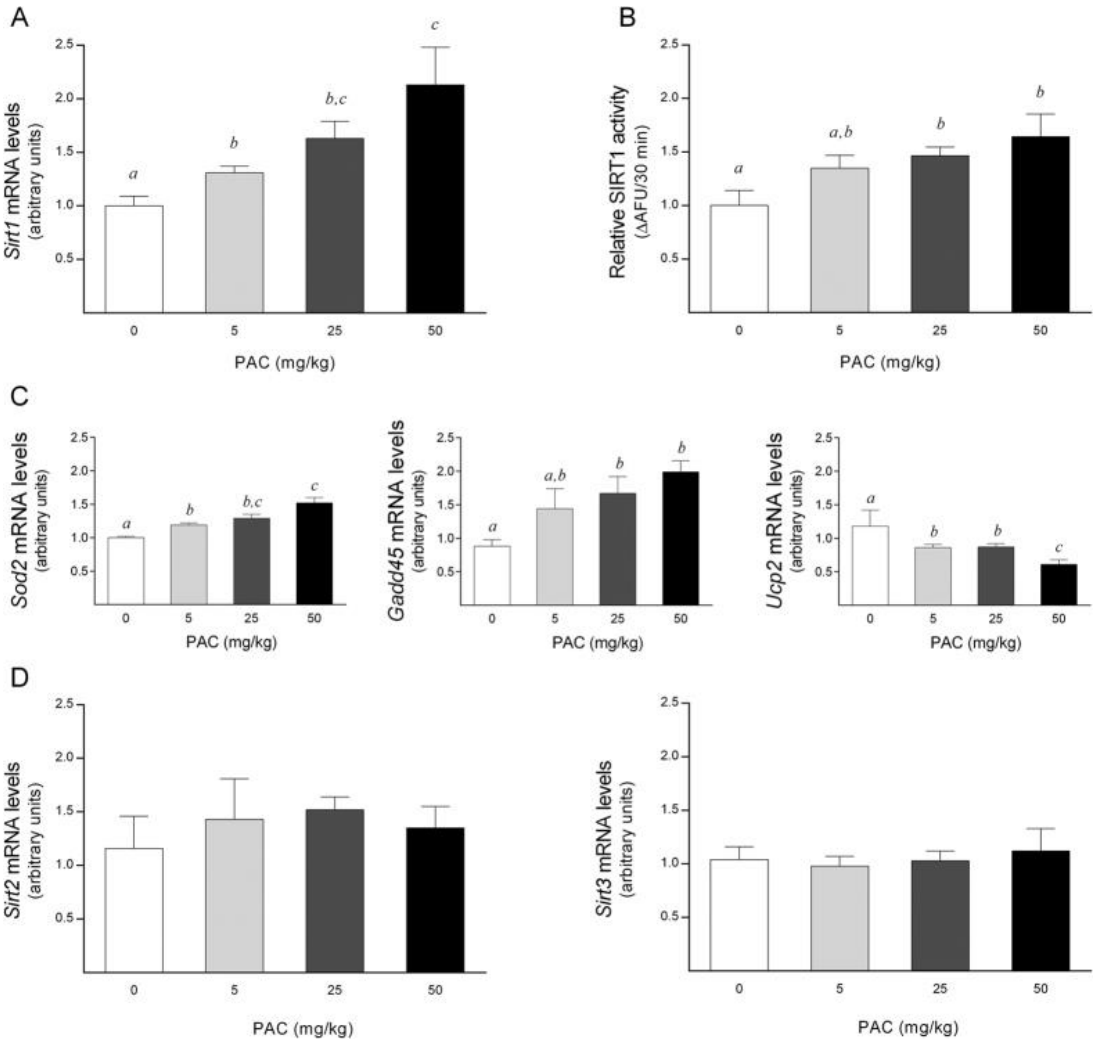

Figure 5. Hepatic SIRT activity in healthy rats supplemented with different doses of PACs. (A-B) PAC consumption dose-dependently increased the hepatic *Sirt1* mRNA levels (A) and SIRT1 activity (B). (C) The mRNA levels of *Gadd45* and *Sod2* were significantly up-regulated in a dose-dependent manner, whereas the *Ucp2* mRNA levels were down-regulated in a similar manner. (D) There was no statistically significant effect of PAC consumption on the hepatic *Sirt2* or *Sirt3* mRNA levels. SIRT activity was indirectly assessed by determining both the mRNA levels of *Sirt1*, *Sirt2* and *Sirt3* and the mRNA levels of selected specific genes modulated by FOXO1 activity. The mRNA levels of the selected genes were normalized to the *Ppia* mRNA levels. The animals were fed a standard chow diet supplemented with 0 (control group), 5, 25 or 50 mg of PACs/kg bw for 21 days. The values shown are the means ± SEM of 10 animals per group. The letters denote a significant difference between groups ($P < 0.05$; one-way ANOVA). AFU: arbitrary fluorescence units; Gadd45: growth arrest and DNA damage-inducible 45; PACs: proanthocyanidins; Sirt*:* sirtuin; Sod2: superoxide dismutase 2; Ucp2: uncoupling protein 2.

3.5 PAC consumption improved the hepatic lipid content in both healthy rats and cultured cells in a SIRT1-mediated manner

Given the promising role of SIRT1 in protecting against metabolic liver diseases, we next evaluated the effects of PAC consumption on the liver fat content in animals subjected to an acute dietary fat challenge. Confirming our hypotheses, PACs significantly reduced the hepatic lipid accumulation in a dose-dependent manner (**Figure 6A**), and this was associated with a significant decrease in both the hepatic cholesterol and triglyceride concentrations (**Figure 6B**). Notably, the hepatic lipid concentrations were negatively and significantly related to the *Sirt1* mRNA levels in the PAC-supplemented animals (*rho* = -0.678, $P = 0.002$). Moreover, when we applied a multivariate linear regression model to assess the role of the variables known to influence the hepatic lipid concentrations, the *Sirt1* mRNA levels were the only variable that was significantly associated with fat accumulation in the liver, with a *rho* coefficient of -0.896 ($P = 0.003$), highlighting the major role of SIRT1 in reducing the hepatic fat depots in the PAC-supplemented animals.

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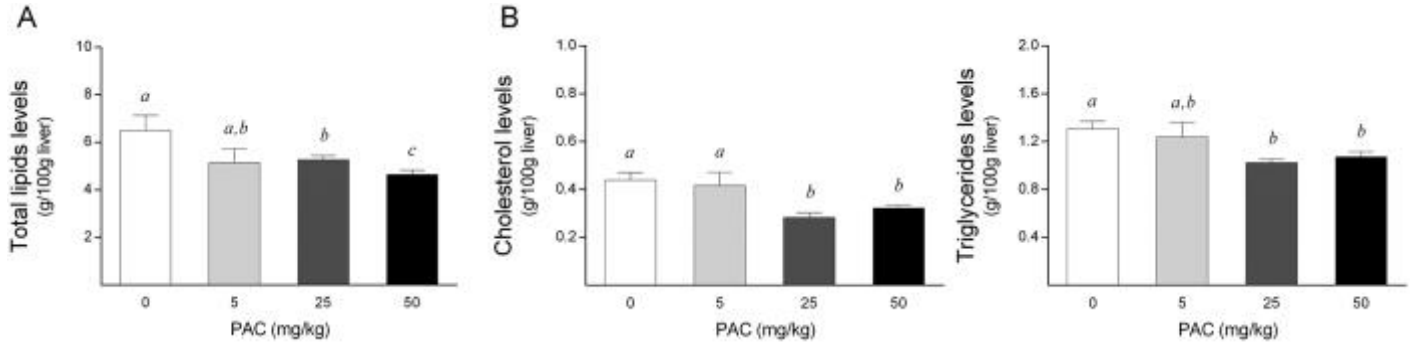

Figure 6. Hepatic lipid profile in healthy rats supplemented with different doses of PACs. (A-B) PAC consumption decreased the total lipid accumulation in the liver (A) by accordingly diminishing the hepatic cholesterol and triglyceride concentrations (B). The animals were fed a standard chow diet supplemented with 0 (control group), 5, 25 or 50 mg of PACs/kg bw for 21 days. The values shown are the means ± SEM of 10 animals per group. The letters denote a significant difference between groups ($P < 0.05$; one-way ANOVA). PACs: proanthocyanidins.

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To further solidify our data, we also evaluated the efficacy of PACs in protecting against fat overaccumulation in an experimental *in vitro* model. Thus, HepG2 cells were incubated with a mixture of 0.5 mM palmitate and 30 mM glucose for 48 h to induce fat-overloading. Then, the cells were treated with 100 mg/L PACs for the last 24 h of incubation. Indeed, when the cultured cells were treated with PACs, the intracellular concentrations of triglycerides decreased significantly to basal levels, reversing the fat accumulation observed in the untreated cells (**Figure 7A**). In addition, to confirm the specificity of SIRT1 in attenuating the triglyceride accumulation in cultured cells, the HepG2 cells were also treated with sirtinol, a SIRT1 inhibitor, for 24 h. Notably, the protective effect of PACs on cellular triglyceride accumulation was significantly abrogated when the cells were treated with 100 μM sirtinol, and the triglyceride levels did not significantly decrease after the PAC treatment (**Figure 7A**). Finally, when the mRNA levels of the key genes involved in lipid metabolism were assessed by real time quantitative polymerase chain reaction (RT-qPCR), PAC treatment resulted in decreased *Fatty acid synthase* (*Fas*) and *Acetyl-coA carboxylase* (*Acc*) mRNA levels compared to the untreated cells, indicating that the delipidating effect of the PAC treatment may be mediated by limiting the capacity for *de novo* lipogenesis (**Figure 7B-C**). In addition, when the cultured cells were treated with 100 μM sirtinol, the levels of the *Fas* and *Acc* mRNAs were again not affected by the PAC treatment, thus confirming that SIRT1 activation plays a critical role in regulating hepatic *de novo* lipogenesis and, consequently, fat accumulation in this experimental model.

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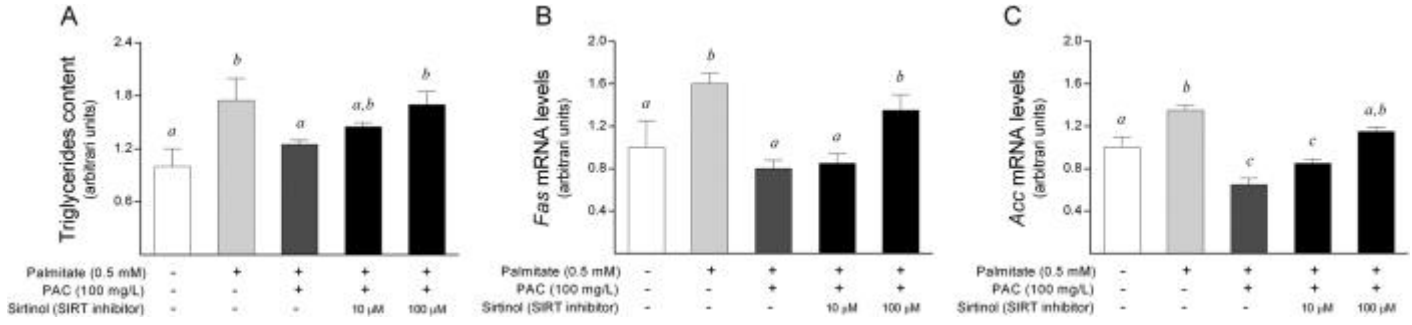

Figure 7. Effect of PACs on an experimental model of hepatocellular steatosis in HepG2 cells. (A) The PAC-treated cells exhibited decreased triglyceride levels. (B-C) The mRNA levels of lipogenic enzymes were assessed by RT-qPCR, and the *Fas* (B) and *Acc* (C) mRNA levels were significantly down-regulated in the treated cells compared to the untreated cells. (A-B) When the cultured cells were treated with sirtinol, the protective effect of PACs on our experimental model was significantly attenuated. The HepG2 cells were incubated with a mixture of palmitate 0.5 mM and glucose 30 mM for 48 hours to induce fat-overloading. Then, the cells were treated with 100 mg/L PACs for the last 24 hours of incubation in the presence or absence of sirtinol, a SIRT1 inhibitor. All values represent the means of three independent experiments. The letters denote a significant difference between groups ($P < 0.05$; one-way ANOVA). Acc: acetyl-coA carboxylase; Fas: fatty acid synthase; PACs: proanthocyanidins.

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4. Discussion

Several molecular mechanisms have been described to account for the metabolic benefits of PAC consumption [8,10-13]. However, all of these mechanisms only partially account for all the physiological and biochemical effects of PACs. Therefore, in this study, we have used a combination of metabolomics and gene expression analyses to identify the potential global mechanism that could completely explain the diverse metabolic effects of PAC consumption. Interestingly, comparative liver metabolomics revealed that NAD^+ and its metabolites are clear targets of PAC consumption. Certainly, PAC administration dose-dependently increased both the hepatic NAD^+ content and mRNA levels of the genes involved in the NAD^+ biosynthesis pathway, highlighting NAD^+ homeostasis as potential PACs target in the livers of healthy rats.

Notably, other studies of our group indicate that an acute single dose of PACs is also able to modulate hepatic NAD^+ levels [22]. Thus, altogether, these results robustly reveal NAD^+ homeostasis as a major target of PACs in liver.

NAD^+ plays a pivotal role in cells, as it is indispensable for energy production and acts as reusable coenzyme in metabolic redox reactions. The roles of NAD^+, however, have expanded beyond its role as a coenzyme, as NAD^+ and its metabolites also act as degradation substrates for a wide range of enzymatic reactions that catalyze ADP-ribosylation and deacetylation of proteins [23]. Through these activities, NAD^+ links the cellular metabolism to changes in signalling and transcriptional events. In

addition, different studies have unequivocally demonstrated the ability of NAD^+ to dynamically respond to physiological stimuli, such as exercise [24] and caloric restriction [25]. Thus, the modulation of NAD^+ homeostasis by PAC consumption empowers these compounds to modify a large number of metabolic processes in the liver. Furthermore, the liver exports NAD^+ precursors to other organs, such as the brain and muscle, which have a lower capacity to synthesize NAD^+ [26]. Thus, as several organs depend on NAD^+ homeostasis in the liver, the modulation of NAD^+ homeostasis by PACs could be extended as a general effect in the whole organism.

NAD^+ availability is determined by the relative rates of NAD^+ biosynthesis and degradation. Although NAD^+ is primarily synthesized by the *salvage* pathway in the liver [27], our results indicate that PAC consumption increased the NAD^+ levels by enhancing the *de novo* NAD^+ biosynthesis pathway. Indeed, PAC increased the flux through the *de novo* biosynthesis pathway by overexpressing some of the key enzymes involved in this pathway and by increasing the precursor's levels. In fact, the hepatic levels of Trp were robustly increased in a dose-response pattern, and it has been described that liver can increase the flux through the *de novo* pathway 40-fold when the levels of Trp or other NAD^+ precursors increase [27]. Together, these results strongly suggest that PAC consumption fosters the use of Trp in the liver as the main precursor of NAD^+, while maintaining the activity of the *salvage* biosynthetic pathway at basal levels (**Figure 8**). Nampt expression was not altered by PACs at any dose. However, NMN levels, the product of the reaction catalyzed by Nampt, increased significantly at 50 mg of PACs, indicating that the actual Nampt activity in

the liver of rats treated with PACs was sufficient to support the increased flux through the *salvage* pathway.

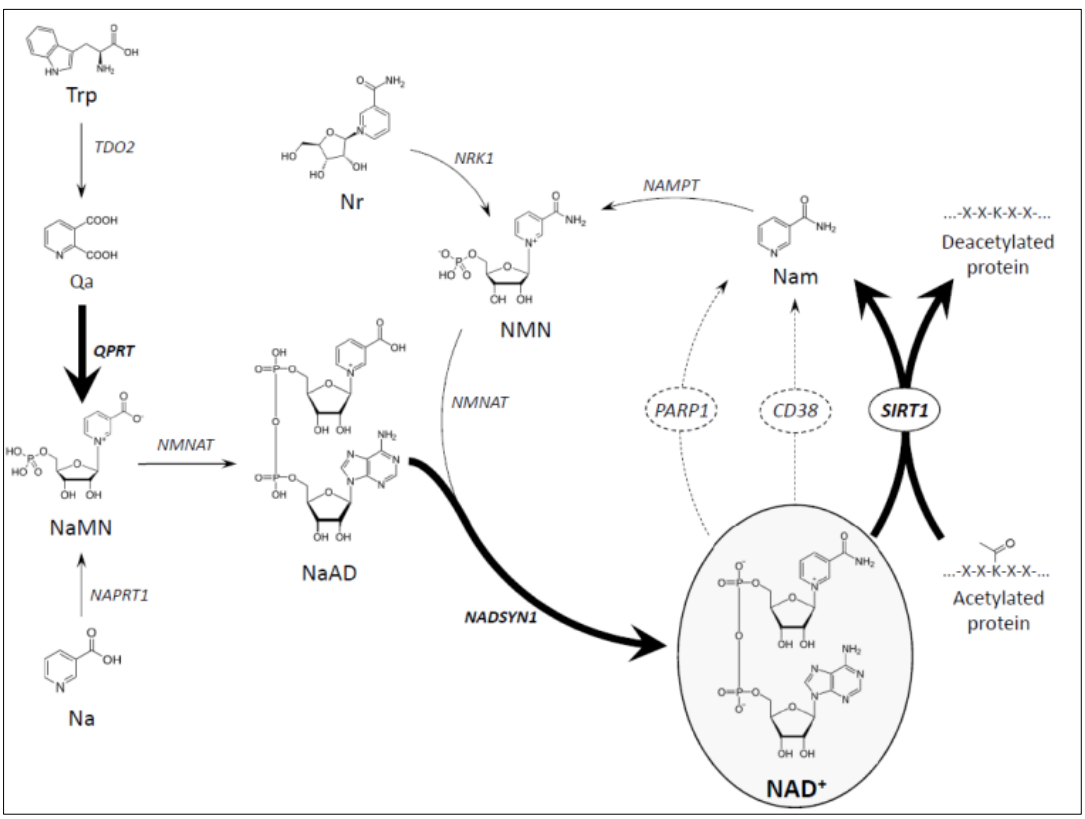

Figure 8. Schematic representation of mammalian NAD$^+$ metabolism and its modulation by PACs. Bold lines: activated reaction; dashed lines: repressed reaction.

Na: nicotinic acid; NAD$^+$: nicotinamide adenosine dinucleotide; Naprt1: nicotinate phosphoribosyltransferase 1; Nam: nicotinamide; Nampt: nicotinamide phosphoribosyltransferase; Nadsyn1: NAD$^+$ synthetase; NMN: nicotinamide mononucleotide; Nrk1: nicotinamide riboside kinase1; PACs: proanthocyanidins; Qprt: quinolinate phosphoribosyltransferase; Tdo2: tryptophan 2,3-dioxygenase; Trp: tryptophan.

Interestingly, SIRTs use NAD$^+$ as a co-substrate to remove acetyl moieties from lysines on histones and proteins, releasing Nam and *O*-acetyl-ADP-ribose. Generally, most SIRTs are activated when there is an energy deficiency and reduced

carbohydrate energy sources, triggering cellular adaptations that improve metabolic efficiency. For example, SIRT1 activity increases during exercise [24], caloric restriction or fasting [25], all of which correlate with higher NAD^+ levels, suggesting that NAD^+ availability is the most important regulator of SIRT1 activity. In this study, PAC consumption increased SIRT1 activity in a dose-dependent pattern, according to the dose-response effect of PACs on the hepatic NAD^+ levels. Notably, despite the levels of Nam, which is a potent inhibitor of SIRT1 [28], increasing in a dose-response pattern with PACs consumption, SIRT1 activity was not inhibited because NAD^+ levels increased higher than Nam levels, in the way that the ratio between NAD^+/Nam remains in favor of NAD^+ as the dose of PACs was raised.

In addition, PACs significantly reduced the expression of other non-SIRT NAD^+-consuming enzymes in the liver, such as PARP1 and CD38. In fact, diverse lines of research indicate that the pharmacological repression of PARP1 drives an increase in the NAD^+ content and activation of SIRT1 [29,30], indicating that the competition between PARPs and SIRTs for NAD^+ could be considered a metabolic determinant. Illustrating the opposing roles of both enzymes, PARP1 is required for the transcriptional co-activation of nuclear factor kappa B (NF-κB) [31], while SIRT1 inhibits NF-κB activity through the deacetylation of RelA/p65 [32]. Furthermore, SIRT1 also negatively regulates PARP1 transcription [33]. Thus, the results of this study indicate that PAC-mediated PARP1 and CD38 repression could primarily contribute to SIRT1 activation.

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Remarkably, PAC consumption also promoted *Sirt1* expression in a dose-response pattern. Several mechanisms control *Sirt1* expression, such as FOXO1, peroxisome proliferator-activated receptor (PPARα and PPARγ), among others. Together, these results indicate that the activation of SIRT1 by PACs was the result of increasing both the cellular NAD^+ and SIRT1 concentrations. Therefore, PACs significantly foster SIRT1 activity. This property is not exclusive of PACs, and other polyphenols, such as resveratrol, can also potentially modulate SIRT1 activity both *in vitro* and *in vivo* [34,35]. However, the mechanism of SIRT1 activation by resveratrol has been debated, and recent studies show that resveratrol could not be a specific activator of SIRT1 [36,37].

In mammals, the SIRT family comprises seven proteins, SIRT1-7, each one with a specific cellular localization and function. Thus, we also evaluated whether PAC consumption could modulate the expression of *Sirt2* and *Sirt3*, which are primarily localized to the cytosol [38] and the mitochondrial matrix [39], respectively. However, the expression levels of *Sirt2* and *Sirt3* were not modified, indicating that PAC-mediated regulation of *Sirt1* expression is specific and not a general effect of PACs on the SIRT family.

SIRT1 is an important modulator of hepatic lipid metabolism by enhancing fatty acid oxidation, decreasing lipogenesis and modulating cholesterol levels through the activation of 5' AMP-activated protein kinase (AMPK) and the deacetylation of both steroid response element binding protein 1 (SREBP1) and farnesoid X receptor (FXR) [40]. Accordingly, our group previously demonstrated that PACs reduced

triglyceridemia in wild type mice but not in FXR-null mice, revealing that FXR is an essential mediator of the hypotriglyceridemic actions of these compounds *in vivo* [11]. In the liver, PACs down-regulated the expression of the transcription factor SREBP1 and several SREBP1-target genes involved in lipogenesis in an FXR-dependent manner. In addition, in cultured mammalian cells, PAC treatment increased the transactivation activity of FXR only when its natural agonist, the chenodeoxycholic acid, was present in the media [11]. Therefore, we can speculate that the direct transactivation of FXR by PACs could be mediated, at least in part, by the consecutive increase in the NAD^+ content and SIRT1 activation, although further studies are needed to confirm this hypothesis. In accord with this role, it has been widely demonstrated that hepatocyte-specific deletion of SIRT1 induces hepatic lipid accumulation by up-regulating the expression of lipogenic genes and reducing fatty acid oxidation [41,42], whereas overexpression of SIRT1 protects against hepatic lipid accumulation and inflammation in response to moderate- and high-fat diets [43]. Interestingly, our results showed that PAC administration dose-dependently attenuated the hepatic lipid accumulation induced by an acute dietary fat challenge, due to the increased NAD^+ levels and SIRT1 activity. Furthermore, we demonstrated that when the HepG2 cells were treated with an SIRT1 inhibitor, the triglyceride levels did not significantly decrease after the PAC treatment. Together, these results strongly suggest that PACs prevented the hepatic lipid accumulation through a SIRT1-mediated mechanism.

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In conclusion, our current study provides the first *in vivo* evidence that the intracellular NAD^+ levels, SIRT1 activity and lipid content in liver are globally modulated in a dose-dependent manner by chronic dietary supplementation with PACs. In view of these results, PACs activated *Sirt1* by both i) promoting NAD^+ synthesis and ii) overexpressing *Sirt1*. These results suggest that PAC consumption could be a new valid strategy to enhance SIRT1 activity through the modulation of the *Parp1*, *Cd38* and *Sirt1* mRNA levels (major NAD^+-consuming enzymes) and the activation of the *de novo* NAD^+ biosynthesis pathway. Interestingly, the PAC doses used in this experiment in rats are doses achievable in a normal human diet [44]. However, to translate these results from both rats and cells to humans, further studies are needed.

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5. Supplementary information

Table 1S. A summary of the rat-specific primer sequences used for qRT-PCR analysis. Ten nanograms of the cDNAs were subjected to quantitative amplification using the SYBR Green PCR Master Mix from Bio-Rad (Barcelona, Spain). The reactions were run on a CFX96 real-time system-C1000 Touch Thermal Cycler (Bio-Rad). The thermal profile settings were 50 °C for 2 min, 95 ºC for 2 min, and then 40 cycles at 95 °C for 15 s and 60 °C for 2 min.

Rat genes	Alignment sequence	Direction	Primer sequences (5'-3')	Primer length (nucleotides)	Tm (ºC)
Cd38	NM_013127.1	Fw	CAG CAC CTT TGG AAG TGT GG	20	53
		Rv	CAG GTC GGT AGT TAT CCT GGC	21	53
Gadd45	NM_024127.2	Fw	TAC ATG GAT CAG TGG GTG CC	20	53
		Rv	TGG GGA GTG ACT GCT TGA GT	20	52
Nadsyn1	NM_181480.1	Fw	GCC CTT TCG GTC AGT GAA GA	20	54
		Rv	GTC GAA GGA AGT CCC AGA GC	20	53
Nampt	NM_177928.3	Fw	CTC TTC ACA AGA GAC TGC CG	20	53
		Rv	TTC ATG GTC TTT CCC CCA CG	20	52
Naprt1	NM_207609.1	Fw	TAG CCC AAA AGG GCA GTG AG	20	54

		Rv	CAG CTT GTA GAC ACA GCC CA	20	51
Nrk1	NM_001024292.1	Fw	CCT GGA CGG AAC AAG GTC TG	20	54
		Rv	CCT TCA AGG GTC CGA ATC CA	20	56
Parp1	NM_013063.2	Fw	ACC GAG TGG AGT ACG CTA AG	20	49
		Rv	GAA CAT GGG TGA CTG CAC CA	20	54
Qprt	NM_001009646.1	Fw	CAC CAT GGA CCC TGA AGG TCT G	22	58
		Rv	TAG CCC GGT AAC GGA CGC AA	20	59
Sirt1	XM_006256146.2	Fw	TTG GCA CCG ATC CTC GAA	18	54
		Rv	ACA GAA ACC CCA GCT CCA	18	52
Sirt2	NM_001008368.1	Fw	AAA CCT CCC ACC TTC ACT GC	20	53
		Rv	GGA TAA GGT AGC GAG TGC GA	20	52
Sirt3	NM_001106313.2	Fw	TGT GGG GTC CGG GAG TAT TA	20	54
		Rv	CCA CCA TGA CCA CAA CCC TA	20	53
Sod2	NM_017051.2	Fw	CAC CGA GGA GAA GTA CCA CG	20	52
		Rv	TGG GTT CTC CAC CAC CCT TA	20	54

Tdo2	NM_022403.2	Fw	CTC CTG GGA CGC ATC ACT AC	20	52
		Rv	AAG TCC TCC TTT GCT GGC TC	20	53
Ucp2	NM_019354.3	Fw	GAG AGT CAA GGG CTA GCG C	19	54
		Rv	GCT TCG ACA GTG CTC TGG TA	20	51

Abbreviations: *Cd38*, CD38 molecule; *Gadd45*, growth arrest and DNA-damage-inducible; *Nadsyn1*, NAD synthetase 1; *Nampt*, nicotinamide phosphoribosyltransferase; *Naprt1*, nicotinate phosphoribosyltransferase; *Nrk1*, nicotinamide riboside kinase 1; *Parp1*, poly (ADP-ribose) polymerase 1; *Qprt*, quinolinate phosphoribosyltransferase; *Sirt1*, sirtuin 1; *Sirt2*, sirtuin 2; *Sirt3*, sirtuin 3; *Sod2*, superoxide dismutase 2; *Tdo2*, tryptophan 2,3-dioxygenase; *Ucp2*, uncoupling protein 2.

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6. Acknowledgments

The authors gratefully acknowledge Yaiza Tobajas for her excellent technical assistance. This work was financially supported by grant AGL2013-40707-R from the Spanish Ministry of Economy and Competitiveness.

7. Author contributions

X.C., L.A. and C.B. conceived and designed the research; G.A., M.S., A.A-R., M.V and M.A.R. carried out the experimental procedures and the molecular analysis; G.A. M.S. and C.B. performed the statistical analyses. All the authors participated in the interpretation and discussion of data. G.A and C.B. wrote the manuscript. All the authors contributed to and approved the final manuscript.

8. Additional information

Competing financial interests: The authors declare no competing financial interests.

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Obesity is a current and worldwide extended problem and one of the main factors related with other chronic pathologies. Conventional therapies, normally based on increasing the exercise and reducing the consumption of energy-dense food used to prevent or palliate obesity, are ineffective. In this sense, the use of bioactive compounds as polyphenols, a group of plant secondary metabolites with a wide range of beneficial healthy effects, arises as a novel strategy to combat obesity and its related pathologies.

Leptin is a key hormone secreted proportionally by the amount of adipocytes that acts primarily in the central nervous system controlling the energy balance. In this process, leptin transport across the blood-brain barrier is especially important. In addition, leptin is implicated in the regulation of peripheral homeostasis, mainly modulating the lipid and carbohydrate metabolism, in organs such as liver, muscle and white adipose tissue. However, obesity is related with an impaired action of leptin, namely leptin resistance, causing hyperleptinemia and an increase in the energy intake.

In this context, the aim of this thesis is to identify phenolic compounds with the capacity to restore the obesogenic-leptin resistance condition caused in peripheral tissues (liver, skeletal muscle and epididymal white adipose tissue) and to increase the leptin transport across the blood-brain barrier.

Our results demonstrate the effects of resveratrol and its metabolites acting in the peripheral leptin signalling pathway on reducing body fat accumulation in an obesogenic rat model. Moreover, resveratrol restores the leptin sensitivity in a palmitate-induced model of steatotic human hepatocellular carcinoma cell line by increasing the leptin receptor content. Finally, the capacity of different phenolic compounds to increase the leptin receptor content and to protect against pro-inflammatory cytokine-induced damage in rat brain endothelial cells is described.

This research provides novel information that can be useful for the functional food industry identifying bioactive compounds that can be used to potentially treat obesity and its related pathologies.