Universidad de Navarra

Facultad de Farmacia

Role of cardiotrophin-1 in adipocyte lipolysis and adipokine production, intestinal sugar uptake and the regulation of circadian clocks

---

Implicación de cardiotrofina-1 en la lipólisis y la secreción de adipoquinas por adipocitos, en el transporte intestinal de azúcares y en la regulación de relojes circadianos

Miguel López Yoldi

Pamplona, 2015

Universidad de Navarra

Facultad de Farmacia

Memoria presentada por D. Miguel López Yoldi para aspirar al grado de Doctor por la Universidad de Navarra.

Fdo. Miguel López Yoldi

El presente trabajo ha sido realizado bajo nuestra dirección en el **Departamento de Ciencias de la Alimentación y Fisiología** de la Facultad de Farmacia y en el **Área de Hepatología y Terapia Génica** del CIMA de la Universidad de Navarra y autorizamos su presentación ante el Tribunal que lo ha de juzgar.

VºBº Directora
María Jesús Moreno Aliaga

VºBº Co-Directora
Matilde Bustos de Abajo

Este trabajo ha sido posible gracias a la financiación de diversas entidades: Asociación de Amigos de la Universidad de Navarra (beca predoctoral 2011-2015), Gobierno de Navarra (Departamento de Salud); Fundación Mutua Madrileña; FIS [PI10/01516 y PI13/01851]; Línea Especial ''Nutrición, Obesidad y Salud Universidad de Navarra"; y por el acuerdo entre FIMA y el ''UTE proyecto CIMA''.

Tras más de cuatro años de duro trabajo, llega el momento de expresar mis agradecimientos a todas aquellas personas que siempre han estado conmigo incondicionalmente, con las que he compartido todas mis alegrías y también han sabido levantar mi ánimo en los momentos más complicados. Sin duda la tesis me ha dado infinitas lecciones científicas pero por encima de todo me ha aportado una gran formación personal, experiencias inolvidables y la oportunidad de conocer a personas que me llevaré para siempre.

En primer lugar, me gustaría agradecer a la Universidad de Navarra y a la Facultad de Farmacia el haberme formado tanto profesional como personalmente durante este periplo. En especial, expresar mi gratitud al Departamento de Ciencias de la Alimentación y Fisiología por darme la oportunidad de desarrollar el presente trabajo de investigación e iniciar mi camino en la carrera investigadora y docente.

Además, quisiera reconocer a la Asociación de Amigos de la Universidad de Navarra no sólo las ayudas recibidas que han hecho posible la realización de este proyecto, sino todo el esfuerzo que realizan para que muchos nos iniciemos en el mundo de la investigación.

Especial gratitud a mis directoras, la Profesora María Jesús Moreno y Dra. Matilde Bustos, por haber confiado en mí para llevar a cabo el presente proyecto. Debo agradecer su paciencia y sus numerosos consejos. Es un orgullo poder compartir vuestros conocimientos y comenzar mi carrera científica de la mano de dos grandes personas y profesionales. Gracias por vuestro apoyo y disposición siempre que lo he necesitado.

Me gustaría hacer extensivos mis agradecimientos al Profesor Alfredo Martínez por su tiempo y dedicación. Gracias por tus muestras de apoyo y por tu gran cercanía. Siempre me llevaré tus consejos prácticos y frases para el recuerdo.

Al resto de profesores de la Universidad de Navarra, y más en concreto del Departamento de Ciencias de la Alimentación y Fisiología, agradezco su entusiasmo, dedicación y esfuerzo que ponen en todo lo que hacen y por su gran interés hacia mi trabajo y hacia mi persona. En este sentido debo agradecer especialmente al Dr. Pedro González Muniesa y a la Dra. Silvia Lorente Cebrián por estar siempre a mi lado,

pendientes de la evolución de mi trabajo y por su gran apoyo científico pero sobre todo moral.

A Paula Oteiza y Beatriz Jimeno, así como a todo el personal de administración y servicios de la Universidad de Navarra, muchísimas gracias por vuestra disponibilidad y gran efectividad siempre que he necesitado algo.

A Eduardo e Idoia, así como a todos mis compañeros que han pasado a lo largo de estos años por el departamento y la sala de ordenadores debo agradecerles su gran acogida en los inicios y su espectacular solidaridad durante todo este tiempo. De todos ellos he aprendido cosas muy interesantes que me llevaré para toda la vida. A pesar de la gran carga de trabajo individual que conlleva la tesis, es una satisfacción poder compartir conversaciones de poyata con gente que sabe transmitir su alegría y sus ganas de comerse el mundo. En este sentido, un reconocimiento especial merecen mis compañeras del "protein team" por haber aportado savia nueva, alegría y diversión (además de mucho conocimiento) a mis últimos meses de trabajo en el laboratorio. Un enorme agradecimiento también a mis compañeros de la VI edición del máster E-MENU. Con ellos empecé el camino de la investigación y nunca olvidaré los buenos momentos pasamos juntos (cenas, viajes, almuerzos…).

I would like to express my gratitude to Professor Peter J. Havel and Dr. Kimber L. Stanhope. Thank you very much for the opportunity to share a great experience in your laboratory and for your confidence and hospitality during my internship in the Department of Molecular Biosciences, at the University of California in Davis. Thank you very much Guoxia, Ella, Vivien and Hazel for your expert supervision and for being so friendly and patient as well as for all your help in many ways.

Por el tiempo compartido y por haber sido un fiel apoyo durante este tiempo debo agradecer especialmente a Verónica Ciaúrriz y Ana Lorente los buenos momentos que hemos vivido juntos así como su paciencia en el laboratorio con los que estamos empezando en este mundo de la ciencia. No puedo olvidarme de mi querida Asunción Redín. Es una suerte que existan personas con esa alegría, esa sonrisa imborrable y ese sentido del humor que te levantan el ánimo hasta en los días más complicados. Seguro que nos seguiremos viendo en tus paseos al monte del Perdón y tus visitas a Zizur. Y de la mano de Asunción viene mi amiga Jaione Barrentxe. Gracias "Jaiobar" por tu

cercanía y saber estar. Eres una gran experta de la absorción intestinal, y siempre he sentido tu apoyo desde el principio hasta el final.

Ha llegado el momento de acordarme de las personas más importantes que me llevo de esta etapa de mi vida y que estoy seguro que serán amigos para siempre. Gracias a mis “maestros” Pedrito y Marta. Sin duda sois una pieza fundamental de este trabajo. Todos mis primeros pasos en el laboratorio los di a vuestro lado y creo que sois los mejores maestros que he podido tener. Después han venido muchos momentos inolvidables fuera del laboratorio y estoy seguro que sois dos de las mejores personas que conozco. Pedrito es para mí mucho más que un amigo y contigo he pasado varios de los momentos más divertidos de los últimos años. Gracias a los dos por sentir vuestro apoyo diario desde el otro lado del charco. Dentro de este selecto club un lugar privilegiado lo ocupa mi gran amiga Mery. Es un gusto increíble trabajar contigo por tu gran alegría y por saber que siempre estás dispuesta a ayudar. Tu sentido del humor y tu gran comportamiento conmigo han sido uno de mis mayores apoyos. Gracias también a “tu David” porque en nuestros ratos de ocio por Zizur siempre sabe darnos lecciones de ciencia vista desde el otro lado de la barrera, que nunca viene mal… Agradecer especialmente a Usune estos 5 años de gran amistad. Sin duda una de las grandes alegrías que me ha dado la tesis es poder conocer una amiga como tú. Dentro de nuestro nerviosismo y estrés siempre hemos sacado fuerzas para reírnos y animarnos en todo momento. Gracias también a Beñat por los pocos pero buenos momentos que hemos pasado los tres juntos por vuestra querida Donostia.

Y por supuesto y con especial cariño gracias a Idoyica. La tesis te da la oportunidad de conocer grandes personas pero tú eres la más increíble que he conocido nunca. Gracias por tu apoyo, compresión, paciencia… pero sobre todo gracias por los momento únicos que hemos pasado juntos y por los muchos que están por venir.

Agradecer a mis amigos de la cuadrilla de Zizur Mayor y a mis compañeros del equipo de fútbol por hacerme pasar grandes momentos y sentirme muy querido a lo largo de este tiempo. Por encima de todos agradezco a Luis, Borjita, Imanol, Galin, Blankiya, Anica y Natalia vuestra amistad sincera y vuestras constantes muestras de cariño. Es una gran suerte tener amigos como vosotros con los que cada minuto juntos es un gran regalo.

Gracias a toda mi familia por el apoyo recibido y por sus continuas muestras de afecto. Especialmente quiero expresar mi admiración a mis abuelas Corpus y Manuela por darme constantes lecciones de vida, esfuerzo y superación. Sois la voz de la experiencia y siempre es un placer tener conversaciones con dos mujeres tan encantadoras. Gracias también a mi tía Milagros por su continuo interés hacia mi evolución tanto personal como académica, así como por la gran ayuda y cariño que siempre recibo por su parte.

Finalmente quiero dedicar este trabajo a mis padres, Paco y Maribel, y a mi querida hermana Teresa. Gracias por vuestro apoyo incondicional y por estar siempre en los buenos y en los malos momentos demostrándome vuestro gran amor. Cada día tengo la suerte de sentirme la persona más querida del mundo. Ojalá algún día llegue a ser como vosotros. Todo lo que soy os lo debo a vosotros y me siento tremendamente afortunado de la educación que he recibido y de tener unos padres y una hermana que constantemente me dan un ejemplo de cómo debe ser una gran persona. Gracias a mi hermana porque desde la distancia siempre está dándome ánimos y diciéndome lo orgullosa que se siente de mí. MUCHAS GRACIAS FAMILIA.

“Lo que con mucho trabajo se adquiere, más se ama”

Aristóteles

A mis padres

A mi hermana

TABLE OF CONTENTS

I. INTRODUCTION...1

1. OBESITY AND COMORBIDITIES 3

1.1 Definition 3

1.2 Consequences of obesity.. 4

1.2.1 Pathologies associated to obesity 4

1.2.2 Obesity and Metabolic Syndrome 5

2. ADIPOSE TISSUE 7

2.1 White adipose tissue 8

2.2 Brown adipose tissue.. 9

2.3 Beige adipocytes 10

3. WHITE ADIPOSE TISSUE FUNCTIONS 11

3.1 Adipose tissue as a storage organ 11

3.1.1 Lipolysis in adipocytes 11

3.1.2 Lipogenesis: Triglycerides storage 18

3.2 Adipose tissue as an endocrine organ 20

3.2.1 Leptin 21

3.2.2 Resistin.. 24

3.2.3 Visfatin 26

3.2.4 Apelin 28

4. INTESTINAL SUGAR ABSORPTION 31

4.1 Transporters involved in intestinal sugar absorption 31

4.2 Regulation of intestinal glucose absorption 33

5. CHRONOBIOLOGY OF OBESITY AND METABOLIC SYNDROME 35

5.1 Molecular mechanisms of the biological clock 35

5.2 Circadian rhythm and metabolic syndrome 38

6. CARDIOTROPHIN-1 39

6.1 Gene and protein structure 39

6.2 Binding and signaling 40

6.3 Expression and biological effects of Cardiotrophin-1 43

6.3.1 Cardiotrophin-1 in energy homeostasis and intermediate metabolism... 44

6.3.2 Cardiotrophin-1 and cardiovascular system 47
6.3.3 Cardiotrophin-1 and nervous system 49
6.3.4 Cardiotrophin-1 and liver 50
II. HYPOTHESIS AND AIMS 73
III.EXPERIMENTAL DESIGN AND METHODS 83
CHAPTER 1. 85
CHAPTER 2. 89
CHAPTER 3. 92
CHAPTER 4. 96
IV. RESULTS 103
CHAPTER 1 105
CHAPTER 2 133
CHAPTER 3 155
CHAPTER 4 175
V. GENERAL DISCUSSION 205
VI. CONCLUSIONS 219
VII. THESIS SUMMARY 225
APPENDICES 231

-A-

AC:	Adenylyl cyclase
ACC:	Acetyl CoA carboxylase
cAMP:	cyclic Adenosine monophosphate
AgRP:	Agouti-related peptide
AMPK:	Adenosine monophosphate-activated protein kinase
AdPLA:	Adipocyte phospholipase A2
β-AR:	β-Adrenergic receptors
ANP:	Atrial natriuretic peptide
ANRT:	Aryl hydrocarbon receptor nuclear translocator
ATGL/desnutrin:	Adipose triglyceride lipase
ATP:	Adenosin triphosphate
ATP-III:	Adult treatment panel-III

-B-

BAT:	Brown adipose tissue
BBM:	Brush border membrane
BBMV:	Brush border membrane vesicles
BMAL1:	ANRT-like protein 1
BMI:	Body mass index

-C-

CBS:	Calf bovine serum
CD:	Chronodisruption
CNTF:	Ciliary neurotrophic factor
CGI-58:	Comparative gene identification-58
CRYs:	Cryptochromes
CT-1:	Cardiotrophin-1

-D-

DAG:	Diacylglycerol
DGAT:	Diacylglycerol acyltransferase

-E-

EHT: Engineered heart tissue

ERK: Extracellular regulated kinase

ET-1: Endothelin-1

-F-

FA: Fatty acids

FABP: Fatty acid binding protein

FAP: Fatty acid translocase

FATP: Fatty acids transport proteins

FAS: Fatty acid sinthase

FBS: Fetal bovine serum

FFA: Free fatty acids

-G-

GC: Guanylyl ciclase

$G_s\alpha$: G protein alpha S

Gi: G Protein alpha Inhibitor 1+2

GLP: gp130-like protein

GLUT: Glucose transporter

cGMP: cyclic Guanosine monophosphate

gp130: Glycoprotein 130

G0S2: G0/G1 switch gene 2

GPAT: Glycerol-phosphate acyltransferase

GPCR: G-protein-coupled receptor

GTP: Guanosine triphosphate

-H-

HDL-C: High-density lipoprotein-cholesterol

HFD: High fat diet

HOMA: Homeostasis model assessment

HSL: Hormone sensitive lipase

-I-

IL: Interleukin

IR: Insulin receptor

IRS: Insulin receptor substrates

-J-

JAK: Janus-activated Kinase

-L-

LD: Lipid droplet

LDL-C: Low-density lipoprotein-cholesterol

LIF: Leukemia inhibitory factor

LIFR: Leukemia inhibitory factor receptor

LPL: Lipoprotein lipase

LPS: Lipopolysaccharide

-M-

MAPK: Mitogen-activated protein kinase

MAG: Monoacylglycerol

α-MG: α-Methyl-D-glucoside

MGL: Monoacylglycerol lipase

MetS: Metabolic syndrome

MUFA: Monounsaturated fatty acids

Myf 5: Myogenic factor 5

-N-

NAD: Nicotinamide adenine dinucleotide

NALFD: Non-alcoholic fatty liver disease

NAMPT: Nicotinamide phosphoribosyltransferase

NASH: Non-alcoholic steatohepatitis

eNOS: endothelial NO synthase

NP: Natriuretic peptides

-O-

ObR: Leptin receptor

-P-

PBEF: Pre-B cell colony enhancing factor

PDE3B: Phosphodiesterase 3B

PERs: Periods

PF: Pair-fed

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PGE_2: Prostaglandin E_2

PIAS: Protein inhibitor of activated STAT

PI3K: Phosphatidylinositol3-kinase

PKA: Protein kinase A

PKG: Protein kinase G

PKC: Protein kinase C

POMC: Proopiomelanocortin

PPARγ: Peroxisome proliferator-activated receptor γ

PPH: Phosphatidyl acyltransferase phosphohydrolase

PTP1B: Protein tyrosine phosphatase 1B

-Q-

Qm: Chylomicrons

-R-

rCT-1: recombinant CT-1

RELM: Resistin-like molecule

RHT: Retinohypothalamic tract

ROS: Reactive oxygen species

-S-

SFA: Saturated fatty acid

SCD1: Stearoyl CoA desaturase

SCN: Suprachiasmatic nucleus

SGLT-1: Sodium-dependent glucose transporter 1

SOCS: Suppressor of cytokine signaling

STAT: Signal transducer and activator of transcription

-T-

TAG: Triacylglycerol

TLR4: Toll-like receptor 4

TNF-α: Tumor necrosis factor alpha

-U-

UCP1: Uncoupling protein 1

-V-

VLDL: Very low density lipoproteins

VO_2: Oxygen consumption

-W-

WAT: White adipose tissue

WHO: World Health Organization

WT: Wild type

I. INTRODUCTION

1. OBESITY AND COMORBIDITIES

1.1 Definition

Obesity (body mass index (BMI) ≥ 30 kg/m^2) has emerged as one of the major global epidemics of the 21st century and is now reaching epidemic proportions in many developed countries (Barcelo-Batllori *et al.* 2009). Throughout the history, obesity has been interpreted in different ways. Currently, obesity is defined as a multifactorial chronic disease, characterized by an excess of adipose tissue due to a positive energy imbalance between calories consumed and calories expended (Chatzigeorgiou *et al.* 2014). Both increased food intake and unhealthy habits such as sedentary lifestyle of developed societies have undoubtedly contributed to the great expansion of obesity. However, the incidence of obesity cannot be only attributed to environmental factors and a role for genetic factors in the susceptibility to develop obesity has also been suggested (Apalasamy *et al.* 2015) **(Figure 1)**.

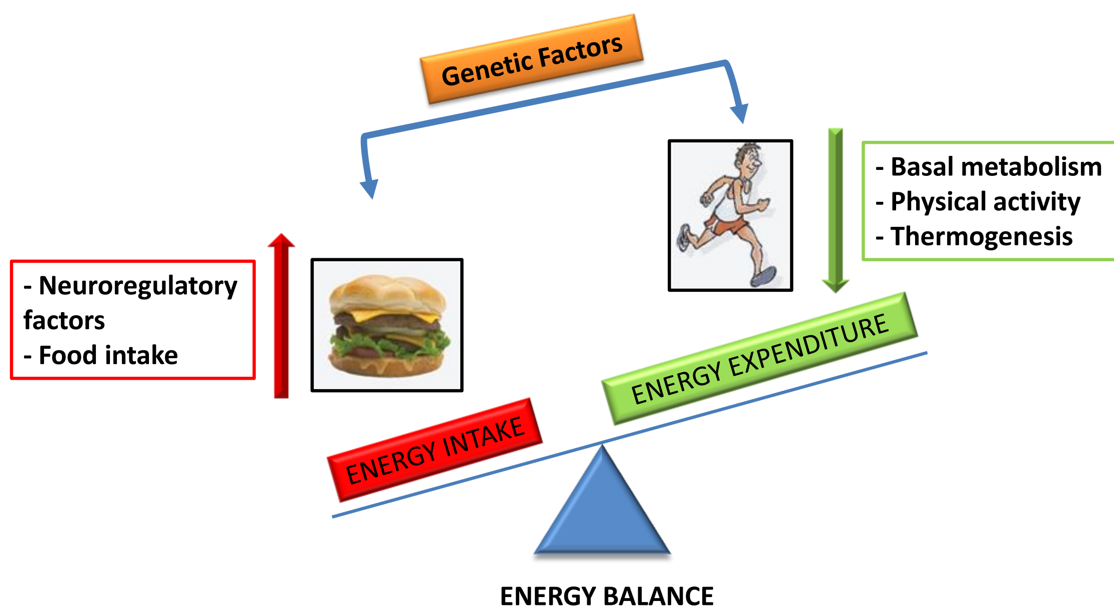


Figure 1: Physiopathology of obesity.

The prevalence of worldwide obesity has more than doubled since 1980. The World Health Organization (WHO, 2015) has recently published that 39% of adults aged 18 years and over were overweight in 2014 and 13% were obese.

Despite adipose tissue plays a key role as the main source of fatty acids (FA) in the postprandial state for energy requirements and heat production, excess adipose tissue accumulation represent a risk that contributes to the development of many pathologies associated such as type 2 diabetes mellitus, cardiovascular diseases, hypertension, dyslipidemia, liver steatosis and even certain types of cancer (Dixon 2010, Prieto-Hontoria *et al.* 2011, Martin-Rodriguez *et al.* 2015). Moreover, treatment of these pathologies represents a big health care cost (Ballesta *et al.* 2006).

All these facts justify the efforts of the scientific community to investigate the causes of the obesity and look for strategies to prevent or reduce the risk of obesity and its associated complications to improve health and to reduce the direct economic costs arising from the treatment of the pathologies associated to obesity. Consequently, identifying the causal factors and understanding their role in the regulation of both energy intake and expenditure is a key step toward the development of obesity treatments.

1.2 Consequences of obesity

1.2.1 Pathologies associated to obesity

The clinical importance of obesity relies on its associated complications as it has been previously noted. Most epidemiological studies observed that mortality increases when BMI exceeds 25 kg/m^2. People with a BMI equal to or greater than 30 kg/m^2 present an increase of more than 50% of the rate of mortality compared to normal weight people. The risk depends especially on excess weight, body fat distribution, interactions between genes and environment and the presence of other cardiovascular risk factors and co-morbidities (Salas-Salvado *et al.* 2007). **Table 1** summarizes the most frequent pathologies associated with excess body weight.

Table 1: Disorders associated with excess body weight. Modified from Salas-Salvado *et al.* (2007).

Metabolic disorders	- Insulin resistance and type 2 diabetes - Hypertension - Atherogenic dyslipidemia - Hyperuricemia
Cardiovascular diseases	- Arteriosclerotic cardiovascular disease - Cardiorespiratory alterations
Digestive disorders	- Hepatic steatosis - Nonalcoholic steatohepatitis, cirrhosis - Gastroesophageal reflux, hiatal hernia - Cholelithiasis
Musculoskeletal disorders	- Bone deformities - Osteoarthritis - Joint injuries
Female urogenital disorders	- Menstrual dysfuncition - Polycystic Ovary Syndrome - Infertility - Increased perinatal risk - Urinary incontinence
Other pathologies	- Certain types of cancer - Psychological and psychosocial distress - Eating disorders - Decreased quality of life - Immunological disorders - Skin disorders

1.2.2 Obesity and Metabolic Syndrome

The metabolic syndrome (MetS) is characterized by a cluster of risk factors associated with obesity that include visceral adiposity, insulin resistance, hypertension, hypertriglyceridemia and low high-density lipoprotein cholesterol (HDL-C); all of which increase the risk for the development of type 2 diabetes and cardiovascular disease (Welty *et al.* 2015). The accumulation of body fat, particularly in the abdominal region, represents the major contributing factor for the development of MetS. In addition to central obesity, other metabolic disorders must be considered for the diagnoses of MetS such as dyslipidemia, increased blood pressure, insulin resistance and a systemic proinflammatory state (Reaven 2002).

MetS or X syndrome was described by Reaven (1988), and receives today a health care due to the high number of people who suffer and who are at high risk of various metabolic complications. The prevalence of MetS correlates with the global epidemic of obesity and is rapidly increasing in most countries, affecting more than 20% of the global adult population (Onat 2011).

There are several criteria for diagnosis of MetS. According to the most used definition, the revised Adult Treatment Panel-III (ATP-III), a diagnosis of MetS can be made when at least three of five of the following alterations are present (Grundy *et al.* 2005) **(Table 2)**.

Table 2: ATP III Clinical Identification of the Metabolic Syndrome.

Risk factor	Definition level
Abdominal obesity	
Men	> 102 cm
Women	> 88 cm
Triglycerides	≥ 1.7 mmol/L
HDL cholesterol	
Men	< 40 mg/dL
Women	< 50 mg/dL
Blood pressure	≥ 130/≥85 mm Hg
Fasting glucose	≥ 6.1 mmol/L

The etiology is very complex and multifactorial **(Figure 2)**. The determining factors include weight, genetics, aging, lifestyle and excess caloric intake (Katzmarzyk *et al.* 2003). The link between the different manifestations of the MetS has been attributed to insulin resistance, which may be the major underlying risk factor that leads to other complications involving cardiovascular risk factors (Balkau *et al.* 1999, Perrone-Filardi *et al.* 2015). This is why the MetS is also called the insulin resistance syndrome (Grundy *et al.* 2005).

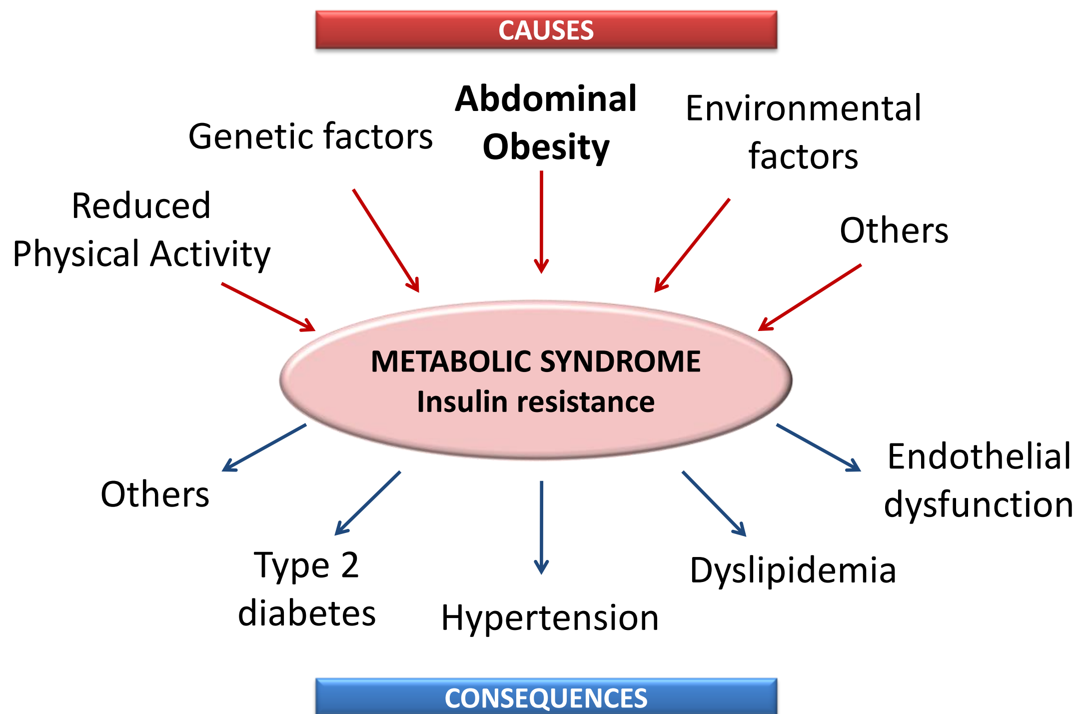


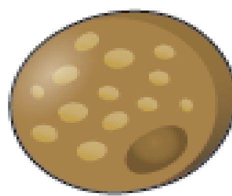
Figure 2: Pathogenesis and consequences of metabolic syndrome.

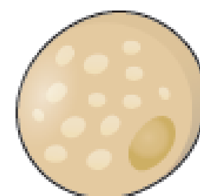
2. ADIPOSE TISSUE

In mammalian two types of adipose tissue are well distinguished: white adipose tissue (WAT) and brown adipose tissue (BAT). According to their differential physiological functions, there are anatomical and morphological differences between both tissues. WAT has been long considered as an energy storage organ in the form of triacylglycerol (TAG), while BAT is responsible for homeostatic maintenance of body temperature through regulation of fat burning and energy expenditure (Hutcheson 2015). However, recently, a third type of adipose tissue known as beige or “brite” (brown in white) adipose tissue has been described. Beige adipocytes appear in WAT depots after permanent thermogenic induction, exhibiting similar molecular and functional features as brown adipocytes (Wu *et al.* 2012). The main characteristics of the three types of adipose tissue depots are displayed in **Table 3**.

Table 3: Characteristics of white, brown and beige adipose tissues.

White adipose tissue (WAT)	Brown adipose tissue (BAT)	Beige adipocytes
Storage organ - Single large lipid droplet - Few mitochondria - Minimal cytoplasm - Adipokine secretion	**Thermogenesis** - Multiple lipid droplets - Many mitochondria - UCP1 expression - Shares developmental origin with muscle	**Thermogenesis** - Multiple lipid droplets - Many mitochondria - Inducible UCP1 expression





White
UCP1$^-$

Brown
UCP1$^+$

Beige
inducible UCP1

2.1 White adipose tissue

Adipose tissue is a very heterogeneous tissue. Mature white adipocytes represent between one-third and two-thirds of total WAT; remaining cells are part of the stromal vascular fraction enclosing preadipocytes, fibroblasts, endothelial cells, histiocytes and macrophages (Scherberich *et al.* 2013). White adipocytes present a unique morphology, having a prominent lipid droplet that occupies almost the entire cell volume, therefore, pushing other intracellular compartments to the cell periphery (Le Lay *et al.* 2009). WAT predominantly arise from non myogenic factor 5 (Myf 5) lineages (Shan *et al.* 2013).

WAT is the most important energy reserve in evolutionary higher organisms and in their adipocytes store excess energy as TAG, allowing rapid mobilization to be used at any time. Adipose tissue has traditionally been considered a passive organ for energy storage or accumulation in a single lipid droplet (Kopecky *et al.* 2004, Church *et al.* 2012), and has relatively low mitochondrial content and lack of uncoupling protein 1 (UCP1) (Lee *et al.* 2014). However, this idea has been challenged from a few years ago.

The identification and further characterization of leptin in 1994 confirmed firmly the adipose tissue as an important endocrine organ (Zhang *et al.* 1994). However, leptin is not the only protein factor secreted by WAT. Indeed, there is a growing list of protein signals and factors that are released from white adipocytes including adipokines that may act in an autocrine, paracrine or an endocrine manner (Catalan *et al.* 2009). Through the action of adipokines, the adipose tissue regulates a wide range of important physiological functions including appetite, satiety, energy expenditure, activity, insulin sensitivity and secretion, glucose and lipid metabolism, fat distribution, endothelial function, hemostasis, blood pressure, neuroendocrine regulation, and function of the immune system (Bluher *et al.* 2015).

The increase in body fat that accompanies obesity may be due to a change in the number and or size of adipocytes, mainly in the subcutaneous fat. In this context, body fat distribution appears to be even more important than the amount of fat. Adipose tissue distribution is a determinant factor of mortality and morbidity associated to obesity (Britton *et al.* 2013). Central adiposity with intra-abdominal deposition of visceral fat, in particular, has been closely linked to cardiometabolic consequences of obesity (Farb *et al.* 2015). However, such association is less consistent in subcutaneous fat mass (Palou *et al.* 2010).

2.2 Brown adipose tissue

BAT is probably the outcome of a single evolutionary development, occurring very early during the evolution of mammals as a natural defense system against hypothermia. This tissue is composed of brown adipocytes that contain numerous lipid droplets (multilocular lipids), which contain a large number of mitochondria, organelles responsible for most of the metabolic processes of transformation and energy production in the form of Adenosin Triphosphate (ATP) (Landsberg *et al.* 2009) and very rich in cytochromes that resemble the characteristic brown of this tissue. BAT is derived from a Myf 5-expressing cell lineage (Seale *et al.* 2008).

For long, BAT has been considered without physiological relevance in adult humans. Recently, this view was radically changed by identification of significant amounts of metabolically active BAT in healthy adults (Cypess *et al.* 2009, Virtanen *et al.* 2009, van Marken Lichtenbelt *et al.* 2009). As previously mentioned, BAT is

specialized in adaptive thermogenesis due to the presence of a BAT-specific mitochondrial proton transport protein, UCP1 (Dempersmier *et al.* 2015). UCP1 is localized in the inner membrane of mitochondria and diminishes the proton gradient by uncoupling cellular respiration and mitochondrial ATP synthesis. Activation of UCP1 in response to cold exposure or food intake results in increased glucose and free fatty acids (FFA) oxidation in order to sustain high levels of uncoupled respiration within BAT, that is, mitochondrial respiration that leads to heat generation (Cannon *et al.* 2004).

In the last years, several independent groups clearly demonstrated the functional and molecular evidence that active BAT depots are present in adult humans and can be readily activated when exposed to mild cold (Cypess *et al.* 2009, Virtanen *et al.* 2009, Chondronikola *et al.* 2014). These BAT depots could contribute to significant energy expenditure upon activation in humans. Moreover, number of prospective studies have reported that many adult humans possess metabolically active BAT, and its mass shows an inverse correlation with BMI and adiposity, indicating that TAG stored in brown adipocytes are the major source of energy for the enhanced metabolic activity of BAT (Saito *et al.* 2009, Orava *et al.* 2013, Chondronikola *et al.* 2014).

2.3 Beige adipocytes

Recent years have seen an intense interest in the ability to ‘‘brown’’ what have traditionally been considered as white adipose tissue depots. Beige adipocytes sporadically reside within WAT, and their development is dramatically induced in response to certain external cues, such as chronic cold exposure, exercise, and long-term treatment with peroxisome proliferator-activated receptor γ (PPARγ) agonists. This process by which the certain white depots could develop brownish characteristics is known as “browning” (Wu *et al.* 2012). In contrast to BAT adipocytes, beige adipocytes are not derived from the Myf 5 lineage (Wu *et al.* 2012). Recent studies indicate the existence of beige adipocytes in adult humans, making this cell type an attractive therapeutic target for obesity and obesity-related diseases, including type 2 diabetes (Schrauwen *et al.* 2015).

There is no evidence suggesting that the functions of brown and brite adipocytes might be any different from each other. Moreover, classical brown and beige

adipocytes *in vivo* are remarkably alike both with regard to molecular and morphological markers. Thus, beige adipocytes are also multilocular, express inducible UCP1 having therefore thermogenic characteristics and have increased mitochondrial respiratory machinery (Shabalina *et al.* 2013).

3. WHITE ADIPOSE TISSUE FUNCTIONS

3.1 Adipose tissue as a storage organ

White adipose tissue is the main fuel reservoir in the body. The traditional role attributed to white adipose tissue is energy storage in adipocytes as TAG when there is an excess of energy (lipogenesis); white adipocytes hydrolyze these TAG into FFA, and glycerol in times of energy need (lipolysis). Both processes occur simultaneously and determine the direction of lipid metabolism.

3.1.1 Lipolysis in adipocytes

Adipose tissue lipolysis has often been considered as a simple and well-understood metabolic pathway. However, in the last years novel lipases and lipid droplet proteins involved in the lipolytic process have been identified. During lipolysis, intracellular TAG undergoes hydrolysis into FFA and glycerol through the action of three major lipases: adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) (Zechner *et al.* 2012). ATGL and HSL are responsible for 95 % of TAG lipase activity, thereby suggesting a complementary relationship between the two lipases (Zechner *et al.* 2009, Thompson *et al.* 2010). FFA and glycerol release from the fat cells is followed by transport of these metabolites through the bloodstream to other tissues for β-oxidation and subsequent ATP generation.

An imbalance between the hydrolysis and synthesis of TAG can be important for the development of obesity. Altered lipolysis could be an element leading to obesity and inter-individual variations in adipose tissue lipolysis are of importance for the rate of weight loss (Lafontan *et al.* 2005). However, excessive lipolytic rates, together with impairment in FFA utilization by muscle and liver, may be major contributors to many detrimental metabolic consequences such as insulin resistance (Lafontan *et al.* 2009).

Nevertheless, several studies have shown that stimulated lipolysis is usually associated with increased FFA uptake by other organs for β-oxidation supporting the idea that increased adipocyte lipolysis may represent potentially useful therapeutic target for treating or preventing obesity (Ahmadian *et al.* 2009).

Adipose triglyceride lipase (ATGL)

In 2004, three groups independently reported an enzyme capable of hydrolyzing TAG. This novel TAG hydrolase, was initially named adipose triglyceride lipase (ATGL) (Zimmermann *et al.* 2004), desnutrin (Villena *et al.* 2004), and iPLA2 (Jenkins *et al.* 2004). The murine ATGL gene codes for a 486-amino acid-long with a molecular mass of 54 kDa protein, while the human homologue, displaying 86% identity, codes for a 506-amino acid protein. ATGL exhibits 10-fold higher substrate specificity for TAG than DAG and selectively assumes the first step in TAG hydrolysis resulting in the formation of DAG and FA (Zimmermann *et al.* 2004). Studies in rodents have shown that ATGL is predominantly expressed in white and brown adipose tissue, although significant levels are also expressed in cardiac and skeletal muscle (Villena *et al.* 2004, Kershaw *et al.* 2006).

ATGL has been shown to be regulated by nutritional status. Thus, studies performed in murine models, have described that ATGL expression is increased by fasting (Villena *et al.* 2004) while is lowered by feeding (Kershaw *et al.* 2006).

Structural domains of human ATGL have been described in studies on mutated and truncated human and murine ATGL. The N-terminal region of the enzyme contains the catalytic patatin domain (Duncan *et al.* 2010). In addition, two phosphorylation sites were localized in the C-terminal region of the enzyme (Ser404 and Ser428 in human ATGL; corresponding to Ser406 and Ser430 in murine ATGL). Although the nature of the putative protein-kinase and the functional role of such sites are unknown, it has been suggested that Ser406 could be a consensus site for AMP-dependent protein kinase (AMPK). Moreover, it has been described that pharmacological stimulation of lipolysis with the AMPK activator AICAR, was dependent on ATGL Ser406 phosphorylation (Ahmadian *et al.* 2011). However, studies performed in human adipose tissue explants described that ATGL phosphorylation at Ser404 is induced by β-adrenergic stimulation. Moreover, phosphorylation of murine

ATGL Ser406 was increased with fasting, exercise, and *ex vivo* stimulation of the cAMP-dependent pathway suggesting a role of protein kinase A (PKA) pathway in the phosphorylation of ATGL (Pagnon *et al.* 2012). Hence, further investigations are necessary to clarify the role of AMPK and/or PKA in the regulation of ATGL by phosphorylation.

Finally, two proteins have been identified as important regulators of ATGL hydrolase activity: comparative gene identification-58 (CGI-58, also known as Abhydrolase Domain Containing 5, ABHD5), which stimulates the activity of ATGL whereas the protein G0S2, encoded by the G(0)/G(1) switch gene 2, inhibits ATGL activity (Lass *et al.* 2006, Yang *et al.* 2010, Gruber *et al.* 2010).

Activation of ATGL is regulated by the interaction of CGI-58 with the patatin domain of ATGL (Schweiger *et al.* 2008, Cornaciu *et al.* 2011) and requires direct protein-protein interactions between ATGL and CGI-58 (Granneman *et al.* 2007, Cornaciu *et al.* 2011). Although the molecular mechanism by which CGI-58 activates ATGL remains to be elucidated, it could potentially be mediated by conformational changes, presentation of substrate, or removal of reaction products (Nielsen *et al.* 2014).

Recently, the protein G0S2 was identified as an inhibitor of ATGL (Yang *et al.* 2010). Like CGI-58, G0S2 interacts directly with the catalytic patatin domain of ATGL (Yang *et al.* 2010, Cornaciu *et al.* 2011). The interaction of G0S2 with ATGL does not depend on the ATGL-CGI-58 interaction, and inhibition by G0S2 has been described to be dominant to activation by CGI-58 (Schweiger *et al.* 2012). It has been proposed that G0S2 is a long-term regulator of lipolysis, as its protein levels decrease during prolonged lipolytic stimulation leading to an increase of ATGL activity (Yang *et al.* 2010, Scherberich *et al.* 2013).

Hormone Sensitive Lipase (HSL)

HSL has long been considered as the key enzyme catalysing the rate-limiting step of adipose tissue lipolysis. In adipose tissue, HSL exhibits 10-fold higher substrate specificity for diacylglicerides (DAG) than TAG and mainly catalyzes the hydrolysis of DAG (Raclot *et al.* 1997). Several isoforms of HSL ranging in size from 88 to 130 kDa are produced by a single gene and by the use of alternative promoters in different tissues (Grober *et al.* 2003, Mairal *et al.* 2002). Adipocyte HSL is composed of an N-terminal domain and a C-terminal catalytic domain that is identical in all known HSL isoforms. This catalytic domain contains the active site, including residues of the catalytic triad (Ser, Asp, His), as well as a regulatory module with all the known phosphorylation sites of HSL (Watt *et al.* 2006b). HSL activity is controlled through reversible phosphorylation (Langin *et al.* 1996).

β-adrenergic activation increases cyclic adenosine monophosphate (cAMP), promoting the phosphorylation of mouse HSL by PKA at three residues (Ser563, Ser659 and Ser660; corresponding to human Ser552, Ser649 and Ser 650) (Anthonsen *et al.* 1998). Phosphorylation of Ser563 is thought to promote the translocation of HSL from the cytosol to the lipid droplet surface (Daval *et al.* 2005), while phosphorylation of Ser659 and Ser660 is critical for activation of the intrinsic enzymatic activity (Anthonsen *et al.* 1998). HSL is also a substrate of the extracellular regulated kinase (ERK), which can phosphorylate HSL at Ser 600, thus increasing lipolysis (Greenberg *et al.* 2001). In addition, HSL is also phosporylated by AMPK at Ser565 (Ser554 in human). Although the role of HSL phosphorylation at Ser565 remains unclear, it is believed to prevent HSL activation, most likely by steric hindrance of phosphorylation of the adjacent Ser563, thus preventing the translocation of HSL to the lipid droplets (Daval *et al.* 2005).

Perilipin

There are many proteins associated to the lipid droplet that constitute a protein-decorated phospholipid monolayer that envelops a neutral lipid core. Proteins of the perilipin family are covering the lipid droplet in adipocytes (Londos *et al.* 1999). This

family of proteins, known as PAT (Perilipin, ADRP and TIP47) is the best-studied lipid droplet-associated proteins (Bickel *et al.* 2009).

Perilipin 1 is highly expressed in white adipocytes and is essential for the hydrolysis of TAG stored in the fat cell by changes of its phosphorylation status and its interaction with HSL and lipase activators (CGI-58 for ATGL). Under basal conditions, perilipin is complexed with CGI-58 avoiding the interaction with ATGL and, thereby the lipolytic activity of this lipase is low (Lass *et al.* 2006). Thus, in the basal state, perilipin is blocking the access of lipases to the triglycerides.

Upon lipolytic stimulation, perilipin is phosphorylated by PKA, releasing CGI-58 to activate ATGL and initiate TAG breakdown (Lass *et al.* 2006). In addition, PKA-dependent phosphorylation of perilipin also promotes HSL translocation from the cytosol to the lipid droplet, allowing the interaction between catalytically active HSL and neutral lipid stores (Brasaemle *et al.* 2000).

Regulation of lipolysis

Regulation of lipolysis is a complex and tightly regulated process **(Figure 3)**. Among the classic regulators the major activators of lipolysis are catecholamines and natriuretic peptides (NP) whereas insulin exerts an antilipolytic effect.

Catecholamines (epinephrine and norepinephrine) are the main stimulators of lipolysis via β-adrenergic receptors (β-AR), however, lipolysis is inhibited through α2-adrenergic receptor and prostaglandins (Lafontan 2008). During the fasting state, catecholamines stimulate lipolysis after binding to the β-AR on the surface of the adipocyte. β-ARs belong to the G-protein-coupled receptor (GPCR) family and are associated with G-protein containing the stimulating Gs subunit (Lafontan *et al.* 1993). Thus, the activation of these receptors promotes the interaction with adenylyl cyclase (AC) which is activated by Gs leading to an increase in intracellular cyclic cAMP concentrations which, in turns results in an increase of c-AMP mediated activation of PKA (Langin *et al.* 2006). Activated-PKA increases lipolysis through the phosphorylation of lipid droplet (LD)-associated protein perilipin and cytoplasmatic HSL promoting its access to triglycerides (Brasaemle *et al.* 2000). Phosphorylation of HSL by PKA occurs at three sites, the serines 563, 659 and 660, enhancing its lipolytic activity (Holm 2003).

In addition to catecholamines, other important positive regulators of lipolysis are the cardiac hormones NPs, which stimulate the guanylyl ciclase (GC) receptor leading to the activation of GC which converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP) resulting in the activation of the cGMP-dependent protein kinase, also known as protein kinase G (PKG). This kinase activates lipolysis by phosphorylation of HSL and perilipin, just like PKA (Sengenes *et al.* 2003).

Moreover, there are also other kinases that regulate lipolysis, such as AMPK. AMPK is a heterotrimeric complex of catalytic α, and regulatory β and γ subunits. α-AMPK acts as an energy sensor, modulating both glucose and lipid homeostasis in adipocytes and also regulates a wide array of physiological events by phosphorylating both key enzymes and transcriptional activators and coactivators (Hardie 2007). AMPK activation is dependent on reversible phosphorylation of the Thr172 within the kinase domain of the α-catalytic subunit (Oakhill *et al.* 2012). Several studies have reported that AMPK activation promotes an anti-lipolytic effect (Daval *et al.* 2005, Anthony *et al.* 2009). In this context, it has been described that AMPK phosphorylates HSL on Ser565, thereby preventing PKA-stimulated activation of HSL (Holm 2003, Daval *et al.* 2005). On the contrary, other studies suggested that AMPK stimulates lipolysis (Yin *et al.* 2003, Koh *et al.* 2008). Thus, *in vitro* studies support the idea that β-adrenergic agents activate AMPK via an intermediary rise in cAMP and that this serves to enhance the lipolytic rate (Yin *et al.* 2003, Djouder *et al.* 2010). Interestingly, AMPK is also capable of increasing ATGL lipolytic activity through the phosphorylation at Ser406 both *in vitro* and *in vivo* (Ahmadian *et al.* 2011). All these findings suggest that AMPK may phosphorylate ATGL and HSL to regulate lipolysis, but the overall effect on lipolyisis remains to be elucidated.

There is also evidence that mitogen-activated protein kinases (MAPKs) can also regulate lipolytic activity in adipocytes. These serine-threonine kinases are involved in the intracellular signaling associated to several cellular activities such as cell proliferation, differentiation, survival, death and transformation (Yun *et al.* 2011, Choi *et al.* 2013). Activation of the MAPK/extracellular regulated kinase (ERK) pathway induces HSL phosphorylation at Ser600, located in the middle of the regulatory module (Greenberg *et al.* 2001) and this phosphorylation increases the hydrolytic activity of HSL similar to that observed when HSL is phosphorylated by PKA (Carmen *et al.* 2006).

In this context, tumor necrosis factor alpha (TNF-α) has been demonstrated to activate MAPK including ERK and JNK in different cell types (Ryden *et al.* 2002, Ryden *et al.* 2004). Thus, it was observed that TNF-α-mediated activation of ERK pathway increases adipose tissue lipolysis (Souza *et al.* 2003).

On the contrary, insulin is the most antilipolytic hormone in adipose tissue. During the fed state, insulin induces the autophosphorylation of insulin receptor (IR) through binding to its receptor, and recruits insulin receptor substrates (IRS 1/2) for tyrosine phosphorylation, which initiates a cascade of signaling events that inhibit lipolysis. This signaling cascade, promotes the activation of phosphatidylinositol3-kinase (PI3K), which leads to an increase of phosphodiesterase 3B (PDE3B) activity which degrades cAMP to 5´AMP (Berggreen *et al.* 2009). This inactivates PKA leading to a reduction of HSL and perilipin phosphorylation and causing a suppression of lipolysis. Insulin is also a major regulator of ATGL in adipocytes by downregulating ATGL mRNA (Kralisch *et al.* 2005, Kershaw *et al.* 2006). In addition to insulin, ATGL gene expression is negatively regulated by TNF-α-mediated p42/44 MAPK activation (Kim *et al.* 2006).

Finally, other molecules have been also described as regulators of lipolysis. Thus, adipose-specific phospholipase A2 (AdPLA) is highly expressed in adipocytes and releases FA (mainly arachidonic acid) from phosphatidiylcholine in a Ca^{2+}-dependent manner (Duncan *et al.* 2008, Wolf 2009). Arachidonic acid is known to be involved in prostaglandin E_2 (PGE_2) production, which leads to the downregulation of lipolysis by decreasing cAMP levels, highlighting the importance of AdPLA in adipose tissue lipolysis (Duncan *et al.* 2008). Moreover, further studies revealed that AdPLA-null mice had increased HSL-phosphorylation, suggesting that HSL phosphoryation through cAMP-mediated activation of PKA is probably a key mediator of increased lipolysis in these mice (Jaworski *et al.* 2009).

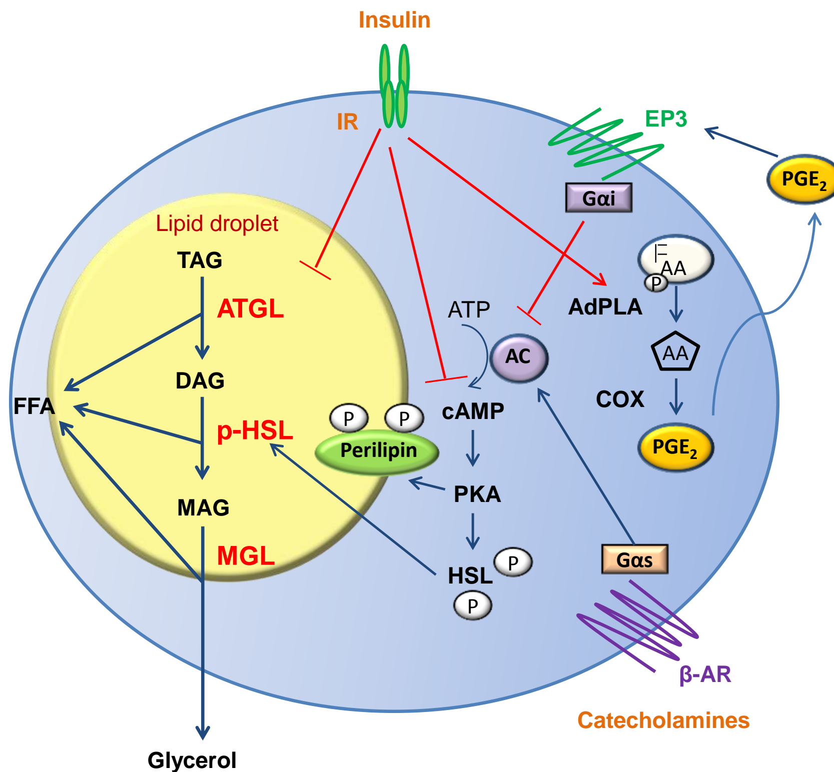


Figure 3: Regulation of lipolysis in adipocytes. Modified from Ahmadian *et al.* (2010).

3.1.2 Lipogenesis: Triglycerides storage

Lipogenesis includes the uptake of plasma FFA and their subsequent re-esterification into TAG within the adipocyte. After dietary food intake, the main source of TAG comes from circulating chylomicrons (Qm) and very low density lipoproteins (VLDL) in circulation. These TAG require the hydrolysis to FFA and monoglycerol by lipoprotein lipase (LPL) located in the vascular endothelium of adipose tissue. Circulating FFA can be picked up by adipocytes through the action of specific fatty acids transport proteins (FATP), fatty acid binding protein (FABP), fatty acid translocase (FAP) among others (Bernlohr *et al.* 1999, Garcia-Arcos *et al.* 2013).

Once inside the cell, fatty acids are reesterified to form TAG and stored in the lipid droplet. The synthesis of triglycerides involves three enzymes: glycerol-phosphate acyltransferase (GPAT), phosphatidyl acyltransferase phosphohydrolase (PPH) and diacylglycerol acyltransferase (DGAT), each of which catalyzes the transfer of a fatty acid molecule to the skeleton of glycerol-3-phosphate. The formation of glycerol-3-phosphate in adipocytes occurs by reduction of dihydroxyacetone phosphate formed

from glucose via glycolysis, which, in turn, is picked up from the bloodstream by specific transporters (GLUT4) and insulin action. (Proenca *et al.* 2014) **(Figure 4)**.

Tryglicerides storage in adipocytes can be also consequence of *de novo* lipogenesis. The term "lipogenesis *de novo*" specifically refers to the formation of TAG from carbon skeletons. Although it is also important in adipocytes, *de novo* synthesis of fatty acids takes place primarily in the liver, where FFA are synthesized by the action of Acetyl CoA Carboxylase (ACC), a rate-limiting enzyme necessary for the conversion of acetyl-CoA to malonyl CoA. Then Fatty Acid Sinthase (FAS) is required for the conversion of actyl-CoA and malonyl-Coa to saturated fatty acid (SFA). Another enzyme that contributes to lipogenesis is Stearoyl CoA Desaturase (SCD1) which catalyzes the synthesis of monounsaturated fatty acids (MUFA) from SFA, playing a key role in the synthesis of TAG, cholesterol esters and diacylglycerol (Ntambi *et al.* 2002).

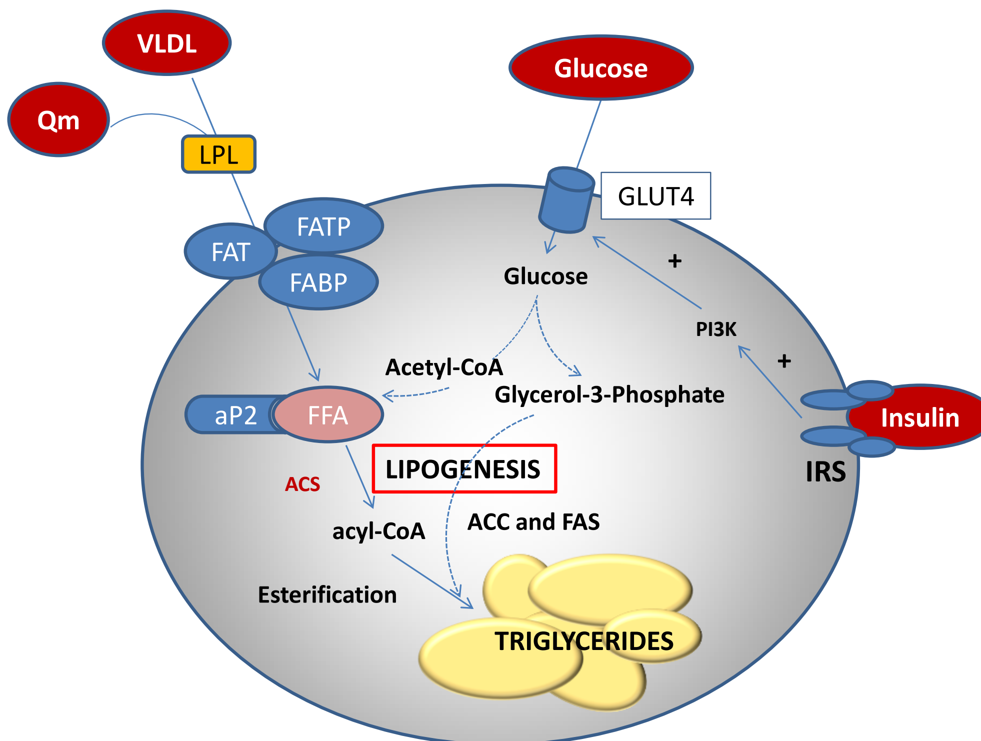


Figure 4: Lipogenesis process in adipocytes.

3.2 Adipose tissue as an endocrine organ

Adipose tissue has long been considered an organ for energy storage. However, the metabolic role of WAT changed in perspective after the discovery of leptin (Zhang *et al.* 1994). WAT has been established as a major secretory and endocrine organ capable of secreting a wide range of bioactive factors known as adipokines (Bluher 2014) **(Figure 5)**.

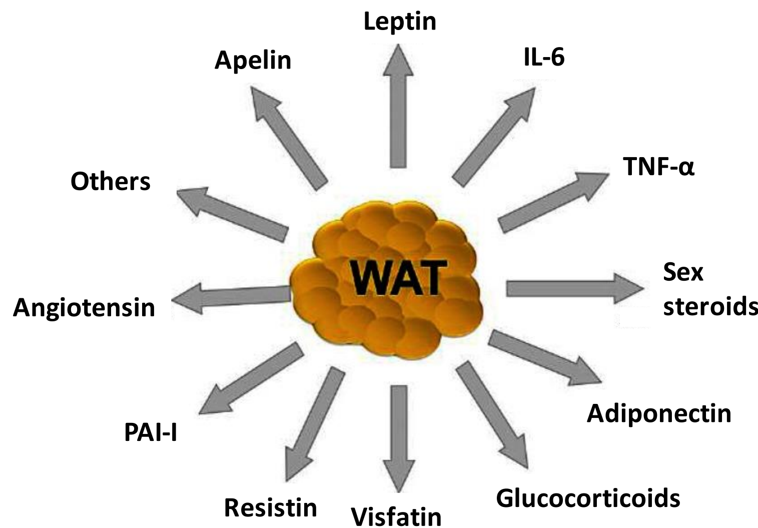


Figure 5: Adipokines secreted by white adipose tissue. Modified from Coelho *et al.* (2013).

Adipokines are involved in the regulation of several functions in target organs (brain, liver, skeletal muscle, vasculature, heart, immune system and pancreatic β-cells), such as appetite and satiety control, fat distribution, insulin sensitivity and insulin secretion, energy expenditure, inflammation, blood pressure, hemostasis, and endothelial function (Bluher 2014, Catalan *et al.* 2009). In addition to an endocrine mode of action, adipokines contribute to the modulation of adipogenesis, immune cell migration into adipose tissue, adipocyte metabolism and function, in an autocrine and paracrine manner (Bluher 2014).

It is well known that a rapid modification of the mechanism that control energy balance leads to the development of obesity and insulin resistance. Dissection of the molecular mechanisms underlying obesity and associated metabolic alterations are essential for the development of new strategies that could contribute to the prevention and treatment of these disorders. Among the different mechanism for the control of energy balance, it has been well recognized that the secretion of adipokines from adipose tissue plays a key role. Alteration of the production (excess or deficit) of

these adipose tissue secreted factors could lead to the development of metabolic disorders.

The key challenges in establishing the secretory functions of WAT are to identify the secreted proteins, to identify the role of each secreted protein, and to assess the pathophysiological consequences of changes in adipocyte protein production with alterations in adiposity. In this context, it is well known that leptin, resistin, visfatin and apelin are key adipokines involved in the control of body weight and/or glucose and lipid metabolism.

3.2.1 Leptin

Leptin, discovered in 1994 (Zhang *et al.* 1994), is a 16 kDa protein mainly secreted by WAT, which regulates body weight homeostasis by inhibiting food intake and increasing thermogenesis via sympathetic innervations of BAT (Ahima *et al.* 1996, Scarpace *et al.* 1998, Zeng *et al.* 2015)**.**

In humans, leptin gene (*LEP*) is located at chromosome 7q31.3 and comprises three exons separated by two introns, being the coding region between exons 2 and 3 (He *et al.* 1995, Isse *et al.* 1995). Leptin is translated as a 167 amino acid protein with an amino-terminal secretory signal sequence. The signal sequence is functional, and results in the translocation of leptin into microsomes with the subsequent removal of the signal peptide. Its sequence is highly conserved, showing homology between different species. Thus, human leptin has 84% homology with mouse and 83% with rat (Zhang *et al.* 1994). Its tertiary structure consisting of four α helices and two β sheets with a simple disulfide bridge between residues 96 and 146 appears to be necessary for activity (Grasso *et al.* 1997) and has structural similarity with other cytokines (Madej *et al.* 1995, Zhang *et al.* 1997).

Leptin is produced in several organs additional to WAT, including brown fat, the placenta and fetal tissues, skeletal muscle and stomach among others (Wang *et al.* 1998, Bado *et al.* 1998, Marti *et al.* 1999, Cervero *et al.* 2006).

The gene for human leptin receptor (*ObR*) is located in chromosome 1q31 and belongs to a member of the class I cytokine receptor superfamily (Fruhbeck 2006). Ob-Re is a soluble receptor secreted in the blood that binds to circulating leptin in order to maintain the concentration of free leptin, while isoform ObRb has the longest

intracellular domain and is considered the main functional receptor of leptin, since it is the isoform with the greater signaling capacity (Murakami *et al.* 1997, Chen *et al.* 1996). The binding of leptin to Ob-Rb activates a number of signaling pathways, such as JAK2/STAT3 and STAT5, IRS/PI3K, SHP2/MAPK, and AMPK/ACC. The leptin signaling cascade is terminated by the induction of a suppressor of cytokine signaling 3 (SOCS3) and protein tyrosine phosphatase 1B (PTP1B) (Ahima *et al.* 2004, Sainz *et al.* 2015b).

The key role of leptin as an adipose signal mainly involved in the regulation of energy balance, was first demonstrated in leptin deficient *ob/ob* mice, in which the mutation caused marked hyperphagia and obesity (Zhang *et al.* 1994). Leptin was first linked to obesity by demonstrating its action on the hypothalamic centers increasing anorexigenic and decreasing orexigenic peptide synthesis through the regulation of proopiomelanocortin (POMC) neurons and Agouti-related peptide (AgRP) neurons, respectively; subsequently resulting in reduced appetite (Friedman *et al.* 1998, Elmquist *et al.* 1999).

Initially, it was hoped that the discovery of leptin would resolve human obesity. In this context, it has been found that administration of leptin decreased food intake and reduces body weight in leptin deficient patients (Rosenbaum *et al.* 2002). Unfortunately, for the majority of overweight and obese subjects, hyperleptinemia is a common characteristic of obesity in human and rodents, where a resistance to the action of leptin has been suggested (Frederich *et al.* 1995, Maffei *et al.* 1995). For this reason, the concept of leptin as the anti-obesity hormone must be reconsidered due to the inability of exogenous leptin to influence body weigh in obese patients. The mechanisms that lead to the development of leptin resistance need to be further elucidated, but it is well established that leptin resistance define most states of obesity (Savage *et al.* 2002). In this situation, elevated levels of circulating leptin are secondary to the increased adipose tissue mass in obesity, which contributes to the increased production of leptin (Schwartz *et al.* 1996, Schwartz *et al.* 1997). The development of leptin resistance characterized by the lack of response to leptin may lead to a dysregulation of central and peripheral actions of leptin, including food intake, nutrient intestinal absorption, intermediate metabolism and insulin sensitivity, thus causing a dysregulation of the energy balance and further contributing to weight gain. Hence,

hyperleptinemia associated to obesity may be a potential marker for recognizing the development metabolic alterations in obese subjects (Sainz *et al.* 2015a).

The discovery of the leptin receptor, ObR (Tartaglia *et al.* 1995), revealed the importance of leptin in the control not only of obesity but also diabetes. This fact was first characterized after observing that a mutation in the leptin receptor induced obesity and diabetes in *db/db* mice (Chen *et al.* 1996). Interestingly, it was observed that *ob/ob* mice treated with leptin exhibited reduced insulin and glucose levels (Pelleymounter *et al.* 1995). Thus, leptin may exert insulin-sensitizing effects and is considered an important regulator of β cell mass and survival (Dunmore *et al.* 2013). Moreover, it has been described that leptin increases FA oxidation and decreases TAG storage in liver and skeletal muscle through a combination of direct activation of AMPK and indirect actions mediated through central neural pathways, thereby improving insulin sensitivity (Minokoshi *et al.* 2002). Importantly, hyperleptinemia predicts a worsening of the features of MetS independently of obesity. This association is mainly related to the development of insulin resistance and glucose intolerance through the upregulation of proinflammatory cytokines such as TNF-α and interleukin (IL)-6 (Franks *et al.* 2005). In this context, both cytokines are closely related to obesity and insulin resistance (Wellen *et al.* 2005). Thus, obese and insulin resistant subjects have increased TNF-α plasma concentration (Mishima *et al.* 2001), and is overexpressed in adipose tissue (Hotamisligil *et al.* 1995, Kern *et al.* 2001) and skeletal muscle (Saghizadeh *et al.* 1996). Moreover, it has been shown that TNF-α infusion leads to impaired glucose uptake in human skeletal muscle by altering insulin signal transduction (Plomgaard *et al.* 2005). Regarding to the role of IL-6 in the development of insulin resistance, several studies have established a positive correlation between plasma IL-6 levels with obesity and insulin resistance, and might be a predictor for the development of type 2 diabetes (Hu *et al.* 2004, Pradhan *et al.* 2001).

Several studies have shown that the functional leptin receptor is also found in a variety of organs such as the heart, liver, kidneys, and pancreas. It is locate on cardiomyocytes, allowing leptin to have a role in the myocardial structure remodeling with some researchers believing that leptin promotes left ventricular hypertrophy, while others strongly believing that leptin attenuates it (Barouch *et al.* 2003, Leifheit-Nestler *et al.* 2013, Allison *et al.* 2013). Leptin receptor is also found in vascular smooth

muscle cells and endothelial cells contributing to vascular remodeling, hypertrophy, and angiogenesis. It also plays an important role in hypertension through the secretion of proinflammatory cytokines, such as TNF-α and IL-6, and to the generation of reactive oxygen species (ROS) in endothelial cells (Bouloumie *et al.* 1999, Yamagishi *et al.* 2001). Leptin has also been shown to augment the release of the vasoconstrictor endothelin-1 (ET-1) (Quehenberger *et al.* 2002). The involvement of leptin in promoting atherosclerosis is still controversial, with many researchers supporting leptin's role as an atherogenic factor, while others studying its antiatherogenic properties (O'Rourke *et al.* 2002, Dubey *et al.* 2006, Chiba *et al.* 2008). Regarding the role in the immune system, leptin acts as an immune mediator where it promotes activation, chemotaxis and survival of both innate and adaptive immune cells (Fernandez-Riejos *et al.* 2010). Therefore, this hormone has a wide range of pleiotropic effects, affecting the cardiovascular, nervous, immune, and reproductive systems.

3.2.2 Resistin

Resistin was discovered in 2001 as a protein which was specifically expressed and secreted by adipocytes and showed increased serum levels in genetic and diet-induced models of obesity in mice (Steppan *et al.* 2001a). Resistin, is a member of the resistin-like molecule (RELM) family of cysteine-rich proteins together with RELM-[alpha], RELM-[beta] and RELM-[gamma] (Steppan *et al.* 2001b, Gerstmayer *et al.* 2003).

At the genomic level resistin gene (*Retn*) is located in the human chromosome 19p13.3 and in the synthetic regions of the mouse chromosome 8A1. Mouse resistin is translated as a 11 kDa cysteine-rich protein that consists of 94 amino acids that is synthesized as a longer precursor containing a 20 amino acid signal sequence. On the other hand, human resistin is composed of 108 amino acid and shares only 59 % homology with its murine counterpart. Hence, although human and murine resistin presents common three-dimensional structure, protein and genomic differences between species have been described for resistin (Ghosh *et al.* 2003, Yang *et al.* 2003).

In rodents, resistin is specifically expressed in adipocytes, whereas this adipokine is expressed at very low levels, if at all, in human adipose cells, but at high levels in peripheral blood mononuclear cells, macrophages, spleen, and bone marrow cells (Nagaev *et al.* 2001, Lu *et al.* 2002, Patel *et al.* 2003, Popovic *et al.* 2014).

Although it has not been well established the receptor by which restistin exerts its signaling actions, some studies have suggested that resistin binds to toll-like receptor 4 (TLR4) receptor in human myeloid, epithelial and endothelial cells (Hsieh *et al.* 2014). Similar results have been observed in *in vivo* studies showing that resistin binds to TLR4 in hypothalamus (Benomar *et al.* 2013). Although TLR4 has been widely proposed as a possible resistin receptor, further investigations are necessary to clarify the relevances of these results. After binding to its yet unknown receptor, resistin activates several signaling cascades including PI3K/AKT, MAPK/ERK and AMPK/ protein kinase C (PKC)/NADPH oxidase/ROS pathways, that in turn lead to the activation of NF-kB leading to the activation of several related genes, including proinflammatory mediators (Codoner-Franch *et al.* 2015).

Throughout the history, resistin has exhibited controversial effects regarding to its role in the pathogenesis of obesity-mediated insulin resistance and type 2 diabetes. In 2001 resistin was discovered as a protein that was inhibited in mature adipocytes during exposure to TZD with an impact on insulin sensitivity and glucose transport (Steppan *et al.* 2001a). After, the description of resistin as a cytokine secreted by rodent adipocytes (Holcomb *et al.* 2000) resistin has been suggested as a possible explanation for the pathogenic sequence of obesity and insulin resistance (Shuldiner *et al.* 2001). Thus, it was observed that circulating levels of resistin were increased in obesity (Rajala *et al.* 2004) and several studies have evidenced the important involvement of resistin in mediating hepatic or skeletal muscle insulin resistance (Pravenec *et al.* 2003, Satoh *et al.* 2004, Banerjee *et al.* 2004). Nevertheless, several studies performed in humans have reported contradictory findings regarding to the association of hyperresistinemia with insulin resistance. In this context, initial investigations suggested a positive correlation between resistin levels with obesity and insulin resistance (Degawa-Yamauchi *et al.* 2003, Hivert *et al.* 2008, Gharibeh *et al.* 2010). In contrast, other groups failed to identify any correlation between resistin and obesity, insulin resistance or type 2 diabetes (Lee *et al.* 2003, Amirhakimi *et al.* 2011, Boyraz *et al.* 2013). However, despite the controversial results regarding resistin´s involvement in the development of insulin resistance and obesity, it seems that resistin is a biomarker that predicts or develop insulin resistance in specific situations (Syed Ikmal *et al.* 2013).

In addition to its function on glucose metabolism, resistin also plays a role in the regulation of the inflammatory processes. Thus, resistin is a potent stimulator of oxidative/nitrosative stress (Codoner-Franch *et al.* 2014) and promotes the expression of TNF-α and IL-6 in WAT and in peripheral-blood mononuclear cells (Bokarewa *et al.* 2005, Nagaev *et al.* 2006, Jiang *et al.* 2013). Conversely, it has been shown a potent increase in the expression of resistin in peripheral blood mononuclear cells mediated by the action proinflammatory mediators such as TNF-α, IL-1β, IL-6, or lipopolysaccharide (LPS) (Kaser *et al.* 2003, Anderson *et al.* 2007). Moreover, C-reactive protein also increases resistin mRNA expression and protein secretion in a dose- and time-dependent manner in peripheral-blood mononuclear cells (Hu *et al.* 2007). Altogether these data suggest a role for resistin in the process of inflammation.

Finally, resistin may contribute to the accumulation of cholesterol and TAG in macrophages, arterial inflammation, endothelial dysfunction, and angiogenesis leading to accelerated atherogenesis and coronary heart disease (Rae *et al.* 2006, Gherman *et al.* 2012, Cabrera de Leon *et al.* 2014). Moreover, resistin has been suggested as a marker of coronary atherosclerosis and the severity of myocardium ischemic injury (Fontana *et al.* 2015).

3.2.3 Visfatin

Visfatin also known as PBEF (pre-B cell colony enhancing factor) or NAMPT (nicotinamide phosphoribosyltransferase) is a 52 kDa protein that was first isolated from a human peripheral blood lymphocyte cDNA library (Samal *et al.* 1994).

Visfatin gene is located on the long arm of chromosome 7 between 7q22.1 and 7q31.33 (Jia *et al.* 2004) and encodes a polypeptide of 491 amino acids with a molecular mass of 52 kDa which is highly conserved between species from bacteria to human. There is no signal sequence in the primary structure of visfatin.

It is now well established that visfatin is expressed by adipose tissue as well as skeletal muscle, liver, and immune cells (Fukuhara *et al.* 2005, Stephens *et al.* 2006). Visfatin has also been found to be produced within the myocardium, localized to cardiomyocytes and fibroblasts (Chang *et al.* 2012), and within the brain localized to neuronal cells, with particularly high expression during ischemia. The widespread

distribution of this molecule points to a broad function of visfatin in both health and disease.

The mechanism of action of extracellular visfatin needs to be further investigated, although it is well accepted as the rate-limiting enzyme in the salvage pathway for nicotinamide adenine dinucleotide (NAD) formation from nicotinamide and nicotinic acid, using ATP as the activator and this biosynthetic activity of NAD is responsible of modulating the activity of the NAD-dependent enzymes such as sirtuins (Guan *et al.* 2014), and poly (ADP-ribose) polymerases (Pillai *et al.* 2005), thereby regulating metabolism (Dahl *et al.* 2012).

Visfatin, originally described as an adipokine with insulin mimetic effects (Fukuhara *et al.* 2005), is currently one of the target molecules involved in the regulation of glucose-stimulated insulin secretion in pancreatic β cells (Friebe *et al.* 2011). Indeed, there is growing evidence about the role of visfatin in the development of type 2 diabetes in both obese and lean subjects (Chen *et al.* 2006, Hajianfar *et al.* 2012). In addition to its positive correlation with the onset of type 2 diabetes, visfatin is also responsible for the development of the complications associated to diabetes such as endothelial dysfunction, diabetic nephropathy and impairment of lipid metabolism (Yilmaz *et al.* 2008, Hajianfar *et al.* 2012). The effects of visfatin on glucose metabolism were first associated to its insulin mimetic properties (Fukuhara *et al.* 2005). However, these properties were retracted and are still controversial and under debate (Fukuhara *et al.* 2007) Thus, other authors also reported that visfatin has insulin mimetic effects in cultured osteoblasts (Xie *et al.* 2007), but another theory proved that not the insulin mimetic activity, but its robust NAD biosynthetic activity in pancreatic β cells is more important in glucose homeostasis *in vivo* (Revollo *et al.* 2007). This hypothesis has been widely confirmed by several *in vitro* and *in vivo* studies, demonstrating the role of visfatin and NAD intermediates in ameliorating β-cells function and cellular homeostasis, glucose metabolism and stress responses (Caton *et al.* 2011, Spinnler *et al.* 2013).

In addition to the regulatory action on type 2 diabetes, several studies have focused on the possible actions of visfatin in obesity. Although there is still some controversy regarding to a positive correlation or not correlation of visfatin with obesity, a meta-analysis, which included human investigations, reported that visfatin

levels were generally increased in obese subjects (Chang *et al.* 2011). Moreover, some investigations have described that some SNPs in visfatin gene have been associated with obesity (Blakemore *et al.* 2009) and obesity-related co-morbidities such as coronary artery disease (Saddi-Rosa *et al.* 2013), as well as glucose and lipid parameters (Jian *et al.* 2006).

Regarding to the role of visfatin on obesity, it has also been described potent actions of this cytokine on the regulation of food intake and behavior exhibiting opposite effects. Thus, intracerebroventriuclar injection of visfatin in chicks caused an increase in food intake (Cline *et al.* 2008), whereas it reduced hypothalamic expression of anorexigenic peptides after injection in the arcuate nucleus of the hypothalamus of rats (Brunetti *et al.* 2012).

Finally, visfatin may also represent a pro-inflammatory cytokine on different cell types. Thus it has been described a role of visfatin in the stimulation of inducible nitric oxide synthase and activation of ERK1/2 pathway promoting angiogenesis (Kim *et al.* 2007). Its pro-inflammatory functions are also mediated by inducing potently various other pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β (Moschen *et al.* 2010, Hector *et al.* 2007), transforming growth factor-β (Song *et al.* 2008a) and monocyte chemoattractant protein 1 (Sommer *et al.* 2010).

3.2.4 Apelin

Apelin, discovered in 1988, was isolated from bovine stomach extract and identified as the endogenous ligand of the G protein-coupled receptor APJ (*APLNR*) (Tatemoto *et al.* 1998). In 2005 Boucher *et al.* described this protein as an adipokine produced by human and murine adipocytes (Boucher *et al.* 2005).

The human apelin gene has been localized on chromosome Xq25-q26.1, and encodes an initially translated 77-amino acid pre-proprotein of 16 kDa. The 41 N-terminal amino acids represent secretory signal sequences, while the remaining 36 C-terminal amino acids comprise apelin-36 (Lee *et al.* 2005). There are several forms of apelin derived from the common 77-amino acids pre-propeptide precursor (Tatemoto *et al.* 1998). Apelin-36 is the most abundant form *in vivo*, which is cleaved by proteases to shorter forms, such as apelin-17 and apelin-13 (Kawamata *et al.* 2001).

The sequencing of the human, bovine, rat, and mouse preproapelin has shown that there is a high homology among the four species, and 100% identity in the C-terminal 13 amino acids, which encodes the mature apelin peptide. In addition, a comparison of the mature apelin peptide with angiotensin II revealed several conserved amino acids (Lee *et al.* 2000).

Apelin exerts its actions through the interaction with the G-protein coupled receptor APJ (Tatemoto *et al.* 1998). APJ-apelin interactions activate several signaling pathways in different cell types (Kleinz *et al.* 2005). APJ signaling activates kinases (MAPK/ERK and AKT) that are associated with cell survival and that such signaling is associated with protection from injury in at least some cell types. Apelin and its receptor APJ are widely expressed in several tissues (stomach, heart, lung, skeletal muscle, adipose tissue, etc.) and in different regions of the brain, including the hypothalamus (O'Carroll *et al.* 2013).

The regulation of apelin has been widely studied in several models of obesity in mice and human, suggesting a potential role of apelin in obesity and related metabolic disorders. The work published by Boucher *et al.* (2005) showed that high-fat diet did not change apelin gene expression in mice resistant to the development of obesity and insulin resistance induced by diet. Similarly, neither the expression of this adipokine changed in animals that developed a diet-induced obesity, but their insulin and glucose levels remained within normal limits. However, a significant increase was detected in the expression of apelin in adipose tissue in animals where the development of obesity was accompanied by insulin resistance. Furthermore, studies performed in humans have demonstrated that plasma apelin correlates positively with body mass index and show a dramatic increase in morbidly obese patients (Boucher *et al.* 2005, Heinonen *et al.* 2005). However, other studies have suggested that apelin is not necessary correlated with body mass index (Soriguer *et al.* 2009, Telejko *et al.* 2010, Reinehr *et al.* 2011). Since plasma apelin levels are increased in obesity and hyperinsulinemia, it suggests that this adipokine could mediate the development of metabolic dysfunction associated to obesity. However, several studies have demonstrated that apelin plays a key role in the regulation of glucose and lipid metabolism, and that it could be a beneficial adipokine. Thus, Dray *et al.* (2008) observed that apelin exhibited glucose-lowering properties both in fasted conditions and during a glucose tolerance test. This

decreased glycemia was mainly mediated by the promoting glucose uptake in target tissues such as skeletal muscle and adipose tissue. Interestingly, apelin increased glucose uptake in muscle of obese and insulin resistance mice, favoring glucose-lowering properties and improved insulin sensitivity. Regarding to the signaling pathways involved in the regulatory effects of apelin on glucose uptake it was observed that this adipokine promoted the activation by phosphorylation of AMPK but also the endothelial NO synthase (eNOS) (Dray *et al.* 2008). Subsequently, a study performed in C2C12 muscle cells observed that apelin was able to stimulate glucose transport *in vitro* through a pathway involving AMPK but not eNOS (Yue *et al.* 2010). The contradictory effect observed in the study of Yue *et al.* could be due to the use of NOS inhibitors that are efficient to decrease glucose uptake *in vivo* in muscle cells but not *in vitro* (Roy *et al.* 1998).

In line with the results observed in the study performed by Dray *et al*. it has also been described that apelin stimulates glucose transport in an AMPK-dependent way in human adipose tissue explants (Attane *et al.* 2011). Moreover, similar results were observed in 3T3-L1 adipocytes, in which the effects were mediated through the activation of the PI3K/AKT pathway (Zhu *et al.* 2011). Interestingly, a recent study has demonstrated that oral administration of apelin decreased the amount of Sodium-dependent Glucose Transporter-1 (SGLT-1) in enterocytes, but increase glucose transporter (GLUT)-12. This effect was secondary to the activation of AMPK pathway and resulted in an increase of glucose uptake in enterocytes (Dray *et al.* 2013) demonstrating the potential role of apelin in the regulation of glucose metabolism, by promoting glucose absorption by the enterocytes.

In addition to its role on glucose metabolism, some studied have demonstrated a role of apelin in the regulation of lipid metabolism. Thus, Higuchi *et al.* (2007) observed that treatment with apelin decreased TAG content and adiposity in standard and obese mice. In parallel with this result, apelin-transgenic mice exhibit a resistance against diet-induced obesity (Yamamoto *et al.* 2011). However, other studies have described conflicting results regarding to lipolysis. Thus, apelin shows inhibitory effects on isoproterenol-stimulated lipolysis in 3T3-L1 adipocytes. These effects are mediated through the activation of the AMPK pathway (Yue *et al.* 2011). Similar results were observed in the study performed by Than *et al.* (2012), in which apelin decreased FFA

through AMPK activation and by increasing the amount of perilipin surrounding the lipid droplet, avoiding the access of lipases.

Recently, it has been reported that apelin-APJ signaling promotes brown adipocyte differentiation by increasing the expressions of brown adipogenic and thermogenic transcriptional factors via PI3K/AKT and AMPK signaling pathways. Apelin increases the basal activity of brown adipocytes and it is able to increase the brown-like characteristics in white adipocytes (Than *et al.* 2015).

Taking into account the relevant effects of apelin on numerous organs and tissues, apelin is considered as an important regulator of whole body metabolism. Thus, it has been proposed that the over-production of apelin in obesity, could prevent the development of obesity-linked disorders such as type 2 diabetes and cardiovascular diseases (Castan-Laurell *et a*l. 2005; Habchi *et al.* 2014).

4. INTESTINAL SUGAR ABSORPTION

Small intestine is the organ where most nutrients from the diet are absorbed. In this context, carbohydrates are an important component of the diet (Barrenetxe *et al.* 2013a). Among these carbohydrates glucose is a crucial energy source, and it is important in many cellular processes (Bergeron *et al.* 2008). In order to get the nutritional benefit, carbohydrates are digested by salivary and pancreatic amylases, and finally broken down into monosaccharides (glucose, galactose and fructose) by disaccharidases in the brush border membrane (BBM) of enterocytes. Finally, monosaccharides are absorbed toward the blood circulation through specific nutrient transporters located in the apical and basolateral plasma membrane of mature enterocytes.

4.1 Transporters involved in intestinal sugar absorption

The intestinal glucose absorption into enterocytes is a secondary active process mediated by SGLT-1. SGLT-1 family protein structure contains 482–718 amino acid residues and a secondary structure that consists of 14 transmembrane α-helices (Turk *et al.* 1996). SGLT-1 is constitutively expressed in the BBM of mature enterocytes in the small intestine but can be rapidly mobilized from the apical membrane to the

cytoplasm storages and *vice versa* in response to different intracellular signals (Wright *et al.* 1997). SGLT-1 is an example of a secondary active transport protein that involves co-transport of sodium and glucose with a coupling ratio of 2:1. Thus, SGLT-1 uses the electrochemical gradient maintained by the basolateral Na^+-K^+ATPase to accumulate glucose in the cell against its concentration gradient (Wright 2001). SGTL-1 is a high affinity/low capacity glucose transporter that becomes saturated at a concentration around 30 mM (Wright *et al.* 2011). In this context, when the glucose concentration in the lumen of the small intestine goes above 30 mM, such as occurs during postprandial state, the low affinity/high capacity glucose transporter GLUT-2 is up-regulated at the BBM, providing a major route for moving glucose into the blood across the basolateral membrane (Kellett *et al.* 2005).

Galactose is also transported into the enterocytes by SGLT-1 (Wright *et al.* 2011), whereas GLUT-5 is a facilitative transporter on the BBM which transports fructose from the lumen of intestine into enterocytes down its concentration gradient. GLUT-7, another member of the GLUT facilitative transporter family can also be responsible for the uptake of glucose and fructoste through BBM (Cheeseman 2008).

Finally, the three monosaccharides (glucose, galactose and fructose) are transported from the enterocytes into the portal circulation by basolateral GLUT-2 (Kellett *et al.* 2008) **(Figure 6)**.

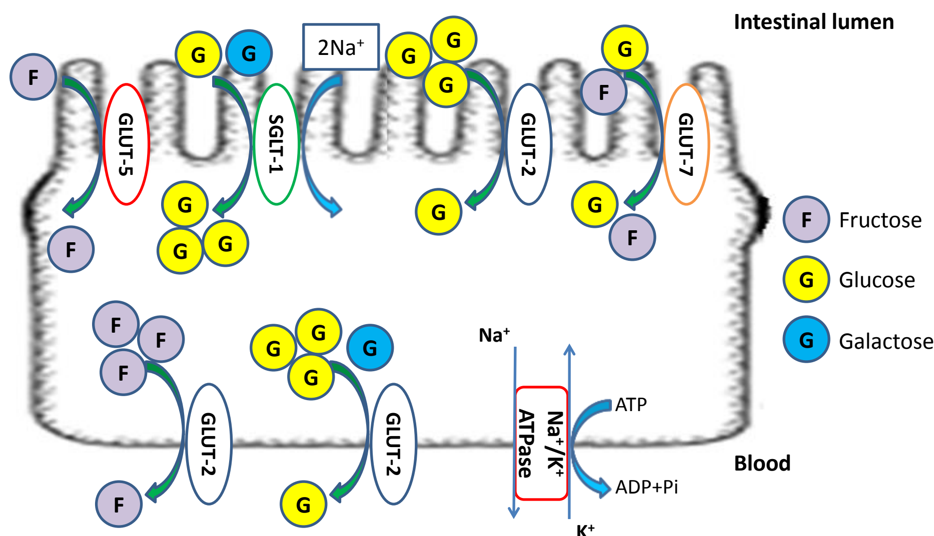


Figure 6: Transporters implicated on intestinal sugar absorption in enterocytes. Modified from Barrenetxe *et al.* (2013a).

4.2 Regulation of intestinal glucose absorption

Regulation of intestinal glucose absorption can be mediated by several specific or nonspecific mechanisms (Ferraris *et al.* 2000). Unspecific mechanisms make reference to those that mediate changes in the absorptive surface of enterocytes by increasing the number (hyperplasia) or the size (hypertrophy), thus regulating glucose absorption. In this context, previous studies have demonstrated that a galactose enriched diet can increase the length of the intestinal microvilli enhancing the absorptive surface (Smith *et al.* 1991), whereas rats fed with a high fat diet showed increased sugar absorption due to increased intestinal mass (Planas *et al.* 1992). Likewise, dietary sugars and artificial sweeteners enhance the expression of the intestinal glucose transporter SGLT-1 and the capacity of the gut to absorb glucose (Shirazi-Beechey *et al.* 2011). Dietary lipids can also modify the composition of the plasma membrane in the small intestine, altering the lipid environment of the transporters and, in turn, their structure and activity. In this sense, an altered saturated/unsaturated fat ratio induces modifications of SGLT-1 function (Alessandri *et al.* 1990). Another unspecific mechanism would be modifications of the Na^+/K^+ ATPase activity that would change the sodium electrochemical gradient inducing changes in the membrane potential and on SGLT-1 activity (Ferraris *et al.* 1997).

On the other hand, specific mechanisms refer to those that comprise transcriptional and/or post-transcriptional mechanisms modifying the number of transporters in the plasma membrane of enterocytes (Ferraris *et al.* 1993), through modifications of their synthesis or degradation (Tsang *et al.* 1994), regulating intestinal glucose absorption. It has been observed in the majority of species, a wide range of monosaccharides effective in enhancing the expression of SGLT-1 (Solberg *et al.* 1987, Dyer *et al.* 1997). Furthermore, the effects of different cytokines on the regulation of SGLT-1 such as TNF-α (Barrenetxe *et al.* 2013b), IL-6 (Lee *et al.* 2007) or IL-1β (Vinuales *et al.* 2013), among others have been described. Similarly some adipokines such as leptin and apelin have also shown regulatory effects on SGLT-1 expression (Dray *et al.* 2013, Fanjul *et al.* 2012).

Post-transcriptional regulation of SGLT-1 also includes the activation of several protein kinases-dependent pathways involved in the regulation of SGLT-1 activity, as

well as in the insertion and recruitment of SGLT-1 in the plasma membrane (Wright *et al.* 1997). Although there are several studies about the regulation of SGLT-1 by different signal transduction pathways, effects of serine/threonine PKA and PKC are the most widely characterized at the level of both transcription and translation in the intestinal epithelial cells (Hirsch *et al.* 1996, Castaneda-Sceppa *et al.* 2010). SGLT-1 contains five PKC phosphorylation sites in rat and human and four in rabbit; and one consensus PKA site in rabbit and human, and none in rat (Kennelly *et al.* 1991). Protein kinases can regulate membrane transport in a direct manner by promoting changes in the transporter or changes in the carrier turnover number; and in an indirect manner leading to an increased amount of the transporters present in the plasma membrane (Vayro *et al.* 1999). In oocytes expressing rabbit SGLT-1, it has been established that the activation of PKA increased the maximum rate of Na+/glucose co-transport by 30%, whereas the activation of PKC decreased the maximum rate of transport by 60% (Wright *et al.* 1997). These effects are mediated by changes in the number of co-transporters in the plasma membrane and by changes in the area of the membrane. Moreover, it has been observed that PKA-mediated phosphorylation of SGLT-1 promotes changes on the conformation of the transporter, leading to the stimulation of glucose uptake (Subramanian *et al.* 2009). In addition, PKC can also indirectly decrease SGLT-1 activity by regulating vesicle trafficking (Wright *et al.* 1997). Interestingly, regarding to the underlying mechanisms that contribute to the PKC-mediated phosphorylation of SGLT-1, it has been demonstrated the involvement of both MAPK and PI3K/AKT/mTOR signaling pathways (Castaneda-Sceppa *et al.* 2010).

It has been also described that AMPK pathway activity may enhance cellular glucose uptake promoting membrane translocation of SGLT-1 (Sopjani *et al.* 2010). Furthermore, inhibition of cGMP production at the level of guanylyl cyclase or inhibition of PKG also showed reduced SGLT-1 activity demonstrating the involvement of PKG pathway in the regulation of SGLT-1 activity (Arthur *et al.* 2014). Finally, it has also been demonstrated that several hormones such as GLP-2 (Cheeseman 1997), thyroid hormone (Matosin-Matekalo *et al.* 1998), Glucagon-37 (Stumpel *et al.* 1997), epidermal growth factor (Chung *et al.* 2002) or cholecystokinin (Hirsh *et al.* 1998) can regulate SGLT-1 activity in a short-term manner by regulating its expression in the BBM.

Interestingly, considering the important physiological action of SGLT-1 absorbing glucose from the intestinal lumen, several agents that inhibit this co-transporter (SGLT inhibitors) have been proposed as potential anti-diabetic drugs due to its ability to increase glucose excretion and to control hyperglycemia through an insulin-independent mechanism (Oku *et al.* 2000, Fujimori *et al.* 2008, Chao 2014).

5. CHRONOBIOLOGY OF OBESITY AND METABOLIC SYNDROME

Chronobiology is a relatively new science that was first observed in the 18th century due to experiments in which the Mimosa plant was placed in a dark box showing that its leaves continued to open and close every 24 h, demonstrating the existence of an internal clock. Nowadays, it is well known that many living organisms have developed intrinsic 24 h clocks that enable to sequester reactions, pathways, and behaviors at the appropriate time to optimize biological functions during specific phases of the day (Oike *et al.* 2014). Several physiological processes such as feeding behavior, lipid/carbohydrate metabolism, hormone release and sleep display day-night rhythms, being controlled by the circadian biological clock (Shi *et al.* 2013). The term circadian comes from the Latin *circa diem*, meaning around a day.

5.1 Molecular mechanisms of the biological clock

In mammals, the circadian system responsible for the synchronization between circadian rhythms and environment is hierarchically organized. At the top of the hierarchy the master clock located at the suprachiasmatic nucleus (SCN) of the hypothalamus receives photic input through the retinohypothalamic tract (RHT) that initiates gene expression in the SCN (Hastings *et al.* 2004) and serves as a central pacemaker adjusting the period and phase of circadian rhythms to a 24 h cycle (Khalsa *et al.* 2003). In addition to photic input, which represent the main signal, other external cues such as feeding time or scheduled exercise can also reset the phase and the period of the SCN, regulating the circadian rhythms (Lax *et al.* 1999, Garaulet *et al.* 2009). The central pacemaker sends this information through neuronal and humoral signals to other brain areas and peripheral oscillators present in most tissues and cells

including adipose tissue and synchronize them to the same phase (Mohawk *et al.* 2012) **(Figure 7)**.

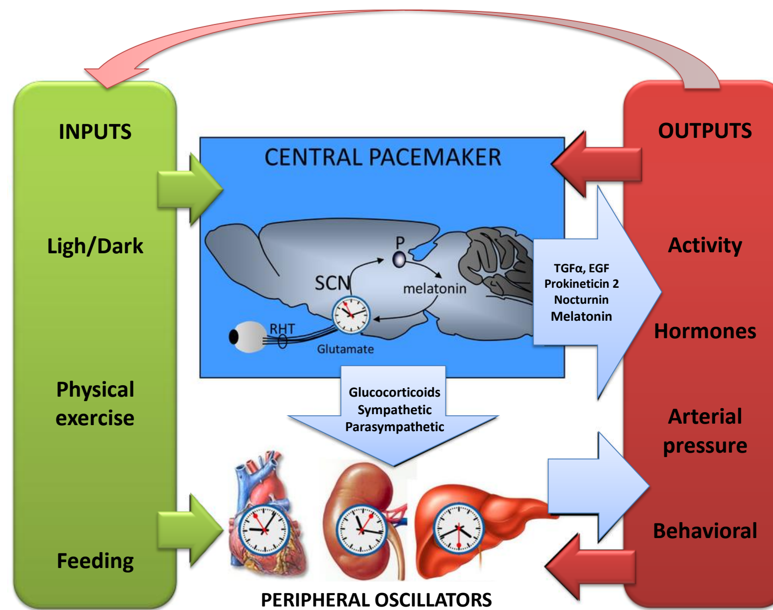


Figure 7: Central and peripheral clock coordinate the circadian system in mammals. Modified from Garaulet *et al.* (2009).

The molecular circadian clockwork consists on a set of proteins that regulate circadian oscillations through positive and negative transcriptional/translational feedback loops (Garaulet *et al.* 2009). The positive limb is composed of CLOCK and brain and muscle aryl hydrocarbon receptor nuclear translocator (ANRT)-like protein 1 (BMAL1), whereas periods (PERs) and cryptochromes (CRYs) are components of the negative feedback loop. CLOCK and BMAL1 heterodimerize to activate the rhythmic expression of the negative regulators *Per* and *Cry* genes through E-box elements (5'-CACGTG-3') (Kume *et al.* 1999) present in clock and clock-controlled genes. On translation, the PER and CRY proteins form heterodimers in the cytosol that eventually translocate into the nucleus following phosphorylation, to repress their own transcription by negatively regulating transcriptional activity of CLOCK:BMAL1 heterodimer (Garaulet *et al.* 2013) allowing the circuit to start again. This negative feedback leads to a cycle in gene expression that takes approximately 24 h to complete (Ukai *et al.* 2010). The positive and negative limbs are connected by additional feedback pathways established by the negative (REV-ERB) and positive (ROR) effects

on *Bmal1* transcription (Sato *et al.* 2004, Cho *et al.* 2012). Moreover, the action of PPAR-γ (Yang *et al.* 2012) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which is rhythmically expressed and stimulates *Bmal1* transcription (Liu *et al.* 2007), provide fine tuning to this process.

The clock genes also receive reciprocal input from the NAMPT gene. In this context, CLOCK:BMAL1 promote the synthesis of NAD^+, involving its regeneration from NAMPT (Ramsey *et al.* 2009, Bass *et al.* 2010). NAD^+ levels show circadian oscillations across the light/dark cycle, supporting its potential role as a metabolite linking metabolic cycles with the core clock (Bass *et al.* 2010). NAD^+ is capable of regulating the clock machinery through the modulation of its transcriptional activity via SIRT1 (Nakahata *et al.* 2008) evidencing that clock output elements of can affect the core clock machinery itself **(see Figure 8)**.

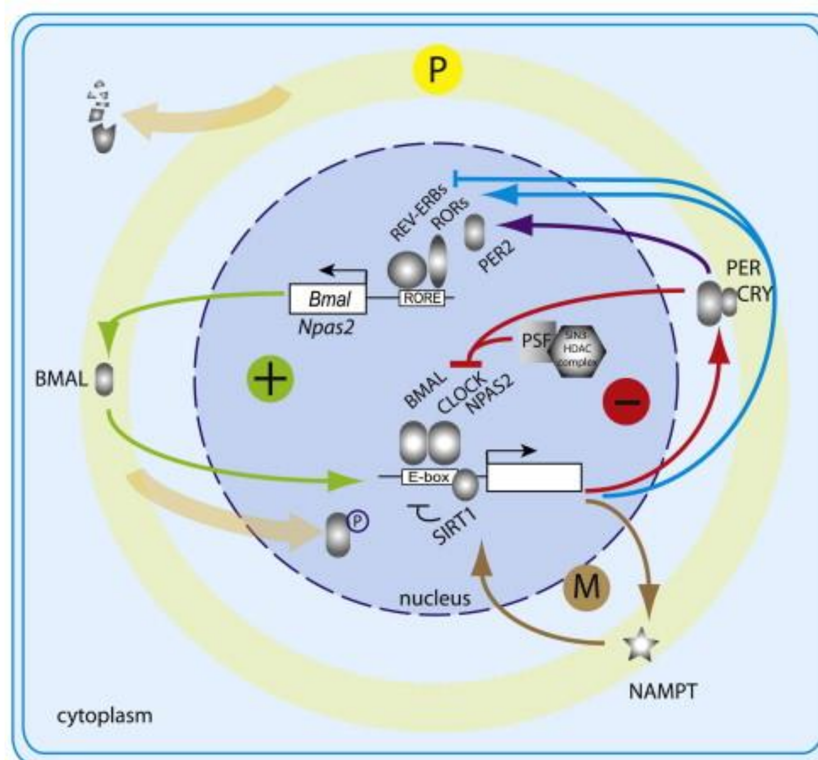


Figure 8: Mammalian molecular circadian clock mechanism (Albrecht 2012).

Clock genes are known to be involved in the regulation of adipose tissue metabolism. Thus, BMAL1 and PER2 play a role in the control of adipocyte differentiation, *de novo* lipogenesis, and fatty acid oxidation in mice (Shimba *et al.* 2005, Grimaldi *et al.* 2010). Moreover, a study performed by Shostak *et al.* (2013),

demonstrated that clock genes are important regulators of adipose tissue lipolysis and help to control the rhythmic release of glycerol and FFA from adipocytes which is of physiological relevance. Furthermore, a trial in humans showed that clock genes are expressed in both subcutaneous and visceral fat and their association with abdominal fat content and cardiovascular risk factors could be an indicator of the potential role of clock genes in the development of metabolic syndrome disturbances (Gomez-Abellan *et al.* 2008).

5.2 Circadian rhythm and metabolic syndrome

Recent studies have demonstrated the interaction between the circadian clock and energy regulation and metabolism. Indeed, disruption of circadian rhythms (chronodisruption) may cause desynchrony, which compromises metabolic homeostasis and contributes to the onset of obesity and MetS (Antunes *et al.* 2010, Bass *et al.* 2010, Okura *et al.* 2014). Several situations of modern lifestyle create misalignment between internal and external timing leading to the development of obesity and MetS (Garaulet *et al.* 2010). Light and food intake represent the main inputs that synchronize the central and peripheral pacemakers. In this context, social jet-lag which represents the discrepancy between circadian and social clocks, leads to the appearance of adverse profiles for the development of metabolic diseases (Rutters *et al.* 2014). It has been observed that the rate of obesity is higher among night-shift workers comparing with day workers, and chronic shift work is associated with increased BMI and risk of type 2 diabetes (Karlsson *et al.* 2001, Parkes 2002, Pan *et al.* 2011). Moreover, there is growing evidence that sleep deprivation, which is widespread in modern society, may represent a major risk factor for the development of metabolic diseases (Maury *et al.* 2010, Garaulet *et al.* 2011, Bass 2012, Owens 2013). Sleep duration and timing, as well as sleep efficiency have been associated with the development of MetS (Corbalan-Tutau *et al.* 2012). It this line, it has been also observed that eating during nocturnal time and high fat feeding and constant snacking contribute to the desynchronization of the rhythms controlled by the SCN leading to the appearance of metabolic disorders (Mendoza *et al.* 2008).

As previously mentioned, adipose tissue contains the machinery necessary to be recognized as a peripheral circadian oscillator. Interestingly, a wide range of molecules

produced and secreted by adipose tissue such as leptin, adipsin, resistin, adiponectin or visfatin are involved in the onset of the complications associated to MetS. All of these molecules exhibit circadian oscillation contributing to the protection or to the development of different disturbances associated to MetS (Garaulet *et al.* 2007, Barnea *et al.* 2009, Garaulet *et al.* 2009, Barnea *et al.* 2015).

6. CARDIOTROPHIN-1

Cardiotrophin-1 is a protein that was originally isolated from the supernatant of mouse embryonic corpuscles and is named for its ability to induce a hypertrophic response in neonatal cardiac myocytes as judged by myocyte enlargement, organization of myosin light chain into sarcomeric units, and atrial natriuretic peptide (ANP) secretion (Pennica *et al.* 1995a). The initial study of this cytokine showed that its structure was very similar to the IL-6 family of cytokines being considered a member of this family (Pennica *et al.* 1995b).

6.1 Gene and protein structure

The coding regions of human and mouse CT-1 are contained on three separate exons that span 6-7 kbp of genomic DNA. When nucleotide sequences of the coding regions of exons were compared between human and mouse it was observed that exon 1, 2 and 3 shared 96%, 84% and 81% homology, respectively (Funamoto *et al.* 2000). The 5'-flanking region of the human CT-1 gene has been cloned and sequenced. Data bank search revealed several *cis*-active DNA elements (SP1, CREB, C/EBP, AP1 and AP- 2 like and GATA) in the proximal 1.1 kb region (Erdmann *et al.* 1998). The human CT-1 gene is located on chromosome 16p11.1-16p11.2 (Pennica *et al.* 1996b). The 5'-flanking region of the mouse CT-1 gene contains a variety of transcription factor binding motif (e.g. CREB, MyoD, NF-IL6, Nkx2.5, GATA). Fluorescent *in situ* hybridization analysis demonstrated that the mouse CT-1 gene was located on chromosome 7F3 (Funamoto *et al.* 2000) **(Figure 9)**.

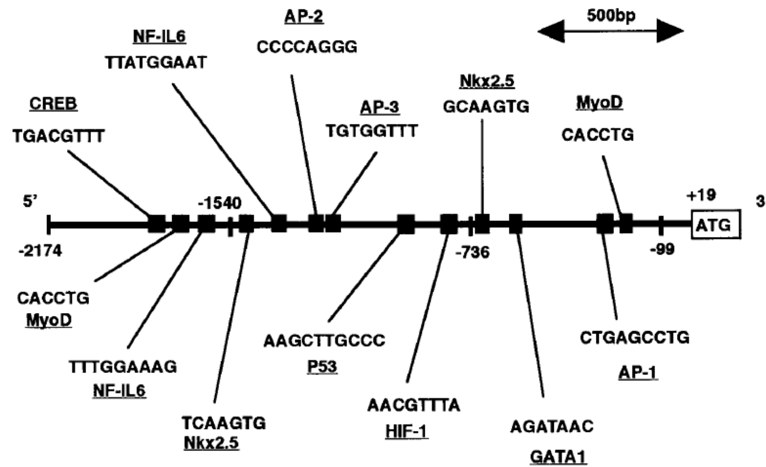


Figure 9: Characterization of the murine cardiotrophin-1 gene (Funamoto *et al.* 2000).

CT-1 protein consists of 203 amino acids and has a molecular weight of 21.5 kDa (Pennica *et al.* 1995a) also presenting a sequence well conserved between species. Thus the protein homology between human and mouse is 78%, and 80% between human and rat. Amino acid sequence of rat CT-1 was 94% identical to that of mouse CT-1 (Ishikawa *et al.* 1996). Both human and mouse protein lacks a conventional, hydrophobic, N-terminal amino acid sequence indicative of a secretion signal, though it has been found that is secreted outside the cell (Pennica *et al.* 1996b). It is noteworthy that the group of Pemberton *et al.* (2005) has described in samples of human and rat plasma the existence of isoforms of this protein with a molecular weight of 2 to 3 times higher than that for the monomeric protein. In this paper the authors suggest that these isoforms correspond to the formation of dimers and trimers with complex tertiary or quaternary structures, which may even include post-translational modifications such as glycosylation.

6.2 Binding and signaling

CT-1 belongs to the IL-6 family of cytokines, which includes other cytokines such as IL-11, IL-6, IL-30, ciliary nerutrophic factor (CNTF), cardiotrophin-like cytokine (CLC, also known as novel neurotopin-1 (NNT-1) or B cell stimulating factor 3 (BSF3)), neuropoietin (NP), leukemia inhibitory factor (LIF), oncostatin M (OSM) and IL-31. All members of this family belong to the four-helical bundle cytokine family sharing little sequence homology being only recognized as family member by prediction of their

protein fold (Pennica *et al.* 1995a). IL-6 type cytokines signals through the gp130 receptor, which causes that some of them can share certain features; however, both the different distribution of this receptor and the fact that each cytokine binds to a more specific receptor, makes that these cytokines are not considered in many of its functions as redundant (Gadient *et al.* 1999).

IL-6 binds the IL-6 receptor a (IL-6Ra) to form an IL-6/IL-6Ra complex, which can now interact with the signal transducing subunit gp130 to form a high-affinity receptor complex. Very similar to IL-6, IL-11 acts through an IL-11Ra/gp130 receptor complex. On the other hand, CNTF, CLC and NP utilize CNTFRα. The ligand/CNTFRα complexes signal through gp130/LIFR heterodimers. Additionally, CNTF operates through binding IL-6Rα and a gp130/LIFR heterodimer (Rousseau *et al.* 2008). CT-1 signals through gp130 and LIFR. Although CT-1 can mimic LIF in binding and activation of the gp130/LIFR complex, it has been reported to recruit a yet uncharacterized α receptor (CT-1Rα) (Robledo *et al.* 1997), which confers CT-1 high potency trophic signaling for motor neurons (Pennica *et al.* 1995b, Pennica *et al.* 1996b, Robledo *et al.* 1997). However, this additional membrane component is apparently not required for CT-1 effects on other cells (Cheng *et al.* 1997). LIF and OSM do not require specific α-receptors for receptor complex formation and signal through gp130/LIFR heterodimeric complexes (Gearing *et al.* 1992, Pennica *et al.* 1995a, Kuropatwinski *et al.* 1997). OSM additionally activates gp130/OSMR complexes (Mosley *et al.* 1996). However, OSM signaling through the gp130/LIFR complex has so far been exclusively found in human cells (Ichihara *et al.* 1997, Wang *et al.* 2000). The OSMR is also utilized by IL-31, which signals through gp130-like protein (GLP)/OSMR heterodimers **(Figure 10)**.

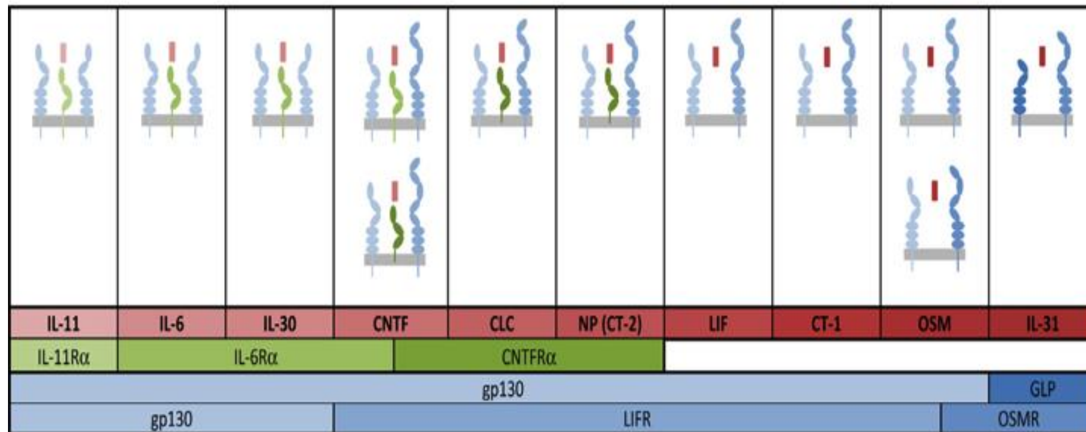


Figure 10: IL-6 type cytokines and receptor complexes. Ligands are colored in red; α-receptors or receptor-like accessory proteins of composite cytokines are colored in green, signaling receptor subunits are colored in blue. Immuno-globulin like domains are indicated as vertical ellipses, fibronectin type III domains are indicated as horizontal ellipses, cytokine receptor homology domains are depicted as a pair of two angled ellipses. Transmembrane and cytoplasmic regions are drafted as lines. Lines not spanning the plasma membrane indicate GPI-anchored receptors. Reading the figure in vertical direction gives information on the specific composition of the cytokine (in case of composite cytokines) and the subunits of the respective receptor complex. Alternative receptor compositions are considered (Rose-John *et al.* 2015).

Binding of gp130 ligand to the corresponding receptors leads to activation of three major downstream pathways: the JAK/STAT axis, the Ras-Raf mitogen-activated protein kinase (MAPK, MEK/ERK) signaling cascade, and the PI3K/AKT (protein kinase B:PKB) pathway **(Figure 11)**. CT-1 binds LIFR with low affinity and this binding stimulates its heterodimerization with the gp130 into a high affinity-binding complex (Pennica *et al.* 1995b). Gp130/LIF activates JAK1, JAK2 and the tyrosine kinase (TYK2) associated with gp130, which phosphorylates tyrosine residues in the cytoplasmic domain of gp130. These phosphotyrosines subsequently serve as the docking sites to recruit STAT proteins, which are phosphorylated (Kurdi *et al.* 2007) allowing STATs to translocate to the nucleus where they bind specific DNA sequences to modulate gene expression. Their activity can be further regulated through serine phosphorylation (Hendry *et al.* 2004) and inhibited by intracellular SOCS (suppressor of cytokine signaling proteins) and PIAS (protein inhibitor of activated STAT) (Liu *et al.* 1998, Chen *et al.* 2000). Phosphorylated receptor subunits also serve as docking points for other Src homology 2 (SH2) domain-containing proteins, such as PI3K, growth factor

receptor-bound protein 2, adapter protein (Shc), Ceacam1 (pp129) and the cytoplasmic SH2 domain containing protein tyrosine phosphatase (SHP2). Recruitment of Shc to phosphorylated subunits of gp130 leads to complex formation with Grb2 and SOS, which activates Ras-Raf mitogen-activated pritein kinase (MAPK, MEK/ERK) pathway (Nakafuku *et al.* 1992, Kumar *et al.* 1994). It has been reported that recruitment of SHP2 to the phosphotyrosine 759 of gp130 allows Gab1-SHP2 interaction, which modify the conformation or phosphorylation status of Gab1, allowing Gab1 to interact with PI-3 kinase and activate downstream signaling pathways (Takahashi-Tezuka *et al.* 1997).

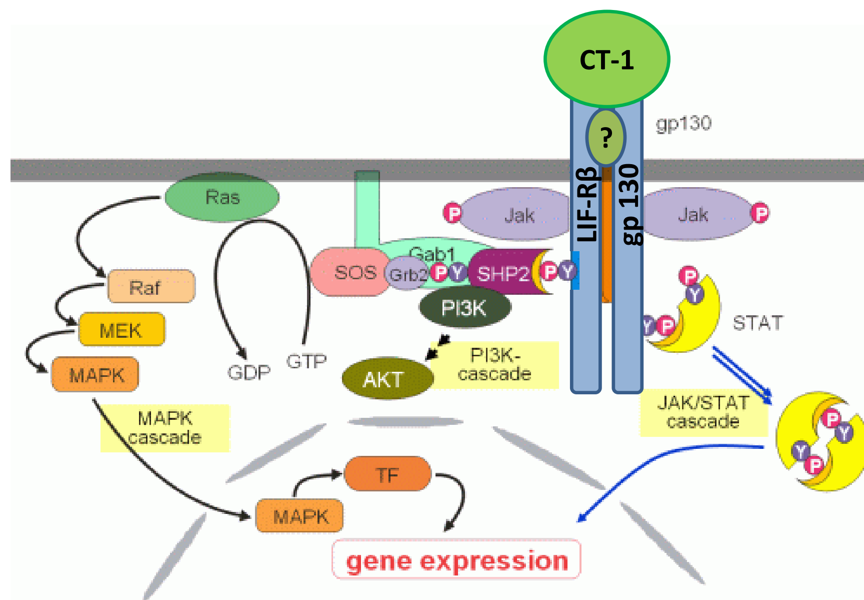


Figure 11: Summary of proposed signaling for cardiotrophin-1 as representative of the cytokines that use gp130 as a receptor. Modified from Heinrich *et al.* (2003).

6.3 Expression and biological effects of Cardiotrophin-1

CT-1 is expressed in many tissues although the role playing in most of them still remains to be elucidated. In adult humans, CT-1 is highly expressed in heart, skeletal muscle, liver, lung and kidney. Lower levels of CT-1 expression are also seen in testis and brain (Pennica *et al.* 1996b). A previous study has identified adipose tissue as a source of CT-1 (Natal *et al.* 2008). Several studies have shown that CT-1 has a variety of effects in these tissues and that could act not only in a paracrine manner but also as an endocrine factor in these organs.

Given the ubiquity of gp130, it is the presence of CT-1Rα and LIFRβ which determines the responsiveness to CT-1. Finally, CT-1 acts on multiple cell types and target organs.

6.3.1 Cardiotrophin-1 in energy homeostasis and intermediate metabolism

In the last years several studies from our group and others have pointed out that CT-1 might also play a key role in the regulation of body weight and intermediate metabolism. The role of CT-1 in the regulation of body weight became clear by two observations: 1) the lack of CT-1 in mice leads to the development of adult-onset obesity (Moreno-Aliaga *et al.* 2011); 2) chronic rCT-1 administration is able to reduce body weight and fat accumulation in diet-induced and genetically obese rodents (Moreno-Aliaga *et al.* 2011, Wang *et al.* 2015). The body weight lowering actions of CT-1 can be explained by its inhibitory action on food intake and its ability to stimulate energy expenditure acting both at central level and on peripheral metabolic organs, including adipose tissue, liver and muscle. Regarding food intake regulation, studies from our group have revealed that CT-1 activates hypothalamic anorexigenic pathways, including STAT3 and S6 ribosomal proteins (Moreno-Aliaga *et al.* 2011). A similar effect has been described for CNTF, which also activates leptin-like anorexigenic pathways (Lambert *et al.* 2001). However, CT-1 deficient mice constitute a model of hypophagic obesity, although the cause of anorexia in these mice could be a compensatory mechanism to slow down exercise-weight gain as a result of low energy expenditure (Moreno-Aliaga *et al.* 2011). Therefore, it is unclear whether CT-1 can act as a physiological regulator of calorie intake, which would be an interesting scientific question to further pursue in the context of obesity.

CT-1 seems to play an important role in the regulation of energy expenditure, the administration of CT-1 increases whole-body oxygen consumption as compared with pair-fed animals (Moreno-Aliaga *et al.* 2011). A striking feature of mature CT-1$^{-/-}$ mice is the presence of a high respiratory quotient (RQ), indicative of impaired fat utilization. Interestingly, CT-1 treatment decreases both fasting and postprandial RQ, causes a rapid clearance of circulating fatty acids after a lipid load, reducing postprandial hypertriglyceridemia (Moreno-Aliaga *et al.* 2011). Taken together these facts support the pivotal role of CT-1 in the regulation of lipid homeostasis. In fact, several studies

from our group have suggested that CT-1 promotes metabolic pathways involved in fat breakdown and oxidation while reduces lipid storage pathways. In line with this, a recent study of our group found that in liver of obese mice, chronic CT-1 administration inhibits *de novo* lipogenesis, stimulates FA oxidation and promotes lipoautophagy, which could also contribute to the delipidating and anti-steatotic effects of CT-1 in liver (Castano *et al.* 2014). In muscle, CT-1 also increases FA oxidation (Moreno-Aliaga *et al.* 2011) and AMPK is responsible in mediating both the inhibitory effect of CT-1 on hepatic lipogenesis and the stimulatory action of the cytokine on FA oxidation in liver and muscle (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). Similar actions have been described for other-related cytokines such as leptin (Minokoshi *et al.* 2002) and CNTF (Watt *et al.* 2006a).

WAT is also a target organ for CT-1 actions; indeed, CT-1 is a potent regulator of signaling in adipocytes *in vitro* and *in vivo* (Zvonic *et al.* 2004). CT-1 administration not only causes a decrease in fat mass, but also induces a switch of WAT lipid metabolism, downregulating lipogenic genes and stimulating lipolysis and fatty acid oxidation. In fact, adipocytes from CT-1-treated mice exhibited increased lipolytic response to isoproterenol (Moreno-Aliaga *et al.* 2011).

Another interesting property of CT-1 is its ability to promote browning/britening of white adipose tissue (Moreno-Aliaga *et al.* 2011). Taking together, all of these observations support that CT-1 induces a dramatic remodeling of adipose tissue. These facts could be also contributing to the anti-obesity actions of CT-1, since stimulating conversion of white fat to metabolically active brite (brown-in-white) adipocytes has been proposed as a promising strategy against obesity and its deleterious associated disorders (Bartelt *et al.* 2014) **(Figure 12)**.

CT-1 has also profound actions on glucose metabolism. Indeed, chronic CT-1 administration to obese mice reduced the associated hyperglycemia and hyperinsulinemia and improves the insulin tolerance test and insulin signaling in muscle (Moreno-Aliaga *et al.* 2011). An interesting finding of our group is that the glucose-lowering properties of CT-1 are also observed in mice with streptozotocin (STZ)-induced insulin deficiency, demonstrating an insulin-independent effect of CT-1 on glucose homeostasis (Moreno-Aliaga *et al.* 2011). In line with this, the study of Jimenez-Gonzalez *et al.* (2013) found that CT-1 has a protective effect from apoptosis

in MIN6B1 cells and murine pancreatic islets, and that CT-1 enhances glucose-stimulated insulin secretion in MIN6B1 cells. Studies *in vitro* and *in vivo* also support the insulin-sensitizing effect of CT-1 in muscle (Moreno-Aliaga *et al.* 2011). However, some controversial data regarding CT-1-induced insulin resistance have been described after chronic treatment with the cytokine in cultured adipocytes (Moreno-Aliaga *et al.* 2011, Zvonic *et al.* 2004). Taking into account that muscle accounts for 80–90% of whole-body glucose uptake, it has been suggested that insulin resistance in WAT may not necessarily cause systemic insulin resistance.

It is important to note that CT-1 is a nutritionally regulated gene, which levels are induced during fasting and decreased by refeeding, suggesting that CT-1 may have a role in the biological adaptation to starvation (Moreno-Aliaga *et al.* 2011). However, little is known about the main regulators of CT-1 production during obesity and metabolic syndrome. In this context, several studies have suggested that circulating levels of CT-1 are higher in obese subjects (Malavazos *et al.* 2008) with metabolic syndrome (Natal *et al.* 2008). Interestingly obese children subjected to a weight loss program exhibited a reduction of CT-1 plasma levels in parallel to the decrease in body weight and body fat mass (Rendo-Urteaga *et al.* 2013). Although WAT was proposed to be a main source of this cytokine (Natal *et al.* 2008), this contention is discordant with data showing reduced CT-1 mRNA abundance in WAT from obese mice (Sanchez-Infantes *et al.* 2014). Other tissues like liver, skeletal muscle and heart may be alternative origins of the increased plasma CT-1 concentration in obesity. Indeed, it has been recently reported that CT-1 is upregulated in steatotic livers from both obese mice and humans (Castano *et al.* 2014). Skeletal muscle is an important source of circulating CT-1. Muscle expression levels of CT-1 exceed those in WAT and are more elevated in obese mice than in wild type animals (our unpublished observations). CT-1 seemingly behaves as a myokine endowed with autocrine, paracrine and/or endocrine effects. In fact, it has been reported that physical exercise increases the levels of CT-1 in serum both in healthy human subjects and elite athletes (Limongelli *et al.* 2010).

Altogether, the beneficial effects of CT-1 on energy homeostasis as well as on glucose and lipid metabolism suggest that the overproduction of CT-1 in obesity, metabolic syndrome and non-alcoholic fatty liver disease (NALFD) could be considered as a compensatory phenomenon to promote fat oxidation and to counteract the

development of the metabolic disturbances associated to obesity. Interestingly, a recent study analyzing the impact of genetic variants of the CT-1 gene locus CTF1 on insulin sensitivity in humans showed a robust association of the CTF1 SNP rs8046707 with the response to insulin (Lutz *et al.* 2014).

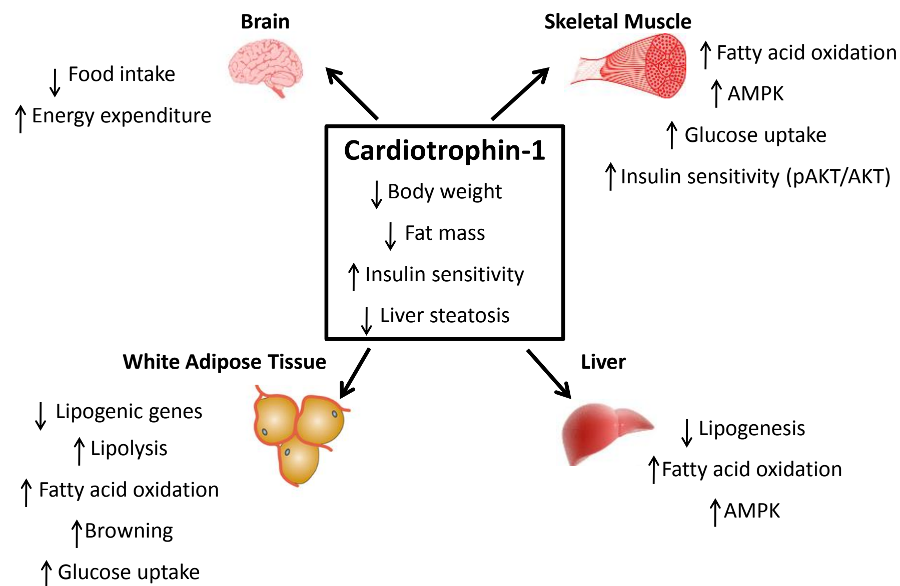


Figure 12: Summary of metabolic effects of cardiotrophin-1 in different tissues (Lopez-Yoldi *et al.* 2015).

6.3.2 Cardiotrophin-1 and cardiovascular system

Gp130 receptor is essential in mediating cardioprotective effects against physiological and pathophysiological stress by promoting cardiomyocyte survival, inducing compensatory hypertrophy and preserving cardiac function. Thus, in mice lacking CT-1 receptor, gp130, only in the heart, aortic banding-induced pressure overload results in massive cardiac cell apoptosis and death of the animals form heart failure (Hirota *et al.* 1999).

The protective effects of CT-1 on cardiac myocytes were described near two decades ago. It was shown that CT-1 is required for cardiac myocyte maturation and was capable of promoting cell survival in neonatal rat cardiomyocytes subjected to serum deprivation through an antiapoptotic pathway mediated by MAPK/ERK1/2 (Sheng *et al.* 1996). The first study on the cardioprotective activity of recombinant protein CT-1 (rCT-1) was reported by Stephanou *et al.* (1998). They demonstrated resistance to ischemia-reperfusion injury in neonatal rat cardiomyocytes with improved cell survival and less apoptotic cell death (Stephanou *et al.* 1998). Since then

several experiments studies have confirmed the defensive role of rCT-1 on cultured neonatal rat cardiomyocytes (Brar *et al.* 2001) and isolated perfused animal hearts (Liao *et al.* 2002).

CT-1 mRNA expression has been observed in normal and disease states (Freed *et al.* 2005). Increased expression of CT-1 has been reported in the ventricles of genetically hypertensive rats (Ishikawa *et al.* 1999) and in rats subjected to pressure overload (Pan *et al.* 1998). In humans, elevated levels of CT-1 have been observed in patients with heart failure (Talwar *et al.* 2003) and hypertensive rats (Lopez *et al.* 2005). While some authors have reported an association between circulating CT-1 and left ventricular structure and function (Lopez *et al.* 2005, Lopez *et al.* 2007, Lopez *et al.* 2009, Monserrat *et al.* 2011, Lopez-Andres *et al.* 2012, Ravassa *et al.* 2013), other studies could not confirm these findings (Zile *et al.* 2011).

CT-1 and LIF are the gp130 ligands reported to be hypertrophic agents for cardiac muscle (Jacoby *et al.* 2003). Both of them are able to activate the three major signaling pathways linked to gp130/LIFR activation that stimulate cardiac hypertrophy both in cultured cells and *in vivo* (Pennica *et al.* 1995b, Chien *et al.* 2002). Similar to LIF, CT-1 induces myocardial hypertrophy with a predominant increase in myocardial cell length by addition of new sarcomeric units in series without increase in cell width (Wollert *et al.* 1996). The contribution of CT-1 to either physiological or maladaptive cardiac hypertrophy is unclear. In numerous experimental studies CT-1 acts as a potent cardiac survival factor, and promotes cardiomyocyte hypertrophy *in vitro* (Sheng *et al.* 1997, Railson *et al.* 2000), but its *in vivo* effects are more difficult to define. It has been reported that chronically augmented CT-1 has detrimental effects on cardiac centrality. However, the experiments have been done in engineered heart tissue (EHT) (Zolk *et al.* 2005) and although EHTs are well suitable as a test system its physiological relevance is not well established.

Recently, Kanazawa *et al.* (2010) using a Dahl salt sensitive rat to provoke HF under chronic salt-induced hypertension, showed that gp130 cytokines, LIF and CT-1 play pivotal role in cholinergic transdifferentiation of cardiac sympathetic nerves. This cholinergic transdifferentiation appears to be beneficial in the failing hearts where it represents a critical endogenous protective mechanism of cardiac myocytes.

6.3.3 Cardiotrophin-1 and nervous system

CT-1 has been shown to play an essential role in neural tissue development and also in the protection of the mature nervous system against a variety of injuries and dysfuntions (Lopez-Yoldi *et al.* 2015).

CT-1 is also expressed in the nervous system and shares some features with other IL-6 family of cytokines such as the regulation of neurotransmitter phenotype, induction of differentiation of neuronal precursors and regulating the development of astrocytes (Nakashima *et al.* 1999, Ochiai *et al.* 2001).

CT-1 promotes the survival of differentiated neurons and can rescue sensory and motoneurons from induced death (Pennica *et al.* 1996a, Bordet *et al.* 2001) having therapeutic applications in neurodegenerative diseases and improved neuromuscular functions (Bordet *et al.* 1999). Notably, it has been reported that administration of rCT-1 by means of an adenovirus encoding CT-1 prevented motoneuron cell death and long-term motor axonal degeneration in a mouse model of progressive motor neuropathy. CT-1 also provided therapeutic benefit in both functional and morphological parameters in a mouse model of spinal muscular atrophy (Bordet *et al.* 2001, Lesbordes *et al.* 2003). Furthermore, adenovirus-mediated gene transfer of CT-1 or rCT-1 delayed neurogenic muscular atrophy and progressive neuromuscular deficiency in an experimental model of amyotrophic lateral sclerosis (Bordet *et al.* 2001, Mitsumoto *et al.* 2001). Finally, adenoviral CT-1 gene transfer into the injured cord promoted survival and regeneration of rubrospinal neurons in adult rats (Zhang *et al.* 2003).

Recently, it has been shown that Alzheimer's disease transgenic mouse models exhibit a marked reduction of CT-1 expression in hippocampus and that restoration of CT-1 tissue levels in these animals resulted in a marked improvement on cognitive function (Wang *et al.* 2013). Moreover, intracerebroventricular administration of CT-1 for 14 days improved learning memory deficits and alleviated neuroinflammation in high-fat diet-induced cognitive deficits in mice (Wang *et al.* 2015).

6.3.4 Cardiotrophin-1 and liver

Soon after its discovery, CT-1 was shown to be a potent inducer of the acute phase response in rat primary hepatocytes (Pennica *et al.* 1996a, Peters *et al.* 1995) and subsequent studies showed that CT-1 is an essential factor in the defense of the liver against a variety of insults (Bustos *et al.* 2000, Beraza *et al.* 2005, Iniguez *et al.* 2006, Marques *et al.* 2007, Tunon *et al.* 2011). In the liver, CT-1 mRNA is expressed by both hepatocytes and non-parenchymal cells (Marques *et al.* 2007). Interestingly it has been shown that exposure to oxidants such as the H_2O_2 analogue tert-butyl-hydroperoxide causes hepatocytes to release CT-1 (Iniguez *et al.* 2006). The fact that CT-1 deficient mice are susceptible to ischemia/reperfusion liver injury and other pro-apoptotic insults indicates that CT-1 acts as natural defense of the liver against damage. Consistently, it has been shown that administration of rCT-1 affords protection against ischemia/reperfusion liver damage (Iniguez *et al.* 2006), galactosamine (Ho *et al.* 2006) and hepatitis of viral origin (Tunon *et al.* 2011). An adenovirus encoding CT-1 protected rats against fulminant hepatitis induced by massive hepatic resection (Bustos *et al.* 2000). CT-1 enhanced regeneration of cirrhotic livers through promotion of angiogenesis and cell proliferation (Yang *et al.* 2008). Furthermore administration of CT-1 to donors decreased ischemia/reperfusion damage in liver transplantation in an experimental pig model (Aguilar-Melero *et al.* 2013). Finally the administration of adenovirus encoding CT-1 improved liver function and the survival of small-for-size grafts (Song *et al.* 2008b). These data indicates that CT-1 deserves testing as a hepatoprotective molecule in situations of acute severe liver damage. As previously mentioned, chronic treatment with rCT-1 was also able to eliminate steatosis from the liver of mice with NAFLD (Castano *et al.* 2014), pointing to CT-1 as a potential therapy for non-alcoholic steatohepatitis (NASH).

REFERENCES

-A-

Aguilar-Melero, P., Luque, A., Machuca, M. M., Perez de Obanos, M. P., Navarrete, R., Rodriguez-Garcia, I. C., Briceno, J., Iniguez, M., Ruiz, J., Prieto, J., de la Mata, M., Gomez-Villamandos, R. J., Muntane, J. & Lopez-Cillero, P. 2013. Cardiotrophin-1 reduces ischemia/reperfusion injury during liver transplant. *J Surg Res,* **181,** e83-91.

Ahima, R. S. & Osei, S. Y. 2004. Leptin signaling. *Physiol Behav,* **81,** 223-41.

Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E. & Flier, J. S. 1996. Role of leptin in the neuroendocrine response to fasting. *Nature,* **382,** 250-2.

Ahmadian, M., Abbott, M. J., Tang, T., Hudak, C. S., Kim, Y., Bruss, M., Hellerstein, M. K., Lee, H. Y., Samuel, V. T., Shulman, G. I., Wang, Y., Duncan, R. E., Kang, C. & Sul, H. S. 2011. Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. *Cell Metab,* **13,** 739-48.

Ahmadian, M., Duncan, R. E. & Sul, H. S. 2009. The skinny on fat: lipolysis and fatty acid utilization in adipocytes. *Trends Endocrinol Metab,* **20,** 424-8.

Ahmadian, M., Wang, Y. & Sul, H. S. 2010. Lipolysis in adipocytes. *Int J Biochem Cell Biol,* **42,** 555-9.

Albrecht, U. 2012. Timing to perfection: the biology of central and peripheral circadian clocks. *Neuron,* **74,** 246-60.

Alessandri, J. M., Arfi, T. S., Thevenoux, J. & Leger, C. L. 1990. Diet-induced alterations of lipids during cell differentiation in the small intestine of growing rats: effect of an essential fatty acid deficiency. *J Pediatr Gastroenterol Nutr,* **10,** 504-15.

Allison, M. A., Bluemke, D. A., McClelland, R., Cushman, M., Criqui, M. H., Polak, J. F. & Lima, J. A. 2013. Relation of leptin to left ventricular hypertrophy (from the Multi-Ethnic Study of Atherosclerosis). *Am J Cardiol,* **112,** 726-30.

Amirhakimi, A., Karamifar, H., Moravej, H. & Amirhakimi, G. 2011. Serum resistin level in obese male children. *J Obes,* **2011,** 953410.

Anderson, P. D., Mehta, N. N., Wolfe, M. L., Hinkle, C. C., Pruscino, L., Comiskey, L. L., Tabita-Martinez, J., Sellers, K. F., Rickels, M. R., Ahima, R. S. & Reilly, M. P. 2007. Innate immunity modulates adipokines in humans. *J Clin Endocrinol Metab,* **92,** 2272-9.

Anthonsen, M. W., Ronnstrand, L., Wernstedt, C., Degerman, E. & Holm, C. 1998. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J Biol Chem,* **273,** 215-21.

Anthony, N. M., Gaidhu, M. P. & Ceddia, R. B. 2009. Regulation of visceral and subcutaneous adipocyte lipolysis by acute AICAR-induced AMPK activation. *Obesity (Silver Spring),* **17,** 1312-7.

Antunes, L. C., Levandovski, R., Dantas, G., Caumo, W. & Hidalgo, M. P. 2010. Obesity and shift work: chronobiological aspects. *Nutr Res Rev,* **23,** 155-68.

Apalasamy, Y. D. & Mohamed, Z. 2015. Obesity and genomics: role of technology in unraveling the complex genetic architecture of obesity. *Hum Genet,* **134,** 361-74.

Arthur, S., Coon, S., Kekuda, R. & Sundaram, U. 2014. Regulation of sodium glucose co-transporter SGLT1 through altered glycosylation in the intestinal epithelial cells. *Biochim Biophys Acta,* **1838,** 1208-14.

Attane, C., Daviaud, D., Dray, C., Dusaulcy, R., Masseboeuf, M., Prevot, D., Carpene, C., Castan-Laurell, I. & Valet, P. 2011. Apelin stimulates glucose uptake but not lipolysis in human adipose tissue ex vivo. *J Mol Endocrinol,* **46,** 21-8.

-B-

Bado, A., Levasseur, S., Attoub, S., Kermorgant, S., Laigneau, J. P., Bortoluzzi, M. N., Moizo, L., Lehy, T., Guerre-Millo, M., Le Marchand-Brustel, Y. & Lewin, M. J. 1998. The stomach is a source of leptin. *Nature,* **394,** 790-3.

Balkau, B. & Charles, M. A. 1999. Comment on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance (EGIR). *Diabet Med,* **16,** 442-3.

Ballesta, M., Carral, F., Olveira, G., Giron, J. A. & Aguilar, M. 2006. Economic cost associated with type II diabetes in Spanish patients. *Eur J Health Econ,* **7,** 270-5.

Banerjee, R. R., Rangwala, S. M., Shapiro, J. S., Rich, A. S., Rhoades, B., Qi, Y., Wang, J., Rajala, M. W., Pocai, A., Scherer, P. E., Steppan, C. M., Ahima, R. S., Obici, S., Rossetti, L. & Lazar, M. A. 2004. Regulation of fasted blood glucose by resistin. *Science,* **303,** 1195-8.

Barcelo-Batllori, S. & Gomis, R. 2009. Proteomics in obesity research. *Proteomics Clin Appl,* **3,** 263-78.

Barnea, M., Chapnik, N., Genzer, Y. & Froy, O. 2015. The circadian clock machinery controls adiponectin expression. *Mol Cell Endocrinol,* **399,** 284-7.

Barnea, M., Madar, Z. & Froy, O. 2009. High-fat diet delays and fasting advances the circadian expression of adiponectin signaling components in mouse liver. *Endocrinology,* **150,** 161-8.

Barouch, L. A., Berkowitz, D. E., Harrison, R. W., O'Donnell, C. P. & Hare, J. M. 2003. Disruption of leptin signaling contributes to cardiac hypertrophy independently of body weight in mice. *Circulation,* **108,** 754-9.

Barrenetxe, J., Fanjul, C., Ducroc, R. & Lostao, M. P. 2013a. *Regulation of intestinal glucose uptake by leptin*, Nova Science Publishers, Inc.

Barrenetxe, J., Sanchez, O., Barber, A., Gascon, S., Rodriguez-Yoldi, M. J. & Lostao, M. P. 2013b. TNFalpha regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokine,* **64,** 181-7.

Bartelt, A. & Heeren, J. 2014. Adipose tissue browning and metabolic health. *Nat Rev Endocrinol,* **10,** 24-36.

Bass, J. 2012. Circadian topology of metabolism. *Nature,* **491,** 348-56.

Bass, J. & Takahashi, J. S. 2010. Circadian integration of metabolism and energetics. *Science,* **330,** 1349-54.

Benomar, Y., Gertler, A., De Lacy, P., Crepin, D., Ould Hamouda, H., Riffault, L. & Taouis, M. 2013. Central resistin overexposure induces insulin resistance through Toll-like receptor 4. *Diabetes,* **62,** 102-14.

Beraza, N., Marques, J. M., Martinez-Anso, E., Iniguez, M., Prieto, J. & Bustos, M. 2005. Interplay among cardiotrophin-1, prostaglandins, and vascular endothelial growth factor in rat liver regeneration. *Hepatology,* **41,** 460-9.

Bergeron, M. J., Simonin, A., Burzle, M. & Hediger, M. A. 2008. Inherited epithelial transporter disorders--an overview. *J Inherit Metab Dis,* **31,** 178-87.

Berggreen, C., Gormand, A., Omar, B., Degerman, E. & Goransson, O. 2009. Protein kinase B activity is required for the effects of insulin on lipid metabolism in adipocytes. *Am J Physiol Endocrinol Metab,* **296,** E635-46.

Bernlohr, D. A., Coe, N. R. & LiCata, V. J. 1999. Fatty acid trafficking in the adipocyte. *Semin Cell Dev Biol,* **10,** 43-9.

Bickel, P. E., Tansey, J. T. & Welte, M. A. 2009. PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. *Biochim Biophys Acta,* **1791,** 419-40.

Blakemore, A. I., Meyre, D., Delplanque, J., Vatin, V., Lecoeur, C., Marre, M., Tichet, J., Balkau, B., Froguel, P. & Walley, A. J. 2009. A rare variant in the visfatin gene (NAMPT/PBEF1) is associated with protection from obesity. *Obesity (Silver Spring),* **17,** 1549-53.

Bluher, M. 2014. Adipokines - removing road blocks to obesity and diabetes therapy. *Mol Metab,* **3,** 230-40.

Bluher, M. & Mantzoros, C. S. 2015. From leptin to other adipokines in health and disease: facts and expectations at the beginning of the 21st century. *Metabolism,* **64,** 131-45.

Bokarewa, M., Nagaev, I., Dahlberg, L., Smith, U. & Tarkowski, A. 2005. Resistin, an adipokine with potent proinflammatory properties. *J Immunol,* **174,** 5789-95.

Bordet, T., Lesbordes, J. C., Rouhani, S., Castelnau-Ptakhine, L., Schmalbruch, H., Haase, G. & Kahn, A. 2001. Protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic ALS mice. *Hum Mol Genet,* **10,** 1925-33.

Bordet, T., Schmalbruch, H., Pettmann, B., Hagege, A., Castelnau-Ptakhine, L., Kahn, A. & Haase, G. 1999. Adenoviral cardiotrophin-1 gene transfer protects pmn mice from progressive motor neuronopathy. *J Clin Invest,* **104,** 1077-85.

Boucher, J., Masri, B., Daviaud, D., Gesta, S., Guigne, C., Mazzucotelli, A., Castan-Laurell, I., Tack, I., Knibiehler, B., Carpene, C., Audigier, Y., Saulnier-Blache, J. S. & Valet, P. 2005. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology,* **146,** 1764-71.

Bouloumie, A., Marumo, T., Lafontan, M. & Busse, R. 1999. Leptin induces oxidative stress in human endothelial cells. *FASEB J,* **13,** 1231-8.

Boyraz, M., Cekmez, F., Karaoglu, A., Cinaz, P., Durak, M. & Bideci, A. 2013. Serum adiponectin, leptin, resistin and RBP4 levels in obese and metabolic syndrome children with nonalcoholic fatty liver disease. *Biomark Med,* **7,** 737-45.

Brar, B. K., Stephanou, A., Liao, Z., O'Leary, R. M., Pennica, D., Yellon, D. M. & Latchman, D. S. 2001. Cardiotrophin-1 can protect cardiac myocytes from injury when added both prior to simulated ischaemia and at reoxygenation. *Cardiovasc Res,* **51,** 265-74.

Brasaemle, D. L., Levin, D. M., Adler-Wailes, D. C. & Londos, C. 2000. The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim Biophys Acta,* **1483,** 251-62.

Britton, K. A., Massaro, J. M., Murabito, J. M., Kreger, B. E., Hoffmann, U. & Fox, C. S. 2013. Body fat distribution, incident cardiovascular disease, cancer, and all-cause mortality. *J Am Coll Cardiol,* **62,** 921-5.

Brunetti, L., Recinella, L., Di Nisio, C., Chiavaroli, A., Leone, S., Ferrante, C., Orlando, G. & Vacca, M. 2012. Effects of visfatin/PBEF/NAMPT on feeding behaviour and hypothalamic neuromodulators in the rat. *J Biol Regul Homeost Agents,* **26,** 295-302.

Bustos, M., Sangro, B., Alzuguren, P., Gil, A. G., Ruiz, J., Beraza, N., Qian, C., Garcia-Pardo, A. & Prieto, J. 2000. Liver damage using suicide genes. A model for oval cell activation. *Am J Pathol,* **157,** 549-59.

-C-

Cabrera de Leon, A., Almeida Gonzalez, D., Gonzalez Hernandez, A., Juan Aleman Sanchez, J., Brito Diaz, B., Dominguez Coello, S., Marcelino Rodriguez, I., Gregorio Oliva Garcia, J., Aguirre Jaime, A. & Rodriguez Perez Mdel, C. 2014. The association of resistin with coronary disease in the general population. *J Atheroscler Thromb,* **21,** 273-81.

Cannon, B. & Nedergaard, J. 2004. Brown adipose tissue: function and physiological significance. *Physiol Rev,* **84,** 277-359.

Carmen, G. Y. & Victor, S. M. 2006. Signalling mechanisms regulating lipolysis. *Cell Signal,* **18,** 401-8.

Castaneda-Sceppa, C., Subramanian, S. & Castaneda, F. 2010. Protein kinase C mediated intracellular signaling pathways are involved in the regulation of sodium-dependent glucose co-transporter SGLT1 activity. *J Cell Biochem,* **109,** 1109-17.

Castano, D., Larequi, E., Belza, I., Astudillo, A. M., Martinez-Anso, E., Balsinde, J., Argemi, J., Aragon, T., Moreno-Aliaga, M. J., Muntane, J., Prieto, J. & Bustos, M. 2014. Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation. *J Hepatol,* **60,** 1017-25.

Catalan, V., Gomez-Ambrosi, J., Rodriguez, A., Salvador, J. & Fruhbeck, G. 2009. Adipokines in the treatment of diabetes mellitus and obesity. *Expert Opin Pharmacother,* **10,** 239-54.

Caton, P. W., Kieswich, J., Yaqoob, M. M., Holness, M. J. & Sugden, M. C. 2011. Nicotinamide mononucleotide protects against pro-inflammatory cytokine-mediated impairment of mouse islet function. *Diabetologia,* **54,** 3083-92.

Cervero, A., Dominguez, F., Horcajadas, J. A., Quinonero, A., Pellicer, A. & Simon, C. 2006. The role of the leptin in reproduction. *Curr Opin Obstet Gynecol,* **18,** 297-303.

Cline, M. A., Nandar, W., Prall, B. C., Bowden, C. N. & Denbow, D. M. 2008. Central visfatin causes orexigenic effects in chicks. *Behav Brain Res,* **186,** 293-7.

Codoner-Franch, P. & Alonso-Iglesias, E. 2015. Resistin: insulin resistance to malignancy. *Clin Chim Acta,* **438,** 46-54.

Codoner-Franch, P., Tavarez-Alonso, S., Porcar-Almela, M., Navarro-Solera, M., Arilla-Codoner, A. & Alonso-Iglesias, E. 2014. Plasma resistin levels are associated with homocysteine, endothelial activation, and nitrosative stress in obese youths. *Clin Biochem,* **47,** 44-8.

Coelho, M., Oliveira, T. & Fernandes, R. 2013. Biochemistry of adipose tissue: an endocrine organ. *Arch Med Sci,* **9,** 191-200.

Corbalan-Tutau, M. D., Madrid, J. A. & Garaulet, M. 2012. Timing and duration of sleep and meals in obese and normal weight women. Association with increase blood pressure. *Appetite,* **59,** 9-16.

Cornaciu, I., Boeszoermenyi, A., Lindermuth, H., Nagy, H. M., Cerk, I. K., Ebner, C., Salzburger, B., Gruber, A., Schweiger, M., Zechner, R., Lass, A., Zimmermann, R. & Oberer, M. 2011. The minimal domain of adipose triglyceride lipase (ATGL) ranges until leucine 254 and can be activated and inhibited by CGI-58 and G0S2, respectively. *PLoS One,* **6,** e26349.

Cypess, A. M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A. B., Kuo, F. C., Palmer, E. L., Tseng, Y. H., Doria, A., Kolodny, G. M. & Kahn, C. R. 2009. Identification and importance of brown adipose tissue in adult humans. *N Engl J Med,* **360,** 1509-17.

Chang, L., Yang, R., Wang, M., Liu, J., Wang, Y., Zhang, H. & Li, Y. 2012. Angiotensin II type-1 receptor-JAK/STAT pathway mediates the induction of visfatin in angiotensin II-induced cardiomyocyte hypertrophy. *Am J Med Sci,* **343,** 220-6.

Chang, Y. H., Chang, D. M., Lin, K. C., Shin, S. J. & Lee, Y. J. 2011. Visfatin in overweight/obesity, type 2 diabetes mellitus, insulin resistance, metabolic syndrome and cardiovascular diseases: a meta-analysis and systemic review. *Diabetes Metab Res Rev,* **27,** 515-27.

Chao, E. C. 2014. SGLT-2 Inhibitors: A New Mechanism for Glycemic Control. *Clin Diabetes,* **32,** 4-11.

Chatzigeorgiou, A., Kandaraki, E., Papavassiliou, A. G. & Koutsilieris, M. 2014. Peripheral targets in obesity treatment: a comprehensive update. *Obes Rev,* **15,** 487-503.

Cheeseman, C. 2008. GLUT7: a new intestinal facilitated hexose transporter. *Am J Physiol Endocrinol Metab,* **295,** E238-41.

Cheeseman, C. I. 1997. Upregulation of SGLT-1 transport activity in rat jejunum induced by GLP-2 infusion in vivo. *Am J Physiol,* **273,** R1965-71.

Chen, H., Charlat, O., Tartaglia, L. A., Woolf, E. A., Weng, X., Ellis, S. J., Lakey, N. D., Culpepper, J., Moore, K. J., Breitbart, R. E., Duyk, G. M., Tepper, R. I. & Morgenstern, J. P. 1996. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell,* **84,** 491-5.

Chen, M. P., Chung, F. M., Chang, D. M., Tsai, J. C., Huang, H. F., Shin, S. J. & Lee, Y. J. 2006. Elevated plasma level of visfatin/pre-B cell colony-enhancing factor in patients with type 2 diabetes mellitus. *J Clin Endocrinol Metab,* **91,** 295-9.

Chen, X. P., Losman, J. A. & Rothman, P. 2000. SOCS proteins, regulators of intracellular signaling. *Immunity,* **13,** 287-90.

Cheng, J. G., Pennica, D. & Patterson, P. H. 1997. Cardiotrophin-1 induces the same neuropeptides in sympathetic neurons as do neuropoietic cytokines. *J Neurochem,* **69,** 2278-84.

Chiba, T., Shinozaki, S., Nakazawa, T., Kawakami, A., Ai, M., Kaneko, E., Kitagawa, M., Kondo, K., Chait, A. & Shimokado, K. 2008. Leptin deficiency suppresses progression of atherosclerosis in apoE-deficient mice. *Atherosclerosis,* **196,** 68-75.

Chien, K. R. & Olson, E. N. 2002. Converging pathways and principles in heart development and disease: CV@CSH. *Cell,* **110,** 153-62.

Cho, H., Zhao, X., Hatori, M., Yu, R. T., Barish, G. D., Lam, M. T., Chong, L. W., DiTacchio, L., Atkins, A. R., Glass, C. K., Liddle, C., Auwerx, J., Downes, M., Panda, S. & Evans, R. M. 2012. Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. *Nature,* **485,** 123-7.

Choi, I., Kim, J., Jeong, H. K., Kim, B., Jou, I., Park, S. M., Chen, L., Kang, U. J., Zhuang, X. & Joe, E. H. 2013. PINK1 deficiency attenuates astrocyte proliferation through mitochondrial dysfunction, reduced AKT and increased p38 MAPK activation, and downregulation of EGFR. *Glia,* **61,** 800-12.

Chondronikola, M., Volpi, E., Borsheim, E., Porter, C., Annamalai, P., Enerback, S., Lidell, M. E., Saraf, M. K., Labbe, S. M., Hurren, N. M., Yfanti, C., Chao, T., Andersen, C. R., Cesani, F., Hawkins, H. & Sidossis,

L. S. 2014. Brown adipose tissue improves whole-body glucose homeostasis and insulin sensitivity in humans. *Diabetes,* **63,** 4089-99.

Chung, B. M., Wallace, L. E., Hardin, J. A. & Gall, D. G. 2002. The effect of epidermal growth factor on the distribution of SGLT-1 in rabbit jejunum. *Can J Physiol Pharmacol,* **80,** 872-8.

Church, C., Horowitz, M. & Rodeheffer, M. 2012. WAT is a functional adipocyte? *Adipocyte,* **1,** 38-45.

-D-

Dahl, T. B., Holm, S., Aukrust, P. & Halvorsen, B. 2012. Visfatin/NAMPT: a multifaceted molecule with diverse roles in physiology and pathophysiology. *Annu Rev Nutr,* **32,** 229-43.

Daval, M., Diot-Dupuy, F., Bazin, R., Hainault, I., Viollet, B., Vaulont, S., Hajduch, E., Ferre, P. & Foufelle, F. 2005. Anti-lipolytic action of AMP-activated protein kinase in rodent adipocytes. *J Biol Chem,* **280,** 25250-7.

Degawa-Yamauchi, M., Bovenkerk, J. E., Juliar, B. E., Watson, W., Kerr, K., Jones, R., Zhu, Q. & Considine, R. V. 2003. Serum resistin (FIZZ3) protein is increased in obese humans. *J Clin Endocrinol Metab,* **88,** 5452-5.

Dempersmier, J. & Sul, H. S. 2015. Shades of brown: a model for thermogenic fat. *Front Endocrinol (Lausanne),* **6,** 71.

Dixon, J. B. 2010. The effect of obesity on health outcomes. *Mol Cell Endocrinol,* **316,** 104-8.

Djouder, N., Tuerk, R. D., Suter, M., Salvioni, P., Thali, R. F., Scholz, R., Vaahtomeri, K., Auchli, Y., Rechsteiner, H., Brunisholz, R. A., Viollet, B., Makela, T. P., Wallimann, T., Neumann, D. & Krek, W. 2010. PKA phosphorylates and inactivates AMPKalpha to promote efficient lipolysis. *EMBO J,* **29,** 469-81.

Dray, C., Knauf, C., Daviaud, D., Waget, A., Boucher, J., Buleon, M., Cani, P. D., Attane, C., Guigne, C., Carpene, C., Burcelin, R., Castan-Laurell, I. & Valet, P. 2008. Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab,* **8,** 437-45.

Dray, C., Sakar, Y., Vinel, C., Daviaud, D., Masri, B., Garrigues, L., Wanecq, E., Galvani, S., Negre-Salvayre, A., Barak, L. S., Monsarrat, B., Burlet-Schiltz, O., Valet, P., Castan-Laurell, I. & Ducroc, R. 2013. The intestinal glucose-apelin cycle controls carbohydrate absorption in mice. *Gastroenterology,* **144,** 771-80.

Dubey, L. & Hesong, Z. 2006. Role of leptin in atherogenesis. *Exp Clin Cardiol,* **11,** 269-75.

Duncan, R. E., Sarkadi-Nagy, E., Jaworski, K., Ahmadian, M. & Sul, H. S. 2008. Identification and functional characterization of adipose-specific phospholipase A2 (AdPLA). *J Biol Chem,* **283,** 25428-36.

Duncan, R. E., Wang, Y., Ahmadian, M., Lu, J., Sarkadi-Nagy, E. & Sul, H. S. 2010. Characterization of desnutrin functional domains: critical residues for triacylglycerol hydrolysis in cultured cells. *J Lipid Res,* **51,** 309-17.

Dunmore, S. J. & Brown, J. E. 2013. The role of adipokines in beta-cell failure of type 2 diabetes. *J Endocrinol,* **216,** T37-45.

Dyer, J., Hosie, K. B. & Shirazi-Beechey, S. P. 1997. Nutrient regulation of human intestinal sugar transporter (SGLT1) expression. *Gut,* **41,** 56-9.

-E-

Elmquist, J. K., Elias, C. F. & Saper, C. B. 1999. From lesions to leptin: hypothalamic control of food intake and body weight. *Neuron,* **22,** 221-32.

Erdmann, J., Hassfeld, S., Kallisch, H., Fleck, E. & Regitz-Zagrosek, V. 1998. Cloning and characterization of the 5'-flanking region of the human cardiotrophin-1 gene. *Biochem Biophys Res Commun,* **244,** 494-7.

-F-

Fanjul, C., Barrenetxe, J., Inigo, C., Sakar, Y., Ducroc, R., Barber, A. & Lostao, M. P. 2012. Leptin regulates sugar and amino acids transport in the human intestinal cell line Caco-2. *Acta Physiol (Oxf),* **205,** 82-91.

Farb, M. G. & Gokce, N. 2015. Visceral adiposopathy: a vascular perspective. *Horm Mol Biol Clin Investig,* **21,** 125-36.

Fernandez-Riejos, P., Najib, S., Santos-Alvarez, J., Martin-Romero, C., Perez-Perez, A., Gonzalez-Yanes, C. & Sanchez-Margalet, V. 2010. Role of leptin in the activation of immune cells. *Mediators Inflamm,* **2010,** 568343.

Ferraris, R. P. & Carey, H. V. 2000. Intestinal transport during fasting and malnutrition. *Annu Rev Nutr,* **20,** 195-219.

Ferraris, R. P. & Diamond, J. 1997. Regulation of intestinal sugar transport. *Physiol Rev,* **77,** 257-302.

Ferraris, R. P. & Diamond, J. M. 1993. Crypt/villus site of substrate-dependent regulation of mouse intestinal glucose transporters. *Proc Natl Acad Sci U S A,* **90,** 5868-72.

Fontana, A., Spadaro, S., Copetti, M., Spoto, B., Salvemini, L., Pizzini, P., Frittitta, L., Mallamaci, F., Pellegrini, F., Trischitta, V. & Menzaghi, C. 2015. Association between resistin levels and all-cause and cardiovascular mortality: a new study and a systematic review and meta-analysis. *PLoS One,* **10,** e0120419.

Franks, P. W., Brage, S., Luan, J., Ekelund, U., Rahman, M., Farooqi, I. S., Halsall, I., O'Rahilly, S. & Wareham, N. J. 2005. Leptin predicts a worsening of the features of the metabolic syndrome independently of obesity. *Obes Res,* **13,** 1476-84.

Frederich, R. C., Hamann, A., Anderson, S., Lollmann, B., Lowell, B. B. & Flier, J. S. 1995. Leptin levels reflect body lipid content in mice: evidence for diet-induced resistance to leptin action. *Nat Med,* **1,** 1311-4.

Freed, D. H., Cunnington, R. H., Dangerfield, A. L., Sutton, J. S. & Dixon, I. M. 2005. Emerging evidence for the role of cardiotrophin-1 in cardiac repair in the infarcted heart. *Cardiovasc Res,* **65,** 782-92.

Friebe, D., Neef, M., Kratzsch, J., Erbs, S., Dittrich, K., Garten, A., Petzold-Quinque, S., Bluher, S., Reinehr, T., Stumvoll, M., Bluher, M., Kiess, W. & Korner, A. 2011. Leucocytes are a major source of circulating nicotinamide phosphoribosyltransferase (NAMPT)/pre-B cell colony (PBEF)/visfatin linking obesity and inflammation in humans. *Diabetologia,* **54,** 1200-11.

Friedman, J. M. & Halaas, J. L. 1998. Leptin and the regulation of body weight in mammals. *Nature,* **395,** 763-70.

Fruhbeck, G. 2006. Intracellular signalling pathways activated by leptin. *Biochem J,* **393,** 7-20.

Fujimori, Y., Katsuno, K., Nakashima, I., Ishikawa-Takemura, Y., Fujikura, H. & Isaji, M. 2008. Remogliflozin etabonate, in a novel category of selective low-affinity sodium glucose cotransporter (SGLT2) inhibitors, exhibits antidiabetic efficacy in rodent models. *J Pharmacol Exp Ther,* **327,** 268-76.

Fukuhara, A. Matsuda, M. Nishizawa, M. Segawa, K. Tanaka, M. Kishimoto, K. Matsuki, Y. Murakami, M. Ichisaka, T. Murakami, H. Watanabe, E. Takagi, T. Akiyoshi, M. Ohtsubo, T. Kihara, S. Yamashita, S. *et al.* 2005. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science,* **307,** 426-30.

Fukuhara, A. Matsuda, M. Nishizawa, M. Segawa, K. Tanaka, M. Kishimoto, K. Matsuki, Y. Murakami, M. Ichisaka, T. Murakami, H. Watanabe, E. Takagi, T. Akiyoshi, M. Ohtsubo, T. Kihara, S. Yamashita, S. *et al.* 2007. Retraction. *Science,* **318,** 565.

Funamoto, M., Hishinuma, S., Fujio, Y., Matsuda, Y., Kunisada, K., Oh, H., Negoro, S., Tone, E., Kishimoto, T. & Yamauchi-Takihara, K. 2000. Isolation and characterization of the murine cardiotrophin-1 gene: expression and norepinephrine-induced transcriptional activation. *J Mol Cell Cardiol,* **32,** 1275-84.

-G-

Gadient, R. A. & Patterson, P. H. 1999. Leukemia inhibitory factor, Interleukin 6, and other cytokines using the GP130 transducing receptor: roles in inflammation and injury. *Stem Cells,* **17,** 127-37.

Garaulet, M. & Gomez-Abellan, P. 2013. Chronobiology and obesity. *Nutr Hosp,* **28 Suppl 5,** 114-20.

Garaulet, M., Hernandez-Morante, J. J., de Heredia, F. P. & Tebar, F. J. 2007. Adiponectin, the controversial hormone. *Public Health Nutr,* **10,** 1145-50.

Garaulet, M. & Madrid, J. A. 2009. Chronobiology, genetics and metabolic syndrome. *Curr Opin Lipidol,* **20,** 127-34.

Garaulet, M., Ordovas, J. M. & Madrid, J. A. 2010. The chronobiology, etiology and pathophysiology of obesity. *Int J Obes (Lond),* **34,** 1667-83.

Garaulet, M., Ortega, F. B., Ruiz, J. R., Rey-Lopez, J. P., Beghin, L., Manios, Y., Cuenca-Garcia, M., Plada, M., Diethelm, K., Kafatos, A., Molnar, D., Al-Tahan, J. & Moreno, L. A. 2011. Short sleep duration is associated with increased obesity markers in European adolescents: effect of physical activity and dietary habits. The HELENA study. *Int J Obes (Lond),* **35,** 1308-17.

Garcia-Arcos, I., Hiyama, Y., Drosatos, K., Bharadwaj, K. G., Hu, Y., Son, N. H., O'Byrne, S. M., Chang, C. L., Deckelbaum, R. J., Takahashi, M., Westerterp, M., Obunike, J. C., Jiang, H., Yagyu, H., Blaner, W. S. & Goldberg, I. J. 2013. Adipose-specific lipoprotein lipase deficiency more profoundly affects brown than white fat biology. *J Biol Chem,* **288,** 14046-58.

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B. & et al. 1992. The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science,* **255,** 1434-7.

Gerstmayer, B., Kusters, D., Gebel, S., Muller, T., Van Miert, E., Hofmann, K. & Bosio, A. 2003. Identification of RELMgamma, a novel resistin-like molecule with a distinct expression pattern. *Genomics,* **81,** 588-95.

Gharibeh, M. Y., Al Tawallbeh, G. M., Abboud, M. M., Radaideh, A., Alhader, A. A. & Khabour, O. F. 2010. Correlation of plasma resistin with obesity and insulin resistance in type 2 diabetic patients. *Diabetes Metab,* **36,** 443-9.

Gherman, C. D. & Mironiuc, A. I. 2012. Evaluation of serum adipokines in peripheral arterial occlusive disease. *Mediators Inflamm,* **2012,** 257808.

Ghosh, S., Singh, A. K., Aruna, B., Mukhopadhyay, S. & Ehtesham, N. Z. 2003. The genomic organization of mouse resistin reveals major differences from the human resistin: functional implications. *Gene,* **305,** 27-34.

Gomez-Abellan, P., Hernandez-Morante, J. J., Lujan, J. A., Madrid, J. A. & Garaulet, M. 2008. Clock genes are implicated in the human metabolic syndrome. *Int J Obes (Lond),* **32,** 121-8.

Granneman, J. G., Moore, H. P., Granneman, R. L., Greenberg, A. S., Obin, M. S. & Zhu, Z. 2007. Analysis of lipolytic protein trafficking and interactions in adipocytes. *J Biol Chem,* **282,** 5726-35.

Grasso, P., Leinung, M. C., Ingher, S. P. & Lee, D. W. 1997. In vivo effects of leptin-related synthetic peptides on body weight and food intake in female ob/ob mice: localization of leptin activity to domains between amino acid residues 106-140. *Endocrinology,* **138,** 1413-8.

Greenberg, A. S., Shen, W. J., Muliro, K., Patel, S., Souza, S. C., Roth, R. A. & Kraemer, F. B. 2001. Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. *J Biol Chem,* **276,** 45456-61.

Grimaldi, B., Bellet, M. M., Katada, S., Astarita, G., Hirayama, J., Amin, R. H., Granneman, J. G., Piomelli, D., Leff, T. & Sassone-Corsi, P. 2010. PER2 controls lipid metabolism by direct regulation of PPARgamma. *Cell Metab,* **12,** 509-20.

Grober, J., Lucas, S., Sorhede-Winzell, M., Zaghini, I., Mairal, A., Contreras, J. A., Besnard, P., Holm, C. & Langin, D. 2003. Hormone-sensitive lipase is a cholesterol esterase of the intestinal mucosa. *J Biol Chem,* **278,** 6510-5.

Gruber, A., Cornaciu, I., Lass, A., Schweiger, M., Poeschl, M., Eder, C., Kumari, M., Schoiswohl, G., Wolinski, H., Kohlwein, S. D., Zechner, R., Zimmermann, R. & Oberer, M. 2010. The N-terminal region of comparative gene identification-58 (CGI-58) is important for lipid droplet binding and activation of adipose triglyceride lipase. *J Biol Chem,* **285,** 12289-98.

Grundy, S. M., Cleeman, J. I., Daniels, S. R., Donato, K. A., Eckel, R. H., Franklin, B. A., Gordon, D. J., Krauss, R. M., Savage, P. J., Smith, S. C., Jr., Spertus, J. A. & Costa, F. 2005. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. *Circulation,* **112,** 2735-52.

Guan, X., Lin, P., Knoll, E. & Chakrabarti, R. 2014. Mechanism of inhibition of the human sirtuin enzyme SIRT3 by nicotinamide: computational and experimental studies. *PLoS One,* **9,** e107729.

-H-

Habchi, M., Duvillard, L., Cottet, V., Brindisi, M. C., Bouillet, B., Beacco, M., Crevisy, E., Buffier, P., Baillot-Rudoni, S., Verges, B. & Petit, J. M. 2014. Circulating apelin is increased in patients with type 1 or type 2 diabetes and is associated with better glycaemic control. *Clin Endocrinol (Oxf),* **81,** 696-701.

Hajianfar, H., Bahonar, A., Entezari, M. H., Askari, G. & Yazdani, M. 2012. Lipid Profiles and Serum Visfatin Concentrations in Patients with Type II Diabetes in Comparison with Healthy Controls. *Int J Prev Med,* **3,** 326-31.

Hardie, D. G. 2007. AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat Rev Mol Cell Biol,* **8,** 774-85.

Hastings, M. H. & Herzog, E. D. 2004. Clock genes, oscillators, and cellular networks in the suprachiasmatic nuclei. *J Biol Rhythms,* **19,** 400-13.

He, Y., Chen, H., Quon, M. J. & Reitman, M. 1995. The mouse obese gene. Genomic organization, promoter activity, and activation by CCAAT/enhancer-binding protein alpha. *J Biol Chem,* **270,** 28887-91.

Hector, J., Schwarzloh, B., Goehring, J., Strate, T. G., Hess, U. F., Deuretzbacher, G., Hansen-Algenstaedt, N., Beil, F. U. & Algenstaedt, P. 2007. TNF-alpha alters visfatin and adiponectin levels in human fat. *Horm Metab Res,* **39,** 250-5.

Heinonen, M. V., Purhonen, A. K., Miettinen, P., Paakkonen, M., Pirinen, E., Alhava, E., Akerman, K. & Herzig, K. H. 2005. Apelin, orexin-A and leptin plasma levels in morbid obesity and effect of gastric banding. *Regul Pept,* **130,** 7-13.

Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Muller-Newen, G. & Schaper, F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J,* **374,** 1-20.

Hendry, L. & John, S. 2004. Regulation of STAT signalling by proteolytic processing. *Eur J Biochem,* **271,** 4613-20.

Higuchi, K., Masaki, T., Gotoh, K., Chiba, S., Katsuragi, I., Tanaka, K., Kakuma, T. & Yoshimatsu, H. 2007. Apelin, an APJ receptor ligand, regulates body adiposity and favors the messenger ribonucleic acid expression of uncoupling proteins in mice. *Endocrinology,* **148,** 2690-7.

Hirota, H., Chen, J., Betz, U. A., Rajewsky, K., Gu, Y., Ross, J., Jr., Muller, W. & Chien, K. R. 1999. Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress. *Cell,* **97,** 189-98.

Hirsch, J. R., Loo, D. D. & Wright, E. M. 1996. Regulation of Na+/glucose cotransporter expression by protein kinases in Xenopus laevis oocytes. *J Biol Chem,* **271,** 14740-6.

Hirsh, A. J. & Cheeseman, C. I. 1998. Cholecystokinin decreases intestinal hexose absorption by a parallel reduction in SGLT1 abundance in the brush-border membrane. *J Biol Chem,* **273,** 14545-9.

Hivert, M. F., Sullivan, L. M., Fox, C. S., Nathan, D. M., D'Agostino, R. B., Sr., Wilson, P. W. & Meigs, J. B. 2008. Associations of adiponectin, resistin, and tumor necrosis factor-alpha with insulin resistance. *J Clin Endocrinol Metab,* **93,** 3165-72.

Ho, D. W., Yang, Z. F., Lau, C. K., Tam, K. H., To, J. Y., Poon, R. T. & Fan, S. T. 2006. Therapeutic potential of cardiotrophin 1 in fulminant hepatic failure: dual roles in antiapoptosis and cell repair. *Arch Surg,* **141,** 1077-84; discussion 1084.

Holcomb, I. N., Kabakoff, R. C., Chan, B., Baker, T. W., Gurney, A., Henzel, W., Nelson, C., Lowman, H. B., Wright, B. D., Skelton, N. J., Frantz, G. D., Tumas, D. B., Peale, F. V., Jr., Shelton, D. L. & Hebert, C. C. 2000. FIZZ1, a novel cysteine-rich secreted protein associated with pulmonary inflammation, defines a new gene family. *EMBO J,* **19,** 4046-55.

Holm, C. 2003. Molecular mechanisms regulating hormone-sensitive lipase and lipolysis. *Biochem Soc Trans,* **31,** 1120-4.

Hotamisligil, G. S., Arner, P., Caro, J. F., Atkinson, R. L. & Spiegelman, B. M. 1995. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. *J Clin Invest,* **95,** 2409-15.

Hsieh, Y. Y., Shen, C. H., Huang, W. S., Chin, C. C., Kuo, Y. H., Hsieh, M. C., Yu, H. R., Chang, T. S., Lin, T. H., Chiu, Y. W., Chen, C. N., Kuo, H. C. & Tung, S. Y. 2014. Resistin-induced stromal cell-derived factor-1 expression through Toll-like receptor 4 and activation of p38 MAPK/ NFkappaB signaling pathway in gastric cancer cells. *J Biomed Sci,* **21,** 59.

Hu, F. B., Meigs, J. B., Li, T. Y., Rifai, N. & Manson, J. E. 2004. Inflammatory markers and risk of developing type 2 diabetes in women. *Diabetes,* **53,** 693-700.

Hu, W. L., Qiao, S. B. & Li, J. J. 2007. Decreased C-reactive protein-induced resistin production in human monocytes by simvastatin. *Cytokine,* **40,** 201-6.

Hutcheson, J. 2015. Adipokines influence the inflammatory balance in autoimmunity. *Cytokine*.

-I-

Ichihara, M., Hara, T., Kim, H., Murate, T. & Miyajima, A. 1997. Oncostatin M and leukemia inhibitory factor do not use the same functional receptor in mice. *Blood,* **90,** 165-73.

Iniguez, M., Berasain, C., Martinez-Anso, E., Bustos, M., Fortes, P., Pennica, D., Avila, M. A. & Prieto, J. 2006. Cardiotrophin-1 defends the liver against ischemia-reperfusion injury and mediates the protective effect of ischemic preconditioning. *J Exp Med,* **203,** 2809-15.

Ishikawa, M., Saito, Y., Miyamoto, Y., Harada, M., Kuwahara, K., Ogawa, E., Nakagawa, O., Hamanaka, I., Kajiyama, N., Takahashi, N., Masuda, I., Hashimoto, T., Sakai, O., Hosoya, T. & Nakao, K. 1999. A heart-specific increase in cardiotrophin-1 gene expression precedes the establishment of ventricular hypertrophy in genetically hypertensive rats. *J Hypertens,* **17,** 807-16.

Ishikawa, M., Saito, Y., Miyamoto, Y., Kuwahara, K., Ogawa, E., Nakagawa, O., Harada, M., Masuda, I. & Nakao, K. 1996. cDNA cloning of rat cardiotrophin-1 (CT-1): augmented expression of CT-1 gene in ventricle of genetically hypertensive rats. *Biochem Biophys Res Commun,* **219,** 377-81.

Isse, N., Ogawa, Y., Tamura, N., Masuzaki, H., Mori, K., Okazaki, T., Satoh, N., Shigemoto, M., Yoshimasa, Y., Nishi, S. & et al. 1995. Structural organization and chromosomal assignment of the human obese gene. *J Biol Chem,* **270,** 27728-33.

-J-

Jacoby, J. J., Kalinowski, A., Liu, M. G., Zhang, S. S., Gao, Q., Chai, G. X., Ji, L., Iwamoto, Y., Li, E., Schneider, M., Russell, K. S. & Fu, X. Y. 2003. Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age. *Proc Natl Acad Sci U S A,* **100,** 12929-34.

Jaworski, K., Ahmadian, M., Duncan, R. E., Sarkadi-Nagy, E., Varady, K. A., Hellerstein, M. K., Lee, H. Y., Samuel, V. T., Shulman, G. I., Kim, K. H., de Val, S., Kang, C. & Sul, H. S. 2009. AdPLA ablation increases lipolysis and prevents obesity induced by high-fat feeding or leptin deficiency. *Nat Med,* **15,** 159-68.

Jenkins, C. M., Mancuso, D. J., Yan, W., Sims, H. F., Gibson, B. & Gross, R. W. 2004. Identification, cloning, expression, and purification of three novel human calcium-independent phospholipase A2 family members possessing triacylglycerol lipase and acylglycerol transacylase activities. *J Biol Chem,* **279,** 48968-75.

Jia, S. H., Li, Y., Parodo, J., Kapus, A., Fan, L., Rotstein, O. D. & Marshall, J. C. 2004. Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. *J Clin Invest,* **113,** 1318-27.

Jian, W. X., Luo, T. H., Gu, Y. Y., Zhang, H. L., Zheng, S., Dai, M., Han, J. F., Zhao, Y., Li, G. & Luo, M. 2006. The visfatin gene is associated with glucose and lipid metabolism in a Chinese population. *Diabet Med,* **23,** 967-73.

Jiang, C. Y., Wang, W., Tang, J. X. & Yuan, Z. R. 2013. The adipocytokine resistin stimulates the production of proinflammatory cytokines TNF-alpha and IL-6 in pancreatic acinar cells via NF-kappaB activation. *J Endocrinol Invest,* **36,** 986-92.

Jimenez-Gonzalez, M., Jaques, F., Rodriguez, S., Porciuncula, A., Principe, R. M., Abizanda, G., Iniguez, M., Escalada, J., Salvador, J., Prosper, F., Halban, P. A. & Barajas, M. 2013. Cardiotrophin 1 protects beta cells from apoptosis and prevents streptozotocin-induced diabetes in a mouse model. *Diabetologia,* **56,** 838-46.

-K-

Kanazawa, H. Ieda, M. Kimura, K. Arai, T. Kawaguchi-Manabe, H. Matsuhashi, T. Endo, J. Sano, M. Kawakami, T. Kimura, T. Monkawa, T. Hayashi, M. Iwanami, A. Okano, H. Okada, Y. Ishibashi-Ueda, H. *et al.* 2010. Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents. *J Clin Invest,* **120,** 408-21.

Karlsson, B., Knutsson, A. & Lindahl, B. 2001. Is there an association between shift work and having a metabolic syndrome? Results from a population based study of 27,485 people. *Occup Environ Med,* **58,** 747-52.

Kaser, S., Kaser, A., Sandhofer, A., Ebenbichler, C. F., Tilg, H. & Patsch, J. R. 2003. Resistin messenger-RNA expression is increased by proinflammatory cytokines in vitro. *Biochem Biophys Res Commun,* **309,** 286-90.

Katzmarzyk, P. T., Leon, A. S., Wilmore, J. H., Skinner, J. S., Rao, D. C., Rankinen, T. & Bouchard, C. 2003. Targeting the metabolic syndrome with exercise: evidence from the HERITAGE Family Study. *Med Sci Sports Exerc,* **35,** 1703-9.

Kawamata, Y., Habata, Y., Fukusumi, S., Hosoya, M., Fujii, R., Hinuma, S., Nishizawa, N., Kitada, C., Onda, H., Nishimura, O. & Fujino, M. 2001. Molecular properties of apelin: tissue distribution and receptor binding. *Biochim Biophys Acta,* **1538,** 162-71.

Kellett, G. L. & Brot-Laroche, E. 2005. Apical GLUT2: a major pathway of intestinal sugar absorption. *Diabetes,* **54,** 3056-62.

Kellett, G. L., Brot-Laroche, E., Mace, O. J. & Leturque, A. 2008. Sugar absorption in the intestine: the role of GLUT2. *Annu Rev Nutr,* **28,** 35-54.

Kennelly, P. J. & Krebs, E. G. 1991. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J Biol Chem,* **266,** 15555-8.

Kern, P. A., Ranganathan, S., Li, C., Wood, L. & Ranganathan, G. 2001. Adipose tissue tumor necrosis factor and interleukin-6 expression in human obesity and insulin resistance. *Am J Physiol Endocrinol Metab,* **280,** E745-51.

Kershaw, E. E., Hamm, J. K., Verhagen, L. A., Peroni, O., Katic, M. & Flier, J. S. 2006. Adipose triglyceride lipase: function, regulation by insulin, and comparison with adiponutrin. *Diabetes,* **55,** 148-57.

Khalsa, S. B., Jewett, M. E., Cajochen, C. & Czeisler, C. A. 2003. A phase response curve to single bright light pulses in human subjects. *J Physiol,* **549,** 945-52.

Kim, J. Y., Tillison, K., Lee, J. H., Rearick, D. A. & Smas, C. M. 2006. The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-alpha in 3T3-L1 adipocytes and is a target for transactivation by PPARgamma. *Am J Physiol Endocrinol Metab,* **291,** E115-27.

Kim, S. R., Bae, S. K., Choi, K. S., Park, S. Y., Jun, H. O., Lee, J. Y., Jang, H. O., Yun, I., Yoon, K. H., Kim, Y. J., Yoo, M. A., Kim, K. W. & Bae, M. K. 2007. Visfatin promotes angiogenesis by activation of extracellular signal-regulated kinase 1/2. *Biochem Biophys Res Commun,* **357,** 150-6.

Kleinz, M. J. & Davenport, A. P. 2005. Emerging roles of apelin in biology and medicine. *Pharmacol Ther,* **107,** 198-211.

Koh, H. J., Brandauer, J. & Goodyear, L. J. 2008. LKB1 and AMPK and the regulation of skeletal muscle metabolism. *Curr Opin Clin Nutr Metab Care,* **11,** 227-32.

Kopecky, J., Rossmeisl, M., Flachs, P., Brauner, P., Sponarova, J., Matejkova, O., Prazak, T., Ruzickova, J., Bardova, K. & Kuda, O. 2004. Energy metabolism of adipose tissue--physiological aspects and target in obesity treatment. *Physiol Res,* **53 Suppl 1,** S225-32.

Kralisch, S., Klein, J., Lossner, U., Bluher, M., Paschke, R., Stumvoll, M. & Fasshauer, M. 2005. Isoproterenol, TNFalpha, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. *Mol Cell Endocrinol,* **240,** 43-9.

Kumar, G., Gupta, S., Wang, S. & Nel, A. E. 1994. Involvement of Janus kinases, p52shc, Raf-1, and MEK-1 in the IL-6-induced mitogen-activated protein kinase cascade of a growth-responsive B cell line. *J Immunol,* **153,** 4436-47.

Kume, K., Zylka, M. J., Sriram, S., Shearman, L. P., Weaver, D. R., Jin, X., Maywood, E. S., Hastings, M. H. & Reppert, S. M. 1999. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. *Cell,* **98,** 193-205.

Kurdi, M. & Booz, G. W. 2007. Jak inhibition, but not Stat1 knockdown, blocks the synergistic effect of IFN-gamma on Fas-induced apoptosis of A549 human non-small cell lung cancer cells. *J Interferon Cytokine Res,* **27,** 23-31.

Kuropatwinski, K. K., De Imus, C., Gearing, D., Baumann, H. & Mosley, B. 1997. Influence of subunit combinations on signaling by receptors for oncostatin M, leukemia inhibitory factor, and interleukin-6. *J Biol Chem,* **272,** 15135-44.

-L-

Lafontan, M. 2008. Advances in adipose tissue metabolism. *Int J Obes (Lond),* **32 Suppl 7,** S39-51.

Lafontan, M. & Berlan, M. 1993. Fat cell adrenergic receptors and the control of white and brown fat cell function. *J Lipid Res,* **34,** 1057-91.

Lafontan, M. & Langin, D. 2009. Lipolysis and lipid mobilization in human adipose tissue. *Prog Lipid Res,* **48,** 275-97.

Lafontan, M., Moro, C., Sengenes, C., Galitzky, J., Crampes, F. & Berlan, M. 2005. An unsuspected metabolic role for atrial natriuretic peptides: the control of lipolysis, lipid mobilization, and systemic nonesterified fatty acids levels in humans. *Arterioscler Thromb Vasc Biol,* **25,** 2032-42.

Lambert, P. D., Anderson, K. D., Sleeman, M. W., Wong, V., Tan, J., Hijarunguru, A., Corcoran, T. L., Murray, J. D., Thabet, K. E., Yancopoulos, G. D. & Wiegand, S. J. 2001. Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity. *Proc Natl Acad Sci U S A,* **98,** 4652-7.

Landsberg, L., Young, J. B., Leonard, W. R., Linsenmeier, R. A. & Turek, F. W. 2009. Do the obese have lower body temperatures? A new look at a forgotten variable in energy balance. *Trans Am Clin Climatol Assoc,* **120,** 287-95.

Langin, D. & Arner, P. 2006. Importance of TNFalpha and neutral lipases in human adipose tissue lipolysis. *Trends Endocrinol Metab,* **17,** 314-20.

Langin, D., Holm, C. & Lafontan, M. 1996. Adipocyte hormone-sensitive lipase: a major regulator of lipid metabolism. *Proc Nutr Soc,* **55,** 93-109.

Lass, A., Zimmermann, R., Haemmerle, G., Riederer, M., Schoiswohl, G., Schweiger, M., Kienesberger, P., Strauss, J. G., Gorkiewicz, G. & Zechner, R. 2006. Adipose triglyceride lipase-mediated lipolysis of cellular fat stores is activated by CGI-58 and defective in Chanarin-Dorfman Syndrome. *Cell Metab,* **3,** 309-19.

Lax, P., Zamora, S. & Madrid, J. A. 1999. Food-entrained feeding and locomotor circadian rhythms in rats under different lighting conditions. *Chronobiol Int,* **16,** 281-91.

Le Lay, S., Blouin, C. M., Hajduch, E. & Dugail, I. 2009. Filling up adipocytes with lipids. Lessons from caveolin-1 deficiency. *Biochim Biophys Acta,* **1791,** 514-8.

Lee, D. K., Cheng, R., Nguyen, T., Fan, T., Kariyawasam, A. P., Liu, Y., Osmond, D. H., George, S. R. & O'Dowd, B. F. 2000. Characterization of apelin, the ligand for the APJ receptor. *J Neurochem,* **74,** 34-41.

Lee, D. K., Saldivia, V. R., Nguyen, T., Cheng, R., George, S. R. & O'Dowd, B. F. 2005. Modification of the terminal residue of apelin-13 antagonizes its hypotensive action. *Endocrinology,* **146,** 231-6.

Lee, J. H., Chan, J. L., Yiannakouris, N., Kontogianni, M., Estrada, E., Seip, R., Orlova, C. & Mantzoros, C. S. 2003. Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration: cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects. *J Clin Endocrinol Metab,* **88,** 4848-56.

Lee, Y. H., Mottillo, E. P. & Granneman, J. G. 2014. Adipose tissue plasticity from WAT to BAT and in between. *Biochim Biophys Acta,* **1842,** 358-69.

Lee, Y. J., Heo, J. S., Suh, H. N., Lee, M. Y. & Han, H. J. 2007. Interleukin-6 stimulates alpha-MG uptake in renal proximal tubule cells: involvement of STAT3, PI3K/Akt, MAPKs, and NF-kappaB. *Am J Physiol Renal Physiol,* **293,** F1036-46.

Leifheit-Nestler, M., Wagner, N. M., Gogiraju, R., Didie, M., Konstantinides, S., Hasenfuss, G. & Schafer, K. 2013. Importance of leptin signaling and signal transducer and activator of transcription-3 activation in mediating the cardiac hypertrophy associated with obesity. *J Transl Med,* **11,** 170.

Lesbordes, J. C., Cifuentes-Diaz, C., Miroglio, A., Joshi, V., Bordet, T., Kahn, A. & Melki, J. 2003. Therapeutic benefits of cardiotrophin-1 gene transfer in a mouse model of spinal muscular atrophy. *Hum Mol Genet,* **12,** 1233-9.

Liao, Z., Brar, B. K., Cai, Q., Stephanou, A., O'Leary, R. M., Pennica, D., Yellon, D. M. & Latchman, D. S. 2002. Cardiotrophin-1 (CT-1) can protect the adult heart from injury when added both prior to ischaemia and at reperfusion. *Cardiovasc Res,* **53,** 902-10.

Limongelli, G., Calabro, P., Maddaloni, V., Russo, A., Masarone, D., D'Aponte, A., Roselli, T., Bonauro, R., D'Alessandro, R., D'Andrea, A., Pacileo, G., Limongelli, F. M. & Calabro, R. 2010. Cardiotrophin-1 and TNF-alpha circulating levels at rest and during cardiopulmonary exercise test in athletes and healthy individuals. *Cytokine,* **50,** 245-7.

Liu, B., Liao, J., Rao, X., Kushner, S. A., Chung, C. D., Chang, D. D. & Shuai, K. 1998. Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci U S A,* **95,** 10626-31.

Liu, C., Li, S., Liu, T., Borjigin, J. & Lin, J. D. 2007. Transcriptional coactivator PGC-1alpha integrates the mammalian clock and energy metabolism. *Nature,* **447,** 477-81.

Londos, C., Brasaemle, D. L., Schultz, C. J., Adler-Wailes, D. C., Levin, D. M., Kimmel, A. R. & Rondinone, C. M. 1999. On the control of lipolysis in adipocytes. *Ann N Y Acad Sci,* **892,** 155-68.

Lopez-Andres, N., Rousseau, A., Akhtar, R., Calvier, L., Inigo, C., Labat, C., Zhao, X., Cruickshank, K., Diez, J., Zannad, F., Lacolley, P. & Rossignol, P. 2012. Cardiotrophin 1 is involved in cardiac, vascular, and renal fibrosis and dysfunction. *Hypertension,* **60,** 563-73.

Lopez-Yoldi, M., Moreno-Aliaga, M. J. & Bustos, M. 2015. Cardiotrophin-1: A multifaceted cytokine. *Cytokine Growth Factor Rev*.

Lopez, B., Castellano, J. M., Gonzalez, A., Barba, J. & Diez, J. 2007. Association of increased plasma cardiotrophin-1 with inappropriate left ventricular mass in essential hypertension. *Hypertension,* **50,** 977-83.

Lopez, B., Gonzalez, A., Lasarte, J. J., Sarobe, P., Borras, F., Diaz, A., Barba, J., Tomas, L., Lozano, E., Serrano, M., Varo, N., Beloqui, O., Fortuno, M. A. & Diez, J. 2005. Is plasma cardiotrophin-1 a marker of hypertensive heart disease? *J Hypertens,* **23,** 625-32.

Lopez, B., Gonzalez, A., Querejeta, R., Barba, J. & Diez, J. 2009. Association of plasma cardiotrophin-1 with stage C heart failure in hypertensive patients: potential diagnostic implications. *J Hypertens,* **27,** 418-24.

Lu, S. C., Shieh, W. Y., Chen, C. Y., Hsu, S. C. & Chen, H. L. 2002. Lipopolysaccharide increases resistin gene expression in vivo and in vitro. *FEBS Lett,* **530,** 158-62.

Lutz, S. Z., Franck, O., Bohm, A., Machann, J., Schick, F., Machicao, F., Fritsche, A., Haring, H. U. & Staiger, H. 2014. Common genetic variation in the human CTF1 locus, encoding cardiotrophin-1, determines insulin sensitivity. *PLoS One,* **9,** e100391.

-M-

Madej, T., Boguski, M. S. & Bryant, S. H. 1995. Threading analysis suggests that the obese gene product may be a helical cytokine. *FEBS Lett,* **373,** 13-8.

Maffei, M., Halaas, J., Ravussin, E., Pratley, R. E., Lee, G. H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S. & et al. 1995. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med,* **1,** 1155-61.

Mairal, A., Melaine, N., Laurell, H., Grober, J., Holst, L. S., Guillaudeux, T., Holm, C., Jegou, B. & Langin, D. 2002. Characterization of a novel testicular form of human hormone-sensitive lipase. *Biochem Biophys Res Commun,* **291,** 286-90.

Malavazos, A. E., Ermetici, F., Morricone, L., Delnevo, A., Coman, C., Ambrosi, B. & Corsi, M. M. 2008. Association of increased plasma cardiotrophin-1 with left ventricular mass indexes in normotensive morbid obesity. *Hypertension,* **51,** e8-9; author reply e10.

Marques, J. M., Belza, I., Holtmann, B., Pennica, D., Prieto, J. & Bustos, M. 2007. Cardiotrophin-1 is an essential factor in the natural defense of the liver against apoptosis. *Hepatology,* **45,** 639-48.

Marti, A., Berraondo, B. & Martinez, J. A. 1999. Leptin: physiological actions. *J Physiol Biochem,* **55,** 43-9.

Martin-Rodriguez, E., Guillen-Grima, F., Marti, A. & Brugos-Larumbe, A. 2015. Comorbidity associated with obesity in a large population: The APNA study. *Obes Res Clin Pract*.

Matosin-Matekalo, M., Mesonero, J. E., Delezay, O., Poiree, J. C., Ilundain, A. A. & Brot-Laroche, E. 1998. Thyroid hormone regulation of the Na+/glucose cotransporter SGLT1 in Caco-2 cells. *Biochem J,* **334 (Pt 3),** 633-40.

Maury, E., Ramsey, K. M. & Bass, J. 2010. Circadian rhythms and metabolic syndrome: from experimental genetics to human disease. *Circ Res,* **106,** 447-62.

Mendoza, J., Pevet, P. & Challet, E. 2008. High-fat feeding alters the clock synchronization to light. *J Physiol,* **586,** 5901-10.

Minokoshi, Y., Kim, Y. B., Peroni, O. D., Fryer, L. G., Muller, C., Carling, D. & Kahn, B. B. 2002. Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature,* **415,** 339-43.

Mishima, Y., Kuyama, A., Tada, A., Takahashi, K., Ishioka, T. & Kibata, M. 2001. Relationship between serum tumor necrosis factor-alpha and insulin resistance in obese men with Type 2 diabetes mellitus. *Diabetes Res Clin Pract,* **52,** 119-23.

Mitsumoto, H., Klinkosz, B., Pioro, E. P., Tsuzaka, K., Ishiyama, T., O'Leary, R. M. & Pennica, D. 2001. Effects of cardiotrophin-1 (CT-1) in a mouse motor neuron disease. *Muscle Nerve,* **24,** 769-77.

Mohawk, J. A., Green, C. B. & Takahashi, J. S. 2012. Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci,* **35,** 445-62.

Monserrat, L., Lopez, B., Gonzalez, A., Hermida, M., Fernandez, X., Ortiz, M., Barriales-Villa, R., Castro-Beiras, A. & Diez, J. 2011. Cardiotrophin-1 plasma levels are associated with the severity of hypertrophy in hypertrophic cardiomyopathy. *Eur Heart J,* **32,** 177-83.

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* **14,** 242-53.

Moschen, A. R., Gerner, R. R. & Tilg, H. 2010. Pre-B cell colony enhancing factor/NAMPT/visfatin in inflammation and obesity-related disorders. *Curr Pharm Des,* **16,** 1913-20.

Mosley, B., De Imus, C., Friend, D., Boiani, N., Thoma, B., Park, L. S. & Cosman, D. 1996. Dual oncostatin M (OSM) receptors. Cloning and characterization of an alternative signaling subunit conferring OSM-specific receptor activation. *J Biol Chem,* **271,** 32635-43.

Murakami, T., Yamashita, T., Iida, M., Kuwajima, M. & Shima, K. 1997. A short form of leptin receptor performs signal transduction. *Biochem Biophys Res Commun,* **231,** 26-9.

-N-

Nagaev, I., Bokarewa, M., Tarkowski, A. & Smith, U. 2006. Human resistin is a systemic immune-derived proinflammatory cytokine targeting both leukocytes and adipocytes. *PLoS One,* **1,** e31.

Nagaev, I. & Smith, U. 2001. Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. *Biochem Biophys Res Commun,* **285,** 561-4.

Nakafuku, M., Satoh, T. & Kaziro, Y. 1992. Differentiation factors, including nerve growth factor, fibroblast growth factor, and interleukin-6, induce an accumulation of an active Ras.GTP complex in rat pheochromocytoma PC12 cells. *J Biol Chem,* **267,** 19448-54.

Nakahata, Y., Kaluzova, M., Grimaldi, B., Sahar, S., Hirayama, J., Chen, D., Guarente, L. P. & Sassone-Corsi, P. 2008. The NAD+-dependent deacetylase SIRT1 modulates CLOCK-mediated chromatin remodeling and circadian control. *Cell,* **134,** 329-40.

Nakashima, K., Wiese, S., Yanagisawa, M., Arakawa, H., Kimura, N., Hisatsune, T., Yoshida, K., Kishimoto, T., Sendtner, M. & Taga, T. 1999. Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation. *J Neurosci,* **19,** 5429-34.

Natal, C., Fortuno, M. A., Restituto, P., Bazan, A., Colina, I., Diez, J. & Varo, N. 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. *Am J Physiol Endocrinol Metab,* **294,** E52-60.

Nielsen, T. S., Jessen, N., Jorgensen, J. O., Moller, N. & Lund, S. 2014. Dissecting adipose tissue lipolysis: molecular regulation and implications for metabolic disease. *J Mol Endocrinol,* **52,** R199-222.

Ntambi, J. M., Miyazaki, M., Stoehr, J. P., Lan, H., Kendziorski, C. M., Yandell, B. S., Song, Y., Cohen, P., Friedman, J. M. & Attie, A. D. 2002. Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity. *Proc Natl Acad Sci U S A,* **99,** 11482-6.

-O-

O'Carroll, A. M., Lolait, S. J., Harris, L. E. & Pope, G. R. 2013. The apelin receptor APJ: journey from an orphan to a multifaceted regulator of homeostasis. *J Endocrinol,* **219,** R13-35.

O'Rourke, L., Gronning, L. M., Yeaman, S. J. & Shepherd, P. R. 2002. Glucose-dependent regulation of cholesterol ester metabolism in macrophages by insulin and leptin. *J Biol Chem,* **277,** 42557-62.

Oakhill, J. S., Scott, J. W. & Kemp, B. E. 2012. AMPK functions as an adenylate charge-regulated protein kinase. *Trends Endocrinol Metab,* **23,** 125-32.

Ochiai, W., Yanagisawa, M., Takizawa, T., Nakashima, K. & Taga, T. 2001. Astrocyte differentiation of fetal neuroepithelial cells involving cardiotrophin-1-induced activation of STAT3. *Cytokine,* **14,** 264-71.

Oike, H., Oishi, K. & Kobori, M. 2014. Nutrients, Clock Genes, and Chrononutrition. *Curr Nutr Rep,* **3,** 204-212.

Oku, A., Ueta, K., Nawano, M., Arakawa, K., Kano-Ishihara, T., Matsumoto, M., Saito, A., Tsujihara, K., Anai, M. & Asano, T. 2000. Antidiabetic effect of T-1095, an inhibitor of Na(+)-glucose cotransporter, in neonatally streptozotocin-treated rats. *Eur J Pharmacol,* **391,** 183-92.

Okura, T. & Higaki, J. 2014. [Metabolic syndrome]. *Nihon Rinsho,* **72,** 1424-8.

Onat, A. 2011. Metabolic syndrome: nature, therapeutic solutions and options. *Expert Opin Pharmacother,* **12,** 1887-1900.

Orava, J., Nuutila, P., Noponen, T., Parkkola, R., Viljanen, T., Enerback, S., Rissanen, A., Pietilainen, K. H. & Virtanen, K. A. 2013. Blunted metabolic responses to cold and insulin stimulation in brown adipose tissue of obese humans. *Obesity (Silver Spring),* **21,** 2279-87.

Owens, B. 2013. Obesity: heavy sleepers. *Nature,* **497,** S8-9.

-P-

Pagnon, J., Matzaris, M., Stark, R., Meex, R. C., Macaulay, S. L., Brown, W., O'Brien, P. E., Tiganis, T. & Watt, M. J. 2012. Identification and functional characterization of protein kinase A phosphorylation sites in the major lipolytic protein, adipose triglyceride lipase. *Endocrinology,* **153,** 4278-89.

Palou, M., Sanchez, J., Priego, T., Rodriguez, A. M., Pico, C. & Palou, A. 2010. Regional differences in the expression of genes involved in lipid metabolism in adipose tissue in response to short- and medium-term fasting and refeeding. *J Nutr Biochem,* **21,** 23-33.

Pan, A., Schernhammer, E. S., Sun, Q. & Hu, F. B. 2011. Rotating night shift work and risk of type 2 diabetes: two prospective cohort studies in women. *PLoS Med,* **8,** e1001141.

Pan, J., Fukuda, K., Kodama, H., Sano, M., Takahashi, T., Makino, S., Kato, T., Manabe, T., Hori, S. & Ogawa, S. 1998. Involvement of gp130-mediated signaling in pressure overload-induced activation of the JAK/STAT pathway in rodent heart. *Heart Vessels,* **13,** 199-208.

Parkes, K. R. 2002. Shift work and age as interactive predictors of body mass index among offshore workers. *Scand J Work Environ Health,* **28,** 64-71.

Patel, L., Buckels, A. C., Kinghorn, I. J., Murdock, P. R., Holbrook, J. D., Plumpton, C., Macphee, C. H. & Smith, S. A. 2003. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem Biophys Res Commun,* **300,** 472-6.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T. & Collins, F. 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science,* **269,** 540-3.

Pemberton, C. J., Raudsepp, S. D., Yandle, T. G., Cameron, V. A. & Richards, A. M. 2005. Plasma cardiotrophin-1 is elevated in human hypertension and stimulated by ventricular stretch. *Cardiovasc Res,* **68,** 109-17.

Pennica, D., Arce, V., Swanson, T. A., Vejsada, R., Pollock, R. A., Armanini, M., Dudley, K., Phillips, H. S., Rosenthal, A., Kato, A. C. & Henderson, C. E. 1996a. Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons. *Neuron,* **17,** 63-74.

Pennica, D., King, K. L., Shaw, K. J., Luis, E., Rullamas, J., Luoh, S. M., Darbonne, W. C., Knutzon, D. S., Yen, R., Chien, K. R. & et al. 1995a. Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy. *Proc Natl Acad Sci U S A,* **92,** 1142-6.

Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D. L., Zioncheck, K. A., Rosenthal, A., Taga, T., Paoni, N. F. & Wood, W. I. 1995b. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem,* **270,** 10915-22.

Pennica, D., Swanson, T. A., Shaw, K. J., Kuang, W. J., Gray, C. L., Beatty, B. G. & Wood, W. I. 1996b. Human cardiotrophin-1: protein and gene structure, biological and binding activities, and chromosomal localization. *Cytokine,* **8,** 183-9.

Perrone-Filardi, P., Paolillo, S., Costanzo, P., Savarese, G., Trimarco, B. & Bonow, R. O. 2015. The role of metabolic syndrome in heart failure. *Eur Heart J*.

Peters, M., Roeb, E., Pennica, D., Meyer zum Buschenfelde, K. H. & Rose-John, S. 1995. A new hepatocyte stimulating factor: cardiotrophin-1 (CT-1). *FEBS Lett,* **372,** 177-80.

Pillai, J. B., Isbatan, A., Imai, S. & Gupta, M. P. 2005. Poly(ADP-ribose) polymerase-1-dependent cardiac myocyte cell death during heart failure is mediated by NAD+ depletion and reduced Sir2alpha deacetylase activity. *J Biol Chem,* **280,** 43121-30.

Planas, B., Pons, S., Nicolau, M. C., Lopez-Garcia, J. A. & Rial, R. 1992. Morphofunctional changes in gastrointestinal tract of rats due to cafeteria diet. *Rev Esp Fisiol,* **48,** 37-43.

Plomgaard, P., Bouzakri, K., Krogh-Madsen, R., Mittendorfer, B., Zierath, J. R. & Pedersen, B. K. 2005. Tumor necrosis factor-alpha induces skeletal muscle insulin resistance in healthy human subjects via inhibition of Akt substrate 160 phosphorylation. *Diabetes,* **54,** 2939-45.

Popovic, D. S., Tomic-Naglic, D. & Stokic, E. 2014. Relation of resistin, leptin and adiponectin--trinity of adipose tissue dysfunction assessment. *Eur J Intern Med,* **25,** e80-1.

Pradhan, A. D., Manson, J. E., Rifai, N., Buring, J. E. & Ridker, P. M. 2001. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. *JAMA,* **286,** 327-34.

Pravenec, M., Kazdova, L., Landa, V., Zidek, V., Mlejnek, P., Jansa, P., Wang, J., Qi, N. & Kurtz, T. W. 2003. Transgenic and recombinant resistin impair skeletal muscle glucose metabolism in the spontaneously hypertensive rat. *J Biol Chem,* **278,** 45209-15.

Prieto-Hontoria, P. L., Perez-Matute, P., Fernandez-Galilea, M., Bustos, M., Martinez, J. A. & Moreno-Aliaga, M. J. 2011. Role of obesity-associated dysfunctional adipose tissue in cancer: a molecular nutrition approach. *Biochim Biophys Acta,* **1807,** 664-78.

Proenca, A. R., Sertie, R. A., Oliveira, A. C., Campana, A. B., Caminhotto, R. O., Chimin, P. & Lima, F. B. 2014. New concepts in white adipose tissue physiology. *Braz J Med Biol Res,* **47,** 192-205.

-Q-

Quehenberger, P., Exner, M., Sunder-Plassmann, R., Ruzicka, K., Bieglmayer, C., Endler, G., Muellner, C., Speiser, W. & Wagner, O. 2002. Leptin induces endothelin-1 in endothelial cells in vitro. *Circ Res,* **90,** 711-8.

-R-

Raclot, T., Langin, D., Lafontan, M. & Groscolas, R. 1997. Selective release of human adipocyte fatty acids according to molecular structure. *Biochem J,* **324 (Pt 3),** 911-5.

Rae, C. & Graham, A. 2006. Human resistin promotes macrophage lipid accumulation. *Diabetologia,* **49,** 1112-4.

Railson, J., Lawrence, K., Stephanou, A., Brar, B., Pennica, D. & Latchman, D. 2000. Cardiotrophin-1 reduces stress-induced heat shock protein production in cardiac myocytes. *Cytokine,* **12,** 1741-4.

Rajala, M. W., Qi, Y., Patel, H. R., Takahashi, N., Banerjee, R., Pajvani, U. B., Sinha, M. K., Gingerich, R. L., Scherer, P. E. & Ahima, R. S. 2004. Regulation of resistin expression and circulating levels in obesity, diabetes, and fasting. *Diabetes,* **53,** 1671-9.

Ramsey, K. M., Yoshino, J., Brace, C. S., Abrassart, D., Kobayashi, Y., Marcheva, B., Hong, H. K., Chong, J. L., Buhr, E. D., Lee, C., Takahashi, J. S., Imai, S. & Bass, J. 2009. Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science,* **324,** 651-4.

Ravassa, S., Beloqui, O., Varo, N., Barba, J., Lopez, B., Beaumont, J., Zalba, G., Diez, J. & Gonzalez, A. 2013. Association of cardiotrophin-1 with left ventricular systolic properties in asymptomatic hypertensive patients. *J Hypertens,* **31,** 587-94.

Reaven, G. 2002. Metabolic syndrome: pathophysiology and implications for management of cardiovascular disease. *Circulation,* **106,** 286-8.

Reaven, G. M. 1988. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes,* **37,** 1595-607.

Reinehr, T., Woelfle, J. & Roth, C. L. 2011. Lack of association between apelin, insulin resistance, cardiovascular risk factors, and obesity in children: a longitudinal analysis. *Metabolism,* **60,** 1349-54.

Rendo-Urteaga, T., Garcia-Calzon, S., Martinez-Anso, E., Chueca, M., Oyarzabal, M., Azcona-Sanjulian, M. C., Bustos, M., Moreno-Aliaga, M. J., Martinez, J. A. & Marti, A. 2013. Decreased cardiotrophin-1 levels are associated with a lower risk of developing the metabolic syndrome in overweight/obese children after a weight loss program. *Metabolism,* **62,** 1429-36.

Revollo, J. R., Korner, A., Mills, K. F., Satoh, A., Wang, T., Garten, A., Dasgupta, B., Sasaki, Y., Wolberger, C., Townsend, R. R., Milbrandt, J., Kiess, W. & Imai, S. 2007. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab,* **6,** 363-75.

Robledo, O., Fourcin, M., Chevalier, S., Guillet, C., Auguste, P., Pouplard-Barthelaix, A., Pennica, D. & Gascan, H. 1997. Signaling of the cardiotrophin-1 receptor. Evidence for a third receptor component. *J Biol Chem,* **272,** 4855-63.

Rose-John, S., Scheller, J. & Schaper, F. 2015. "Family reunion" - A structured view on the composition of the receptor complexes of interleukin-6-type and interleukin-12-type cytokines. *Cytokine Growth Factor Rev*.

Rosenbaum, M., Murphy, E. M., Heymsfield, S. B., Matthews, D. E. & Leibel, R. L. 2002. Low dose leptin administration reverses effects of sustained weight-reduction on energy expenditure and circulating concentrations of thyroid hormones. *J Clin Endocrinol Metab,* **87,** 2391-4.

Rousseau, F., Chevalier, S., Guillet, C., Ravon, E., Diveu, C., Froger, J., Barbier, F., Grimaud, L. & Gascan, H. 2008. Ciliary neurotrophic factor, cardiotrophin-like cytokine, and neuropoietin share a conserved binding site on the ciliary neurotrophic factor receptor alpha chain. *J Biol Chem,* **283,** 30341-50.

Roy, D., Perreault, M. & Marette, A. 1998. Insulin stimulation of glucose uptake in skeletal muscles and adipose tissues in vivo is NO dependent. *Am J Physiol,* **274,** E692-9.

Rutters, F., Lemmens, S. G., Adam, T. C., Bremmer, M. A., Elders, P. J., Nijpels, G. & Dekker, J. M. 2014. Is social jetlag associated with an adverse endocrine, behavioral, and cardiovascular risk profile? *J Biol Rhythms,* **29,** 377-83.

Ryden, M., Arvidsson, E., Blomqvist, L., Perbeck, L., Dicker, A. & Arner, P. 2004. Targets for TNF-alpha-induced lipolysis in human adipocytes. *Biochem Biophys Res Commun,* **318,** 168-75.

Ryden, M., Dicker, A., van Harmelen, V., Hauner, H., Brunnberg, M., Perbeck, L., Lonnqvist, F. & Arner, P. 2002. Mapping of early signaling events in tumor necrosis factor-alpha -mediated lipolysis in human fat cells. *J Biol Chem,* **277,** 1085-91.

-S-

Saddi-Rosa, P., Oliveira, C., Crispim, F., Giuffrida, F. M., de Lima, V., Vieira, J., Doria, A., Velho, G. & Reis, A. 2013. Association of circulating levels of nicotinamide phosphoribosyltransferase (NAMPT/Visfatin) and of a frequent polymorphism in the promoter of the NAMPT gene with coronary artery disease in diabetic and non-diabetic subjects. *Cardiovasc Diabetol,* **12,** 119.

Saghizadeh, M., Ong, J. M., Garvey, W. T., Henry, R. R. & Kern, P. A. 1996. The expression of TNF alpha by human muscle. Relationship to insulin resistance. *J Clin Invest,* **97,** 1111-6.

Sainz, N., Barrenetxe, J., Moreno-Aliaga, M. J. & Martinez, J. A. 2015a. Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. *Metabolism,* **64,** 35-46.

Sainz, N., Gonzalez-Navarro, C. J., Martinez, J. A. & Moreno-Aliaga, M. J. 2015b. Leptin signaling as a therapeutic target of obesity. *Expert Opin Ther Targets,* **19,** 893-909.

Saito, M., Okamatsu-Ogura, Y., Matsushita, M., Watanabe, K., Yoneshiro, T., Nio-Kobayashi, J., Iwanaga, T., Miyagawa, M., Kameya, T., Nakada, K., Kawai, Y. & Tsujisaki, M. 2009. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes,* **58,** 1526-31.

Salas-Salvado, J., Rubio, M. A., Barbany, M. & Moreno, B. 2007. [SEEDO 2007 Consensus for the evaluation of overweight and obesity and the establishment of therapeutic intervention criteria]. *Med Clin (Barc),* **128,** 184-96; quiz 1 p following 200.

Samal, B., Sun, Y., Stearns, G., Xie, C., Suggs, S. & McNiece, I. 1994. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. *Mol Cell Biol,* **14,** 1431-7.

Sanchez-Infantes, D., White, U. A., Elks, C. M., Morrison, R. F., Gimble, J. M., Considine, R. V., Ferrante, A. W., Ravussin, E. & Stephens, J. M. 2014. Oncostatin m is produced in adipose tissue and is regulated in conditions of obesity and type 2 diabetes. *J Clin Endocrinol Metab,* **99,** E217-25.

Sato, T. K., Panda, S., Miraglia, L. J., Reyes, T. M., Rudic, R. D., McNamara, P., Naik, K. A., FitzGerald, G. A., Kay, S. A. & Hogenesch, J. B. 2004. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron,* **43,** 527-37.

Satoh, H., Nguyen, M. T., Miles, P. D., Imamura, T., Usui, I. & Olefsky, J. M. 2004. Adenovirus-mediated chronic "hyper-resistinemia" leads to in vivo insulin resistance in normal rats. *J Clin Invest,* **114,** 224-31.

Savage, D. B. & O'Rahilly, S. 2002. Leptin: a novel therapeutic role in lipodystrophy. *J Clin Invest,* **109,** 1285-6.

Scarpace, P. J. & Matheny, M. 1998. Leptin induction of UCP1 gene expression is dependent on sympathetic innervation. *Am J Physiol,* **275,** E259-64.

Scherberich, A., Di Maggio, N. D. & McNagny, K. M. 2013. A familiar stranger: CD34 expression and putative functions in SVF cells of adipose tissue. *World J Stem Cells,* **5,** 1-8.

Schrauwen, P., van Marken Lichtenbelt, W. D. & Spiegelman, B. M. 2015. The future of brown adipose tissues in the treatment of type 2 diabetes. *Diabetologia,* **58,** 1704-7.

Schwartz, M. W., Peskind, E., Raskind, M., Boyko, E. J. & Porte, D., Jr. 1996. Cerebrospinal fluid leptin levels: relationship to plasma levels and to adiposity in humans. *Nat Med,* **2,** 589-93.

Schwartz, M. W. & Seeley, R. J. 1997. The new biology of body weight regulation. *J Am Diet Assoc,* **97,** 54-8; quiz 59-60.

Schweiger, M., Paar, M., Eder, C., Brandis, J., Moser, E., Gorkiewicz, G., Grond, S., Radner, F. P., Cerk, I., Cornaciu, I., Oberer, M., Kersten, S., Zechner, R., Zimmermann, R. & Lass, A. 2012. G0/G1 switch gene-2 regulates human adipocyte lipolysis by affecting activity and localization of adipose triglyceride lipase. *J Lipid Res,* **53,** 2307-17.

Schweiger, M., Schoiswohl, G., Lass, A., Radner, F. P., Haemmerle, G., Malli, R., Graier, W., Cornaciu, I., Oberer, M., Salvayre, R., Fischer, J., Zechner, R. & Zimmermann, R. 2008. The C-terminal region of human adipose triglyceride lipase affects enzyme activity and lipid droplet binding. *J Biol Chem,* **283,** 17211-20.

Seale, P., Bjork, B., Yang, W., Kajimura, S., Chin, S., Kuang, S., Scime, A., Devarakonda, S., Conroe, H. M., Erdjument-Bromage, H., Tempst, P., Rudnicki, M. A., Beier, D. R. & Spiegelman, B. M. 2008. PRDM16 controls a brown fat/skeletal muscle switch. *Nature,* **454,** 961-7.

Sengenes, C., Bouloumie, A., Hauner, H., Berlan, M., Busse, R., Lafontan, M. & Galitzky, J. 2003. Involvement of a cGMP-dependent pathway in the natriuretic peptide-mediated hormone-sensitive lipase phosphorylation in human adipocytes. *J Biol Chem,* **278,** 48617-26.

Shabalina, I. G., Petrovic, N., de Jong, J. M., Kalinovich, A. V., Cannon, B. & Nedergaard, J. 2013. UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. *Cell Rep,* **5,** 1196-203.

Shan, T., Liang, X., Bi, P., Zhang, P., Liu, W. & Kuang, S. 2013. Distinct populations of adipogenic and myogenic Myf5-lineage progenitors in white adipose tissues. *J Lipid Res,* **54,** 2214-24.

Sheng, Z., Knowlton, K., Chen, J., Hoshijima, M., Brown, J. H. & Chien, K. R. 1997. Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy. *J Biol Chem,* **272,** 5783-91.

Sheng, Z., Pennica, D., Wood, W. I. & Chien, K. R. 1996. Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. *Development,* **122,** 419-28.

Shi, S. Q., Ansari, T. S., McGuinness, O. P., Wasserman, D. H. & Johnson, C. H. 2013. Circadian disruption leads to insulin resistance and obesity. *Curr Biol,* **23,** 372-81.

Shimba, S., Ishii, N., Ohta, Y., Ohno, T., Watabe, Y., Hayashi, M., Wada, T., Aoyagi, T. & Tezuka, M. 2005. Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc Natl Acad Sci U S A,* **102,** 12071-6.

Shirazi-Beechey, S. P., Moran, A. W., Batchelor, D. J., Daly, K. & Al-Rammahi, M. 2011. Glucose sensing and signalling; regulation of intestinal glucose transport. *Proc Nutr Soc,* **70,** 185-93.

Shostak, A., Meyer-Kovac, J. & Oster, H. 2013. Circadian regulation of lipid mobilization in white adipose tissues. *Diabetes,* **62,** 2195-203.

Shuldiner, A. R., Yang, R. & Gong, D. W. 2001. Resistin, obesity and insulin resistance--the emerging role of the adipocyte as an endocrine organ. *N Engl J Med,* **345,** 1345-6.

Smith, M. W., James, P. S. & Peacock, M. A. 1991. Galactose effects on enterocyte differentiation in the mouse jejunum. *Biochim Biophys Acta,* **1093,** 144-6.

Solberg, D. H. & Diamond, J. M. 1987. Comparison of different dietary sugars as inducers of intestinal sugar transporters. *Am J Physiol,* **252,** G574-84.

Sommer, G., Kralisch, S., Kloting, N., Kamprad, M., Schrock, K., Kratzsch, J., Tonjes, A., Lossner, U., Bluher, M., Stumvoll, M. & Fasshauer, M. 2010. Visfatin is a positive regulator of MCP-1 in human adipocytes in vitro and in mice in vivo. *Obesity (Silver Spring),* **18,** 1486-92.

Song, H. K., Lee, M. H., Kim, B. K., Park, Y. G., Ko, G. J., Kang, Y. S., Han, J. Y., Han, S. Y., Han, K. H., Kim, H. K. & Cha, D. R. 2008a. Visfatin: a new player in mesangial cell physiology and diabetic nephropathy. *Am J Physiol Renal Physiol,* **295,** F1485-94.

Song, J., Zhang, Y. W., Yao, A. H., Yu, Y., Hua, Z. Y., Pu, L. Y., Li, G. Q., Li, X. C., Zhang, F., Sheng, G. Q. & Wang, X. H. 2008b. Adenoviral cardiotrophin-1 transfer improves survival and early graft function after ischemia and reperfusion in rat small-for-size liver transplantation model. *Transpl Int,* **21,** 372-83.

Sopjani, M., Bhavsar, S. K., Fraser, S., Kemp, B. E., Foller, M. & Lang, F. 2010. Regulation of Na+-coupled glucose carrier SGLT1 by AMP-activated protein kinase. *Mol Membr Biol,* **27,** 137-44.

Soriguer, F., Garrido-Sanchez, L., Garcia-Serrano, S., Garcia-Almeida, J. M., Garcia-Arnes, J., Tinahones, F. J. & Garcia-Fuentes, E. 2009. Apelin levels are increased in morbidly obese subjects with type 2 diabetes mellitus. *Obes Surg,* **19,** 1574-80.

Souza, S. C., Palmer, H. J., Kang, Y. H., Yamamoto, M. T., Muliro, K. V., Paulson, K. E. & Greenberg, A. S. 2003. TNF-alpha induction of lipolysis is mediated through activation of the extracellular signal related kinase pathway in 3T3-L1 adipocytes. *J Cell Biochem,* **89,** 1077-86.

Spinnler, R., Gorski, T., Stolz, K., Schuster, S., Garten, A., Beck-Sickinger, A. G., Engelse, M. A., de Koning, E. J., Korner, A., Kiess, W. & Maedler, K. 2013. The adipocytokine Nampt and its product NMN have no effect on beta-cell survival but potentiate glucose stimulated insulin secretion. *PLoS One,* **8,** e54106.

Stephanou, A., Brar, B., Heads, R., Knight, R. D., Marber, M. S., Pennica, D. & Latchman, D. S. 1998. Cardiotrophin-1 induces heat shock protein accumulation in cultured cardiac cells and protects them from stressful stimuli. *J Mol Cell Cardiol,* **30,** 849-55.

Stephens, J. M. & Vidal-Puig, A. J. 2006. An update on visfatin/pre-B cell colony-enhancing factor, an ubiquitously expressed, illusive cytokine that is regulated in obesity. *Curr Opin Lipidol,* **17,** 128-31.

Steppan, C. M., Bailey, S. T., Bhat, S., Brown, E. J., Banerjee, R. R., Wright, C. M., Patel, H. R., Ahima, R. S. & Lazar, M. A. 2001a. The hormone resistin links obesity to diabetes. *Nature,* **409,** 307-12.

Steppan, C. M., Brown, E. J., Wright, C. M., Bhat, S., Banerjee, R. R., Dai, C. Y., Enders, G. H., Silberg, D. G., Wen, X., Wu, G. D. & Lazar, M. A. 2001b. A family of tissue-specific resistin-like molecules. *Proc Natl Acad Sci U S A,* **98,** 502-6.

Stumpel, F., Scholtka, B. & Jungermann, K. 1997. A new role for enteric glucagon-37: acute stimulation of glucose absorption in rat small intestine. *FEBS Lett,* **410,** 515-9.

Subramanian, S., Glitz, P., Kipp, H., Kinne, R. K. & Castaneda, F. 2009. Protein kinase-A affects sorting and conformation of the sodium-dependent glucose co-transporter SGLT1. *J Cell Biochem,* **106,** 444-52.

Syed Ikmal, S. I., Zaman Huri, H., Vethakkan, S. R. & Wan Ahmad, W. A. 2013. Potential biomarkers of insulin resistance and atherosclerosis in type 2 diabetes mellitus patients with coronary artery disease. *Int J Endocrinol,* **2013,** 698567.

-T-

Takahashi-Tezuka, M., Hibi, M., Fujitani, Y., Fukada, T., Yamaguchi, T. & Hirano, T. 1997. Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK. *Oncogene,* **14,** 2273-82.

Talwar, S. & Choudhary, S. K. 2003. Tuberculous aneurysms of the aorta. *J Thorac Cardiovasc Surg,* **125,** 1184.

Tartaglia, L. A. Dembski, M. Weng, X. Deng, N. Culpepper, J. Devos, R. Richards, G. J. Campfield, L. A. Clark, F. T. Deeds, J. Muir, C. Sanker, S. Moriarty, A. Moore, K. J. Smutko, J. S. Mays, G. G. *et al.* 1995. Identification and expression cloning of a leptin receptor, OB-R. *Cell,* **83,** 1263-71.

Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M. X., Kawamata, Y., Fukusumi, S., Hinuma, S., Kitada, C., Kurokawa, T., Onda, H. & Fujino, M. 1998. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem Biophys Res Commun,* **251,** 471-6.

Telejko, B., Kuzmicki, M., Wawrusiewicz-Kurylonek, N., Szamatowicz, J., Nikolajuk, A., Zonenberg, A., Zwierz-Gugala, D., Jelski, W., Laudanski, P., Wilczynski, J., Kretowski, A. & Gorska, M. 2010. Plasma apelin levels and apelin/APJ mRNA expression in patients with gestational diabetes mellitus. *Diabetes Res Clin Pract,* **87,** 176-83.

Than, A., Cheng, Y., Foh, L. C., Leow, M. K., Lim, S. C., Chuah, Y. J., Kang, Y. & Chen, P. 2012. Apelin inhibits adipogenesis and lipolysis through distinct molecular pathways. *Mol Cell Endocrinol,* **362,** 227-41.

Than, A., He, H. L., Chua, S. H., Xu, D., Sun, L., Leow, M. K. & Chen, P. 2015. Apelin Enhances Brown Adipogenesis and Browning of White Adipocytes. *J Biol Chem,* **290,** 14679-91.

Thompson, B. R., Lobo, S. & Bernlohr, D. A. 2010. Fatty acid flux in adipocytes: the in's and out's of fat cell lipid trafficking. *Mol Cell Endocrinol,* **318,** 24-33.

Tsang, R., Ao, Z. & Cheeseman, C. 1994. Influence of vascular and luminal hexoses on rat intestinal basolateral glucose transport. *Can J Physiol Pharmacol,* **72,** 317-26.

Tunon, M. J., San Miguel, B., Crespo, I., Riezu-Boj, J. I., Larrea, E., Alvarez, M., Gonzalez, I., Bustos, M., Gonzalez-Gallego, J. & Prieto, J. 2011. Cardiotrophin-1 promotes a high survival rate in rabbits with lethal fulminant hepatitis of viral origin. *J Virol,* **85,** 13124-32.

Turk, E., Kerner, C. J., Lostao, M. P. & Wright, E. M. 1996. Membrane topology of the human Na+/glucose cotransporter SGLT1. *J Biol Chem,* **271,** 1925-34.

-U-

Ukai, H. & Ueda, H. R. 2010. Systems biology of mammalian circadian clocks. *Annu Rev Physiol,* **72,** 579-603.

-V-

van Marken Lichtenbelt, W. D., Vanhommerig, J. W., Smulders, N. M., Drossaerts, J. M., Kemerink, G. J., Bouvy, N. D., Schrauwen, P. & Teule, G. J. 2009. Cold-activated brown adipose tissue in healthy men. *N Engl J Med,* **360,** 1500-8.

Vayro, S. & Silverman, M. 1999. PKC regulates turnover rate of rabbit intestinal Na+-glucose transporter expressed in COS-7 cells. *Am J Physiol,* **276,** C1053-60.

Villena, J. A., Roy, S., Sarkadi-Nagy, E., Kim, K. H. & Sul, H. S. 2004. Desnutrin, an adipocyte gene encoding a novel patatin domain-containing protein, is induced by fasting and glucocorticoids: ectopic expression of desnutrin increases triglyceride hydrolysis. *J Biol Chem,* **279,** 47066-75.

Vinuales, C., Gascon, S., Barranquero, C., Osada, J. & Rodriguez-Yoldi, M. J. 2013. Interleukin-1beta reduces galactose transport in intestinal epithelial cells in a NF-kB and protein kinase C-dependent manner. *Vet Immunol Immunopathol,* **155,** 171-81.

Virtanen, K. A., Lidell, M. E., Orava, J., Heglind, M., Westergren, R., Niemi, T., Taittonen, M., Laine, J., Savisto, N. J., Enerback, S. & Nuutila, P. 2009. Functional brown adipose tissue in healthy adults. *N Engl J Med,* **360,** 1518-25.

-W-

Wang, D., Li, X., Gao, K., Lu, D., Zhang, X., Ma, C., Ye, F. & Zhang, L. 2013. Cardiotrophin-1 (CTF1) ameliorates glucose-uptake defects and improves memory and learning deficits in a transgenic mouse model of Alzheimer's disease. *Pharmacol Biochem Behav,* **107,** 48-57.

Wang, D., Liu, L., Yan, J., Wu, W., Zhu, X. & Wang, Y. 2015. Cardiotrophin-1 (CT-1) improves high fat diet-induced cognitive deficits in mice. *Neurochem Res,* **40,** 843-53.

Wang, J., Liu, R., Hawkins, M., Barzilai, N. & Rossetti, L. 1998. A nutrient-sensing pathway regulates leptin gene expression in muscle and fat. *Nature,* **393,** 684-8.

Wang, Y., Robledo, O., Kinzie, E., Blanchard, F., Richards, C., Miyajima, A. & Baumann, H. 2000. Receptor subunit-specific action of oncostatin M in hepatic cells and its modulation by leukemia inhibitory factor. *J Biol Chem,* **275,** 25273-85.

Watt, M. J., Dzamko, N., Thomas, W. G., Rose-John, S., Ernst, M., Carling, D., Kemp, B. E., Febbraio, M. A. & Steinberg, G. R. 2006a. CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK. *Nat Med,* **12,** 541-8.

Watt, M. J., Holmes, A. G., Pinnamaneni, S. K., Garnham, A. P., Steinberg, G. R., Kemp, B. E. & Febbraio, M. A. 2006b. Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. *Am J Physiol Endocrinol Metab,* **290,** E500-8.

Welty, F. K., Alfaddagh, A. & Elajami, T. K. 2015. Targeting inflammation in metabolic syndrome. *Transl Res*.

Wellen, K. E. & Hotamisligil, G. S. 2005. Inflammation, stress, and diabetes. *J Clin Invest,* **115,** 1111-9.

Wolf, G. 2009. Adipose-specific phospholipase as regulator of adiposity. *Nutr Rev,* **67,** 551-4.

Wollert, K. C., Taga, T., Saito, M., Narazaki, M., Kishimoto, T., Glembotski, C. C., Vernallis, A. B., Heath, J. K., Pennica, D., Wood, W. I. & Chien, K. R. 1996. Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy. Assembly of sarcomeric units in series VIA gp130/leukemia inhibitory factor receptor-dependent pathways. *J Biol Chem,* **271,** 9535-45.

Wright, E. M. 2001. Renal Na(+)-glucose cotransporters. *Am J Physiol Renal Physiol,* **280,** F10-8.

Wright, E. M., Hirsch, J. R., Loo, D. D. & Zampighi, G. A. 1997. Regulation of Na+/glucose cotransporters. *J Exp Biol,* **200,** 287-93.

Wright, E. M., Loo, D. D. & Hirayama, B. A. 2011. Biology of human sodium glucose transporters. *Physiol Rev,* **91,** 733-94.

Wu, J. Bostrom, P. Sparks, L. M. Ye, L. Choi, J. H. Giang, A. H. Khandekar, M. Virtanen, K. A. Nuutila, P. Schaart, G. Huang, K. Tu, H. van Marken Lichtenbelt, W. D. Hoeks, J. Enerback, S. Schrauwen, P. *et al.* 2012. Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. *Cell,* **150,** 366-76.

-X-

Xie, H., Tang, S. Y., Luo, X. H., Huang, J., Cui, R. R., Yuan, L. Q., Zhou, H. D., Wu, X. P. & Liao, E. Y. 2007. Insulin-like effects of visfatin on human osteoblasts. *Calcif Tissue Int,* **80,** 201-10.

-Y-

Yamagishi, S. I., Edelstein, D., Du, X. L., Kaneda, Y., Guzman, M. & Brownlee, M. 2001. Leptin induces mitochondrial superoxide production and monocyte chemoattractant protein-1 expression in aortic endothelial cells by increasing fatty acid oxidation via protein kinase A. *J Biol Chem,* **276,** 25096-100.

Yamamoto, T., Habata, Y., Matsumoto, Y., Yasuhara, Y., Hashimoto, T., Hamajyo, H., Anayama, H., Fujii, R., Fuse, H., Shintani, Y. & Mori, M. 2011. Apelin-transgenic mice exhibit a resistance against diet-induced obesity by increasing vascular mass and mitochondrial biogenesis in skeletal muscle. *Biochim Biophys Acta,* **1810,** 853-62.

Yang, G., Jia, Z., Aoyagi, T., McClain, D., Mortensen, R. M. & Yang, T. 2012. Systemic PPARgamma deletion impairs circadian rhythms of behavior and metabolism. *PLoS One,* **7,** e38117.

Yang, R. Z., Huang, Q., Xu, A., McLenithan, J. C., Eisen, J. A., Shuldiner, A. R., Alkan, S. & Gong, D. W. 2003. Comparative studies of resistin expression and phylogenomics in human and mouse. *Biochem Biophys Res Commun,* **310,** 927-35.

Yang, X., Lu, X., Lombes, M., Rha, G. B., Chi, Y. I., Guerin, T. M., Smart, E. J. & Liu, J. 2010. The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. *Cell Metab,* **11,** 194-205.

Yang, Z. F., Lau, C. K., Ngai, P., Lam, S. P., Ho, D. W., Poon, R. T. & Fan, S. T. 2008. Cardiotrophin-1 enhances regeneration of cirrhotic liver remnant after hepatectomy through promotion of angiogenesis and cell proliferation. *Liver Int,* **28,** 622-31.

Yilmaz, M. I., Saglam, M., Carrero, J. J., Qureshi, A. R., Caglar, K., Eyileten, T., Sonmez, A., Cakir, E., Yenicesu, M., Lindholm, B., Stenvinkel, P. & Axelsson, J. 2008. Serum visfatin concentration and endothelial dysfunction in chronic kidney disease. *Nephrol Dial Transplant,* **23,** 959-65.

Yin, W., Mu, J. & Birnbaum, M. J. 2003. Role of AMP-activated protein kinase in cyclic AMP-dependent lipolysis In 3T3-L1 adipocytes. *J Biol Chem,* **278,** 43074-80.

Yue, P., Jin, H., Aillaud, M., Deng, A. C., Azuma, J., Asagami, T., Kundu, R. K., Reaven, G. M., Quertermous, T. & Tsao, P. S. 2010. Apelin is necessary for the maintenance of insulin sensitivity. *Am J Physiol Endocrinol Metab,* **298,** E59-67.

Yue, P., Jin, H., Xu, S., Aillaud, M., Deng, A. C., Azuma, J., Kundu, R. K., Reaven, G. M., Quertermous, T. & Tsao, P. S. 2011. Apelin decreases lipolysis via G(q), G(i), and AMPK-Dependent Mechanisms. *Endocrinology,* **152,** 59-68.

Yun, E. S., Park, S. S., Shin, H. C., Choi, Y. H., Kim, W. J. & Moon, S. K. 2011. p38 MAPK activation is required for esculetin-induced inhibition of vascular smooth muscle cells proliferation. *Toxicol In Vitro,* **25,** 1335-42.

-Z-

Zechner, R., Kienesberger, P. C., Haemmerle, G., Zimmermann, R. & Lass, A. 2009. Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J Lipid Res,* **50,** 3-21.

Zechner, R., Zimmermann, R., Eichmann, T. O., Kohlwein, S. D., Haemmerle, G., Lass, A. & Madeo, F. 2012. FAT SIGNALS--lipases and lipolysis in lipid metabolism and signaling. *Cell Metab,* **15,** 279-91.

Zeng, W., Pirzgalska, R. M., Pereira, M. M., Kubasova, N., Barateiro, A., Seixas, E., Lu, Y. H., Kozlova, A., Voss, H., Martins, G. G., Friedman, J. M. & Domingos, A. I. 2015. Sympathetic Neuro-adipose Connections Mediate Leptin-Driven Lipolysis. *Cell,* **163,** 84-94.

Zhang, F., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., Clawson, D. K., DiMarchi, R. D., Furman, T. C., Hale, J. E., Hsiung, H. M., Schoner, B. E., Smith, D. P., Zhang, X. Y., Wery, J. P. & Schevitz, R. W. 1997. Crystal structure of the obese protein leptin-E100. *Nature,* **387,** 206-9.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* **372,** 425-32.

Zhang, Z. F., Liao, W. H., Yang, Q. F., Li, H. Y., Wu, Y. M. & Zhou, X. F. 2003. Protective effects of adenoviral cardiotrophin-1 gene transfer on rubrospinal neurons after spinal cord injury in adult rats. *Neurotox Res,* **5,** 539-48.

Zhu, S., Sun, F., Li, W., Cao, Y., Wang, C., Wang, Y., Liang, D., Zhang, R., Zhang, S., Wang, H. & Cao, F. 2011. Apelin stimulates glucose uptake through the PI3K/Akt pathway and improves insulin resistance in 3T3-L1 adipocytes. *Mol Cell Biochem,* **353,** 305-13.

Zile, M. R., Desantis, S. M., Baicu, C. F., Stroud, R. E., Thompson, S. B., McClure, C. D., Mehurg, S. M. & Spinale, F. G. 2011. Plasma biomarkers that reflect determinants of matrix composition identify the presence of left ventricular hypertrophy and diastolic heart failure. *Circ Heart Fail,* **4,** 246-56.

Zimmermann, R., Strauss, J. G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A. & Zechner, R. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science,* **306,** 1383-6.

Zolk, O., Engmann, S., Munzel, F. & Krajcik, R. 2005. Chronic cardiotrophin-1 stimulation impairs contractile function in reconstituted heart tissue. *Am J Physiol Endocrinol Metab,* **288,** E1214-21.

Zvonic, S., Hogan, J. C., Arbour-Reily, P., Mynatt, R. L. & Stephens, J. M. 2004. Effects of cardiotrophin on adipocytes. *J Biol Chem,* **279,** 47572-9.

II. HYPOTHESIS AND AIMS

In the last years, several studies from our group and others have pointed out that CT-1 might play a key role in the regulation of body weight and intermediate metabolism (Natal *et al.* 2008, Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). In fact, CT-1 deficiency causes a late-onset adult obesity in mice, accompanied by insulin resistance and hypercholesterolemia. Moreover chronic administration of recombinant CT-1 to mice reduces food intake and increases energy expenditure leading to a reduction in body weight and fat mass, improving insulin sensitivity in obese animals (Moreno-Aliaga *et al.* 2011, Wang *et al.* 2013). CT-1 seems to be also a pivotal regulator of lipid metabolism by reducing hepatic lipogenesis while promoting fatty acid oxidation in both liver and muscle through AMPK activation (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). CT-1 also has a relevant glucose-lowering effect, in part by promoting glucose uptake and increasing insulin signaling in muscle (Moreno-Aliaga *et al.* 2011).

White adipose tissue (WAT) is also a target organ for CT-1 action, which is able to activate major signaling pathways involved in the control of metabolism in this tissue (Zvonic *et al.* 2004, Natal *et al.* 2008). Thus, our previous data revealed that chronic CT-1 administration induced a dramatic remodeling of WAT characterized by a reduction of adipocyte size accompanied by the downregulation of lipogenic genes and upregulation of genes involved in lipid catabolism. In this context, our previous data in mice suggest lipolytic properties for CT-1. However, the mechanism underlying the lipolytic action of CT-1 still remains unknown. Moreover, CT-1 promotes mitochondrial biogenesis and adipose tissue browning (Moreno-Aliaga *et al.* 2011).

During the last decade, WAT has also been established as an important endocrine organ with a key relevance in the regulation of food intake, energy expenditure and glucose and lipid homeostasis (Romacho *et al.* 2014). Indeed, WAT is responsible for the expression and secretion of an array of molecules known as adipokines that can regulate many physiological processes (Kloting *et al.* 2014). However, the potential ability of CT-1 to regulate adipocyte secretory function as well as their potential involvement in the metabolic actions of CT-1 has not been yet described.

In recent years, the intestine has also emerged as an important metabolic organ with a role in the pathophysiology of various metabolic diseases including obesity, insulin resistance and diabetes (Bradley *et al.* 2011). Recently, the sodium-glucose

co-transporters that mediate intestinal glucose absorption have been considered to be new therapeutic targets to reduce hyperglycemia in diabetes. Agents that inhibit those co-transporters (SGLT inhibitors) increase glucose excretion and help control hyperglycemia through an insulin-independent mechanism, introducing a new concept to the diabetes treatment (Chao 2014). There is not information available about the physiological/pharmacological actions of CT-1 on intestinal absorptive function. Therefore, it is important to characterize if the glucose-lowering effects of CT-1 are also related to its potential actions in the regulation of intestinal glucose absorption.

The circadian system has a great influence on metabolic disturbances and *vice versa*. Although the exact mechanisms linking metabolic syndrome with chronodisruption are still unknown, most hypotheses point to an internal desynchronization of circadian rhythms involved in metabolism (Garaulet *et al.* 2009). Some studies have implicated various cytokines such as IL-6 in the regulation of certain clock genes as human PER1 (Motzkus *et al.* 2002). The control of the circadian rhythms relies on a complex, highly integrated network of clocks distributed throughout the organism. In this context, adipose tissue contains the machinery necessary to be recognized as a peripheral circadian oscillator that contributes to synchronize the central pacemaker to the same phase (Mohawk *et al.* 2012). Interestingly, several adipokines secreted by adipose exhibit profound day/night circadian rhythms, and accumulating evidence links disruption of these rhythms to the development of metabolic disturbances (van der Spek *et al.* 2012). However, the potential involvement of CT-1 on the control of clock genes and circadian rhythm machinery, especially in obesity remains still unknown.

Based on all the previously mentioned observations, the **hypothesis** of the present study is that the anti-obesity and anti-diabetic actions of CT-1 could be also related to its potential ability to regulate lipolysis, adipokine secretion and intestinal sugar absorption as well as its involvement in the control of circadian clocks.

The **main objective** of the present study was to analyze the role of CT-1 in the regulation of lipolysis and the secretion of adipokines in adipocytes as well as to characterize its potential implication in intestinal sugar absorption and its role in the regulation of circadian metabolic clocks.

The **specific aims** were:

1. To determine the effects of CT-1 on lipolysis by characterizing its actions on the main lipases and lipid droplet proteins involved in the control of the lipolytic pathway *in vitro* and *in vivo*.

2. To analyze the actions of CT-1 on the production of four adipokines (leptin, resistin, visfatin and apelin) involved in the regulation of body weight, insulin sensitivity and inflammation, and to characterize the major signaling pathways implicated.

3. To characterize the ability of CT-1 to regulate intestinal sugar absorption, as well as to identify its actions on the sodium-dependent glucose transporter-1 (SGLT-1) and the cellular mechanisms that could underlie these effects.

4. To analyze whether CT-1 (deficiency or treatment) could be regulating metabolic circadian rhythm and the expression of core circadian clock genes in white adipose tissue of mice and to characterize the potential circadian rhythm/diurnal variations of plasma CT-1 profile in normal weight and overweight/obese subjects.

En los últimos años, varios estudios de nuestro grupo y otros han señalado que CT-1 podría jugar un papel clave en la regulación del peso corporal y el metabolismo (Natal *et al.* 2008, Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). De hecho, la deficiencia de CT-1 provoca el desarrollo de obesidad en ratones adultos, acompañada por insulino-resistencia e hipercolesterolemia. Además, se vio que la administración crónica de CT-1 recombinante en ratones reduce la ingesta de alimentos y aumenta el gasto energético provocando una reducción en el peso corporal y la masa grasa, mejorando la sensibilidad a la insulina en los animales obesos (Moreno-Aliaga *et al.* 2011, Wang *et al.* 2013). CT-1 parece ser también un importante regulador del metabolismo lipídico al reducir la lipogénesis hepática mientras que promueve la oxidación de ácidos grasos en el hígado y el músculo a través de la activación de la AMPK (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). CT-1 también tiene un importante efecto reductor de la glucemia, en parte gracias al aumento de la captación de glucosa y de la señalización de insulina en el músculo (Moreno-Aliaga *et al.* 2011).

El tejido adiposo blanco es también un órgano diana para la acción de CT-1, ya que es capaz de activar las principales vías de señalización implicadas en el control del metabolismo en este tejido (Zvonic *et al.* 2004, Natal *et al.* 2008). En este sentido, nuestros datos previos revelaron que la administración crónica de CT-1 promueve una remodelación de tejido adiposo blanco caracterizada por una reducción de tamaño del adipocito acompañada por la inhibición de genes lipogénicos y la estimulación de genes implicados en el catabolismo de los lípidos. En este contexto, a pesar de que los estudios en ratones sugieren que CT-1 podría tener propiedades lipolíticas, los mecanismos que subyacen a estas acciones siguen siendo desconocidos. Por otra parte, CT-1 promueve la biogénesis mitocondrial y el pardeamiento del tejido adiposo (Moreno-Aliaga *et al.* 2011).

Durante la última década, el tejido adiposo blanco también se ha postulado como un importante órgano endocrino con una gran relevancia en la regulación de la ingesta de alimentos, el gasto energético y la homeostasis de la glucosa y los lípidos (Romacho *et al.* 2014). De hecho, el tejido adiposo blanco es responsable de la expresión y secreción de un amplio espectro de moléculas conocidas como adipoquinas que pueden regular importantes procesos fisiológicos (Kloting *et al.* 2014). Sin embargo, la

potencial capacidad de CT-1 para regular la función secretora de los adipocitos, así como su implicación en las acciones metabólicas de CT-1 aún no han sido analizadas.

En los últimos años, el intestino también se ha postulado como un órgano metabólico importante con un papel en la fisiopatología de varias enfermedades metabólicas como la obesidad, la insulino-resistencia y la diabetes tipo 2 (Bradley *et al.* 2011). Recientemente, los co-transportadores de sodio-glucosa que median la absorción intestinal de glucosa se han considerado como nuevas dianas terapéuticas para reducir la hiperglucemia en la diabetes. Los agentes que inhiben a estos co-transportadores (inhibidores de SGLT) aumentan la excreción de glucosa y ayudan a controlar la hiperglucemia a través de mecanismos independientes de la insulina, introduciendo un nuevo concepto para el tratamiento de la diabetes (Chao 2014). Sin embargo, no hay información disponible acerca de las acciones fisiológicas/farmacológicas de CT-1 sobre la absorción intestinal. Por lo tanto, es importante caracterizar si los efectos hipoglucemiantes de CT-1 podrían estar también relacionados con sus posibles acciones en la regulación de la absorción intestinal de glucosa.

El sistema circadiano tiene una gran influencia sobre los trastornos metabólicos y viceversa. Aunque los mecanismos exactos que relacionan la cronodisrupción con el síndrome metabólico son aún poco conocidos, la mayoría de las hipótesis apuntan a una desincronización de los ritmos circadianos internos implicados en el metabolismo (Garaulet *et al.* 2009). Algunos estudios han demostrado el papel de diversas citoquinas tales como IL-6 en la regulación de ciertos genes reloj como PER1 en humanos (Motzkus *et al.* 2002). El control de los ritmos circadianos está formado por una compleja red, altamente integrada de relojes distribuidos por todo el organismo. En este sentido, el tejido adiposo contiene la maquinaria necesaria para ser reconocido como un oscilador circadiano periférico que contribuye a sincronizar el marcapasos central (Mohawk *et al.* 2012). Curiosamente, varias adipoquinas segregadas por el tejido adiposo presentan profundos ritmos circadianos, y varios estudios han demostrado que la interrupción de estos ritmos podría estar relacionada con el desarrollo de trastornos metabólicos (van der Spek *et al.* 2012). Sin embargo, la posible participación de CT-1 sobre el control de los genes del reloj y la maquinaria del ritmo circadiano en la obesidad sigue siendo desconocida.

En base a todas las observaciones mencionadas anteriormente, la **hipótesis** del presente estudio es que las acciones contra la obesidad y anti-diabéticas de CT-1 podrían ser subyacentes a su capacidad para regular la lipólisis, la secreción de adipoquinas, así como la absorción intestinal de azúcares y su implicación en la regulación circadiana del metabolismo.

El **objetivo principal** del presente estudio fue analizar el papel de CT-1 en la regulación de la lipólisis y la secreción de adipoquinas, así como caracterizar su potencial implicación en la absorción intestinal de azúcares y su función en el control circadiano del metabolismo.

Los **objetivos específicos** fueron:

1. Determinar los efectos de CT-1 en la lipólisis, y caracterizar sus acciones sobre las principales lipasas y proteínas asociadas a la gota lipídica involucradas en el control de la ruta lipolítica *in vitro* e *in vivo*.

2. Analizar las acciones de CT-1 en la producción de cuatro adipoquinas (leptina, resistina, visfatina y apelina) que participan en la regulación del peso corporal, la sensibilidad a la insulina y la inflamación, y caracterizar las principales vías de señalización implicadas.

3. Evaluar la capacidad de CT-1 para regular la absorción intestinal de azúcares, así como identificar sus acciones en la expresión del transportador de glucosa dependiente de sodio-1 (SGLT-1) y los mecanismos celulares que podrían estar mediando estos efectos.

4. Analizar si CT-1 (deficiencia o tratamiento) podría estar regulando el ritmo circadiano y la expresión de los principales genes del reloj en el tejido adiposo blanco de ratón, así como caracterizar el potencial ritmo circadiano/variaciones diurnas plasmáticas de CT-1 en sujetos con normopeso y con sobrepeso/obesidad.

REFERENCES

-B-

Bradley, W. D., Zwingelstein, C. & Rondinone, C. M. 2011. The emerging role of the intestine in metabolic diseases. *Arch Physiol Biochem,* **117,** 165-76.

-C-

Castano, D., Larequi, E., Belza, I., Astudillo, A. M., Martinez-Anso, E., Balsinde, J., Argemi, J., Aragon, T., Moreno-Aliaga, M. J., Muntane, J., Prieto, J. & Bustos, M. 2014. Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation. *J Hepatol,* **60,** 1017-25.

Chao, E. C. 2014. SGLT-2 Inhibitors: A New Mechanism for Glycemic Control. *Clin Diabetes,* **32,** 4-11.

-G-

Garaulet, M. & Madrid, J. A. 2009. Chronobiology, genetics and metabolic syndrome. *Curr Opin Lipidol,* **20,** 127-34.

-K-

Kloting, N. & Bluher, M. 2014. Adipocyte dysfunction, inflammation and metabolic syndrome. *Rev Endocr Metab Disord,* **15,** 277-87.

-M-

Mohawk, J. A., Green, C. B. & Takahashi, J. S. 2012. Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci,* **35,** 445-62.

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* **14,** 242-53.

Motzkus, D., Albrecht, U. & Maronde, E. 2002. The human PER1 gene is inducible by interleukin-6. *J Mol Neurosci,* **18,** 105-9.

-N-

Natal, C., Fortuno, M. A., Restituto, P., Bazan, A., Colina, I., Diez, J. & Varo, N. 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. *Am J Physiol Endocrinol Metab,* **294,** E52-60.

-R-

Romacho, T., Elsen, M., Rohrborn, D. & Eckel, J. 2014. Adipose tissue and its role in organ crosstalk. *Acta Physiol (Oxf),* **210,** 733-53.

-V-

van der Spek, R., Kreier, F., Fliers, E. & Kalsbeek, A. 2012. Circadian rhythms in white adipose tissue. *Prog Brain Res,* **199,** 183-201.

-W-

Wang, D., Li, X., Gao, K., Lu, D., Zhang, X., Ma, C., Ye, F. & Zhang, L. 2013. Cardiotrophin-1 (CTF1) ameliorates glucose-uptake defects and improves memory and learning deficits in a transgenic mouse model of Alzheimer's disease. *Pharmacol Biochem Behav,* **107,** 48-57.

-Z-

Zvonic, S., Hogan, J. C., Arbour-Reily, P., Mynatt, R. L. & Stephens, J. M. 2004. Effects of cardiotrophin on adipocytes. *J Biol Chem,* **279,** 47572-9.

III.EXPERIMENTAL DESIGN AND METHODS

CHAPTER 1. "Cardiotrophin-1 stimulates lipolysis through the regulation of main adipose tissue lipases"

In vitro studies

- Cell model and experimental design

3T3-L1 mouse embryo fibroblast (American Type Culture Collection; Rockville, MD) were cultured and differentiated to adipocytes as previously described (Fernandez-Galilea *et al.* 2012). 3T3-L1 adipocytes were serum starved for 4 h and then treated with recombinant CT-1 (rCT-1) at several concentrations (1-40 ng/mL) for different time intervals (1-24 h). Comparative studies with IL-6 (20 ng/mL, 24 h) were also carried out. For the study of the potential signaling pathways involved in the actions of rCT-1 on lipolysis, pretreatment for 1 h with specific inhibitors or activators of these pathways was performed.

Figure 13 summarizes the experimental design and assays performed in chapter 1 to analyze the lipolytic actions of rCT-1 in adipocytes, including the evaluation of its actions in the regulation of the major lipases and lipid droplet proteins involved in the hydrolysis of TAG and the characterization of the major signaling pathways implicated (more detailed information about the protocols is described in the section of Methods of the corresponding manuscript).

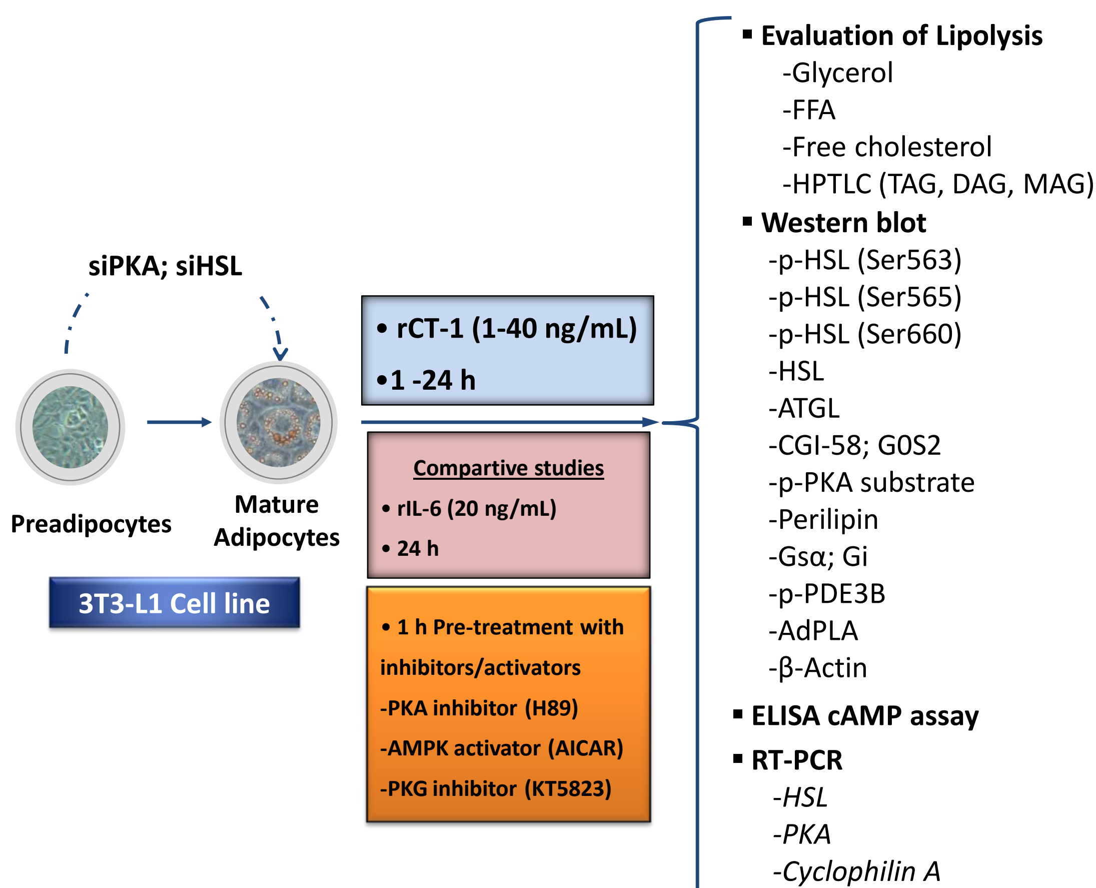


Figure 13: *In vitro* experimental design for Chapter 1.

- <u>Evaluation of lipolysis in 3T3-L1 adipocytes</u>

  - **Glycerol** measurements in cell culture media were performed using the Pentra C200 autoanalyzer.

  - **Free fatty acids (FFA)** in cell culture media were evaluated by using the Lipolysis Assay KIT for Free Fatty Acids Detection.

  - **Triacylglycerol (TAG), Diacylglycerol (DAG) and monoacylglycerol** (MAG) levels were determined by high performance thin-layer chromatography (HPTLC). **Free cholesterol** was determined by the Wako Free Cholesterol E test.

- <u>Western Blots</u>

Analysis of the major lipases and regulatory proteins involved in lipolysis was carried out by **Western blot** technique. The following antibodies were used: phospho-HSL (Ser563), phospho-HSL (Ser565), phospho-HSL (Ser660), HSL, ATGL, CGI-58, G0S2, phospho (Ser/Thr) PKA substrate, perilipin, G protein alpha S (Gsα), G Protein alpha

Inhibitor 1+2 (Gi), Phospho-phosphodiesterase 3B (p-PDE3B), adipocyte phospholipase A2 (AdPLA) and β-Actin **(Table 4)**.

- cAMP assay

The amount of intracellular cAMP in 3T3-L1 adipocytes was quantified using the cAMP Direct ELISA kit **(Table 5)**.

- Small interfering RNA experiments

Cells were transfected with small-interfering RNA (siRNA) specific to PKA and HSL. Transfection of 3T3-L1 adipocytes was performed using the Amaxa® Cell Line Nucleofector® kit L with the Nucleofector® II system.

- Analyses of mRNA levels

mRNA expression levels of *HSL*, *PKA and Cyclophilin A* (as housekeeping gene) were measured by real-time-PCR using iCycler and iQ SYBR Green Supermix **(Table 6)**.

In vivo studies

- Animal model and experimental design

Eight-week-old male *ob/ob* (C57BL/6J background) mice were supplied by the Janvier Laboratory (Le Genest St. Isle, France). The animals were established at the Animal facilities of the Center for Applied Medical Research (CIMA) of the University of Navarra (UN). All experiments were performed according to national and institutional animal care and use guidelines and with the approval of the Ethics Committee for Animal Experimentation (CEEA) of the UN.

The animals were randomly divided into two experimental groups **(Figure 14)**:

- ***ob/ob* + saline** (n = 4): Treated with vehicle (saline) intravenously (i.v.) (retro-orbital injection) for 30 min.
- ***ob/ob* + rCT-1-treated** (n = 5): Treated with rCT-1 (0.2 mg/kg of body weight) i.v. for 30 min.

Mice were euthanized and epididymal fat was snap frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

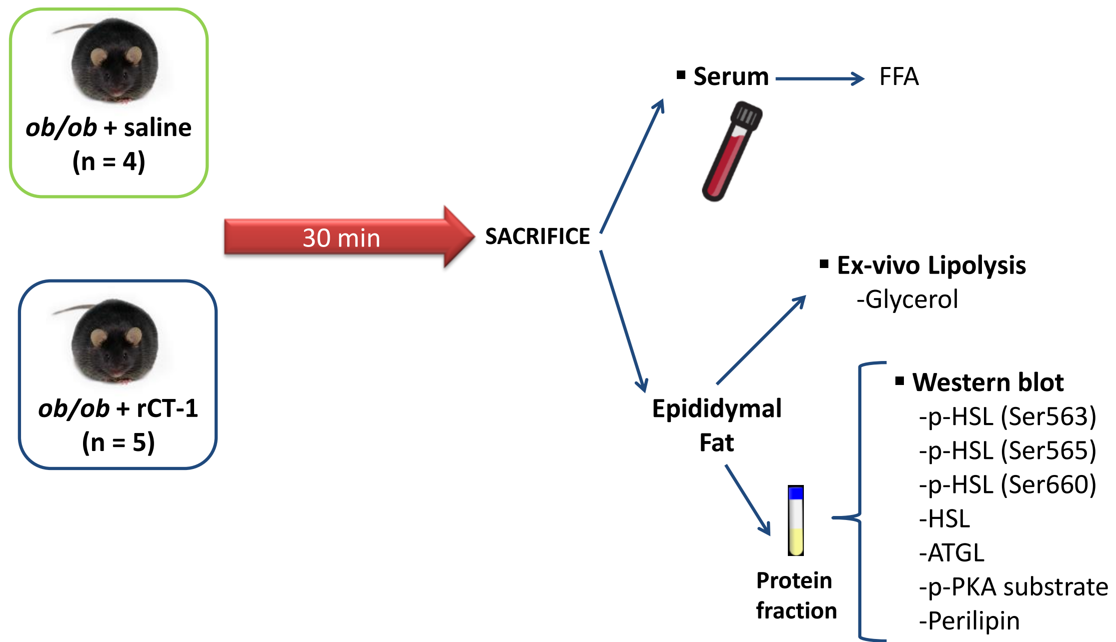


Figure 14: *In vivo* experimental design for Chapter 1.

- Evaluation of lipolysis in mice

  - **FFA in serum samples** were quantified using a Pentra C200 autoanalyzer.

  - ***Ex vivo* lipolysis** was assessed in epididymal adipose tissue explants from control and rCT-1-treated mice, by the quantification of the amount of glycerol released to media (quantified as described above and normalized by protein content).

- Western Blots

  - As described before.

CHAPTER 2. "Cardiotrophin-1 regulates adipokine production in 3T3-L1 adipocytes and adipose tissue from obese mice"

In vitro studies

- Cell model and experimental design

Serum-deprived mature 3T3-L1 adipocytes were treated with rCT-1 (1-40 ng/mL) for 18 h in other to study the effects of this cytokine on the secretion of several adipokines (leptin, resistin, visfatin and apelin). Short-term treatments (1 h) with rCT-1 (20 ng/mL) and comparative studies with IL-6 (20 ng/mL, 18 h) were also carried out. For the study of the potential signaling pathways involved in the actions of rCT-1 on adipokine´s expression, pretreatment for 1h with specific inhibitors or activators of several pathways was performed.

Figure 15 illustrates the experimental design and the techniques carried out to evaluate the effects of rCT-1 on the regulation of adipokine production.

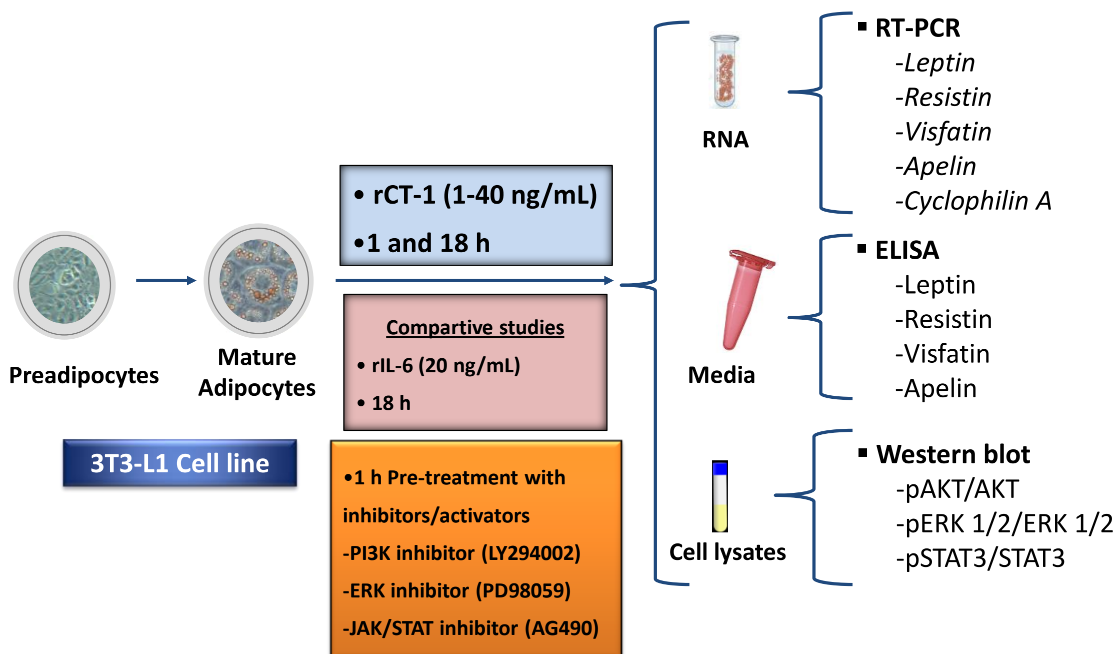


Figure 15: *In vitro* experimental design for Chapter 2.

- Analyses of mRNA levels

mRNA expression levels of *Cyclophilin A*, *Leptin, Resistin, Visfatin* and *Apelin,* were measured by real-time PCR using iCycler (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad) **(Table 6)**.

- Enzyme Immunoassay (ELISA).

Leptin, resistin, visfatin and apelin released to the cell culture media were measured using the appropriate ELISA **(Table 5)**.

- Western Blots

Analysis of the major signaling pathways involved in the stimulatory effects of rCT-1 on apelin production was carried out by **Western blot** technique. The following antibodies were used: phospho-AKT (Ser473), AKT, phospho-ERK 1/2 (Thr202/Tyr204), ERK, phospho-STAT3 (Tyr705) and STAT3 **(Table 4)**.

***In vivo* studies**

- Animal models and experimental design

Eight-week-old male C57BL/6J mice were obtained from Harlan Laboratories (Barcelona, Spain) and were placed on high fat diet (HFD) (60% of kcal from fat, 20% from carbohydrates and 20% from protein, Research Diets, New Brunswick, NJ) *ad libitum* for 12 weeks. The animals were established at the Animal facilities of CIMA (UN) where they were kept in cages under the same controlled conditions of light, temperature and humidity as previously described.

After 12 weeks, the obese HFD-fed animals were randomly divided into four experimental groups **(Figure 16)**:

- **HFD + saline** (n = 4): Treated with saline i.v. (retro-orbital) for 30 min.
- **HFD + rCT-1-treated** (n = 4): Treated with rCT-1 (0.2 mg/kg of body weight) i.v. (retro-orbital) for 30 min.
- **HFD + Saline** (n = 4): Treated with saline i.v. (retro-orbital) for 3h.
- **HFD + rCT-1 treated** (n = 4): Treated with rCT-1 (0.2 mg/kg of body weight) i.v. (retro-orbital injection) for 3 h.

At the indicated time point, mice were euthanized and epididymal fat was snap frozen in liquid nitrogen and stored at -80 °C for subsequent analysis.

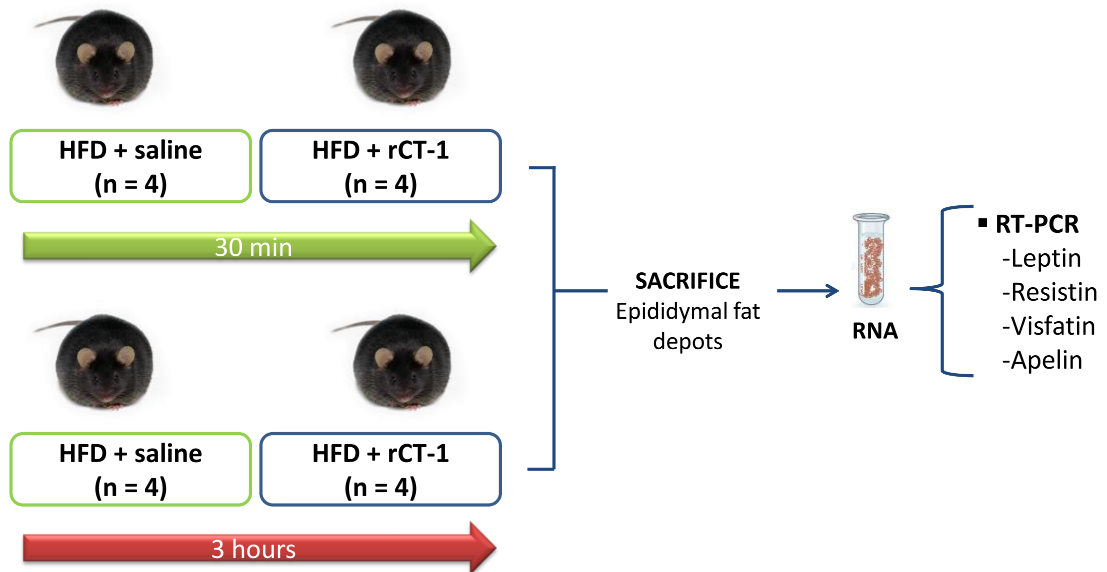


Figure 16: *In vivo* experimental design for Chapter 2.

- Analyses of mRNA levels

mRNA expression levels of *Cyclophilin A*, *Leptin, Resistin, Visfatin* and *Apelin,* were measured by real-time PCR using iCycler and iQ SYBR Green Supermix **(Table 6).**

CHAPTER 3. "Cardiotrophin-1 decreases intestinal sugar uptake in mice and in Caco-2 cells"

In vitro studies

- *In vitro* models for intestinal sugar uptake studies and experimental design

Studies in Caco-2 cells

The Caco-2 cell line is a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma cells, which are widely used for intestinal physiology studies because of its ability to grow as a monolayer with the morphological and phenotypical characteristics of the enterocytes. Human epithelial colorectal adenocarcinoma cell line, Caco-2 cells, was kindly provided by Dr. Edith Brot-Laroche (Université Pierre et Marie Curie, INSERM U505, Paris). Caco-2 cells were cultured and treated with or without rCT-1 (1 and 20 ng/mL) for different times (1 or 24 h). To analyze the signaling pathways involved in the actions of rCT-1 on sugar uptake, cells were pre-incubated for 30 min in presence or absence of specific inhibitors or activators before the addition of rCT-1 to the treated cells.

The following techniques, whose protocols are deeply described in the corresponding manuscripts, were carried out to characterize the actions of *in vitro* rCT-1 treatment on intestinal sugar absorption, as well as the cellular mechanisms that could be underlying these effects **(Figure 17)**.

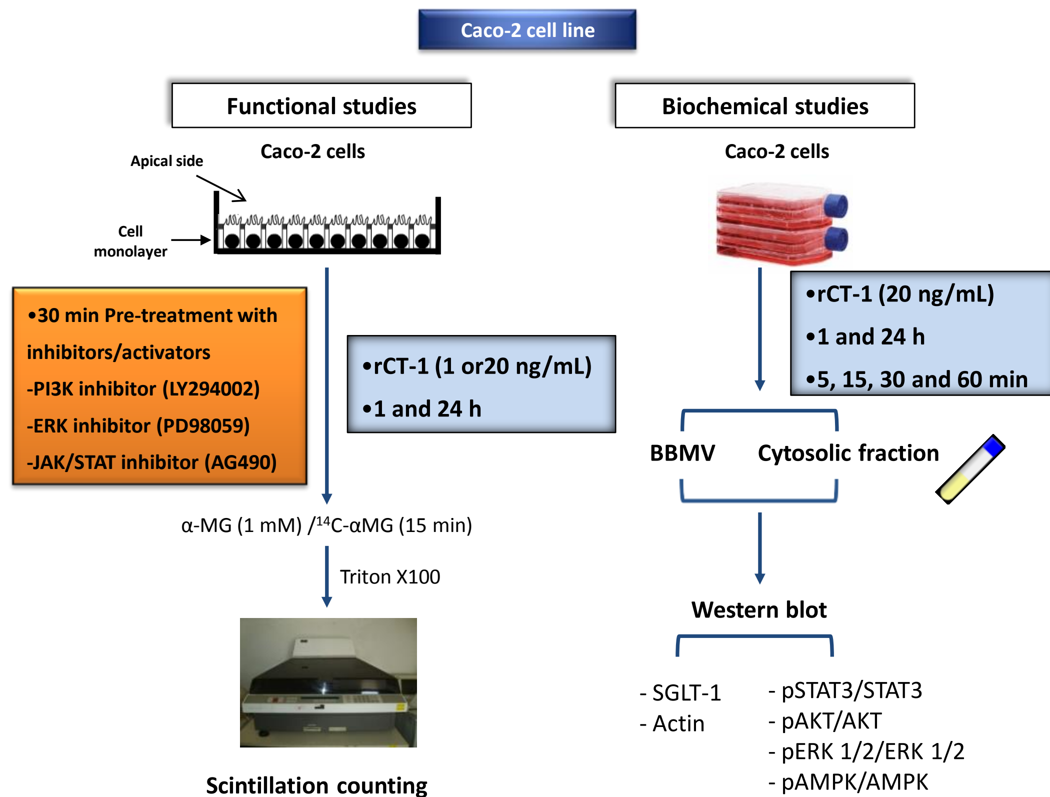


Figure 17: *In vitro* experimental design for Chapter 3. BBMV (Brush Border Membrane Vesicles).

- Functional studies in Caco-2 cells

α-methyl-D-glucoside (α-MG) uptake experiments were performed in cell monolayers grown on culture plates for 17–21 days post-seeding. Cells were pre-incubated in serum and glucose-free DMEM during 2 h and incubated with rCT-1 (1 or 20 ng/mL) for 1 or 24 h; then, cells were incubated for 15 min with 0.1 mM α-MG and traces of [^{14}C]-α-MG. In order to evaluate the uptake of α-MG, cellular radioactivity was measured by liquid scintillation counting.

- Western Blots

Analysis of the levels of SGLT-1, the main transporter of α-MG, as well as the levels of several proteins (total and phosphorylated) involved in the regulatory effects of rCT-1 were carried out by **Western blot** technique. The following antibodies were used: SGLT-1, phospho-AKT (Ser473), AKT, phospho-ERK 1/2 (Thr202/Tyr204), ERK, phospho-AMPK (Thr183), AMPK, phospho-STAT3 (Tyr705), STAT3 and β-actin **(Table 4)**.

Studies in everted intestinal rings

Everted jejunal rings were obtained from C57BL/6J mice as previously described (Lostao *et al.* 1998) and incubated in presence or absence of rCT-1 (1-50 ng/mL) for 1 h. The uptake of α-MG (1 mM, 15 min), a SGLT-1 specific substrate, was determined on everted jejunal rings according to the protocol of (Lostao *et al.* 1998). A comparative study with CNTF (20 ng/mL, 1 h), another cytokine of the same family, was also carried out.

Animal studies

- Animal models and experimental design

Acute treatment of mice with rCT-1.

Eight-week-old male C57BL/6J mice were purchased from Harlan Laboratories (Barcelona, Spain). Fasted mice for 18 h were injected i.v. either with saline or rCT-1 (0.2 mg/kg) and sacrificed at 3 h after administration.

Long-term treatment of high fat diet (HFD)-fed wild type and CT-1$^{-/-}$ mice with rCT-1

Wild type (WT) and CT-1 null (CT-1$^{-/-}$) mice, generated as described by Oppenheim *et al.* (2001), were used. We analyzed mice backcrossed into a C57BL/6J background for 11 generations (provided by Diane Pennica, Genentech, and Bettina Holtmann, University of Wuerzburg, Germany) (Moreno-Aliaga *et al.* 2011). WT and CT-1$^{-/-}$ animals were placed on high fat diet (HFD) (60% of kcal from fat, 20% from carbohydrates and 20% from protein, Research Diets, New Brunswick, NJ) *ad libitum* for 12 weeks. At the end, HFD-fed WT and CT-1$^{-/-}$ animals were divided into four experimental subgroups **(Figure 18)**:

- **HFD-WT + saline** (n = 6): Treated with saline i.v. (retro-orbital) for 6 days.
- **HFD-WT + rCT-1** (n = 6): Treated with rCT-1 (0.2 mg/kg of body weight) i.v for 6 days.
- **HFD-CT-1$^{-/-}$ + saline** (n = 6): Treated with saline i.v. for 6 days.
- **HFD-CT-1$^{-/-}$ + rCT-1** (n = 6): Treated with rCT-1 (0.2 mg/kg/day of body weight) i.v. for 6 days.

At the indicated time point, mice were euthanized and the effects of *in vivo* rCT-1 administration on the uptake of α-MG were determined on everted intestinal rings.

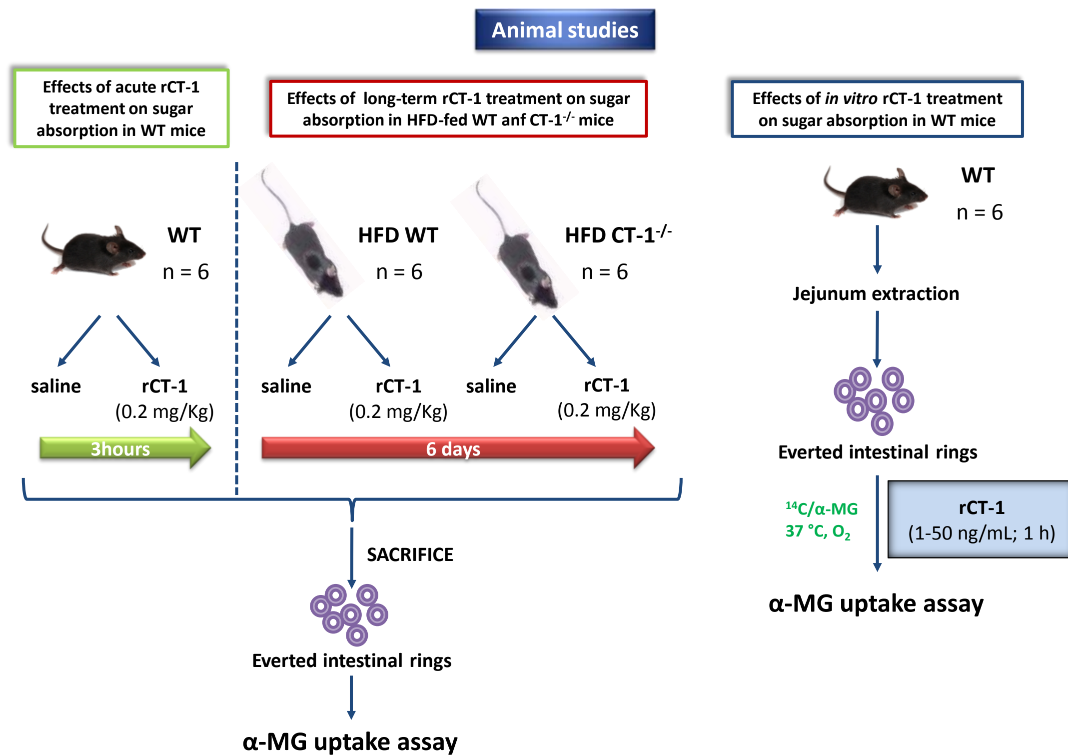


Figure 18: Experimental design for assessing the *in vivo* and *in vitro* effects of CT-1 on α-MG uptake in everted intestinal rings (Chapter 3).

- <u>Everted intestinal rings uptake assays</u>

The *ex vivo* uptake of α-MG, a SGLT-1 specific substrate, was determined on everted jejunal rings obtained from un-treated and treated animals.

CHAPTER 4. "Role of cardiotrophin-1 (CT-1) in the regulation of metabolic circadian rhythm and adipose core clock genes in mice and characterization of 24-h circulating CT-1 profile in normal weight and overweight/obese subjects"

Animal studies

- Animal models and experimental design

Experimental design 1: WT and CT-1$^{-/-}$ mice were housed under the same controlled conditions of light, temperature and humidity as described in the previous studies for different experimental periods (2 and 12 months) **(Figure 19)**.

Experimental design 2: eight weeks old *ob/ob* (C57BL/6J background) and WT mice were obtained from the Janvier Laboratory (Le Genest St Isle). *ob/ob* mice were divided into three subgroups, one that received rCT-1 intravenously (0.2 mg/kg/day) for 10 consecutive days, another given the same volume of saline and fed *ad libitum* and other saline-treated mice pair-fed (PF) to the amount of food consumed by the rCT-1-treated group **(Figure 19)**:

- ***ob/ob*** (n = 6): Treated with saline, i.v. (retro-orbital injection) for 10 days and fed *ad libitum*.
- ***ob/ob* + rCT-1** (n = 7): Treated with rCT-1 (0.2 mg/kg/day of body weight) i.v. for 10 days and fed *ad libitum*.
- ***ob/ob*-PF** (n = 5): Treated with saline, i.v. for 10 days and fed with the same amount of food consumed by the rCT-1-treated group the day before.

Animals were sacrificed after an overnight fast. Epididymal fat depots were excised, weighed and kept at −80 °C for subsequent analysis.

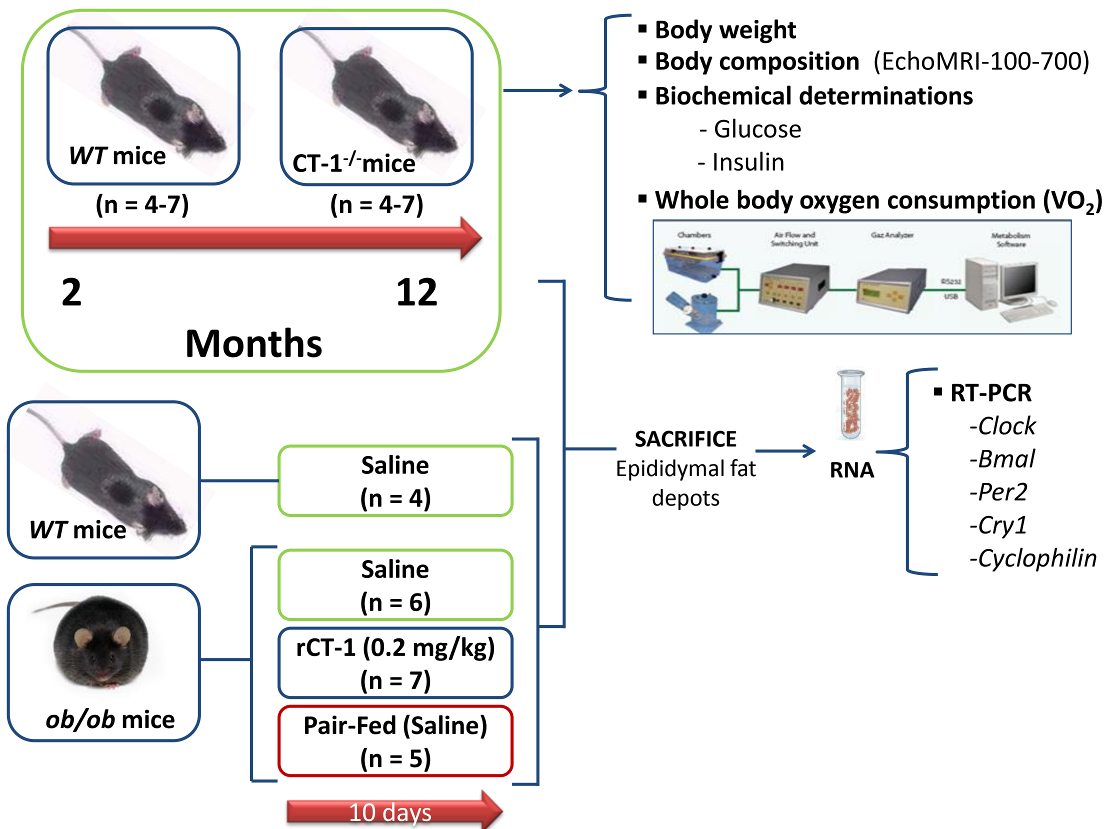


Figure 19: Animal experimental design for Chapter 4.

- Body weight and body composition analysis

Body weight was measured three times per week in WT and *CT-1*$^{-/-}$ mice from 2 to 12 months old. Whole animal body composition was measured in live conscious animals using Quantum Molecular Resonance (QMR) technology (EchoMRI-100-700).

- Biochemistry

All serum measurements were done on mice fasted for 16 h, using a Cobas Mira Autoanalyzer. Insulin was analyzed using mouse ELISA kits.

- Whole body oxygen consumption

Twenty-four hours whole body oxygen consumption (VO_2) was measured in WT and CT-1$^{-/-}$ mice (2 and 12 months-old) using the Oxylet System.

- Analyses of mRNA levels

mRNA expression levels of *Clock*, *Bmal1, Per2,* and *Cry1,* were measured by real-time PCR using predesigned TaqMan® Assays-on-Demand **(Table 7).**

Human Study

- Subjects and experimental design

The eighty-four participants in this study (37 lean and 47 overweight/obese) are a subgroup from an NIH-funded investigation (in which a total of 187 participants were studied). This investigation reports only the results of baseline samples collected prior to the dietary intervention. 24-hour serial blood collections (every 1 hour) (Stanhope *et al.* 2011) were conducted on the 3rd day of the baseline (0 week) inpatient period **(Figure 20).**

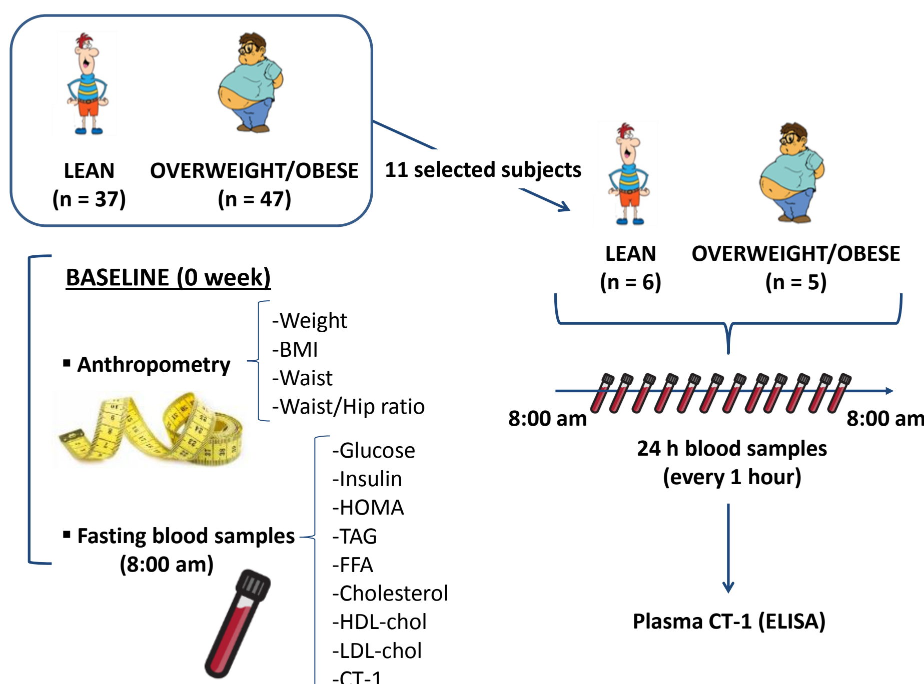


Figure 20: Subjects and experimental design for Chapter 4.

- Anthropometry

Weight, body mass index (BMI), waist circumference, hip and waist/hip ratio were determined at baseline.

- Blood biochemical parameters

Fasting glucose, insulin, TAG, FFA, cholesterol, HDL-C, LDL-C and CT-1 were measured in serum/plasma samples at baseline.

- 24-h CT-1 plasma concentration

CT-1 plasma levels were determined by ELISA **(see Table 5)** in 11 selected patients (6 lean and 5 overweight/obese). Blood was obtained every hour during 24 h.

Table 4: List of primary antibodies used for Western blot studies in chapters 1, 2 and 3.

Antibody	Manufacturer	Ref	Source
Phospho HSL (Ser563)	Cell signaling technology	4139	Rabbit
Phospho HSL (Ser565)	Cell signaling technology	4137	Rabbit
Phospho HSL (Ser660)	Cell signaling technology	4126	Rabbit
HSL	Cell signaling technology	4107	Rabbit
ATGL/Desnutrin	Cell signaling technology	2138	Rabbit
G0S2	Santa Cruz Biotechnologies	sc-133423	Rabbit
CGI58	Santa Cruz Biotechnologies	sc-130934	Rabbit
Phospho PKA substrate	Cell signaling technology	9624	Rabbit
Perilipin	Cell signaling technology	9349	Rabbit
G protein alpha S (Gsα)	Abcam	ab83735	Rabbit
G Protein alpha Inhibitor 1+2 (Gi)	Abcam	ab3522	Rabbit
Phospho-PDE3B	Acris	AP10211PU-N	Rabbit
Adipocyte Phospholipase A2 (AdPLA)	Cayman Chemical	10337	Rabbit
Phospho AKT (Ser473)	Cell signaling technology	4058	Rabbit
AKT	Cell signaling technology	9272	Rabbit
Phospho ERK 1/2 (Thr202/Tyr204)	Cell signaling technology	4370	Rabbit
MAPK (ERK 1/2)	Cell signaling technology	9102	Rabbit
Phospho STAT3 (Tyr705)	Cell signaling technology	9131	Rabbit
STAT3	Cell signaling technology	9139	Mouse
Phospho AMPK (Thr172)	Cell signaling technology	2535	Rabbit
AMPK	Cell signaling technology	2532	Rabbit
SGLT-1	Santa Cruz Biotechnologies	sc-98974	Rabbit
Actin	Sigma-Aldrich	A3853	Rabbit

Table 5: List of ELISA kits used in chapters 1, 2 and 4.

Antibody	Manufacturer	Ref	Sample type
cAMP	Arbor Assays	K019-H1	Cell lysates
Leptin	Invitrogen	KMC2281	Mouse
Resistin	RayBiotech	EIAM-RES	Mouse
Visfatin	RayBiotech	EIAM-VIS	Mouse
Apelin-12	Phoenix Peptide	EK-057-23	Mouse
CT-1	Manual		Human

Table 6: List of primers for real-time PCR using iCycler (Bio-Rad) in chapters 1 and 2.

Gene Symbol	Sequence	bp	Genebank Number	Specie
Cyclophilin A	Sense 5`-GTCTCCTTCGAGCTGTTTGC- 3` Antisense 5`-CGTGTAAAGTCACCACCCTG- 3`	148	NM_008907.1	Mouse
HSL	Sense 5`-TACAAACGCAACGAGACAGG-3’ Antisense 5`-AGAAGGCTTTCCAGAAGTGC-3’	142	NM_001039507.2	Mouse
Pka	Sense 5`- GGAAGAGAAGAAGCCTGGAT -3` Antisense 5`-GCGATCCGAGTCTGGATCTT - 3`	154	NM_025506	Mouse
Cyclophilin A	Sense 5`-GTCTCCTTCGAGCTGTTTGC- 3` Antisense 5`-CGTGTAAAGTCACCACCCTG- 3`	148	NM_008907.1	Mouse
Leptin	Sense 5`-TCAATGACATTTCACACACGC- 3` Antisense 5`-TTTGAAACTTCAGCATTCAGG- 3`	139	NM_008493.3	Mouse
Resistin	Sense 5`-CTGTGTCCCATCGATGAAGC- 3` Antisense 5`-TGGAGGAGACTGTCCAGCAA- 3`	108	NM_022984.4	Mouse
Visfatin	Sense 5`-CAGAGCACAGTACCATAACG-3` Antisense 5`-GAGGATTTCCAGAGTCAGGT-3`	219	NM_021524.2	Mouse
Apelin	Sense 5`-CTCTGGCTCTCCTTGACTGC- 3` Antisense 5`- CCTGGTCCAGTCCTCGAAGT- 3`	122	NM_013912.3	Mouse

Table 7: List of TaqMan probes used for real time-PCR in chapter 4.

Gene Symbol	Ref	Gene Symbol	Specie
Clock	Mm00455959_m1	*CLOCK*	Mouse
Bmal1	Mm00500226_m1	*ARNTL*	Mouse
Per2	Mm00478113_m1	*PER2*	Mouse
Cry1	Mm00514392_m1	*CRY1*	Mouse
Cyclophilin	Mm02342430_g1	*CYCLOPHILIN A*	Mouse

REFERENCES

-F-

Fernandez-Galilea, M., Perez-Matute, P., Prieto-Hontoria, P. L., Martinez, J. A. & Moreno-Aliaga, M. J. 2012. Effects of lipoic acid on lipolysis in 3T3-L1 adipocytes. *J Lipid Res,* **53,** 2296-306.

-L-

Lostao, M. P., Urdaneta, E., Martinez-Anso, E., Barber, A. & Martinez, J. A. 1998. Presence of leptin receptors in rat small intestine and leptin effect on sugar absorption. *FEBS Lett,* **423,** 302-6.

-M-

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* **14,** 242-53.

-O-

Oppenheim, R. W., Wiese, S., Prevette, D., Armanini, M., Wang, S., Houenou, L. J., Holtmann, B., Gotz, R., Pennica, D. & Sendtner, M. 2001. Cardiotrophin-1, a muscle-derived cytokine, is required for the survival of subpopulations of developing motoneurons. *J Neurosci,* **21,** 1283-91.

-S-

Stanhope, K. L., Bremer, A. A., Medici, V., Nakajima, K., Ito, Y., Nakano, T., Chen, G., Fong, T. H., Lee, V., Menorca, R. I., Keim, N. L. & Havel, P. J. 2011. Consumption of fructose and high fructose corn syrup increase postprandial triglycerides, LDL-cholesterol, and apolipoprotein-B in young men and women. *J Clin Endocrinol Metab,* **96,** E1596-605.

IV. RESULTS

CHAPTER 1

Cardiotrophin-1 stimulates lipolysis through the regulation of main adipose tissue lipases

López-Yoldi M[1,2], Fernández-Galilea M[1], Laiglesia LM[1,2], Larequi E[3], Prieto J[3,4], Martínez JA[1,2,5], Bustos M[3*], Moreno-Aliaga MJ[1,2,5]

[1]Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Navarra, Spain.

[2] Centre for Nutrition Research. University of Navarra, Pamplona, Navarra, Spain.

[3]Department of Hepatology and Gene Therapy, CIMA, University of Navarra, Pamplona, Navarra, Spain.

[4]CIBERehd. Institute of Health Carlos III, Madrid, Spain.

[5]CIBERobn, Physiopathology of Obesity and Nutrition, Institute of Health Carlos III, Madrid, Spain.

*Equal senior authorship

Published in: J Lipid Res. 2014; 55(12):2634-43.

Impact Factor (2014): 4.421.

65/290 in Biochemistry & Molecular Biology (Q1)

ABSTRACT

Cardiotrophin-1 (CT-1) is a cytokine with antiobesity properties and with a role in lipid metabolism regulation and adipose tissue function. The aim of this study was to analyze the molecular mechanisms involved in the lipolytic actions of CT-1 in adipocytes. Recombinant CT-1 (rCT-1) effects on the main proteins and signaling pathways involved in the regulation of lipolysis were evaluated in 3T3-L1 adipocytes and in mice. rCT-1 treatment stimulated basal glycerol release in a concentration and time-dependent manner in 3T3-L1 adipocytes. rCT-1 (20 ng/mL for 24 h) raised cAMP levels and in parallel increased PKA-mediated phosphorylation of perilipin and HSL at Ser660. siRNA knock down of HSL or PKA as well as pretreatment with the PKA inhibitor H89 blunted the CT-1-induced lipolysis, suggesting that the lipolytic action of CT-1 in adipocytes is mainly mediated by activation of HSL through PKA pathway. In *ob/ob* mice, acute rCT-1 treatment also promoted PKA-mediated phosphorylation of perilipin and HSL at Ser660 and Ser563, and increased ATGL content in adipose tissue. These results showed that the ability of CT-1 to regulate the activity of the main lipases underlies the lipolytic action of this cytokine *in vitro* and *in vivo*, and could contribute to CT-1 antiobesity effects.

Supplementary key words: *Adipocytes . Adipose triglyceride lipase. Cell signaling . Cytokines . Hormone-sensitive lipase. Obesity . Perilipin . Protein kinase A .*

INTRODUCTION

Cardiotrophin-1 (CT-1) belongs to the interleukin-6 (IL)-6 family of cytokines. These cytokines exert their cellular effects by interacting with the glycoprotein 130 (gp130)/leukemia inhibitory factor receptor (LIFR) heterodimer (Pennica *et al.* 1995). Adipose tissue has been identified as a source of CT-1 (Natal *et al.* 2008), being this cytokine capable of activating major signaling pathways involved in metabolic control in adipocytes (Zvonic *et al.* 2004). Moreover, it has been reported that CT-1 levels are raised in obesity and metabolic syndrome (Natal *et al.* 2008), suggesting that CT-1 could be a new marker for obesity and related diseases. A recent study by our group has revealed that CT-1 is a key regulator of energy homeostasis, as well as of glucose and lipid metabolism (Moreno-Aliaga *et al.* 2011). Thus, chronic recombinant CT-1 (rCT-1) treatment reduced body weight and corrected insulin resistance in *ob/ob* and in high-fat-fed obese mice by reducing food intake and enhancing energy expenditure. Moreover, rCT-1 induced dramatic white adipose tissue remodeling characterized by the upregulation of genes implicated in the control of fatty acid oxidation, mitochondrial biogenesis and lipolysis. In this context, it has been reported that adipocytes from rCT-1-treated mice exhibited an increased lipolytic response to isoproterenol, while adipocytes from old obese *CT-1* null mice responded poorly to isoproterenol, suggesting that CT-1 might play a role in the regulation of lipolysis (Moreno-Aliaga *et al.* 2011). However, the mechanism underlying the lipolytic action of CT-1 still remains unknown.

During lipolysis, intracellular triacylglycerol (TAG) are hydrolyzed through the consecutive action of three major lipases: adipose triglyceride lipase (ATGL/desnutrin), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL). ATGL exhibits higher substrate specificity for TAG than diacylglycerol (DAG) and selectively assumes the first step in TAG hydrolysis resulting in the formation of DAG and fatty acid (Zimmermann *et al.* 2004). ATGL lipolytic activity is co-activated by the protein comparative gene identification-58 (CGI-58), whereas it is inhibited by the protein G0/G1 switch gene 2 (G0S2) (Lu *et al.* 2010, Cornaciu *et al.* 2011). Moreover, recent findings describe how ATGL can be also regulated through phosphorylation by

adenosine monophosphate-activated protein kinase (AMPK) at Ser406, stimulating its lipolytic activity (Ahmadian *et al.* 2011).

It is well known that the activity of HSL is controlled postranscriptionally through reversible phosphorylation. Experiments in murine adipocytes have demonstrated that Ser563, Ser659 and Ser660 are the major protein kinase A (PKA) phosphorylation sites, which are essential for the translocation of HSL to the lipid droplet surface and for stimulation of HSL (Watt *et al.* 2006). Besides PKA phosphorylation, HSL can also be phosphorylated by other kinases, such as extracellular regulated kinase (ERK), which phosphorylates HSL at Ser600, increasing lipolysis (Greenberg *et al.* 2001). Another serine residue (Ser565) is a substrate of AMPK, which phosphorylates HSL preventing PKA-mediated activation of HSL by phosphorylation (Anthonsen *et al.* 1998).

Perilipin A is a protein associated with the cytoplasm side of the lipid droplets (Brasaemle 2007). Under basal conditions, perilipin A maintains a low rate of basal lipolysis by sequestering CGI-58 (Granneman *et al.* 2009) and by restricting the access of cytosolic lipases to the lipid droplet. However, cyclic adenosine monophosphate (cAMP) mediated activation of PKA induces conformational changes in perilipin A, facilitating the translocation of phosphorylated HSL from the cytoplasm to the lipid droplet surface and enhancing the lipolytic process (Miyoshi *et al.* 2007).

Based on these previous findings, we aimed at analyzing if the lipolytic actions of rCT-1 in adipocytes are mediated by changes in the regulation of the major lipases and lipid droplet proteins involved in the hydrolysis of TAG, and to characterize the major signaling pathways implicated.

MATERIAL AND METHODS

Cell culture and differentiation of 3T3-L1 cells

Mouse embryo fibroblast 3T3-L1 (American Type Culture Collection; Rockville, MD) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) containing 25 mM glucose, 10% (v/v) calf bovine serum (CBS) (Invitrogen, Carlsbad, CA), and 1% (v/v) penicillin/streptomycin (Invitrogen) and maintained in an incubator set to 37°C and 5% of carbon dioxide. At confluence, pre-adipocytes were cultured for 48 h in DMEM (Invitrogen) containing 25 mM glucose, 10% fetal bovine serum (FBS) (Invitrogen), and antibiotics, and supplemented with dexamethasone (1 mM; Sigma, St. Louis, MO),

isobutylmethylxantine (0.5 mM; Sigma) and insulin (10 µg/mL; Sigma). After that, cells were cultured with 10% FBS and insulin for 48 h and then media were replaced with 10% FBS in DMEM and antibiotics, but without insulin, and these media were changed every 2 days until day 8 post-confluence when cells attained the morphology and typical features of mature adipocytes (Fernandez-Galilea *et al.* 2012).

Recombinant Protein CT-1 (rCT-1)

rCT-1 was obtained as described elsewhere (Beraza *et al.* 2005) and contained < 0.04 ng LPS *per* 1 µg of the protein as determined by the Limulus amoebocyte lysate assay (Cambrex, East Rutherford, NJ).

***In vitro* treatments**

The inhibitors H89 (Santa Cruz Biotechnology, Santa Cruz, CA), and KT5823 (Calbiochem, La Jolla, CA) were dissolved in DMSO. The AMPK activator AICAR (Sigma) was dissolved in ultra-purified water. All compounds were prepared as 1000x stock solutions and added to the culture medium. Control cells were treated with the same amount of vehicle (DMSO and/or Ethanol).

Prior to the addition of the appropriate treatments, cells were serum starved for 4 h using the medium DMEM supplemented with 0.1% FBS and then treated with or without rCT-1 (1-40 ng/mL) during different time intervals (1-24 h). To analyze the signaling pathways involved in the lipolytic actions of rCT-1, adipocytes were preincubated for 1 h in presence or absence of specific inhibitors or activators (1 µM of PKA inhibitor H89, 2 mM of AMPK activator AICAR and 1 µM of protein kinase G (PKG) inhibitor KT5823 before the addition of rCT-1 to the treated wells as described elsewhere (Fernandez-Galilea *et al.* 2012).

Animal experiments

Eight-week-old male *ob/ob* (C57BL/6J background) mice were supplied by the Janvier Laboratory (Le Genest St. Isle, France). rCT-1 (0.2 mg/kg of body weight) was administered intravenously (retro-orbital injection) and animals were euthanized 30 min after administration. Control mice were injected with vehicle (saline). At the indicated time point, mice were euthanized and epididymal fat was snap frozen in liquid nitrogen and stored at -80 °C for subsequent analysis. All experimental

procedures were performed according to the institutional guidelines for the use of laboratory animals and approved by the University of Navarra Ethics Committee.

Determination of lipolysis in 3T3-L1 adipocytes

Lipolysis was evaluated by the biochemical determination of the amount of glycerol and free fatty acids (FFA) released into the culture media. Glycerol measurements were performed after 1, 2, 12, 18 and 24 h of rCT-1 treatment using the Pentra C200 autoanalyzer (Roche Diagnostic, Basel, Switzerland), following manufacturer´s instructions. FFA were evaluated after 3 h of rCT-1 treatment by using the Lipolysis Assay KIT for Free Fatty Acids Detection (Zen-Bio Inc, Research Triangle Park, NC) according to the manufacturer's instructions. TAG, DAG and monoacylglycerol (MAG) levels were also determined by thin-layer chromatography. Briefly, cell lysates were mixed with an equal amount of chloroform/methanol (2:1; v/v). After vortexing for 1 min and resting for 10 min, the samples were centrifuged for 10 min. Organic layers were collected and vacuum dried. The pellets resolved in 40 µL chloroform/methanol (1:1, v/v) were applied as 5-mm spots to high-performance thin-layer chromatography (HPTLC)-silica gel with an aluminum backing (Merck, Darmstadt, Germany). The HPTLC plates were developed with a solvent system (hexane:dietil ether:acetic acid ; 70:30:1, v/v/v) at room temperature. The plate was dried and placed in a system with iodine salts vapor (Panreac, Barcelona, Spain) until the lipids were visible. Identities of the stained lipids were determined by referring to standards. Bands corresponding to TAG, DAG and MAG were scraped from the plate and quantified using the ABX Pentra Triglyceride CP (Horiba, Montpellier, France). For the determination of free cholesterol, the pellets were resolved using isopropanol, and free cholesterol was determined by the Wako Free Cholesterol E test (Wako, Neuss, Germany) (Takahashi *et al.* 2013).

***Ex vivo* lipolysis assay in epididymal adipose tissue explants**

Epididymal fat pads were surgically removed from overnight fasted mice treated with either rCT-1 (0.2 mg/kg of body weight) or vehicle for 30 min. Fat pads of approximately 100 mg were minced into small pieces and incubated in 6 well plates with 1 mL of HEPES phosphate buffer (pH 7.4; containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM $CaCl_2.2H_2O$, 1.25 mM $MgSO_4.7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM

Na_2HPO_4, and 10 mM HEPES) at 37 °C. Media samples were collected at 1 h and 2 h of incubation. Glycerol content was quantified as described above and normalized by protein content.

Determination of FFA in serum samples

Serum FFA were measured in mice fasted for 16 h, before and after 30 min treatment with vehicle- or rCT-1 (0.2 mg/kg of body weight). FFA were quantified using a Pentra C200 autoanalyzer (Roche Diagnostic, Basel, Switzerland), following manufacturer´s instructions.

Western Blot analysis

3T3-L1 cell lysates were obtained by the addition of a buffer containing 2 mM Tris HCl (pH 8), 137 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail 1 (Sigma), 2 mM orthovanadate, and 1 mM PMSF. In *ob/ob* mice, tissues samples were thawed and homogenized in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton-X-100, 0.1 M sodium fluoride, 10 mM EDTA, 50 mM sodium chloride, 10 mM orthovanadate, 0.1% SDS and protease inhibitors cocktail (Roche)]. In both cases, samples were centrifuged and protein concentrations were determined by BCA method according to the supplier´s instructions (Pierce-Thermo Scientific, Rockford, IL). Briefly, equivalent amounts of total protein (25-50 μg) were electrophoretically separated by 12-15% SDS-PAGE in the presence of a reducing agent (2-mercaptoethanol). Proteins were electroblotted from the gel to polyvinylidene difluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Following the transfer of proteins, the membranes were blocked and probed with specific primary antibodies against phospho-HSL (Ser563), phospho-HSL (Ser565), phospho-HSL (Ser660), ATGL, phospho (Ser/Thr) PKA substrate, perilipin (Cell signaling technologies, Danvers, MA), G protein alpha S ($G_s\alpha$), G Protein alpha Inhibitor 1+2 (Gi) (Abcam, Cambridge, UK), phosphodiesterase 3B (PDE3B) (Phospho) (Acris Antibodies, Herford, Germany), adipocyte phospholipase A2 (AdPLA) (Cayman Chemical, Ann Arbor, MI) and β-actin (Sigma). After that, membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (Sigma) for 1 h and then were revealed with the SuperSignal kit revelation solution (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. The results were analyzed by

densitometry using the GS-800 Calibrated densitometer (Bio-Rad, München, Germany).

cAMP assay

The cAMP Direct EIA kit (Arbor Assay, Ann Arbor, MI) was used to quantify the amount of intracellular cAMP after 24 h of control or rCT-1 treatment (20 ng/mL) in 3T3-L1 adipocytes.

Small interfering RNA experiments

The predesigned small-interfering RNA (siRNA) specific to PKA (Silencer Select siRNA) and control siRNA (Silencer Select Control siRNA) were purchased from Ambion (Ambion Inc., Austin, Texas, USA) and siRNA specific to HSL and control were obtained from Santa Cruz. Transfection of 3T3-L1 adipocytes was performed using the Amaxa® Cell Line Nucleofector® kit L with the Nucleofector® II system (Lonza, Basel, Switzerland) using recommended settings according to manufacturer's protocol. The transfected cells were seeded in 6-well plates, and experiments were conducted after 24 h incubation.

Analyses of mRNA levels

Total RNA was extracted with TRIZOL (Invitrogen) and real-time PCR was performed using iCycler (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). For relative quantitation of gene expression we used the comparative Ct method [$2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the target and control genes (cyclophilin)]. Primers were designed according to published complementary DNA or genomic sequences.

Statistical analysis

Data are presented as mean ± standard error (SEM). Comparisons between the values for different variables were analyzed by one-way ANOVA followed by Bonferroni post hoc tests or by Student's t-test or Mann-Whitney U-test once the normality had been screened using Kolmogorov-Smirnoff and Shapiro-Wilk tests. Statistical analyses and graphs were carried out using GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA). Overall, a P value <0.05 was considered significant.

RESULTS

Effects of rCT-1 on lipolysis in 3T3-L1 adipocytes

The incubation with rCT-1 (1-20 ng/mL) significantly increased basal glycerol released to the media in a dose-dependent manner after 18 h of treatment. Similar effects on glycerol release were observed for IL-6 (Fig. 1A). Furthermore, the effect of rCT-1 (20 ng/mL) on lipolysis was time-dependent. As shown in Fig. 1B, a significant increase in glycerol release was seen in the rCT-1-treated group after 12 h ($P < 0.01$) of incubation onwards, and this increase continued for up to at least 24 h ($P < 0.001$). However, after 3 h treatment a significant concentration-dependent increase in the amount of FFA released was already observed in rCT-1 treated adipocytes ($P < 0.05$) (Fig. 1C).

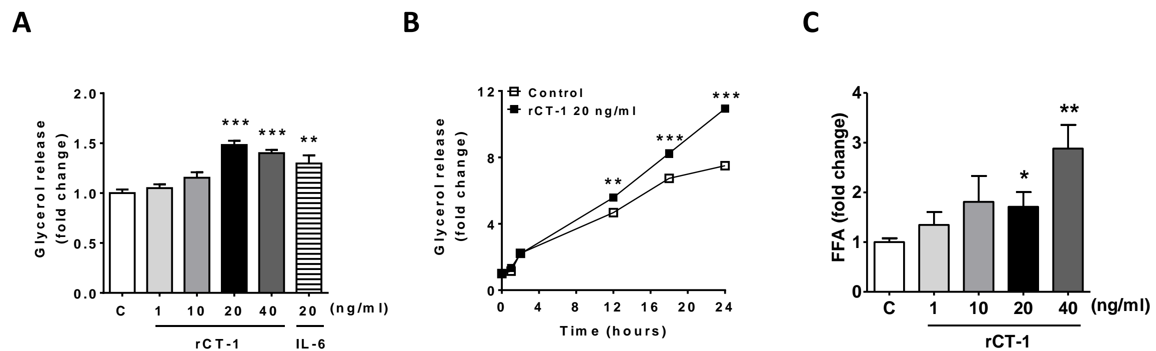
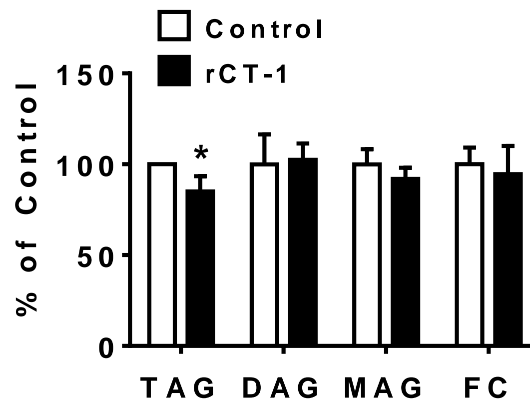


Fig. 1. Cardiotrophin-1 induces lipolysis in differentiated 3T3-L1 adipocytes. (A) Fully differentiated 3T3-L1 adipocytes were serum starved for 4 h and then treated with various concentrations of rCT-1 (1, 10, 20 and 40 ng/mL) or IL-6 (20 ng/mL) for 18 h, and the amount of glycerol release into the media was measured. (B) Time-response effects of rCT-1 (20 ng/mL) treatment on glycerol release. (C) Dose-dependent effects of rCT-1 on FFA release in adipocytes after 3 h of treatment. Data are mean ± SEM (n=5-7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control (C) group.

Moreover, we also measured the intermediary metabolites after total lipid separation by HPTLC. Our data revealed that rCT-1 treatment did not induce any significant change in DAG and MAG levels, suggesting that the major products of rCT-1-induced TAG hydrolysis are FFA and glycerol. Finally, because it has been described that in some tissues HSL has also lipolytic activity against cholesteryl esters (Yeaman 2004), intracellular free cholesterol levels were tested. However, rCT-1-treated adipocytes showed no changes in intracellular free cholesterol (Supplementary Fig. I).



Supplementary Fig. I. Effects of rCT-1 treatment on TAG, DAG, MAG and free cholesterol (FC) content. Mature adipocytes were treated with rCT-1 (20 ng/mL) for 24 h. TAG, DAG, MAG content was quantified after lipid separation by HPTLC. Intracellular FC was determined by an enzymatic assay as described in Methods. Data are expressed as mean ± SEM. (n = 4-5). *$P < 0.05$.

Effects of rCT-1 on the main proteins involved in lipolysis control

For a better understanding of the mechanisms involved in CT-1 lipolytic actions, we first tested in 3T3-L1 adipocytes the effects of rCT-1 on ATGL, the enzyme that predominantly catalyzes the initial step in TAG hydrolysis (Zimmermann *et al.* 2004). rCT-1 treatment for 1 and 2 h did not modify ATGL levels, but at 24 h the adipocytes treated with the cytokine exhibited a significant ($P < 0.05$) decrease in ATGL protein levels (Fig. 2A, B). ATGL activity is regulated by CGI-58 (activator) and G0S2 (inhibitor) via non competing mechanisms (Lu *et al.* 2010). Our data provided evidence that in parallel with the inhibition of ATGL, rCT-1 treatment increased the content of the ATGL inhibitor G0S2 ($P < 0.05$) after 24 h of treatment (Fig. 2A, D), while it did not significantly modify CGI-58 levels (Fig. 2A, C).

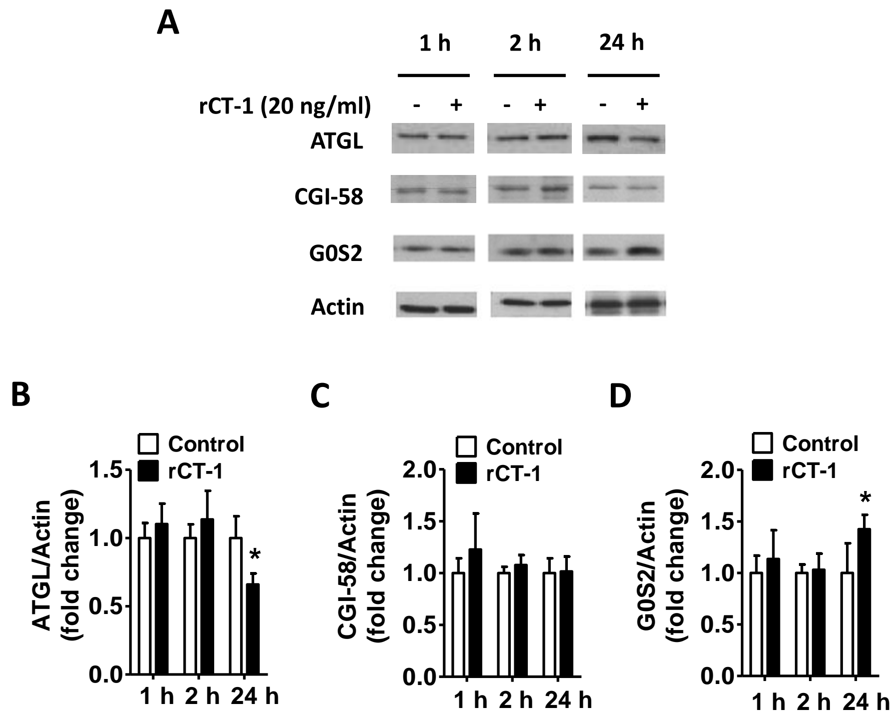


Fig. 2. ATGL is downregulated after 24 h of rCT-1 treatment. (A) Representative Western Blot and (B-D) densitometric analysis of ATGL (B), CGI-58, activator of ATGL (C) and G0S2, inhibitor of ATGL (D) in differentiated 3T3-L1 adipocytes treated with rCT-1 (20 ng/mL) or vehicle (Control) for 24 h. Band intensities for ATGL, CGI-58, and G0S2 were normalized to actin. Data are expressed as mean ± SEM (n = 4-7). **P* < 0.05, compared with vehicle-treated cells.

We next evaluated the effects of rCT-1 on HSL, a key lipase regulated by reversible phosphorylation (Watt *et al.* 2006). Our data revealed that rCT-1 (20 ng/mL) treatment increased the phosphorylation of HSL at Ser660 (which promotes its lipolytic activity), being significant after 2 ($P < 0.05$) and 24 h ($P < 0.05$) of treatment. However, rCT-1 did not modify the phosphorylation of HSL at Ser563 or Ser565 (Fig. 3A). To better characterize the involvement of HSL activation in the lipolytic actions of rCT-1, we tested the effect of the cytokine in HSL depleted adipocytes using siRNA. Our data revealed that rCT-1-induced glycerol release was almost completely abolished after silencing of HSL expression in adipocytes (Fig. 3B).

Finally, we also analyzed the effects of rCT-1 on perilipin A, an essential lipid droplet-associated protein, whose phosphorylation (PKA-dependent) is essential for the translocation of HSL from the cytosol to the lipid droplet surface (Sztalryd *et al.* 2003). Using a perilipin-specific antibody and a phospho-PKA-motif specific substrate antibody (Choi *et al.* 2010, Fernandez-Galilea *et al.* 2012), our data showed that rCT-1

caused a significant increase ($P < 0.05$) in the phospho-PKA substrate/perilipin ratio after 24 h of treatment (Fig. 3C), suggesting the involvement of PKA activation in the lipolytic actions of CT-1, in cultured adipocytes.

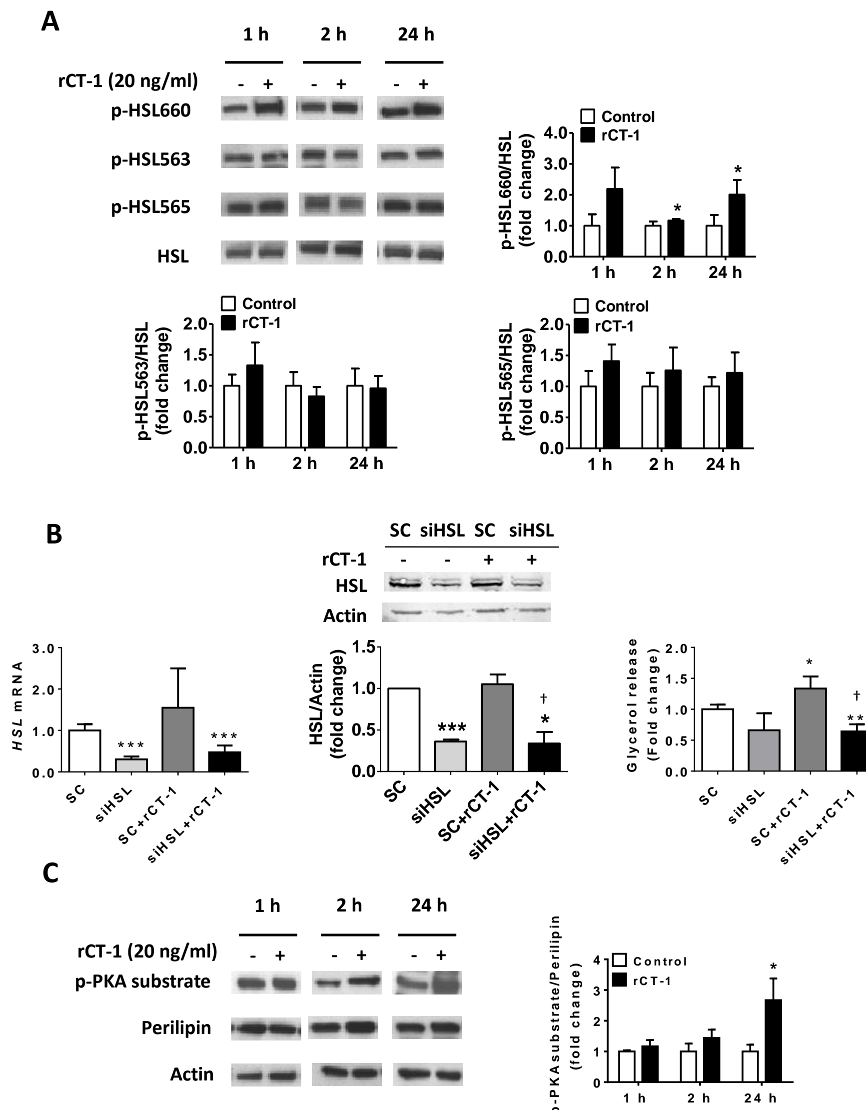


Fig. 3. HSL is involved in rCT-1-induced lipolysis. (A) Representative Western Blot and densitometric analysis of HSL phosphorylation at Ser660, Ser563 and Ser565 in differentiated 3T3-L1 adipocytes normalized by total HSL protein. (B) rCT-1-stimulated lipolysis is prevented by siRNA knock-down of HSL. mRNA levels, protein expression of HSL and glycerol release in 3T3-L1 adipocytes transfected with control siRNA (SC) or siRNA targeting endogenous HSL in the presence or absence of rCT-1 for 24 h. (C) Adipocyte lysates were immunoblotted using a phospho-PKA-motif-specific antibody, and the blots were stripped and reprobed with antiperilipin antibodies to detect native perilipins. Band density was quantified, and data were expressed as p-PKA substrate/perilipin ratio. Results are expressed as mean ± SEM. (n = 4-7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs control (vehicle-treated cells); †$P < 0.05$ compared with CT-1-treated cells.

Characterization of the signaling pathways involved in the lipolytic actions of CT-1

Several signaling pathways have been found to be involved in the regulation of lipolysis, including cAMP/PKA, AMPK and cGMP-dependent protein kinase (cGK)-I by different lipolytic/antilipolytic agents (Nishikimi *et al.* 2009). Our data demonstrated that lipolytic actions of rCT-1 were completely reversed ($P < 0.001$) by pre-treatment with the PKA inhibitor H89 (Fig. 4A). Moreover, the blocking of PKA also reversed rCT-1-induced phosphorylation of perilipin and HSL at Ser660 ($P < 0.001$) (Fig. 4B). The involvement of PKA in the lipolytic action of rCT-1 was further supported by the fact that silencing of PKA expression using siRNA dramatically decrease rCT-1-stimulated glycerol release in adipocytes (Fig. 4C).

Because these data suggest that CT-1 lipolytic actions take place by activation of cAMP/PKA pathway, we also tested the effects of rCT-1 on cAMP, showing a significant ($P < 0.01$) increase in cAMP levels in rCT-1-treated adipocytes (Fig. 5A). In order to elucidate the mechanisms by which rCT-1 increases cAMP levels in adipocytes, we tested the effects of the cytokine on G-protein receptor complexes regulating adenylate cyclase. As shown in Fig. 5B treatment with rCT-1 induced a significant increase in protein expression of $G_S\alpha$, a protein which couples stimulatory receptors to adenylyl cyclase, whereas no changes where observed in Gi protein, which inhibits adenylyl cyclase. Moreover, neither the levels of p-PDE3B nor the levels of AdPLA were modified by rCT-1 (Supplementary Fig. IIA and IIB).

Treatment with the AMPK activator AICAR was also able to prevent the stimulation of glycerol release ($P < 0.001$) as well as the phosphorylation of Ser660 HSL ($P < 0.05$) and perilipin ($P < 0.01$) (Fig. 4A, B). On the other hand, pretreatment with PKG inhibitor KT5823 did not modify the stimulatory action of rCT-1 on glycerol release (Fig. 4A) or the phosphorylation of HSL and perilipin (Fig. 4B).

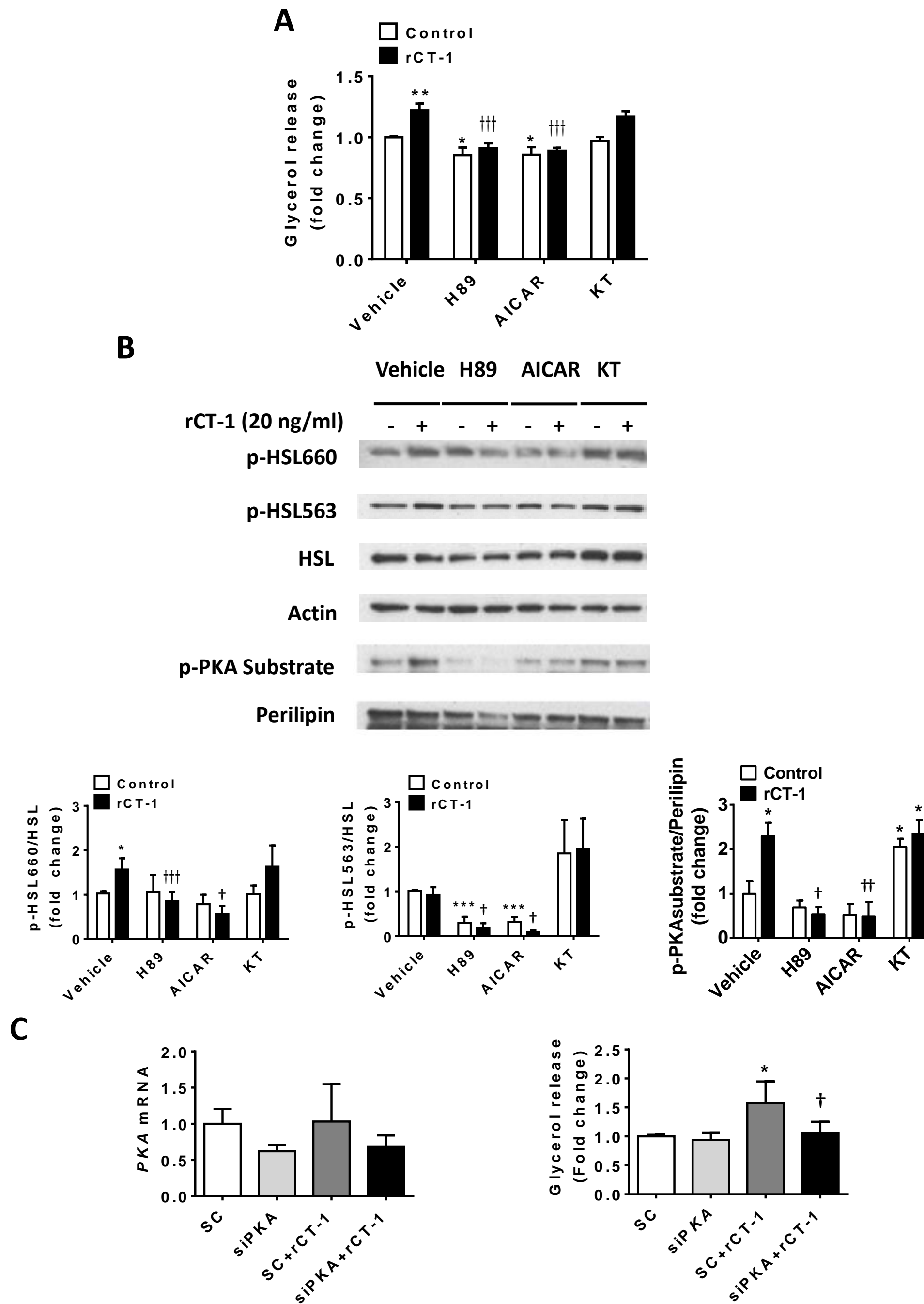
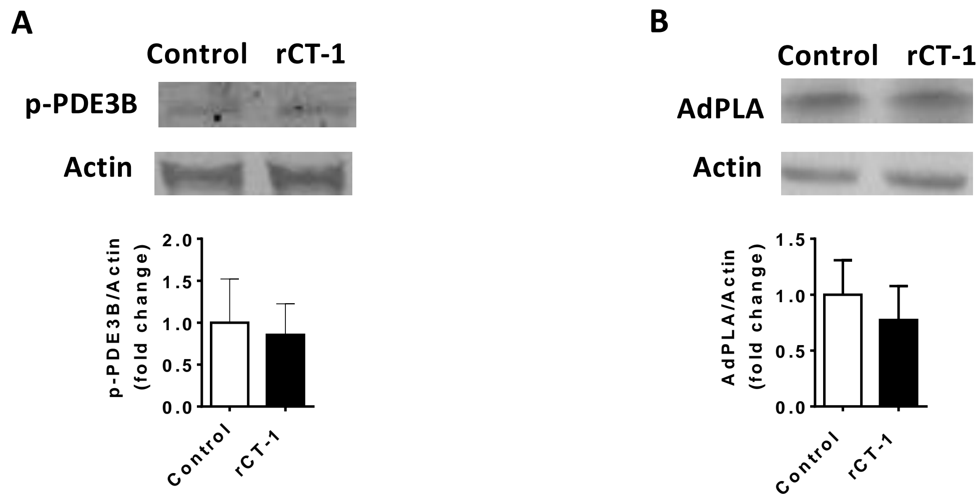


Fig. 4. PKA signaling pathway is involved in the lipolytic action of rCT-1. (A) Effects of rCT-1 (20 ng/mL) treatment on glycerol release in differentiated 3T3-L1 adipocytes pretreated for 1 h in the presence or absence of PKA inhibitor H89 (1 μM), AMPK activator AICAR (2 mM) and PKG inhibitor KT5823 (1 μM), and then exposed to rCT-1 (20 ng/mL) or vehicle (DMSO) for 24 h. (B) Representative Western Blot and densitometric analysis of phospho-HSL660, phospho-HSL563 normalized by total HSL protein and phospho-PKA substrate/perilipin ratio. (C) rCT-1-stimulated lipolysis is prevented by siRNA knock-down of PKA. mRNA levels of PKA and glycerol release in samples transfected with control siRNA (SC) or siPKA in either the absence or presence of rCT-1 for 24 h. Data are expressed as mean ± SEM (n = 3-7). *P < 0.05, **P < 0.01, ***P < 0.001 compared with control (vehicle-treated cells); †P < 0.05, ††P < 0.01, †††P < 0.001 compared with rCT-1-treated cells.



Supplementary Fig. II. Representative Western blot and densitometric analysis of (A) p-PDE3B and (B) AdPLA protein levels in rCT-1-treated (20 ng/ml) 3T3-L1 adipocytes for 24 h. Data are expressed as mean ± SEM (n = 4-7).

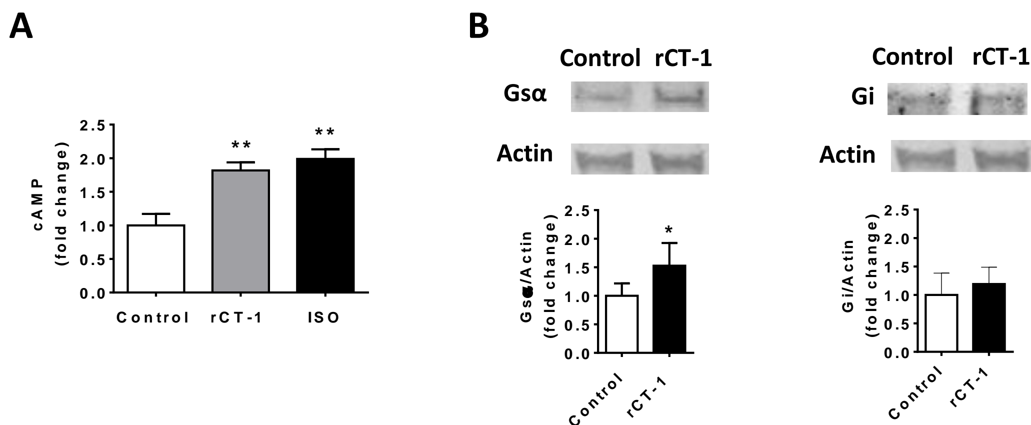


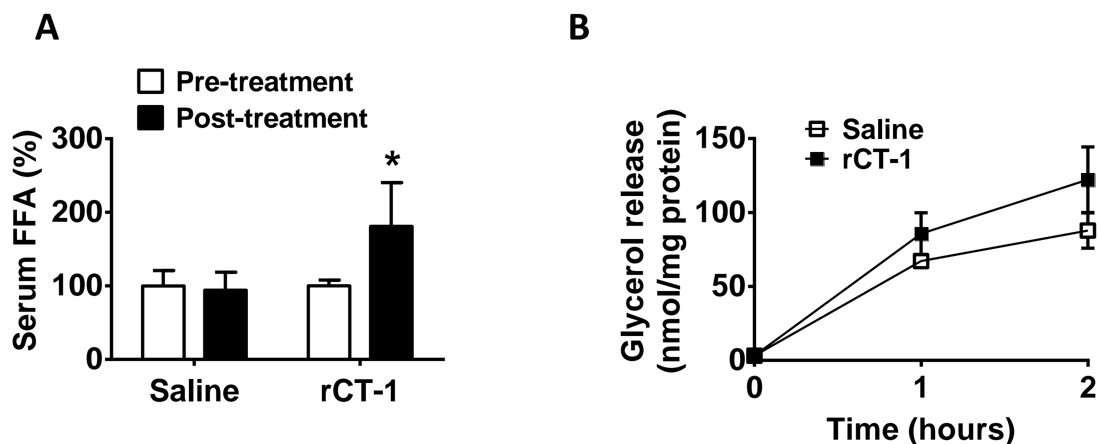
Fig. 5. rCT-1 stimulates $G_s\alpha$ and cAMP levels. (A) Intracellular cAMP levels after 24 h of treatment with rCT-1 (20 ng/mL). (B) Representative Western blot and densitometric analysis of $G_s\alpha$ and Gi protein levels in rCT-1-treated 3T3-L1 adipocytes. Data are expressed as mean ± SEM (n = 4-7) *$P < 0.05$, **$P < 0.01$ compared with control (vehicle-treated cells).

In vivo effects of rCT-1 treatment on HSL, Perilipin, ATGL, CGI-58 and G0S2 in adipose tissue of mice

Finally, we aimed to analyze whether the effects of rCT-1 on lipolysis observed in cultured adipocytes were also reproduced in adipose tissue after *in vivo* administration of the cytokine in mice. Interestingly, we found that rCT-1 treatment (0.2 mg/kg) for 30 min induced a significant increase in HSL phosphorylation at both Ser660 ($P < 0.05$) and Ser563 ($P < 0.001$) as compared to control mice. However, AMPK-mediated

phosphorylation of HSL on Ser565, which prevents HSL activation, was significantly ($P < 0.001$) decreased. Moreover, rCT-1 treatment boosted ($P < 0.05$) PKA-mediated phosphorylation of perilipin (Fig. 6A). These results further support the key role of the PKA pathway in the lipolytic action of CT-1. A statistically significant increase ($P < 0.05$) in ATGL protein levels was also found in epididymal fat of *ob/ob* mice after 30 min of rCT-1 treatment, whereas neither the activator (CGI-58), nor the inhibitor (G0S2) of ATGL activity showed any changes after rCT-1 treatment (Fig. 6B). These facts suggest the ability of rCT-1 to promote lipolysis *in vivo*.

In support of this, we found that the administration of rCT-1 (0.2 mg/kg) for 30 min to lean mice caused an increase in the levels of plasma FFAs in comparison with saline-treated mice (Supplementary Fig. IIIA). Moreover, adipose tissue explants from rCT-1-treated mice exhibited increased lipolytic response as compared with vehicle-treated group (Supplementary Fig. IIIB).



Supplementary Fig. III. (A) Effect of rCT-1 administration (0.2 mg/kg for 30 min) on serum FFA in mice. (B) Glycerol release in adipose tissue explants from vehicle or rCT-1-treated mice. Results are expressed as mean ± SEM. (n = 3). *$P < 0.05$, compared with vehicle-treated mice.

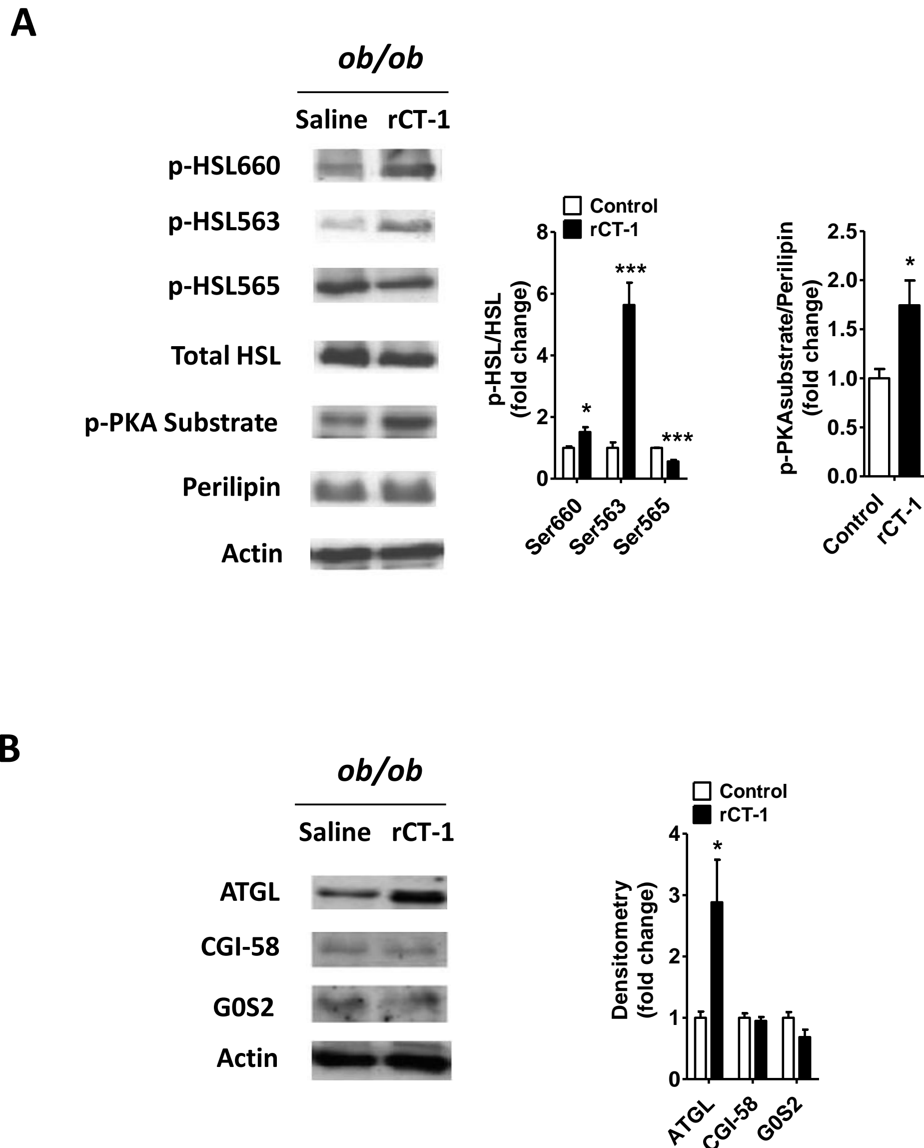


Fig. 6. *In vivo* administration of rCT-1 (0.2 mg/kg of body weight) for 30 min stimulates the main lipolytic enzymes in adipose tissue of *ob/ob* mice. (A) Representative Western Blot and densitometric analysis of HSL phosphorylation (Ser563, Ser660, Ser565) and perilipin in epididymal fat. Phospho-HSL bands intensities were normalized to total HSL, and phospho-PKA substrate bands were normalized to perilipin. (B) Representative Western Blot and densitometric analysis of ATGL, CGI-58 and G0S2 in epididymal fat from *ob/ob* mice treated with rCT-1 or vehicle. Band intensities for ATGL, CGI-58, and G0S2 were normalized to actin. Results are expressed as mean ± SEM. (n = 4-7). *$P < 0.05$, ***$P < 0.001$.

DISCUSSION

The present study demonstrates the lipolytic activity of CT-1 in adipocytes both *in vitro* and *in vivo*. In fact, in cultured adipocytes CT-1 treatment promoted a decrease in intracellular TAG in parallel with an increase in the release of glycerol and FFA, suggesting the ability of CT-1 to promote TAG catabolism. Interestingly, we found that CT-1 stimulates lipolysis in adipocytes through the regulation of the major lipases and lipid droplet proteins involved in the hydrolysis of TAG. Indeed, CT-1 promotes HSL phosphorylation at Ser660, a residue that is involved in the activation of this lipase. It is well known that PKA is the major kinase involved in the phosphorylation of HSL at the sites that cause HSL activation, including Ser563, Ser659 and Ser660. However, in the present study we observed that CT-1 selectively induces the phosphorylation of Ser660 without affecting Ser563 in cultured adipocytes. In this context, it has been described that phosphorylation sites Ser659 and Ser660 are the critical activity controlling sites, whereas Ser563 plays a minor role in direct activation of HSL *in vitro* (Anthonsen *et al.* 1998). Importantly, activation of HSL seems to be a key factor for the lipolytic action of CT-1, since silencing of HSL expression in adipocytes completely abolished CT-1-induced glycerol release, and partly prevent basal lipolysis.

Nevertheless, our *in vivo* studies revealed that acute administration of CT-1 to mice was able to stimulate adipose tissue lipolysis and to phosphorylate HSL not only at Ser660, but also a dramatic stimulation at Ser563 was also observed. It is important to take into account that *in vivo*, the regulation of PKA-stimulated HSL Ser563 and Ser660 phosphorylation seems to be time- and tissue-dependent. In fact, a different time-response pattern for Ser563 and Ser660 phosphorylation has been described at diverse times during/after exercise in human adipose tissue. Moreover, a differential response in HSL phosphorylation was observed for Ser563 and Ser660 after treatment with β-adrenergic and AMPK stimulation in 3T3-L1 adipocytes (Watt *et al.* 2006). These facts suggest that although PKA is able to stimulate the phosphorylation of both Ser residues of HSL, the magnitude and time-response pattern could be different.

Other findings also support the view that the CT-1-induced lipolysis is secondary to the activation of cAMP/PKA pathway: i) CT-1 increases cAMP intracellular content, ii) CT-1 promotes PKA-mediated phosphorylation of perilipin, enabling the translocation

of phosphorylated HSL from the cytoplasm to the lipid droplet surface (Brasaemle *et al.* 2000), iii) the PKA inhibitor H89 blunts the phosphorylation of the two main PKA-targets perilipin and HSL (at Ser660) and the subsequent increase in glycerol release induced by CT-1, and iv) silencing of PKA expression in adipocytes almost completely abrogated CT-1-induced glycerol release. Increased cAMP levels could be the consequence of increased adenylyl cyclase activity or reduced cAMP degradation (mainly mediated by PDE3B action). It is well established that the stimulation of G_s-coupled receptors induces the activation of adenylyl cyclase, leading to increased intracellular cAMP levels and subsequence activation of PKA and phosphorylation and translocation of HSL to fat droplets (Belfrage *et al.* 1981, Chaves *et al.* 2011). Here, we demonstrate that CT-1 increased the levels of $G_s\alpha$, without affecting Gi, the inhibitory protein of adenylyl cyclase. Our findings also revealed that neither the levels of PDE3B, an enzyme that catalyses the breakdown of cAMP to its inactive form, nor the levels of AdPLA, which inhibits cAMP production by regulating prostaglandin E_2 (PGE_2) abundance, were modified by CT-1. Taken together, these findings suggest that CT-1-induced increase of cAMP is secondary to Gs-mediated stimulation of adenylyl cyclase. CT-1 has the gp130 receptor as effector signaling. This receptor shares a large degree of sequence homology with leptin receptor, and both activate the JAK/STAT and ERK signaling pathways (Febbraio 2007). However, the lipolytic effect of leptin has been related with the downregulation of the adenylyl cyclase-inhibitory G protein pathway (Fruhbeck *et al.* 2014). In this context, growing evidence exists for a cross-talk of signaling cascades initiated by G-protein-coupled receptors (GPCRs) and the IL-6 family of cytokines signaling pathway (Garbers *et al.* 2012). In contrast to CT-1, TNF-α stimulates lipolysis in adipocytes by decreasing Gi without affecting Gs levels (Gasic *et al.* 1999) or by down-regulating the expression of PDE3B (Rahn Landstrom *et al.* 2000).

Our present data indicate that besides the stimulatory effect of CT-1 on lipolysis, ATGL protein levels are inhibited in long-term (24 h) CT-1-treated cultured adipocytes, in parallel with the increase of the ATGL inhibitor G0S2 (Yang *et al.* 2010). This apparently surprising finding of down-regulation of ATGL together with increased lipolysis has been described after treatment with some lipolytic molecules such as TNF-α in cultured adipocytes (Lorente-Cebrian *et al.* 2012, Kim *et al.* 2006). This may suggest that an interaction between these two regulatory processes (activity and

expression) occurs (high ATGL activity might be compensated by low expression) (Kralisch *et al.* 2005). Similarly to HSL, ATGL activity is also stimulated by catecholamines, but in contrast to HSL, ATGL activity is not directly regulated post-translationally via protein kinase A-mediated phosphorylation (Kershaw *et al.* 2007). It is well known that ATGL protein is mainly co-activated by CGI-58 and inhibited by G0S2. However, the transcriptional regulation of ATGL is poorly characterized. In this context, peroxisome proliferator-activated receptor γ (PPARγ) has been identified as a regulator of ATGL levels (Kershaw *et al.* 2007). It has been described that CT-1 induces a transient decrease in PPARγ in adipocytes at 24 h (Zvonic *et al.* 2004). In concordance, our data revealed that the decrease in ATGL observed after 24 h of treatment with CT-1 in cultured adipocytes paralleled with the drop in PPARγ levels (data not shown), suggesting a potential association between both events. In contrast with the lack of effect of short-term treatments (1-2 h) with CT-1 on ATGL in cultured adipocytes, the *in vivo* acute administration of CT-1 induced a marked increase in ATGL protein levels at 30 min, suggesting that putatively some mechanisms may regulate ATGL protein expression *in vivo* that do not exist in the *in vitro* model. In this context, several studies of our group have revealed that acute administration of CT-1 to mice have profound peripheral and central effects acting on neurohormonal regulators that could also secondarily affect adipose tissue lipolysis. Thus, CT-1 stimulate insulin signaling and sensitivity, decrease blood glucose, activates AMPK and promotes fatty acid oxidation in muscle and liver, and modulate hypothalamic pathways involved in energy intake (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). Interestingly, growing evidence suggests a role of hypothalamic regulation of adipose tissue function and metabolism (Stefanidis *et al.* 2014).

AMPK has also been shown as an important regulator of lipolysis by regulating both HSL and ATGL activity by phosphorylation. Nevertheless, the effects of AMPK activation on lipolysis are complex since both antilipolytic (Dagon *et al.* 2006, Boon *et al.* 2008, Anthony *et al.* 2009) and lipolytic (Koh *et al.* 2007, Yamaguchi *et al.* 2005) actions have been reported. In fact, AMPK effects on lipolysis seem to be time-dependent, involving antagonistic modulation of HSL and ATGL (Gaidhu *et al.* 2009). In this context, several trials have demonstrated that AMPK induces phosphorylation of HSL at Ser565, which prevents phosphorylation of HSL by cyclic AMP-dependent

protein kinase (PKA), causing suppression of PKA-stimulated lipolysis (Garton *et al.* 1990). On the other hand, AMPK phosphorylates ATGL at Ser406, increasing TAG hydrolase activity, and providing evidence for increased lipolysis (Ahmadian *et al.* 2011). The present data show that AMPK activation totally abolishes the lipolytic effect of CT-1 and suggest that AICAR-induced phosphorylation of HSL at Ser565 is able to prevent CT-1 induced-PKA-mediated phosphorylation and activation of HSL.

Activation of cGMP pathway has also been shown to promote lipolysis. A downstream effector of cGMP, cGMP-dependent protein kinase, also called protein kinase G (PKG), was shown to induce perilipin and HSL phosphorylation and to be at the origin of atrial natriuretic peptide-induced lipolysis (Sengenes *et al.* 2005). We tested the potential involvement of cGMP/PKG pathway in the lipolytic actions of CT-1. The results suggest that this pathway is not involved in CT-1 stimulated lipolysis since treatment with PKG inhibitor KT5823 was not able to reverse CT-1 induced glycerol release and did not cause any significant changes in HSL or perilipin activation.

Several studies have described lipolytic actions in adipocytes for IL-6 (Ji *et al.* 2011) and other members of the gp130 ligand family of cytokines such as leukemia inhibitory factor (LIF) (Marshall *et al.* 1994). However, differential effects have been found among cytokines of this family. For example, previous studies by our group have shown that chronic administration of CT-1 to obese mice stimulated the lipolytic response to isoproterenol in adipocytes (Moreno-Aliaga *et al.* 2011). On the other hand, no significant changes in lipolysis were found after the administration of CNTF to high-fat-fed mice (Crowe *et al.* 2008). The study by Wolsk *et al*. (Wolsk *et al.* 2010) showed that an acute increase in IL-6 selectively stimulates lipolysis in skeletal muscle, whereas adipose tissue was unaffected in humans. Our present study clearly shows an increase of the main lipases in adipose tissue after an acute administration of CT-1. Additional findings also suggest differential mechanisms of action underlying the lipolytic properties of CT-1 and IL-6. Thus, it has been observed that IL-6 increased lipolysis in differentiated porcine adipocytes by activation of ERK, which was inhibited by specific ERK inhibitor, while IL-6 treatment did not elevate intracellular cAMP, and specific PKA inhibitor (H89) did not affect IL-6-induced lipolysis, suggesting that PKA pathway was not involved in IL-6 lipolytic effects (Yang *et al.* 2008). On the contrary,

our present data clearly suggest the involvement of the cAMP/PKA pathway in the lipolytic action of CT-1.

Activation of lipolysis has been proposed as a promising therapeutic target for the treatment of obesity (Ahmadian *et al.* 2009). Our results suggest that the ability of CT-1 to activate the pathway in adipocytes could also contribute to its anti-obesity properties. In line with this, we have previously reported that chronic administration of CT-1 to *ob/ob* mice increased adipocytes lipolytic response to isoproterenol (Moreno-Aliaga *et al.* 2011). It is important to mention that increased lipolysis and FFA release from adipose tissue have been associated with the development of metabolic disturbances in obesity (Ormseth *et al.* 2011). However, several studies have suggested that increasing lipolysis in adipose tissue does not necessarily increase serum FFA levels because increasing lipolysis in adipose tissue causes a shift within adipocytes toward increased FFA utilization and energy expenditure and thus protects against obesity (Ahmadian *et al.* 2009). In this context, our previous data revealed that CT-1 is able to promote FFA oxidation not only in adipose tissue but also in muscle, reducing insulin resistance in obese mice (Moreno-Aliaga *et al.* 2011).

In summary, the present data demonstrate that the ability of CT-1 to regulate the activity of the main lipases underlies the lipolytic action of this cytokine *in vitro* and *in vivo*, and may account for the anti-obesity effects of CT-1.

ACKNOWLEDGEMENTS

This study was supported by grants from the Government of Navarra (Department of Health); Mutua Madrileña Foundation; FIS [PI10/01516 and PI13/01851]; Línea Especial ‘‘Nutrición, Obesidad y Salud Universidad de Navarra”; and by the agreement between FIMA and the ‘‘UTE project CIMA’’. Miguel López-Yoldi was supported by a Doctoral grant from Asociación de Amigos University of Navarra.

REFERENCES

-A-

Ahmadian, M., Abbott, M. J., Tang, T., Hudak, C. S., Kim, Y., Bruss, M., Hellerstein, M. K., Lee, H. Y., Samuel, V. T., Shulman, G. I., Wang, Y., Duncan, R. E., Kang, C. & Sul, H. S. 2011. Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. *Cell Metab,* **13,** 739-48.

Ahmadian, M., Duncan, R. E. & Sul, H. S. 2009. The skinny on fat: lipolysis and fatty acid utilization in adipocytes. *Trends Endocrinol Metab,* **20,** 424-8.

Anthonsen, M. W., Ronnstrand, L., Wernstedt, C., Degerman, E. & Holm, C. 1998. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J Biol Chem,* **273,** 215-21.

Anthony, N. M., Gaidhu, M. P. & Ceddia, R. B. 2009. Regulation of visceral and subcutaneous adipocyte lipolysis by acute AICAR-induced AMPK activation. *Obesity (Silver Spring),* **17,** 1312-7.

-B-

Belfrage, P., Fredrikson, G., Nilsson, N. O. & Stralfors, P. 1981. Regulation of adipose-tissue lipolysis by phosphorylation of hormone-sensitive lipase. *Int J Obes,* **5,** 635-41.

Beraza, N., Marques, J. M., Martinez-Anso, E., Iniguez, M., Prieto, J. & Bustos, M. 2005. Interplay among cardiotrophin-1, prostaglandins, and vascular endothelial growth factor in rat liver regeneration. *Hepatology,* **41,** 460-9.

Boon, H., Bosselaar, M., Praet, S. F., Blaak, E. E., Saris, W. H., Wagenmakers, A. J., McGee, S. L., Tack, C. J., Smits, P., Hargreaves, M. & van Loon, L. J. 2008. Intravenous AICAR administration reduces hepatic glucose output and inhibits whole body lipolysis in type 2 diabetic patients. *Diabetologia,* **51,** 1893-900.

Brasaemle, D. L. 2007. Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *J Lipid Res,* **48,** 2547-59.

Brasaemle, D. L., Levin, D. M., Adler-Wailes, D. C. & Londos, C. 2000. The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim Biophys Acta,* **1483,** 251-62.

-C-

Castano, D., Larequi, E., Belza, I., Astudillo, A. M., Martinez-Anso, E., Balsinde, J., Argemi, J., Aragon, T., Moreno-Aliaga, M. J., Muntane, J., Prieto, J. & Bustos, M. 2014. Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation. *J Hepatol,* **60,** 1017-25.

Cornaciu, I., Boeszoermenyi, A., Lindermuth, H., Nagy, H. M., Cerk, I. K., Ebner, C., Salzburger, B., Gruber, A., Schweiger, M., Zechner, R., Lass, A., Zimmermann, R. & Oberer, M. 2011. The minimal domain of adipose triglyceride lipase (ATGL) ranges until leucine 254 and can be activated and inhibited by CGI-58 and G0S2, respectively. *PLoS One,* **6,** e26349.

Crowe, S., Turpin, S. M., Ke, F., Kemp, B. E. & Watt, M. J. 2008. Metabolic remodeling in adipocytes promotes ciliary neurotrophic factor-mediated fat loss in obesity. *Endocrinology,* **149,** 2546-56.

Chaves, V. E., Frasson, D. & Kawashita, N. H. 2011. Several agents and pathways regulate lipolysis in adipocytes. *Biochimie,* **93,** 1631-40.

Choi, S. M., Tucker, D. F., Gross, D. N., Easton, R. M., DiPilato, L. M., Dean, A. S., Monks, B. R. & Birnbaum, M. J. 2010. Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. *Mol Cell Biol,* **30,** 5009-20.

-D-

Dagon, Y., Avraham, Y. & Berry, E. M. 2006. AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2alpha in adipocytes. *Biochem Biophys Res Commun,* **340,** 43-7.

-F-

Febbraio, M. A. 2007. gp130 receptor ligands as potential therapeutic targets for obesity. *J Clin Invest,* **117,** 841-9.

Fernandez-Galilea, M., Perez-Matute, P., Prieto-Hontoria, P. L., Martinez, J. A. & Moreno-Aliaga, M. J. 2012. Effects of lipoic acid on lipolysis in 3T3-L1 adipocytes. *J Lipid Res,* **53,** 2296-306.

Fruhbeck, G., Mendez-Gimenez, L., Fernandez-Formoso, J. A., Fernandez, S. & Rodriguez, A. 2014. Regulation of adipocyte lipolysis. *Nutr Res Rev,* **27,** 63-93.

-G-

Gaidhu, M. P., Fediuc, S., Anthony, N. M., So, M., Mirpourian, M., Perry, R. L. & Ceddia, R. B. 2009. Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. *J Lipid Res,* **50,** 704-15.

Garbers, C., Hermanns, H. M., Schaper, F., Muller-Newen, G., Grotzinger, J., Rose-John, S. & Scheller, J. 2012. Plasticity and cross-talk of interleukin 6-type cytokines. *Cytokine Growth Factor Rev,* **23,** 85-97.

Garton, A. J. & Yeaman, S. J. 1990. Identification and role of the basal phosphorylation site on hormone-sensitive lipase. *Eur J Biochem,* **191,** 245-50.

Gasic, S., Tian, B. & Green, A. 1999. Tumor necrosis factor alpha stimulates lipolysis in adipocytes by decreasing Gi protein concentrations. *J Biol Chem,* **274,** 6770-5.

Granneman, J. G., Moore, H. P., Krishnamoorthy, R. & Rathod, M. 2009. Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *J Biol Chem,* **284,** 34538-44.

Greenberg, A. S., Shen, W. J., Muliro, K., Patel, S., Souza, S. C., Roth, R. A. & Kraemer, F. B. 2001. Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. *J Biol Chem,* **276,** 45456-61.

-J-

Ji, C., Chen, X., Gao, C., Jiao, L., Wang, J., Xu, G., Fu, H., Guo, X. & Zhao, Y. 2011. IL-6 induces lipolysis and mitochondrial dysfunction, but does not affect insulin-mediated glucose transport in 3T3-L1 adipocytes. *J Bioenerg Biomembr,* **43,** 367-75.

-K-

Kershaw, E. E., Schupp, M., Guan, H. P., Gardner, N. P., Lazar, M. A. & Flier, J. S. 2007. PPARgamma regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. *Am J Physiol Endocrinol Metab,* **293,** E1736-45.

Kim, J. Y., Tillison, K., Lee, J. H., Rearick, D. A. & Smas, C. M. 2006. The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-alpha in 3T3-L1 adipocytes and is a target for transactivation by PPARgamma. *Am J Physiol Endocrinol Metab,* **291,** E115-27.

Koh, H. J., Hirshman, M. F., He, H., Li, Y., Manabe, Y., Balschi, J. A. & Goodyear, L. J. 2007. Adrenaline is a critical mediator of acute exercise-induced AMP-activated protein kinase activation in adipocytes. *Biochem J,* **403,** 473-81.

Kralisch, S., Klein, J., Lossner, U., Bluher, M., Paschke, R., Stumvoll, M. & Fasshauer, M. 2005. Isoproterenol, TNFalpha, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. *Mol Cell Endocrinol,* **240,** 43-9.

-L-

Lorente-Cebrian, S., Bustos, M., Marti, A., Fernandez-Galilea, M., Martinez, J. A. & Moreno-Aliaga, M. J. 2012. Eicosapentaenoic acid inhibits tumour necrosis factor-alpha-induced lipolysis in murine cultured adipocytes. *J Nutr Biochem,* **23,** 218-27.

Lu, X., Yang, X. & Liu, J. 2010. Differential control of ATGL-mediated lipid droplet degradation by CGI-58 and G0S2. *Cell Cycle,* **9,** 2719-25.

-M-

Marshall, M. K., Doerrler, W., Feingold, K. R. & Grunfeld, C. 1994. Leukemia inhibitory factor induces changes in lipid metabolism in cultured adipocytes. *Endocrinology,* **135,** 141-7.

Miyoshi, H., Perfield, J. W., 2nd, Souza, S. C., Shen, W. J., Zhang, H. H., Stancheva, Z. S., Kraemer, F. B., Obin, M. S. & Greenberg, A. S. 2007. Control of adipose triglyceride lipase action by serine 517 of perilipin A globally regulates protein kinase A-stimulated lipolysis in adipocytes. *J Biol Chem,* **282,** 996-1002.

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* **14,** 242-53.

-N-

Natal, C., Fortuno, M. A., Restituto, P., Bazan, A., Colina, I., Diez, J. & Varo, N. 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. *Am J Physiol Endocrinol Metab,* **294,** E52-60.

Nishikimi, T., Iemura-Inaba, C., Akimoto, K., Ishikawa, K., Koshikawa, S. & Matsuoka, H. 2009. Stimulatory and Inhibitory regulation of lipolysis by the NPR-A/cGMP/PKG and NPR-C/G(i) pathways in rat cultured adipocytes. *Regul Pept,* **153,** 56-63.

-O-

Ormseth, M. J., Swift, L. L., Fazio, S., Linton, M. F., Chung, C. P., Raggi, P., Rho, Y. H., Solus, J., Oeser, A., Bian, A., Gebretsadik, T., Shintani, A. & Stein, C. M. 2011. Free fatty acids are associated with insulin resistance but not coronary artery atherosclerosis in rheumatoid arthritis. *Atherosclerosis,* **219,** 869-74.

-P-

Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D. L., Zioncheck, K. A., Rosenthal, A., Taga, T., Paoni, N. F. & Wood, W. I. 1995. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem,* **270,** 10915-22.

-R-

Rahn Landstrom, T., Mei, J., Karlsson, M., Manganiello, V. & Degerman, E. 2000. Down-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumour necrosis factor alpha and cAMP. *Biochem J,* **346 Pt 2,** 337-43.

-S-

Sengenes, C., Moro, C., Galitzky, J., Berlan, M. & Lafontan, M. 2005. [Natriuretic peptides: a new lipolytic pathway in human fat cells]. *Med Sci (Paris),* **21,** 61-5.

Stefanidis, A., Wiedmann, N. M., Adler, E. S. & Oldfield, B. J. 2014. Hypothalamic control of adipose tissue. *Best Pract Res Clin Endocrinol Metab,* **28,** 685-701.

Sztalryd, C., Xu, G., Dorward, H., Tansey, J. T., Contreras, J. A., Kimmel, A. R. & Londos, C. 2003. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J Cell Biol,* **161,** 1093-103.

-T-

Takahashi, Y., Shinoda, A., Furuya, N., Harada, E., Arimura, N., Ichi, I., Fujiwara, Y., Inoue, J. & Sato, R. 2013. Perilipin-mediated lipid droplet formation in adipocytes promotes sterol regulatory element-binding protein-1 processing and triacylglyceride accumulation. *PLoS One,* **8,** e64605.

-W-

Watt, M. J., Holmes, A. G., Pinnamaneni, S. K., Garnham, A. P., Steinberg, G. R., Kemp, B. E. & Febbraio, M. A. 2006. Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. *Am J Physiol Endocrinol Metab,* **290,** E500-8.

Wolsk, E., Mygind, H., Grondahl, T. S., Pedersen, B. K. & van Hall, G. 2010. IL-6 selectively stimulates fat metabolism in human skeletal muscle. *Am J Physiol Endocrinol Metab,* **299,** E832-40.

-Y-

Yamaguchi, S., Katahira, H., Ozawa, S., Nakamichi, Y., Tanaka, T., Shimoyama, T., Takahashi, K., Yoshimoto, K., Imaizumi, M. O., Nagamatsu, S. & Ishida, H. 2005. Activators of AMP-activated protein kinase enhance GLUT4 translocation and its glucose transport activity in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab,* **289,** E643-9.

Yang, X., Lu, X., Lombes, M., Rha, G. B., Chi, Y. I., Guerin, T. M., Smart, E. J. & Liu, J. 2010. The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. *Cell Metab,* **11,** 194-205.

Yang, Y., Ju, D., Zhang, M. & Yang, G. 2008. Interleukin-6 stimulates lipolysis in porcine adipocytes. *Endocrine,* **33,** 261-9.

Yeaman, S. J. 2004. Hormone-sensitive lipase--new roles for an old enzyme. *Biochem J,* **379,** 11-22.

-Z-

Zimmermann, R., Strauss, J. G., Haemmerle, G., Schoiswohl, G., Birner-Gruenberger, R., Riederer, M., Lass, A., Neuberger, G., Eisenhaber, F., Hermetter, A. & Zechner, R. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science,* **306,** 1383-6.

Zvonic, S., Hogan, J. C., Arbour-Reily, P., Mynatt, R. L. & Stephens, J. M. 2004. Effects of cardiotrophin on adipocytes. *J Biol Chem,* **279,** 47572-9.

CHAPTER 2

Cardiotrophin-1 regulates adipokine production in 3T3-L1 adipocytes and adipose tissue from obese mice

López-Yoldi M[1,2], Marcos-Gomez B[1], Romero-Lozano MA[3], Prieto J[3,4], Martínez JA[1,2,5,6], Bustos M[3*], Moreno-Aliaga MJ[1,2,5,6*]

[1]Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Navarra, Spain.

[2]Centre for Nutrition Research. University of Navarra, Pamplona, Navarra, Spain.

[3]Department of Hepatology and Gene Therapy, CIMA, University of Navarra, Pamplona, Navarra, Spain.

[4]CIBERehd. Institute of Health Carlos III, Madrid, Spain.

[5]CIBERobn, Physiopathology of Obesity and Nutrition, Institute of Health Carlos III, Madrid, Spain.

[6]IdiSNA, Navarra's Health Research Institute, Pamplona, Spain.

*Equal senior authorship

ABSTRACT

Cardiotrophin-1 (CT-1) is a member of the IL-6 family of cytokines. A recent study of our group has revealed that CT-1 is a key regulator of glucose and lipid metabolism. The aim of the present study was to analyze the *in vitro* and *in vivo* effects of CT-1 on the production of several adipokines involved in body weight regulation, nutrient metabolism and inflammation. For this purpose, 3T3-L1 adipocytes were incubated with recombinant protein CT-1 (rCT-1) (1-40 ng/mL) for 1 and 18 h. Moreover, the acute effects of rCT-1 administration (0.2 mg/kg, i.v.) for 30 min and 3 h on adipokines levels were also evaluated in high-fat fed obese mice. In 3T3-L1 adipocytes, rCT-1 treatment downregulated the expression and secretion of leptin, resistin and visfatin. However, rCT-1 significantly stimulated apelin mRNA and apelin secretion. rCT-1 treatment (18 h) also promoted the activation by phosphorylation of AKT, ERK 1/2 and STAT3. Interestingly, pre-treatment with the PI3K inhibitor LY294002 and with the JAK/STAT inhibitor AG490 reversed the stimulatory effects of CT-1 on apelin gene expression, suggesting that both pathways could be mediating the effects of CT-1 on the production of this adipokine. On the other hand, acute administration of rCT-1 (30 min and 3 h) to diet-induced obese mice downregulated leptin and resistin, without significantly modifying apelin or visfatin mRNA expression in white adipose tissue. The present study demonstrates the ability of rCT-1 to modulate the production of adipokines in *vitro* and *in vivo*, suggesting that the regulation of the secretory function of adipocytes could be also involved in the metabolic actions of this cytokine.

Supplementary key words: *Adipokines . Cardiotrophin-1 . Adipose tissue . Leptin . Resistin . Visfatin . Apelin .*

INTRODUCTION

Obesity is considered a low-grade chronic inflammatory state with an increase in systemic markers of inflammation. It is thought that this increase in inflammation is one of the mechanisms that could contribute to the development of the pathologies associated to obesity such as metabolic diseases (type 2 diabetes mellitus, fatty liver disease, and dyslipidemia), cardiovascular disorders, diseases of the central nervous system (dementia) and different types of cancer (Van Gaal *et al.* 2006, LeRoith *et al.* 2008). In addition to its primary role as a fuel reservoir, white adipose tissue (WAT) has also been established as a metabolically important endocrine organ with a key relevance in the regulation of energy expenditure and glucose homeostasis (Fonseca-Alaniz *et al.* 2006). Indeed, WAT is responsible for the expression and secretion of an array of molecules known as adipokines that can regulate many physiological processes. Within WAT, adipokines have been shown to modulate adipogenesis, immune cell migration into adipose tissue, and adipocyte metabolism and function (Kloting *et al.* 2014). At the systemic level, adipokines modulate different biological processes in target organs, including the brain, liver, muscle, vasculature, heart, pancreas, immune system, among others (Bluher 2014).

Leptin is one of the most abundant and important adipokines. The most well-known effect of leptin is to regulate body weight and energy balance but it also has fundamental roles in glucose and lipid homeostasis, reproduction, immunity, inflammation, bone physiology, and tissue remodeling (Paz-Filho *et al.* 2015, Sainz *et al.* 2015a, Sainz *et al.* 2015b). Resistin is a pro-inflammatory cytokine produced by adipose tissue. Its exact role in insulin resistance and obesity has not yet been determined, but in mice resistin has been shown to increase blood glucose and insulin concentrations by promoting hepatic insulin resistance and gluconeogenesis (Pagano *et al.* 2006). Although its function in humans is still unclear, some studies have demonstrated that obese individuals display higher serum resistin values than lean subjects (Meier *et al.* 2004). Regarding visfatin, it has been described that it is upregulated by pro-inflammatory cytokines and under inflammatory conditions. Also, the findings of McGee et al. (McGee *et al.* 2011) have suggested that visfatin may represent a pro-inflammatory cytokine that is affected by insulin sensitivity through

NF-κB and JNK pathways. Apelin is another member of the adipokine family (Boucher *et al.* 2005) and it is known for its anti-obesity and anti-diabetic properties (Castan-Laurell *et al.* 2011). Apelin promotes insulin sensitivity and glucose utilization in adipose and muscle tissues (Yue *et al.* 2010, Zhu *et al.* 2011). Because of this and other beneficial effects of apelin, it has been suggested that over-production of apelin could be one of the last protective defense before the onset of disorders related to obesity such as type 2 diabetes or cardiovascular dysfunction (Castan-Laurell *et al.* 2005, Carpene *et al.* 2007).

Cardiotrophin-1 (CT-1) belongs to the interleukin-6 (IL)-6 family of cytokines. These cytokines exert their cellular effects by interacting with the glycoprotein 130 (gp130)/leukemia inhibitory factor receptor (LIFR) heterodimer (Pennica *et al.* 1995). Adipose tissue has been identified as a source of CT-1 (Natal *et al.* 2008), being this cytokine capable of activating major signaling and metabolic pathways in adipocytes (Zvonic *et al.* 2004, Lopez-Yoldi *et al.* 2014). Recent studies by our group have revealed that CT-1 is a key regulator of energy homeostasis, as well as of glucose and lipid metabolism. Thus, chronic recombinant CT-1 (rCT-1) treatment reduced body weight and corrected insulin resistance and hepatic steatosis in *ob/ob* and in high-fat-diet (HFD)-fed obese mice (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). CT-1 induces a dramatic metabolic remodeling of adipose tissue reducing the expression of lipogenic genes while promoting lipolysis, fatty acid oxidation and mitochondrial biogenesis (Moreno-Aliaga *et al.* 2011, Lopez-Yoldi *et al.* 2014). However, the actions of CT-1 on adipocyte secretory function remain still unknown. Therefore, we aimed at analyzing the direct effects of rCT-1 on adipokine´s gene expression and secretion in 3T3-L1 adipocytes, and to characterize the major signaling pathways implicated. Furthermore, we also investigated the effects of acute rCT-1 treatments on adipokines mRNA levels in WAT from obese mice.

MATERIAL AND METHODS

Cell culture and differentiation of 3T3-L1 cells

Mouse embryo fibroblast 3T3-L1 (American Type Culture Collection; Rockville, MD) were cultured in Dulbecco´s modified Eagle´s medium (DMEM) containing 25 mM glucose, 10% (v/v) calf bovine serum (CBS) (Invitrogen, Carlsbad, CA), and 1% (v/v)

penicillin/streptomycin (Invitrogen) and maintained in an incubator set to 37°C and 5% of carbon dioxide. At confluence, pre-adipocytes were cultured for 48 h in DMEM (Invitrogen) containing 25 mM glucose, 10% fetal bovine serum (FBS) (Invitrogen), and antibiotics, and supplemented with dexamethasone (1 mM; Sigma, St. Louis, MO), isobutylmethylxantine (0.5 mM; Sigma) and insulin (10 μg/mL; Sigma). After that, cells were cultured with 10% FBS and insulin for 48 h and then media were replaced with 10% FBS in DMEM and antibiotics, but without insulin, and these media were changed every 2 days until day 8 post-confluence when cells attained the morphology and typical features of mature adipocytes (Fernandez-Galilea *et al.* 2012).

Recombinant Protein CT-1 (rCT-1)

rCT-1 was obtained as described elsewhere (Beraza *et al.* 2005) and contained < 0.04 ng LPS per 1 μg of the protein as determined by the Limulus amoebocyte lysate assay (Cambrex, East Rutherford, NJ).

***In vitro* treatments**

The day of the experiment, adipocytes were serum starved during 4 h using the medium DMEM supplemented with 0.1 % FBS and then treated with rCT-1 (1-40 ng/mL) or IL-6 (20 ng/mL) for 1 and 18 h. Adipocytes were treated in the presence or absence of different specific inhibitors of the cellular signaling pathways involved in the actions of rCT-1 on adipokine production. Selective PI3K inhibitor LY294002 (50 μM; Sigma), MAPK/ERK inhibitor PD98059 (50 μM; Sigma) and JAK/STAT inhibitor AG490 (20 μM; Calbiochem, San Diego, CA) were added to the medium for 1 h prior to the addition of rCT-1 (20 ng/mL) to the treated wells. Then, adipocytes were incubated 18 h at 37 º C and 5% CO_2.

Acute treatments with rCT-1 in HFD-fed mice

Eight-week-old male C57BL/6J mice were obtained from Harlan Laboratories (Barcelona, Spain) and were placed on high fat diet (HFD) (60% of kcal from fat, 20% from carbohydrates and 20% from protein, Research Diets, New Brunswick, NJ) *ad libitum* for 12 weeks. At the end, mice were fasted for 18 h and injected intravenously (i.v.) with saline or rCT-1 (0.2 mg/kg) before they were euthanized after 30 min or 3 h. Epididymal fat depots were excised, weighed and kept at −80 °C for subsequent

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

Analyses of mRNA levels

Total RNA was extracted with TRIZOL (Invitrogen) and real-time PCR was performed using iCycler (Bio-Rad, München, Germany) and iQ SYBR Green Supermix (Bio-Rad), as previously described (Castano *et al.* 2014). For relative quantification of gene expression we used the comparative Ct method [$2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the target and control genes (cyclophilin)]. Primers were designed according to published complementary DNA or genomic sequences.

Biochemical analysis

The total amount of adipokines released to the cell culture media was measured using the appropriate Enzyme Immunoassay (ELISA) kit and following the manufacturer´s protocol. Total apelin, was determined using the ELISA for mouse/rat apelin from Phoenix Peptide (Burlingame, CA) as elsewhere (Lorente-Cebrian *et al.* 2010). Visfatin and resistin were assessed with the mouse ELISA kits of RayBiotech, Inc (Norcross, GA). Leptin was estimated by the mouse ELISA kit (Invitrogen) as described (Prieto-Hontoria *et al.* 2011).

Western Blot analysis

3T3-L1 cell lysates were obtained by the addition of a buffer containing 2 mM TrisHCl (pH 8), 137 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail 1 (Sigma), 2 mM orthovanadate, and 1 mM PMSF. Briefly, equivalent amounts of total protein (25-50 µg) were electrophoretically separated by 12-15% SDS-PAGE in the presence of a reducing agent (2-mercaptoethanol). Proteins were electroblotted from the gel to polyvinylidenedifluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Following the transfer of proteins, the membranes were blocked and probed with specific primary antibodies against phospho-AKT (Ser473), AKT, phospho-ERK 1/2 (Thr202/Tyr204), ERK, phospho-STAT3 (Tyr705) and STAT3 (Cell Signaling Technologies, Beverly, MA) and β-actin (Sigma). After that, membranes were hybridized with

horseradish peroxidase-conjugated secondary antibody (Sigma) for 1 h and then revealed with the SuperSignal kit revelation solution (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. The results were analyzed by densitometry using the GS-800 Calibrated densitometer (Bio-Rad).

Statistical analysis

Data are presented as mean ± standard error (SEM). Comparisons between the values for different variables were analyzed by one-way ANOVA followed by Bonferroni post hoc tests or by Student’s t-test or Mann-Whitney U-test once the normality had been screened using Kolmogorov-Smirnoff and Shapiro-Wilk tests. Statistical analyses and graphs were carried out using GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA). Overall, a P value < 0.05 was considered significant.

RESULTS

CT-1 downregulates the expression of leptin, resistin and visfatin in 3T3-L1 adipocytes

Because hiperleptinemia as well as high levels of resistin and visfatin have been related to the development of insulin resistance and other metabolic complications in obesity, we first analyzed the regulatory effects of rCT-1 on the production of these adipokines.

As shown in Fig. 1A (top panel), rCT-1 reduced leptin mRNA levels in a concentration dependent manner, being significant from 1 ng/mL. Similar results were observed on the gene expression levels of resistin after the treatment with rCT-1. Treatment with this cytokine induced a dose-dependent downregulation on resistin gene expression that was significant at the doses of 20 and 40 ng/mL (Fig. 1B, top panel). Finally, visfatin gene levels were also significantly downregulated after the incubation with rCT-1 (Fig. 1C, top panel). We next analyzed the effects of rCT-1 on the secretion of these adipokines to the media. In parallel with the results observed on gene expression, CT-1 induced an inhibition of the secretion of both leptin and resistin in a dose-dependent manner (Fig. 1A and 1B, bottom panels). Regarding to visfatin,

treatment with rCT-1 promoted a decrease on the secretion of this adipokine but this effect was not dose-dependent (Fig. 1C, bottom panels).

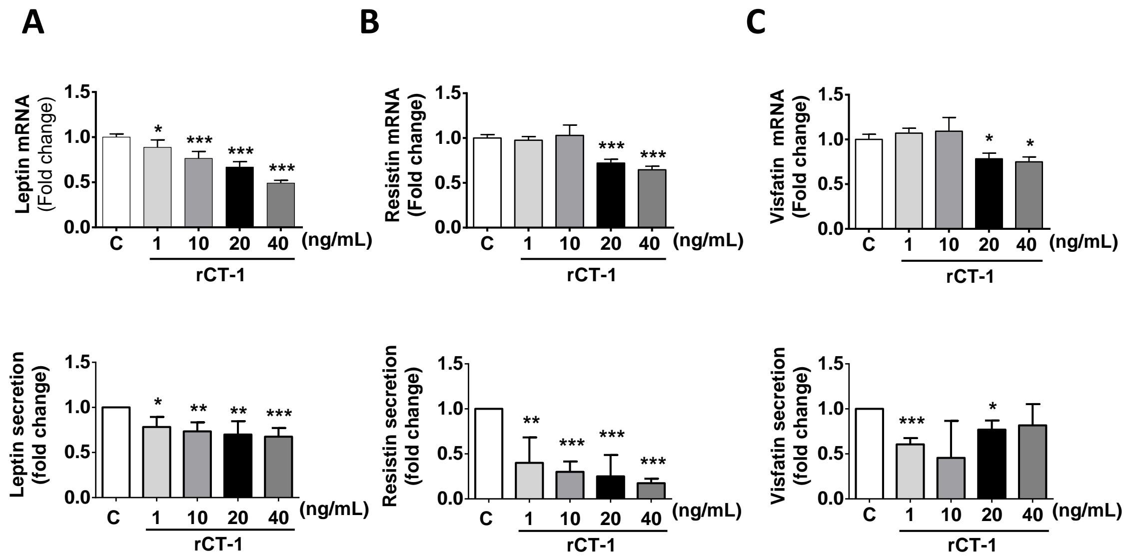
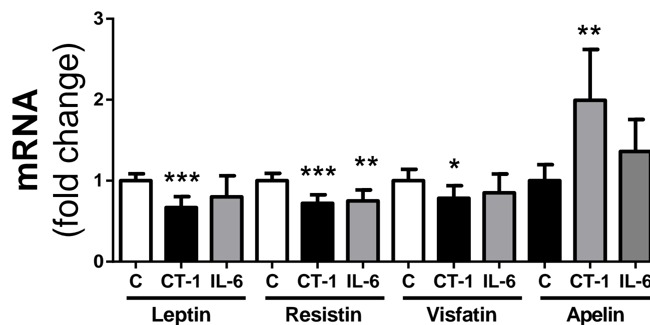


Fig. 1. Cardiotrophin-1 regulates adipokines production in 3T3-L1 adipocytes. Effects of rCT-1 (1-40 ng/mL) on leptin (A), resistin (B) and visfatin (C) gene expression (top panels) and protein secretion (botton panels) after 18 h of treatment. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group.

A comparative study with IL-6 (a member of the same family of cytokines) was carried out. Our data revealed that IL-6 (20 ng/mL) was also able to downregulate resistin mRNA levels, while it does not significantly affect the gene expression of leptin and visfatin (Supplementary Fig. I).



Supplementary Fig. I. Comparative effects of CT-1 (20 ng/mL) and IL-6 (20 ng/mL) on leptin, resistin, visfatin and apelin gene expression after 18 h of treatment. Data are mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group.

CT-1 stimulates apelin gene expression and secretion in 3T3-L1 adipocytes

In contrast to the effect observed in the rest of the adipokines analyzed, treatment of adipocytes with rCT-1 (20 ng/mL) promoted a significant increase in apelin gene expression. This stimulatory effect was observed after short (1 h) (*P* < 0.001) and long-term (18 h) (*P* < 0.01) treatments (Fig. 2A). Moreover, we observed that the upregulation of apelin expression by rCT-1 treatment (18 h) occurred in a concentration-dependent manner, being significant at the doses of 20 and 40 ng/mL (Fig. 2B, left panel). Similar results were observed on the secretion of this adipokine to the media after the treatment with rCT-1 (Fig. 2B, right panel). Interestingly, IL-6 did not mimic the stimulatory effect of CT-1 on the expression of apelin (Supplementary Fig. I).

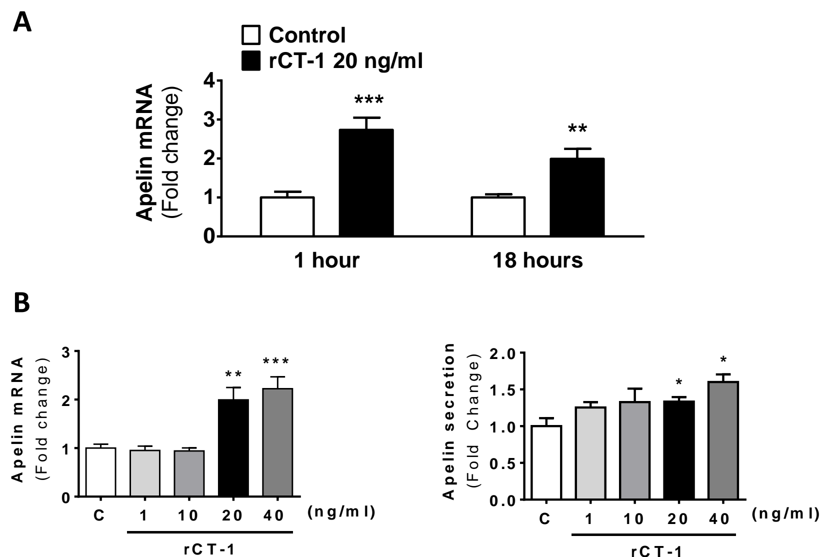


Fig. 2. Cardiotrophin-1 regulates apelin production in 3T3-L1 adipocytes. **(**A) Apelin gene expression after 1 and 18 h treatment with rCT-1 (20 ng/mL). **(**B) Effects of rCT-1 (1-40 ng/mL) on apelin gene expression (left panel) and secretion (right panel) after 18 h of treatment. Data are mean ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with control group.

Signaling pathways involved in the rCT-1 stimulatory effects on apelin production

To further characterize the signaling pathways involved in the stimulatory effects of rCT-1 on apelin production, we examined whether MAPK/ERK1/2 and/or PI3K/AKT (two major signaling pathways involved on apelin production) could be potential mediators of rCT-1 actions in the up-regulation of apelin. We also analyzed the effects of rCT-1 on STAT3 phosphorylation, a pathway highly regulated by CT-1. As shown in Fig. 3A, treatment of mature 3T3-L1 adipocytes with rCT-1 (20 ng/mL) induced the

phosphorylation of ERK 1/2 ($P < 0.05$), the main protein involved in MAPK signaling pathway. Moreover, our data showed that rCT-1 treatment also promoted the activation of AKT by phosphorylation at Ser473 ($P < 0.01$), suggesting that rCT-1 actions could be secondary to the activation of both pathways. Finally, rCT-1 also induced the activation of STAT3 by phosphorylation ($P < 0.001$).

Furthermore, for a better characterization of the signaling pathways involved in apelin regulation by rCT-1, we tested the effects of specific inhibitors these three pathways in 3T3-L1 adipocytes. Treatment with the PI3K inhibitor LY294002 completely reversed the stimulatory effects of rCT-1 on apelin mRNA levels ($P < 0.001$). Moreover, treatment with the JAK/STAT inhibitor AG490 blocked both basal and r-CT-1 mediated stimulation on apelin expression ($P < 0.001$). However, pretreatment with the MAPK/ERK inhibitor PD98059 did not modify the significant increase in apelin mRNA levels mediated by rCT-1, while significantly inhibit basal apelin gene expresion (Fig. 3B).

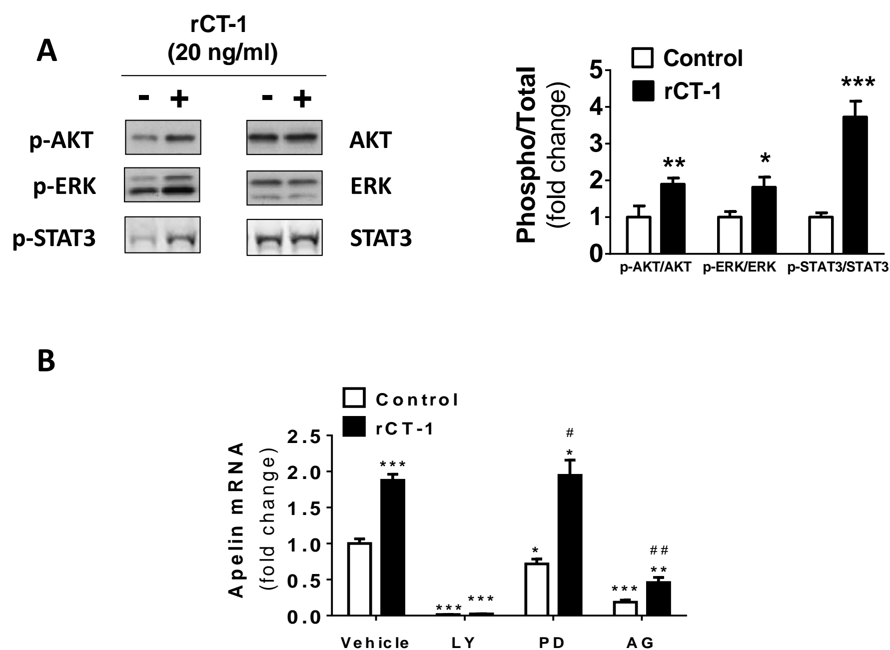
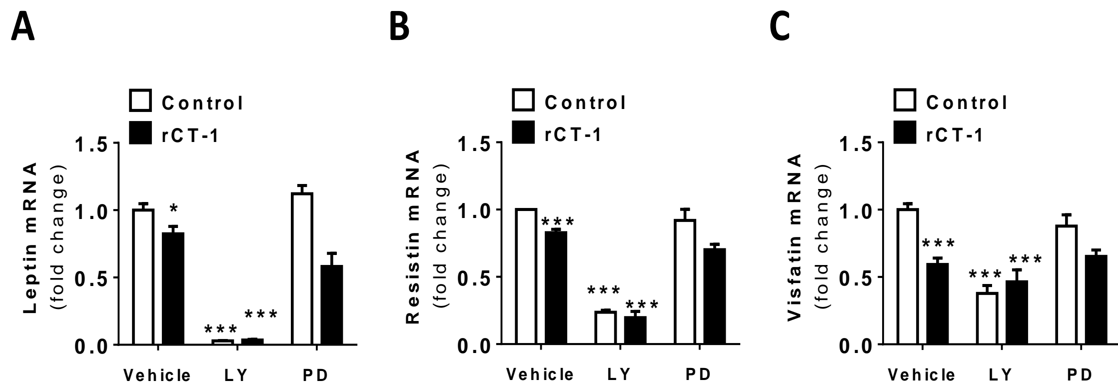


Fig. 3. Signaling pathways involved in CT-1 effects on apelin. **(**A) Representative Western blot and densitometric analysis of p-AKT (Ser473), p-ERK 1/2 (Thr202/Tyr204) and p-STAT3 (Tyr705) activation in mature 3T3-L1 adipocytes after treatment for 18 h with rCT-1 (20 ng/mL). (B) Effects of rCT-1 on apelin mRNA after 18 h of rCT-1 treatment (20 ng/mL) in the presence or absence of PI3K inhibitor LY294002 (50 µM), MAPK/ERK1/2 inhibitor PD98059 (50 µM) and JAK/STAT inhibitor AG490 (20 µM). Data are mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with rCT-1-treated group.

Because MAPK/ERK1/2 and PI3K have also been involved in the regulation of the expression of leptin (Ott *et al.* 2004), resistin (Stan *et al.* 2011) and visfatin (Kralisch *et al.* 2005), the effects of specific inhibitors of these pathways on the actions of CT-1 on these three adipokines were also tested. However, pretreatment with PD98059 or with LY294002 were unable to reverse the downregulation of leptin, resistin and visfatin induced by CT-1, suggesting that other pathways different from MAPK/ERK and PI3K are mediating the inhibitory effects of CT-1 on the expression of these adipokines (Supplementary Fig. II).



Supplementary Fig. II. Effects of rCT-1 on leptin (A), resistin (B) and visfatin (C) mRNA after 18 h of rCT-1 treatment (20 ng/mL) in the presence or absence of PI3K inhibitor LY294002 (50 µM) and MAPK/ERK1/2 inhibitor PD98059 (50 µM). Data are mean ± SEM. *P < 0.05, ***P < 0.001 compared with control group.

Effects of acute rCT-1 treatments on the expression of adipokines in HFD-obese mice

Finally, we analyzed the effects of acute *in vivo* administration of rCT-1 on the expression of leptin, resistin, visfatin and apelin in WAT from HFD-fed mice with the aim of identifying the actions of CT-1 on WAT adipokine expression that occurs independently of the reduction in adiposity caused when CT-1 is chronically administrated. As shown in Fig. 4A and 4B, short-term treatments with rCT-1 (30 min and 3 h, respectively) induced a significant downregulation of mRNA expression of leptin (P < 0.01) and resistin (P < 0.05) in obese mice. However, no significant changes were observed on the mRNA expression of visfatin and apelin.

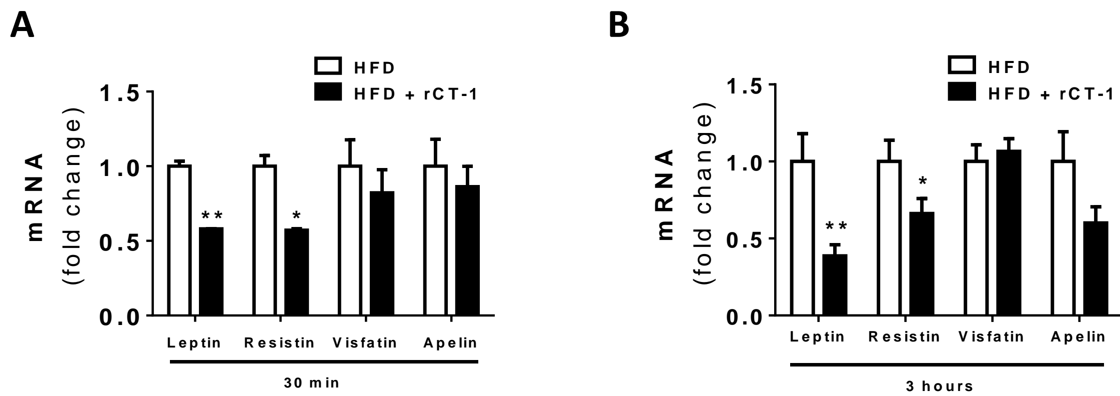


Fig. 4. *In vivo* CT-1 administration regulates adipokines expression in mice. A-B: Effects of rCT-1 (0.2 mg/kg) acute administration for 30 min (A) and 3 h (B) on adipokines gene expression in epididymal WAT from diet-induced obese mice. Data are mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$ compared with HFD group.

DISCUSSION

It has been well established that adipose tissue plays an important role as an endocrine organ capable of secreting several adipokines involved in the regulation of appetite and satiety, fat distribution, insulin secretion and sensitivity, energy expenditure, endothelial function, inflammation, blood pressure, and hemostasis (Fasshauer *et al.* 2015). On the other hand, in the last years several studies have pointed out that CT-1, a member of IL-6 family of cytokines, could have potential applications for treatment of obesity and associated co-morbidities such as insulin resistance and fatty liver (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). In order to gain more insight into the beneficial effects of CT-1 in these pathologies, we studied the direct effects of rCT-1 on expression and secretion of several adipokines. The present study demonstrates the ability of CT-1 to modulate endocrine adipocyte function by regulating the production of several adipokines involved in the control of energy metabolism in cultured adipocytes as well as in adipose tissue of treated animals.

Our data show that rCT-1 downregulates adipocyte-secreted hormones implicated in obesity and insulin resistance with pro-inflammatory properties such as leptin, resistin and visfatin in cultured adipocytes. Leptin has an important role in the regulation of appetite and energy homeostasis (Zhang *et al.* 1994) and leptin levels are increased in obesity in a manner proportional to adipose tissue mass and nutritional

status (Fain *et al.* 2004). Hyperleptinemia has been related to the development of several obesity-associated disorders such as type 2 diabetes mellitus, hypertension, cardiovascular disease, and cancer (Patel *et al.* 2008, Prieto-Hontoria *et al.* 2011). The present study clearly demonstrates that CT-1 administration to HFD obese mice reduced leptin expression in WAT. Interestingly, this drop in leptin is not secondary to reduced fat mass as the inhibitory action of CT-1 on leptin mRNA levels is observed after acute treatment (30 min and 3 h) with the cytokine. Moreover, CT-1 can inhibit leptin in cultured adipocytes, suggesting a direct action of the cytokine on leptin production machinery in these cells. It has been described that CNTF, another member of IL-6 family of cytokines, also downregulates leptin expression in mice and humans (Ott *et al.* 2004). Although CNTF as well as CT-1 do not attenuate adipocyte differentiation (Zvonic *et al.* 2004), both cytokines can decrease the levels of leptin in cultured adipocytes. The inhibitory effects of CNTF on leptin expression are mediated by PI3K signaling pathway as treatment with the PI3K inhibitor LY294002 completely abrogated the CNTF-induced impairment of leptin expression (Ott *et al.* 2004). In contrast, our current data show that inhibiting PI3K pathway is not able to reverse the reduction of leptin mRNA levels induced by this cytokine. In this context, it is important to note that the effects of some gp130 cytokines on leptin seem to be differential. Thus, in divergence with the downregulation of leptin mRNA mediated by CT-1 and CNTF, we did not observed significant effects of IL-6 on leptin expression at the tested dose. We have reported that CT-1 is an anorexigenic cytokine (Moreno-Aliaga *et al.* 2011) as well as CNTF (Febbraio 2007) and metformin (Klein *et al.* 2004), all of them able to decrease the levels of leptin. Ott *et al.* (2004) speculated that the downregulation of leptin secretion by anorexigenic substances could be a mechanism to overcome leptin resistance by causing an upregulation of leptin-receptors in response to decreased leptin levels. Actually, it has been suggested that high leptin levels do indeed cause leptin resistance characterized by attenuation of the anorexigenic effect of leptin and, after a longer time-period, an attenuated thermogenic effect in brown fat (Scarpace *et al.* 2002).

On the other hand, resistin is an adipose tissue secretory factor, which has been reported to aggravate metabolic syndrome through impairment of glucose metabolism (Ikeda *et al.* 2013). It has been found that various glucose-lowering agents such as

metformin reduce resistin gene expression in isolated adipocytes (Rea *et al.* 2006). We have previously reported that CT-1 administration to *ob/ob* mice for 6 days reduced resistin circulating levels in parallel with the reduction in fat mass, suggesting that the beneficial effects of CT-1 on glucose metabolism could be also related to its ability to reduce resistin (Moreno-Aliaga *et al.* 2011). Our current data suggest direct actions of CT-1 on resistin independently of changes in fat mass, since the inhibitory effects of CT-1 on resistin were observed after acute treatment with the cytokine (30 min and 3 h) and also in cultured adipocytes. Regarding the mechanisms underlying the inhibitory effect of CT-1 on resistin, our data suggest that is not mediated through the PI3K/AKT or MAPK pathway, since inhibition of both pathways did not modify CT-1 actions on resistin.

Current data also showed that CT-1 decreased visfatin in cultured adipocytes, an adipokine with pro-inflammatory properties. Elevated circulation levels of this adipokine have been observed in individuals with inflammatory diseases, with a clear participation in a wide range of inflammatory conditions (Valentini *et al.* 2009). Recently, it has been reported that the cardiovascular protective molecules, quercetin and resveratrol (Derdemezis *et al.* 2011) and the antioxidant oleanolic acid (Kim *et al.* 2014) can reduce visfatin secretion in cultured adipocytes suggesting an anti-inflammatory role of these compounds. In concordance with the inhibitory effects of CT-1 on visfatin production, it has been previously described that IL-6 also downregulates visfatin gene expression in a dose and time-dependent manner (Kralisch *et al.* 2005). However, in the present study we did not observe any significant effects of IL-6 on visfatin mRNA levels at the conditions tested (20 ng/mL, 18 h), suggesting that the effect of this cytokine is highly dependent on the concentration and the duration of treatment. The inhibitory effects of IL-6 on visfatin gene expression are mediated, at least in part, by MAPK pathway as inhibition of this pathway by PD98059 significantly reversed the downregulation of visfatin mRNA levels (Kralisch *et al.* 2005). On the contrary, our data revealed that PD98059 did not modify the inhibitory effect of CT-1 on visfatin expression, evidencing that differential pathways are involved in the actions of both gp130 cytokines on visfatin. Altogether, the present study shows the capacity of CT-1 to decrease pro-inflammatory adipokines such as resistin, leptin and visfatin in adipocytes, suggesting that CT-1 could have

anti-inflammatory properties and that is beneficial role in glucose metabolism could take place in part through the regulation of adipokines.

Apelin is an adipokine involved in the regulation of various aspects of energy metabolism through APJ receptors and has been known for its anti-obesity and anti-diabetic properties (Castan-Laurell *et al.* 2011). Indeed, apelin promotes insulin sensitivity and glucose utilization in adipose and muscle tissues (Yue *et al.* 2010, Zhu *et al.* 2011, Dray *et al.* 2008). Apelin injection decreases WAT mass and serum triglyceride levels in obese mice, whereas apelin knockout mice have augmented body adiposity and serum free-fatty acid levels (Yue *et al.* 2010). Recently, it has been shown that apelin enhances brown adipogenesis and browning of white adipocytes (Than *et al.* 2015). As apelin production in adipocytes and its plasma levels are elevated in obesity associated to hyper-insulinemia (Boucher *et al.* 2005), it has been suggested that the over-production of apelin in obesity could be one of the last protections before the emergence of the obesity-related disorders such as type 2 diabetes (Perez-Echarri *et al.* 2009, Castan-Laurell *et al.* 2011). Interestingly, our current data demonstrate the ability of CT-1 to increase apelin secretion in cultured adipocytes. In elucidating the signaling cascade responsible for the upregulation and secretion of apelin by CT-1, we found that PI3K inhibition by LY294002 abrogated basal apelin gene expression, as reported (Boucher *et al.* 2005, Lorente-Cebrian *et al.* 2010), and more importantly completely reversed the stimulatory effects of CT-1 treatment on apelin gene expression. This fact together with the observation that CT-1 treatment increases AKT phosphorylation, suggests that PI3K might be a main signaling pathway mediating the effects of CT-1 on apelin production. In contrast, inhibition of MAPK, other major signaling pathway involved in apelin production (Boucher *et al.* 2005), had no discernible effect on CT-1-induced upregulation of apelin expression. However, blockade of the JAK/STAT signaling pathway by pretreatment with AG490 blunted the CT-1-stimulated apelin expression indicating that this pathway might play a role in mediating the increase on apelin production by CT-1. Interestingly, blockade of the JAK/STAT signaling pathway also diminished basal apelin mRNA levels, suggesting a potential role of these proteins in the physiological production of apelin. Based on our current data in cultured adipocytes, it could be suggested that the beneficial effects of CT-1 on glucose and lipid metabolism could be also related to its ability to upregulate

apelin production from adipocytes, as it has been suggested for other anti-obesogenic and insulin-sensitizer compounds (Lorente-Cebrian *et al.* 2010, Fernandez-Galilea *et al.* 2011, Perez-Echarri *et al.* 2009). However, the acute treatment of HFD-obese mice with rCT-1 was not able to promote upregulation of apelin gene in WAT. Several reasons may account for these differential outcomes between the *in vitro* and *in vivo* effects of CT-1 on apelin production. First, when administered *in vivo* acutely, CT-1 has a potent glucose lowering and insulin-sensitizing properties (Moreno-Aliaga *et al.* 2011) which could secondarily affect transcriptional regulation of apelin in WAT, since hyperinsulinemia is a key regulator of apelin production by adipocytes in obesity (Boucher *et al.* 2005). It is also established that CT-1 administration caused a decrease in mean arterial pressure (Jin *et al.* 1998), which could also influence the production of apelin, an adipokine that has been involved in blood pressure regulation (Galanth *et al.* 2012). To get a better inside of the *in vivo* regulation of apelin by rCT-1, we also analyzed its expression in WAT after chronic treatment with the cytokine during 6 days. Chronic treatment with CT-1 caused a marked reduction in food intake, body and fat mass weight (Moreno-Aliaga *et al.* 2011), accompanied a parallel reduction in leptin levels, including leptin mRNA in WAT. However, no significant changes were observed on apelin mRNA levels (Supplemental Fig. III). It is well known that apelin production by fat cells is augmented in obesity (Castan-Laurell *et al.* 2005), and that it is strongly inhibited by fasting (Boucher *et al.* 2005) and weight and fat mass loss (Soriguer *et al.* 2009). Based on these observations, it would be expected a downregulation of apelin mRNA levels in WAT of mice chronically treated with CT-1 in parallel with the drop in weight and fat mass. However, apelin mRNA levels in WAT are similar to those of untreated animals suggesting that CT-1 may have a stimulatory action on the production of this adipokine (as observed *in vitro*), counteracting the decrease in apelin that accompanies adipocyte size reduction and fat mass loss.

In summary, data of the current study demonstrate that CT-1 is capable to regulate the secretory pattern of adipokines by adipocytes, decreasing the production of pro-inflammatory adipokines, such as leptin, resistin and visfatin, while stimulating the secretion of apelin. These suggest that the beneficial actions of CT-1 on glucose and lipid metabolism could be also related to its ability to control adipokine secretion and therefore the cross-talk between adipose tissue and other key metabolic organs.

This highlights the relevance of performing future studies to better characterize the *in vivo* actions of CT-1 on the production of other adipokines and the subsequence metabolic implications.

ACKNOWLEDGEMENTS

This study was supported by grants from the Government of Navarra (Department of Health); Mutua Madrileña Foundation; FIS [PI10/01516 and PI13/01851]; Línea Especial ‘‘Nutrición, Obesidad y Salud Universidad de Navarra”; and by the agreement between FIMA and the ‘‘UTE project CIMA’’. Miguel López-Yoldi was supported by a Doctoral grant from Asociación de Amigos University of Navarra.

REFERENCES

-B-

Beraza, N., Marques, J. M., Martinez-Anso, E., Iniguez, M., Prieto, J. & Bustos, M. 2005. Interplay among cardiotrophin-1, prostaglandins, and vascular endothelial growth factor in rat liver regeneration. *Hepatology,* **41,** 460-9.

Bluher, M. 2014. Adipokines - removing road blocks to obesity and diabetes therapy. *Mol Metab,* **3,** 230-40.

Boucher, J., Masri, B., Daviaud, D., Gesta, S., Guigne, C., Mazzucotelli, A., Castan-Laurell, I., Tack, I., Knibiehler, B., Carpene, C., Audigier, Y., Saulnier-Blache, J. S. & Valet, P. 2005. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology,* **146,** 1764-71.

-C-

Carpene, C., Dray, C., Attane, C., Valet, P., Portillo, M. P., Churruca, I., Milagro, F. I. & Castan-Laurell, I. 2007. Expanding role for the apelin/APJ system in physiopathology. *J Physiol Biochem,* **63,** 359-73.

Castan-Laurell, I., Boucher, J., Dray, C., Daviaud, D., Guigne, C. & Valet, P. 2005. Apelin, a novel adipokine over-produced in obesity: friend or foe? *Mol Cell Endocrinol,* **245,** 7-9.

Castan-Laurell, I., Dray, C., Attane, C., Duparc, T., Knauf, C. & Valet, P. 2011. Apelin, diabetes, and obesity. *Endocrine,* **40,** 1-9.

Castano, D., Larequi, E., Belza, I., Astudillo, A. M., Martinez-Anso, E., Balsinde, J., Argemi, J., Aragon, T., Moreno-Aliaga, M. J., Muntane, J., Prieto, J. & Bustos, M. 2014. Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation. *J Hepatol,* **60,** 1017-25.

-D-

Derdemezis, C. S., Kiortsis, D. N., Tsimihodimos, V., Petraki, M. P., Vezyraki, P., Elisaf, M. S. & Tselepis, A. D. 2011. Effect of Plant Polyphenols on Adipokine Secretion from Human SGBS Adipocytes. *Biochem Res Int,* **2011,** 285618.

Dray, C., Knauf, C., Daviaud, D., Waget, A., Boucher, J., Buleon, M., Cani, P. D., Attane, C., Guigne, C., Carpene, C., Burcelin, R., Castan-Laurell, I. & Valet, P. 2008. Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab,* **8,** 437-45.

-F-

Fain, J. N., Madan, A. K., Hiler, M. L., Cheema, P. & Bahouth, S. W. 2004. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology,* **145,** 2273-82.

Fasshauer, M. & Bluher, M. 2015. Adipokines in health and disease. *Trends Pharmacol Sci,* **36,** 461-70.

Febbraio, M. A. 2007. gp130 receptor ligands as potential therapeutic targets for obesity. *J Clin Invest,* **117,** 841-9.

Fernandez-Galilea, M., Perez-Matute, P., Prieto-Hontoria, P., Martinez, J. A. & Moreno-Aliaga, M. J. 2011. Effects of lipoic acid on apelin in 3T3-L1 adipocytes and in high-fat fed rats. *J Physiol Biochem,* **67,** 479-86.

Fernandez-Galilea, M., Perez-Matute, P., Prieto-Hontoria, P. L., Martinez, J. A. & Moreno-Aliaga, M. J. 2012. Effects of lipoic acid on lipolysis in 3T3-L1 adipocytes. *J Lipid Res,* **53,** 2296-306.

Fonseca-Alaniz, M. H., Takada, J., Alonso-Vale, M. I. & Lima, F. B. 2006. [The adipose tissue as a regulatory center of the metabolism]. *Arq Bras Endocrinol Metabol,* **50,** 216-29.

-G-

Galanth, C., Hus-Citharel, A., Li, B. & Llorens-Cortes, C. 2012. Apelin in the control of body fluid homeostasis and cardiovascular functions. *Curr Pharm Des,* **18,** 789-98.

-I-

Ikeda, Y., Tsuchiya, H., Hama, S., Kajimoto, K. & Kogure, K. 2013. Resistin affects lipid metabolism during adipocyte maturation of 3T3-L1 cells. *FEBS J,* **280,** 5884-95.

-J-

Jin, H., Yang, R., Ko, A., Pennica, D., Wood, W. I. & Paoni, N. F. 1998. Effects of cardiotrophin-1 on haemodynamics and cardiac function in conscious rats. *Cytokine,* **10,** 19-25.

-K-

Kim, H. S., Han, S. Y., Sung, H. Y., Park, S. H., Kang, M. K., Han, S. J. & Kang, Y. H. 2014. Blockade of visfatin induction by oleanolic acid via disturbing IL-6-TRAF6-NF-kappaB signaling of adipocytes. *Exp Biol Med (Maywood),* **239,** 284-92.

Klein, J., Westphal, S., Kraus, D., Meier, B., Perwitz, N., Ott, V., Fasshauer, M. & Klein, H. H. 2004. Metformin inhibits leptin secretion via a mitogen-activated protein kinase signalling pathway in brown adipocytes. *J Endocrinol,* **183,** 299-307.

Kloting, N. & Bluher, M. 2014. Adipocyte dysfunction, inflammation and metabolic syndrome. *Rev Endocr Metab Disord,* **15,** 277-87.

Kralisch, S., Klein, J., Lossner, U., Bluher, M., Paschke, R., Stumvoll, M. & Fasshauer, M. 2005. Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab,* **289,** E586-90.

-L-

LeRoith, D., Novosyadlyy, R., Gallagher, E. J., Lann, D., Vijayakumar, A. & Yakar, S. 2008. Obesity and type 2 diabetes are associated with an increased risk of developing cancer and a worse prognosis; epidemiological and mechanistic evidence. *Exp Clin Endocrinol Diabetes,* **116 Suppl 1,** S4-6.

Lopez-Yoldi, M., Fernandez-Galilea, M., Laiglesia, L. M., Larequi, E., Prieto, J., Martinez, J. A., Bustos, M. & Moreno-Aliaga, M. J. 2014. Cardiotrophin-1 stimulates lipolysis through the regulation of main adipose tissue lipases. *J Lipid Res,* **55,** 2634-43.

Lorente-Cebrian, S., Bustos, M., Marti, A., Martinez, J. A. & Moreno-Aliaga, M. J. 2010. Eicosapentaenoic acid up-regulates apelin secretion and gene expression in 3T3-L1 adipocytes. *Mol Nutr Food Res,* **54 Suppl 1,** S104-11.

-M-

McGee, K. C., Harte, A. L., da Silva, N. F., Al-Daghri, N., Creely, S. J., Kusminski, C. M., Tripathi, G., Levick, P. L., Khanolkar, M., Evans, M., Chittari, M. V., Patel, V., Kumar, S. & McTernan, P. G. 2011. Visfatin is regulated by rosiglitazone in type 2 diabetes mellitus and influenced by NFkappaB and JNK in human abdominal subcutaneous adipocytes. *PLoS One,* **6,** e20287.

Meier, U. & Gressner, A. M. 2004. Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clin Chem,* **50,** 1511-25.

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* **14,** 242-53.

-N-

Natal, C., Fortuno, M. A., Restituto, P., Bazan, A., Colina, I., Diez, J. & Varo, N. 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. *Am J Physiol Endocrinol Metab,* **294,** E52-60.

-O-

Ott, V., Fasshauer, M., Meier, B., Dalski, A., Kraus, D., Gettys, T. W., Perwitz, N. & Klein, J. 2004. Ciliary neurotrophic factor influences endocrine adipocyte function: inhibition of leptin via PI 3-kinase. *Mol Cell Endocrinol,* **224,** 21-7.

-P-

Pagano, C., Soardo, G., Pilon, C., Milocco, C., Basan, L., Milan, G., Donnini, D., Faggian, D., Mussap, M., Plebani, M., Avellini, C., Federspil, G., Sechi, L. A. & Vettor, R. 2006. Increased serum resistin in nonalcoholic fatty liver disease is related to liver disease severity and not to insulin resistance. *J Clin Endocrinol Metab,* **91,** 1081-6.

Patel, S. B., Reams, G. P., Spear, R. M., Freeman, R. H. & Villarreal, D. 2008. Leptin: linking obesity, the metabolic syndrome, and cardiovascular disease. *Curr Hypertens Rep,* **10,** 131-7.

Paz-Filho, G., Mastronardi, C. A. & Licinio, J. 2015. Leptin treatment: facts and expectations. *Metabolism,* **64,** 146-56.

Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D. L., Zioncheck, K. A., Rosenthal, A., Taga, T., Paoni, N. F. & Wood, W. I. 1995. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem,* **270,** 10915-22.

Perez-Echarri, N., Perez-Matute, P., Marcos-Gomez, B., Martinez, J. A. & Moreno-Aliaga, M. J. 2009. Effects of eicosapentaenoic acid ethyl ester on visfatin and apelin in lean and overweight (cafeteria diet-fed) rats. *Br J Nutr,* **101,** 1059-67.

Prieto-Hontoria, P. L., Perez-Matute, P., Fernandez-Galilea, M., Martinez, J. A. & Moreno-Aliaga, M. J. 2011. Lipoic acid inhibits leptin secretion and Sp1 activity in adipocytes. *Mol Nutr Food Res,* **55,** 1059-69.

-R-

Rea, R. & Donnelly, R. 2006. Effects of metformin and oleic acid on adipocyte expression of resistin. *Diabetes Obes Metab,* **8,** 105-9.

-S-

Sainz, N., Barrenetxe, J., Moreno-Aliaga, M. J. & Martinez, J. A. 2015a. Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. *Metabolism,* **64,** 35-46.

Sainz, N., Gonzalez-Navarro, C. J., Martinez, J. A. & Moreno-Aliaga, M. J. 2015b. Leptin signaling as a therapeutic target of obesity. *Expert Opin Ther Targets,* **19,** 893-909.

Scarpace, P. J., Matheny, M., Zhang, Y., Shek, E. W., Prima, V., Zolotukhin, S. & Tumer, N. 2002. Leptin-induced leptin resistance reveals separate roles for the anorexic and thermogenic responses in weight maintenance. *Endocrinology,* **143,** 3026-35.

Soriguer, F., Garrido-Sanchez, L., Garcia-Serrano, S., Garcia-Almeida, J. M., Garcia-Arnes, J., Tinahones, F. J. & Garcia-Fuentes, E. 2009. Apelin levels are increased in morbidly obese subjects with type 2 diabetes mellitus. *Obes Surg,* **19,** 1574-80.

Stan, D., Calin, M., Manduteanu, I., Pirvulescu, M., Gan, A. M., Butoi, E. D., Simion, V. & Simionescu, M. 2011. High glucose induces enhanced expression of resistin in human U937 monocyte-like cell line by MAPK- and NF-kB-dependent mechanisms; the modulating effect of insulin. *Cell Tissue Res,* **343,** 379-87.

-T-

Than, A., He, H. L., Chua, S. H., Xu, D., Sun, L., Leow, M. K. & Chen, P. 2015. Apelin Enhances Brown Adipogenesis and Browning of White Adipocytes. *J Biol Chem,* **290,** 14679-91.

-V-

Valentini, L., Wirth, E. K., Schweizer, U., Hengstermann, S., Schaper, L., Koernicke, T., Dietz, E., Norman, K., Buning, C., Winklhofer-Roob, B. M., Lochs, H. & Ockenga, J. 2009. Circulating adipokines and the protective effects of hyperinsulinemia in inflammatory bowel disease. *Nutrition,* **25,** 172-81.

Van Gaal, L. F., Mertens, I. L. & De Block, C. E. 2006. Mechanisms linking obesity with cardiovascular disease. *Nature,* **444,** 875-80.

-Y-

Yue, P., Jin, H., Aillaud, M., Deng, A. C., Azuma, J., Asagami, T., Kundu, R. K., Reaven, G. M., Quertermous, T. & Tsao, P. S. 2010. Apelin is necessary for the maintenance of insulin sensitivity. *Am J Physiol Endocrinol Metab,* **298,** E59-67.

-Z-

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* **372,** 425-32.

Zhu, S., Sun, F., Li, W., Cao, Y., Wang, C., Wang, Y., Liang, D., Zhang, R., Zhang, S., Wang, H. & Cao, F. 2011. Apelin stimulates glucose uptake through the PI3K/Akt pathway and improves insulin resistance in 3T3-L1 adipocytes. *Mol Cell Biochem,* **353,** 305-13.

Zvonic, S., Hogan, J. C., Arbour-Reily, P., Mynatt, R. L. & Stephens, J. M. 2004. Effects of cardiotrophin on adipocytes. *J Biol Chem,* **279,** 47572-9.

CHAPTER 3

Cardiotrophin-1 decreases intestinal sugar uptake in mice and in Caco-2 cells

López-Yoldi M[1,2], Castilla-Madrigal R[1,2], Lostao MP[1,2,6], Barber A[1], Prieto J[3,4], Martínez JA[1,2,5,6], Bustos M[3]*, Moreno-Aliaga MJ[1,2,5,6]*

[1]Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Navarra, Spain.

[2]Centre for Nutrition Research. University of Navarra, Pamplona, Navarra, Spain. [3]Department of Hepatology and Gene Therapy, CIMA, University of Navarra, Pamplona, Navarra, Spain.

[4]CIBERehd. Institute of Health Carlos III, Madrid, Spain.

[5]CIBERobn, Physiopathology of Obesity and Nutrition, Institute of Health Carlos III, Madrid, Spain.

[6]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain.

*Equal senior authorship

Under review in: Acta Physiologica

Impact Factor (2014): 4.382

10/83 in Physiology (Q1)

ABSTRACT

Cardiotrophin-1 (CT-1) is a member of the IL-6 family of cytokines with a key role in glucose and lipid metabolism. In the current investigation, we examined the *in vivo* and *in vitro* effects of CT-1 treatment on intestinal sugar absorption in different experimental models. rCT-1 effects on α-Methyl-D-glucoside uptake were assessed in everted intestinal rings from wild-type and CT-$1^{-/-}$ mice and in Caco-2 cells. rCT-1 actions on SGLT-1 expression in brush border membrane vesicles and the identification of the potential signaling pathways involved were determined by Western blot. *In vivo* administration (0.2 mg/kg) of rCT-1 caused a significant decrease on α-Methyl-D-glucoside uptake in everted intestinal rings from wild-type and CT-$1^{-/-}$ mice after short and long-term treatments. Similarly, *in vitro* treatment (1-50 ng/mL) with rCT-1 reduced α-Methyl-D-glucoside uptake in everted intestinal rings. In Caco-2 cells, rCT-1 treatment (20 ng/mL, 1 and 24 h) lowered apical uptake of α-Methyl-D-glucoside in parallel with a decrease on SGLT-1 protein expression. rCT-1 promoted the phosphorylation of STAT3 after 5 and 15 min treatment, but inhibited the activation by phosphorylation of AMPK after 30 and 60 min. Interestingly, pre-treatment with the JAK/STAT inhibitor (AG490) and with the AMPK activator (AICAR) reversed the inhibitory effects of rCT-1 on α-Methyl-D-glucoside uptake. AICAR also prevented the inhibition of SGLT-1 observed in rCT-1-treated cells. CT-1 inhibits intestinal sugar absorption by the reduction of SGLT-1 levels through the AMPK pathway, which could also contribute to explain the hypoglycemic and anti-obesity properties of CT-1.

Supplementary key words: *Cardiotrophin-1 . Sugar uptake . SGLT-1 . Caco-2 cells . Everted intestinal rings . AMPK .*

INTRODUCTION

A main function of the small intestine is the selective absorption of dietary nutrients. Among these nutrients, glucose represents an essential source of energy for most cells (Barrenetxe *et al.* 2013a).

Glucose transport into the enterocytes is an active process mediated by the sodium-dependent glucose co-transporter-1 (SGLT-1), which is responsible for the co-transport of one sugar molecule with two sodium molecules. Thereby, this transporter is electrogenic and can transport glucose inside the enterocyte against its concentration gradient using the sodium electrochemical gradient provided by the Na^+/K^+ ATPase (Wright *et al.* 2007, Wright *et al.* 2011).

SGLT-1 is constitutively expressed in the brush border membrane of enterocytes, but can be regulated post-transcriptionally through the activation of protein kinases-dependent pathways, which control the insertion and recruitment of this transporter in the membrane (Wright *et al.* 1997, Ishikawa *et al.* 1997, Vayro *et al.* 1999). The effects of different cytokines on the regulation of SGLT-1 such as TNF-α (Barrenetxe *et al.* 2013b), interleukin (IL)-6 (IL-6) (Lee *et al.* 2007) or IL-1β (Vinuales *et al.* 2013), among others have been described.

Cardiotrophin-1 (CT-1) belongs to the IL-6 family of cytokines. These cytokines exert their cellular effects by interacting with the glycoprotein 130 (gp130)/leukemia inhibitory factor receptor (LIFR) heterodimer (Pennica *et al.* 1995). A previous study from our group revealed that CT-1 is a key regulator of energy homeostasis, as well as of glucose and lipid metabolism (Moreno-Aliaga *et al.* 2011). Thus, chronic recombinant CT-1 (rCT-1) treatment reduced body weight and corrected insulin resistance in *ob/ob* and in high-fat-fed obese mice by reducing food intake and enhancing energy expenditure. Moreover, CT-1 administration induced a significant decrease of plasma glucose levels and enhanced insulin sensitivity by the activation of AKT (Moreno-Aliaga *et al.* 2011). Also it has been shown that CT-1 regulates glucose uptake in several cell types, including myocytes and adipocytes (Moreno-Aliaga *et al.* 2011). However, the effects of CT-1 on intestinal sugar absorption remain unknown.

For this reason, the purpose of the present study was to characterize the actions of both *in vivo* and *in vitro* CT-1 treatment on intestinal sugar absorption, as well as the cellular mechanisms that could underlie these effects.

MATERIAL AND METHODS

Recombinant Protein CT-1 (rCT-1)

rCT-1 was obtained as described elsewhere (Beraza *et al.* 2005) and contained < 0.04 ng LPS per 1 µg of protein as determined by the Limulus amoebocyte lysate assay (Cambrex, East Rutherford, NJ).

CT-1$^{-/-}$ mice

CT-1 null mice were generated as described by Oppenheim et al. (Oppenheim *et al.* 2001). We analyzed mice backcrossed into a C57BL/6J background for 11 generations (provided by Diane Pennica, Genentech, and Bettina Holtmann, University of Wuerzburg, Germany) (Moreno-Aliaga *et al.* 2011). All experimental procedures here presented were approved by the University of Navarra Ethics Committee.

Acute treatment of wild type mice with rCT-1

C57BL/6J male at age of 8 weeks were purchased from Harlan Laboratories (Barcelona, Spain). Fasted mice for 18 h were injected intravenously (i.v.) either with saline or rCT-1 (0.2 mg/kg) and sacrificed at 3 h after administration.

Long-term treatment of high fat diet (HFD)-fed wild type and CT-1$^{-/-}$ mice with rCT-1

Young male wild-type (WT) and CT-1$^{-/-}$ mice (8 weeks of age) were fed with a HFD (D12492 60% kcal diet, Research Diets Inc, Brunswick, NJ) *ad libitum* for 12 weeks. After this period, WT and CT-1$^{-/-}$ HFD-fed animals were divided into two subgroups, one that received rCT-1 i.v. (0.2 mg/kg/day) for 6 days and other given saline instead of rCT-1.

Everted intestinal rings uptake assays

The effects of *in vivo* rCT-1 administration on the uptake of α-Methyl-D-Glucoside (α-MG), a SGLT-1 specific substrate, was determined on everted jejunal rings obtained from the treated animals as previously described (Lostao *et al.* 1998). Briefly, at the end of treatments, animals were sacrificed and groups of 6 rings were incubated at 37 °C for 15 min under continuous shaking in Krebs-Ringer-Tris (KRT) solution continuously gassed with O_2. The solution contained 1 mM α-MG and 0.0025 µCi/mL of [^{14}C] α-MG (GE Healthcare, Little Chaldfont, UK).

For the *in vitro* studies, rCT-1 was added to the solution containing the everted intestinal rings from untreated-wild-type (WT) mice at 1-50 ng/mL. After the incubation period, rings were washed in ice-cold KRT solution and then incubated for 24 h in a solution containing 0.1 M HNO_3 to denature the proteins and to allow the exit of the cellular radioactivity which was finally determined by liquid scintillation counting.

Cell culture of Caco-2 cells and treatments

The human epithelial cell line Caco-2 PD7 clone was kindly provided by Dr. Edith Brot-Laroche. Caco-2 cells were cultured in Dulbecco´s modified Eagle´s medium (DMEM) (Gibco-Invitrogen, Paisley, UK) containing 10% fetal bovine serum (FBS, Gibco), 1% non-essential amino acids (Lonza, Basel, Switzerland), 1% penicillin (10000 U/mL)–streptomycin (10000 µg/mL) (Gibco) and 1% amphotericin B (250 µg/mL, Gibco) and maintained in an incubator set to 37 °C and 5% of carbon dioxide. When cells reached 80% confluence, confirmed by microscopic observance, they were dissociated with 0.05% trypsin-EDTA (0.25% trypsin 1X, Gibco), and sub-cultured in 75 cm^2 flask. For transport studies, cells were seeded in 24-well culture plates. Culture medium was replaced every 2 days until the day of the experiment.

Caco-2 cells were treated with/without rCT-1 (1 or 20 ng/mL). To analyze the signaling pathways involved in the actions of rCT-1 on sugar uptake, cells were pre-incubated for 30 min in presence or absence of specific inhibitors or activators (the MAPK/ERK inhibitor PD98059 (50 µM; Sigma, St. Louis, MO), the selective PI3K inhibitor LY294002 (50 µM; Sigma), the AMPK activator AICAR (1 mM; Sigma) and the JAK/STAT pathway inhibitor AG490 (20 µM, Calbiochem, San Diego, CA)) before the addition of rCT-1 to the treated cells. All compounds were prepared as 1000x stock solutions and added to the culture medium. Control cells were treated with the same amount of vehicle (DMSO and/or ethanol).

α-MG uptake experiments in Caco-2 cells

Uptake experiments were performed in cell monolayers grown on culture plates 17–21 days post-seeding. Prior to the addition of the treatments cells were pre-incubated in serum and glucose-free DMEM for 2 h and then treated with or without rCT-1 (1 or 20 ng/mL) during different time intervals. After the preincubation period, plates were incubated for 15 min with 0.1 mM α-MG (Sigma) and traces of [^{14}C]-α-MG (0.6 μCi/mL; 303 mCi/mmol, American Radiolabeled Chemicals, St Louis, MO) diluted in DMEM.

After the incubation (1 or 24 h) period at 37 °C and 5% of carbon dioxide, uptake was stopped by adding ice-cold Phosphate Buffered Saline with calcium and magnesium (PBS, Sigma). Cells were again washed twice with ice-cold buffer to eliminate non-specific radioactivity fixation and were finally solubilized in 500 μL 1% Triton X-100 in 0.1 M NaOH. Samples (100 μL) were taken to measure radioactivity by liquid scintillation counting. Protein concentration was determined by Bradford method (Bio-Rad Protein Assay; Bio-Rad laboratories, Hercules, CA, USA).

Western Blot analysis

For the signaling studies, Caco-2 cell were treated with rCT-1 for 5, 15, 30 and 60 min. Then Caco-2 cell lysates were obtained by the addition of a buffer containing 2 mM TrisHCl (pH 8), 137 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail (Sigma), 2 mM orthovanadate, and 1 mM PMSF as previously described (Barrenetxe *et al.* 2013b). For SGLT-1 determination, cells (17-21 days post seeding) were treated with or without rCT-1 (20 ng/mL) for 1 or 24 h, and then incubated for 15 min with 1 mM α-MG. After the incubation period, brush border membrane vesicles (BBMV) were isolated from each flask by $MgCl_2$ precipitation method (Kessler *et al.* 1978, Barrenetxe *et al.* 2013b).

In both cases, samples were centrifuged and protein concentrations were determined by the Bradford method. Briefly, equivalent amounts of total protein (25-50 μg) were electrophoretically separated by 12-15% SDS-PAGE in the presence of a reducing agent (2-mercaptoethanol). Proteins were electroblotted from the gel to polyvinylidenedifluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Following the transfer of proteins, the membranes were blocked in bovine serum

albumin (BSA) and probed with specific primary antibodies against SGLT-1 (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-AKT (Ser473), AKT, phospho-ERK 1/2 (Thr202/Tyr204) , ERK, phospho-AMPK (Thr183), AMPK, phospho-STAT3 (Tyr705), STAT3 (Cell SignalingTechnologies, Beverly, MA) and β-actin (Sigma). After that, membranes were hybridized for 1 h with infrared fluorescent secondary antibodies (Cell Signaling) and quantified using an Odyssey® Sa infrared imaging system (LI-COR Biosciences, Lincoln, USA).

Statistical analysis

Data are presented as mean ± standard error (SEM). Comparisons between the values for different variables were analyzed by one-way ANOVA followed by Bonferroni post-hoc tests, or by Student's t-test or Mann-Whitney U-test, once the normality had been screened using Kolmogorov-Smirnoff and Shapiro-Wilk tests. Statistical analyses and graphs were carried out using GraphPad Prism 6 software (Graph-Pad Software Inc., San Diego, CA). Overall, a P value < 0.05 was considered significant.

RESULTS

Effects of *in vivo* rCT-1 treatment on sugar absorption in mice

The effect of *in vivo* rCT-1 administration on *ex vivo* sugar absorption was analyzed by measuring α-MG uptake in intestinal everted rings from WT mice, 3 h after a single dose of rCT-1 (0.2 mg/kg) or saline. We also tested the effects of long-term treatment with rCT-1 (0.2 mg/kg/day; 6 days) on intestinal α-MG uptake in WT and CT-1$^{-/-}$ mice. rCT-1 short-term treatment (3 h) significantly (P < 0. 01) decreased the absorption of 1 mM α-MG in WT mice (Fig. 1A). As shown in Fig. 1B, similar effects on α-MG uptake were observed after rCT-1 long-term treatment (6 days) in both WT (P < 0. 001) and CT-1$^{-/-}$ (P < 0. 001) mice previously fed on HFD during 12 weeks. It is important to mention that no significant changes in α-MG uptake were observed in intestinal rings from WT and CT-1 deficient mice fed on a HFD (Fig. 1B), suggesting that the lack of this cytokine did not significantly impair intestinal sugar glucose absorption.

Effects of *in vitro* rCT-1 treatment on sugar absorption in mice

We next evaluated the *in vitro* effects of rCT-1 on sugar absorption after the incubation of everted jejunal rings in a solution containing rCT-1 with a range of concentrations from 1 to 50 ng/mL. Fig. 1C shows that rCT-1 inhibits intestinal α-MG absorption, being significant at the doses of 1, 20 and 50 ng/mL. We also compared the effect of rCT-1 (20 ng/mL) on α-MG uptake with the effect caused by CNTF (20 ng/mL), a cytokine of the same family. As shown in Fig. 1D, rCT-1 ($P < 0.001$) but not CNTF induced a significant decrease on α-MG uptake, suggesting that the inhibitory actions on sugar uptake were specific of rCT-1.

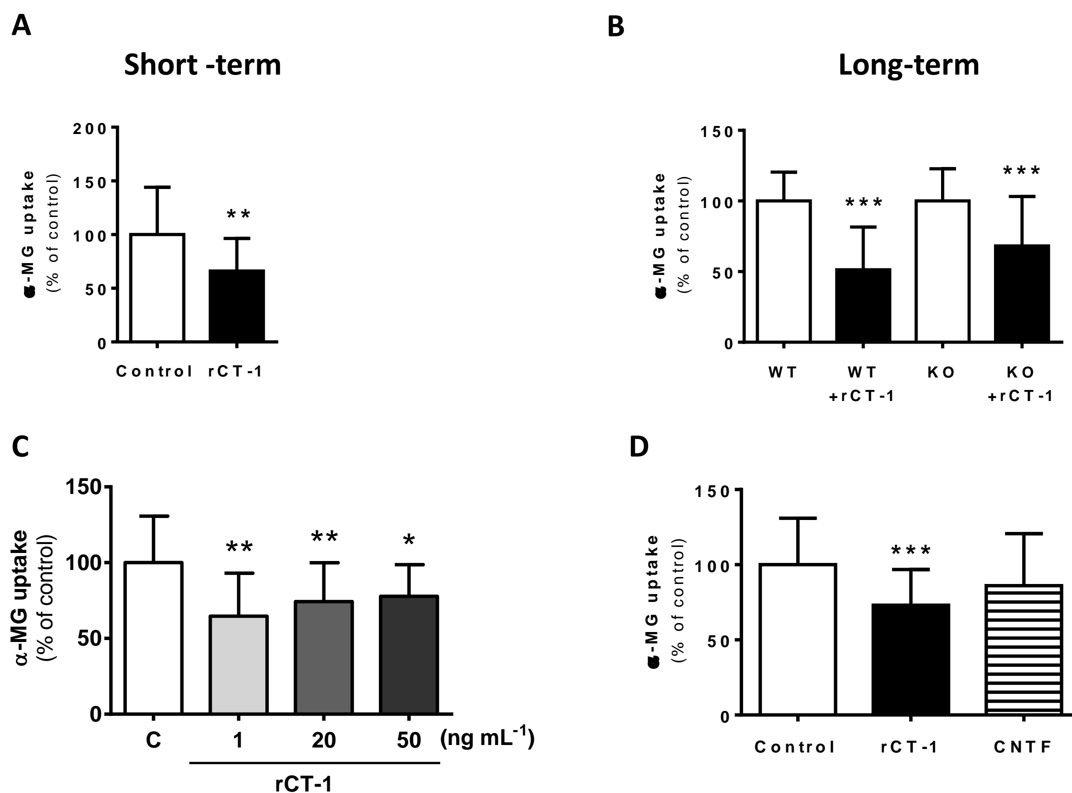


Fig. 1. α-Methylglucoside (α-MG) uptake in intestinal everted rings after *in vivo* and *in vitro* treatment with rCT-1. (A) α-MG (1 mM) uptake for 15 min after short-term (3 h) *in vivo* rCT-1 treatment (0.2 mg/kg) in WT mice (n = 5). (B) α-MG (1 mM) uptake for 15 min after long-term (6 days) rCT-1 treatment (0.2 mg/kg/day) in WT and CT-$1^{-/-}$ (KO) mice-fed on HFD for 12 weeks (n = 6). (C) Effect of *in vitro* rCT-1 treatment (1-50 ng/mL; 1 h) on α-MG (1 mM) uptake for 15 min. (D) Comparative effect of *in vitro* treatment with rCT-1 (20 ng/mL) and CNTF (20 ng/mL), another cytokine of the IL-6 family, on α-MG (1 mM; 15 min) uptake. Data are expressed as mean ± SEM. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$ compared with control group. (n = 6-20 rings from 5-7 independent experiments).

Inhibition of sugar uptake by rCT-1 in Caco-2 cells

Next we evaluated the effects of rCT-1 (1 and 20 ng/mL) on 0.1 mM α-MG uptake (15 min) in Caco-2 cells that were pre-incubated with the cytokine for 1 or 24 h. As shown in Fig. 2A, rCT-1 (20 ng/mL) significantly inhibited α-MG uptake after short (1 h) ($P < 0.01$) and long-time (24 h) ($P < 0.001$) treatments.

To further investigate whether the effects of rCT-1 on α-MG uptake were mediated by changes on the membrane expression levels of the specific α-MG transporter SGLT-1, we measured, by Western blot, the expression of this transporter in BBMV after incubating the cells in the same conditions of the functional studies. Interestingly, the inhibitory effect of rCT-1 on α-MG uptake was accompanied by a significant decrease ($P < 0.05$) on the expression level of SGLT-1 in the apical membrane of enterocytes treated with rCT-1 for 1 ($P < 0.05$) and 24 h ($P < 0.01$) (Fig. 2B).

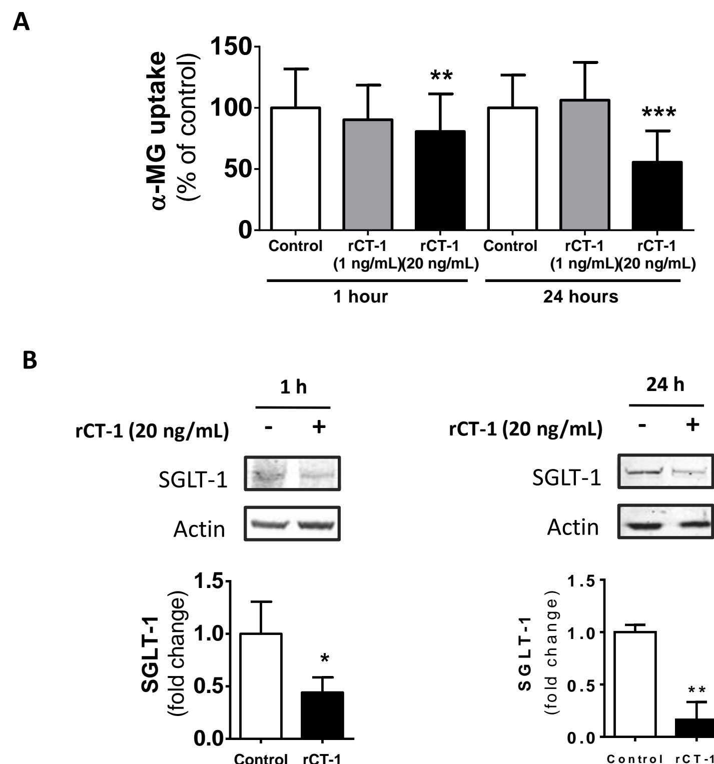


Fig. 2. Effects of rCT-1 on α-MG uptake and SGLT-1 protein levels in Caco-2 cells.(A) Cells were pre-incubated with rCT-1 (1 or 20 ng/mL) for 1 or 24 h before measuring the uptake of 0.1 mM α-MG for 15 min (n = 16-44). (B) Protein expression of SGLT-1 in brush border membrane vesicles of Caco-2 cells obtained after 1 or 24 h incubation of the cells with rCT-1 (20 ng/mL) followed by 15 min incubation with 0.1 mM α-MG. Data are expressed as mean ± SEM. (n = at least 3 independent experiments). $*P < 0.05$; $**P < 0.01$, $***P < 0.001$ compared with control group.

Taking together, these results demonstrate that rCT-1 inhibits, in a short and long-term manner, α-MG uptake by regulating SGLT-1 expression level at the brush border membrane of the enterocytes.

Signaling pathways involved in the inhibitory effect of rCT-1 on sugar uptake

We next aimed to study the different signaling pathways that could be potentially implicated in the inhibitory effect of rCT-1 on sugar absorption and SGLT-1 expression at the apical membrane in Caco-2 cells. For this purpose, phosphorylation levels of STAT3, ERK 1/2, AKT and AMPK were determined at 5, 15, 30 and 60 min after rCT-1 treatment. As expected, rCT-1induced an increase in the phosphorylation of STAT3 at Tyr705, that was significant after 5 ($P < 0.05$) and 15 min ($P < 0.01$) of treatment (Fig. 3A top left panel). Moreover, rCT-1 also promoted the phosphorylation of AKT (Ser473) after 5 ($P < 0.01$) and 15 min ($P < 0.05$) incubation (Fig. 3A top right panel) suggesting that both pathways could be early implicated in the cascade of events triggered by rCT-1 on Caco-2 cells. However, the stimulatory effect of rCT-1 on the phosphorylation of STAT3 and AKT disappeared after 30 and 60 min of treatment. We also evaluated the effects of rCT-1 on ERK phosphorylation, but not significant changes were observed (Fig. 3A bottom left panel). Interestingly, rCT-1 treatment for 15 min induced the phosphorylation of AMPK (Thr172) ($P < 0.01$). However, this effect was reversed after 30 and 60 min incubation with rCT-1, causing a significant decrease ($P < 0.01$ and $P < 0.05$) on the phosphorylation of AMPK (Fig. 3A bottom right panel). To further characterize the signaling pathways involved in the inhibitory effects of rCT-1 on sugar uptake ($P < 0.01$), we measured α-MG uptake in cells previously exposed to specific inhibitors of the different signaling pathways analyzed. As shown in Fig. 3B, the PI3K inhibitor LY294002 alone reduced α-MG uptake, but was not able to prevent the inhibitory effect of rCT-1. Interestingly, the AMPK activator AICAR completely blunted the reduction of rCT-1 of α-MG uptake induced by rCT-1. Similar results were observed after pre-treatment with the JAK/STAT inhibitor AG490, indicating that these pathways could be, at least in part, mediating the inhibitory action of rCT-1 on α-MG uptake after 1 hour incubation.

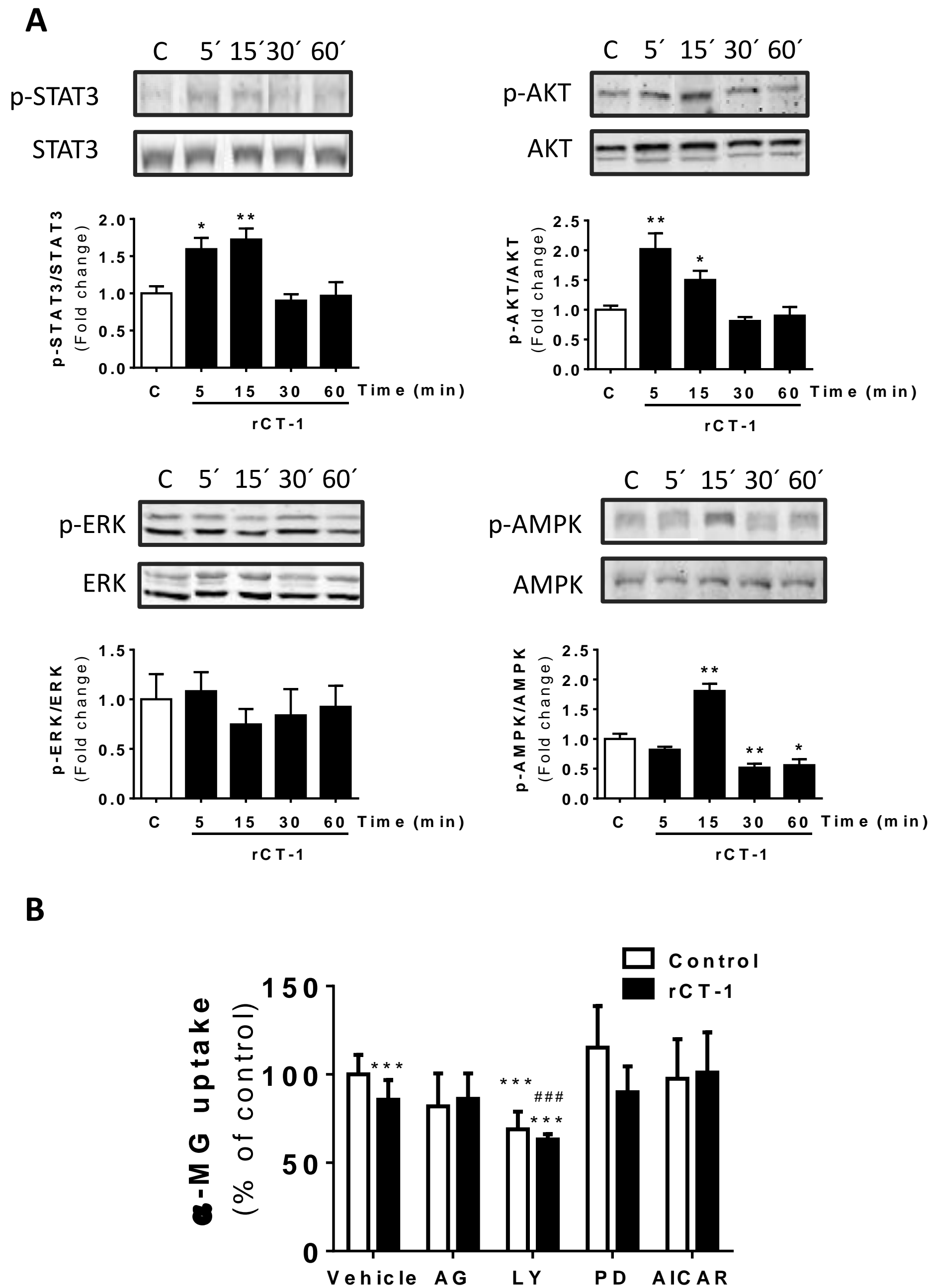


Fig. 3. Characterization of signaling pathways involved in the inhibitory effect of rCT-1 on α-MG uptake in Caco-2 cells. (A) Representative Western blot and densitometric analysis of phosphorylation levels of STAT3 (Tyr705), AKT (Ser473), ERK 1/2 (Thr202/Tyr204) and AMPK (Thr172) at 5, 15, 30 and 60 min of treatment with rCT-1 (20 ng/mL). Phosphorylation data were normalized by total protein content of STAT3, AKT, ERK and AMPK respectively (n = 4). (B) Effects of pretreatment (30 min) with the JAK/STAT inhibitor AG490 (50 µM), the PI3K inhibitor LY294002 (50 µM),the ERK inhibitor PD98059 (50 µM) or the AMPK activator AICAR (1 mM) on the inhibitory effect of rCT-1 (20 ng/mL, 1 h) on α-MG uptake (0.1 mM, 15 min) in Caco-2 cells (n = 16-44). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 compared with control group. ###P < 0.001 compared with rCT-1-treated cells.

Effect of AICAR on the inhibitory effect of rCT-1 on SGLT-1

Previous studies suggested that AICAR enhanced the translocation of SGLT-1 to the luminal membrane of Caco-2 cells (Sopjani *et al.* 2010). In these sense, we wondered whether the incubation with this AMPK activator could reverse the effects of rCT-1 on SGLT-1 expression levels in BBMV of Caco-2 cells. We observed that AICAR abolished the inhibition induced by rCT-1 on SGLT-1 expression after 1 and 24 h of treatment, suggesting that alterations in AMPK pathway could be mediating the effects of rCT-1 on SGLT-1 expression (Fig. 4A). Moreover, the inhibitory effect of rCT-1 on α-MG uptake was reversed when cells were incubated in presence of AICAR (Fig. 4B).

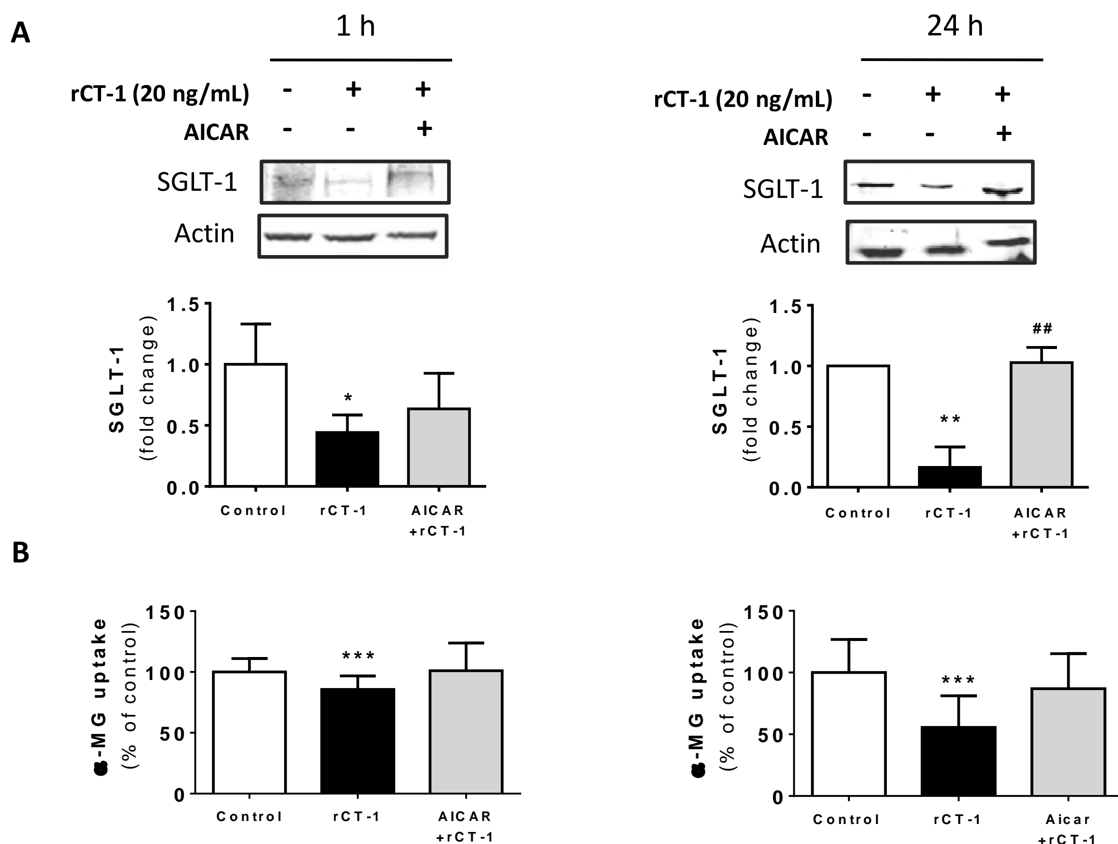


Fig. 4. AICAR restores the inhibitory effect of rCT-1 on (A) SGLT-1 expression and (B) α-MG uptake in Caco-2 cells. (A) Representative Western Blot and densitometric analysis of SGLT-1 in BBMV of Caco-2 cells obtained after 1 or 24 h incubation with rCT-1 (20 ng/mL) followed by a 15 min incubation with 0.1 mM α-MG, in the presence or absence of the AMPK activator AICAR (1 mM) (n = 3-6 independent experiments). (B) Effect of rCT-1 treatment (20 ng/mL; 1 and 24 h) on α-MG (0.1 mM) uptake for 15 min in Caco-2 cells (n = 4-30). Data are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.01 compared with control group. ##P < 0.01 compared with rCT-1-treated cells.

DISCUSSION

A previous study from our group revealed that CT-1 is a key regulator of energy homeostasis, and glucose and lipid metabolism (Moreno-Aliaga *et al.* 2011). Indeed, chronic CT-1 administration to obese mice reduced hyperglycemia and hyperinsulinemia and improves insulin sensitivity. Interestingly, we also found that the glucose-lowering properties of CT-1 were in part independent of insulin, as it could be observed in mice with streptozotocin (STZ)-induced insulin deficiency (Moreno-Aliaga *et al.* 2011).

A main finding of this research is that *in vivo* administration of rCT-1 inhibits intestinal sugar uptake after short and long-term treatments. These observations suggest that the hypoglycemic and anti-obesity properties of CT-1 could be in part secondary to its ability to reduce intestinal sugar absorption. CT-1 has the gp130 receptor as effector of intracellular signaling, which shares a large degree of sequence homology with the leptin receptor, and both activate the JAK/STAT and ERK signaling pathways (Febbraio 2007). In line with the results observed for CT-1, previous trials have demonstrated that leptin also inhibits sugar intestinal uptake *in vitro* and *in vivo* (Barrenetxe *et al.* 2004, Fanjul *et al.* 2012). Interestingly, our data also revealed that the inhibitory effect of CT-1 on α-MG uptake observed in human Caco-2 cells was accompanied by a decrease on the abundance of SGLT-1 in the brush border membrane of the enterocytes. Similarly, leptin also decreases SGLT-1 expression levels on enterocyte brush border membrane in Caco-2 cells (Fanjul *et al.* 2012). However, the inhibitory effect of CT-1 on intestinal glucose transport is not shared by other cytokine of the same family, since we have found that CNTF does not significantly inhibits α-MG uptake *in vitro*. Other experiment revealed that IL-6, another gp130 cytokine, increased 3-O-methyl glucose intestinal transport in rabbits (Hardin *et al.* 2000). Taken together, these data suggest that inhibition of intestinal sugar uptake seems to be specific feature for CT-1 and it is not common for gp130 cytokines.

Several studies have suggested that the effects of CT-1 on sugar uptake are cell/tissue-specific. Thus, a role of CT-1 in the regulation of myocardial glucose metabolism have been described in cardiomyocytes, showing inhibitory or stimulatory effects on 2-deoxyglucose (2-DG) transport at low (1 nM) or high (10 nM)

concentrations respectively (Asrih *et al.* 2013). In contrast to the inhibitory effect of CT-1 on intestinal sugar absorption, stimulatory effects of similar concentrations of the cytokine (20 ng/mL) on both basal and insulin stimulated 2-DG uptake have been described in soleus muscle and in L6E9 myotubes (Moreno-Aliaga *et al.* 2011). A similar outcome was observed in 3T3-L1 adipocytes after 3 and 24 h of treatment with the cytokine (20 ng/mL). Interestingly, AKT activation is a major mediator in the stimulatory effect of CT-1 on glucose uptake by muscle and adipose cells (Moreno-Aliaga *et al.* 2011). In the present study, CT-1 also induces a time-dependent transient activation of PI3K/AKT pathway. Nevertheless, this pathway is not likely to play a role in CT-1 inhibitory action on α-MG uptake, because treatment with a PI3K inhibitor did not reverse this effect.

It is well established that activation of AMPK increases glucose uptake in muscle and heart (Coughlan *et al.* 2014). Previous studies from our group and others have demonstrated that CT-1 modulate AMPK activation in muscle, liver and cardiomyocytes (Moreno-Aliaga *et al.* 2011, Asrih *et al.* 2013, Castano *et al.* 2014). Interestingly, chronic activation of AMPK appeared to mediate the stimulation of basal and insulin-stimulated glucose uptake in cardiomyocytes treated with the high doses of CT-1(Asrih *et al.* 2013). AMPK plays also a role in the regulation of SGLT-1-mediated transport in mice (Walker *et al.* 2005). In this way, previous studies have described that AMPK activation increases glucose uptake by stimulating SGLT-1-mediated Na^+-coupled transport in Caco-2 cells (Sopjani *et al.* 2010). Likewise, other authors have demonstrated that increased AMPK activity leads to the up-regulation of SGLT-1, which promotes increased cardiac glucose uptake in mice (Banerjee *et al.* 2010).

In the present study, a time-dependent effect of CT-1 on AMPK regulation in Caco-2 cells was found. Thus, although a transient activation of AMPK was observed at 15 min of incubation with CT-1, longer periods of treatment with the cytokine promoted an inhibition of AMPK activation. In fact, several of our present observations suggest the implication of the downregulation of the AMPK pathway in the inhibitory effect of CT-1 on sugar absorption in enterocytes: 1) The decrease on AMPK activation induced by CT-1 temporarily coincides with the decrease of SGLT-1 expression and the inhibition on α-MG uptake at 1 h; 2) AICAR, an activator of AMPK pathway, completely abolishes

the inhibitory effects of CT-1 on glucose uptake, and importantly 3) AICAR is able to reverse the decrease induced by the cytokine on SGLT-1 expression level.

The activation of the gp130 receptor leads to phosphorylation of STAT3, the signaling effector of rCT-1 (Heinrich *et al.* 2003). In this context, we show that CT-1 promotes a transient activation of STAT3 in Caco-2 cells. Moreover, our functional studies demonstrate that the reduction of sugar uptake caused by rCT-1 is abolished when cells are pre-treated with the JAK/STAT inhibitor (AG490), supporting the idea that early activation of STAT3 is required for the inhibitory effects of CT-1 on α-MG uptake. However, the fact that STAT3 phosphorylation is unchanged at 60 min when the inhibition of SGLT-1 and α-MG uptake is observed, suggest that is not the main determinant of these actions, although its early activation seems to be required. In line with these observations, it has also been described that leptin, a potent activator of STAT3 signaling pathway (Bates *et al.* 2003) also decreases the hypothalamic activity of AMPK (Minokoshi *et al.* 2004), suggesting that this inhibition could be secondary to the activation of STAT3 pathway. However, the relationship of this early STAT3 activation with the later changes in AMPK phosphorylation remains to be determined.

Previous studies have demonstrated that CT-1 is capable of activating the ERK1/2 pathway (Robledo *et al.* 1997, Bustos *et al.* 2003), which has been shown to play key roles in CT-1 intracellular signal transductions (Fukada *et al.* 1996). However, we did not observe any changes on the activation of ERK1/2 by phosphorylation in Caco-2 cells after treatment with CT-1. It is important to note that ERK1/2 was constitutively activated in this cell line, and that probably because of this CT-1 was not able to further increase the phosphorylation levels of this kinase.

Although the administration of pharmacological doses of CT-1 clearly modulates intestinal sugar absorption, our current data revealed that the lack of CT-1 did not lead to significant changes in intestinal sugar uptake. Therefore, the physiological relevance of this cytokine in the regulation of the gut function needs to be further characterized.

In summary, data of the present investigation demonstrate the ability of CT-1 to inhibit intestinal sugar absorption by mechanisms implicating the down-regulation of the SGLT-1 transporter expression at the apical membrane of the cells. Several signaling pathways, especially STAT3 and AMPK, might be involved in the effects of

CT-1 on sugar uptake. These effects of CT-1 on intestinal sugar absorption could contribute to the hypoglycemic and anti-obesity properties of this cytokine.

ACKNOWLEDGEMENTS

This study was supported by grants from the Government of Navarra (Department of Health); Mutua Madrileña Foundation; FIS [PI10/01516 and PI13/01851]; CIBERobn; Línea Especial ‘‘Nutrición, Obesidad y Salud” and Centre for Nutrition Research, Universidad de Navarra; and by the agreement between FIMA and the ‘‘UTE project CIMA’’. Miguel López-Yoldi was supported by a fellowship from Asociación de Amigos University of Navarra.

REFERENCES

-A-

Asrih, M., Gardier, S., Papageorgiou, I. & Montessuit, C. 2013. Dual effect of the heart-targeting cytokine cardiotrophin-1 on glucose transport in cardiomyocytes. *J Mol Cell Cardiol,* 56, 106-15.

-B-

Banerjee, S. K., Wang, D. W., Alzamora, R., Huang, X. N., Pastor-Soler, N. M., Hallows, K. R., McGaffin, K. R. & Ahmad, F. 2010. SGLT1, a novel cardiac glucose transporter, mediates increased glucose uptake in PRKAG2 cardiomyopathy. *J Mol Cell Cardiol,* 49, 683-92.

Barrenetxe, J., Fanjul, C., Ducroc, R. & Lostao, M. P. 2013a. Regulation of intestinal glucose uptake by leptin.) *Glucose Uptake.* Nova Science Publishers, Inc.

Barrenetxe, J., Sainz, N., Barber, A. & Lostao, M. P. 2004. Involvement of PKC and PKA in the inhibitory effect of leptin on intestinal galactose absorption. *Biochem Biophys Res Commun,* 317, 717-21.

Barrenetxe, J., Sanchez, O., Barber, A., Gascon, S., Rodriguez-Yoldi, M. J. & Lostao, M. P. 2013b. TNFalpha regulates sugar transporters in the human intestinal epithelial cell line Caco-2. *Cytokine,* 64, 181-7.

Bates, S. H., Stearns, W. H., Dundon, T. A., Schubert, M., Tso, A. W., Wang, Y., Banks, A. S., Lavery, H. J., Haq, A. K., Maratos-Flier, E., Neel, B. G., Schwartz, M. W. & Myers, M. G., Jr. 2003. STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature,* 421, 856-9.

Beraza, N., Marques, J. M., Martinez-Anso, E., Iniguez, M., Prieto, J. & Bustos, M. 2005. Interplay among cardiotrophin-1, prostaglandins, and vascular endothelial growth factor in rat liver regeneration. *Hepatology,* 41, 460-9.

Bustos, M., Beraza, N., Lasarte, J. J., Baixeras, E., Alzuguren, P., Bordet, T. & Prieto, J. 2003. Protection against liver damage by cardiotrophin-1: a hepatocyte survival factor up-regulated in the regenerating liver in rats. *Gastroenterology,* 125, 192-201.

-C-

Castano, D., Larequi, E., Belza, I., Astudillo, A. M., Martinez-Anso, E., Balsinde, J., Argemi, J., Aragon, T., Moreno-Aliaga, M. J., Muntane, J., Prieto, J. & Bustos, M. 2014. Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation. *J Hepatol,* 60, 1017-25.

Coughlan, K. A., Valentine, R. J., Ruderman, N. B. & Saha, A. K. 2014. AMPK activation: a therapeutic target for type 2 diabetes? *Diabetes Metab Syndr Obes,* 7, 241-53.

-F-

Fanjul, C., Barrenetxe, J., Inigo, C., Sakar, Y., Ducroc, R., Barber, A. & Lostao, M. P. 2012. Leptin regulates sugar and amino acids transport in the human intestinal cell line Caco-2. *Acta Physiol (Oxf),* 205, 82-91.

Febbraio, M. A. 2007. gp130 receptor ligands as potential therapeutic targets for obesity. *J Clin Invest,* 117, 841-9.

Fukada, T., Hibi, M., Yamanaka, Y., Takahashi-Tezuka, M., Fujitani, Y., Yamaguchi, T., Nakajima, K. & Hirano, T. 1996. Two signals are necessary for cell proliferation induced by a cytokine receptor gp130: involvement of STAT3 in anti-apoptosis. *Immunity,* 5, 449-60.

-H-

Hardin, J., Kroeker, K., Chung, B. & Gall, D. G. 2000. Effect of proinflammatory interleukins on jejunal nutrient transport. *Gut,* 47, 184-91.

Heinrich, P. C., Behrmann, I., Haan, S., Hermanns, H. M., Muller-Newen, G. & Schaper, F. 2003. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. *Biochem J,* 374, 1-20.

-I-

Ishikawa, Y., Eguchi, T. & Ishida, H. 1997. Mechanism of beta-adrenergic agonist-induced transmural transport of glucose in rat small intestine. Regulation of phosphorylation of SGLT1 controls the function. *Biochim Biophys Acta,* 1357, 306-18.

-K-

Kessler, M., Acuto, O., Storelli, C., Murer, H., Muller, M. & Semenza, G. 1978. A modified procedure for the rapid preparation of efficiently transporting vesicles from small intestinal brush border membranes. Their use in investigating some properties of D-glucose and choline transport systems. *Biochim Biophys Acta,* 506, 136-54.

-L-

Lee, Y. J., Heo, J. S., Suh, H. N., Lee, M. Y. & Han, H. J. 2007. Interleukin-6 stimulates alpha-MG uptake in renal proximal tubule cells: involvement of STAT3, PI3K/Akt, MAPKs, and NF-kappaB. *Am J Physiol Renal Physiol,* 293, F1036-46.

Lostao, M. P., Urdaneta, E., Martinez-Anso, E., Barber, A. & Martinez, J. A. 1998. Presence of leptin receptors in rat small intestine and leptin effect on sugar absorption. *FEBS Lett,* 423, 302-6.

-M-

Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y. B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferre, P., Birnbaum, M. J., Stuck, B. J. & Kahn, B. B. 2004. AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus. *Nature,* 428, 569-74.

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* 14, 242-53.

-O-

Oppenheim, R. W., Wiese, S., Prevette, D., Armanini, M., Wang, S., Houenou, L. J., Holtmann, B., Gotz, R., Pennica, D. & Sendtner, M. 2001. Cardiotrophin-1, a muscle-derived cytokine, is required for the survival of subpopulations of developing motoneurons. *J Neurosci,* 21, 1283-91.

-P-

Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D. L., Zioncheck, K. A., Rosenthal, A., Taga, T., Paoni, N. F. & Wood, W. I. 1995. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J Biol Chem,* 270, 10915-22.

-R-

Robledo, O., Fourcin, M., Chevalier, S., Guillet, C., Auguste, P., Pouplard-Barthelaix, A., Pennica, D. & Gascan, H. 1997. Signaling of the cardiotrophin-1 receptor. Evidence for a third receptor component. *J Biol Chem,* 272, 4855-63.

-S-

Sopjani, M., Bhavsar, S. K., Fraser, S., Kemp, B. E., Foller, M. & Lang, F. 2010. Regulation of Na+-coupled glucose carrier SGLT1 by AMP-activated protein kinase. *Mol Membr Biol,* 27, 137-44.

-V-

Vayro, S. & Silverman, M. 1999. PKC regulates turnover rate of rabbit intestinal Na+-glucose transporter expressed in COS-7 cells. *Am J Physiol,* 276, C1053-60.

Vinuales, C., Gascon, S., Barranquero, C., Osada, J. & Rodriguez-Yoldi, M. J. 2013. Interleukin-1beta reduces galactose transport in intestinal epithelial cells in a NF-kB and protein kinase C-dependent manner. *Vet Immunol Immunopathol,* 155, 171-81.

-W-

Walker, J., Jijon, H. B., Diaz, H., Salehi, P., Churchill, T. & Madsen, K. L. 2005. 5-aminoimidazole-4-carboxamide riboside (AICAR) enhances GLUT2-dependent jejunal glucose transport: a possible role for AMPK. *Biochem J,* 385, 485-91.

Wright, E. M., Hirayama, B. A. & Loo, D. F. 2007. Active sugar transport in health and disease. *J Intern Med,* 261, 32-43.

Wright, E. M., Hirsch, J. R., Loo, D. D. & Zampighi, G. A. 1997. Regulation of Na+/glucose cotransporters. *J Exp Biol,* 200, 287-93.

Wright, E. M., Loo, D. D. & Hirayama, B. A. 2011. Biology of human sodium glucose transporters. *Physiol Rev,* 91, 733-94.

CHAPTER 4

Role of cardiotrophin-1 (CT-1) in the regulation of metabolic circadian rhythm and adipose core clock genes in mice and characterization of 24-h circulating CT-1 profile in normal weight and overweight/obese subjects

López-Yoldi M[1,2], Stanhope KL[3], Garaulet M[4], Chen XG[3], Marcos-Gómez B[1], Carrasco-Benso MP[4], Lee V[3], Nunez MV[3], Martínez-Ansó E[5], Sáinz N[1,2], Huerta AE[1,2], Prieto J[5,6], Martínez JA[1,2,7,8], Bustos M[5*], Havel PJ[3], Moreno-Aliaga MJ[1,2,7,8*]

[1]Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Navarra, Spain.

[2]Centre for Nutrition Research. University of Navarra, Pamplona, Navarra, Spain.

[3]Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, Davis, CA.

[4] Department of Physiology, Faculty of Biology, University of Murcia, Murcia, Spain.

[5]Department of Hepatology and Gene Therapy, CIMA, University of Navarra, Pamplona, Navarra, Spain.

[6]CIBERehd. Institute of Health Carlos III, Madrid, Spain

[7]CIBERobn, Physiopathology of Obesity and Nutrition, Institute of Health Carlos III, Madrid, Spain.

[8]IdiSNA, Navarra Institute for Health Research, Pamplona, Spain.

*Equal senior authorship

ABSTRACT

Cardiotrophin-1 (CT-1) is a regulator of glucose and lipid homeostasis and adipose tissue function. The present study analyzed whether CT-1 could also act as a peripheral regulator of metabolic rhythms and adipose tissue core clock genes in mice. Moreover, the circadian rhythmicity of plasma CT-1 levels was also evaluated in normal weight and overweight/obese subjects. Interestingly, circadian rhythmicity of the rate of oxygen consumption (VO_2) was totally disrupted in aged CT-1 deficient (CT-1$^{-/-}$) obese mice (12 months). Although circadian rhythms of VO_2 were conserved in young-lean CT-1$^{-/-}$ mice (2 months), CT-1 deficiency resulted in a phase shift of the acrophase for VO_2. Moreover, the lack of CT-1 also induced marked alterations of *Bmal1* and *Cry1* mRNA levels in young CT-1 null mice, which was also evident for *Clock* and *Per2* in 12-month-old CT-1$^{-/-}$ mice. Moreover, treatment with recombinant CT-1 attenuated the decreased expression of *Clock* mRNA in adipose tissue in *ob/ob* mice. In humans, CT-1 plasma profile exhibited a 24-h rhythm in lean, but not in overweight/obese subjects. The 24-h pattern of CT-1 was characterized by a pronounced increase in CT-1 levels during the night (from 2:00 to 8:00 am), with the achrophase at 8:00 am, and followed by a drop between 8:00 am to 9:00 am. All these observations suggest a potential role of CT-1 as a peripheral regulator of metabolic circadian rhythms.

Supplementary key words: *Cardiotrophin-1 . Circadian rhythm . Clock genes . Adipose tissue . Oxygen consumption . Obesity .*

INTRODUCTION

Obesity (defined as a body mass index [BMI] > 30 kg/m^2) is a growing public health problem that has reached epidemic proportions in many developed countries (Reichman *et al.* 2015). Among the different causes of obesity, modern human lifestyle can be responsible for a desynchrony between internal and external human timing, largely contributing to the onset of metabolic syndrome (MetS), obesity and type 2 diabetes (Garaulet *et al.* 2009, Gimble *et al.* 2011, Morris *et al.* 2012). In this context, it has been described that different phenomena such as short-time sleep duration (Buxton *et al.* 2012); shift work (Peplonska *et al.* 2015) or jet lag (Kumar Jha *et al.* 2015) among others, could be related to the development of obesity and type 2 diabetes.

Recent studies have demonstrated the interaction between circadian clock and energy metabolism regulation. Indeed, disruption of circadian rhythms (chronodisruption) may cause desynchrony, which impairs metabolism homeostasis and contributes to the onset of obesity and MetS (Antunes *et al.* 2010, Bass *et al.* 2010). In mammals, circadian molecular complex is integrated by a central pacemaker at the suprachiasmatic nucleus (SCN) of the hypothalamus and peripheral oscillators present in most tissues and cells including adipose tissue (Eckel-Mahan *et al.* 2013). “Peripheral” clocks are controlled by the “central” clock to ensure a temporally synchronized physiology (Reppert *et al.* 2002, Sahar *et al.* 2012). The positive limb of the molecular core clock machinery is composed and directed by CLOCK and BMAL1 which heterodimerize to regulate the expression of the negative elements and circadian genes. On the other hand, the negative limb comprised PERs and CRYs which form heterodimers and repress their own transcription by negatively regulating the transcriptional activity of CLOCK:BMAL1 heterodimer (Garaulet *et al.* 2010).

Cardiotrophin-1 (CT-1) belongs to the interleukin-6 (IL)-6 family of cytokines and has been described to be elevated in obesity and metabolic syndrome (Natal *et al.* 2008), suggesting that CT-1 could be a new marker for obesity and related diseases. A study by our group has revealed that CT-1 is a key regulator of energy homeostasis, as well as of glucose and lipid metabolism (Moreno-Aliaga *et al.* 2011). Thus, CT-1 null ($CT\text{-}1^{-/-}$) mice develop mature-onset obesity, insulin resistance, and hypercholesterolemia despite reduced calorie intake (Moreno-Aliaga *et al.* 2011).

Moreover, chronic recombinant CT-1 (rCT-1) treatment reduced body weight and corrected insulin resistance in *ob/ob* and in high-fat-fed obese mice by reducing food intake and enhancing energy expenditure.

Adipose tissue has been established as a peripheral oscillator capable of modulating central core clock genes (Mohawk *et al.* 2012). However, the regulation of the peripheral clock and the clock-controlled genes present in the adipose depots remains unclear (Sukumaran *et al.* 2010). In this context, it has been proposed that several molecules secreted by adipose tissue such as leptin and adiponectin, that are important metabolic mediators, could be peripheral regulators of the circadian clocks in the brain and peripheral organs (Froy 2012, Hashinaga *et al.* 2013). Interestingly, some of these adipokines are clock controlled and its adipose expression exhibits diurnal variation (Ando *et al.* 2005, Bray *et al.* 2007, Garaulet *et al.* 2011). Moreover, these and other hormones involved in metabolic regulation such as ghrelin and IL-6 exhibit 24-h variation in plasma (Bodosi *et al.* 2004, Johnston 2012, Froy 2012). To date, there is no information available about the potential involvement of CT-1 in the regulation of core clock genes in adipose tissue and the potential diurnal variations of CT-1 in humans have not been studied.

Therefore, the aim of the present study was to analyze whether CT-1 (both deficiency and treatment) could be regulating circadian rhythmicity and the expression of core circadian clock genes in white adipose tissue in mice and to evaluate a potential circadian rhythm of CT-1 levels in plasma of normal weight and overweight/obese human subjects.

MATERIAL AND METHODS

Animal Studies:

Recombinant Protein CT-1 (rCT-1)

rCT-1 was obtained as described elsewhere (Beraza *et al.* 2005) and contained < 0.04 ng LPS per 1 µg of the protein as determined by the Limulus amoebocyte lysate assay (Cambrex, East Rutherford, NJ).

Animal models

CT-1$^{-/-}$ mice

CT-1 null mice were generated as described by Oppenheim *et al.* (2001). We analyzed mice backcrossed into a C57BL/6J background for 11 generations (provided by Diane Pennica, Genentech, and Bettina Holtmann, University of Wuerzburg, Germany). C57BL/6J mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Body weight and food intake were measured in wild type (WT) and CT-1$^{-/-}$ mice from 2 to 12 months old. At the end of the different experimental periods (2 and 12 months), WT and CT-1$^{-/-}$ animals were sacrificed after 16 h of fasting. Epididymal fat depots were excised, weighed and kept at −80 °C for subsequent analysis. All experimental procedures were approved by the University of Navarra Ethics Committee.

Long-term treatment with rCT-1 in ob/ob mice

Eight weeks old *ob/ob* mice were obtained from the Janvier Laboratory (Le Genest St Isle). C57BL/6J mice were obtained from Harlan Laboratories (Barcelona, Spain) and were placed on high fat diet (HFD) (60% of kcal from fat, 20% from carbohydrates and 20% from protein, Research Diets, New Brunswick, NJ) *ad libitum*, and monitored for food intake and body weight for 12 weeks. *Ob/ob* mice were divided into three subgroups, one that received rCT-1 intravenously (0.2 mg/kg/day) for 10 days and fed *ad libitum*, another given saline instead of rCT-1 and fed *ad libitum*, and a pair fed (PF) group given saline and receiving the same amount of food ingested by the rCT-1 treated group. This group is necessary to distinguish what proportion of the rCT-1 actions is independent of rCT-1 effects on food intake. Food intake and body weight were measured daily. Animals were sacrificed after an overnight fast, unless indicated otherwise. Epididymal fat depots were excised, weighed and kept at −80 °C for subsequent analysis. All experimental procedures were approved by the University of Navarra Ethics Committee.

Body Composition Analysis

Whole animal body composition was measured in live conscious animals using Quantum Molecular Resonance (QMR) technology (EchoMRI-100-700, Echo Medical Systems, Houston, TX).

Biochemistry

All serum measurements were done on mice fasted for 16 h, unless otherwise indicated, using a Cobas Mira Autoanalyzer (Roche Diagnostic, Basel, Switzerland) (Moreno-Aliaga et al. 2011). Insulin was analyzed using mouse ELISA kits from Linco Research and Crystal Chem. Inc.

Whole body oxygen consumption

Twenty-four hours whole body oxygen consumption (VO_2) was measured in WT and CT-$1^{-/-}$ mice (2 and 12 months-old) using the Oxylet System (Panlab, Spain) as previously described (Moreno-Aliaga *et al.* 2011).

Real time PCR

Total RNA was extracted from epididymal fat depots using TRIzol® reagent (Invitrogen, CA) according to the manufacturer's instructions. RNA concentrations and quality were measured by Nanodrop Spectrophotometer 1000 (Thermo Scientific, DE). RNA was then incubated with a RNAse-free kit DNase (Ambion, Austin, TX) for 30 min at 37 °C. RNA (2 µg) was reverse transcribed into complementary DNA using MMLV (Moloney murine leukemia virus) reverse transcriptase (Invitrogen). mRNA levels were determined using predesigned TaqMan® Assays-on-Demand (*Clock*: Mm00455959-_m1; *Bmal*: Mm00500226_m1; *Per2*: Mm00478113_m1; *Cry1*: Mm00514392_m1). Taqman Universal Master Mix was also provided by the same supplier. The reaction conditions were followed as described by the manufacturer's instructions. Amplification and detection of specific products were performed using ABI PRISM 7900HT (Applied Biosystems). mRNA levels were normalized by the housekeeping gene Cyclophilin (Mm02342430_g1), obtained from Applied Biosystems. All samples were analyzed in duplicate. The relative expression level of each gene was calculated as $2^{-\Delta\Delta Ct}$.

Human Study:

Subjects

Participants in this study are a subgroup from an NIH-funded investigation in which a total of 187 participants assigned to 8 experimental groups were studied. This paper report only the results of baseline samples collected prior to the dietary sugar intervention.

Participants were recruited through an internet listing (Craigslist.com) and local postings of flyers, and underwent telephone and in-person interviews with medical history, complete blood count and serum biochemistry panel to assess eligibility. Inclusion criteria included age 18-40 years and body mass index (BMI) 18-35 kg/m^2 with a self-report of stable body weight during the prior six months. Exclusion criteria included: diabetes (fasting glucose>125 mg/dL), evidence of renal or hepatic disease, fasting plasma triacylglcycerol (TAG) >400 mg/dL, hypertension (>140/90 mm Hg), hemoglobin < 8.5 g/dL and surgery for weight loss. Individuals who smoked, habitually ingested more than two alcoholic beverages/day, exercised more than 3.5 hours/week at a level more vigorous than walking, or used thyroid, lipid-lowering, glucose-lowering, anti-hypertensive, anti-depressant, or weight loss medications were also excluded.

Methods

The first phase of this study consisted of a 3.5-day inpatient baseline period during which subjects resided at the University of California Davis Clinical and Translational Science Center's Clinical Research Center (CCRC), consumed a standardized baseline diet and participated in experimental procedures.

Inpatient diets: During days 2 and 3 of the baseline inpatient period, subjects consumed energy-balanced meals consisting of conventional foods. Daily energy requirements were calculated by the Mifflin equation. With this equation, the resting energy expenditure (REE) is calculated by using the following formula: REE (males) = 10 x weight (kg) + 6.25 x height (cm) - 5 x age (y) + 5; REE (females) = 10 x weight (kg) + 6.25 x height (cm) - 5 x age (y) – 161 (Mifflin *et al.* 1990) with adjustment of 1.3 for activity on the days of the 24-h serial blood collections, and adjustment of 1.5 for the

other days. The baseline diet contained 55% energy requirements mainly as low-fiber complex carbohydrate (i.e. white bread, white rice, regular pasta), 30% from fat, 15% from protein, and 22 g fiber/2000 kcal. The timing of inpatient meals and the energy distribution were: Breakfast-09:00 h (25%); Lunch-13:00 h (35%); Dinner-18:00 h (40%).

Procedures: 24-hour serial blood collections (Stanhope *et al.* 2011) were conducted on the 3rd day of the baseline (0 week) inpatient periods. Three fasting blood samples were collected at 08:00, 08:30 and 09:00 h. Twenty-nine postprandial blood samples were collected at 30-60 minute intervals until 08:00 h the following morning. Fasting plasma concentrations of TAG, total, low and high density lipoprotein-cholesterol (LDL-C and HDL-C) were determined with a Polychem Chemistry Analyzer (PolyMedCo, Inc., Cortland, NY) with reagents from MedTest DX (Cortlandt Manor, NY). Glucose concentrations were measured with an automated glucose analyzer (YSI, Inc. Yellow Springs, OH) and insulin by radioimmunoassay (Millipore, St.Charles, MO). Free fatty acids were assayed with an enzymatic colorimetric assay (Wako Chemicals, Richmond, VA) adapted to a microtiter plate.

Ethics: The study was conducted in accordance with experimental protocol that was approved by the UC Davis Institutional Review Board and participants provided written informed consent.

Determination of serum CT-1 plasma concentration

Plasma CT-1 concentrations were measured by ELISA as described elsewhere (Rendo-Urteaga *et al.* 2013). Briefly, plasma samples were added in triplicates to 96-well plates pre-coated overnight with a rat monoclonal anti-CT-1 antibody (R&D Systems), washed with PBS and blocked in 1% BSA/PBS. Then, human plasma samples diluted/pretreated in dilution buffer (1:20 in PBST-0.25% containing BSA and FBS) were incubated o/n at 4 ^{o}C. Following incubation, plates were washed three times with PBST-0.05% and incubated with a Rabbit anti-human CT-1 antibody (R&D Systems) for 2 h at room temperature. After incubation, unbound antibodies were washed off with PBST and incubated with a peroxidase conjugated rabbit specific antibody added for detection of the bound anti-CT-1. Finally, plates were washed, and labeled antibody

binding determined using 3,3,5,5-tetramethylbenzidine substrate. Absorbance was read at 650 nm and CT-1 plasma concentrations determined by comparison with a standard curve of serially diluted recombinant CT-1 in dilution buffer.

Statistical analysis

Data are presented as mean ± standard error (SEM). A repeated-measures ANOVA was carried out to analyze significant time-dependent changes in CT-1 plasma levels during 24 h, as well as during the light period (from 8:00 to 20:00 h) and the dark period (from 20:00 to 8:00 h). Comparisons between the values for different variables were analyzed by Student's t-test or Mann-Whitney U-test once the normality had been screened using Kolmogorov-Smirnoff and Shapiro-Wilk tests. Statistical analyses and graphs were carried out using GraphPad Prism 6 software (Graph-Pad Software Inc., San Diego, CA) and Stata Statistical Software (Release 12. College Station, TX: StataCorp LP). Overall, a P value < 0.05 was considered significant.

To investigate the presence of circadian rhythm in VO_2 in WT and CT-1$^{-/-}$ animal models, and in humans plasma CT-1 concentrations, a least squares periodic regression (Batschelet 1981) was used to fit a sinusoidal function to the data (T = 24 h). Cosinor analysis was used to characterize the rhythm, calculating its mesor (mean value of VO_2 rhythm fitted to a cosine function), amplitude (difference between the maximum [or minimum] value of the cosine function and mesor), acrophase (timing of the maximum value of the cosine fitted curve relative to local 00:00 h). These parameters are represented graphically in a polar plot (hourly) to visualize the characteristics of the rhythm by a vector (cosinor) whose length corresponds to the amplitude and the direction to the acrophase. All the data from all individuals were used simultaneously to directly estimate the population parameters (one-step method) using a model that includes a sinusoid and a constant for each individual, which corresponds to the individual mesor, eliminating the effect of interindividual variation in the estimated global rhythm.

RESULTS

CT-1 null mice exhibit altered metabolic circadian rhythm and expression of core clock genes in adipose tissue

As previously reported (Moreno-Aliaga *et al.* 2011), CT-1 deficient and WT mice showed similar body weight at 2 months of age, but CT-1 deficient mice developed adult onset obesity (Supplementary Table I). However, the CT-1 null mice already exhibited decreased energy expenditure and oxygen consumption at 2 months, which preceded and accompanied the development of obesity (Moreno-Aliaga *et al.* 2011).

Supplementary Table I. Body weight and biochemical parameters in WT and CT-1$^{-/-}$ mice at 2 and 12 months of age.

	2 Months		12 Months	
	WT	**CT-1$^{-/-}$**	**WT**	**CT-1$^{-/-}$**
Body weight (g)	19.23 ± 0.80	18.33 ± 0.60	31.33 ±1.25	39.75 ± 2.13**
Total Fat Mass (%)	1.57 ± 0.12	1.56 ± 0.24	4.29 ± 0.53	10.86 ± 1.35***
Glucose (mM)	5.48 ± 0.10	6.18 ± 0.37	7.81 ± 1.46	10.33 ± 1.17*
Insulin (pM)	30.69 ± 3.34	30.85 ± 4.59	52.63 ± 15.87	57.01 ± 12.89*

*P < 0.05, **P < 0.01, ***P < 0.001 compared with WT control group (n = 5-7).

With the aim of studying if CT-1 deficiency could also cause circadian disturbances, we analyzed the daily rhythm of 24-h oxygen consumption rate (VO_2) (Rubal *et al.* 1992), in WT and CT-1$^{-/-}$ mice at 2 and 12 months of age. Parameters imputed from each group obtained by cosinor analysis, defining the characteristics of the rhythms as mesor, amplitude, acrophase, and percent rhythm are reported in Table 1. Although both young WT and CT-1 deficient mice (2 months-old) exhibited robust circadian rhythms for VO_2 values (P < 0.05), it is important to note that CT-1 deficiency caused a phase shift characterized by an advance of approximately 1 h of achrophase (Table 1).

Interestingly, aging *per se* (comparisons between WT mice at 2 and 12 months of age) lead to chronodisturbances characterized by an advance of the acrophase together with a reduction of the amplitude of the rhythm (Table 1).

Table 1. Parameters imputed from each group obtained by cosinor analysis defining the circadian rhythms for oxygen consumption rate (VO_2) as mesor, amplitude, acrophase, and PR in WT and CT-1$^{-/-}$ mice at 2 and 12 months.

	2 Months		12 Months	
	WT	**CT-1$^{-/-}$**	**WT**	**CT-1$^{-/-}$**
Mesor (AU)	76.4	65.53	53.34	33.28
Amplitude (AU)	5.93	6.01	3.86	2.65
Acrophase (h)	3:27	2:24	0:19	0:27
PR (%)	36.56	38.07	32.68	15.85

AU, arbitrary units.

On the other hand, while the 24-h VO_2 circadian rhythmicity was maintained in 12 months old WT mice (Fig. 1A), in CT-1 deficient obese mice the VO_2 circadian rhythms were completely lost (Fig. 1B), suggesting that CT-1 deficiency not only promotes obesity but also could aggravate chronodisruption associated with aging.

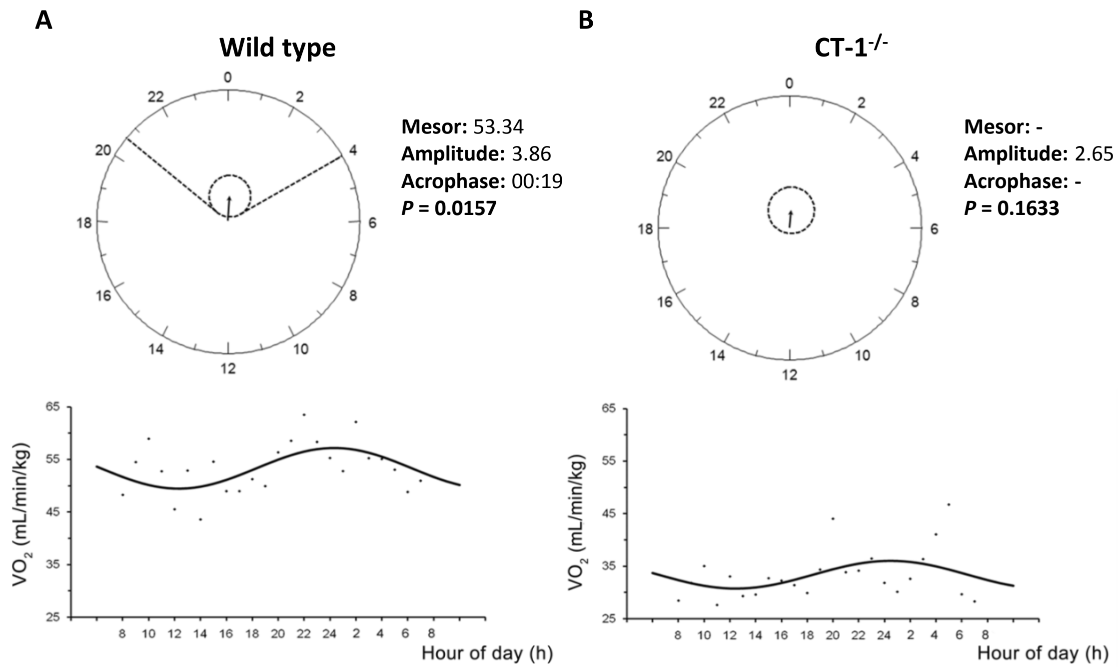


Fig. 1. Cosinor analysis of the circadian rhythm for oxygen consumption rate (VO_2). Top: polar (clock-like) representation of the estimates of the parameters of the rhythm for VO_2 in the form of a 24-h clock in (A) WT and (B) CT1$^{-/-}$ mice at 12 months of age. Bottom: x-y plot showing the best fitting waveform profile of the rhythm.

To test whether CT-1 deficiency could also modulate mRNA levels of core clock genes in peripheral organs, we analyzed the gene expression of *Clock*, *Bmal1*, *Per2* and *Cry1* in adipose tissue of WT and CT-1$^{-/-}$ mice at 2 and 12 months of age. As shown in Fig. 2, the pattern of mRNA levels of the core clock genes during aging (from 2 to 12 months) was markedly different between WT and CT-1$^{-/-}$ mice. Thus, in WT mice, an increase in the gene expression levels of *Clock* (P < 0.05), *Bmal1* (P < 0.01), *Per2* (P < 0.05) and *Cry1* (P < 0.05) was observed in old (12 months-old) in comparison with young (2 months-old). Interestingly, this pattern was disrupted in CT-1 deficient mice. Specifically, a significant decrease in *Clock* (P < 0.05) and *Per2* (P < 0.05) mRNA was observed in obese CT-1 deficient mice in comparison to WT mice at 12 months (Fig. 2A, C respectively), but no significant changes were observed between young lean WT and CT-1$^{-/-}$ animals at 2 months of age. This suggests that the disruption in adipose tissue *Clock* and *Per2* mRNA levels observed in old CT-1 null mice could be secondary to the higher adiposity of these animals. An important finding of our study was the observation that the gene expression of the positive element *Bmal1* and the negative

element *Cry1* was significantly different ($P < 0.01$) between WT and CT-1 knockout mice at 2 months of age, despite the similarity in body composition that was observed between the two groups at this age. This suggests that the decrease in *Bmal1* and *Cry1* is related to CT-1 deficiency and it is not a consequence of increased adiposity. Although the mRNA levels of *Bmal* ($P < 0.01$) and *Cry1* increased at 12 months of age in comparison with those observed at 2 months, they never reached the levels of those observed in WT mice (Fig. 2B, D). All these observations suggest an involvement of CT-1 in the regulation of the expression of clock genes in adipose tissue.

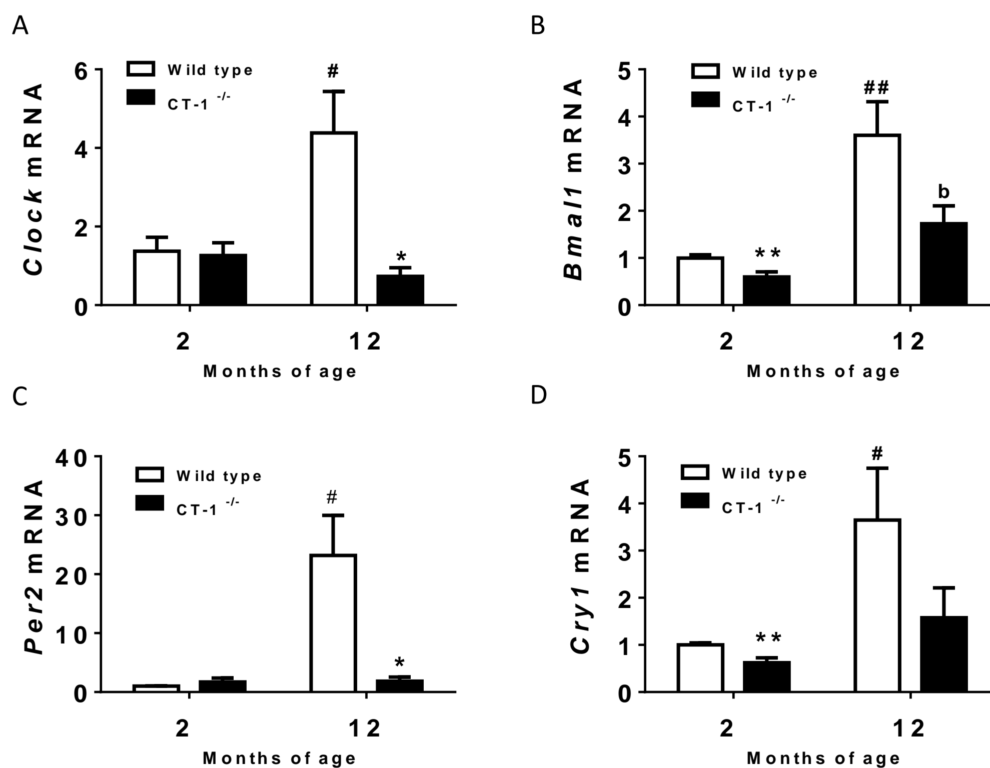


Fig. 2. Changes in clock genes with aging in WT and CT-1$^{-/-}$ mice. Gene expression levels of the core clock genes *Clock* (A), *Bmal1* (B), *Per2* (C) and *Cry1* (D) at 2 and 12 months of age in WT and CT-1$^{-/-}$ mice, expressed as fold change relative to WT mice mRNA expression at 2 months of age for each gene. Data are means ± SEM. (n = 4-7). *$P < 0.05$, **$P < 0.01$ compared with WT control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with WT group at 2 months. $^{b}P < 0.01$ compared with CT-1$^{-/-}$ group at 2 months.

Effects of CT-1 treatment on clock genes profile in adipose tissue of *ob/ob* mice

We next evaluated whether treatment with rCT-1 could modulate the expression of the core clock genes *Clock*, *Bmal*, *Per2* and *Cry1* in adipose tissue of *ob/ob* mice. With regard to the positive elements, *Clock* gene expression was significantly reduced ($P < 0.05$) in *ob/ob* mice comparing with WT mice, whereas in CT-1 treated animals this effect was less pronounced and not significant differences with WT mice were observed. Importantly, this action on *Clock* expression was not observed in PF-animals (Fig. 3A), suggesting that it is due to a direct effect of the cytokine and that it is not a consequence of the anorexic and body weight lowering properties of CT-1 (see Supplementary Table II). In contrast to what was observed for *Clock*, adipose *Bmal* expression was similar in *ob/ob* mice than in WT mice. However, *Bmal* mRNA levels were significantly increased in *ob/ob* mice treated with rCT-1 and in the PF-group in comparison with WT animals, suggesting that this effect could be secondary to the anorexigenic properties of CT-1 (Fig. 3B).

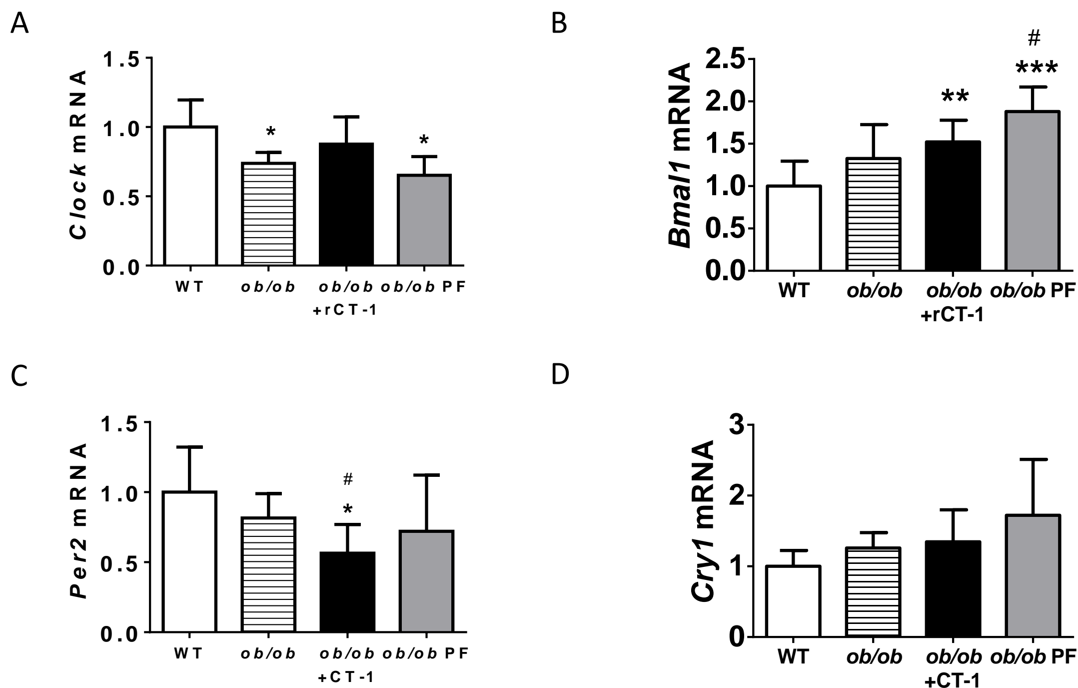


Fig. 3. Effects of rCT-1 treatment on clock genes expression in *ob/ob* mice. mRNA levels of the positive elements *Clock* (A), *Bmal1* (B) and the negative elements *Per2* (C), *Cry1* (D) in *ob/ob* mice treated with rCT-1 (0.2 mg/Kg/day; 10 days). Data (means ± SEM) are expressed as fold change relative to WT mice mRNA expression for each gene. (n = 5-7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with WT control group. $^{\#}P < 0.05$ compared with *ob/ob* group. PF: pair-fed.

In relation to the negative element *Per2*, rCT-1 treatment caused a significant reduction of *Per2* gene expression ($P < 0.05$) in *ob/ob* mice, which was not observed in the PF-group (Fig. 3C). Finally, neither congenital leptin deficiency nor rCT-1 treatment caused any change on the mRNA levels of *Cry1*, the other negative element (Fig. 3D).

Supplementary Table II. Body weight and biochemical parameters in WT, and in *ob/ob* mice before and after treatment with rCT-1 (0.2 mg/kg/day) or saline (*ad libitum* controls and PF) during 10 days.

	ob/ob	***ob/ob* + rCT-1**	***ob/ob*-PF**
Body weight (% change)	4.94 ± 0.33	11.30± 1.03***	7.58 ± 0.68###
Glucose (mM)	6.50 ± 0.54	4.43 ± 0.47*	6.38 ± 0.39##
Insulin (pM)	836.8 ± 140.1	400.4 ± 60.41*	552.5 ± 113.5

**P* < 0.05, ***P* < 0.01, ****P* < 0.001 compared with *ob/ob* group. ##*P* < 0.01, ###*P* < 0.001 compared with *ob/ob* + rCT-1 group (n = 5-7).

24-h CT-1 plasma profile in normal weight and overweight/obese subjects

Table 2 shows the basal characteristics of the subjects included in the study. Overweight/obese individuals exhibited hyperinsulinemia ($P < 0.05$) and higher levels of cholesterol ($P < 0.01$) than the lean subjects, due to increased levels of LDL-C ($P < 0.001$). CT-1 levels were moderately higher in overweight/obese subjects, but no significant differences were observed as compared to normal weight individuals (Table 2).

Table 2. Baseline anthropometric and biochemical parameters of normal weight *vs* overweight/obese subjects.

Variables	All subjects (n=84)	Normal weight (n=37)	Overweight/Obese (n=47)	*P* value
Anthropometry				
Weight (Kg)	74.70 ± 1.45	65.05 ± 1.53	82.29 ± 1.59	**<0.0001**
BMI (Kg/m^2)	25.36 ± 0.40	22.15 ± 0.30	27.88 ± 0.40	**<0.0001**
Waist (cm)	77.94 ± 1.08	70.28 ± 1.00	83.96 ± 1.17	**<0.0001**
Waist/Hip ratio	0.79 ± 0.01	0.76 0.01	0.81 ± 0.01	**0.002**
Blood routine variables				
Glucose (mg/dL)	90.39 ± 0.72	89.19 ± 0.97	91.38 ± 1.03	0.127
Insulin (mU/L)	13.6 ± 1.02	11.31 ± 0.67	15.37 ± 1.67	**0.046**
HOMA	2.91 ± 0.24	2.39 ± 0.15	3.30 ± 0.39	0.057
TAG (mg/dL)	102.9 ± 4.82	88.52 ± 5.12	114.2 ± 7.25	**0.007**
FFA (mmol/L)	0.39 ± 0.01	0.37 ± 0.02	0.4 ± 0.02	0.236
Cholesterol (mg/dL)	156.7 ± 3.31	145 ± 4.62	165.9 ± 4.25	**0.001**
HDL-C (mg/dL)	45.21 ± 1.39	46.77 ± 1.78	43.99 ± 2.06	0.325
LDL-C (mg/dL)	91.35 ± 2.73	80.99 ± 3.58	99.51 ± 3.58	**0.0005**
CT-1 baseline (ng/mL)	10.61 ± 0.96	9.67 ± 1.19	11.34 ± 1.44	0.390

Data are mean ± SEM. BMI: body mass index; HOMA: homeostasis model assessment; TAG: triacylglycerol; FFA: free fatty acids; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; CT-1: cardiotrophin-1

When the potential correlations between plasma CT-1 concentrations and anthropometrical and biochemical parameters were analyzed, Spearman's correlation analysis revealed that CT-1 levels were positively correlated with weight (rho = 0.349; *P* = 0.034), waist circumference (rho = 0.372; *P* = 0.023) and waist/hip ratio (rho = 0.398; *P* = 0.015), while negatively associated with LDL-C (rho = -0.341; *P* = 0.039) in normal weight subjects. Interestingly, these associations were not observed in overweight/obese individuals (Table 3).

Table 3. Correlation analysis between plasma CT-1 and anthropometric and biochemical variables in normal weight and overweight/obese subjects at baseline.

	All subjects (n = 84)		Normal weight (n = 37)		Overweight/Obese (n = 47)	
	rho	***P***	**rho**	***P***	**Rho**	***P***
Weight	0.134	0.225	0.349	**0.034**	-0.003	0.982
BMI	0.129	0.244	0.112	0.509	0.101	0.500
Waist	0.149	0.175	0.372	**0.023**	-0.048	0.750
Waist/Hip ratio	0.172	0.118	0.398	**0.015**	0.018	0.905
Glucose	0.088	0.434	0.157	0.362	-0.003	0.985
Insulin	0.019	0.889	0.043	0.840	0.004	0.983
HOMA	0.081	0.561	0.196	0.371	0.063	0.738
TAG	0.021	0.849	0.253	0.130	-0.131	0.378
FFA	-0.011	0.924	-0.058	0.732	-0.009	0.952
Cholesterol	-0.083	0.454	-0.298	0.073	-0.102	0.497
HDL-C	-0.078	0.480	-0.058	0.732	-0.078	0.601
LDL-C	-0.093	0.398	-0.341	**0.039**	-0.034	0.823

BMI: Body mass index; HOMA: homeostasis model assessment; TAG: triacylglycerol; FFA: free fatty acids; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol. **All:** glucose (n = 82) insulin (n = 55) HOMA (n = 54); **Normal weight:** glucose (n = 36) insulin (n = 24) HOMA (n = 23); **Overweight/Obese:** glucose (n = 46) insulin (n = 31) HOMA (n = 31).

We next focused on analyzing the possible circadian pattern of CT-1 secretion in plasma samples from human subjects. For that purpose, 24-h profile CT-1 plasma levels were determined in 11 selected subjects (6 normal weight and 5 overweight/obese) matched for similar CT-1 plasma levels at baseline (characteristics of these 11 subjects are summarized in Supplementary Table III).

Supplementary Table III. Baseline anthropometric and biochemical variables of subjects selected for the circadian rhythm analysis.

Variables	All subjects (n=11)	Normal weight (n=6)	Overweight/Obese (n=5)	*P* value
Anthropometry				
Age	23.73± 1.20	23.33 ± 1.93	24.20 ± 1.50	0.740
Weight (Kg)	74.22 ± 4.08	63.92 ± 2.23	86.58 ± 3.77	**0.0004**
BMI (Kg/m^2)	25.42 ± 1.10	22.47 ± 0.55	28.96 ± 0.66	**<0.0001**
Waist (cm)	78.06 ± 2.77	70.98 ± 1.62	86.57± 2.25	**0.0003**
Waist/Hip ratio	0.79 ± 0.01	0.77 ± 0.01	0.81 ± 0.02	0.109
Blood routine variables				
Glucose (mg/dL)	87.37 ± 2.43	86.07 ± 3.05	88.94 ± 2.84	0.588
Insulin (mU/L)	18.47 ± 4.64	11.09 ± 1.59	25.84 ± 8.20	0.119
HOMA	3.61 ± 0.99	1.90 ± 0.46	5.67 ± 1.77	0.097
TAG (mg/dL)	92.42 ± 6.86	85.56 ± 6.32	100.63 ± 12.96	0.299
FFA (mmol/L)	0.40 ± 0.05	0.48 ± 0.08	0.30 ± 0.04	0.080
Cholesterol (mg/dL)	151.66 ± 10.14	141.21 ± 15.73	164.20 ± 11.07	0.281
HDL-C (mg/dL)	47.36 ± 4.51	51.33 ± 7.34	42.60 ± 4.54	0.362
LDL-C (mg/dL)	85.78 ± 8.21	72.94 ± 11.21	101.18 ± 8.58	0.085
CT-1 baseline (ng/mL)	14.68 ± 1.29	16.10 ± 2.07	12.99 ± 1.21	0.251

Data are mean ± SE. BMI: body mass index; HOMA: homeostasis model assessment; TAG: tricylglycerol; FFA: free fatty acids; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol.

Fig. 4A shows the 24-h plasma CT-1 profile expressed as percentage relative to 24 h mean. Parameters obtained by the cosinor analysis which define the circadian rhythms characteristics as mesor, amplitude, relative amplitude, acrophase, and percent rhythm are shown in Fig. 4 and Supplementary Table IV. According to these results, a significant circadian secretion pattern was found for CT-1 plasma levels when considering the 11 selected subjects (including normal weight and overweight/obese) studied (Fig. 4B) (P = 0.0081), the achrophase was at 8:00 am. Data were confirmed with further repeated-measures analyses (ANOVA) which showed significant time-dependent changes in CT-1 plasma levels during both the light-period (from 8:00 to 20:00 h, P = 0.0005), and the dark period (from 20:00 to 8:00 h, P = 0.0001) when considering all subjects (normal weight and overweight/obese together) (Fig. 4A). Interestingly, the analyses of the CT-1 plasma pattern during the dark period revealed a minimum mean value of CT-1 around 2:00 am, followed by a pronounced increase in

CT-1 plasma levels (from 2:00 to 8:00 am), reaching maximal values at 8:00 am. Interestingly, a significant drop (P = 0.0046) in CT-1 occurred between 8:00 am to 9:00 am (Fig. 4A, B).

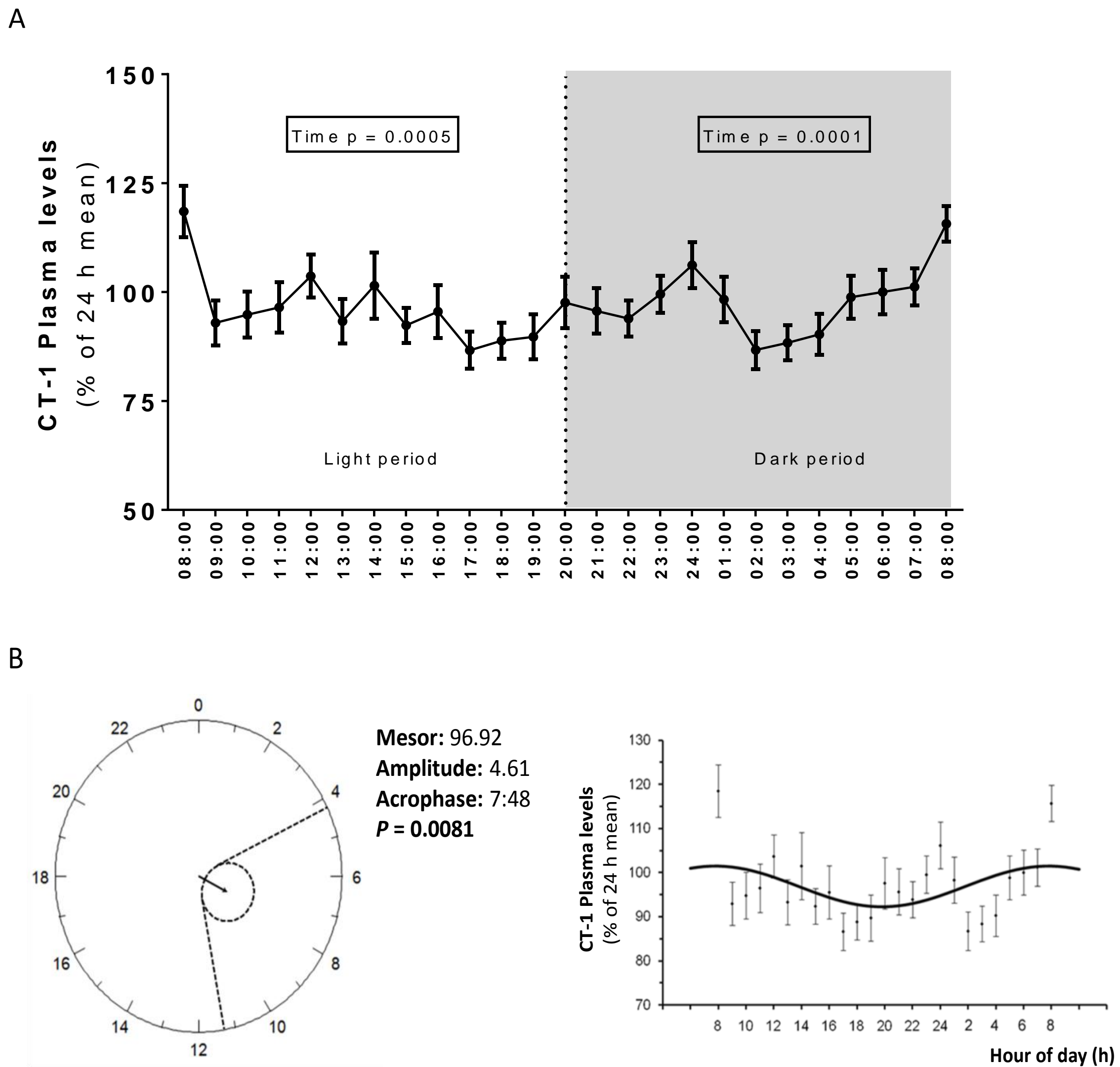


Fig. 4. Circadian CT-1 plasma profile in humans. (*A*) Twenty-four hours plasma CT-1 profile in a selected group of 11 subjects. Plasma CT-1 levels are expressed as percentage relative to 24 h mean. *B*) Polar (clock-like) representation (left panel) and the best fitting waveform (right panel) of the rhythm for CT-1 in human plasma samples.

We next aimed to identify potential differences in CT-1 profile excursions between normal weight and overweight/obese subjects. Importantly, our data revealed that the circadian rhythmicity of CT-1 was only observed in normal weight but not in overweight/obese subjects (Fig. 5 and Supplementary Table IV).

Supplementary Table IV. Estimates of the parameters that characterize the rhythm (mesor, amplitude, and acrophase) calculated for population, including the significance level and the percentage of variance explained by the rhythm of plasma CT-1. Upper and lower limits (95% of confidence) are also shown. Mesor population estimates were obtained directly from the individual mesor. Acrophase data are referred to hour of the day.

Plasma CT-1					
All subjects (n = 11)	**Mesor (AU)**	**Amplitude (AU)**	**Acrophase (hh:mm)**	**PR (%)**	***P***
Estimated	96.92	4.61	7:48	3.5	**0.0081**
Lower limit	94.83	0.99	4:16		
Upper limit	99.01	8.24	11:18		
Normal weight (n = 6)	**Mesor (AU)**	**Amplitude (AU)**	**Acrophase (hh:mm)**	**PR (%)**	***P***
Estimated	98.65	6.73	5:39	9.67	**0.0006**
Lower limit	96.25	2.52	3:04		
Upper limit	101.04	10.94	8:18		
Overweight/Obese (n = 5)	**Mesor (AU)**	**Amplitude (AU)**	**Acrophase (hh:mm)**	**PR (%)**	***P***
Estimated	94.86	5.46	11:16	3.69	0.103
Lower limit	91.33	0.83			
Upper limit	98.40	11.76			

AU: Arbitrary units, hh: hours, mm: minutes.

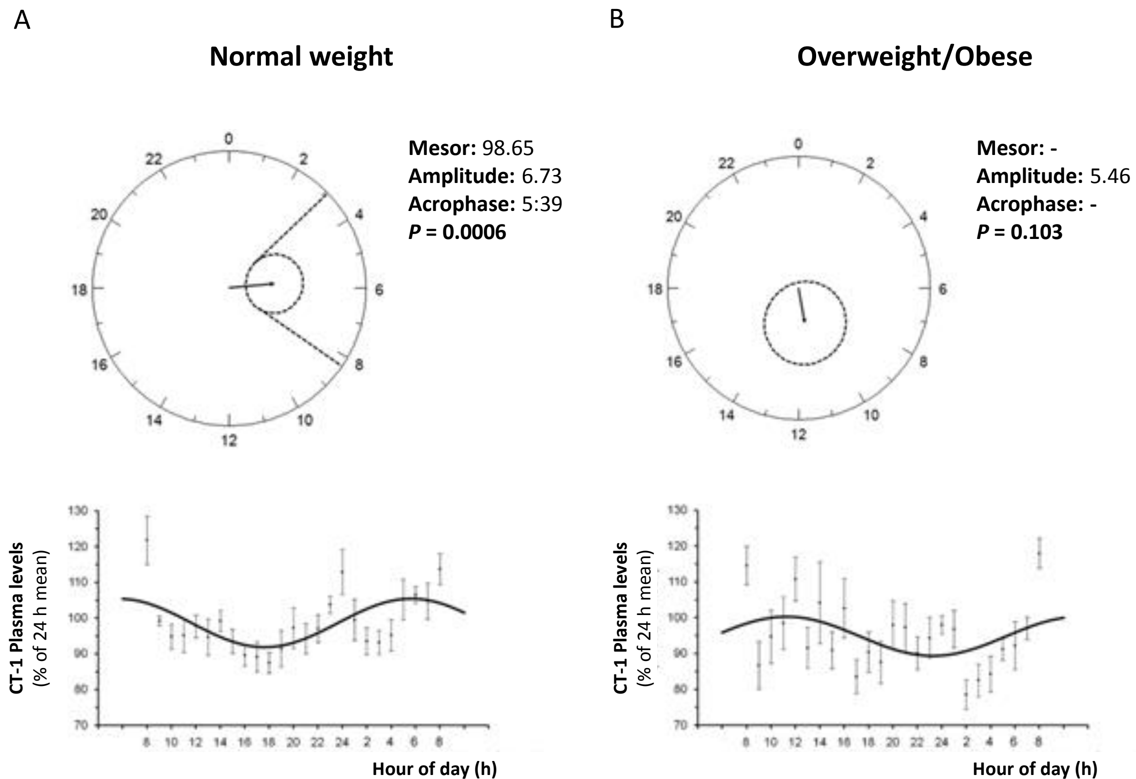


Fig. 5. Cosinor analysis of the circadian rhythm for plasma CT-1 in normal weight and overweight/obese subjects. Top: polar (clock-like) representation of the estimates of the parameters of the rhythm for plasma CT-1 in the form of a 24-h clock in (*A*) normal weight and (*B*) overweight/obese subjects. Bottom: x-y plots showing the best fitting waveform profile of the rhythm in (*A*) normal weight (n = 6) and (*B*) overweight/obese subjects (n = 5).

DISCUSSION

The current study strongly suggests that CT-1 could be a peripheral regulator of circadian rhythms and core clock genes in adipose tissue. Thus, a novel observation from our study is that circadian rhythmicity was altered in CT-1$^{-/-}$ mice compared to the wild type mice at 12 months of age, which could contribute to the mature onset obesity and metabolic disturbances observed in these animals (Moreno-Aliaga *et al.* 2011). Although it cannot be concluded whether the chronodisruption observed in old CT-1 deficient mice is a cause or a consequence of obesity, the fact that young-lean CT-1$^{-/-}$ mice (2 months) already exhibit a phase shift of acrophase suggests that chronodisruption could precede and accompany the development of obesity in the adult state in these animals.

Moreover, the lack of CT-1 dramatically alters the expression pattern of core clock genes in adipose tissue. Interestingly, several observations suggest that disruption of adipose tissue clock genes could precede the development of obesity in CT-1 deficient mice. Gene expression of the positive element *Bmal* and the negative element *Cry1* were already significantly downregulated at 2 months of age when WT and CT-1$^{-/-}$ mice exhibited similar body weight and percentage of fat mass, suggesting that the alterations of these clock genes are specific of CT-1 deficiency and not secondary to the changes in adiposity observed in adult mice. It is important to note that CT-1 deficient mice exhibit decreased energy expenditure and oxygen consumption at 2 months, which precedes and accompanies the development of obesity (Moreno-Aliaga *et al.* 2011). Hence, it can be proposed that alterations in energy expenditure and oxygen consumption observed in young CT-1$^{-/-}$ mice (Moreno-Aliaga *et al.* 2011) could be, at least in part, secondary to the disruption of clock machinery characterized by the significant decrease of *Bmal* and *Cry1* mRNA observed in the adipose tissue of these animals. In this context, it has also been described that impairment of peripheral circadian clocks in adipose tissue precedes metabolic abnormalities in *ob/ob* mice (Ando *et al.* 2011). Interestingly, it has been reported that *Cry1/2* deficiency results in an increased vulnerability to HFD-induced obesity, despite reduced food intake, that might be related to more efficient lipid storage in adipose tissues (Barclay *et al.* 2013). Importantly, CT-1 null mice are also a model of hypophagic obesity that exhibit increased expression of lipogenic genes (Moreno-Aliaga *et al.* 2011). It has been also reported that *Bmal1* null male and female mice also displayed increased adiposity on the normal diet, but the high fat diet did not exaggerate these differences (Kennaway *et al.* 2013). However, other studies have reported conflicting outcomes regarding the role of Bmal1 and Cry in obesity susceptibility (Hemmeryckx *et al.* 2011, Griebel *et al.* 2014).

The alterations in core clock genes observed in young CT-1 null mice become more evident in the 12-month-old obese CT-1$^{-/-}$ mice, which also exhibited a marked reduction in the expression of *Clock* and *Per2 mRNA* levels that was not observed in young CT-1 deficient mice, suggesting that the increased adiposity of these animals could aggravate the disruption of adipose tissue clock genes induced by CT-1 deficiency. Although from the current data we cannot deduce if the reduced levels of

clock genes mRNA are due to a reduced amplitude of the rhythm or to a phase shift, our data suggest that CT-1 deficiency disrupted the expression of core clock genes in adipose tissue, which preceded and accompanied the development of mature obesity in these animals. Further studies should analyze the daily rhythms of these clock genes in obese CT-1$^{-/-}$ mice.

Interestingly, other important outcome of the present study is that the significant disruption of *Clock* gene expression in adipose tissue of *ob/ob* mice was not observed in mice treated with CT-1. It is important to highlight that this effect is not secondary to the anorexic and body weight lowering properties of CT-1, as the PF-group exhibited *Clock* mRNA levels similar to *ob/ob* mice. CT-1-treated mice showed a reduction of *Per2* gene expression compared with the *ob/ob* untreated group and PF mice, suggesting a direct effect of this cytokine on the regulation of core clock genes in adipose tissue. This result reinforces that CT-1 is a cytokine capable of affecting the clock machinery. Similarly, treatment with other cytokines has also exhibited potent effects on the regulation of clock genes. TNF-α suppressed the expression of the period genes *Per1*, *Per2*, and *Per3* in fibroblasts *in vitro* and *in vivo* in the liver of mice infused with the cytokine (Cavadini *et al.* 2007). In contrast, *Per1* gene activity was stimulated by IL-6 in human hepatoma (HuH-7) cells (Motzkus *et al.* 2002). Furthermore, *in vivo* and *in vitro* leptin treatments have been shown to increase the expression of the clock controlled gene erythroblastosis virus α (Rev-Erbα) in pancreatic isolated islets through a MAPK pathway (Vieira *et al.* 2012).

In line with the potential role of CT-1 as a regulator of adipose tissue clock gene machinery, a previous study have suggested that cardiotrophin-like cytokine is probably a SCN output signal important for shaping daily rhythms of behavior, suggesting an unexpected role for the cytokine in adult brain function (Kraves *et al.* 2006).

Previous studies have suggested that circulating IL-6 concentrations present circadian and even ultradian variations, with generally lower levels during daytime and higher during the night (Bauer *et al.* 1994, Vgontzas *et al.* 2005, Agorastos *et al.* 2014). The present study revealed that CT-1 plasma levels also exhibit diurnal variations and a 24-h circadian rhythm. The most remarkable changes in CT-1 along the day includes

the rise of its plasma levels during the night reaching maximum peak at 8:00 am, followed by a marked fall that occurs between 8:00 and 9:00 am.

It has been proposed the role of circadian system in the regulation of appetite during sleep/wake cycles, as a mechanism that may facilitate the typical overnight fast (Scheer *et al.* 2013). Indeed, it has been described a sleep-induced release of leptin, an anorexigenic signal (Simon *et al.* 1998). This property of the circadian system may help explain the relatively reduced hunger in the morning despite a prolonged overnight fast. Taking into account the previously mentioned anorexigenic properties of CT-1, it can be hypothesized that the nocturnal rise of this cytokine during the late night hours might be also contributing to control hunger and appetite rhythms in humans. The fact that CT-1 starts to increase some hours after the sleep period and fall after the awake could also suggest a potential relation with the sleep-wake cycles.

Interestingly, the nocturnal rise of CT-1 also resembles the increase in FFA during the night (Saloranta *et al.* 1993). This could suggest that the changes of CT-1 plasma levels might be also related to metabolic situations, such as prolonged fasting that occurs along the late hours of night and the early morning. In this regard, a previous study of our group revealed that CT-1 is a nutritionally regulated gene that increased markedly when mice were subjected to 48 h fasting and decreased promptly upon refeeding in several tissues (Moreno-Aliaga *et al.* 2011). However, it is important to mention that the current data suggest that plasma CT-1 levels do not seem to be acutely regulated by meals since levels of this cytokine remain without remarkable changes after breakfast, lunch or dinner.

An interesting observation is that the circadian rhythmicity of CT-1 observed in normal weight subjects was lost in overweight/obese individuals. Indeed, obesity has been explained as a fault in the circadian system, as pathology associated with "chronodisruption" (Garaulet *et al.* 2010a). Chronodisruption (CD) is a term that suggests that rhythms can become desynchronized and that this may have adverse effects on health (Colas *et al.* 2004, Stewart *et al.* 2007, Erren *et al.* 2009). CD can be assessed in physiological studies as a reduction in rhythm amplitude, sometimes as a total loss of the rhythmicity, while other times can be characterized by a delayed or advanced phase between different peripheral clocks and the SCN and also as phase inversion of circadian rhythms, as seen in night workers (Erren *et al.* 2009). The current

results in plasma CT-1, that show a loss of rhythmicity in overweight/obese subjects, parallel previous results obtained with adipocytokines produced by adipose tissue, such as leptin, adiponectin and resistin, that not only show clear circadian rhythms in their plasma concentration but their rhythmicity is strongly attenuated or even absent in obese individuals (Mingrone *et al.* 2005, Yildiz *et al.* 2004). Several factors could be contributing to this, including a differential metabolic response to overnight fast between normal weight and obese (Yu *et al.* 2011).

Altogether, data of the present study suggest a potential role of CT-1 as a peripheral regulator of circadian metabolic rhythms and core clock genes in adipose tissue, being able to attenuate the disruption of some core clock genes observed in obesity. Moreover, our current results also suggest that CT-1 plasma levels exhibit 24-h rhythmicity in normal weight subjects, and that these rhythms are lost with obesity. Further research is needed to better define the factors (physiologic and pathophysiologic) that could be influencing CT-1 secretory rhythms.

ACKNOWLEDGEMENTS

This study was supported by grants from the Government of Navarra (Department of Health); Mutua Madrileña Foundation; FIS [PI10/01516 and PI13/01851]; CIBERobn; Línea Especial ‘‘Nutrición, Obesidad y Salud” and Centre for Nutrition Research, Universidad de Navarra; and by the agreement between FIMA and the ‘‘UTE project CIMA’’ and for the Spanish Government of Science and Innovation (SAF2014-52480-R) and European Regional Development Fund (ERDF) to Marta Garaulet. Miguel López-Yoldi was supported by a fellowship from Asociación de Amigos University of Navarra.

The human study was supported by National Institutes of Health (NIH)/National Heart, Lung and Blood Institute 1R01 HL09133 (PI: Havel) and 1R01 HL107256 (PI: Havel); National Center for Research Resources, a component of the NIH, and NIH Roadmap for Medical Research UL1 RR024146 (PI: Berglund). Kimber Stanhope is supported by a Building Interdisciplinary Research Careers in Women’s Health award (K12 HD051958; PI: Gold) funded by the National Institute of Child Health and Human Development, Office of Research on Women's Health, Office of Dietary Supplements, and the National Institute of Aging.

REFERENCES

-A-

Agorastos, A., Hauger, R. L., Barkauskas, D. A., Moeller-Bertram, T., Clopton, P. L., Haji, U., Lohr, J. B., Geracioti, T. D., Jr., Patel, P. M., Chrousos, G. P. & Baker, D. G. 2014. Circadian rhythmicity, variability and correlation of interleukin-6 levels in plasma and cerebrospinal fluid of healthy men. *Psychoneuroendocrinology,* **44,** 71-82.

Ando, H., Kumazaki, M., Motosugi, Y., Ushijima, K., Maekawa, T., Ishikawa, E. & Fujimura, A. 2011. Impairment of peripheral circadian clocks precedes metabolic abnormalities in ob/ob mice. *Endocrinology,* **152,** 1347-54.

Ando, H., Yanagihara, H., Hayashi, Y., Obi, Y., Tsuruoka, S., Takamura, T., Kaneko, S. & Fujimura, A. 2005. Rhythmic messenger ribonucleic acid expression of clock genes and adipocytokines in mouse visceral adipose tissue. *Endocrinology,* **146,** 5631-6.

Antunes, L. C., Levandovski, R., Dantas, G., Caumo, W. & Hidalgo, M. P. 2010. Obesity and shift work: chronobiological aspects. *Nutr Res Rev,* **23,** 155-68.

-B-

Barclay, J. L., Shostak, A., Leliavski, A., Tsang, A. H., Johren, O., Muller-Fielitz, H., Landgraf, D., Naujokat, N., van der Horst, G. T. & Oster, H. 2013. High-fat diet-induced hyperinsulinemia and tissue-specific insulin resistance in Cry-deficient mice. *Am J Physiol Endocrinol Metab,* **304,** E1053-63.

Bass, J. & Takahashi, J. S. 2010. Circadian integration of metabolism and energetics. *Science,* **330,** 1349-54.

Batschelet, E. 1981. *Circular Statistics in Biology,* London, Academic Press.

Bauer, J., Hohagen, F., Ebert, T., Timmer, J., Ganter, U., Krieger, S., Lis, S., Postler, E., Voderholzer, U. & Berger, M. 1994. Interleukin-6 serum levels in healthy persons correspond to the sleep-wake cycle. *Clin Investig,* **72,** 315.

Beraza, N., Marques, J. M., Martinez-Anso, E., Iniguez, M., Prieto, J. & Bustos, M. 2005. Interplay among cardiotrophin-1, prostaglandins, and vascular endothelial growth factor in rat liver regeneration. *Hepatology,* **41,** 460-9.

Bodosi, B., Gardi, J., Hajdu, I., Szentirmai, E., Obal, F., Jr. & Krueger, J. M. 2004. Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. *Am J Physiol Regul Integr Comp Physiol,* **287,** R1071-9.

Bray, M. S. & Young, M. E. 2007. Circadian rhythms in the development of obesity: potential role for the circadian clock within the adipocyte. *Obes Rev,* **8,** 169-81.

Buxton, O. M., Cain, S. W., O'Connor, S. P., Porter, J. H., Duffy, J. F., Wang, W., Czeisler, C. A. & Shea, S. A. 2012. Adverse metabolic consequences in humans of prolonged sleep restriction combined with circadian disruption. *Sci Transl Med,* **4,** 129ra43.

-C-

Cavadini, G., Petrzilka, S., Kohler, P., Jud, C., Tobler, I., Birchler, T. & Fontana, A. 2007. TNF-alpha suppresses the expression of clock genes by interfering with E-box-mediated transcription. *Proc Natl Acad Sci U S A,* **104,** 12843-8.

Colas, D., London, J., Gharib, A., Cespuglio, R. & Sarda, N. 2004. Sleep-wake architecture in mouse models for Down syndrome. *Neurobiol Dis,* **16,** 291-9.

-E-

Eckel-Mahan, K. & Sassone-Corsi, P. 2013. Metabolism and the circadian clock converge. *Physiol Rev,* **93,** 107-35.

Erren, T. C. & Reiter, R. J. 2009. Defining chronodisruption. *J Pineal Res,* **46,** 245-7.

-F-

Froy, O. 2012. Circadian rhythms and obesity in mammals. *ISRN Obes,* **2012,** 437198.

-G-

Garaulet, M. & Madrid, J. A. 2009. Chronobiology, genetics and metabolic syndrome. *Curr Opin Lipidol,* **20,** 127-34.

Garaulet, M. & Madrid, J. A. 2010a. Chronobiological aspects of nutrition, metabolic syndrome and obesity. *Adv Drug Deliv Rev,* **62,** 967-78.

Garaulet, M., Ordovas, J. M., Gomez-Abellan, P., Martinez, J. A. & Madrid, J. A. 2011. An approximation to the temporal order in endogenous circadian rhythms of genes implicated in human adipose tissue metabolism. *J Cell Physiol,* **226,** 2075-80.

Garaulet, M., Ordovas, J. M. & Madrid, J. A. 2010. The chronobiology, etiology and pathophysiology of obesity. *Int J Obes (Lond),* **34,** 1667-83.

Gimble, J. M., Sutton, G. M., Bunnell, B. A., Ptitsyn, A. A. & Floyd, Z. E. 2011. Prospective influences of circadian clocks in adipose tissue and metabolism. *Nat Rev Endocrinol,* **7,** 98-107.

Griebel, G., Ravinet-Trillou, C., Beeske, S., Avenet, P. & Pichat, P. 2014. Mice deficient in cryptochrome 1 (cry1 (-/-)) exhibit resistance to obesity induced by a high-fat diet. *Front Endocrinol (Lausanne),* **5,** 49.

-H-

Hashinaga, T., Wada, N., Otabe, S., Yuan, X., Kurita, Y., Kakino, S., Tanaka, K., Sato, T., Kojima, M., Ohki, T., Nakayama, H., Egashira, T., Tajiri, Y. & Yamada, K. 2013. Modulation by adiponectin of circadian clock rhythmicity in model mice for metabolic syndrome. *Endocr J,* **60,** 483-92.

Hemmeryckx, B., Himmelreich, U., Hoylaerts, M. F. & Lijnen, H. R. 2011. Impact of clock gene Bmal1 deficiency on nutritionally induced obesity in mice. *Obesity (Silver Spring),* **19,** 659-61.

-J-

Johnston, J. D. 2012. Adipose circadian rhythms: translating cellular and animal studies to human physiology. *Mol Cell Endocrinol,* **349,** 45-50.

-K-

Kennaway, D. J., Varcoe, T. J., Voultsios, A. & Boden, M. J. 2013. Global loss of bmal1 expression alters adipose tissue hormones, gene expression and glucose metabolism. *PLoS One,* **8,** e65255.

Kraves, S. & Weitz, C. J. 2006. A role for cardiotrophin-like cytokine in the circadian control of mammalian locomotor activity. *Nat Neurosci,* **9,** 212-9.

Kumar Jha, P., Challet, E. & Kalsbeek, A. 2015. Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals. *Mol Cell Endocrinol*.

-M-

Mifflin, M. D., St Jeor, S. T., Hill, L. A., Scott, B. J., Daugherty, S. A. & Koh, Y. O. 1990. A new predictive equation for resting energy expenditure in healthy individuals. *Am J Clin Nutr,* **51,** 241-7.

Mingrone, G., Manco, M., Granato, L., Calvani, M., Scarfone, A., Mora, E. V., Greco, A. V., Vidal, H., Castagneto, M. & Ferrannini, E. 2005. Leptin pulsatility in formerly obese women. *FASEB J,* **19,** 1380-2.

Mohawk, J. A., Green, C. B. & Takahashi, J. S. 2012. Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci,* **35,** 445-62.

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* **14,** 242-53.

Morris, C. J., Yang, J. N. & Scheer, F. A. 2012. The impact of the circadian timing system on cardiovascular and metabolic function. *Prog Brain Res,* **199,** 337-58.

Motzkus, D., Albrecht, U. & Maronde, E. 2002. The human PER1 gene is inducible by interleukin-6. *J Mol Neurosci,* **18,** 105-9.

-N-

Natal, C., Fortuno, M. A., Restituto, P., Bazan, A., Colina, I., Diez, J. & Varo, N. 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. *Am J Physiol Endocrinol Metab,* **294,** E52-60.

-O-

Oppenheim, R. W., Wiese, S., Prevette, D., Armanini, M., Wang, S., Houenou, L. J., Holtmann, B., Gotz, R., Pennica, D. & Sendtner, M. 2001. Cardiotrophin-1, a muscle-derived cytokine, is required for the survival of subpopulations of developing motoneurons. *J Neurosci,* **21,** 1283-91.

-P-

Peplonska, B., Bukowska, A. & Sobala, W. 2015. Association of Rotating Night Shift Work with BMI and Abdominal Obesity among Nurses and Midwives. *PLoS One,* **10,** e0133761.

-R-

Reichman, T. W., Therapondos, G., Serrano, M. S., Seal, J., Evers-Meltzer, R., Bohorquez, H., Cohen, A., Carmody, I., Ahmed, E., Bruce, D. & Loss, G. E. 2015. "Weighing the risk": Obesity and outcomes following liver transplantation. *World J Hepatol,* **7,** 1484-93.

Rendo-Urteaga, T., Garcia-Calzon, S., Martinez-Anso, E., Chueca, M., Oyarzabal, M., Azcona-Sanjulian, M. C., Bustos, M., Moreno-Aliaga, M. J., Martinez, J. A. & Marti, A. 2013. Decreased cardiotrophin-1 levels are associated with a lower risk of developing the metabolic syndrome in overweight/obese children after a weight loss program. *Metabolism,* **62,** 1429-36.

Reppert, S. M. & Weaver, D. R. 2002. Coordination of circadian timing in mammals. *Nature,* **418,** 935-41.

Rubal, A., Choshniak, I. & Haim, A. 1992. Daily rhythms of metabolic rate and body temperature of two murids from extremely different habitats. *Chronobiol Int,* **9,** 341-9.

-S-

Sahar, S. & Sassone-Corsi, P. 2012. Regulation of metabolism: the circadian clock dictates the time. *Trends Endocrinol Metab,* **23,** 1-8.

Saloranta, C., Taskinen, M. R., Widen, E., Harkonen, M., Melander, A. & Groop, L. 1993. Metabolic consequences of sustained suppression of free fatty acids by acipimox in patients with NIDDM. *Diabetes,* **42,** 1559-66.

Scheer, F. A., Morris, C. J. & Shea, S. A. 2013. The internal circadian clock increases hunger and appetite in the evening independent of food intake and other behaviors. *Obesity (Silver Spring),* **21,** 421-3.

Simon, C., Gronfier, C., Schlienger, J. L. & Brandenberger, G. 1998. Circadian and ultradian variations of leptin in normal man under continuous enteral nutrition: relationship to sleep and body temperature. *J Clin Endocrinol Metab,* **83,** 1893-9.

Stanhope, K. L., Bremer, A. A., Medici, V., Nakajima, K., Ito, Y., Nakano, T., Chen, G., Fong, T. H., Lee, V., Menorca, R. I., Keim, N. L. & Havel, P. J. 2011. Consumption of fructose and high fructose corn syrup increase postprandial triglycerides, LDL-cholesterol, and apolipoprotein-B in young men and women. *J Clin Endocrinol Metab,* **96,** E1596-605.

Stewart, L. S., Persinger, M. A., Cortez, M. A. & Snead, O. C., 3rd 2007. Chronobiometry of behavioral activity in the Ts65Dn model of Down syndrome. *Behav Genet,* **37,** 388-98.

Sukumaran, S., Xue, B., Jusko, W. J., Dubois, D. C. & Almon, R. R. 2010. Circadian variations in gene expression in rat abdominal adipose tissue and relationship to physiology. *Physiol Genomics,* **42A,** 141-52.

-V-

Vgontzas, A. N., Bixler, E. O., Lin, H. M., Prolo, P., Trakada, G. & Chrousos, G. P. 2005. IL-6 and its circadian secretion in humans. *Neuroimmunomodulation,* **12,** 131-40.

Vieira, E., Marroqui, L., Batista, T. M., Caballero-Garrido, E., Carneiro, E. M., Boschero, A. C., Nadal, A. & Quesada, I. 2012. The clock gene Rev-erbalpha regulates pancreatic beta-cell function: modulation by leptin and high-fat diet. *Endocrinology,* **153,** 592-601.

-Y-

Yildiz, B. O., Suchard, M. A., Wong, M. L., McCann, S. M. & Licinio, J. 2004. Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. *Proc Natl Acad Sci U S A,* **101,** 10434-9.

Yu, H., Xia, F., Lam, K. S., Wang, Y., Bao, Y., Zhang, J., Gu, Y., Zhou, P., Lu, J., Jia, W. & Xu, A. 2011. Circadian rhythm of circulating fibroblast growth factor 21 is related to diurnal changes in fatty acids in humans. *Clin Chem,* **57,** 691-700.

V. GENERAL DISCUSSION

CT-1 has been proposed as an important regulator of glucose and lipid metabolism with potential applications for the treatment of obesity and insulin resistance (Moreno-Aliaga *et al.* 2011). Indeed, chronic rCT-1 administration reduces body weight, by decreasing food intake and increasing energy expenditure, and corrects insulin resistance and fatty liver in obese mice (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014). Moreover, previous studies have demonstrated that CT-1 is an adipokine (Natal *et al.* 2008) capable of activating major signaling pathways involved in the control of metabolism in adipocytes (Zvonic *et al.* 2004). In this context, our group has demonstrated that chronic administration of CT-1 in mice promotes remodeling of WAT which is characterized by the upregulation of genes involved in the control of lipolysis, fatty acid oxidation, mitochondrial biogenesis and genes that promote the change to brown fat phenotype (Moreno-Aliaga *et al.* 2011).

In the present studies we provide novel information about the ability of CT-1 to regulate different metabolic processes that could also account for its anti-obesity and insulin sensitizing properties. In this context, one of the major outcomes is the observation that CT-1 stimulates lipolysis *in vitro* and *in vivo* through the regulation of the main lipases and lipid droplet proteins involved in the hydrolysis of TAG. Indeed, CT-1 promotes HSL phosphorylation at Ser660 (*in vitro* and *in vivo*) and at Ser563 (*in vivo*), two residues that are involved in the activation of this lipase. Importantly, activation of HSL seems to be a key factor for the lipolytic action of CT-1, since silencing of HSL expression in cultured adipocytes completely abolished CT-1-induced glycerol release, and partly prevent basal lipolysis.

In addition, several findings lead us to the conclusion that CT-1-induced lipolysis is secondary to the activation of cAMP/PKA pathway. Thus, CT-1 increases cAMP intracellular content, promoting the PKA-mediated phosphorylation of perilipin, subsequently enabling the translocation of HSL from the cytoplasm to the lipid droplet surface. Then, CT-1 leads to the activation of HSL by phosphorylation. Importantly, the PKA inhibitor H89 blunts the phosphorylation of the two main PKA-targets, perilipin and HSL (at Ser660), and the subsequent increase in glycerol release promoted by CT-1. Furthermore, silencing of PKA expression in adipocytes almost completely abrogated CT-1-induced glycerol release, highlighting the important role of PKA in mediating the stimulatory effects of CT-1 on lipolysis.

Regarding the potential mechanisms that could be contributing to the increase of cAMP levels in adipocytes treated with CT-1, we demonstrate for the first time that CT-1 increased the levels of G protein α S, Gsα, in cultured adipocytes, which has been reported to rise intracellular cAMP through the activation of adenylyl cyclase (Chaves *et al.* 2011), without affecting Gi, the inhibitory protein of adenylyl cyclase, leading to the subsequent activation of PKA and phosphorylation and translocation of HSL to fat droplets. Our findings also revealed that neither the activation of PDE3B, an enzyme that catalyses the breakdown of cAMP to its inactive form, nor the levels of AdPLA, which inhibits cAMP production by regulating prostaglandin E_2 (PGE_2) abundance (Jaworski *et al.* 2009), were modified by CT-1.

However, our *in vitro* results show that despite the lipolytic effects of CT-1, ATGL is reduced after chronic treatment with the cytokine, together with an increase of the ATGL inhibitory protein G0S2. This apparently surprising effect of CT-1 has also been observed after treatment with other lipolytic molecules such as TNF-α (Kim *et al.* 2006, Lorente-Cebrian *et al.* 2012), suggesting that increased ATGL might be compensated by low expression (Kralisch *et al.* 2005b). In contrast with the effects observed in cultured adipocytes, acute rCT-1 administration to obese mice promotes an increase of ATGL protein expression suggesting that putatively some mechanisms may regulate ATGL *in vivo* that do not exist in the *in vitro* model.

In summary, the ability of CT-1 to regulate the activity of the main lipases underlies the lipolytic action of this cytokine, and may account for the anti-obesity effects of CT-1. It is important to mention that increased lipolysis and FFA release from adipose tissue have been associated with the development of metabolic disturbances in obesity (Ormseth *et al.* 2011). However, several studies have suggested that increasing lipolysis in adipose tissue does not necessarily increase serum FFA levels because increasing lipolysis in adipose tissue causes a shift within adipocytes toward increased FFA utilization and energy expenditure and thus protects against obesity (Ahmadian *et al.* 2009). In this context, our previous data revealed that CT-1 is able to promote FFA oxidation not only in adipose tissue but also in muscle and liver, reducing insulin resistance in obese mice (Moreno-Aliaga *et al.* 2011, Castano *et al.* 2014).

In addition, the current study also suggest that the beneficial effects of CT-1 in obesity and associated co-morbidities, such as insulin resistance and fatty liver disease,

could be secondary to changes on the secretory pattern of adipokines by adipose tissue. In this context, it is important to remind that in the last years, far from being a mere energy storage organ, WAT has emerged as a key endocrine organ that plays a critical role in metabolism through the secretion of hormones and adipokines that participate in the regulation of whole-body energy homeostasis (Fasshauer *et al.* 2015).

In the present study we demonstrate the ability of CT-1 to modulate the pattern of secretion of four adipokines (leptin, resistin, visfatin and apelin) involved in the control of energy metabolism, insulin sensitivity and inflammation. Thus, CT-1 reduces the production of leptin, a hormone that plays a key role in the regulation of food intake and energy homeostasis (Zhang *et al.* 1994); however, the hyperleptinemia observed in a majority of obese people has been related to the development of several obesity-associated disorders such as type 2 diabetes mellitus, hypertension, cardiovascular disease, and cancer (Prieto-Hontoria *et al.* 2011, Sainz *et al.* 2015). Interestingly, our current data suggest a direct action of CT-1 on leptin production machinery in adipocytes, and not only secondary to the reduction of body weight and fat mass, since the inhibitory action of CT-1 on leptin mRNA levels is observed after acute treatment with the cytokine and also in cultured adipocytes. The ability to decrease leptin production does not seem to be a common feature for all the cytokines of the gp130 family. Thus, we did not find significant effects of IL-6 on leptin, while CNTF has been shown to downregulate leptin expression in adipocytes (Ott *et al.* 2004). This inhibitory effect of CNTF on leptin seems to be mediated by the PI3K pathway (Ott *et al.* 2004). In contrast, our data demonstrate that inhibition of PI3K is not able to reverse the inhibitory action of CT-1 on leptin expression, leading us to the hypothesis that gp130 cytokines regulate leptin production in a differential manner.

Previous studies of our group found a reduction of circulating resistin after chronic treatment with CT-1 in *ob/ob* mice, in parallel with the reduction in fat mass (Moreno-Aliaga *et al.* 2011). Our current data suggest that CT-1 could have direct regulatory actions on resistin independently of changes in fat mass, since CT-1 inhibits resistin in cultured adipocytes, similarly to what was described for leptin. Resistin has been reported to aggravate metabolic syndrome through impairment of glucose metabolism

(Ikeda *et al.* 2013); therefore, the beneficial effects of CT-1 on glucose metabolism (Moreno-Aliaga *et al.* 2011) could be in part mediated by its ability to reduce resistin.

The present study also shows that CT-1 causes a reduction of visfatin in cultured adipocytes. Visfatin is an adipokine able to modulate processes involved in the pathogenesis of obesity and related disorders by influencing the oxidative stress response, apoptosis, lipid and glucose metabolism, inflammation and insulin resistance (Garten *et al.* 2015). Several studies have suggested that visfatin has proinflammatory properties and that is elevated in individuals with inflammatory disease (Valentini *et al.* 2009). However, the physiological relevance of visfatin in obesity, insulin sensitivity and inflammation is still under debate (Garten *et al.* 2015). In this context, visfatin-lowering properties have been described for other cardiovascular protective molecules, such as quercetin and resveratrol (Derdemezis *et al.* 2011) and the antioxidant oleanolic acid (Kim *et al.* 2014) in adipocytes. However, other studies have observed that omega-3 fatty acids and some insulin-sensitizing drugs such as rosiglitazone promote visfatin release (Haider *et al.* 2006, Lorente-Cebrian *et al.* 2009). A previous study have described inhibitory effects of IL-6 (at a highest dose that the tested in our current study) on visfatin through the MAPK/ERK pathway (Kralisch *et al.* 2005a). However, our present study suggest that other pathways different from MAPK/ERK are mediating the inhibitory effects of CT-1 on visfatin expression in cultured adipocytes, evidencing that different mechanisms are involved in the actions of both gp130 cytokines on the regulation of this adipokine.

In contrast to the inhibitory effect on the production of leptin, resistin and visfatin, CT-1 promotes a dose-dependent increase on apelin expression and secretion in cultured adipocytes. It has been described that apelin is an adipokine with important anti-obesity and insulin sensitizing properties (Castan-Laurell *et al.* 2011). Indeed, apelin promotes insulin sensitivity and glucose utilization in adipose and muscle tissues (Dray *et al.* 2008, Yue *et al.* 2010, Zhu *et al.* 2011). Moreover, it has been observed that apelin injection decreases WAT mass and serum triglyceride levels in obese mice, whereas apelin knockout mice have augmented body adiposity and serum free-fatty acid levels (Yue *et al.* 2010). Because apelin production in adipocytes and its plasma levels are elevated in obesity associated to hyperinsulinemia (Boucher *et al.* 2005), it has been suggested that the over-production of apelin in obesity could be one of the

last protections before the emergence of the obesity-related disorders such as type 2 diabetes (Perez-Echarri *et al.* 2009, Castan-Laurell *et al.* 2011). In elucidating the signaling cascade responsible for the upregulation and secretion of apelin by CT-1, we found that PI3K and JAK/STAT inhibitors but not MAPK/ERK blockade reversed the stimulatory effects of CT-1 on apelin gene expression. This fact, together with the observation that CT-1 treatment increases both STAT3 and AKT phosphorylation, suggests that these pathways might be mediating the effects of CT-1 on apelin production. Based on our current data in cultured adipocytes, it could be proposed that the beneficial effects of CT-1 on glucose and lipid metabolism could be also related to its ability to upregulate apelin production from adipocytes. However, the acute treatment of HFD-obese mice with rCT-1 was not able to promote upregulation of apelin gene in WAT. Several reasons may account for these differential outcomes between the *in vitro* and *in vivo* effects of CT-1 on apelin production. First, when administered *in vivo* acutely, CT-1 has potent glucose lowering and insulin-sensitizing properties (Moreno-Aliaga *et al.* 2011) which could secondarily affect transcriptional regulation of apelin in WAT, since hyperinsulinemia is a key regulator of apelin production by adipocytes in obesity (Boucher *et al.* 2005). It is well known that apelin production by fat cells is augmented in obesity (Castan-Laurell *et al.* 2005), and that it is strongly inhibited by fasting (Boucher *et al.* 2005) and weight and fat mass loss (Soriguer *et al.* 2009). Based on these observations, it would be expected a downregulation of apelin mRNA levels in WAT of mice chronically treated with CT-1 in parallel with the drop in weight and fat mass. However, apelin mRNA levels in WAT of mice chronically treated with CT-1 (unpublished observations) are similar to those of untreated animals suggesting that CT-1 may have a stimulatory action on the production of this adipokine (as observed *in vitro*), counteracting the decrease in apelin that accompanies adipocyte size reduction and fat mass loss.

Altogether, the present study shows the ability of CT-1 to decrease some pro-inflammatory adipokines such as leptin, resistin and visfatin while increase the production of apelin, suggesting that the beneficial metabolic effects of CT-1 could be also related to its ability to control adipokine secretion and therefore the cross-talk between adipose tissue and other key metabolic organs. Further studies are needed to

better elucidate CT-1 actions on other relevant adipokines and their role in mediating the metabolic effects of CT-1.

Nowadays, the sodium-glucose co-transporters have been proposed as new therapeutic targets to reduce hyperglycemia in diabetes. Agents that inhibit those co-transporters (SGLT inhibitors) increase glucose excretion and help to control hyperglycemia through an insulin independent mechanism, introducing a new concept to the diabetes treatment (Mittermayer *et al.* 2015). In contrast to other anti-diabetic therapies, these agents promote a negative energy balance and weight loss, which is also beneficial for insulin-resistant and type 2 diabetic subjects (Hardman *et al.* 2011). A previous study of our group revealed that the hypoglycemic effects of CT-1 were mediated by insulin-dependent and insulin-independent mechanisms. In fact, the glucose-lowering properties of CT-1 were also observed in mice with streptozotocin-induced insulin deficiency, demonstrating clearly an insulin-independent effect of CT-1 on glucose homeostasis. A main finding of the present study is that *in vivo* administration of CT-1 inhibits intestinal sugar uptake after short and long-term treatments. These observations suggest that the hypoglycemic and anti-obesity properties of CT-1 (Moreno-Aliaga *et al.* 2011) could be in part secondary to its ability to reduce intestinal sugar absorption. Our data also revealed that the inhibitory effect of CT-1 on α-MG uptake observed in human Caco-2 cells was accompanied by a decrease on the abundance of SGLT-1 in the brush border membrane of the enterocytes. Interestingly, the inhibitory action of CT-1 on intestinal glucose transport is not observed for similar doses of CNTF, other cytokine of the same family with anti-obesity and anti-diabetic properties.

The inhibition of CT-1 on SGLT-1 and sugar uptake seems to be mediated by disruption of AMPK. In fact, CT-1 promoted a time-dependent inhibition of AMPK activation in Caco-2 cells, which temporarily coincides with the decrease of SGLT-1 expression and the inhibition on α-MG uptake at 1 h. Moreover, treatment with AICAR, an activator of AMPK, completely abolishes the inhibitory effects of CT-1 on glucose uptake, and reverses the decrease induced by the cytokine on SGLT-1 expression level. Our functional studies also suggest that early activation of STAT3 is required for the inhibitory effects of CT-1 on α-MG uptake, although it is not the main determinant of these actions.

Remarkably, the administration of pharmacological doses of CT-1 clearly modulates intestinal sugar absorption; however, our current data revealed that the lack of CT-1 did not lead to significant changes in intestinal sugar uptake. Therefore, the physiological relevance of this cytokine in the regulation of the gut function needs to be further characterized.

In summary, data of the present investigation strongly suggest that the ability of CT-1 to inhibit intestinal sugar absorption by mechanisms implicating the down-regulation of SGLT-1 at the apical membrane could also contribute to the hypoglycemic and anti-obesity properties of this cytokine.

Recent studies have demonstrated the interaction between circadian clock and energy regulation metabolism. Indeed, disruption of circadian rhythms (chronodisruption) may cause desynchrony, which impairs metabolism homeostasis and contributes to the onset of obesity and metabolic syndrome (Antunes *et al.* 2010, Bass *et al.* 2010). Moreover, adipose tissue has been established as a peripheral oscillator capable of modulating central core clock genes (Mohawk *et al.* 2012). However, the regulation of the peripheral clock and the clock-controlled genes present in the adipose remains unclear (Sukumaran *et al.* 2010). The current study strongly suggests that CT-1 could be a peripheral regulator of circadian rhythms and core clock genes in adipose tissue. Thus, a novel result from our study is the discovery that circadian rhythmicity is altered in CT-1$^{-/-}$ mice as compared to wild type mice at 12 months of age, which could contribute to the mature onset obesity and metabolic disturbances observed in these animals (Moreno-Aliaga *et al.* 2011). Although it is hard to state whether the chronodisruption observed in old CT-1 deficient mice is a cause or a consequence of obesity, the fact that young-lean CT-1$^{-/-}$ mice (2 months) already exhibit a phase shift of acrophase suggests that chronodisruption could precede and accompany the development of obesity in the adult state in these animals.

Moreover, the lack of CT-1 dramatically alters the expression pattern of core clock genes in adipose tissue. Interestingly, several observations suggest that disruption of adipose tissue clock genes could precede the development of obesity in CT-1 deficient mice. Thus, gene expression of the positive element *Bmal* and the negative element *Cry1* was already significantly downregulated at 2 months of age when WT and CT-1$^{-/-}$ mice exhibited similar body weight and percentage of fat mass, evidencing that the

alterations of these clock genes are specific of CT-1 deficiency and not secondary to the changes in adiposity observed in adult mice. Therefore, it can be proposed that the alterations in energy expenditure and oxygen consumption observed in young CT-1$^{-/-}$ mice (Moreno-Aliaga *et al.* 2011) could be, at least in part, related to the disruption of clock machinery in the adipose tissue of these animals.

Interestingly, other important outcome of the present study is the observation that the disruption of *Clock* mRNA expression in adipose tissue of *ob/ob* mice was not observed in mice treated with CT-1. It is important to highlight that this effect seems to be specific for CT-1 and that it is not secondary to the anorexigenic and body weight lowering properties of CT-1, as it is not observed in PF-group which exhibits *Clock* mRNA levels similar to *ob/ob* mice. Altogether, data from our experimental studies suggest that CT-1 might be a peripheral regulator of clock genes in adipose tissue.

The study of the diurnal variations of plasma CT-1 levels revealed that this cytokine shows important daytime variations and a 24-h circadian rhythm. In this regard, the most remarkable changes in CT-1 daytime profile include the rise of CT-1 plasma levels during the night reaching a maximum peak at 8:00 am, followed by a dramatic decrease around 9:00 am. A role of circadian system in the regulation of appetite during sleep/wake cycles, as a mechanism that may facilitate the typical overnight fast has been proposed (Scheer *et al.* 2013). This property of the circadian system may help explain the relatively reduced hunger in the morning despite a prolonged overnight fast. Taking into account the previously mentioned anorexigenic properties of CT-1, it can be hypothesized that the nocturnal rise of this cytokine during the late night hours might be also contributing to control hunger and appetite rhythms in humans.

An interesting finding of the present study is that the circadian rhythmicity observed for CT-1 in normal weight subjects is lost in overweight/obese individuals. Indeed, obesity has been explained as a fault in the circadian system, as pathology associated with "chronodisruption" (Garaulet *et al.* 2010). In this context, disruption of the 24-h profiles and rhythmicity of other adipocytokines such as leptin, adiponectin and resistin has been also described in obese compared to healthy lean subjects (Yildiz *et al.* 2004, Mingrone *et al.* 2005). Several factors could be contributing to this,

including a differential metabolic response to overnight fast between lean and obese individuals (Yu *et al.* 2011).

Altogether, data of the present study suggest a potential role of CT-1 as a peripheral regulator of circadian metabolic rhythms and core clock genes in adipose tissue, being able to attenuate the disruption of some core clock genes observed in obesity. Moreover, our current results also suggest that CT-1 plasma levels exhibit 24-h rhythmicity in normal weight subjects, and that these rhythms are lost with obesity. Further research is needed to better define the mechanisms (physiologic and pathophysiologic) that could be influencing CT-1 secretory rhythms.

In conclusion, the present study provides novel findings regarding the mechanisms underlying the metabolic effects of CT-1 on obesity and related disorders. The most remarkable outcomes included: 1) the ability of CT-1 to stimulate lipolysis by regulating the main lipases and proteins involved in the control of this catabolic pathway, which could also contribute to the anti-obesity properties of this cytokine; 2) the modulation of the production of bioactive adipokines by white adipocytes, which could affect the cross-talk between adipose tissue and other metabolic organs; 3) the capability of CT-1 to inhibit SGLT-1 and intestinal sugar absorption, which could participate in the glucose lowering properties of this cytokine; 4) the finding that CT-1 might be a peripheral regulator of circadian metabolic rhythms and clock gene machinery in adipose tissue, suggesting that CT-1 could help to attenuate the chronodisruption and metabolic disturbances associated to obesity.

REFERENCES

-A-

Ahmadian, M., Duncan, R. E. & Sul, H. S. 2009. The skinny on fat: lipolysis and fatty acid utilization in adipocytes. *Trends Endocrinol Metab,* **20,** 424-8.

Antunes, L. C., Levandovski, R., Dantas, G., Caumo, W. & Hidalgo, M. P. 2010. Obesity and shift work: chronobiological aspects. *Nutr Res Rev,* **23,** 155-68.

-B-

Bass, J. & Takahashi, J. S. 2010. Circadian integration of metabolism and energetics. *Science,* **330,** 1349-54.

Boucher, J., Masri, B., Daviaud, D., Gesta, S., Guigne, C., Mazzucotelli, A., Castan-Laurell, I., Tack, I., Knibiehler, B., Carpene, C., Audigier, Y., Saulnier-Blache, J. S. & Valet, P. 2005. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology,* **146,** 1764-71.

-C-

Castan-Laurell, I., Boucher, J., Dray, C., Daviaud, D., Guigne, C. & Valet, P. 2005. Apelin, a novel adipokine over-produced in obesity: friend or foe? *Mol Cell Endocrinol,* **245,** 7-9.

Castan-Laurell, I., Dray, C., Attane, C., Duparc, T., Knauf, C. & Valet, P. 2011. Apelin, diabetes, and obesity. *Endocrine,* **40,** 1-9.

Castano, D., Larequi, E., Belza, I., Astudillo, A. M., Martinez-Anso, E., Balsinde, J., Argemi, J., Aragon, T., Moreno-Aliaga, M. J., Muntane, J., Prieto, J. & Bustos, M. 2014. Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation. *J Hepatol,* **60,** 1017-25.

Chaves, V. E., Frasson, D. & Kawashita, N. H. 2011. Several agents and pathways regulate lipolysis in adipocytes. *Biochimie,* **93,** 1631-40.

-D-

Derdemezis, C. S., Kiortsis, D. N., Tsimihodimos, V., Petraki, M. P., Vezyraki, P., Elisaf, M. S. & Tselepis, A. D. 2011. Effect of Plant Polyphenols on Adipokine Secretion from Human SGBS Adipocytes. *Biochem Res Int,* **2011,** 285618.

Dray, C., Knauf, C., Daviaud, D., Waget, A., Boucher, J., Buleon, M., Cani, P. D., Attane, C., Guigne, C., Carpene, C., Burcelin, R., Castan-Laurell, I. & Valet, P. 2008. Apelin stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab,* **8,** 437-45.

-F-

Fasshauer, M. & Bluher, M. 2015. Adipokines in health and disease. *Trends Pharmacol Sci,* **36,** 461-70.

-G-

Garaulet, M. & Madrid, J. A. 2010. Chronobiological aspects of nutrition, metabolic syndrome and obesity. *Adv Drug Deliv Rev,* **62,** 967-78.

Garten, A., Schuster, S., Penke, M., Gorski, T., de Giorgis, T. & Kiess, W. 2015. Physiological and pathophysiological roles of NAMPT and NAD metabolism. *Nat Rev Endocrinol,* **11,** 535-46.

-H-

Haider, D. G., Mittermayer, F., Schaller, G., Artwohl, M., Baumgartner-Parzer, S. M., Prager, G., Roden, M. & Wolzt, M. 2006. Free fatty acids normalize a rosiglitazone-induced visfatin release. *Am J Physiol Endocrinol Metab,* **291,** E885-90.

Hardman, T. C. & Dubrey, S. W. 2011. Development and potential role of type-2 sodium-glucose transporter inhibitors for management of type 2 diabetes. *Diabetes Ther,* **2,** 133-45.

-I-

Ikeda, Y., Tsuchiya, H., Hama, S., Kajimoto, K. & Kogure, K. 2013. Resistin affects lipid metabolism during adipocyte maturation of 3T3-L1 cells. *FEBS J,* **280,** 5884-95.

-J-

Jaworski, K., Ahmadian, M., Duncan, R. E., Sarkadi-Nagy, E., Varady, K. A., Hellerstein, M. K., Lee, H. Y., Samuel, V. T., Shulman, G. I., Kim, K. H., de Val, S., Kang, C. & Sul, H. S. 2009. AdPLA ablation increases lipolysis and prevents obesity induced by high-fat feeding or leptin deficiency. *Nat Med,* **15,** 159-68.

-K-

Kim, H. S., Han, S. Y., Sung, H. Y., Park, S. H., Kang, M. K., Han, S. J. & Kang, Y. H. 2014. Blockade of visfatin induction by oleanolic acid via disturbing IL-6-TRAF6-NF-kappaB signaling of adipocytes. *Exp Biol Med (Maywood),* **239,** 284-92.

Kim, J. Y., Tillison, K., Lee, J. H., Rearick, D. A. & Smas, C. M. 2006. The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-alpha in 3T3-L1 adipocytes and is a target for transactivation by PPARgamma. *Am J Physiol Endocrinol Metab,* **291,** E115-27.

Kralisch, S., Klein, J., Lossner, U., Bluher, M., Paschke, R., Stumvoll, M. & Fasshauer, M. 2005a. Interleukin-6 is a negative regulator of visfatin gene expression in 3T3-L1 adipocytes. *Am J Physiol Endocrinol Metab,* **289,** E586-90.

Kralisch, S., Klein, J., Lossner, U., Bluher, M., Paschke, R., Stumvoll, M. & Fasshauer, M. 2005b. Isoproterenol, TNFalpha, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. *Mol Cell Endocrinol,* **240,** 43-9.

-L-

Lorente-Cebrian, S., Bustos, M., Marti, A., Fernandez-Galilea, M., Martinez, J. A. & Moreno-Aliaga, M. J. 2012. Eicosapentaenoic acid inhibits tumour necrosis factor-alpha-induced lipolysis in murine cultured adipocytes. *J Nutr Biochem,* **23,** 218-27.

Lorente-Cebrian, S., Bustos, M., Marti, A., Martinez, J. A. & Moreno-Aliaga, M. J. 2009. Eicosapentaenoic acid stimulates AMP-activated protein kinase and increases visfatin secretion in cultured murine adipocytes. *Clin Sci (Lond),* **117,** 243-9.

-M-

Mingrone, G., Manco, M., Granato, L., Calvani, M., Scarfone, A., Mora, E. V., Greco, A. V., Vidal, H., Castagneto, M. & Ferrannini, E. 2005. Leptin pulsatility in formerly obese women. *FASEB J,* **19,** 1380-2.

Mittermayer, F., Caveney, E., De Oliveira, C., Gourgiotis, L., Puri, M., Tai, L. J. & Turner, J. R. 2015. Addressing unmet medical needs in type 2 diabetes: a narrative review of drugs under development. *Curr Diabetes Rev,* **11,** 17-31.

Mohawk, J. A., Green, C. B. & Takahashi, J. S. 2012. Central and peripheral circadian clocks in mammals. *Annu Rev Neurosci,* **35,** 445-62.

Moreno-Aliaga, M. J., Perez-Echarri, N., Marcos-Gomez, B., Larequi, E., Gil-Bea, F. J., Viollet, B., Gimenez, I., Martinez, J. A., Prieto, J. & Bustos, M. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab,* **14,** 242-53.

-N-

Natal, C., Fortuno, M. A., Restituto, P., Bazan, A., Colina, I., Diez, J. & Varo, N. 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. *Am J Physiol Endocrinol Metab,* **294,** E52-60.

-O-

Ormseth, M. J., Swift, L. L., Fazio, S., Linton, M. F., Chung, C. P., Raggi, P., Rho, Y. H., Solus, J., Oeser, A., Bian, A., Gebretsadik, T., Shintani, A. & Stein, C. M. 2011. Free fatty acids are associated with insulin resistance but not coronary artery atherosclerosis in rheumatoid arthritis. *Atherosclerosis,* **219,** 869-74.

Ott, V., Fasshauer, M., Meier, B., Dalski, A., Kraus, D., Gettys, T. W., Perwitz, N. & Klein, J. 2004. Ciliary neurotrophic factor influences endocrine adipocyte function: inhibition of leptin via PI 3-kinase. *Mol Cell Endocrinol,* **224,** 21-7.

-P-

Perez-Echarri, N., Perez-Matute, P., Marcos-Gomez, B., Martinez, J. A. & Moreno-Aliaga, M. J. 2009. Effects of eicosapentaenoic acid ethyl ester on visfatin and apelin in lean and overweight (cafeteria diet-fed) rats. *Br J Nutr,* **101,** 1059-67.

Prieto-Hontoria, P. L., Perez-Matute, P., Fernandez-Galilea, M., Martinez, J. A. & Moreno-Aliaga, M. J. 2011. Lipoic acid inhibits leptin secretion and Sp1 activity in adipocytes. *Mol Nutr Food Res,* **55,** 1059-69.

-S-

Sainz, N., Barrenetxe, J., Moreno-Aliaga, M. J. & Martinez, J. A. 2015. Leptin resistance and diet-induced obesity: central and peripheral actions of leptin. *Metabolism,* **64,** 35-46.

Scheer, F. A., Morris, C. J. & Shea, S. A. 2013. The internal circadian clock increases hunger and appetite in the evening independent of food intake and other behaviors. *Obesity (Silver Spring),* **21,** 421-3.

Soriguer, F., Garrido-Sanchez, L., Garcia-Serrano, S., Garcia-Almeida, J. M., Garcia-Arnes, J., Tinahones, F. J. & Garcia-Fuentes, E. 2009. Apelin levels are increased in morbidly obese subjects with type 2 diabetes mellitus. *Obes Surg,* **19,** 1574-80.

Sukumaran, S., Xue, B., Jusko, W. J., Dubois, D. C. & Almon, R. R. 2010. Circadian variations in gene expression in rat abdominal adipose tissue and relationship to physiology. *Physiol Genomics,* **42A,** 141-52.

-V-

Valentini, L., Wirth, E. K., Schweizer, U., Hengstermann, S., Schaper, L., Koernicke, T., Dietz, E., Norman, K., Buning, C., Winklhofer-Roob, B. M., Lochs, H. & Ockenga, J. 2009. Circulating adipokines and the protective effects of hyperinsulinemia in inflammatory bowel disease. *Nutrition,* **25,** 172-81.

-Y-

Yildiz, B. O., Suchard, M. A., Wong, M. L., McCann, S. M. & Licinio, J. 2004. Alterations in the dynamics of circulating ghrelin, adiponectin, and leptin in human obesity. *Proc Natl Acad Sci U S A,* **101,** 10434-9.

Yue, P., Jin, H., Aillaud, M., Deng, A. C., Azuma, J., Asagami, T., Kundu, R. K., Reaven, G. M., Quertermous, T. & Tsao, P. S. 2010. Apelin is necessary for the maintenance of insulin sensitivity. *Am J Physiol Endocrinol Metab,* **298,** E59-67.

-Z-

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. & Friedman, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* **372,** 425-32.

Zhu, S., Sun, F., Li, W., Cao, Y., Wang, C., Wang, Y., Liang, D., Zhang, R., Zhang, S., Wang, H. & Cao, F. 2011. Apelin stimulates glucose uptake through the PI3K/Akt pathway and improves insulin resistance in 3T3-L1 adipocytes. *Mol Cell Biochem,* **353,** 305-13.

Zvonic, S., Hogan, J. C., Arbour-Reily, P., Mynatt, R. L. & Stephens, J. M. 2004. Effects of cardiotrophin on adipocytes. *J Biol Chem,* **279,** 47572-9.

VI. CONCLUSIONS

1. CT-1 stimulates lipolysis in a concentration- and time-dependent manner in 3T3-L1 cultured adipocytes, mainly through the activation by phosphorylation of the lipid droplet coat protein perilipin and the hormone sensitive lipase (HSL) at Ser660 without affecting Ser563. Despite its lipolytic effects, CT-1 decreases the protein levels of other key lipase, ATGL, in this cellular model, in parallel with an increase in the expression of its inhibitor G0S2.

2. Furthermore, CT-1 increases the levels of G protein alpha S (Gsα), causing augmented intracellular cAMP levels. Moreover, siRNA knock-down of HSL or PKA, as well as pretreatment with the PKA inhibitor H89 are able to reverse the CT-1-induced lipolysis, suggesting that the lipolytic action of CT-1 in adipocytes is mainly mediated by activation of HSL through the cAMP/PKA pathway.

3. Acute *in vivo* CT-1 administration can also promote adipose tissue lipolysis in mice. This lipolytic effect is mediated by increased phosphorylation of phospho-PKA substrate perilipin as well as of HSL at Ser563 and Ser660. Moreover, the *in vivo* CT-1 treatment induces a marked increase in ATGL protein levels without modifying the expression of its activator, CGI-58, or the inhibitor, G0S2.

4. CT-1 modifies the production of adipocyte-secreted hormones in 3T3-L1 adipocytes, characterized by the inhibition of leptin, resistin and visfatin and the stimulation of apelin. Pretreament with the PI3K inhibitor LY294002 or with the JAK/STAT inhibitor AG490 blunts the CT-1-stimulated apelin expression, suggesting that the activation of these pathways might be involved in mediating the stimulatory effect of CT-1 on apelin production.

5. Acute CT-1 administration to diet-induced obese mice does not alter visfatin or apelin but reduces leptin and resistin expression in WAT. These effects are not secondary to reduced fat mass as the inhibitory actions of CT-1 on leptin and resistin gene expression are observed after short treatment periods (30 min and 3 h) with the cytokine.

6. *In vivo* and *in vitro* treatment with CT-1 inhibits α-methyl-D-Glucoside (α-MG) uptake in intestinal everted rings. Furthermore, mechanistic studies performed in Caco-2 cells demonstrate that the ability of CT-1 to reduce α-MG uptake is accompanied by the downregulation of the expression of the SGLT-1 transporter at the apical membrane of the cells. Several signaling pathways, especially STAT3 and AMPK, might be involved in the inhibitory effects of CT-1 on sugar uptake.

7. CT-1 deficiency in mice leads to the disruption of circadian metabolic rhythmicity and core clock genes in adipose tissue, which could contribute to the mature onset obesity and metabolic disturbances observed in these animals. In addition, the administration of CT-1 is able to restore the disruption of *Clock* gene expression in *ob/ob* mice supporting the idea that CT-1 could be a peripheral regulator of clock genes in adipose tissue.

8. The 24-h profile of CT-1 plasma levels shows daytime variations characterized by a pronounced rise during the night period (from 2:00 to 8:00 am), with the acrophase at 8:00 am. Interestingly, the circadian rhythmicity of CT-1 observed in normal weight subjects was lost in overweight/obese individuals.

GENERAL CONCLUSION

The current data demonstrate the ability of CT-1 to stimulate lipolysis, as well as to modulate the secretion of adipokines in white adipocytes suggesting that these effects could contribute to the anti-obesity and glucose-lowering properties of CT-1. Moreover, the capability of CT-1 to inhibit SGLT-1 and intestinal sugar absorption could contribute to the glucose lowering properties of this cytokine. Importantly, CT-1 might be a potential peripheral regulator of metabolic rhythms and clock gene machinery in adipose tissue, suggesting that CT-1 could help to attenuate the chronodisruption and metabolic disturbances associated to obesity.

1. CT-1 causa una estimulación de la lipólisis dosis y tiempo-dependiente en adipocitos 3T3-L1 en cultivo, principalmente a través de la activación por fosforilación de perilipina (una proteína que recubre la gota lipídica) y de la lipasa sensible a hormonas (HSL) en el residuo Ser660 sin afectar al residuo Ser563. A pesar de sus efectos lipolíticos, CT-1 disminuye los niveles proteicos de la otra lipasa clave, ATGL, en este modelo celular, en paralelo con un aumento en la expresión de su inhibidor G0S2.

2. Además, CT-1 promueve la expresión de la proteína G alfa S (Gsα), provocando un aumento de los niveles de AMPc intracelular. Por otra parte, el silenciamiento de HSL y/o PKA, así como el pretratamiento con el inhibidor de PKA H89 son capaces de revertir la lipólisis inducida por CT-1, sugiriendo que la acción lipolítica de CT-1 en los adipocitos está mediada principalmente por la activación de HSL a través de la vía cAMP/PKA.

3. La administración aguda de CT-1 *in vivo* es capaz de promover la lipólisis en tejido adiposo de ratones. Este efecto lipolítico está mediado por un aumento de la fosforilación de los substratos de PKA, perilipina y HSL (en los residuos Ser563 y Ser660). Por otra parte, el tratamiento con CT-1 *in vivo* induce un aumento en los niveles de la proteína ATGL sin modificar la expresión de su activador, CGI-58, o el inhibidor, G0S2.

4. CT-1 modifica el patrón de secreción hormonal en adipocitos 3T3-L1, inhibiendo la producción de leptina, resistina y visfatina y estimulando la producción de apelina. El pretratamiento con el inhibidor de PI3K LY294002 o con el inhibidor de JAK/STAT AG490 revierte la estimulación de la expresión de apelina inducida por CT-1, lo que sugiere que la activación de estas vías podría estar implicada en el efecto estimulador de la CT-1 sobre la producción de apelina.

5. La administración aguda de CT-1 a ratones con obesidad inducida por la dieta no altera la expresión visfatina o apelina, pero reduce la expresión de leptina y de resistina en tejido adiposo blanco. Estos efectos no son secundarios a la pérdida significativa de masa grasa ya que las acciones inhibidoras de CT-1 sobre la

expresión génica de leptina y resistina se observan después de períodos de tratamiento cortos (30 min y 3 h) con la citoquina.

6. El tratamiento *in vivo* e *in vitro* con CT-1 inhibe la captación de α-metil-D-glucósido (α-MG) en anillos intestinales evertidos. Además, los estudios mecanísticos realizados en células Caco-2 demuestran que la capacidad de CT-1 para reducir la absorción de α-MG está acompañada por una disminución de la expresión del transportador SGLT-1 en la membrana apical de estas células. Varias vías de señalización, especialmente STAT3 y AMPK, podrían estar involucradas en los efectos inhibitorios de CT-1 sobre la absorción intestinal de azúcares.

7. La deficiencia de CT-1 en ratones promueve alteraciones del ritmo circadiano metabólico y de los genes reloj en tejido adiposo, lo que podría contribuir al desarrollo tardío de obesidad y trastornos metabólicos observados en estos animales. Además, la administración de CT-1 es capaz de restaurar la alteración de la expresión génica de *Clock* en ratones *ob/ob* reforzando la idea de que CT-1 podría ser un regulador periférico de los genes del reloj en el tejido adiposo.

8. El perfil de 24 h de los niveles circulantes de CT- 1 muestra variaciones a lo largo del día, que se caracterizan por un aumento pronunciado de los niveles de esta citoquina durante la noche (de las 2:00 am a las 8:00 am), con la acrofase a las 8:00 am. Es destacable que el ritmo circadiano de CT-1 observado en los sujetos normopeso no se observó en individuos con sobrepeso/obesidad.

CONCLUSIÓN GENERAL

Los datos del presente estudio demuestran la capacidad de CT-1 para estimular la lipólisis, así como para modular la secreción de adipoquinas en el tejido adiposo blanco, sugiriendo que estos efectos podrían contribuir a las acciones anti-obesidad e hipoglucemiantes descritas para CT-1. Por otra parte, la capacidad de CT-1 para inhibir SGLT-1 y la absorción intestinal de azúcares podría contribuir a las propiedades reductoras de la glucemia descritas para esta citoquina. Es importante destacar que, CT-1 podría ser un potencial regulador periférico de los ritmos metabólicos y de la maquinaria de genes reloj en el tejido adiposo, lo que sugiere que CT-1 podría ayudar a corregir la cronodisrupción y en consecuencia los desórdenes metabólicos asociados a la obesidad.

VII. THESIS SUMMARY

In the last years, several studies from our group and others have pointed out that CT-1 might play a key role in the regulation of body weight and intermediate metabolism. Indeed, our previous data in mice demonstrated that CT-1 is a master regulator of fat and glucose metabolism with potential applications for treatment of obesity and insulin resistance. Thus, these studies suggest lipolytic properties for CT-1 *in vivo*, but the mechanism underlying the lipolytic action of CT-1 still remains unknown.

Moreover, during the last decade, WAT has also been established as an important endocrine organ with a key relevance in the regulation of food intake, energy expenditure and glucose and lipid homeostasis. Indeed, WAT is responsible for the expression and secretion of an array of molecules known as adipokines that can regulate many physiological processes. However, the potential ability of CT-1 to regulate adipocyte secretory function as well as their potential involvement in the metabolic actions of CT-1 has not been yet analyzed.

The intestine has also emerged as an important metabolic organ with a role in the pathophysiology of various metabolic diseases including obesity, insulin resistance and diabetes. Recently, the sodium-glucose co-transporters that mediate intestinal glucose absorption have been considered to be new therapeutic targets to reduce hyperglycemia in diabetes. However, there is still not information available about the physiological/pharmacological actions of CT-1 on intestinal absorptive function.

Moreover, the circadian system has a great influence on metabolic disturbances and vice versa. Although the exact mechanisms linking metabolic syndrome with chronodisruption remain still unknown, most hypotheses point to an internal desynchronization of circadian rhythms involved in metabolism. Interestingly, several adipokines secreted by adipose exhibit profound day/night circadian rhythms, and accumulating evidence links the disruption of these rhythms to the development of metabolic disturbances. However, the potential involvement of CT-1 on the control of clock genes and circadian rhythm machinery, especially in obesity is still unknown.

Based on the previously mentioned observations, this thesis is focused on the analysis of the role of CT-1 in the regulation of lipolysis and the secretion of adipokines in adipocytes as well as the characterization of its potential implication in intestinal sugar absorption and in the chronobiology of obesity.

Firstly, we demonstrated that CT-1 stimulates lipolysis *in vitro* and *in vivo* through the activation of the main lipases and lipid droplet associated proteins. CT-1 treatment stimulated basal glycerol release in a concentration and time-dependent manner in 3T3-L1 adipocytes. Moreover, CT-1 raised cAMP levels and in parallel increased PKA-mediated phosphorylation of perilipin and HSL at Ser660. In this line, siRNA knock down of HSL or PKA as well as pretreatment with the PKA inhibitor H89 blunted the CT-1-induced lipolysis, suggesting that the lipolytic action of CT-1 in adipocytes is mainly mediated by activation of HSL through PKA pathway. In *ob/ob* mice, acute rCT-1 treatment also promoted PKA-mediated phosphorylation of perilipin and HSL at Ser660 and Ser563, and increased ATGL content in adipose tissue. Our results suggest that the ability of CT-1 to regulate the activity of the main lipases underlies the lipolytic action of this cytokine *in vitro* and *in vivo*, and could contribute to CT-1 antiobesity effects.

In addition we observed that CT-1 inhibits the production of adipocyte-secreted hormones implicated in obesity and insulin resistance with pro-inflammatory properties such as leptin, resistin and visfatin in cultured adipocytes, whereas promotes the gene expression and secretion of apelin. This stimulatory action of CT-1 on apelin production is linked to the activation PI3K and STAT3 signaling pathways, since pre-treatment with the PI3K inhibitor LY294002, and with the JAK/STAT inhibitor AG490, reversed the stimulatory effects of CT-1 on apelin gene expression in cultured adipocytes. Moreover, acute CT-1 administration to obese mice reduced leptin and resistin expression in WAT. Thus, the present study demonstrates the ability of rCT-1 to modulate the production of adipokines in *vitro* and *in vivo*, suggesting that the regulation of the secretory function of adipocytes could be also involved in the metabolic actions of this cytokine.

Furthermore, the present investigation demonstrates the ability of CT-1 to inhibit intestinal sugar absorption *in vitro* and *in vivo.* Moreover, mechanistic studies performed in Caco-2 cells demonstrate that the reduction of α-MG uptake induced by CT-1 is accompanied by the downregulation of the expression of the SGLT-1 co-transporter at the apical membrane of the cells. Several signaling pathways, especially STAT3 and AMPK, might be involved in the effects of CT-1 on sugar uptake, since pre-treatment with the JAK/STAT inhibitor AG490, and with the AMPK activator AICAR,

reversed the inhibitory effects of CT-1 on α-Methyl-D-glucoside uptake in Caco-2 cells. These effects of CT-1 on intestinal sugar absorption could contribute to the hypoglycemic and anti-obesity properties of this cytokine.

Finally, the present study demonstrates the potential of CT-1 as a peripheral regulator of metabolic rhythms and adipose tissue core clock genes in mice. Interestingly, circadian rhythmicity of oxygen consumption rate (VO_2) was totally disrupted in old CT-1 deficient ($CT\text{-}1^{-/-}$) obese mice (12 months). Although young-lean $CT\text{-}1^{-/-}$ mice (2 months) conserved circadian rhythms for VO_2 values, CT-1 deficiency already caused a phase shift of acrophase. Moreover, the lack of CT-1 also induced remarkable alterations in *Bmal1* and *Cry* mRNA levels in young CT-1 null mice, which become also evident for *Clock* and *Per2* in $CT\text{-}1^{-/-}$ 12-month-old mice. Moreover, treatment with CT-1 attenuated the drop in adipose *Clock* mRNA observed in *ob/ob* mice. Furthermore, the 24-h profile of CT-1 plasma levels shows daytime variations characterized by a pronounced rise during the night period (from 2:00 to 8:00 am), with the acrophase at 8:00 am. Interestingly, the circadian rhythmicity of CT-1 observed in normal weight subjects was lost in overweight/obese individuals. All these observations suggest a potential role of CT-1 as a peripheral regulator of metabolic circadian rhythms.

Altogether, data of the current study demonstrate the ability of CT-1 to stimulate lipolysis, as well as to modulate the secretion of adipokines in white adipocytes suggesting that these effects could contribute to the anti-obesity and glucose-lowering properties previously described for CT-1. Moreover, the capability of CT-1 to inhibit SGLT-1 and intestinal sugar absorption could contribute to the glucose lowering properties of this cytokine. Importantly, CT-1 might be a potential peripheral regulator of metabolic rhythms and clock gene machinery in adipose tissue, suggesting that CT-1 could help to restore the chronodisruption associated to obesity.

APPENDICES

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

ASBMB
JOURNAL OF LIPID RESEARCH

Cardiotrophin-1 stimulates lipolysis through the regulation of main adipose tissue lipases

Miguel López-Yoldi,*,† Marta Fernández-Galilea,* Laura M. Laiglesia,*,† Eduardo Larequi,§ Jesús Prieto,§, J. Alfredo Martínez,*,†,†† Matilde Bustos,[1,§] and Maria J. Moreno-Aliaga[1,2,]*,†,††**

Departments of Nutrition, Food Science, and Physiology* and Gene Therapy and Hepatology, CIMA,§ and Centre for Nutrition Research,† University of Navarra, Pamplona, Navarra, Spain; and CIBERehd** and CIBERobn, Physiopathology of Obesity and Nutrition,†† Institute of Health Carlos III, Madrid, Spain

Abstract Cardiotrophin-1 (CT-1) is a cytokine with antiobesity properties and with a role in lipid metabolism regulation and adipose tissue function. The aim of this study was to analyze the molecular mechanisms involved in the lipolytic actions of CT-1 in adipocytes. Recombinant CT-1 (rCT-1) effects on the main proteins and signaling pathways involved in the regulation of lipolysis were evaluated in 3T3-L1 adipocytes and in mice. rCT-1 treatment stimulated basal glycerol release in a concentration- and time-dependent manner in 3T3-L1 adipocytes. rCT-1 (20 ng/ml for 24 h) raised cAMP levels, and in parallel increased protein kinase (PK)A-mediated phosphorylation of perilipin and hormone sensitive lipase (HSL) at Ser660. siRNA knock-down of HSL or PKA, as well as pretreatment with the PKA inhibitor H89, blunted the CT-1-induced lipolysis, suggesting that the lipolytic action of CT-1 in adipocytes is mainly mediated by activation of HSL through the PKA pathway. In *ob/ob* mice, acute rCT-1 treatment also promoted PKA-mediated phosphorylation of perilipin and HSL at Ser660 and Ser563, and increased adipose triglyceride lipase (desnutrin) content in adipose tissue. These results showed that the ability of CT-1 to regulate the activity of the main lipases underlies the lipolytic action of this cytokine in vitro and in vivo, and could contribute to CT-1 antiobesity effects.—López-Yoldi, M., M. Fernández-Galilea, L. M. Laiglesia, E. Larequi, J. Prieto, J. A. Martínez, M. Bustos, and M. J. Moreno-Aliaga. **Cardiotrophin-1 stimulates lipolysis through the regulation of main adipose tissue lipases. *J. Lipid Res.* 2014.** 55: **2634–2643.**

Supplementary key words adipocytes • adipose triglyceride lipase • cell signaling • cytokines • hormone-sensitive lipase • obesity • perilipin • protein kinase A

Cardiotrophin-1 (CT-1) belongs to the interleukin (IL)-6 family of cytokines. These cytokines exert their cellular effects by interacting with the glycoprotein 130 (gp130)/leukemia inhibitory factor receptor heterodimer (1). Adipose tissue has been identified as a source of CT-1 (2), and this cytokine is capable of activating major signaling pathways involved in metabolic control in adipocytes (3). Moreover, it has been reported that CT-1 levels are raised in obesity and metabolic syndrome (2), suggesting that CT-1 could be a new marker for obesity and related diseases. A recent study by our group has revealed that CT-1 is a key regulator of energy homeostasis, as well as of glucose and lipid metabolism (4). Thus, chronic recombinant CT-1 (rCT-1) treatment reduced body weight and corrected insulin resistance in *ob/ob* and high-fat-fed obese mice by reducing food intake and enhancing energy expenditure. Moreover, rCT-1 induced dramatic white adipose tissue remodeling characterized by the upregulation of genes implicated in the control of fatty acid oxidation, mitochondrial biogenesis, and lipolysis. In this context, it has been reported that adipocytes from rCT-1-treated mice exhibited an increased lipolytic response to isoproterenol, while adipocytes from old obese *CT-1*-null mice responded poorly to isoproterenol, suggesting that CT-1 might play a role in the regulation of lipolysis (4). However, the mechanism underlying the lipolytic action of CT-1 still remains unknown.

During lipolysis, intracellular triacylglycerol (TAG) is hydrolyzed through the consecutive action of three major lipases: adipose triglyceride lipase (ATGL/desnutrin),

This study was supported by grants from the Government of Navarra (Department of Health); Mutua Madrileña Foundation; FIS (PI10/01516 and PI13/01851); Línea Especial "Nutrición, Obesidad y Salud Universidad de Navarra"; and by the agreement between FIMA and the "UTE project CIMA". M.L-Y. was supported by a doctoral grant from Asociación de Amigos University of Navarra.

Manuscript received 3 October 2014.

Published, JLR Papers in Press, October 28, 2014
DOI 10.1194/jlr.M055335

Abbreviations: AdPLA, adipocyte phospholipase A2; AICAR, aminoimidazole carboxamide ribonucleotide; ATGL, adipose triglyceride lipase (desnutrin); CGI-58, comparative gene identification-58; cGMP, cyclic guanosine monophosphate; CT-1, cardiotrophin-1; DAG, diacylglycerol; Gi, inhibitory guanine nucleotide binding protein; gp130, glycoprotein 130; Gs, stimulatory guanine nucleotide binding protein; G0S2, G0/G1 switch gene 2; HPTLC, high-performance TLC; HSL, hormone sensitive lipase; IL, interleukin; MAG, monoacylglycerol; PDE3B, phosphodiesterase 3B; PK, protein kinase; rCT-1, recombinant cardiotrophin-1; TAG, triacylglycerol.

[1] M. Bustos and M. J. Moreno-Aliaga contributed equally to the work.
[2] To whom correspondence should be addressed.
e-mail: mjmoreno@unav.es

[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three figures.

Copyright © 2014 by the American Society for Biochemistry and Molecular Biology, Inc.

This article is available online at http://www.jlr.org

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

hormone sensitive lipase (HSL), and monoacylglycerol (MAG) lipase. ATGL exhibits higher substrate specificity for TAG than diacylglycerol (DAG), and selectively assumes the first step in TAG hydrolysis resulting in the formation of DAG and fatty acid (5). ATGL lipolytic activity is coactivated by the protein comparative gene identification-58 (CGI-58), whereas it is inhibited by the protein G0/G1 switch gene 2 (G0S2) (6, 7). Moreover, recent findings describe how ATGL can also be regulated through phosphorylation by AMPK at Ser406, stimulating its lipolytic activity (8).

It is well-known that the activity of HSL is controlled postranscriptionally through reversible phosphorylation. Experiments in murine adipocytes have demonstrated that Ser563, Ser659, and Ser660 are the major protein kinase (PK)A phosphorylation sites, which are essential for the translocation of HSL to the lipid droplet surface and for stimulation of HSL (9). Besides PKA phosphorylation, HSL can also be phosphorylated by other kinases, such as ERK, which phosphorylates HSL at Ser600, increasing lipolysis (10). Another serine residue (Ser565) is a substrate of AMPK, which phosphorylates HSL preventing PKA-mediated activation of HSL by phosphorylation (11).

Perilipin A is a protein associated with the cytoplasm side of the lipid droplets (12). Under basal conditions, perilipin A maintains a low rate of basal lipolysis by sequestering CGI-58 (13) and by restricting the access of cytosolic lipases to the lipid droplet. However, cAMP-mediated activation of PKA induces conformational changes in perilipin A, facilitating the translocation of phosphorylated HSL from the cytoplasm to the lipid droplet surface and enhancing the lipolytic process (14).

Based on these previous findings, we aimed at analyzing whether the lipolytic actions of rCT-1 in adipocytes are mediated by changes in the regulation of the major lipases and lipid droplet proteins involved in the hydrolysis of TAG, and to characterize the major signaling pathways implicated.

MATERIALS AND METHODS

Cell culture and differentiation of 3T3-L1 cells

Mouse embryo fibroblast 3T3-L1 cells (American Type Culture Collection, Rockville, MD) were cultured in DMEM containing 25 mM glucose, 10% (v/v) calf bovine serum (Invitrogen, Carlsbad, CA), and 1% (v/v) penicillin/streptomycin (Invitrogen), and maintained in an incubator set to 37°C and 5% of carbon dioxide. At confluence, preadipocytes were cultured for 48 h in DMEM (Invitrogen) containing 25 mM glucose, 10% FBS (Invitrogen), and antibiotics, and supplemented with dexamethasone (1 mM; Sigma, St. Louis, MO), isobutylmethylxantine (0.5 mM; Sigma), and insulin (10 μg/ml; Sigma). After that, cells were cultured with 10% FBS and insulin for 48 h and then media were replaced with 10% FBS in DMEM and antibiotics, but without insulin, and these media were changed every 2 days until day 8 postconfluence, when cells attained the morphology and typical features of mature adipocytes (15).

rCT-1

rCT-1 was obtained as described elsewhere (16) and contained <0.04 ng LPS per 1 μg of the protein as determined by the Limulus amoebocyte lysate assay (Cambrex, East Rutherford, NJ).

In vitro treatments

The inhibitors H89 (Santa Cruz Biotechnology, Santa Cruz, CA) and KT5823 (Calbiochem, La Jolla, CA) were dissolved in DMSO. The AMPK activator, aminoimidazole carboxamide ribonucleotide (AICAR) (Sigma), was dissolved in ultra-purified water. All compounds were prepared as 1,000× stock solutions and added to the culture medium. Control cells were treated with the same amount of vehicle (DMSO and/or ethanol).

Prior to the addition of the appropriate treatments, cells were serum starved for 4 h using the medium DMEM supplemented with 0.1% FBS and then treated with or without rCT-1 (1–40 ng/ml) during different time intervals (1–24 h). To analyze the signaling pathways involved in the lipolytic actions of rCT-1, adipocytes were preincubated for 1 h in presence or absence of specific inhibitors or activators (1 μM of PKA inhibitor H89, 2 mM of AMPK activator AICAR, and 1 μM of PKG inhibitor KT5823) before the addition of rCT-1 to the treated wells, as described elsewhere (15).

Animal experiments

Eight-week-old male *ob/ob* (C57BL/6J background) mice were supplied by the Janvier Laboratory (Le Genest St. Isle, France). rCT-1 (0.2 mg/kg of body weight) was administered intravenously (retro-orbital injection) and animals were euthanized 30 min after administration. Control mice were injected with vehicle (saline). At the indicated time point, mice were euthanized and epididymal fat was snap-frozen in liquid nitrogen and stored at −80°C for subsequent analysis. All experimental procedures were performed according to the institutional guidelines for the use of laboratory animals and approved by the University of Navarra Ethics Committee.

Determination of lipolysis in 3T3-L1 adipocytes

Lipolysis was evaluated by the biochemical determination of the amount of glycerol and FFAs released into the culture media. Glycerol measurements were performed after 1, 2, 12, 18, and 24 h of rCT-1 treatment using the Pentra C200 autoanalyzer (Roche Diagnostic, Basel, Switzerland), following manufacturer's instructions. FFAs were evaluated after 3 h of rCT-1 treatment by using a lipolysis assay kit for FFAs detection (Zen-Bio Inc., Research Triangle Park, NC) according to the manufacturer's instructions. TAG, DAG, and MAG levels were also determined by TLC. Briefly, cell lysates were mixed with an equal amount of chloroform/methanol (2:1; v/v). After vortexing for 1 min and resting for 10 min, the samples were centrifuged for 10 min. Organic layers were collected and vacuum dried. The pellets resolved in 40 μl chloroform/methanol (1:1, v/v) were applied as 5 mm spots to high-performance TLC (HPTLC)-silica gel with an aluminum backing (Merck, Darmstadt, Germany). The HPTLC plates were developed with a solvent system (hexane:diethyl ether:acetic acid, 70:30:1, v/v/v) at room temperature. The plate was dried and placed in a system with iodine salt vapor (Panreac, Barcelona, Spain) until the lipids were visible. Identities of the stained lipids were determined by referring to standards. Bands corresponding to TAG, DAG, and MAG were scraped from the plate and quantified using the ABX Pentra triglyceride CP (Horiba, Montpellier, France). For the determination of free cholesterol, the pellets were resolved using isopropanol, and free cholesterol was determined by the Wako Free Cholesterol E test (Wako, Neuss, Germany) (17).

ASBMB
JOURNAL OF LIPID RESEARCH
Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

ASBMB
JOURNAL OF LIPID RESEARCH

Ex vivo lipolysis assay in epididymal adipose tissue explants

Epididymal fat pads were surgically removed from overnight-fasted mice treated with either rCT-1 (0.2 mg/kg of body weight) or vehicle for 30 min. Fat pads of approximately 100 mg were minced into small pieces and incubated in 6-well plates with 1 ml of HEPES phosphate buffer (pH 7.4) (containing 5 mM D-glucose, 2% BSA, 135 mM NaCl, 2.2 mM $CaCl_2 \cdot 2H_2O$, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.45 mM KH_2PO_4, 2.17 mM Na_2HPO_4, and 10 mM HEPES) at 37°C. Media samples were collected at 1 h and 2 h of incubation. Glycerol content was quantified as described above and normalized by protein content.

Determination of FFAs in serum samples

Serum FFAs were measured in mice fasted for 16 h, before and after 30 min treatment with vehicle or rCT-1 (0.2 mg/kg of body weight). FFAs were quantified using a Pentra C200 autoanalyzer (Roche Diagnostic, Basel, Switzerland), following manufacturer's instructions.

Western blot analysis

3T3-L1 cell lysates were obtained by the addition of a buffer containing 2 mM Tris HCl (pH 8), 137 mM NaCl, 2 mM EDTA, 1% protease inhibitor cocktail 1 (Sigma), 2 mM orthovanadate, and 1 mM PMSF. In *ob/ob* mice, tissue samples were thawed and homogenized in lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 0.1 M sodium fluoride, 10 mM EDTA, 50 mM sodium chloride, 10 mM orthovanadate, 0.1% SDS, and protease inhibitor cocktail (Roche)]. In both cases, samples were centrifuged and protein concentrations were determined by the BCA method according to the supplier's instructions (Pierce-Thermo Scientific, Rockford, IL). Briefly, equivalent amounts of total protein (25–50 μg) were electrophoretically separated by 12–15% SDS-PAGE in the presence of a reducing agent (2-mercaptoethanol). Proteins were electroblotted from the gel to polyvinylidene difluoride membranes (GE Healthcare Europe GmbH, Barcelona, Spain). Following the transfer of proteins, the membranes were blocked and probed with specific primary antibodies against phospho-HSL (Ser563), phospho-HSL (Ser565), phospho-HSL (Ser660), ATGL, phospho (Ser/Thr) PKA substrate, perilipin (Cell Signaling Technologies, Danvers, MA), G protein α S ($G_s\alpha$), G protein α inhibitor 1+2 (Gi) (Abcam, Cambridge, UK), phosphodiesterase 3B (PDE3B) (Phospho) (Acris Antibodies, Herford, Germany), adipocyte phospholipase A2 (AdPLA) (Cayman Chemical, Ann Arbor, MI), and β-actin (Sigma). After that, membranes were hybridized with horseradish peroxidase-conjugated secondary antibody (Sigma) for 1 h and then were revealed with the SuperSignal kit revelation solution (Pierce Biotechnology, Rockford, IL) following the manufacturer's protocol. The results were analyzed by densitometry using the GS-800 calibrated densitometer (Bio-Rad, München, Germany).

cAMP assay

The cAMP Direct EIA kit (Arbor Assay, Ann Arbor, MI) was used to quantify the amount of intracellular cAMP after 24 h of control or rCT-1 treatment (20 ng/ml) in 3T3-L1 adipocytes.

siRNA experiments

The predesigned siRNA specific to PKA (Silencer Select siRNA) and control siRNA (Silencer Select Control siRNA) were purchased from Ambion (Ambion Inc., Austin, TX), and siRNA specific to HSL and control were obtained from Santa Cruz. Transfection of 3T3-L1 adipocytes was performed using the Amaxa® Cell Line Nucleofector® kit L with the Nucleofector® II system (Lonza, Basel, Switzerland) using the recommended settings according to the manufacturer's protocol. The transfected cells were seeded in 6-well plates, and experiments were conducted after 24 h incubation.

Analyses of mRNA levels

Total RNA was extracted with TRIzol (Invitrogen) and real-time PCR was performed using iCycler (Bio-Rad) and iQ SYBR Green Supermix (Bio-Rad). For relative quantitation of gene expression, we used the comparative Ct method [$2^{\Delta Ct}$, where ΔCt represents the difference in threshold cycle between the target and control genes (cyclophilin)]. Primers were designed according to published cDNA or genomic sequences.

Statistical analysis

Data are presented as mean ± SEM. Comparisons between the values for different variables were analyzed by one-way ANOVA followed by Bonferroni post hoc tests or by Student's *t*-tests or Mann-Whitney U-tests once the normality had been screened using Kolmogorov-Smirnoff and Shapiro-Wilk tests. Statistical analyses and graphs were carried out using GraphPad Prism 5 software (Graph-Pad Software Inc., San Diego, CA). Overall, a P value <0.05 was considered significant.

RESULTS

Effects of rCT-1 on lipolysis in 3T3-L1 adipocytes

The incubation with rCT-1 (1–20 ng/ml) significantly increased basal glycerol released to the media in a dose-dependent manner after 18 h of treatment. Similar effects on glycerol release were observed for IL-6 (**Fig. 1A**). Furthermore, the effect of rCT-1 (20 ng/ml) on lipolysis was time-dependent. As shown in Fig. 1B, a significant increase in glycerol release was seen in the rCT-1-treated group after 12 h ($P < 0.01$) of incubation onwards, and this increase continued for up to at least 24 h ($P < 0.001$). However, after 3 h treatment, a significant concentration-dependent increase in the amount of FFAs released was already observed in rCT-1-treated adipocytes ($P < 0.05$) (Fig. 1C). Moreover, we also measured the intermediary metabolites after total lipid separation by HPTLC. Our data revealed that rCT-1 treatment did not induce any significant change in DAG and MAG levels, suggesting that the major products of rCT-1-induced TAG hydrolysis are FFAs and glycerol. Finally, because it has been described that in some tissues HSL also has lipolytic activity against cholesteryl esters (18), intracellular free cholesterol levels were tested. However, rCT-1-treated adipocytes showed no changes in intracellular free cholesterol (supplementary Fig. I).

Effects of rCT-1 on the main proteins involved in lipolysis control

For a better understanding of the mechanisms involved in CT-1 lipolytic actions, we first tested in 3T3-L1 adipocytes the effects of rCT-1 on ATGL, the enzyme that predominantly catalyzes the initial step in TAG hydrolysis (5). rCT-1 treatment for 1 and 2 h did not modify ATGL levels, but at 24 h the adipocytes treated with the cytokine exhibited

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

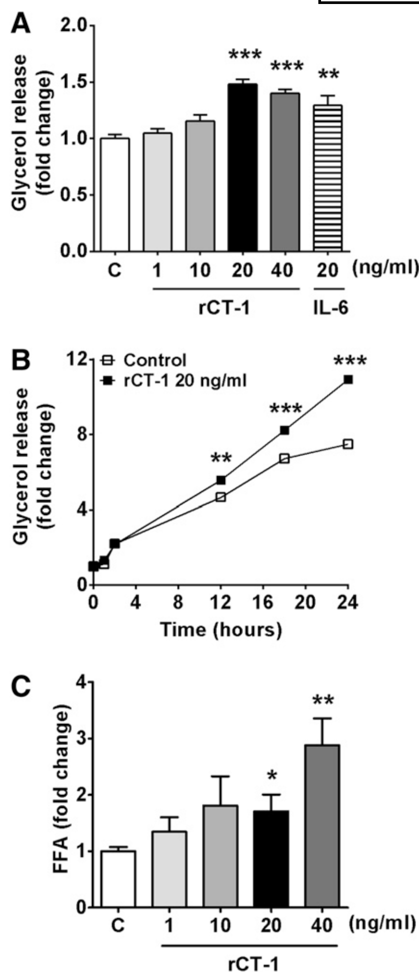


Fig. 1. CT-1 induces lipolysis in differentiated 3T3-L1 adipocytes. A: Fully differentiated 3T3-L1 adipocytes were serum starved for 4 h and then treated with various concentrations of rCT-1 (1, 10, 20, and 40 ng/ml) or IL-6 (20 ng/ml) for 18 h, and the amount of glycerol released into the media was measured. B: Time-response effects of rCT-1 (20 ng/ml) treatment on glycerol release. C: Dose-dependent effects of rCT-1 on FFAs release in adipocytes after 3 h of treatment. Data are mean ± SEM (n = 5–7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control (C) group.

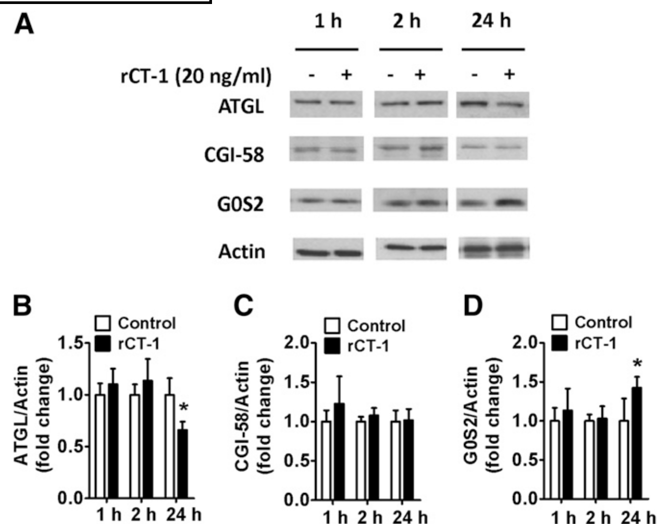


Fig. 2. ATGL is downregulated after 24 h of rCT-1 treatment. A: Representative Western blot and (B–D) densitometric analysis of ATGL (B), CGI-58 activator of ATGL (C), and G0S2 inhibitor of ATGL (D) in differentiated 3T3-L1 adipocytes treated with rCT-1 (20 ng/ml) or vehicle (Control) for 24 h. Band intensities for ATGL, CGI-58, and G0S2 were normalized to actin. Data are expressed as mean ± SEM (n = 4–7). *$P < 0.05$ compared with vehicle-treated cells.

a significant ($P < 0.05$) decrease in ATGL protein levels (**Fig. 2A, B**). ATGL activity is regulated by CGI-58 (activator) and G0S2 (inhibitor) via noncompeting mechanisms (6). Our data provided evidence that in parallel with the inhibition of ATGL, rCT-1 treatment increased the content of the ATGL inhibitor G0S2 ($P < 0.05$) after 24 h of treatment (Fig. 2A, D), while it did not significantly modify CGI-58 levels (Fig. 2A, C).

We next evaluated the effects of rCT-1 on HSL, a key lipase regulated by reversible phosphorylation (9). Our data revealed that rCT-1 (20 ng/ml) treatment increased the phosphorylation of HSL at Ser660 (which promotes its lipolytic activity), being significant after 2 ($P < 0.05$) and 24 h ($P < 0.05$) of treatment. However, rCT-1 did not modify the phosphorylation of HSL at Ser563 or Ser565 (**Fig. 3A**). To better characterize the involvement of HSL activation in the lipolytic actions of rCT-1, we tested the effect of the cytokine in HSL depleted adipocytes using siRNA. Our data revealed that rCT-1-induced glycerol release was almost completely abolished after silencing of HSL expression in adipocytes (Fig. 3B).

Finally, we also analyzed the effects of rCT-1 on perilipin A, an essential lipid droplet-associated protein, whose phosphorylation (PKA-dependent) is essential for the translocation of HSL from the cytosol to the lipid droplet surface (19). Using a perilipin-specific antibody and a phospho-PKA-motif-specific substrate antibody (15, 20), our data showed that rCT-1 caused a significant increase ($P < 0.05$) in the phospho-PKA substrate/perilipin ratio after 24 h of treatment (Fig. 3C), suggesting the involvement of PKA activation in the lipolytic actions of CT-1 in cultured adipocytes.

Characterization of the signaling pathways involved in the lipolytic actions of CT-1

Several signaling pathways have been found to be involved in the regulation of lipolysis, including cAMP/PKA, AMPK, and cyclic guanosine monophosphate (cGMP)-dependent protein kinase-I by different lipolytic/antilipolytic agents (21). Our data demonstrated that lipolytic actions of rCT-1 were completely reversed ($P < 0.001$) by pretreatment with the PKA inhibitor H89 (**Fig. 4A**). Moreover, the blocking of PKA also reversed rCT-1-induced phosphorylation of perilipin and HSL at Ser660 ($P < 0.001$) (Fig. 4B). The involvement of PKA in the lipolytic action of rCT-1 was further supported by the fact that silencing of PKA expression using siRNA dramatically decreases rCT-1-stimulated glycerol release in adipocytes (Fig. 4C). Because these data suggest that CT-1 lipolytic actions take place by activation of the cAMP/PKA pathway, we also tested the effects of rCT-1 on cAMP, showing a significant ($P < 0.01$) increase in cAMP levels in rCT-1-treated

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

ASBMB JOURNAL OF LIPID RESEARCH

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

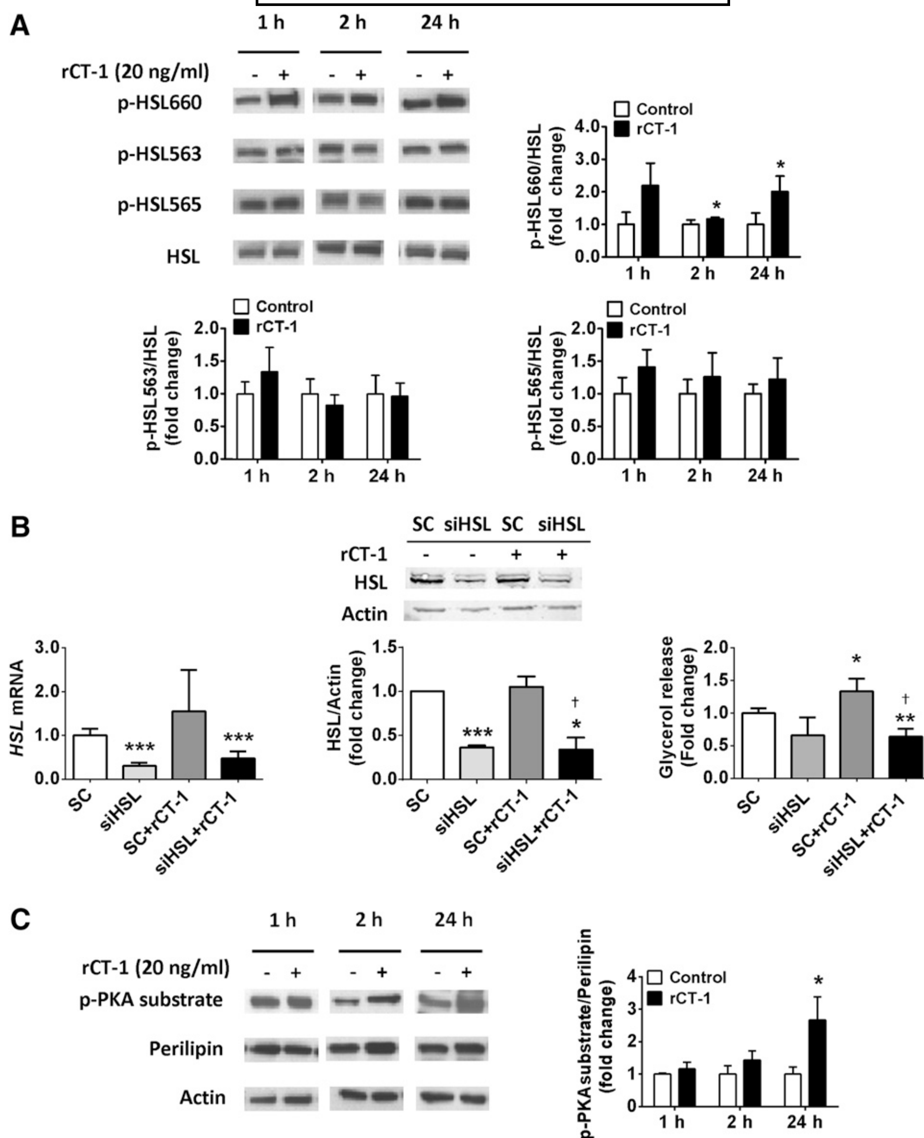


Fig. 3. HSL is involved in rCT-1-induced lipolysis. A: Representative Western blot and densitometric analysis of HSL phosphorylation at Ser660, Ser563, and Ser565 in differentiated 3T3-L1 adipocytes normalized by total HSL protein. B: rCT-1-stimulated lipolysis is prevented by siRNA knock-down of HSL. mRNA levels, protein expression of HSL, and glycerol release in 3T3-L1 adipocytes transfected with control siRNA (SC) or siRNA targeting endogenous HSL in the presence or absence of rCT-1 for 24 h. C: Adipocyte lysates were immunoblotted using a phospho-PKA-motif-specific antibody, and the blots were stripped and reprobed with antiperilipin antibodies to detect native perilipins. Band density was quantified, and data were expressed as phospho-PKA (p-PKA) substrate/perilipin ratio. Results are expressed as mean ± SEM. (n = 4–7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control (vehicle-treated cells); †$P < 0.05$ compared with CT-1-treated cells.

adipocytes (**Fig. 5A**). In order to elucidate the mechanisms by which rCT-1 increases cAMP levels in adipocytes, we tested the effects of the cytokine on G protein-receptor complexes regulating adenylate cyclase. As shown in Fig. 5B, treatment with rCT-1 induced a significant increase in protein expression of $G_s\alpha$, a protein which couples stimulatory receptors to adenylyl cyclase, whereas no changes were observed in Gi protein, which inhibits adenylyl cyclase. Moreover, neither the levels of phospho-PDE3B nor the levels of AdPLA were modified by rCT-1 (supplementary Fig. IIA, B).

Treatment with the AMPK activator, AICAR, was also able to prevent the stimulation of glycerol release ($P < 0.001$), as well as the phosphorylation of Ser660 HSL ($P < 0.05$) and perilipin ($P < 0.01$) (Fig. 4A, B). On the other hand, pretreatment with PKG inhibitor KT5823 did not modify the stimulatory action of rCT-1 on glycerol release (Fig. 4A) or the phosphorylation of HSL and perilipin (Fig. 4B).

In vivo effects of rCT-1 treatment on HSL, perilipin, ATGL, CGI-58, and G0S2 in adipose tissue of mice

Finally, we aimed to analyze whether the effects of rCT-1 on lipolysis observed in cultured adipocytes were also reproduced in adipose tissue after in vivo administration of the cytokine in mice. Interestingly, we found that rCT-1

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

ASBMB JOURNAL OF LIPID RESEARCH

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

ASBMB
JOURNAL OF LIPID RESEARCH

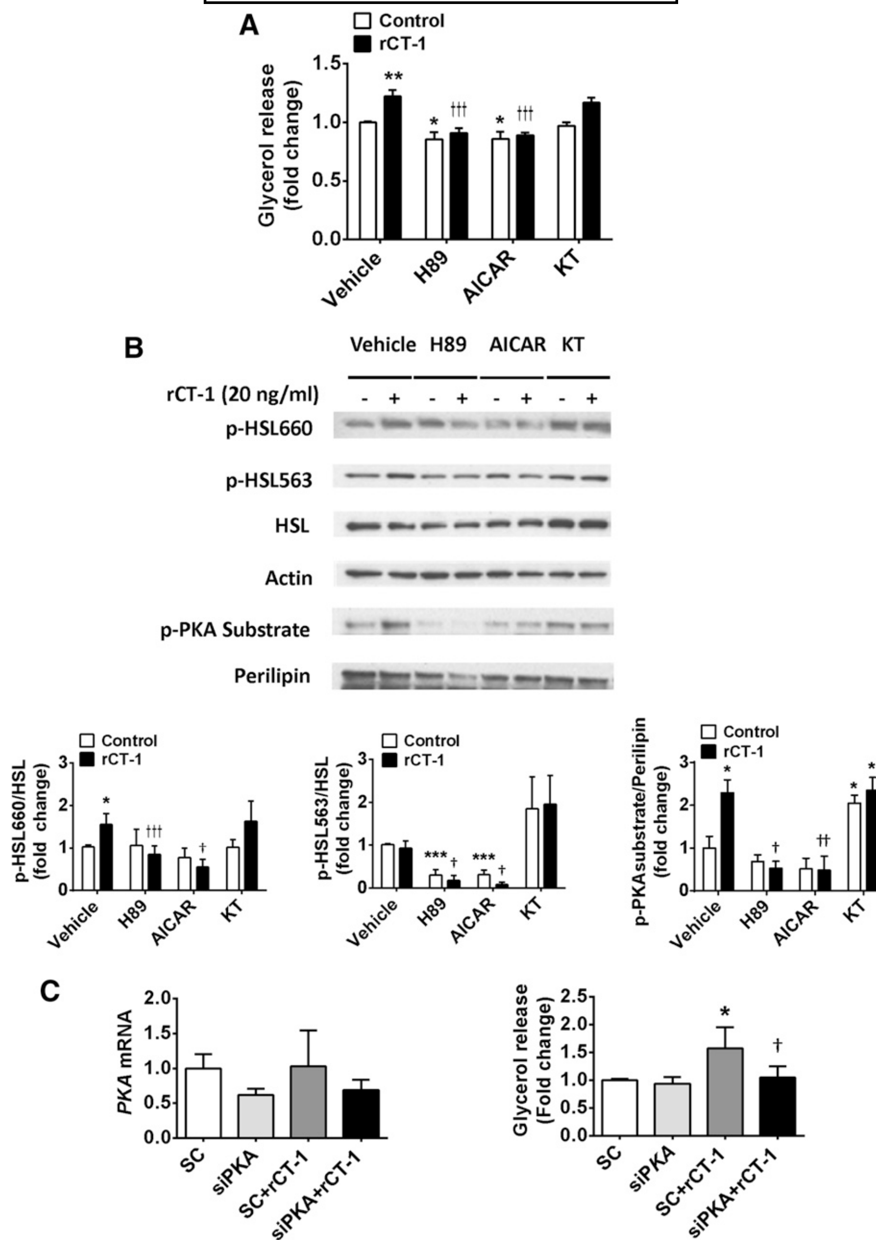


Fig. 4. PKA signaling pathway is involved in the lipolytic action of rCT-1. A: Effects of rCT-1 (20 ng/ml) treatment on glycerol release in differentiated 3T3-L1 adipocytes pretreated for 1 h in the presence or absence of PKA inhibitor H89 (1 μM), AMPK activator AICAR (2 mM), and PKG inhibitor KT5823 (1 μM), and then exposed to rCT-1 (20 ng/ml) or vehicle (DMSO) for 24 h. B: Representative Western blot and densitometric analysis of phospho-HSL660 (p-HSL660), phospho-HSL563 (p-HSL563) normalized by total HSL protein, and phospho-PKA (p-PKA) substrate/perilipin ratio. C: rCT-1-stimulated lipolysis is prevented by siRNA knock-down of PKA. mRNA levels of PKA and glycerol release in samples transfected with control siRNA (SC) or siPKA in either the absence or presence of rCT-1 for 24 h. Data are expressed as mean ± SEM (n = 3–7). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control (vehicle-treated cells); †$P < 0.05$, ††$P < 0.01$, †††$P < 0.001$ compared with rCT-1-treated cells.

treatment (0.2 mg/kg) for 30 min induced a significant increase in HSL phosphorylation at both Ser660 ($P < 0.05$) and Ser563 ($P < 0.001$) as compared with control mice. However, AMPK-mediated phosphorylation of HSL on Ser565, which prevents HSL activation, was significantly ($P < 0.001$) decreased. Moreover, rCT-1 treatment boosted ($P < 0.05$) PKA-mediated phosphorylation of perilipin (**Fig. 6A**). These results further support the key role of the PKA pathway in the lipolytic action of CT-1. A statistically significant increase ($P < 0.05$) in ATGL protein levels was also found in epididymal fat of *ob/ob* mice after 30 min of rCT-1 treatment, whereas neither the activator (CGI-58), nor the inhibitor (G0S2) of ATGL activity showed any changes after rCT-1 treatment (Fig. 6B). These facts suggest the ability of rCT-1 to promote lipolysis in vivo. In support of this, we found that the administration of rCT-1

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

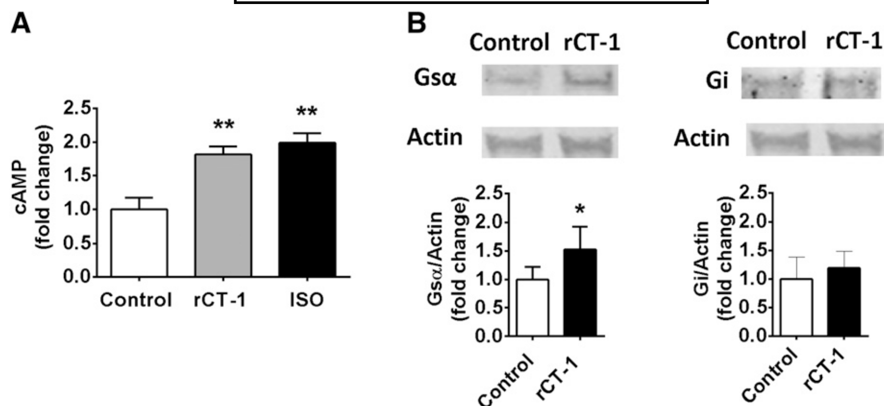


Fig. 5. rCT-1 stimulates $G_s\alpha$ and cAMP levels. A: Intracellular cAMP levels after 24 h of treatment with rCT-1 (20 ng/ml). B: Representative Western blot and densitometric analysis of $G_s\alpha$ and Gi protein levels in rCT-1-treated 3T3-L1 adipocytes. Data are expressed as mean ± SEM (n = 4–7). **P* < 0.05, ***P* < 0.01 compared with control (vehicle-treated cells).

(0.2 mg/kg) for 30 min to lean mice caused an increase in the levels of plasma FFAs in comparison with saline-treated mice (supplementary Fig. IIIA). Moreover, adipose tissue explants from rCT-1-treated mice exhibited increased lipolytic response as compared with the vehicle-treated group (supplementary Fig. IIIB).

DISCUSSION

The present study demonstrates the lipolytic activity of CT-1 in adipocytes both in vitro and in vivo. In fact, in cultured adipocytes, CT-1 treatment promoted a decrease in intracellular TAG in parallel with an increase in the release of glycerol and FFAs, suggesting the ability of CT-1 to promote TAG catabolism. Interestingly, we found that CT-1 stimulates lipolysis in adipocytes through the regulation of the major lipases and lipid droplet proteins involved in the hydrolysis of TAG. Indeed, CT-1 promotes HSL phosphorylation at Ser660, a residue that is involved in the activation of this lipase. It is well-known that PKA is the major kinase involved in the phosphorylation of HSL at the sites that cause HSL activation, including Ser563, Ser659, and Ser660. However, in the present study we observed that CT-1 selectively induces the phosphorylation of Ser660 without affecting Ser563 in cultured adipocytes. In this context, it has been described that phosphorylation sites Ser659 and Ser660 are the critical activity controlling sites, whereas Ser563 plays a minor role in direct activation of HSL in vitro (11). Importantly, activation of HSL seems to be a key factor for the lipolytic action of CT-1, because silencing of HSL expression in adipocytes completely abolished CT-1-induced glycerol release, and partly prevented basal lipolysis.

Nevertheless, our in vivo studies revealed that acute administration of CT-1 to mice was able to stimulate adipose tissue lipolysis and to phosphorylate HSL, not only at Ser660, but also a dramatic stimulation at Ser563 was observed. It is important to take into account that in vivo, the regulation of PKA-stimulated HSL Ser563 and Ser660 phosphorylation seems to be time- and tissue-dependent. In fact, a different time-response pattern for Ser563 and Ser660 phosphorylation has been described at diverse times during/after exercise in human adipose tissue. Moreover, a differential response in HSL phosphorylation was observed for Ser563 and Ser660 after treatment with β-adrenergic and AMPK stimulation in 3T3-L1 adipocytes (9). These facts suggest that although PKA is able to stimulate the phosphorylation of both Ser residues of HSL, the magnitude and time-response pattern could be different.

Other findings also support the view that the CT-1-induced lipolysis is secondary to the activation of the cAMP/PKA pathway: *i*) CT-1 increases cAMP intracellular content; *ii*) CT-1 promotes PKA-mediated phosphorylation of perilipin, enabling the translocation of phosphorylated HSL from the cytoplasm to the lipid droplet surface (22); *iii*) the PKA inhibitor, H89, blunts the phosphorylation of the two main PKA-targets, perilipin and HSL (at Ser660), and the subsequent increase in glycerol release induced by CT-1; and *iv*) silencing of PKA expression in adipocytes almost completely abrogated CT-1-induced glycerol release. Increased cAMP levels could be the consequence of increased adenylyl cyclase activity or reduced cAMP degradation (mainly mediated by PDE3B action). It is well-established that the stimulation of G_s-coupled receptors induces the activation of adenylyl cyclase, leading to increased intracellular cAMP levels and subsequent activation of PKA and phosphorylation and translocation of HSL to fat droplets (23, 24). Here, we demonstrate that CT-1 increased the levels of $G_s\alpha$ without affecting Gi, the inhibitory protein of adenylyl cyclase. Our findings also revealed that neither the levels of PDE3B, an enzyme that catalyses the breakdown of cAMP to its inactive form, nor the levels of AdPLA, which inhibits cAMP production by regulating prostaglandin E_2 abundance, were modified by CT-1. Taken together, these findings suggest that CT-1-induced increase of cAMP is secondary to stimulatory guanine nucleotide binding protein (Gs)-mediated stimulation of adenylyl cyclase. CT-1 exerts its signaling effects by interacting with the gp130 receptor. This receptor shares a large degree of sequence homology with the leptin receptor, and both activate the Janus kinase/signal transducer and

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015
ASBMB JOURNAL OF LIPID RESEARCH

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1.html

ASBMB JOURNAL OF LIPID RESEARCH

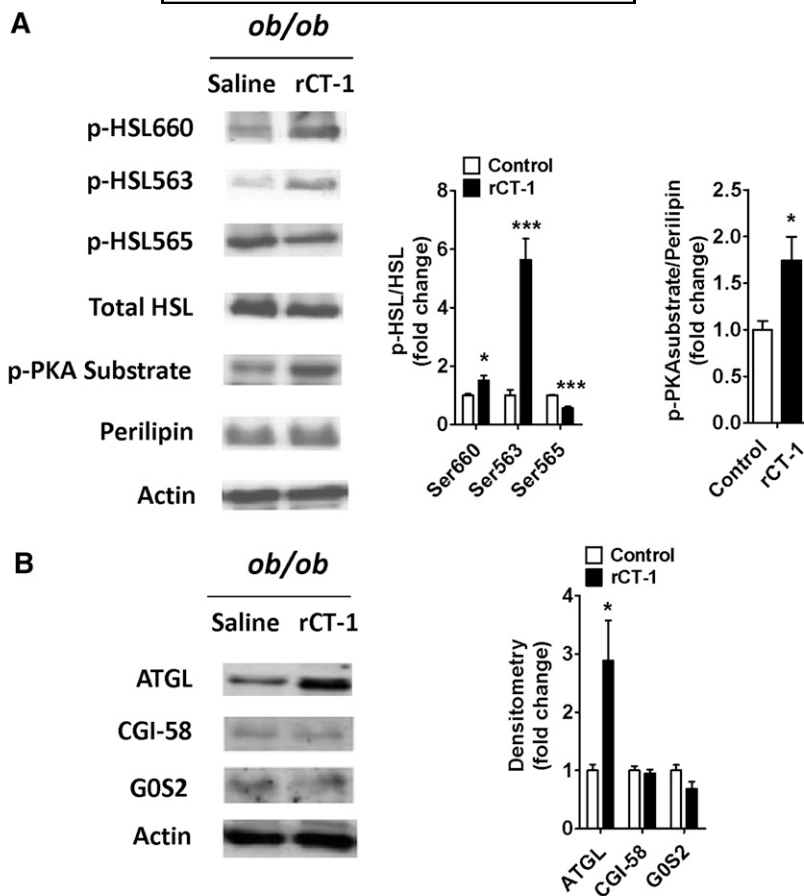


Fig. 6. In vivo administration of rCT-1 (0.2 mg/kg of body weight) for 30 min stimulates the main lipolytic enzymes in adipose tissue of *ob/ob* mice. A: Representative Western blot and densitometric analysis of HSL phosphorylation (Ser563, Ser660, Ser565) and perilipin in epididymal fat. Phospho-HSL (p-HSL) band intensities were normalized to total HSL, and phospho-PKA (p-PKA) substrate bands were normalized to perilipin. B: Representative Western blot and densitometric analysis of ATGL, CGI-58, and G0S2 in epididymal fat from *ob/ob* mice treated with rCT-1 or vehicle. Band intensities for ATGL, CGI-58, and G0S2 were normalized to actin. Results are expressed as mean ± SEM (n = 4–7). $^{*}P < 0.05$, $^{***}P < 0.001$.

activator of transcription (JAK/STAT) and ERK signaling pathways (25). However, the lipolytic effect of leptin has been related to the downregulation of the adenylyl cyclase-inhibitory G protein pathway (26). In this context, growing evidence exists for a cross-talk of signaling cascades initiated by G protein-coupled receptors (GPCRs) and the IL-6 family of cytokines signaling pathway (27). In contrast to CT-1, TNF-α stimulates lipolysis in adipocytes by decreasing Gi without affecting Gs levels (28) or by downregulating the expression of PDE3B (29).

Our present data indicate that besides the stimulatory effect of CT-1 on lipolysis, ATGL protein levels are inhibited in long-term (24 h) CT-1-treated cultured adipocytes, in parallel with the increase of the ATGL inhibitor, G0S2 (30). This apparently surprising finding of downregulation of ATGL together with increased lipolysis has been described after treatment with some lipolytic molecules, such as TNF-α, in cultured adipocytes (31, 32). This may suggest that an interaction between these two regulatory processes (activity and expression) occurs (high ATGL activity might be compensated by low expression) (33). Similarly to HSL, ATGL activity is also stimulated by catecholamines, but in contrast to HSL, ATGL activity is not directly regulated posttranslationally via PKA-mediated phosphorylation (34). It is well-known that ATGL protein is mainly coactivated by CGI-58 and inhibited by G0S2. However, the transcriptional regulation of ATGL is poorly characterized. In this context, PPARγ has been identified as a regulator of ATGL levels (34). It has been described that CT-1 induces a transient decrease in PPARγ in adipocytes at 24 h (3). In concordance, our data revealed that the decrease in ATGL observed after 24 h of treatment with CT-1 in cultured adipocytes paralleled with the drop in PPARγ levels (data not shown), suggesting a potential association between both events. In contrast with the lack of effect of short-term treatments (1–2 h) with CT-1 on ATGL in cultured adipocytes, the in vivo acute administration of CT-1 induced a marked increase in ATGL protein levels at 30 min, suggesting that putatively some mechanisms may regulate ATGL protein expression in vivo that do not exist in the in vitro model. In this context, several studies of our group have revealed that acute administration of CT-1 to mice has profound peripheral and central effects acting on neurohormonal regulators that could

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

also secondarily affect adipose tissue lipolysis. Thus, CT-1 stimulates insulin signaling and sensitivity, decreases blood glucose, activates AMPK and promotes fatty acid oxidation in muscle and liver, and modulates hypothalamic pathways involved in energy intake (4, 35). Interestingly, growing evidence suggests a role of hypothalamic regulation of adipose tissue function and metabolism (36).

AMPK has also been shown as an important regulator of lipolysis by regulating both HSL and ATGL activity by phosphorylation. Nevertheless, the effects of AMPK activation on lipolysis are complex because both antilipolytic (37–39) and lipolytic (40, 41) actions have been reported. In fact, AMPK effects on lipolysis seem to be time-dependent, involving antagonistic modulation of HSL and ATGL (42). In this context, several trials have demonstrated that AMPK induces phosphorylation of HSL at Ser565, which prevents phosphorylation of HSL by cyclic AMP-dependent protein kinase (PKA), causing suppression of PKA-stimulated lipolysis (43). On the other hand, AMPK phosphorylates ATGL at Ser406, increasing TAG hydrolase activity and providing evidence for increased lipolysis (8). The present data show that AMPK activation totally abolishes the lipolytic effect of CT-1 and suggest that AICAR-induced phosphorylation of HSL at Ser565 is able to prevent CT-1 induced-PKA-mediated phosphorylation and activation of HSL.

Activation of the cGMP pathway has also been shown to promote lipolysis. A downstream effector of cGMP, cGMP-dependent protein kinase, also called PKG, was shown to induce perilipin and HSL phosphorylation and to be at the origin of atrial natriuretic peptide-induced lipolysis (44). We tested the potential involvement of the cGMP/PKG pathway in the lipolytic actions of CT-1. The results suggest that this pathway is not involved in CT-1-stimulated lipolysis because treatment with the PKG inhibitor, KT5823, was not able to reverse CT-1-induced glycerol release and did not cause any significant changes in HSL or perilipin activation.

Several studies have described lipolytic actions in adipocytes for IL-6 (45) and other members of the gp130 ligand family of cytokines such as leukemia inhibitory factor (46). However, differential effects have been found among cytokines of this family. For example, previous studies by our group have shown that chronic administration of CT-1 to obese mice stimulated the lipolytic response to isoproterenol in adipocytes (4). On the other hand, no significant changes in lipolysis were found after the administration of ciliary neurotrophic factor to high-fat-fed mice (47). The study by Wolsk et al. (48) showed that an acute increase in IL-6 selectively stimulates lipolysis in skeletal muscle, whereas adipose tissue was unaffected in humans. Our present study clearly shows an increase of the main lipases in adipose tissue after an acute administration of CT-1. Additional findings also suggest differential mechanisms of action underlying the lipolytic properties of CT-1 and IL-6. Thus, it has been observed that IL-6 increased lipolysis in differentiated porcine adipocytes by activation of ERK, which was inhibited by a specific ERK inhibitor, while IL-6 treatment did not elevate intracellular cAMP, and the specific PKA inhibitor (H89) did not affect IL-6-induced lipolysis, suggesting that the PKA pathway was not involved in IL-6 lipolytic effects (49). On the contrary, our present data clearly suggest the involvement of the cAMP/PKA pathway in the lipolytic action of CT-1.

Activation of lipolysis has been proposed as a promising therapeutic target for the treatment of obesity (50). Our results suggest that the ability of CT-1 to activate the pathway in adipocytes could also contribute to its anti-obesity properties. In line with this, we have previously reported that chronic administration of CT-1 to *ob/ob* mice increased the adipocytes' lipolytic response to isoproterenol (4). It is important to mention that increased lipolysis and FFAs release from adipose tissue have been associated with the development of metabolic disturbances in obesity (51). However, several studies have suggested that increasing lipolysis in adipose tissue does not necessarily increase serum FFAs levels, because increasing lipolysis in adipose tissue causes a shift within adipocytes toward increased FFAs utilization and energy expenditure and, thus, protects against obesity (50). In this context, our previous data revealed that CT-1 is able to promote FFAs oxidation not only in adipose tissue but also in muscle, reducing insulin resistance in obese mice (4).

In summary, the present data demonstrate that the ability of CT-1 to regulate the activity of the main lipases underlies the lipolytic action of this cytokine in vitro and in vivo, and may account for the anti-obesity effects of CT-1.

REFERENCES

1. Pennica, D., K. J. Shaw, T. A. Swanson, M. W. Moore, D. L. Shelton, K. A. Zioncheck, A. Rosenthal, T. Taga, N. F. Paoni, and W. I. Wood. 1995. Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. *J. Biol. Chem.* **270:** 10915–10922.
2. Natal, C., M. A. Fortuno, P. Restituto, A. Bazan, I. Colina, J. Diez, and N. Varo. 2008. Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome. *Am. J. Physiol. Endocrinol. Metab.* **294:** E52–E60.
3. Zvonic, S., J. C. Hogan, P. Arbour-Reily, R. L. Mynatt, and J. M. Stephens. 2004. Effects of cardiotrophin on adipocytes. *J. Biol. Chem.* **279:** 47572–47579.
4. Moreno-Aliaga, M. J., N. Perez-Echarri, B. Marcos-Gomez, E. Larequi, F. J. Gil-Bea, B. Viollet, I. Gimenez, J. A. Martinez, J. Prieto, and M. Bustos. 2011. Cardiotrophin-1 is a key regulator of glucose and lipid metabolism. *Cell Metab.* **14:** 242–253.
5. Zimmermann, R., J. G. Strauss, G. Haemmerle, G. Schoiswohl, R. Birner-Gruenberger, M. Riederer, A. Lass, G. Neuberger, F. Eisenhaber, A. Hermetter, et al. 2004. Fat mobilization in adipose tissue is promoted by adipose triglyceride lipase. *Science.* **306:** 1383–1386.
6. Lu, X., X. Yang, and J. Liu. 2010. Differential control of ATGL-mediated lipid droplet degradation by CGI-58 and G0S2. *Cell Cycle.* **9:** 2719–2725.
7. Cornaciu, I., A. Boeszoermenyi, H. Lindermuth, H. M. Nagy, I. K. Cerk, C. Ebner, B. Salzburger, A. Gruber, M. Schweiger, R. Zechner, et al. 2011. The minimal domain of adipose triglyceride lipase (ATGL) ranges until leucine 254 and can be activated and inhibited by CGI-58 and G0S2, respectively. *PLoS ONE.* **6:** e26349.
8. Ahmadian, M., M. J. Abbott, T. Tang, C. S. Hudak, Y. Kim, M. Bruss, M. K. Hellerstein, H. Y. Lee, V. T. Samuel, G. I. Shulman, et al. 2011. Desnutrin/ATGL is regulated by AMPK and is required for a brown adipose phenotype. *Cell Metab.* **13:** 739–748.
9. Watt, M. J., A. G. Holmes, S. K. Pinnamaneni, A. P. Garnham, G. R. Steinberg, B. E. Kemp, and M. A. Febbraio. 2006. Regulation of

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

Supplemental Material can be found at:
http://www.jlr.org/content/suppl/2014/10/28/jlr.M055335.DC1
.html

HSL serine phosphorylation in skeletal muscle and adipose tissue. *Am. J. Physiol. Endocrinol. Metab.* **290:** E500–E508.
10. Greenberg, A. S., W. J. Shen, K. Muliro, S. Patel, S. C. Souza, R. A. Roth, and F. B. Kraemer. 2001. Stimulation of lipolysis and hormone-sensitive lipase via the extracellular signal-regulated kinase pathway. *J. Biol. Chem.* **276:** 45456–45461.
11. Anthonsen, M. W., L. Ronnstrand, C. Wernstedt, E. Degerman, and C. Holm. 1998. Identification of novel phosphorylation sites in hormone-sensitive lipase that are phosphorylated in response to isoproterenol and govern activation properties in vitro. *J. Biol. Chem.* **273:** 215–221.
12. Brasaemle, D. L. 2007. Adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *J. Lipid Res.* **48:** 2547–2559.
13. Granneman, J. G., H. P. Moore, R. Krishnamoorthy, and M. Rathod. 2009. Perilipin controls lipolysis by regulating the interactions of AB-hydrolase containing 5 (Abhd5) and adipose triglyceride lipase (Atgl). *J. Biol. Chem.* **284:** 34538–34544.
14. Miyoshi, H., J. W. Perfield 2nd, S. C. Souza, W. J. Shen, H. H. Zhang, Z. S. Stancheva, F. B. Kraemer, M. S. Obin, and A. S. Greenberg. 2007. Control of adipose triglyceride lipase action by serine 517 of perilipin A globally regulates protein kinase A-stimulated lipolysis in adipocytes. *J. Biol. Chem.* **282:** 996–1002.
15. Fernández-Galilea, M., P. Pérez-Matute, P. L. Prieto-Hontoria, J. A. Martinez, and M. J. Moreno-Aliaga. 2012. Effects of lipoic acid on lipolysis in 3T3–L1 adipocytes. *J. Lipid Res.* **53:** 2296–2306.
16. Beraza, N., J. M. Marques, E. Martinez-Anso, M. Iniguez, J. Prieto, and M. Bustos. 2005. Interplay among cardiotrophin-1, prostaglandins, and vascular endothelial growth factor in rat liver regeneration. *Hepatology.* **41:** 460–469.
17. Takahashi, Y., A. Shinoda, N. Furuya, E. Harada, N. Arimura, I. Ichi, Y. Fujiwara, J. Inoue, and R. Sato. 2013. Perilipin-mediated lipid droplet formation in adipocytes promotes sterol regulatory element-binding protein-1 processing and triacylglyceride accumulation. *PLoS ONE.* **8:** e64605.
18. Yeaman, S. J. 2004. Hormone-sensitive lipase–new roles for an old enzyme. *Biochem. J.* **379:** 11–22.
19. Sztalryd, C., G. Xu, H. Dorward, J. T. Tansey, J. A. Contreras, A. R. Kimmel, and C. Londos. 2003. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. *J. Cell Biol.* **161:** 1093–1103.
20. Choi, S. M., D. F. Tucker, D. N. Gross, R. M. Easton, L. M. DiPilato, A. S. Dean, B. R. Monks, and M. J. Birnbaum. 2010. Insulin regulates adipocyte lipolysis via an Akt-independent signaling pathway. *Mol. Cell. Biol.* **30:** 5009–5020.
21. Nishikimi, T., C. Iemura-Inaba, K. Akimoto, K. Ishikawa, S. Koshikawa, and H. Matsuoka. 2009. Stimulatory and Inhibitory regulation of lipolysis by the NPR-A/cGMP/PKG and NPR-C/G(i) pathways in rat cultured adipocytes. *Regul. Pept.* **153:** 56–63.
22. Brasaemle, D. L., D. M. Levin, D. C. Adler-Wailes, and C. Londos. 2000. The lipolytic stimulation of 3T3-L1 adipocytes promotes the translocation of hormone-sensitive lipase to the surfaces of lipid storage droplets. *Biochim. Biophys. Acta.* **1483:** 251–262.
23. Belfrage, P., G. Fredrikson, N. O. Nilsson, and P. Stralfors. 1981. Regulation of adipose-tissue lipolysis by phosphorylation of hormone-sensitive lipase. *Int. J. Obes.* **5:** 635–641.
24. Chaves, V. E., D. Frasson, and N. H. Kawashita. 2011. Several agents and pathways regulate lipolysis in adipocytes. *Biochimie.* **93:** 1631–1640.
25. Febbraio, M. A. 2007. gp130 receptor ligands as potential therapeutic targets for obesity. *J. Clin. Invest.* **117:** 841–849.
26. Frühbeck, G., L. Méndez-Giménez, J. A. Fernández-Formoso, S. Fernández, and A. Rodríguez. 2014. Regulation of adipocyte lipolysis. *Nutr. Res. Rev.* **27:** 63–93.
27. Garbers, C., H. M. Hermanns, F. Schaper, G. Muller-Newen, J. Grotzinger, S. Rose-John, and J. Scheller. 2012. Plasticity and cross-talk of interleukin 6-type cytokines. *Cytokine Growth Factor Rev.* **23:** 85–97.
28. Gasic, S., B. Tian, and A. Green. 1999. Tumor necrosis factor alpha stimulates lipolysis in adipocytes by decreasing Gi protein concentrations. *J. Biol. Chem.* **274:** 6770–6775.
29. Rahn Landström, T., J. Mei, M. Karlsson, V. Manganiello, and E. Degerman. 2000. Down-regulation of cyclic-nucleotide phosphodiesterase 3B in 3T3-L1 adipocytes induced by tumour necrosis factor alpha and cAMP. *Biochem. J.* **346:** 337–343.
30. Yang, X., X. Lu, M. Lombes, G. B. Rha, Y. I. Chi, T. M. Guerin, E. J. Smart, and J. Liu. 2010. The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. *Cell Metab.* **11:** 194–205.
31. Lorente-Cebrián, S., M. Bustos, A. Marti, M. Fernández-Galilea, J. A. Martinez, and M. J. Moreno-Aliaga. 2012. Eicosapentaenoic acid inhibits tumour necrosis factor-alpha-induced lipolysis in murine cultured adipocytes. *J. Nutr. Biochem.* **23:** 218–227.
32. Kim, J. Y., K. Tillison, J. H. Lee, D. A. Rearick, and C. M. Smas. 2006. The adipose tissue triglyceride lipase ATGL/PNPLA2 is downregulated by insulin and TNF-alpha in 3T3-L1 adipocytes and is a target for transactivation by PPARgamma. *Am. J. Physiol. Endocrinol. Metab.* **291:** E115–E127.
33. Kralisch, S., J. Klein, U. Lossner, M. Bluher, R. Paschke, M. Stumvoll, and M. Fasshauer. 2005. Isoproterenol, TNFalpha, and insulin downregulate adipose triglyceride lipase in 3T3-L1 adipocytes. *Mol. Cell. Endocrinol.* **240:** 43–49.
34. Kershaw, E. E., M. Schupp, H. P. Guan, N. P. Gardner, M. A. Lazar, and J. S. Flier. 2007. PPARgamma regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. *Am. J. Physiol. Endocrinol. Metab.* **293:** E1736–E1745.
35. Castaño, D., E. Larequi, I. Belza, A. M. Astudillo, E. Martínez-Ansó, J. Balsinde, J. Argemi, T. Aragon, M. J. Moreno-Aliaga, J. Muntane, et al. 2014. Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation. *J. Hepatol.* **60:** 1017–1025.
36. Stefanidis, A., N. M. Wiedmann, E. S. Adler, and B. J. Oldfield. 2014. Hypothalamic control of adipose tissue. *Best Pract. Res. Clin. Endocrinol. Metab.* **28:** 685–701.
37. Dagon, Y., Y. Avraham, and E. M. Berry. 2006. AMPK activation regulates apoptosis, adipogenesis, and lipolysis by eIF2alpha in adipocytes. *Biochem. Biophys. Res. Commun.* **340:** 43–47.
38. Boon, H., M. Bosselaar, S. F. Praet, E. E. Blaak, W. H. Saris, A. J. Wagenmakers, S. L. McGee, C. J. Tack, P. Smits, M. Hargreaves, et al. 2008. Intravenous AICAR administration reduces hepatic glucose output and inhibits whole body lipolysis in type 2 diabetic patients. *Diabetologia.* **51:** 1893–1900.
39. Anthony, N. M., M. P. Gaidhu, and R. B. Ceddia. 2009. Regulation of visceral and subcutaneous adipocyte lipolysis by acute AICAR-induced AMPK activation. *Obesity (Silver Spring).* **17:** 1312–1317.
40. Koh, H. J., M. F. Hirshman, H. He, Y. Li, Y. Manabe, J. A. Balschi, and L. J. Goodyear. 2007. Adrenaline is a critical mediator of acute exercise-induced AMP-activated protein kinase activation in adipocytes. *Biochem. J.* **403:** 473–481.
41. Yamaguchi, S., H. Katahira, S. Ozawa, Y. Nakamichi, T. Tanaka, T. Shimoyama, K. Takahashi, K. Yoshimoto, M. O. Imaizumi, S. Nagamatsu, et al. 2005. Activators of AMP-activated protein kinase enhance GLUT4 translocation and its glucose transport activity in 3T3-L1 adipocytes. *Am. J. Physiol. Endocrinol. Metab.* **289:** E643–E649.
42. Gaidhu, M. P., S. Fediuc, N. M. Anthony, M. So, M. Mirpourian, R. L. Perry, and R. B. Ceddia. 2009. Prolonged AICAR-induced AMP-kinase activation promotes energy dissipation in white adipocytes: novel mechanisms integrating HSL and ATGL. *J. Lipid Res.* **50:** 704–715.
43. Garton, A. J., and S. J. Yeaman. 1990. Identification and role of the basal phosphorylation site on hormone-sensitive lipase. *Eur. J. Biochem.* **191:** 245–250.
44. Sengenes, C., C. Moro, J. Galitzky, M. Berlan, and M. Lafontan. 2005. Natriuretic peptides: a new lipolytic pathway in human fat cells [article in French]. *Med. Sci. (Paris).* **21:** 61–65.
45. Ji, C., X. Chen, C. Gao, L. Jiao, J. Wang, G. Xu, H. Fu, X. Guo, and Y. Zhao. 2011. IL-6 induces lipolysis and mitochondrial dysfunction, but does not affect insulin-mediated glucose transport in 3T3–L1 adipocytes. *J. Bioenerg. Biomembr.* **43:** 367–375.
46. Marshall, M. K., W. Doerrler, K. R. Feingold, and C. Grunfeld. 1994. Leukemia inhibitory factor induces changes in lipid metabolism in cultured adipocytes. *Endocrinology.* **135:** 141–147.
47. Crowe, S., S. M. Turpin, F. Ke, B. E. Kemp, and M. J. Watt. 2008. Metabolic remodeling in adipocytes promotes ciliary neurotrophic factor-mediated fat loss in obesity. *Endocrinology.* **149:** 2546–2556.
48. Wolsk, E., H. Mygind, T. S. Grondahl, B. K. Pedersen, and G. van Hall. 2010. IL-6 selectively stimulates fat metabolism in human skeletal muscle. *Am. J. Physiol. Endocrinol. Metab.* **299:** E832–E840.
49. Yang, Y., D. Ju, M. Zhang, and G. Yang. 2008. Interleukin-6 stimulates lipolysis in porcine adipocytes. *Endocrine.* **33:** 261–269.
50. Ahmadian, M., R. E. Duncan, and H. S. Sul. 2009. The skinny on fat: lipolysis and fatty acid utilization in adipocytes. *Trends Endocrinol. Metab.* **20:** 424–428.
51. Ormseth, M. J., L. L. Swift, S. Fazio, M. F. Linton, C. P. Chung, P. Raggi, Y. H. Rho, J. Solus, A. Oeser, A. Bian, et al. 2011. Free fatty acids are associated with insulin resistance but not coronary artery atherosclerosis in rheumatoid arthritis. *Atherosclerosis.* **219:** 869–874.

Downloaded from www.jlr.org at UNA BIBLIOTECA HUMANIDADES, on May 5, 2015

Cytokine & Growth Factor Reviews 26 (2015) 523–532

Contents lists available at ScienceDirect

Cytokine & Growth Factor Reviews

journal homepage: www.elsevier.com/locate/cytogfr

Mini review

Cardiotrophin-1: A multifaceted cytokine

Miguel López-Yoldi [a,b], María J. Moreno-Aliaga [a,b,c,d], Matilde Bustos [e,*]

[a] Department of Nutrition, Food Science and Physiology, University of Navarra, Pamplona, Spain
[b] Centre for Nutrition Research, University of Navarra, Pamplona, Spain
[c] CIBER Fisiopatología de la Obesidad y Nutrición (CIBERobn), Instituto de Salud Carlos III (ISCIII), Madrid, Spain
[d] Instituto de Investigación Sanitaria de Navarra (IdiSNA), Pamplona, Spain
[e] Area of Hepatology and Gene Therapy, CIMA (Center for Applied Medical Research) University of Navarra, Pamplona, Spain

ARTICLE INFO

Article history:
Received 5 June 2015
Accepted 1 July 2015
Available online 4 July 2015

Keywords:
Cardiotrophin-1
Cytokines
IL-6
Cytoprotection
Metabolism

ABSTRACT

Cardiotrophin-1 (CT-1) is a member of the gp130 family of cytokines that have pleiotropic functions on different tissues and cell types. Although many effects of CT-1 have been described on the heart, there is an extensive research showing important protective effects in other organs such as liver, kidney or nervous system. Recently, several studies have pointed out that CT-1 might also play a key role in the regulation of body weight and intermediate metabolism. This paper will review many aspects of CT-1 physiological role in several organs and discuss data for consideration in therapeutic approaches.

© 2015 Elsevier Ltd. All rights reserved.

1. Introduction

Despite increasing interest on cardiotrophin-1 (CT-1) as a cytoprotective molecule, many aspects of its physiological role have not been unambiguously established. Here we review present knowledge of the biological activities of CT-1 on different organs and systems and discuss data on the potential therapeutic applications of its remarkable properties.

1.1. Discovery and cloning

In 1995, Pennica et al. observed that conditioned medium from differentiated embryoid bodies induced a hypertrophic response in neonatal cardiac myocytes as judged by myocyte enlargement, organization of myosin light chain into sarcomeric units, and atrial natriuretic peptide (ANP) secretion. They identified in a screen of a cDNA library derived from the mouse embryoid bodies a new protein named Cardiotrophin-1 (CT-1), based on the observed biological properties [1]. CT-1 was described as a 21.5 kDa protein (203 amino acids long) and, although the amino acid sequence of CT-1 had some similarity with leukemia inhibitory factor (LIF) (24% identity) and ciliary neurotrophic factor (CNTF) (19% identity), structural considerations indicated that CT-1 was a new member of the interleukin (IL)-6 family of cytokines. All cytokines of this family belong to the four-helical bundle cytokine family sharing little sequence homology and being only recognized as family members by prediction of their protein fold [1]. Although most members of the IL-6 family of cytokines present a hydrophobic, N-terminal secretion signal sequence, CT-1, like CNTF, lacks this secretion sequence. However, experiments with cultured myotubes have shown that a significant proportion of CT-1 produced by these cells is found in the culture medium [2] suggesting that the factor might be released by an unconventional mechanism to become available to surrounding responsive cells.

The coding regions of human and mouse CT-1 are contained on three separate exons that span 6–7 kbp of genomic DNA. When nucleotide sequences of the coding regions of exons were compared between human and mouse it was observed that exon 1, 2 and 3 shared 96%, 84% and 81% homology, respectively [3]. Amino-acid sequence of rat CT-1 was 94% identical to that of mouse CT-1 [4]. The 5′-flanking region of the human CT-1 gene has been cloned and sequenced. Data bank search revealed several *cis*-active DNA elements (SP1, CREB, C/EBP, AP-1 and AP-2-like and GATA) in the proximal 1.1 kb region [5]. The human CT-1 gene is located on chromosome 16p11.1–16p11.2 [6]. The 5′-flanking region of the mouse CT-1 gene contains a variety of transcription factor binding motif (*e.g.* CREB, MyoD, NF-IL6, Nkx2.5, GATA). Fluorescent *in situ* hybridization (FISH) analysis demonstrated that the mouse CT-1 gene was located on chromosome 7F3. Other members of IL-6 family of cytokine have been reported to be on chromosome 5 (IL-6), chromosome 11 (LIF, OSM) and chromosome

* Corresponding author: Matilde Bustos, Tel : +34 948 19 47 00 Ext 4015; Fax: +34 948 19 47 17.
E-mail address: mbustos@unav.es (M. Bustos).

http://dx.doi.org/10.1016/j.cytogfr.2015.07.009
1359-6101/© 2015 Elsevier Ltd. All rights reserved.

19 (CNTF). It has been considered that neuropoietin (NP) and CT-1 genes are twin genes, both localized on mouse chromosome 7F3 with only 1.7 kb distant from each other with opposite transcriptional orientations in the similar way that CNTF and CLC genes are closely related and both are localized on chromosome 19. The chromosomal proximity and the general intron exon organization suggest that these cytokines arose from a gene duplication event [7].

1.2. CT-1 receptors and signaling

CT-1 signals through a specific combination of β receptor glycoprotein 130 (gp130) and β receptor leukemia inhibitory factor receptor (LIFR). Although CT-1 can mimic LIF in binding and activation of the gp130/LIFR complex, it has been reported to recruit an as yet uncharacterized α receptor (CT-1Rα) [8], which confers CT-1 high potency trophic signaling for motor neurons [2,8,9]. However, this additional membrane component is apparently not required for CT-1 effects on other cells [10]. Signal transduction *via* gp130 involves three major downstream pathways: the JAK (Janus-activated kinase)–STAT (signal transducer and activator of transcription) axis, the Ras-Raf mitogen-activated protein kinase (MAPK, MEK/ERK) signaling cascade, and the phosphatidylinositol 3-kinase-dependent (PI3K)/AKT (protein kinase B:PKB) pathway. CT-1 signaling produces overlapping biological activities with other members of IL-6 family of cytokines but also displays distinctive biological actions from the other family members. Differences in the biological responses elicited by the diverse cytokines of IL-6 superfamily may relate to the presence or absence of specific cytokine receptor subunits on the cell membrane. CT-1 binds LIFR with low affinity and this binding stimulates its heterodimerization with the gp130 into a high affinity-binding complex [9]. Gp130/LIFR activates JAK1, JAK2 and the tyrosine kinase (TYK2) associated with gp130, which phosphorylates tyrosine residues in the cytoplasmic domain of gp130. These phosphotyrosines subsequently serve as the docking sites to recruit STAT proteins, which are phosphorylated [11] allowing STATs to translocate to the nucleus where they bind specific DNA sequences to modulate gene expression. Their activity can be further regulated through serine phosphorylation [12] and inhibited by intracellular SOCS (suppressors of cytokine signaling proteins) and PIAS (protein inhibitor of activated STAT) [13,14]. Phosphorylated receptor subunits also serve as docking points for other Src homology 2 (SH2) domain-containing proteins, such as phosphatidylinositol 3-kinase (PI3K), growth factor receptor-bound protein 2, adapter proteins (Shc), Ceacam1 (pp120) and the cytoplasmic SH2 domain containing protein tyrosine phosphatase (SHP2). Recruitment of Shc to phosphorylated subunits of gp130 leads to complex formation with Grb2 and SOS, which activates Ras-Raf mitogen-activated protein kinase (MAPK, MEK/ERK) pathway [15,16]. It has been reported that recruitment of SHP2 to the phosphotyrosine 759 of gp130 allows Gab1–SHP-2 interaction, which modify the conformation or phosphorylation status of Gab1, allowing Gab1 to interact with PI-3 kinase and activate downstream signaling pathways [17].

2. Biological effects of CT-1

2.1. Cardiovascular

Gp130 receptor is essential in mediating cardioprotective effects against physiological and pathophysiological stress by promoting cardiomyocyte survival, inducing compensatory hypertrophy and preserving cardiac function. Thus, in mice lacking the CT-1 receptor gp130 only in the heart, aortic banding-induced pressure overload results in massive cardiac cell apoptosis and death of the animals from heart failure [18]. In contrast, control mice undergo a hypertrophic response and survive. These observations imply that gp130 ligands participate in the adaptive processes of the heart in response to stress. Moreover, it seems that protective and hypertrophic effects by gp130 ligands may be functionally interconnected.

The protective effects of CT-1 on cardiac myocytes were described near two decades ago. It was shown that CT-1 is required for cardiac myocyte maturation and was capable of promoting cell survival in neonatal rat cardiomyocytes subjected to serum deprivation through an antiapoptotic pathway mediated by MAPK, ERK1/2 [19]. The first study on the cardioprotective activity of recombinant protein CT-1 (rCT-1) was reported by Stephanou et al. They demonstrated resistance to ischemia–reperfusion injury in neonatal rat cardiomyocytes with improved cell survival and less apoptotic cell death [20]. Since then several experimental studies have confirmed the defensive role of rCT-1 on cultured neonatal rat cardiomycytes [21] and isolated perfused animal hearts [22]. It is worth mentioning that CT-1 was able to limit myocardial injury even when administered at the time of reoxygenation, which implies important clinical applications for CT-1. Many of the mentioned studies emphasized the importance of the anti-apoptotic MAPK, ERK1/2 activation, as a cardioprotective signaling pathway downstream of CT-1 activation [21,22]. Cardioprotection by CT-1 has also been associated with other molecular mediators including activation of heat shock proteins 70 and 90 [20], induction of PI3K-Akt-BAD axis [23], activation of p38MAPK [24] and reduction of oxidative stress [25] *via* manganese superoxide dismutase (MnSOD) an enzyme that scavenges superoxide anion through its transformation into the less damaging H_2O_2. Craig et al. demonstrated that cardioprotection by CT-1 was dependent on the downstream nuclear translocation of the nuclear factor kappa B (NFkB) [24]. Many of the signaling cascades mentioned above inhibit pro-apoptotic proteins such as p53, Fas and Bax, and upregulate anti-apoptotic proteins such as Bcl2 [26]. Importantly, the JAK/STAT pathway, which has been shown to defend cardiomyocytes against ischemia/reperfusion injury by reducing ROS production [27] and to be a mechanism underpinning the protective effects of late preconditioning [27], was largely independent from the survival promoting effects of CT-1 on cardiomyocytes [22,24,28,29].

CT-1 mRNA expression has been observed in normal and disease states [30]. Increased expression of CT-1 has been reported in the ventricles of genetically hypertensive rats [31] and in rats subjected to pressure overload [32]. In humans, elevated serum levels of CT-1 have been observed in patients with heart failure (HF) [33] and hypertensive heart disease [34]. While some authors have reported an association between circulating CT-1 and left ventricular structure and function [34–39], other studies could not confirm these findings [40]. Elevated CT-1 mRNA and protein were observed in the ventricles from rats with myocardial infarction, and in patients with ischemic heart disease, valvular heart disease or after myocardial infarction (MI) [41]. Freed et al. have shown a beneficial effect of CT-1 on infarct scar formation by maintaining the cellularity of the scar, which is associated with improved ventricular performance, even though these cells may not contribute to synchronous ventricular contraction [30].

CT-1 and LIF are the gp130 ligands reported to be hypertrophic agents for cardiac muscle [42]. Both of them are able to activate the three major signaling pathways linked to gp130/LIFR activation that stimulate cardiac hypertrophy, both in cultured cells and *in vivo* [9,43]. Similar to LIF, CT-1 induces myocardial hypertrophy with a predominant increase in myocardial cell length by addition of new sarcomeric units in series without increase in cell width [44]. The mechanisms mediating CT-1-induced cardiomyocyte hypertrophy have not been fully clarified. Initially, activation of the

JAK/STAT3 axis was considered to be responsible for this effect [45,46]. However, recent findings have implicated MEK5-ERK5 pathway in cardiac hypertrophy [47]. It has also been reported that the hypertrophic effects of CT-1 on cardiac cells are at least in part dependent on hsp56 induction downstream of JAK/STAT, MEK/ERK and PI3K/Akt [48]. Furthermore, ERK1/2 is able to inhibit STAT3 phosphorylation, which negatively regulates CT-1-induced hypertrophic responses in cardiomyocytes [46].

Physiological heart hypertrophy is associated with normal or enhanced cardiac function. In contrast, pathological cardiac hypertrophy that occurs in cardiovascular disease (*e.g.*, hypertension) is associated with decreased contractility ultimately leading to heart failure (HF), a condition where the heart cannot sustain the supply of oxygenated blood to the body. Early cardiac hypertrophy (early remodeling) seen in the setting of cardiovascular disease reduces wall stress and helps the heart to maintain cardiac output. Over time, chronic stress resulting from chronic pressure, inadequate vascularization and increased sympathetic tone can lead to further remodeling which involves dilatation of the left ventricle, accumulation of collagen (cardiac fibrosis), and loss of cardiac myocytes (late remodeling) resulting in HF [49]. The contribution of CT-1 to either physiological or maladaptive cardiac hypertrophy is unclear. In numerous experimental studies CT-1 acts as a potent cardiac survival factor, and promotes cardiomyocyte hypertrophy *in vitro* [29,50] but its *in vivo* effects are more difficult to define. Jin et al. explored the consequences of administration of chronic rCT-1 to healthy mice and observed an increased heart weight/body weight ratio [51]. On the contrary, treatment of *ob/ob* mice for 10 days with rCT-1 did not induce cardiac hypertrophy [52]. In line with this study, Rajuetet al. observed that administration of CNTF (using the same receptors than CT-1) regressed the established left ventricular hypertrophy in *ob/ob* mice [53]. Moreover, the protective effects against ventricular remodeling by CT-1 were shown using transplantation of CT-1-expressing skeletal myoblast into myocardium [54]. Likewise, chronic administration of LIF did not cause hypertrophy and improved cardiac performance [55]. LIF as well as CT-1 is upregulated in the hypertrophied adult heart in response to hemodynamic overload [56]. CT-1 upregulation appears to be required for the development of an appropriate myocardial response to injury by activating the gp130 signaling cascade and inhibiting myocyte apoptosis.

The contribution of CT-1 signaling to contractile dysfunction is unclear. It has been reported that chronically augmented CT-1 has detrimental effects on cardiac contractility. However, the experiments have been done in engineered heart tissue (EHT) [57] and although EHTs are well suitable as a test system its physiological relevance is not well established. Recently, Ruiz-Hurtado reported that CT-1 modulates Ca^{2+} handling in cultured cardiac ventricular myocytes. They showed that CT-1 increases L type Ca^{2+} current by a PKA-dependent mechanism [58] but further studies are needed to implicate abnormal Ca^{2+} handling induced by CT-1 in the pathophysiology of cardiovascular diseases. Conversely, other studies point to the idea that gp130 ligands can contribute to heart protection in chronic stress. Thus, it has been reported that long-standing hypertension in the spontaneously hypertensive rat is associated with reduced LIFR expression in the heart and that loss of gp130/LIFR signaling correlates with cardiac myocyte apoptosis and transition from adaptive hypertrophy to HF [59]. In line with this finding, as we mentioned before, cardiac restrictive gp130 null mice under biomechanical stress induced by pressure overload did not show adaptive hypertrophic response and rapidly develop cardiac dysfunction [18]. Importantly, in myocardial biopsies from patients with heart failure gp130 or LIFR are decreased while CT-1 is upregulated [60]. Likely, in these circumstances there is a defective myocardial response to increased CT-1 levels.

Recently, Kanazawa et al. using a Dahl salt sensitive rat to provoke HF under chronic salt-induced hypertension, showed that gp130 cytokines, LIF and CT-1 play pivotal role in cholinergic transdifferentiation of cardiac sympathetic nerves [61]. This cholinergic transdifferentiation appears to be beneficial in the failing hearts where it represents a critical endogenous protective mechanism of cardiac myocytes (Fig. 1).

2.2. Nervous system

CT-1 has been shown to play an essential role in neural tissue development and also in the protection of the mature nervous system against a variety of injuries and dysfunctions.

In the original reports by Pennica and coworkers, the authors showed that CT-1 had a much wider range of activities than its ability to induce cardiac myocyte hypertrophy. They found that CT-1 was able to modulate the transmitter phenotype of sympathetic neurons by inhibiting tyrosine hydroxylase activity (a noradrenergic marker) while stimulating choline actetyltransferase activity (a cholinergic marker) and that CT-1 was able to promote the survival of dopaminergic neurons [9].

Soon after its discovery, it was demonstrated that CT-1-like protein was expressed in fetal mouse choroid plexus (CP) [19] and that CT-1 mRNA could be detected in fetal forebrain and forebrain-neuroepithelial cells cultures [62]. More recently, CT-1 was shown to be expressed by CP cells, ependymal cells (particularly in those lining the ventricular neuroaxis) and leptomeninges in developing postnatal and adult rat brain. It was observed that CT-1 staining was generally more intense at their apical surface with some ependymal cells exhibiting a perinuclear distribution [63]. These authors proposed that CT-1 was released from ependymal cells to act as a CSF-borne signal. Two lines of evidence support this possibility. First, CT-1 was detected at levels (2–5 ng/ml) sufficient for neurotrophic activity in conditioned culture medium from viable CP cells [2] and secondly CT-1 was detected in human CP and CSF. It has been suggested that CT-1 levels within CSF are controlled by a release mechanism dependent on CP dynamics and structural integrity. CP have been sometimes considered as a circumventricular organs (CVOs) [64] containing potential niches of stem/precursor cells that can be activated by injury [65,66]. In line with this observation, Ochiai and coworkers demonstrated that CT-1 was able to signal in fetal neuroepithelial cells (also called neuron stem cells, NSC, in the early-gestational to mid-gestational stages) and to induce premature astrocyte formation in cultured cortical precursors in a synergistic manner with bone morphogenetic protein (BMP)-2 *via* a gp130-JAK–STAT3 pathway.

More recently, it has been shown that CT-1 is expressed in the embryonic cortex being involved in astrogenesis in the mammal brain [67]. Although CT-1 is barely detected in mouse brain at E12 or E13, its presence is prominent at E17.5, coincident with the emergence of astrocytes *in vivo*. At this time point transcripts for receptor components for CT-1, *i.e.* LIFR and gp130, were also detectable in the fetal brain. The authors strongly argue that embryonic neuron–derived CT-1 provides a key extrinsic signal for determining generation of astrocytes at the appropriate time and in the appropriate number in the developing cortex. In fact, they showed a very dramatic decline in astrocyte formation in precursors depleted of CT-1, as well as reduction by 50–75% of astrocytes in the neonatal cortex of CT-1 deficient mice. However, this decrease was not as intense as that seen in mice deficient in LIFR or gp130 [67]. These findings support the model that only when neurogenesis is largely complete (reaching high levels of CT-1 in neurons) gliogenesis can commence. Accumulating evidence indicates that the main factors regulating the mechanism of astrogenesis in the mammalian brain from NSCs is the activation of

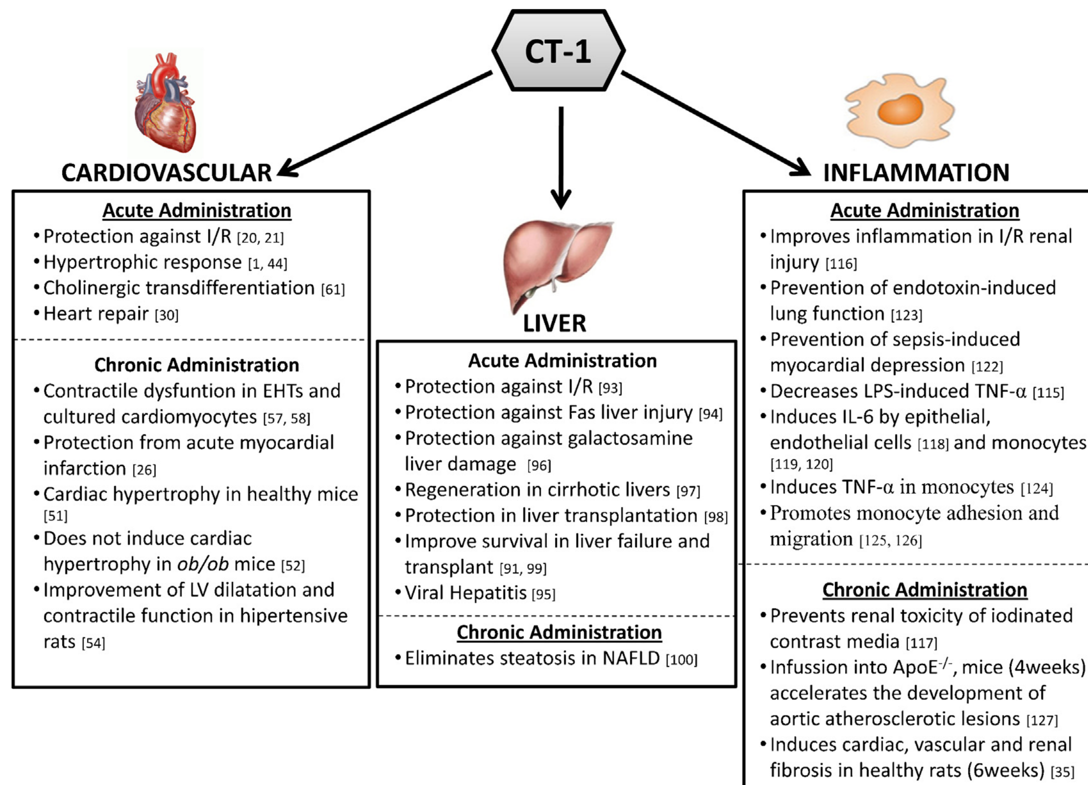


Fig. 1. Schematic representation of biological activities of cardiotrophin-1: ischemic/reperfusion (I/R); engineered heart tissue (EHT); left ventricular (LV); non-alcoholic fatty liver disease (NAFLD).

the gp130- JAK–STAT3 pathway and subsequent induction of astrocytic genes such as glial fibrillary acidic protein (GFAP) [68].

CT-1 has been shown to be a potent survival factor for isolated embryonic motoneurons from rat [2], mouse [69], and chick [70]. In line with this observations Oppenheim and coworkers demonstrated that CT-1 is the only ligand acting on gp130/LIFR receptors that appears to be physiologically required for the survival of motoneurons during embryonic development [70]. CT-1 is released from embryonic limb bud [2] and thus could act as a target-derived neurotrophic factor on responsive motoneurons. Retrograde axonal transport of CT-1 has been documented [71]. The importance of skeletal muscle for motoneurons during embryonic development is manifested by the observation that ablation of skeletal muscle by genetic manipulation in mice leads to complete loss of motoneurons between E14 and birth [72]. In mouse, the deletion of LIFR or gp130 induced a reduction of motoneurons in brainstem nuclei and spinal cord in the range of 40% suggesting that other components from muscle are involved in the survival of motoneurons acting *via* independent of gp130. CT-1 null mice showed less percentage of motoneurons loss than that LIFR [73] or gp130 [74] deficient mice indicating that other factors acting together with CT-1 promote the survival of motoneurons in the embryonic period. In keeping with these findings, measurement of grip strength in 4-month-old CT-1$^{-/-}$ mice revealed a significant reduction of muscle strength in comparison to wild-type controls [70].

Motoneurons and preganglionic sympathetic neurons (PSNs) share common progenitor cells [75]. PSNs participate in the regulation of autonomic functions; their targets are the peripheral autonomic ganglia, intra-adrenal and extra-adrenal chromaffin cells. Oberle et al. showed that CT-1 is necessary to establish and maintain a correct number of PSNs during embryonic development. CT-1 was expressed in both spinal cord and adrenal gland. Whether CT-1 acts on PSNs as a target-derived or local factor remains to be investigated [76].

Notably, it has been reported that administration of rCT-1 by means of an adenovirus encoding CT-1 prevented motoneuron cell death and long-term motor axonal degeneration in a mouse model of progressive motor neuropathy (*pmm*). CT-1 inhibited muscle denervation and improved neuromuscular functions in this mouse model [77]. Similar results were obtained in the same animal model of *pmm* by electroporation with a plasmid encoding CT-1 [78]. CT-1 also provided therapeutic benefit in both functional and morphological parameters in a mouse model of spinal muscular atrophy (SMA) [79,80]. Furthermore, adenovirus-mediated gene transfer of CT-1 or the administration of rCT-1 delayed neurogenic muscular atrophy and progressive neuromuscular deficiency in an experimental model of amyotrophic lateral sclerosis (ALS) [79,81]. Finally, adenoviral CT-1 gene transfer into the injured cord promoted survival and regeneration of rubrospinalneurons in adult rats [82] and promyelinating effects of CT-1 have been demonstrated *in vitro* by Stankoff et al. [83].

The neuroprotective activity of CT-1 is manifested by its ability to reduce the death of cultured cortical neurons caused by free radical-induced oxidative stress [84] and excitatory damage [85]. Also administration of CT-1 prevented postnatal brain injury after focal cerebral ischemic insults [84,86]. Moreover, transplantation of neural stem cells transduced with CT-1 reduced the susceptibility to recurrent seizures by repairing and replacing lost neurons in an experimental model for epilepsy [87]. Importantly, it has been shown that Alzheimer's disease (AD) transgenic mouse models exhibit a marked reduction of CT-1 expression in hippocampus and that restoration of CT-1 tissue levels in these animals resulted in noticeable improvement on cognitive function [88]. Moreover, intracerebroventricular administration of CT-1 for 14 days improved learning memory deficits and alleviated

neuroinflammation in high-fat-diet-induced cognitive deficits in mice [89]. These data provide the rationale for future studies aimed at exploring a potential therapeutic role of CT-1 in cognitive disorders including AD (Fig. 2).

2.3. Liver

Soon after its discovery, CT-1 was shown to be a potent inducer of the acute phase response in rat primary hepatocytes [2,90] and subsequent studies showed that CT-1 is an essential factor in the defense of the liver against a variety of insults [91–95]. In the liver CT-1 mRNA is expressed by both hepatocytes and non-parenchymal cells [94]. Interestingly it has been shown that exposure to pro-oxidants such as the H_2O_2 analogue *tert*-butyl-hydroperoxide causes hepatocytes to release CT-1 [93]. The fact that CT-1 deficient mice are susceptible to ischemia/reperfusion liver injury and other pro-apoptotic insults indicates that CT-1 acts as a natural defense of the liver against damage. Consistently, it has been shown that administration of rCT-1 affords protection against ischemia/reperfusion liver damage [93], galactosamine [96] and hepatitis of viral origin [95]. An adenovirus encoding CT-1 protected rats against fulminant hepatitis induced by massive hepatic resection [91]. CT-1 enhanced regeneration of cirrhotic livers through promotion of angiogenesis and cell proliferation [97]. Furthermore, the administration of CT-1 to donors decreased ischemia/reperfusion damage in liver transplantation in an experimental pig model [98]. Finally, the administration of adenovirus encoding CT-1 improved liver function and the survival of small-for-size grafts [99]. In liver cells, CT-1 activates essential cytoprotective and anti-apopotic pathways such as STAT-3, ERK and AKT. These data indicate that CT-1 deserves testing as a hepatoprotective molecule in situations of acute severe liver damage. Recently, it was found that chronic treatment with rCT-1 was able to eliminate steatosis from the liver of mice with non-alcoholic fatty liver disease (NFALD). This effect was mediated by AMPK activation, suppression of lipogenesis and stimulation of hepatic fatty acid oxidation [100] pointing to CT-1 as a potential therapy for non-alcoholic steatohepatitis (NASH) (Fig. 1).

2.4. Energy homeostasis and intermediate metabolism

In the last years several studies from our group and others have pointed out that CT-1 might also play a key role in the regulation of body weight and intermediate metabolism. The role of CT-1 in the regulation of body weight became clear by two observations: (1) the lack of CT-1 in mice leads to the development of adult-onset obesity [52]; (2) chronic rCT-1 administration is able to reduce body weight and fat accumulation in diet-induced and genetically obese rodents [52,89]. The body weight lowering actions of CT-1 can be explained by its inhibitory action on food intake and its ability to stimulate energy expenditure acting both at central level and on peripheral metabolic organs, including adipose tissue, liver and muscle. Regarding food intake regulation, studies of our group revealed that CT-1 activates hypothalamic anorexigenic pathways, including STAT-3 and S6 ribosomal proteins [52]. A similar effect has been described for CNTF, which also activates leptin-like anorexigenic pathways [101]. It is unclear whether CT-1 can act as a physiological regulator of calorie intake, which would be an interesting scientific question to further pursue in the context of obesity.

CT-1 seems to play an important role in the regulation of energy expenditure, the administration of CT-1 increases whole-body oxygen consumption as compared with *pair-fed* animals [52]. A striking feature of mature CT-$1^{-/-}$ mice is the presence of a high respiratory quotient (RQ), indicative of impaired fat utilization. Interestingly, CT-1 treatment decreases both fasting and post-prandial RQ, causes a rapid clearance of circulating fatty acids after a lipid load, reducing postprandial hypertriglyceridemia [52]. Taken together these facts support the pivotal role of CT-1 in the regulation of lipid homeostasis. In fact, several studies from our group have suggested that CT-1 promotes metabolic pathways involved fat breakdown and oxidation while reduces lipid storage pathways. In line with this, a recent study of our group found that in liver of obese mice, chronic CT-1 administration inhibits *de novo* lipogenesis, stimulates fatty acid (FA) oxidation and promotes lipoautophagy, which could also contribute to the delipidating and anti-steatotic effects of CT-1 in liver [100]. In muscle, CT-1 also

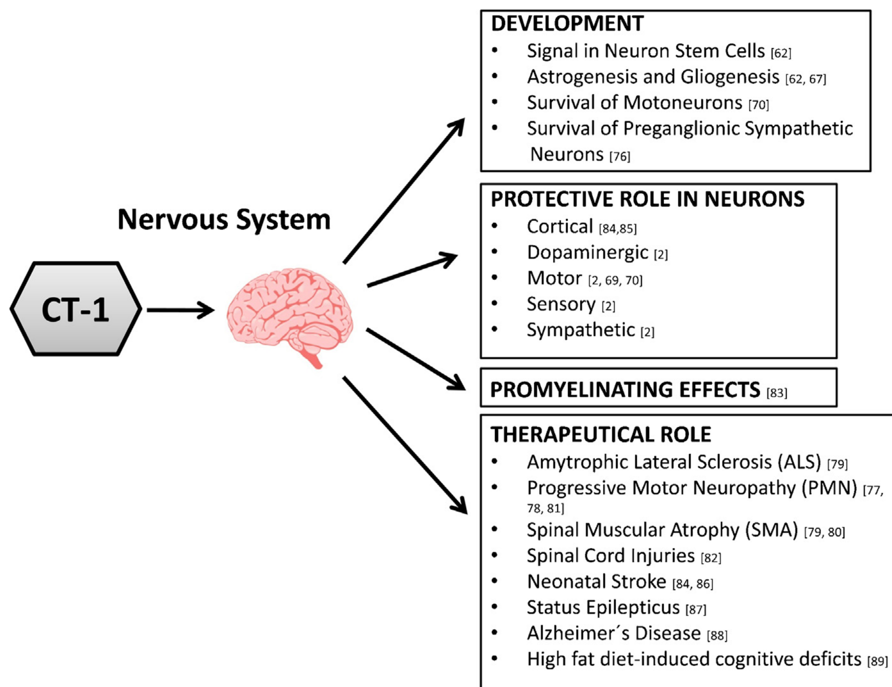


Fig. 2. Schematic representation of the biological effects of cardiotrophin-1 on nervous system.

increases FA oxidation [52] and AMPK is responsible in mediating both the inhibitory effect of CT-1 on hepatic lipogenesis and the stimulatory action of the cytokine on FA oxidation in liver and muscle [52,100]. Similar actions have been described for other-related cytokines such as leptin [102] and CNTF [103].

White adipose tissue (WAT) is also a target organ for CT-1 actions; indeed, CT-1 is a potent regulator of signaling in adipocytes *in vitro* and *in vivo* [104]. CT-1 administration not only causes a decrease in fat mass, but also induces a switch of WAT lipid metabolism, downregulating lipogenic genes and stimulating lipolysis and fatty acid oxidation. In fact, adipocytes from CT-1-treated mice exhibited increased lipolytic response to isoproterenol [52]. Interestingly, we have recently described that the lipolytic actions of CT-1 are mediated by its ability to regulate the levels and activity of some of the main lipases and lipid droplet proteins involved in the lipolytic pathway such as PKA-mediated phosphorylation of perilipin and HSL, and adipose triglyceride lipase (ATGL) [105].

Another interesting property of CT-1 is its ability to promote browning/britening of white adipose tissue [52]. Taking together all of these observations support that CT-1 induces a dramatic remodeling of adipose tissue. These facts could be also contributing to the anti-obesity actions of CT-1, since stimulating conversion of white fat to metabolically active brite (brown-in-white) adipocytes has been proposed as a promising strategy against obesity and its deleterious associateddisorders [106].

CT-1 has also profound actions on glucose metabolism. Indeed, chronic CT-1 administration to obese mice reduced the associated hyperglycemia and hyperinsulinemia and improves the insulin tolerance test and insulin signaling in muscle. An interesting finding of our group is that the glucose-lowering properties of CT-1 are also observed in mice with streptozotocin (STZ)-induced insulin deficiency, demonstrating an insulin-independent effect of CT-1 on glucose homeostasis [52]. In line with this, the study of Jiménez-González et al. [107] found that CT-1 has a protective effect from apoptosis in MIN6B1 cells and murine islets, and that CT-1 enhances glucose-stimulated insulin secretion in MIN6B1 cells. Studies *in vitro* and *in vivo* support the insulin-sensitizing effect of CT-1 in muscle [52]. However, some controversial data regarding CT-1-induced insulin resistance have been described after chronic treatment with the cytokine in cultured adipocytes [52,104]. Taking into account that muscle accounts for 80–90% of whole-body glucose uptake, it has been suggested that insulin resistance in WAT may not necessarily cause systemic insulin resistance.

It is important to note that CT-1 is a nutritionally regulated gene, which levels are induced during fasting and decreased by refeeding, suggesting that CT-1 may have a role in the biological adaptation to starvation [52]. However, little is known about the main regulators of CT-1 production during obesity and metabolic syndrome. In this context, several studies have suggested that circulating levels of CT-1 are higher in obese subjects [108] with metabolic syndrome [109]. Interestingly, obese children subjected to a weight loss program exhibited a reduction of CT-1 plasma levels in parallel to the decrease in body weight and body fat mass [110]. Although WAT was proposed to be a main source of this cytokine [109], this contention is discordant with data showing reduced CT-1 mRNA abundance in WAT from obese mice [111]. Other tissues like liver, skeletal muscle and heart may be alternative origins of the increased plasma CT-1 concentration in obesity. Indeed, we have recently reported that CT-1 is upregulated in steatotic livers from both obese mice and humans [100]. Skeletal muscle is an important source of circulating CT-1. Muscle expression levels of CT-1 exceed those in WAT and are more elevated in obese mice than in wild type animals (our unpublished observations). CT-1 seemingly behaves as a myokine endowed with autocrine, paracrine and/or endocrine effects. In fact, it has been reported that physical exercise increases the levels of CT-1 in serum both in healthy human subjects and elite athletes [112].

Altogether, the beneficial effects of CT-1 on energy homeostasis as well as on glucose and lipid metabolism suggest that the overproduction of CT-1 in obesity, metabolic syndrome and NALFD could be considered as a compensatory phenomenon to promote fat oxidation and to counteract the development of the metabolic disturbances associated to obesity. Interestingly, a recent study analyzing the impact of genetic variants of the CT-1 gene locus *CTF1* on insulin sensitivity in humans showed a robust association of the *CTF1* SNP rs8046707 with the response to insulin [113] (Fig. 3).

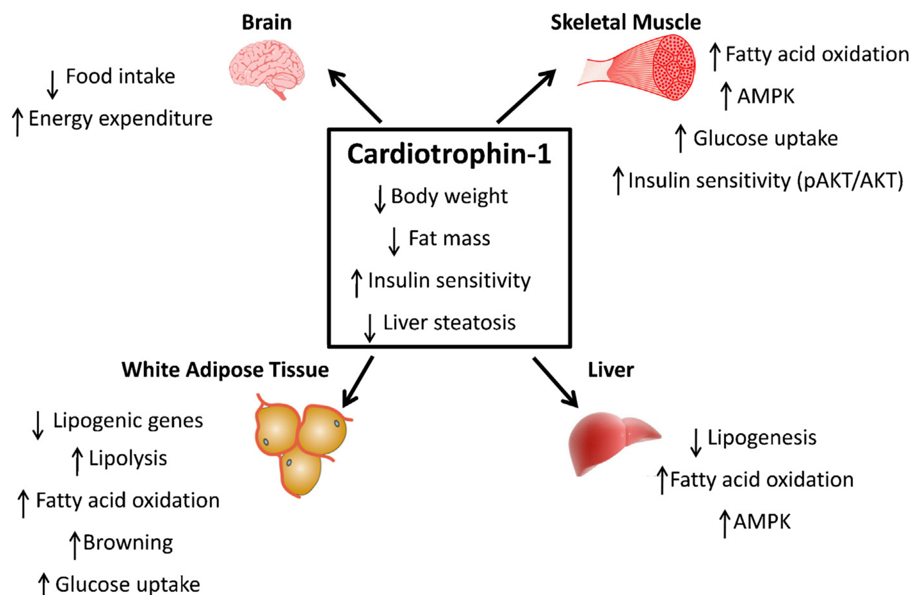


Fig. 3. Metabolic effects of cardiotrophin-1. CT-1 modulates food intake and energy expenditure, as well as several metabolic pathways in liver, muscle and white adipose tissue. In general CT-1 promotes oxidative metabolism, reduces lipid accumulation and improves the sensitivity to insulin.

2.5. Inflammation

It has been shown that cell signaling cascade induced by members of the IL-6 family of cytokines cross talk with pathways (mainly NFkB and p38 MAPK) activated by pro-inflammatory cytokines such as TNF-α and IL-1β [114] anticipating a role of CT-1 in the modulation of inflammation.

Different reports have shown that CT-1 attenuates the expression of pro-inflammatory cytokines in a variety of experimental models of tissue damage. Indeed, it was found that CT-1 lowered TNF-α levels in a model of endotoxemia [115], in orthotopic liver transplant [98], in obese mice [100] and in renal ischemia/reperfusion injury [116]. Also, rCT-1 administration prevented the renal toxicity of iodinated contrast media in rats [117]. Moreover, CT-1 has been reported to induce the production of IL-6 by epithelial [8], endothelial cells [118] and monocytes [119,120] with resulting protective, pro-inflammatory and anti-inflammatory effects [121]. In this line, CT-1 was shown to prevent myocardial depression associated with sepsis [122]. It has also been reported that CT-1 reduced neutrophil accumulation in the lung and reduced edema and pulmonary dysfunction in a model of LPS-induced acute lung injury [123]. In contrast, other studies observed that CT-1 increased TNF-α in human peripheral blood mononuclear cells [124] and stimulated monocyte adhesion and migration [125,126] suggesting a contribution to the inflammatory process [125]. In line with these studies, four-week infusion of rCT-1 into ApoE$^{-/-}$ significantly accelerated the development of aortic atherosclerotic lesions [127]. López-Andres et al. reported that chronic exposure of rCT-1 for six weeks was associated with cardiac, vascular and renal fibrosis resulting in further structural and functional damage [35]. The opposite activities of CT-1 may reflect the dose and time dependency of its mechanism of action.

In different models and experimental settings CT-1 demonstrated cytoprotective activity *via* NFkB activation, as in the protection of cardiomyocytes or the kidney against ischemia/reperfusion injury [24,116]. It should be noted that NFkB activation may mediate both pro-inflammatory and anti-inflammatory effects and that this nuclear factor may participate in the initiation and in the resolution of inflammation depending on time and biological context. In contrast to the anti-apoptotic role of NFkB observed in many cell types [128], it may fulfill anti-inflammatory functions by causing apoptosis of inflammatory cells [129]. Also, NFkB may contribute to attenuate inflammation by stimulating a switch in the biological program of macrophages, from a pro-inflammatory M1 phenotype to an anti-inflammatory, growth-promoting and wound-repairing M2 phenotype. Whether NFkB plays pro or anti-inflammatory effects is influenced in part by its levels, timing and experimental conditions. Thus, opposite effects have been observed depending on its early or later activation. Likewise, partial inhibition of IKKb may be protective while complete abrogation of its activity has the opposite effect [130] (Fig. 1).

3. Conclusions

CT-1 is a multifaceted cytokine with potent cytoprotective properties, which holds promise as a therapeutic agent. Regarding the nervous system, CT-1 is essential for motoneuron maintenance and survival during development and is highly effective in protecting neurons against stressful or noxious stimuli being worth exploring as a potential therapy in a variety of neuronal disorders. CT-1 is a key regulator of energy metabolism, which deeply influences glucose and lipid metabolism in rodents pointing to a potential role of this molecule in the treatment of obesity and the metabolic syndrome. The observed elevation of CT-1 in obese subjects likely represents a compensatory mechanism to attenuate obesity-related metabolic dysfunction. Robust hepatoprotective effects of CT-1 have been described in different experimental models of acute liver damage, like Fas-induced apoptosis or ischemia/reperfusion injury rising hopes for potential application of this cytokine in acute liver failure. Importantly, CT-1 is highly effective at resolving liver steatosis deserving consideration for the treatment of NAFLD.

In the cardiovascular system, CT-1 protects cardiomyocytes against stress-induced apoptosis and induces cardiac hypertrophy in the overloaded myocardium. More work should be performed to characterize the pathophysiological role of CT-1 in heart failure and ischemic myocardiopathies. Finally, CT-1 has been shown to modulate the inflammatory reaction in directions that may differ according to the specific tissue, timing and local concentration. A deeper understanding of the involvement of CT-1 in inflammation is warranted.

In summary, CT-1 has shown to be an interesting cytokine; however, due to its ability to regulate several organs systems the capacity to manipulate its function for therapeutic benefit directing to the target organ is the key challenge that remains to be investigated.

Conflict of interest statement

None.

Acknowledgments

This work was supported by FIS Project Grant (PI13/01851) awarded to MB. ML-Y is a recipient of doctoral grant from Asociacion de Amigos University of Navarra.

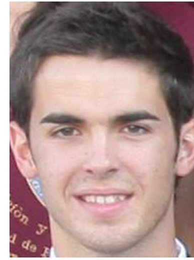
References

[1] D. Pennica, K.L. King, K.J. Shaw, E. Luis, J. Rullamas, S.M. Luoh, et al., Expression cloning of cardiotrophin 1, a cytokine that induces cardiac myocyte hypertrophy, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 1142–1146.
[2] D. Pennica, V. Arce, T.A. Swanson, R. Vejsada, R.A. Pollock, M. Armanini, et al., Cardiotrophin-1, a cytokine present in embryonic muscle, supports long-term survival of spinal motoneurons, Neuron 17 (1996) 63–74.
[3] M. Funamoto, S. Hishinuma, Y. Fujio, Y. Matsuda, K. Kunisada, H. Oh, et al., Isolation and characterization of the murine cardiotrophin-1 gene: expression and norepinephrine-induced transcriptional activation, J. Mol. Cell. Cardiol. 32 (2000) 1275–1284.
[4] M. Ishikawa, Y. Saito, Y. Miyamoto, K. Kuwahara, E. Ogawa, O. Nakagawa, et al., cDNA cloning of rat cardiotrophin-1 (CT-1): augmented expression of CT-1 gene in ventricle of genetically hypertensive rats, Biochem. Biophys. Res. Commun. 219 (1996) 377–381.
[5] J. Erdmann, S. Hassfeld, H. Kallisch, E. Fleck, V. Regitz-Zagrosek, Cloning and characterization of the 5′-flanking region of the human cardiotrophin-1 gene, Biochem. Biophys. Res. Commun. 244 (1998) 494–497.
[6] D. Pennica, T.A. Swanson, K.J. Shaw, W.J. Kuang, C.L. Gray, B.G. Beatty, et al., Human cardiotrophin-1: protein and gene structure, biological and binding activities, and chromosomal localization, Cytokine 8 (1996) 183–189.
[7] D. Derouet, F. Rousseau, F. Alfonsi, J. Froger, J. Hermann, F. Barbier, et al., Neuropoietin, a new IL-6-related cytokine signaling through the ciliary neurotrophic factor receptor, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 4827–4832.
[8] O. Robledo, M. Fourcin, S. Chevalier, C. Guillet, P. Auguste, A. Pouplard-Barthelaix, et al., Signaling of the cardiotrophin-1 receptor. Evidence for a third receptor component, J. Biol. Chem. 272 (1997) 4855–4863.
[9] D. Pennica, K.J. Shaw, T.A. Swanson, M.W. Moore, D.L. Shelton, K.A. Zioncheck, et al., Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex, J. Biol. Chem. 270 (1995) 10915–10922.
[10] J.G. Cheng, D. Pennica, P.H. Patterson, Cardiotrophin-1 induces the same neuropeptides in sympathetic neurons as do neuropoietic cytokines, J. Neurochem. 69 (1997) 2278–2284.
[11] M. Kurdi, G.W. Booz, Jak inhibition, but not Stat1 knockdown, blocks the synergistic effect of IFN-gamma on Fas-induced apoptosis of A549 human non-small cell lung cancer cells, J. Interferon Cytokine Res. 27 (2007) 23–31 (the official journal of the International Society for Interferon and Cytokine Research).
[12] L. Hendry, S. John, Regulation of STAT signalling by proteolytic processing, Eur. J. Biochem./FEBS. 271 (2004) 4613–4620.
[13] X.P. Chen, J.A. Losman, P. Rothman, SOCS proteins, regulators of intracellular signaling, Immunity 13 (2000) 287–290.

[14] B. Liu, J. Liao, X. Rao, S.A. Kushner, C.D. Chung, D.D. Chang, et al., Inhibition of Stat1-mediated gene activation by PIAS1, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 10626–10631.
[15] G. Kumar, S. Gupta, S. Wang, A.E. Nel, Involvement of Janus kinases, p52shc, Raf-1, and MEK-1 in the IL-6-induced mitogen-activated protein kinase cascade of a growth-responsive B cell line, J. Immunol. 153 (1994) 4436–4447.
[16] M. Nakafuku, T. Satoh, Y. Kaziro, Differentiation factors, including nerve growth factor, fibroblast growth factor, and interleukin-6, induce an accumulation of an active Ras. GTP complex in rat pheochromocytoma PC12 cells, J. Biol. Chem. 267 (1992) 19448–19454.
[17] M. Takahashi-Tezuka, M. Hibi, Y. Fujitani, T. Fukada, T. Yamaguchi, T. Hirano, Tec tyrosine kinase links the cytokine receptors to PI-3 kinase probably through JAK, Oncogene 14 (1997) 2273–2282.
[18] H. Hirota, J. Chen, U.A. Betz, K. Rajewsky, Y. Gu, J. Ross Jr., et al., Loss of a gp130 cardiac muscle cell survival pathway is a critical event in the onset of heart failure during biomechanical stress, Cell 97 (1999) 189–198.
[19] Z. Sheng, D. Pennica, W.I. Wood, K.R. Chien, Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival, Development 122 (1996) 419–428.
[20] A. Stephanou, B. Brar, R. Heads, R.D. Knight, M.S. Marber, D. Pennica, et al., Cardiotrophin-1 induces heat shock protein accumulation in cultured cardiac cells and protects them from stressful stimuli, J. Mol. Cell. Cardiol. 30 (1998) 849–855.
[21] B.K. Brar, A. Stephanou, Z. Liao, R.M. O'Leary, D. Pennica, D.M. Yellon, et al., Cardiotrophin-1 can protect cardiac myocytes from injury when added both prior to simulated ischaemia and at reoxygenation, Cardiovasc. Res. 51 (2001) 265–274.
[22] Z. Liao, B.K. Brar, Q. Cai, A. Stephanou, R.M. O'Leary, D. Pennica, et al., Cardiotrophin-1 (CT-1) can protect the adult heart from injury when added both prior to ischaemia and at reperfusion, Cardiovasc. Res. 53 (2002) 902–910.
[23] K. Kuwahara, Y. Saito, I. Kishimoto, Y. Miyamoto, M. Harada, E. Ogawa, et al., Cardiotrophin-1 phosphorylates akt and BAD, and prolongs cell survival via a PI3K-dependent pathway in cardiac myocytes, J. Mol. Cell. Cardiol. 32 (2000) 1385–1394.
[24] R. Craig, A. Larkin, A.M. Mingo, D.J. Thuerauf, C. Andrews, P.M. McDonough, et al., p38 MAPK and NF-kappa B collaborate to induce interleukin-6 gene expression and release. Evidence for a cytoprotective autocrine signaling pathway in a cardiac myocyte model system, J. Biol. Chem. 275 (2000) 23814–23824.
[25] E.C. Lopez-Diez, C.L. Winder, L. Ashton, F. Currie, R. Goodacre, Monitoring the mode of action of antibiotics using Raman spectroscopy: investigating subinhibitory effects of amikacin on Pseudomonas aeruginosa, Anal. Chem. 77 (2005) 2901–2906.
[26] Y. Ruixing, Y. Dezhai, L. Jiaquan, Effects of cardiotrophin-1 on hemodynamics and cardiomyocyte apoptosis in rats with acute myocardial infarction, J. Med. Investig.: JMI 51 (2004) 29–37.
[27] Y. Oshima, Y. Fujio, T. Nakanishi, N. Itoh, Y. Yamamoto, S. Negoro, et al., STAT3 mediates cardioprotection against ischemia/reperfusion injury through metallothionein induction in the heart, Cardiovasc. Res. 65 (2005) 428–435.
[28] D.S. Latchman, Cardiotrophin-1: a novel cytokine and its effects in the heart and other tissues, Pharmacol. Ther. 85 (2000) 29–37.
[29] J. Railson, K. Lawrence, A. Stephanou, B. Brar, D. Pennica, D. Latchman, Cardiotrophin-1 reduces stress-induced heat shock protein production in cardiac myocytes, Cytokine 12 (2000) 1741–1744.
[30] D.H. Freed, R.H. Cunnington, A.L. Dangerfield, J.S. Sutton, I.M. Dixon, Emerging evidence for the role of cardiotrophin-1 in cardiac repair in the infarcted heart, Cardiovasc. Res. 65 (2005) 782–792.
[31] M. Ishikawa, Y. Saito, Y. Miyamoto, M. Harada, K. Kuwahara, E. Ogawa, et al., A heart-specific increase in cardiotrophin-1 gene expression precedes the establishment of ventricular hypertrophy in genetically hypertensive rats, J. Hypertens. 17 (1999) 807–816.
[32] J. Pan, K. Fukuda, H. Kodama, M. Sano, T. Takahashi, S. Makino, et al., Involvement of gp130-mediated signaling in pressure overload-induced activation of the JAK/STAT pathway in rodent heart, Heart Vessels 13 (1998) 199–208.
[33] S. Talwar, S.K. Choudhary, Tuberculous aneurysms of the aorta, J. Thorac. Cardiovasc. Surg. 125 (2003) 1184.
[34] B. Lopez, A. Gonzalez, J.J. Lasarte, P. Sarobe, F. Borras, A. Diaz, et al., Is plasma cardiotrophin-1 a marker of hypertensive heart disease? J. Hypertens. 23 (2005) 625–632.
[35] N. Lopez-Andres, A. Rousseau, R. Akhtar, L. Calvier, C. Inigo, C. Labat, et al., Cardiotrophin 1 is involved in cardiac, vascular, and renal fibrosis and dysfunction, Hypertension 60 (2012) 563–573.
[36] B. Lopez, J.M. Castellano, A. Gonzalez, J. Barba, J. Diez, Association of increased plasma cardiotrophin-1 with inappropriate left ventricular mass in essential hypertension, Hypertension 50 (2007) 977–983.
[37] B. Lopez, A. Gonzalez, R. Querejeta, J. Barba, J. Diez, Association of plasma cardiotrophin-1 with stage C heart failure in hypertensive patients: potential diagnostic implications, J. Hypertens. 27 (2009) 418–424.
[38] L. Monserrat, B. Lopez, A. Gonzalez, M. Hermida, X. Fernandez, M. Ortiz, et al., Cardiotrophin-1 plasma levels are associated with the severity of hypertrophy in hypertrophic cardiomyopathy, Eur. Heart J. 32 (2011) 177–183.
[39] S. Ravassa, O. Beloqui, N. Varo, J. Barba, B. Lopez, J. Beaumont, et al., Association of cardiotrophin-1 with left ventricular systolic properties in asymptomatic hypertensive patients, J. Hypertens. 31 (2013) 587–594.
[40] M.R. Zile, S.M. Desantis, C.F. Baicu, R.E. Stroud, S.B. Thompson, C.D. McClure, et al., Plasma biomarkers that reflect determinants of matrix composition identify the presence of left ventricular hypertrophy and diastolic heart failure, Circ. Heart Fail. 4 (2011) 246–256.
[41] T. Aoyama, Y. Takimoto, D. Pennica, R. Inoue, E. Shinoda, R. Hattori, et al., Augmented expression of cardiotrophin-1 and its receptor component, gp130, in both left and right ventricles after myocardial infarction in the rat, J. Mol. Cell. Cardiol. 32 (2000) 1821–1830.
[42] J.J. Jacoby, A. Kalinowski, M.G. Liu, S.S. Zhang, Q. Gao, G.X. Chai, et al., Cardiomyocyte-restricted knockout of STAT3 results in higher sensitivity to inflammation, cardiac fibrosis, and heart failure with advanced age, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 12929–12934.
[43] K.R. Chien, E.N. Olson, Converging pathways and principles in heart development and disease: CV@CSH, Cell 110 (2002) 153–162.
[44] K.C. Wollert, T. Taga, M. Saito, M. Narazaki, T. Kishimoto, C.C. Glembotski, et al., Cardiotrophin-1 activates a distinct form of cardiac muscle cell hypertrophy. Assembly of sarcomeric units in series VIA gp130/leukemia inhibitory factor receptor-dependent pathways, J. Biol. Chem. 271 (1996) 9535–9545.
[45] J.E. Railson, Z. Liao, B.K. Brar, J.C. Buddle, D. Pennica, A. Stephanou, et al., Cardiotrophin-1 and urocortin cause protection by the same pathway and hypertrophy via distinct pathways in cardiac myocytes, Cytokine 17 (2002) 243–253.
[46] Z.J. Tian, W. Cui, Y.J. Li, Y.M. Hao, J. Du, F. Liu, et al., Different contributions of STAT3, ERK1/2, and PI3-K signaling to cardiomyocyte hypertrophy by cardiotrophin-1, Acta Pharmacol. Sin. 25 (2004) 1157–1164.
[47] N. Takahashi, Y. Saito, K. Kuwahara, M. Harada, K. Tanimoto, Y. Nakagawa, et al., Hypertrophic responses to cardiotrophin-1 are not mediated by STAT3, but via a MEK5-ERK5 pathway in cultured cardiomyocytes, J. Mol. Cell. Cardiol. 38 (2005) 185–192.
[48] Y. Jamshidi, A. Zourlidou, C.J. Carroll, J. Sinclair, D.S. Latchman, Signal-transduction pathways involved in the hypertrophic effect of hsp56 in neonatal cardiomyocytes, J. Mol. Cell. Cardiol. 36 (2004) 381–392.
[49] K.L. Weeks, J.R. McMullen, The athlete's heart vs. the failing heart: can signaling explain the two distinct outcomes? Physiology (Bethesda) 26 (2011) 97–105.
[50] Z. Sheng, K. Knowlton, J. Chen, M. Hoshijima, J.H. Brown, K.R. Chien, Cardiotrophin 1 (CT-1) inhibition of cardiac myocyte apoptosis via a mitogen-activated protein kinase-dependent pathway. Divergence from downstream CT-1 signals for myocardial cell hypertrophy, J. Biol. Chem. 272 (1997) 5783–5791.
[51] H. Jin, R. Yang, G.A. Keller, A. Ryan, A. Ko, D. Finkle, et al., In vivo effects of cardiotrophin-1, Cytokine 8 (1996) 920–926.
[52] M.J. Moreno-Aliaga, N. Perez-Echarri, B. Marcos-Gomez, E. Larequi, F.J. Gil-Bea, B. Viollet, et al., Cardiotrophin-1 is a key regulator of glucose and lipid metabolism, Cell Metab. 14 (2011) 242–253.
[53] S.V. Raju, M. Zheng, K.H. Schuleri, A.C. Phan, D. Bedja, R.M. Saraiva, et al., Activation of the cardiac ciliary neurotrophic factor receptor reverses left ventricular hypertrophy in leptin-deficient and leptin-resistant obesity, Proc. Natl. Acad. Sci. U.S.A. 103 (2006) 4222–4227.
[54] R. Toh, S. Kawashima, M. Kawai, T. Sakoda, T. Ueyama, S. Satomi-Kobayashi, et al., Transplantation of cardiotrophin-1-expressing myoblasts to the left ventricular wall alleviates the transition from compensatory hypertrophy to congestive heart failure in Dahl salt-sensitive hypertensive rats, J. Am. Coll. Cardiol. 43 (2004) 2337–2347.
[55] F.A. Zouein, M. Kurdi, G.W. Booz, LIF and the heart: just another brick in the wall? Eur. Cytokine Netw. 24 (2013) 11–19.
[56] F. Wang, Y. Seta, G. Baumgarten, D.J. Engel, N. Sivasubramanian, D.L. Mann, Functional significance of hemodynamic overload-induced expression of leukemia-inhibitory factor in the adult mammalian heart, Circulation 103 (2001) 1296–1302.
[57] O. Zolk, S. Engmann, F. Munzel, R. Krajcik, Chronic cardiotrophin-1 stimulation impairs contractile function in reconstituted heart tissue, Am. J. Physiol. Endocrinol. Metab. 288 (2005) E1214–E1221.
[58] G. Ruiz-Hurtado, N. Gomez-Hurtado, M. Fernandez-Velasco, E. Calderon, T. Smani, A. Ordonez, et al., Cardiotrophin-1 induces sarcoplasmic reticulum Ca(2+) leak and arrhythmogenesis in adult rat ventricular myocytes, Cardiovasc. Res. 96 (2012) 81–89.
[59] N. Lopez, N. Varo, J. Diez, M.A. Fortuno, Loss of myocardial LIF receptor in experimental heart failure reduces cardiotrophin-1 cytoprotection. A role for neurohumoral agonists? Cardiovasc. Res. 75 (2007) 536–545.
[60] O. Zolk, L.L. Ng, R.J. O'Brien, M. Weyand, T. Eschenhagen, Augmented expression of cardiotrophin-1 in failing human hearts is accompanied by diminished glycoprotein 130 receptor protein abundance, Circulation 106 (2002) 1442–1446.
[61] H. Kanazawa, M. Ieda, K. Kimura, T. Arai, H. Kawaguchi-Manabe, T. Matsuhashi, et al., Heart failure causes cholinergic transdifferentiation of cardiac sympathetic nerves via gp130-signaling cytokines in rodents, J. Clin. Investig. 120 (2010) 408–421.
[62] W. Ochiai, M. Yanagisawa, T. Takizawa, K. Nakashima, T. Taga, Astrocyte differentiation of fetal neuroepithelial cells involving cardiotrophin-1-induced activation of STAT3, Cytokine 14 (2001) 264–271.
[63] A.L. Gard, E. Gavin, V. Solodushko, D. Pennica, Cardiotrophin-1 in choroid plexus and the cerebrospinal fluid circulatory system, Neuroscience 127 (2004) 43–52.

[64] Y. Itokazu, M. Kitada, M. Dezawa, A. Mizoguchi, N. Matsumoto, A. Shimizu, et al., Choroid plexus ependymal cells host neural progenitor cells in the rat, Glia 53 (2006) 32–42.
[65] I. Decimo, F. Bifari, F.J. Rodriguez, G. Malpeli, S. Dolci, V. Lavarini, et al., Nestin- and doublecortin-positive cells reside in adult spinal cord meninges and participate in injury-induced parenchymal reaction, Stem Cells 29 (2011) 2062–2076.
[66] R. Lin, L. Iacovitti, Classic and novel stem cell niches in brain homeostasis and repair, Brain Res. (2015), http://dx.doi.org/10.1016/j.brainres.2015.04.029 [Epub ahead of print].
[67] F. Barnabe-Heider, J.A. Wasylnka, K.J. Fernandes, C. Porsche, M. Sendtner, D.R. Kaplan, et al., Evidence that embryonic neurons regulate the onset of cortical gliogenesis via cardiotrophin-1, Neuron 48 (2005) 253–265.
[68] C. MuhChyi, B. Juliandi, T. Matsuda, K. Nakashima, Epigenetic regulation of neural stem cell fate during corticogenesis, Int. J. Dev. Neurosci. 31 (2013) 424–433 (the official journal of the International Society for Developmental Neuroscience).
[69] V. Arce, A. Garces, B. de Bovis, P. Filippi, C. Henderson, B. Pettmann, et al., Cardiotrophin-1 requires LIFRbeta to promote survival of mouse motoneurons purified by a novel technique, J. Neurosci. Res. 55 (1999) 119–126.
[70] R.W. Oppenheim, S. Wiese, D. Prevette, M. Armanini, S. Wang, L.J. Houenou, et al., Cardiotrophin-1, a muscle-derived cytokine, is required for the survival of subpopulations of developing motoneurons, J. Neurosci. 21 (2001) 1283–1291 (the official journal of the Society for Neuroscience).
[71] H.B. Rind, R. Butowt, C.S. von Bartheld, Synaptic targeting of retrogradely transported trophic factors in motoneurons: comparison of glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, and cardiotrophin-1 with tetanus toxin, J. Neurosci. 25 (2005) 539–549 (the official journal of the Society for Neuroscience).
[72] U. Grieshammer, M. Lewandoski, D. Prevette, R.W. Oppenheim, G.R. Martin, Muscle-specific cell ablation conditional upon Cre-mediated DNA recombination in transgenic mice leads to massive spinal and cranial motoneuron loss, Dev. Biol. 197 (1998) 234–247.
[73] M. Li, M. Sendtner, A. Smith, Essential function of LIF receptor in motor neurons, Nature 378 (1995) 724–727.
[74] K. Nakashima, S. Wiese, M. Yanagisawa, H. Arakawa, N. Kimura, T. Hisatsune, et al., Developmental requirement of gp130 signaling in neuronal survival and astrocyte differentiation, J. Neurosci. 19 (1999) 5429–5434 (the official journal of the Society for Neuroscience).
[75] A. Schober, K. Unsicker, Growth and neurotrophic factors regulating development and maintenance of sympathetic preganglionic neurons, Int. Rev. Cytol. 205 (2001) 37–76.
[76] S. Oberle, A. Schober, V. Meyer, B. Holtmann, C. Henderson, M. Sendtner, et al., Loss of leukemia inhibitory factor receptor beta or cardiotrophin-1 causes similar deficits in preganglionic sympathetic neurons and adrenal medulla, J. Neurosci. 26 (2006) 1823–1832 (the official journal of the Society for Neuroscience).
[77] T. Bordet, H. Schmalbruch, B. Pettmann, A. Hagege, L. Castelnau-Ptakhine, A. Kahn, et al., Adenoviral cardiotrophin-1 gene transfer protects pmn mice from progressive motor neuronopathy, J. Clin. Investig. 104 (1999) 1077–1085.
[78] J.C. Lesbordes, T. Bordet, G. Haase, L. Castelnau-Ptakhine, S. Rouhani, H. Gilgenkrantz, et al., In vivo electrotransfer of the cardiotrophin-1 gene into skeletal muscle slows down progression of motor neuron degeneration in pmn mice, Hum. Mol. Genet. 11 (2002) 1615–1625.
[79] T. Bordet, J.C. Lesbordes, S. Rouhani, L. Castelnau-Ptakhine, H. Schmalbruch, G. Haase, et al., Protective effects of cardiotrophin-1 adenoviral gene transfer on neuromuscular degeneration in transgenic ALS mice, Hum. Mol. Genet. 10 (2001) 1925–1933.
[80] J.C. Lesbordes, C. Cifuentes-Diaz, A. Miroglio, V. Joshi, T. Bordet, A. Kahn, et al., Therapeutic benefits of cardiotrophin-1 gene transfer in a mouse model of spinal muscular atrophy, Hum. Mol. Genet. 12 (2003) 1233–1239.
[81] H. Mitsumoto, B. Klinkosz, E.P. Pioro, K. Tsuzaka, T. Ishiyama, R.M. O'Leary, et al., Effects of cardiotrophin-1 (CT-1) in a mouse motor neuron disease, Muscle Nerve 24 (2001) 769–777.
[82] Z.F. Zhang, W.H. Liao, Q.F. Yang, H.Y. Li, Y.M. Wu, X.F. Zhou, Protective effects of adenoviral cardiotrophin-1 gene transfer on rubrospinal neurons after spinal cord injury in adult rats, Neurotoxicity Res. 5 (2003) 539–548.
[83] B. Stankoff, M.S. Aigrot, F. Noel, A. Wattilliaux, B. Zalc, C. Lubetzki, Ciliary neurotrophic factor (CNTF) enhances myelin formation: a novel role for CNTF and CNTF-related molecules, J. Neurosci. 22 (2002) 9221–9227 (the official journal of the Society for Neuroscience).
[84] T.C. Wen, M.R. Rogido, J.E. Moore, T. Genetta, H. Peng, A. Sola, Cardiotrophin-1 protects cortical neuronal cells against free radical-induced injuries in vitro, Neurosci. Lett. 387 (2005) 38–42.
[85] G. Toth, H. Yang, R.A. Anguelov, J. Vettraino, Y. Wang, G. Acsadi, Gene transfer of glial cell-derived neurotrophic factor and cardiotrophin-1 protects PC12 cells from injury: involvement of the phosphatidylinositol 3-kinase and mitogen-activated protein kinase kinase pathways, J. Neurosci. Res. 69 (2002) 622–632.
[86] A. Sola, H. Peng, M. Rogido, T.C. Wen, Animal models of neonatal stroke and response to erythropoietin and cardiotrophin-1, Int. J. Dev. Neurosci. 26 (2008) 27–35 (the official journal of the International Society for Developmental Neuroscience).
[87] X. Shu, S. Du, X. Chen, Z. Li, Transplantation of neural stem cells overexpressing cardiotrophin-1 inhibits sprouting of hippocampal mossy fiber in a rat model of status epilepticus, Cell Biochem. Biophysics. 61 (2011) 367–370.
[88] D. Wang, X. Li, K. Gao, D. Lu, X. Zhang, C. Ma, et al., Cardiotrophin-1 (CTF1) ameliorates glucose-uptake defects and improves memory and learning deficits in a transgenic mouse model of Alzheimer's disease, Pharmacol. Biochem. Behav. 107 (2013) 48–57.
[89] D. Wang, L. Liu, J. Yan, W. Wu, X. Zhu, Y. Wang, Cardiotrophin-1 (CT-1) improves high fat diet-induced cognitive deficits in mice, Neurochem. Res. 40 (2015) 843–853.
[90] M. Peters, E. Roeb, D. Pennica, K.H. Meyer zum Buschenfelde, S. Rose-John, A new hepatocyte stimulating factor: cardiotrophin-1 (CT-1), FEBS Lett. 372 (1995) 177–180.
[91] M. Bustos, B. Sangro, P. Alzuguren, A.G. Gil, J. Ruiz, N. Beraza, et al., Liver damage using suicide genes. A model for oval cell activation, Am. J. Pathol. 157 (2000) 549–559.
[92] N. Beraza, J.M. Marques, E. Martinez-Anso, M. Iniguez, J. Prieto, M. Bustos, Interplay among cardiotrophin-1, prostaglandins, and vascular endothelial growth factor in rat liver regeneration, Hepatology 41 (2005) 460–469.
[93] M. Iniguez, C. Berasain, E. Martinez-Anso, M. Bustos, P. Fortes, D. Pennica, et al., Cardiotrophin-1 defends the liver against ischemia–reperfusion injury and mediates the protective effect of ischemic preconditioning, J. Exp. Med. 203 (2006) 2809–2815.
[94] J.M. Marques, I. Belza, B. Holtmann, D. Pennica, J. Prieto, M. Bustos, Cardiotrophin-1 is an essential factor in the natural defense of the liver against apoptosis, Hepatology 45 (2007) 639–648.
[95] M.J. Tunon, B. San Miguel, I. Crespo, J.I. Riezu-Boj, E. Larrea, M. Alvarez, et al., Cardiotrophin-1 promotes a high survival rate in rabbits with lethal fulminant hepatitis of viral origin, J. Virol. 85 (2011) 13124–13132.
[96] D.W. Ho, Z.F. Yang, C.K. Lau, K.H. Tam, J.Y. To, R.T. Poon, et al., Therapeutic potential of cardiotrophin 1 in fulminant hepatic failure: dual roles in antiapoptosis and cell repair, Arch. Surg. 141 (2006) 1077–1084 (discussion 84).
[97] Z.F. Yang, C.K. Lau, P. Ngai, S.P. Lam, D.W. Ho, R.T. Poon, et al., Cardiotrophin-1 enhances regeneration of cirrhotic liver remnant after hepatectomy through promotion of angiogenesis and cell proliferation, Liver Int. 28 (2008) 622–631 (official journal of the International Association for the Study of the Liver).
[98] P. Aguilar-Melero, A. Luque, M.M. Machuca, M.P. Perez de Obanos, R. Navarrete, I.C. Rodriguez-Garcia, et al., Cardiotrophin-1 reduces ischemia/reperfusion injury during liver transplant, J. Surg. Res. 181 (2013) e83–e91.
[99] J. Song, Y.W. Zhang, A.H. Yao, Y. Yu, Z.Y. Hua, L.Y. Pu, et al., Adenoviral cardiotrophin-1 transfer improves survival and early graft function after ischemia and reperfusion in rat small-for-size liver transplantation model, Trans. Int. 21 (2008) 372–383 (official journal of the European Society for Organ Transplantation).
[100] D. Castano, E. Larequi, I. Belza, A.M. Astudillo, E. Martinez-Anso, J. Balsinde, et al., Cardiotrophin-1 eliminates hepatic steatosis in obese mice by mechanisms involving AMPK activation, J. Hepatol. 60 (2014) 1017–1025.
[101] P.D. Lambert, K.D. Anderson, M.W. Sleeman, V. Wong, J. Tan, A. Hijarunguru, et al., Ciliary neurotrophic factor activates leptin-like pathways and reduces body fat, without cachexia or rebound weight gain, even in leptin-resistant obesity, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 4652–4657.
[102] Y. Minokoshi, Y.B. Kim, O.D. Peroni, L.G. Fryer, C. Muller, D. Carling, et al., Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase, Nature 415 (2002) 339–343.
[103] M.J. Watt, N. Dzamko, W.G. Thomas, S. Rose-John, M. Ernst, D. Carling, et al., CNTF reverses obesity-induced insulin resistance by activating skeletal muscle AMPK, Nat. Med. 12 (2006) 541–548.
[104] S. Zvonic, J.C. Hogan, P. Arbour-Reily, R.L. Mynatt, J.M. Stephens, Effects of cardiotrophin on adipocytes, J. Biol. Chem. 279 (2004) 47572–47579.
[105] M. Lopez-Yoldi, M. Fernandez-Galilea, L.M. Laiglesia, E. Larequi, J. Prieto, J.A. Martinez, et al., Cardiotrophin-1 stimulates lipolysis through the regulation of main adipose tissue lipases, J. Lipid Res. 55 (2014) 2634–2643.
[106] A. Bartelt, J. Heeren, Adipose tissue browning and metabolic health, Nat. Rev. Endocrinol. 10 (2014) 24–36.
[107] M. Jimenez-Gonzalez, F. Jaques, S. Rodriguez, A. Porciuncula, R.M. Principe, G. Abizanda, et al., Cardiotrophin 1 protects beta cells from apoptosis and prevents streptozotocin-induced diabetes in a mouse model, Diabetologia 56 (2013) 838–846.
[108] A.E. Malavazos, F. Ermetici, L. Morricone, A. Delnevo, C. Coman, B. Ambrosi, et al., Association of increased plasma cardiotrophin-1 with left ventricular mass indexes in normotensive morbid obesity, Hypertension 51 (2008) e8–e9 (author reply e10).
[109] C. Natal, M.A. Fortuno, P. Restituto, A. Bazan, I. Colina, J. Diez, et al., Cardiotrophin-1 is expressed in adipose tissue and upregulated in the metabolic syndrome, Am. J. Physiol. Endocrinol. Metab. 294 (2008) E52–E60.
[110] T. Rendo-Urteaga, S. Garcia-Calzon, E. Martinez-Anso, M. Chueca, M. Oyarzabal, M.C. Azcona-Sanjulian, et al., Decreased cardiotrophin-1 levels are associated with a lower risk of developing the metabolic syndrome in overweight/obese children after a weight loss program, Metabolism 62 (2013) 1429–1436 (clinical and experimental).
[111] D. Sanchez-Infantes, U.A. White, C.M. Elks, R.F. Morrison, J.M. Gimble, R.V. Considine, et al., Oncostatin m is produced in adipose tissue and is regulated in conditions of obesity and type 2 diabetes, J. Clin. Endocrinol. Metab. 99 (2014) E217–E225.
[112] G. Limongelli, P. Calabro, V. Maddaloni, A. Russo, D. Masarone, A. D'Aponte, et al., Cardiotrophin-1 and TNF-alpha circulating levels at rest and during

cardiopulmonary exercise test in athletes and healthy individuals, Cytokine 50 (2010) 245–247.
[113] S.Z. Lutz, O. Franck, A. Bohm, J. Machann, F. Schick, F. Machicao, et al., Common genetic variation in the human CTF1 locus, encoding cardiotrophin-1, determines insulin sensitivity, PLoS ONE 9 (2014) e100391.
[114] C. Ehlting, W.S. Lai, F. Schaper, E.D. Brenndorfer, R.J. Matthes, P.C. Heinrich, et al., Regulation of suppressor of cytokine signaling 3 (SOCS3) mRNA stability by TNF-alpha involves activation of the MKK6/p38MAPK/MK2 cascade, J. Immunol. 178 (2007) 2813–2826.
[115] F. Benigni, S. Sacco, D. Pennica, P. Ghezzi, Cardiotrophin-1 inhibits tumor necrosis factor production in the heart and serum of lipopolysaccharide-treated mice and in vitro in mouse blood cells, Am. J. Pathol. 149 (1996) 1847–1850.
[116] M.B. Garcia-Cenador, M.F. Lorenzo-Gomez, J.J. Herrero-Payo, J. Ruiz, M.P. Perez de Obanos, J. Pascual, et al., Cardiotrophin-1 administration protects from ischemia–reperfusion renal injury and inflammation, Transplantation 96 (2013) 1034–1042.
[117] Y. Quiros, P.D. Sanchez-Gonzalez, F.J. Lopez-Hernandez, A.I. Morales, J.M. Lopez-Novoa, Cardiotrophin-1 administration prevents the renal toxicity of iodinated contrast media in rats, Toxicol. Sci. 132 (2013) 493–501 (an official journal of the Society of Toxicology).
[118] M. Fritzenwanger, K. Meusel, M. Foerster, F. Kuethe, A. Krack, H.R. Figulla, Cardiotrophin-1 induces interleukin-6 synthesis in human umbilical vein endothelial cells, Cytokine 36 (2006) 101–106.
[119] M. Fritzenwanger, K. Meusel, M. Foerster, F. Kuethe, A. Krack, H.R. Figulla, Cardiotrophin-1 induces interleukin-6 synthesis in human monocytes, Cytokine 38 (2007) 137–144.
[120] M.U. Moreno, G. San Jose, A. Pejenaute, M.F. Landecho, J. Diez, O. Beloqui, et al., Association of phagocytic NADPH oxidase activity with hypertensive heart disease: a role for cardiotrophin-1? Hypertension 63 (2014) 468–474.
[121] J. Mauer, J.L. Denson, J.C. Bruning, Versatile functions for IL-6 in metabolism and cancer, Trends Immunol. 36 (2015) 92–101.
[122] S. Hishinuma, M. Funamoto, Y. Fujio, K. Kunisada, K. Yamauchi-Takihara, Hypoxic stress induces cardiotrophin-1 expression in cardiac myocytes, Biochem. Biophys. Res. Commun. 264 (1999) 436–440.
[123] E.J. Pulido, B.D. Shames, D. Pennica, R.M. O'Leary, D.D. Bensard, B.S. Cain, et al., Cardiotrophin-1 attenuates endotoxin-induced acute lung injury, J. Surg. Res. 84 (1999) 240–246.
[124] M. Fritzenwanger, K. Meusel, C. Jung, M. Franz, Z. Wang, M. Foerster, et al., Cardiotrophin-1 induces tumor necrosis factor alpha synthesis in human peripheral blood mononuclear cells, Mediat. Inflam. 2009 (2009) 489802.
[125] T. Ichiki, M. Jougasaki, M. Setoguchi, J. Imamura, H. Nakashima, T. Matsuoka, et al., Cardiotrophin-1 stimulates intercellular adhesion molecule-1 and monocyte chemoattractant protein-1 in human aortic endothelial cells, Am. J. Physiol. Heart Circ. Physiol. 294 (2008) H750–H763.
[126] M. Fritzenwanger, M. Foerster, K. Meusel, C. Jung, H.R. Figulla, Cardiotrophin-1 induces intercellular adhesion molecule-1 expression by nuclear factor kappaB activation in human umbilical vein endothelial cells, Chin. Med. J. 121 (2008) 2592–2598.
[127] H. Konii, K. Sato, S. Kikuchi, H. Okiyama, R. Watanabe, A. Hasegawa, et al., Stimulatory effects of cardiotrophin 1 on atherosclerosis, Hypertension 62 (2013) 942–950.
[128] M. Barkett, T.D. Gilmore, Control of apoptosis by Rel/NF-kappaB transcription factors, Oncogene 18 (1999) 6910–6924.
[129] T. Lawrence, C. Fong, The resolution of inflammation: anti-inflammatory roles for NF-kappaB, Int. J. Biochem. Cell Biol. 42 (2010) 519–523.
[130] C.M. Alvira, Nuclear factor-kappa-B signaling in lung development and disease: one pathway, numerous functions. Birth defects research Part A, Clin. Mol. Teratol. 100 (2014) 202–216.



Miguel López-Yoldi is a Ph.D. student in the Department of Nutrition, Food Sciences and Physiology at the University of Navarra. His research is focus in the role of Cardiotrophin-1 in adipose tissue.

María J. Moreno-Aliaga is Professor of Human Physiology in the Department of Nutrition, Food Sciences and Physiology at the University of Navarra. Dr. Moreno-Aliaga is Coordinator of the Nutridynamic Area of the Centre for Nutrition Research at the University of Navarra. She received her Ph.D. from the School of Pharmacy at the University of Navarra and was a Postdotoral Fellow at the University of California, Davis. During the last 15 years her research has been focused mainly in the study of Nutritional Genomics of adipose tissue, and in the mechanisms underlying obesity, inflammation and insulin resistance.

Matilde Bustos, M.D., Ph.D. is principal researcher in the area of Hepatology and Gene Therapy at CIMA (Center for Applied Medical Research) University of Navarra, Pamplona (Spain), a position she has held since late 1997. She received her Ph.D. from the School of Medicine at the University of Granada (Spain) and she did her post-doctoral studies at Duke University Medical Center (NC, USA). During the last years she has studied the role of cardiotrophin-1 (CT-1) as a protective cytokine in the field of hepatology. Recently, she has analyzed the metabolic properties of CT-1 showing a key role in obesity and associated co-morbidities. Her research is focused mainly in the study of molecular and cellular mechanism of NAFLD (non-alcoholic fatty liver disease) and steatohepatitis, her aim is to develop novel drugs to treat metabolic diseases.