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Regulation of body energy homeostasis by the selected retinoids and fatty acids

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> *"Caminante, son tus huellas*
> *el camino y nada más;*
> **Caminante, no hay camino,**
> **se hace camino al andar.**
> *Al andar se hace el camino,*
> *y al volver la vista atrás*
> *se ve la senda que nunca*
> *se ha de volver a pisar.*
> *Caminante no hay camino*
> *sino estelas en la mar."*
>
> *Proverbios y cantares (XXIX)*
> *Antonio Machado*

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ABSTRACT

Beside their general effects on satiety, ingested nutrients affect body control mechanisms that regulate energy balance and that include different processes involved in the control of food intake, energy expenditure and local tissue processes. Fatty acids (FA) and vitamin A related compounds are both nutrients with lipidic nature and already known to exert different effects on these processes. It is known that FAs are the ones responsible for the trigliceride effects in the central nervous system as well as in the gastrointestinal tract. Similarly vitamin A metabolites are higly active compounds required for a wide range of biological processes. However, due to the great variety of FA structures and their physicochemical properties as well as a number of vitamin A metabolites there are still many gaps in our knowledge on their effects, especially with regard to the underlying molecular mechanism.

Thus, the general objective of this thesis was to study into more depth the effects of these two groups of nutrients on the processes involved in the body energy homeostasis and to profound the understanding of their molecular mechanisms.

The first line of the thesis dealt with the FA effects on food intake. More further on, it revolved around the different satiating capacities of the long-chain saturated palmitic fatty acid and the unsaturated linoleic fatty acid. In particular, we were interested to explain the observed satiety differences through: a) FA central effects on the hipothalamic gene expression and the levels of neuropeptides involved in the short-term control of food intake, and b) FA gastrointestinal effects on the stomach protein expression and secretion. Special attention was given to stomach hormones leptin and ghrelin that are involved in the short-term control of satiety and hunger, respectively.

The second line of the thesis dealing with the vitamin A metabolites had as an objective to investigate their effects on body energy expenditure and local tissue processes especially the ones associated with the biology and function of adipose tissue. In particular we explored: a) the effects of early vitamin A supplementation (in the form of retynil ester, RE) on the white adipose tissue (WAT) development and its consequences on the later propensity to obesity, b) effects of β-carotene on adipose tissue biology in the same life period, during lactation c) effects of all-trans retinoic acid (ATRA) on skeletal muscle secretoma, possible to have direct effects on adipose tissue and as such being another mechanism likely contributing to ATRA activation of WAT, and d) ATRA effects on mitochondriogenesis and the induction of WAT oxidative metabolism and thermogenesis in relation to ATRA induced process of browning.

The results obtained inside the first line show different satiating capacities of palmitic and linoleic acid, which can be explained by their distinctive central effects affecting the expression of hypothalamic neuropeptides included in the control of food intake as well as distinct gastrointestinal effects, including gastrointestinal hormone expression and release. In concrete, stronger satiating capacities of linoleic acid could

be explained by its earlier and stronger activation of anorexigenic signalling in the hipotalamus as well as the decrease in ghrelin levels. However, in addition to these already known effects of ghrelin, our results point out the role of gastric leptin in the FA satiating action and indicate a possibility of this being a safety mechanism to counteract anorexigenic central actions induced by FFAs via inhibition of gastric ghrelin, especially in stress situations such as fasting.

Results from the second line of investigation regarding vitamin A early supplementation on WAT development indicate that such supplementation (as RE) affects adipose cells to retain increased proliferation potential (higher expression of PCNA), which correlated with a reduced expression of adipogenic markers (e.g. PPARγ). These changes facilitate fat storage and thus favor the increased adiposity gain upon a subsequent stimulus in the form of a high fat diet later in life, which was observed previously by our group. On the other hand, although its absorption was confirmed by significantly increased serum and liver levels, β-carotene supplementation in the same period did not affect adipose tissue development in young rats. In fact, β-carotene in the iWAT of these animals was not even detected. Furthermore in the same line of investigation, results of the ATRA treatments showed affected myokine production in the skeletal muscle cells, e.g. induced expression of FNDC5/irisin and FGF21, which were proved to exert effects upon adipose tissue, including browning. These changes in skeletal muscle contribute to the understanding of ATRA anti-obesity and anti-diabetic action and the enhancement of whole body fatty acid catabolism. Additionaly in the same line, our results indicate that ATRA induces mitochondria biogenesis and oxidative phosphorylation/thermogenic capacities in mature white adipocytes. This impact of ATRA could explain the possible remodeling of mature white adipocytes into mitochondria-rich more brown-fat like cells, described previously by our group.

Taken together, these results show various effects of fatty acids and vitamin A metabolites on different body control processes that contribute to the organism's energy homeostasis. Knowledge of FA effects with regard to the food intake can broaden our understanding of the metabolic effects of different diets and their effects on body weight control as well as open up possibilities for the design of new fat replacers with improved efficiency. Vitamin A research is a broad area each time showing more proofs of the importance of related nutrients in the adipose tissue biology and with this associated control of body weight. Results presented here contribute to the understanding of their effects on different tissue processes and the elements of energy expenditure that could be used in the prevention and treatment of obesity and associated metabolic disorders.

Regulación de la homeostasis energética corporal por los retinoides y ácidos grasos seleccionados

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RESUMEN

Además de sus efectos generales sobre la saciedad, los nutrientes ingeridos afectan a los mecanismos de control del cuerpo que regulan el equilibrio de energía y que incluyen diferentes procesos implicados en el control de la ingesta de alimentos, gasto de energía y los procesos tisulares locales. Los ácidos grasos (AG) y los compuestos relacionados con vitamina A son nutrientes con la naturaleza lipídica y es sabido que ejercen efectos diferentes sobre estos procesos. Se sabe que los AG son los responsables de los efectos de los triglicéridos en el sistema nervioso central, así como en el tracto gastrointestinal. Del mismo modo metabolitos de la vitamina A son compuestos altamente activos necesarios para una amplia gama de procesos biológicos. Sin embargo, debido a la gran variedad de estructuras de los AG y sus propiedades físico-químicas, así como una serie de metabolitos de vitamina A todavía hay muchos huecos en el conocimiento sobre sus efectos, especialmente en relación con el mecanismo molecular subyacente.

Por lo tanto, el objetivo general de esta tesis fue estudiar en mayor profundidad los efectos de estos dos grupos de nutrientes en los procesos implicados en la homeostasis de la energía del cuerpo y profundizar en la comprensión de sus mecanismos moleculares.

La primera línea de la tesis aborda los efectos de los AG en la ingesta de alimentos. Así, se centra en torno a las diferentes capacidades saciantes de los AGs de cadena larga, el acido graso saturados palmítico y el ácido graso insaturado linoleico. En particular, nos intereso explicar las diferencias observadas en la saciedad a través de: a) efectos centrales de los AG sobre la expresión génica en el hipothalamo y los niveles de los neuropéptidos implicados en el control de la ingesta de alimentos a corto plazo, y b) efectos gastrointestinales de los AG en la expresión y secreción de las proteínaa del estómago. Se prestó especial atención a las hormonas leptina y grelina en el estómago que están involucrados en el control a corto plazo de la saciedad y el hambre, respectivamente.

La segunda línea de la tesis que se centra en los metabolitos de la vitamina A tuvo como objetivo investigar sus efectos sobre el gasto energetico del cuerpo y los procesos locales en los tejidos especialmente relacionados con la biología y la función del tejido adiposo. En particular, hemos explorado: a) los efectos de la suplementacion con la vitamina A (en forma de retinil éster, RE) en el desarrollo del tejido adiposo blanco (TAB) y sus consecuencias sobre la propensión a la obesidad en la edad adula, b) los efectos de β-caroteno en la biología del tejido adiposo en el mismo período la vida, durante la lactancia, c) efectos del ácido retinoico (ATRA) en el secretoma del músculo esquelético, que posiblemente tiene efectos directos sobre el tejido adiposo y, como tal, constituye otro mecanismo probablemente contribuyendo a la activación de TAB con ATRA, y d) los efectos de ATRA sobre mitochondriogenesis y la inducción del metabolismo oxidativo y la termogénesis del WAT en relación con el proceso de 'browning' inducido por ATRA.

Los resultados obtenidos dentro de la primera línea muestran diferentes capacidades saciantes de ácido palmítico y linoleico, lo que puede explicarse por sus distintos efectos centrales que afectan a la expresión de neuropeptidos hipotalámicos relacionados con el control de la ingesta de alimentos, así como distintos efectos gastrointestinales, incluyendo la expresión y la liberación de las hormonas gastrointestinales. En concreto, las capacidades saciantes más fuertes de ácido linoleico podrían explicarse por su activación más temprana y más fuerte de la señalización anorexígenica en el hipotálamo, así como la disminución en los niveles de grelina. Sin embargo, además de estos efectos ya conocidos de la grelina, nuestros resultados señalan el papel de la leptina gástrica, en el acción saciante de los ácidos grasos e indican una posibilidad de que esto sea un mecanismo de seguridad para contrarrestar las acciones centrales anorexígenicas inducidas por ácidos grasos libres a través de la inhibición de la grelina gástrica, especialmente en situaciones de estrés tales como el ayuno.

Los resultados de la segunda línea de investigación con respecto al efecto de la suplementacion con vitamina A en la edad temprana en el desarrollo de TAB indican que tales suplementos (como RE) afectan a las células adiposas para retener aumentado potencial de proliferación (mayor expresión de PCNA), que se correlaciona con una expresión reducida de marcadores adipogénicos (por ejemplo, PPARγ). Estos cambios facilitan el almacenamiento de grasa y por lo tanto favorecen ganancia de la adiposidad sobre un estímulo posterior en la forma de una dieta alta en grasas, más tarde en la vida, que se observó previamente por nuestro grupo. Por otro lado, a pesar de que su absorción fue confirmada por sus niveles en el suero y el hígado incrementados significativamente, la suplementacion con β-caroteno en el mismo período no afectó el desarrollo del tejido adiposo en ratas jóvenes. De hecho, β-caroteno en la TAB inguinal de estos animales ni siquiera se detectó. Además, en la misma línea de investigación, los resultados de los tratamientos con ATRA mostraron producción de miokinas afectada en las células del músculo esquelético, por ejemplo, inducida expresión de FNDC5/irisin y FGF21, que se ha demostrado que ejercen efectos sobre el tejido adiposo, incluyendo el 'browning'. Estos cambios en el músculo esquelético contribuyen a la comprensión de los efectos de ATRA contra la obesidad y su acción anti-diabética y la mejora del catabolismo general de los ácidos grasos. Adicionalmente, en la misma línea, nuestros resultados indican que ATRA induce la biogénesis de las mitocondrias y las capacidades de fosforilación oxidativa y termogénica en adipocitos blancos maduros. Este impacto de ATRA podría explicar la posible remodelación de los adipocitos blancos maduros en las células más marrones ricas en mitocondrias, que se describió anteriormente por nuestro grupo.

Tomados en conjunto, estos resultados muestran diversos efectos de los ácidos grasos y metabolitos de vitamina A en diferentes procesos corporales que contribuyen a la homeostasis de la energía del organismo. El conocimiento de los efectos de los AG con respecto a la ingesta de alimentos puede ampliar nuestra comprensión de los efectos metabólicos de diferentes dietas y sus efectos sobre el control del peso corporal, así como abrir nuevas posibilidades para el diseño de nuevos sustitutos de la grasa con mayor eficacia. El estudio de vitamina A es un área de investigación cada vez más amplia mostrando la importancia de los diferentes nutrientes relacionados en la biología del tejido adiposo y asi asociados efectos en el control de peso corporal. Los resultados presentados aquí contribuyen a la comprensión de sus efectos en diferentes procesos en los tejidos y en los elementos del gasto energetico que podrían utilizarse en la prevención y el tratamiento de la obesidad y trastornos metabólicos asociados.

List of original articles

This thesis is based on the following original manuscripts:

I. **Mušinović H**, Ribot J, Palou A. REGULATION OF GASTRIC LEPTIN SECRETION BY FREE FATTY ACIDS. Manuscript to be submitted

II. Granados N, Amengual J, Ribot J, **Mušinović H**, Ceresi E, von Lintig J, Palou A, Bonet ML. VITAMIN A SUPPLEMENTATION IN EARLY LIFE AFFECTS LATER RESPONSE TO AN OBESOGENIC DIET IN RATS. Int J Obes (Lond). 2013; 37(9): 1169-76.

III. **Mušinović H**, Bonet ML, Granados N, von Lintig J, Ribot J, Palou A. β-CAROTENE DURING THE SUCKLING PERIOD IS ABSORBED INTACT AND POORLY AFFECTS ADIPOSE TISSUE DEVELOPMENT IN YOUNG RATS. Manuscript to be submitted

IV. Amengual J, Granados N, **Mušinović H**, García-Carrizo FJ, Bonet ML, Palou A, Ribot J. RETINOIC ACID INCREASES FATTY ACID OXIDATION AND FNDC5/IRISIN EXPRESSION IN SKELETAL MUSCLE CELLS. Manuscript to be submitted

V. Tourniaire F, **Mušinović H**, Gouranton E, Astier J, Marcotorchino J, Palou A, Bonet ML, Ribot J, Landrier JF. ALL-TRANS RETINOIC ACID INDUCES OXIDATIVE PHOSPHORYLATION AND MITOCHONDRIA BIOGENESIS IN ADIPOCYTES. Manuscript to be submitted

Abbreviations

ACC: acetyl-CoA carboxylase

ADD: adenosine deaminase

ADP: adenosine diphosphate

AgRP: agouti related peptide

AMPK: AMP (adenosine monophosphate)-kinase

ARC: arcuate nucleus

ATGL: adipose triglyceride lipase, desnutrin

ATP: adenosine triphosphate

ATRA: all-trans retinoic acid

BAT: brown adipose tissue

BMR: basal metabolic rate

C/EBP: CCAAT/enhancer binding protein

cAMP: cyclic AMP (adenosine monophosphate)

CART: cocaine and amphetamine regulated transcript

CCK: cholecystokinin

ChREBP: carbohydrate regulatory element-binding protein

CLA: conjugated linoleic acid

CNS: central nervous system

CPT: carnitine palmitoyltransferase

CRH corticotropinreleasing hormone

DAG: diacylglycerol

eWAT: epididimal WAT

FA: fatty acid

FGF-21: fibroblast growth factor-21

GI: gastrointestinal

GIP: glucose-dependent insulinotropic polypeptide

GLP-1: glucagon like peptide 1

GLUT4: glucose transporter type 4

gWAT: gonadal WAT

HSL: hormone sensitive lipase

IL-6: interleukin-6

ISX: intestine-specific homeobox

iWAT: inginal WAT

LCFA: long chain fatty acid

LCT: long-chain triacylglycerol

LPL: lipoprotein lipase

MAG: monoacylglycerol

MCFA: medium chain fatty acid

MCH melanine-concentrating hormone

MCT: medium-chain triacylglycerol

MSH melanocyte-stimulating hormone

NADPH: nicotinamide adenine dinucleotide phosphate

NPY: neuropeptide Y

OXM: oxyntomodulin

PCNA: proliferating cell nuclear antigen

PGC-1: peroxisome proliferator-activated receptor gamma coactivator 1

PKA: protein kinase A

POMC: pro-opiomelanocortin

PP: pancreatic polypeptide

PPAR: peroxisome proliferator-activated receptor

PUFA: polyunsaturated fatty acid

PYY: peptide YY

RA: retinoic acid

RAR: retinoic acid receptor

RE: retinyl ester

ROL: retinol

ROS: reactive oxygen species

rWAT: retroperitoneal WAT

RXR: retinoid X receptor

SNS: sympathetic nervous system

SREBP: sterol regulatory element-binding protein

TAG: trigliceride, triacylglycerol

TEF: thermic effect of food

TF: transcriptional factor

TNF: tumor necrosis factor

TR: thyroid receptor

TRH: thyrotropin releasing hormone

TSH: thyroid stimulating hormone

UCP: uncoupling protein

WAT: white adipose tissue

α-MSH: alpha melanocyte stimulating hormone

1 INTRODUCTION

1.1 Energy homeostasis and body weight regulatory systems

Living creatures possess a unique capability to maintain the properties of their internal environment stable and relatively constant. This homeostatic phenomena is already known in great detail for a number of organic functions such as temperature, blood pressure, blood nutrient and hormone levels and is lately becoming increasingly recognized in the control of body energy levels i.e. body weight maintainance. Our bodies posses a set of control mechanisms to prevent big and sudden changes in our body energy levels i.e. our body weight, even in situations of energy deprivation or energy excess (Havel, 2001). Being that well tuned, the system of body energy/weight homeostasis is also higly complex, composed of many processes being also metabolically intersected. Body weight, as well as body composition, and thus the obesity (i.e. increased size of fat stores) are influenced by biochemical processes regulating a variety of functions such as: food intake, energy expenditure, nutrient partitioning, glucose and lipid metabolism and the processes involved in adipose tissue maintainance (proliferation of preadipocytes, differentiation and apoptosis of fat cells) in the end influencing number and size of adipocytes. However, this variety of processes is usually grouped into **three main cathegories including: control of energy intake, energy expenditure and adipogenesis and other adipose tissue biology related processes** (Palou et al., 2000)**.** All of them are influenced by the nutritional status and diet composition and the balance between them impacts the final outcome on body weight and body composition. Changes in them, both acquired and genetic, have been studied more and more in the last decades as part of the ever growing research on obesity and its prevention. In general, obesity occurs when energy intake exceeds energy expenditure. However, its progression is caused by an array of heterogeneous alterations in this complex regulatory system making it a multisystem/multifactorial disease/disorder. Each of these processes combines different biochemical paths and metabolic players, each of which will be explained in more detail in the continuation.
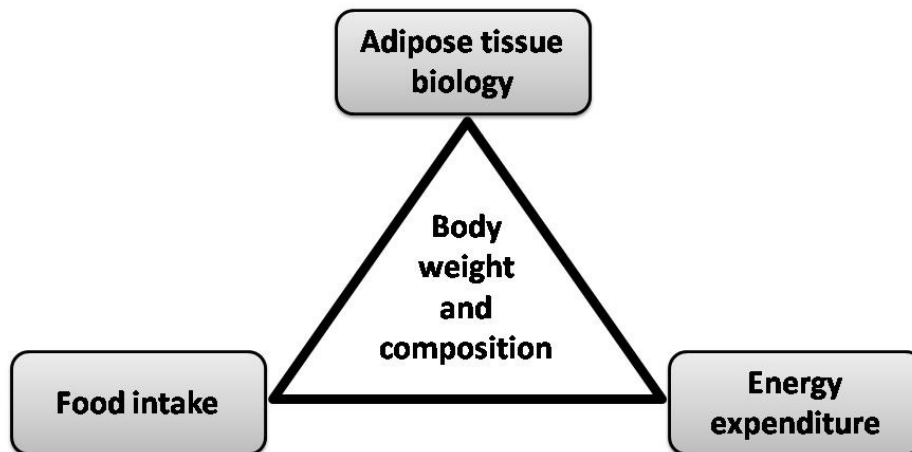
Figure 1: Main groups of processes controlling body weight and composition covered in the thesis

1.1.1 Mechanisms of food intake control

Feeding control mechanisms include biochemical processes that determine **sensations of hunger and satiety** that provoke desire to eat i.e. determine the end of the meal (satiation) and the time until the next meal (satiety) (Palou et al., 2000). These sensations are determined through processes that depend on an interplay between **internal signals and environmental factors**. Internal signals include homeostatic factors such as signals from the gastrointestinal tract, metabolism and storage. Environmental factors, including: availability of foods, palatability, social factors, habits and time of the day, memory, are also called nonhomeostatic factors as are not influenced by and do not contribute to the organism homeostatic state (Mattes, 2008). As such they are recognised as the leading cause of obesity epidemics. Internal signals influencing the sensations of satiety and hunger can be both **short and long termed**, conveying information about recent energy intake and body energy stores, respectively. In general they are provoked by the nutrient and hormonal levels in the gastrointestinal tract, circulation and adipose tissue. Subsequent to meal ingestion, the interactions of nutrients with the receptors in the gastrointestinal (GI) tract modulate a number of its functions, including gastric emptying (Heddle et al., 1989), gastrointestinal hormone secretion (Feltrin et al., 2004) and energy intake (Chapman et al., 1999).

1.1.1.1 Short- term regulation of feeding

Short term signals influencing satiety originate from the GI tract and include physical signals of **gastric distention** i.e. mechanical stretching of the stomach, and an array of **GI peptides** released in response to food (Chaudhri et al., 2008). Gastric load stimulates vagal mechanosensitive fibers dose-dependently but independently of the contents of the gastric load. Stimulation of these fibers suppresses feeding, but the effect of gastric distension alone is insufficient to account for all aspects of postprandial satiety.

Gut content i.e nutritional stimuli provokes the secretion of many GI hormones from the GI tract into the circulation. Major anorexigenic (inhibit food intake) GI peptides that are released after food intake and involved in the sensation of satiety are: cholecystokinin (**CCK**), peptide YY (**PYY**), pancreatic polypeptide (**PP**), glucagon like peptide 1 (**GLP-1**), oxyntomodulin (**OXM**) and especially extensively studied lately **leptin** (Cummings and Overduin, 2007).

CCK is released very quickly after starting food intake by the proximal small intestine in response to the intake of fat, amino acids and small peptides resulting from digestion (Liddle et al., 1985). Lipid effect on decreased food intake is mediated by release of CCK (Greenberg et al., 1987). CCK-A receptors have been identified on the abdominal vagus (Kopin et al., 1999). CCK stimulates gallbladder contraction and exocrine pancreatic secretion, slows down gastric emptying, thereby prolonging postprandial gastric distension and mechanoreceptor stimulation thus contributing to satiety. CCK functions as a positive feedback signal to stimulate digestive processes and negative feedback signal to limit the amount of food consumed during an individual meal. The administration of CCK produces a dose-dependent decrease in the size of food intake (Chandler et al., 2004). CCK release is stimulated by gastric leptin that is secreted into the stomach lumen during meals and then travels to the duodenum (Guilmeau et al., 2003).

PYY is released by the distal small intestine and the large intestine in proportion to the energy content of food (mostly fat) and unlike CCK has later postprandial release with peaking in plasma after 60-90 min, but its anorexigenic effects are more long lasting than those of CCK (Batterham et al., 2002). Fasting plasma PYY levels and its secretion after food intake are lower in obese individuals then in the ones with normal weight (Batterham et al., 2003; Le Roux et al., 2006). **PP** is produced and secreted postprandialy by the pancreas in proportion to the energy content of food and its levels

remain higher up to 6h after food intake (Cummings and Overduin, 2007). **GLP-1 and OXM** derive from the pre-proglucagon in the terminal small intestine (ileum) and its administration reduces food intake (Dakin et al., 2001), most likely at a hepatic site or by inhibiting gastric emptying. Repeated administration of GLP-1 (Meeran et al., 1999) or OXM (Dakin et al., 2002) reduces food intake and body weight i.e. causes less weight gain in rats.

Among gastrointestinal hormones the one that lately attracts more attention is **leptin**. Leptin is vastly recognized as a hormone produced by the adipose tissue and as such involved in the long-term control of energy intake. However, leptin is produced in non-adipose tissues as well such as stomach (Bado et al., 1998; Cinti et al., 2000), placenta (Masuzaki et al., 1997; Ashworth et al., 2000), skeletal muscle (Wang et al., 1998), mammary epithelium (Casabiell et al., 1997). In the stomach leptin is present in the lower half of gastric mucosa, in the chief cells (Cinti et al., 2000). Gastric leptin is released in response to food intake and is involved in the acute feeding control by inhibiting food intake. Increased leptin levels after the meal lead to the feeling of fullness and meal termination. Leptin levels in stomach and serum show positive correlation, are increased after food intake and decreased during fasting (Pico et al., 2002). Feeding increases leptin levels in serum by acutely stimulating its secretion from gastric mucosa. In rats, a short period of feeding following fasting is capable of practically emptying the leptin stores from the stomach mucosa. At the same time as it stimulates leptin release from gastric mucosa, food intake also stimulates leptin mRNA expression, allowing the synthesis of new leptin and recovery of its stomach stores that reach the steady-state levels seen in well-fed rats (Sanchez et al., 2004b).

In healthy individuals **insulin** has dual effects. It down regulates food intake and, as leptin, also exerts peripheral anabolic effects that are crucial for the use of nutrients. Its levels are low during fasting and raise upon food intake to provoke glucose entry into cells and, in the case energy is abundant, its storage in form of fat (insulin peripheral anabolic effects) (Havel, 2001). Insulin and leptin roles are further interconected by the fact that chronic hyperinsulinemia may be one of the causes of leptin resistance (Lustig et al., 2004). In this state leptin levels are increased but it does not show its effect in decreasing food intake. For that reason people with insulin resistance (and leptin resistance) eat more and gain weight. On the other hand, a

decrease in insulinemia improves leptin sensitivity and helps to lose weight (Ruige et al., 1999).

Unlike satiaty, **hunger** is transmitted by one main GI hormone, **ghrelin**. As leptin, ghrelin is also produced by the stomach but mainly in the endocrine cells and has the opposite, inducing effect on food intake (Cummings and Foster, 2003). Thus, as opposed to leptin, ghrelin is implicated in the short-term control of pre-meal hunger and its main role is meal initiation (Cummings et al., 2001). Its administration increases food intake and body weight (Wren et al., 2001) and an antagonist of its receptor reduces food intake and body weight gain in mice (Asakawa et al., 2003). Another difference between leptin and ghrelin is the correlation between their stomach and serum levels. Ghrelin stomach and serum levels are, unlike leptin levels, inversely correlated. The content of total ghrelin in gastric fundus is decreased when fasting and returns to normal when re-feeding (Toshinai et al., 2001). On the other hand, fasting causes elevation of ghrelin plasma levels which is reduced immediately after food intake. This inverse pattern of ghrelin levels may result from increased secretion of ghrelin from the stomach in response to fasting and subsequent decreased secretion upon resumption of feeding. Fasting has more profound stimulation on the secretion of ghrelin from the stomach than on its biosynthesis (Yin et al., 2009). Mechanical signals such as gastric distention do not have influence on hunger as they have on satiety. Namely, the decrease of ghrelin after food intake is not due to stomach distension because it is not produced by water (Tschop et al., 2000) or saline (Gomez et al., 2004) ingestion. Gastric ghrelin expression is related with satiating effects of macronutrients. Ghrelin levels are profoundly decreased after gastric bypass surgery, suggesting that ingested nutrients might be important in inducing ghrelin secretion from the stomach (Cummings et al., 2002). Also, exposure of the small intestinal lumen to lipids is required for ghrelin suppression (Feinle-Bisset et al., 2005), as intravenous administration of lipid is ineffective (Mohlig et al., 2002).

Beside this hormonal signal, sensation of hunger is greately influenced by the blood glucose levels being provoked in the state of **hypoglycemia.** This effect of hypoglycemia on increased hunger sensations and food intake has been termed glucoprivic feeding (Havel, 2001). Thus, another important function of glucose, beside being a nutrient, is as an important regulatory signal that regulates autonomic and central nervous mechanisms to control glucose and energy homeostasis, feeding

behavior, and energy storage and expenditure. Changes in circulating glucose concentrations appear to elicit meal initiation and termination by regulating activity of specific neurons in the central nervous system (CNS) that respond to glucose and regulate glucose and energy homeostasis (Marty et al., 2007). In hypothalamic nuclei and the brain stem is the highest concentration of glucose-excited and glucose-inhibited neurons that modulate their firing activity, increasing or decreasing it, respectively, in response to changes in extracellular glucose levels. It is believed that glucose sensing by these cells goes via similar mechanism as in pancreatic beta-cells for systemic glycemia. The activated neurons activate vagal afferences (autonomic nerves) which activate mechanisms (stimulation/inhibition of glucagon and insulin secretion) that restore normoglycemia.

Gastrointestinal peptides secreted from enteroendocrine cells in response to ingested food exert their effects either directly as hormones entering the bloodstream and reaching the **hypothalamic appetite-regulating nuclei** or by diffusing through interstitial fluids to activate nearby vagal afferent nerve fibers that project to the hypothalamus (Cummings and Overduin, 2007). **Vagus nerve** is the major neuronal connection between the GI tract and the CNS and transfers information about direct mechanical or chemical stimulation of the GI tract by ingesta. Vagus is also a primary site at which a number of circulating satiety factors act and is thought to be a major site of GI hormone signaling (Chaudhri et al., 2008). Afferent signals travel in vagal nerve fibers from stretch receptors and from chemoreceptors activated by the presence of nutrients in the stomach and proximal small intestine. Nutrients arriving via the portal vein may also trigger vagal afferent signals from the liver (Havel, 2001).

Gastric leptin could provide rapid information to the brain by activating leptin-responsive vagal afferent fibers that originate in the gastric and intestinal walls and terminate in the brainstem (Cakir et al., 2007). Similarly, gastric vagal afferents comprise the major pathway conveying ghrelin's starvation signals to the brain (Date et al., 2002). It is likely that the primary site of action of CCK, PYY, and GLP-1 is the vagus nerve, whereas PP may act directly on the brainstem. The site of action of OXM is less clear, with direct actions on hypothalamic and brainstem circuits suggested by some investigators (Chaudhri et al., 2008).

1.1.1.2 Long-term regulation of feeding

The energy density of food and short-term hormonal signals by themselves are insufficient to produce sustained changes in energy balance and body adiposity. Rather, these signals interact with long-term regulators to maintain energy homeostasis (Havel, 2001). In contrast to short term signals (e.g. nutrients and GI hormones) that act primarily as determinants of satiety to limit the size of individual meals, long-term regulators of energy homeostasis are activated in proportion to both body adipose stores and to the amount of energy consumed over a more prolonged period of time. Long-term feeding control is affected by signals that reflect the amount of body energy reserves. These are so called **adiposity** signals such as peptides **leptin** (released from the adipose tissue) and **insulin** (released from pancreas). Their circulating concentrations are proportional to body fat content. Both insulin and leptin act in the CNS to inhibit food intake and to increase energy expenditure.

In addition, these long-term signals interact with the short-term signals in the regulation of energy homeostasis. For example, secretion and circulating levels of long-term signals are influenced by recent energy intake and dietary macronutrient content. Insulin and leptin concentrations decrease during fasting and energy-restricted diets, independent of body fat changes, ensuring that feeding is triggered before body energy stores become depleted (Havel, 2001). On the other hand, long-term signals appear to set sensitivity to the satiety-producing effects of short-term signals such as CCK. In that way they regulate food intake and energy expenditure to ensure that energy homeostasis is maintained and that body weight and adiposity remain relatively constant. This is nicely demonstrated on vagal afferents, being a peripheral sites of integration of both short term and long term changes in nutrient status. Vagal afferent neurons projecting to the gut exhibit distinct neurochemical phenotype depending on food intake over the previous 6–48 h and upon longer alterations in the diet. For example fasting decreases some receptors and hormones associated with the inhibition of food intake and CCK upon refeeding reverses these changes (Dockray and Burdyga, 2011).

Insulin is secreted from β cells in the endocrine pancreas in response to circulating nutrients (glucose and amino acids) and to the incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and GLP-1, which are released during meal ingestion and absorption. Insulin can also act indirectly by stimulating leptin production from adipose tissue via increased glucose metabolism. In contrast, dietary fat and

fructose do not stimulate insulin secretion and therefore do not increase leptin production. This is one of the possible reasons used to explain the particular propensity of high-fat and high-fructose diets to increase body weight. There is also evidence that leptin can inhibit insulin secretion from the pancreas. Insulin signaling in the brain limits food intake and over the long term, insulin secretion functions as a negative feedback signal of recent energy intake and body adiposity. In addition to inhibiting food intake, insulin increases sympathetic neural activity and energy expenditure. Thus, insulin can modulate energy balance by inhibiting energy intake and by increasing thermogenesis (Havel, 2001).
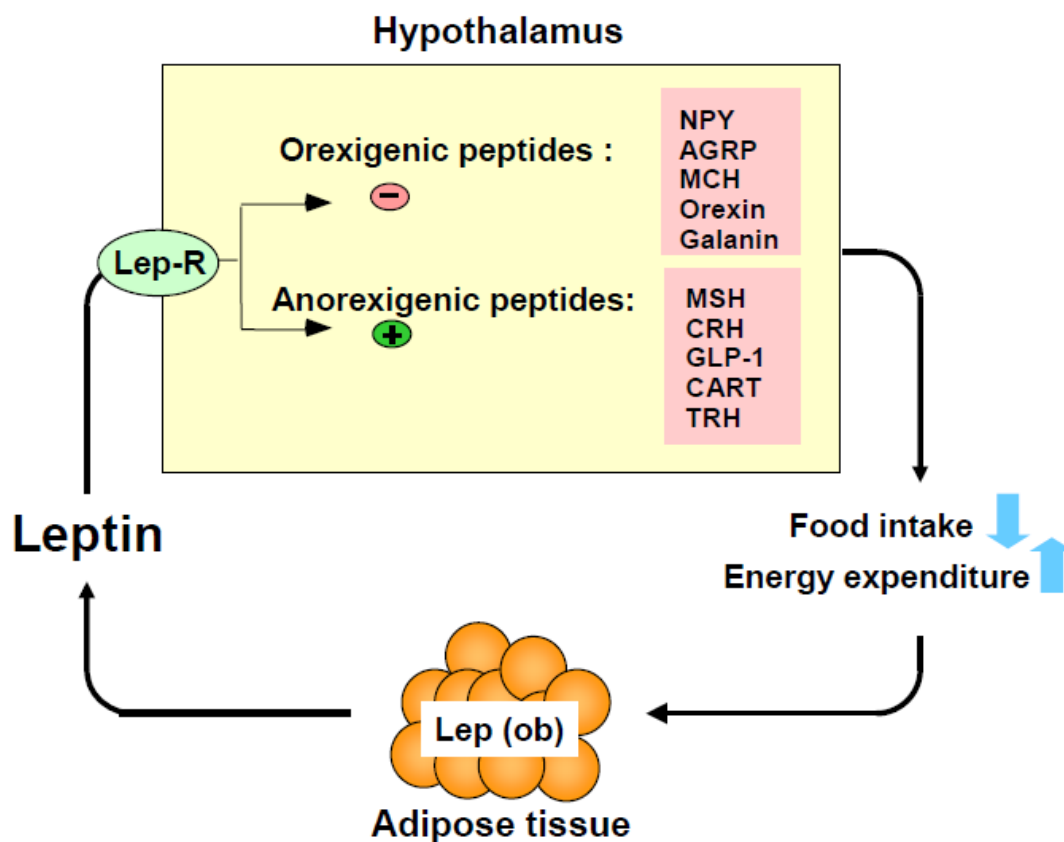
Figure 2. Effects of leptin on the orexigenic and anorexigenic hypothalamic neuropeptides involved in the control of food intake and energy expenditure. NPY neuropeptide Y; AGRP agouti related protein; MCH melanine-concentrating hormone; MSH melanocyte-stimulating hormone; CRH corticotropinreleasing hormone; GLP-1 glucagon like peptide-1; CART cocaine and amphetamine regulated transcript; TRH thyrotropin releasing hormone. Adapted from (Palou et al., 2000).

As seen before, **leptin** released from the stomach mucosa (gastric leptin) after food intake acutely controls/supresses feeding. On the contrary, the one released from

the adipose tissue is involved in chronic feeding control. Leptin expressed by adipocytes and released to the circulation acts on the leptin receptors in the brain regions implicated in the regulation of feeding behaviour and energy balance (Elmquist et al., 1997; Elmquist et al., 1998) providing them the information about the level of body adiposity i.e. body fat reserves. Through the CNS efferent signals leptin regulates energy balance by determining changes in feeding behaviour, with suppression of appetite, and an increase in metabolic activity and energy expenditure (thermogenesis). However, obese people have higher circulating leptin levels but uncapable to produce the satiating effect due to leptin resistance i.e. insensibility of its receptor (Sinha et al., 1996). Moreover, leptin also stimulates the rate of lipolysis and the expression of enzymes of fatty acid oxidation in adipose (Wang et al., 1999; Siegrist-Kaiser et al., 1997) and pancreatic cells (Zhou et al., 1997), causing a reduction of the triglyceride content of these cells which is not accompanied by a parallel release of fatty acids (Wang et al., 1999; Palou et al., 2000). Taken together, these results suggest that leptin favours the internal consumption of fatty acids as thermogenic fuels.

Ghrelin could also be adiposity signal involved in a long-term body-weight regulation as it circulates at levels that are inversely proportional to the size of body-fat stores i.e. are decreased in obese individuals, but increase after weight loss (Kojima et al., 1999; Cummings et al., 2002). Ghrelin blood levels increase in response to weight loss and that may contribute to weight regain. If ghrelin is really an adiposity signal, then we can ask how can cells that produce ghrelin sense the status of body-fat stores, as they are located primarily in the gut, which is not a major site of fat storage in mammals. Some researchers declare that leptin, an unequivocal adiposity signal, regulates ghrelin levels. However, it seems more probable that they act in parallel at an equivalent level in these pathways, and that they are opposing metabolic counterparts to one another. They are regulated reciprocally by changes in energy balance and exert opposite central effects in response to them (Cummings and Foster, 2003).

1.1.1.3 Central regulation of energy homeostasis by the melanocortin system

As mentioned before, information on nutrient-related signals is conveyed to the CNS through circulation by nutrients and secreted hormones and through nervous system by afferent fibers of the vagus nerve and afferents passing into the spinal cord (Jordan et al., 2010). These include signals originated in the GI tract that convey information about

recent energy intake history (satiety signals, ghrelin), signals that convey information about the amount of energy stores in the body (adiposity signals, such as leptin and insulin), and metabolic signals i.e. circulating levels of fuel substrates (fatty acids, glucose) that condition the status of nutrient sensing pathways in hypothalamic neurons (Dowell et al., 2005; Dowell et al., 2005). Key brain regions, such as the hypothalamus and brainstem, receive peripheral signals of short- and long-term energy status to change feeding behaviour. Arcuate nucleus of the **hypothalamus** is crucial for feeding control and is a site of convergence and integration of these nutrient-related signals with other central and peripheral neuronal inputs as well as hormonal and metabolic signals that reflect changes in global energy status (Schwartz, 2006). Hypothalamus is located close to discontinuous blood–brain barrier, allowing direct access of circulating hormones, metabolites and nutrients to the CNS (Jordan et al., 2010; Jordan et al., 2010). They provide information about energy homeostasis and cooperately regulate the activity of neuron populations that control food intake, energy expenditure, and glucose homeostasis. Hypothalamic nutrient and hormone signaling converge in these neurons influencing each other and controlling their excitability. Neuron populations respond to changes in circulating hormone and nutrient concentrations by either activating or inhibiting their firing activity.

Hypothalamus contains two interconnected groups of neurons producing **neurotransmitters** with opposing effects on food intake and energy expenditure. One neuronal group produces orexigenic neurotransmiters neuropeptide Y (NPY) and agouti related peptide (AgRP), and the other produces anorexigenic neurotransmiters pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript peptide (CART). These are the most important neuronal populations that serve as primary sensors for circulating signals in the hypothalamus and in turn regulate energy homeostasis. NPY has been described as the most powerful central enhancer of appetite, and its expression is abundantly co-localized with AgRP expression, which is synthesized exclusively in the arcuate nucleus. POMC is the precursor of several molecules and, importantly, of the anorexigenic alpha melanocyte stimulating hormone (α-MSH), which is the main regulator of energy balance in the melanocortin family and mediates its anorexigenic effects through melanocortin receptors. α-MSH and AGRP target CNS neurons that express melanocortin receptors (MC4R and/or MC3R). α-MSH is their endogenous agonist and AGRP their endogenous antagonist. Binding of α-MSH

provokes intracellular transduction which final effect is inhibition of food intake and increase of energy expenditure. CART is the other anorexigenic important peptide and, similarly to NPY/AgRP neurons, 90% of CART neurons are co-localized with POMC neurons in the arcuate nucleus. This complex hypothalamic system that receives a number of afferent signals on the state of body energy levels and in turn regulates body energy homeostasis is called central melanocortin system (Fan et al., 2005). Its activity is vastly regulated by leptin and ghrelin signalling which affect the balance of orexigenic and anorexigenic neurotransmitters in turn influencing food intake and energy expenditure.

Leptin binds to leptin receptors expressed on neurons in the hypothalamus (Burgos-Ramos et al., 2010). Both NPY/AgRP and POMC/CART arcuate neurons express the long form of the leptin receptor (OB-Rb) (a class I cytokine receptor, crucial for the mediation of leptin effects). Leptin activates neurons that produce MC4R agonist (α-MSH) and inhibits neurons that produce MC4R antagonist (AGRP), in that way stimulating α-MSH signaling pathway. Through its receptors at hypothalamus, leptin activates JAK2-STAT3 signaling, subsequently increasing the synthesis and secretion of anorexigenic (POMC, CART) and decreasing the synthesis and secretion of orexigenic peptides (NPY, AgRP) in that way suppressing food intake and inducing energy expenditure. Such mechanism regulates food intake and energy balance in accordance with the information about total body fat conveyed by the leptin to central nervous system. Leptin also activates neurons that produce thyrotropin releasing hormone (TRH), and thereby the hypothalamic-pituitary-thyroid axis, which leads to increased energy expenditure (Ghamari-Langroudi et al., 2010). **Ghrelin** exerts its action by binding to its receptors in neurons of the vagal system and in the hypothalamus leading to activation of orexigenic NPY/AGRP and inhibition of anorexigenic circuits, in an opposite, competing way to leptin (Abizaid and Horvath, 2012; Cummings and Foster, 2003).

1.1.2   Control of energy expenditure

Control of energy expenditure includes biochemical processes that control the degree to which energy from food is used (Lowell and Spiegelman, 2000). Energy in the body is expended in three main processes: basal metabolism, physical activity and thermogenesis (adaptive thermogenesis and thermic effect of food). Basal metabolism is

an obligatory energy expenditure, required for steady-state performance of cellular and organ function such as cardio-respiratory activity and maintenance of cellular integrity (Henry, 2005). Physical activity is the energy expenditure associated with muscular work.

Of particular interest is the control of energy expenditure through changes in thermogenesis i.e. regulated dissipation of energy as heat in response to cold (cold-induced thermogenesis) and diet (diet-induced thermogenesis), through the oxidation of stored lipids (Lowell and Spiegelman, 2000). Thus, the activation of **adaptive thermogenesis** makes it possible to dissipate part of the energy contained in food as heat instead of accumulating it as fat, in that way reducing the feed efficiency (weight gained per calories consumed). If adaptive thermogenesis is activated than more energy from food consumed will be dissipated as heat and less will be used in the production of energy-rich molecules ATP (adenosine triphosphate) and NADPH (nicotinamide adenine dinucleotide phosphate), or in the case when substrates are abundant, less will be available for storage. This phenomena should not be confused with the **thermic effect of food (TEF)** which is a general effect of feeding on an acute increase of the resting metabolic rate (Reed and Hill, 1996). TEF is the energy required for the processing of the ingested nutrients (digestion, transport, metabolism, storage) and is stable for given nutrients. On the other hand, as it name indicates, adaptive thermogenesis is, as opposed to thermic effect of food, adaptable and can change depending on the amount of energy ingested.

Thermogenesis is mediated by mitochondrial uncoupling proteins (**UCPs**) (Ricquier and Bouillaud, 2000) that separate oxidative phosphorylation from ATP synthesis, so that substrates are oxidized even if there is no need of ATP. UCP1 behaves functionally as a regulated proton transport protein. It is a channel for H^+ ions like ATP-synthase, but unlike it, UCP1 does not produce ATP but instead 'produces' heat. **Brown adipose tissuee (BAT)** is specialized in adaptive thermogenesis and is rich in mitochondria that express uncoupling protein 1; UCP1 (Nedergaard et al., 2007). Brown adipocytes also express high levels of fatty acid oxidation enzymes and respiratory chain components, contributing to a high oxidative capacity. They are also characterized by multilocular fat distribution, as opposed to unilocular lipid depots present in white adipocytes. Typical white adipocytes are also poor in mitochondria that do not express UCP1 and have a low oxidative capacity. They are specialized in storage and release of

energy. Rodents have well-defined BAT depots present throughout the life and for them BAT thermogenesis is critical for thermoregulation and is also a defense against obesity. In rodents, overfeeding activates adaptive thermogenesis in brown adipose tissue, a response that opposes weight gain (Rothwell and Stock, 1979; Rothwell and Stock, 1979). For many years it has been assumed that BAT depots undergo atrophy in humans after birth. However, although it is now recognized that adult humans do have functional BAT depots (Nedergaard et al., 2007; Saito, 2013), the contribution of adaptive termogenesis to human energy balance is still under debate.
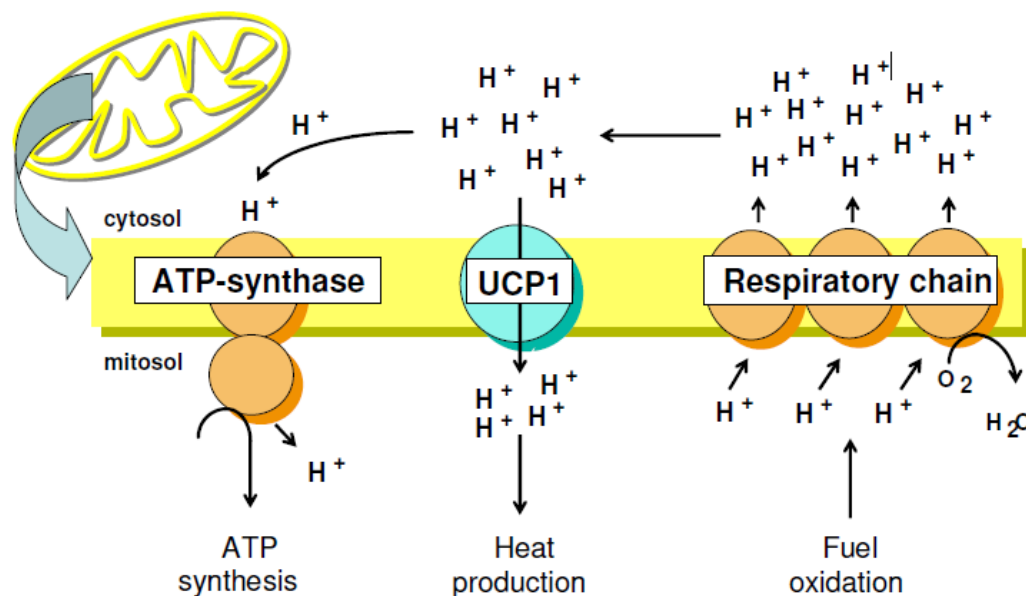
Figure 3. The functioning of UCP1: UCP1 behaves functionally as a regulated proton transport protein. Adapted from (Palou et al., 1998).

Beside **UCP1 other homologues** exist in other tissues such as UCP2 in the liver and UCP3 in the skeletal muscle where they also serve in the adaptive thermogenesis (Ricquier and Bouillaud, 2000). However their primary function in the modulation of energy expenditure is unlikely. Experimental data showing their increased expression during starvation and an absence of obesity in knockout mice suggest novel putative functions which are derived from their uncoupling activity. These include the regulation of ATP-dependent cellular processes (such as insulin secretion in pancreatic beta cells that is mediated by glucose metabolism and increased cellular ATP/ADP ratio, and

which is inhibited by UCP2 activity), reduction of the production of reactive oxygen species (ROS) in mitochondria and a facilitation of fatty acid handling by mitochondria (acting as *fatty acid cyclers*). In fact, with the discovery of the role of UCP proteins, the inicialy great interest for fatty acid cyclers gradualy decreased.

In addition, interest for brown adipose tissue grew even more with the discovery of the inducible property of white adipocytes to acquire more brown fat-like properties in response to appropriate stimuli. Such cells interspersed among typical white adipocytes in the white adipose tissue are called **'brite'** (from brown-in-white) **or 'beige' adipocytes** and have characteristics similar to brown adipocytes, including expression of UCP1 and multilocular intracellular lipid distribution (Oberkofler et al., 1997; Bonet et al., 2013). These isolated cells found in several different adipose depots are characteristic of adult humans and adult ferrets which unlike rodents do not present morphologically well-defined BAT. There is a controversy whether they originate from proliferation and de novo differentiation of a specific pool of precursor cells contained in WAT depots or are derived through the transdifferentiation from pre-existing white adipocytes. It is also not clear if they derive from the same precursor as classical brown adipocytes or are a distinct type of thermogenic fat cells (Bonet et al., 2013).

This process of white to brown fat differentiation is called **browning** and includes enhancing of white adipocytes oxidative metabolism and the capacity for uncoupled respiration through the induction of UCP1 expression. Such changes occur in response to specific stimuli such as chronic cold exposure or β-adrenergic stimulation, and other pharmacological and nutritional agents (Bonet et al., 2013). In fact, many stimuli activating BAT also induce WAT browning. This adaptable property of white adipocytes raises many interesting possibilities of nutritional and pharmacological interventions able to induce their brown adipocyte programming. Moreover, due to the participation of these processes in body energy expenditure their activation can help maintain body weight and prevent metabolic damage, such as insulin resistance and diabetes, caused by excessive fat accumulation and excess circulating fatty acids. For these reasons activation of browning and BAT thermogenesis appear as new potential strategies to treat obesity and related metabolic complications and constitute an active area of research.

Being the center of both oxidative metabolism and also thermogenesis, mitochondria are the master organels of cell fuel utilization. **Mitochondrial biogenesis**

is an active process including synthesis and degradation. Mitochondria are produced and eventually after normal functioning degraded and this turnover is adjusted depending on the cell energy needs (Vina et al., 2009). Mitochondrial synthesis is stimulated by the PGC-1α-NRF1-TFAM pathway (Vina et al., 2009). PGC-1α is the initiator of mitochondrial biogenesis. NRF1 is an intermediate transcription factor which stimulates the synthesis of TFAM which is a final effector activating the duplication of mitochondrial DNA molecules. PGC-1α and PGC-1β belong to peroxisome proliferator-activated receptor-c coactivators (PGCs), nuclear coactivators of mitochondrial biogenesis that control the transcription of genes involved in cellular energy metabolism and are highly expressed in tissues rich in mitochondria. PGC-1α has already been extensively studied and its role is known in a number of processes including adaptive thermogenesis, mitochondrial function, fatty acid β-oxidation and adipocyte differentiation (Finck and Kelly, 2006). Less is known about PGC-1β although its role is confirmed in mitochondrial biogenesis in liver, muscle and brown adipocytes and in myocardial development. PGC-1β modulates the expression of genes controlling mitochondrial function and adipogenesis and its expression increases during brown adipocyte differentiation, as do mitochondrial number and oxidation function (Uldry et al., 2006).

Energy expenditure is, like food intake, under central control by the brain and is in fact tightly linked to and coordinated with the control of food intake. Cold and nutritional status (food intake or starvation) are afferent signals that affect **central melanocortin system**. This system plays a key role in the control of energy balance by controling the output (activity) of efferent pathways regulating food intake and energy expenditure (Fan et al., 2005). Signals from the melanocortin system are transmited further to the two major efferent pathways controlling energy expenditure: sympathetic nervous system (SNS) and hypothalamic-pituitary-thyroid axis. They act in synergy exerting their effects on target thermogenic tissues, BAT, skeletal muscle and liver. As mentioned before **sympathetic nervous system** via its mediator noradrenaline controls BAT thermogenesis through acute effects such as stimulation of lipolysis and activation of UCP1 activity (by lipolysis-derived fatty acids) and through chronic effects including UCP1 gene transcription, mitochondrial biogenesis, BAT hyperplasia and recruitment of brown adipocytes in WAT depots (Bartness et al., 2010). **Hypothalamic-pituitary-thyroid axis** is a hormonal system consisting of thyrotropin releasing hormone (TRH)

released by the hypothalamus that stimulates pituitary gland to release TSH (thyroid stimulating hormone) and TSH stimulates thyroid gland to release thryroid hormones triiodothyronine (T3) and thyroxine (T4) (Silvestri et al., 2005). These hormones are the master regulators of metabolism affecting the basal metabolic rate (BMR) and protein, fat, and carbohydrate metabolism. However their mechanisms of action remain poorly understood. T3 is a regulator of gene expression through the activation of thyroid receptor (TR), through which it also enhances UCP1 gene transcription in brown adipocytes (Ribeiro et al., 2010; Ribeiro et al., 2010) .

1.1.3   Control of local tissue/cellular processes

Balance between adipogenesis and other local processes such as preadipocyte proliferation, adipocyte remodelling (e.g. browning explained before) and adipocyte apoptosis i.e. a balance between pro- and anti-adipogenic transcriptional factors and environmental factors determines whether pre-adipocytes remain quiescent, divide or undergo adipogenesis influencing finally the development of adipose tissue (Feve, 2005). There are many different factors both physiological and environmental that affect the development of adipose tissue, either stimulating or inhibiting adipogenesis. Factors that regulate adipogenesis either promote or block the cascade of transcription factors that co-ordinate the differentiation process. For example, some inflammatory cytokines can inhibit stem cell commitment and differentiation or even induce dedifferentiation.

It is generally accepted that the adipocyte number is a major determinant for the fat mass in adults and that the number of fat cells stays constant in adulthood in lean and obese individuals, even after marked weight loss, indicating that the number of adipocytes is set during childhood and adolescence. However, a certain percentage (10%) of adipocyes is renewed annualy, through the coordination of *de novo* adipogeneis and a death of existing adipocytes (Spalding et al., 2008). New fat cells can be generated throughout life from pre-adipocyte cells present in the tissue depending on the environmental factors, especially nutritional status (Feve, 2005).

Adipogenesis is the process by which cells specialised in energy storage or dissipation (white and brown adipocytes, respectively) are formed through the differentiation of precursor cells into mature adipocytes. Mature adipose cells are characterized by certain abilities such as: transport of large amounts of glucose in

response to insulin, FA synthesis and TAG storage in times of energy excess i.e. TAG hydrolyses in times of energy deprivation and in the case of brown adipocytes a great capacity for thermogenesis. Finally, adipocyte maturation is characterized by the capacity of the cell to synthesize and secrete an array of protein and non-protein factors, some of which are involved in the endocrine control of energy homeostasis. During gestation adipose cells are formed from mesoderm. BAT appears during late gestation, while WAT develops rapidly early after birth as a result of increased size of existing fat cells and proliferation of precursor pre-adipocyte cells (Feve, 2005). This **perinatal origin** of WAT makes this period especially important control point in the regulation of its development and as such especially responsive to dietary influences.

Adipogenesis is controlled by an interplay of many transcription factors, including members of the C/EBP (CCAAT/enhancer binding protein), PPARγ (peroxisome proliferator-activated receptor γ) and ADD (adenosine deaminase) families (Wu et al., 1999; Morrison and Farmer, 1999). **PPARγ** is a master regulator of adipogenesis and adipocyte hyperthrophy that activates genes that encode adipocyte markers and proteins directly or indirectly involved in lipogenesis. PPARγ expression in adipose tissue is induced by insulin.

Apoptosis of adipose cells is emerging as a new target in the treatment of obesity (Alkhouri et al., 2010). Apoptosis is a normal phenomenon of cell death for the purpose of maintaining homeostasis. It is assumed that through the induction of apoptosis we could remove adipocytes and in that way achieve fat loss. It is reported that several adipokines and natural products play roles in the induction of adipocyte apoptosis e.g. leptin, tumor necrosis factor-α (TNF-α), and some natural compounds.

As mentioned before adipose tissue is an important source of molecular signals, as leptin, which can in turn affect development of adipose tissue. Leptin inhibits adipogenesis and also increases lipolysis in the muscle and BAT and FA oxidation in the muscle and liver (Muoio and Lynis, 2002).

Moreover, metabolism and development of adipose tissue are influenced by hormones and other signalling molecules secreted from other tissues such as liver and skeletal muscle. In that way they communicate with each other and orchestrate functions to maintain body homeostasis **Adipose tissue and skeletal muscle** are the largest organs in the body and beside their main functions in energy storage/expenditure i.e. movement both also have endocrine function which provides molecular basis

underlying their cross talk. Both adipocytes and myocytes produce and release a range of bioactive proteins, generally termed adipokines in the case of adipocytes and myokines for muscle cells (Trayhurn et al., 2011). Term **myokine** refers to cytokines and other peptides that are produced, expressed and released by muscle fibres and exert either autocrine, paracrine or endocrine effects. Via myokines that work in a hormone-like fashion exerting specific endocrine effects, muscles communicate with other organs of the body, such as adipose tissue. Some of the myokines proved to exert effects upon adipose tissue are interleukin-6 (IL-6), fibroblast growth factor-21 (FGF21) and FINC5 (Irisin). IL-6, beside acting localy, also stimulates lipolysis in adipose tissue (Pedersen and Febbraio, 2005). FGF21 induces hepatic expression of peroxisome proliferator-activated receptor gamma coactivator protein-1alpha (PGC-1α), a key transcriptional regulator of energy homeostasis, and causes corresponding increases in fatty acid oxidation, tricarboxylic acid cycle flux and gluconeogenesis without increasing glycogenolysis (Potthoff et al., 2009; Domouzoglou and Maratos-Flier, 2011). Irisin is a PGC-1α-dependent myokine which drives brown-fat-like development of white adipose tissue (Pedersen and Febbraio, 2012).

Adipose tissue is one of the key players in the regulatory system exerting an important influence on hormone-regulated fuel partitioning in peripheral tissues, and it relates to many metabolic complications of obesity. Consequently, beside energy intake and energy expenditure, adipogenesis, and in general fuel usage in the body, is vastly influenced by nutrient partitioning, a process of distribution of nutrients among tissues and metabolic pathways (Palou et al., 2000). In the process of **nutrient partitioning between tissues** nutrients are in general partitioned in adipose tissue for storage and in muscle for oxidation. Liver is however the main site of de novo lipogenesis (Jump et al., 2005). In the tissues nutrients are further distributed betweeen different metabolic pathways, namely catabolitic e.g. glycolysis, fat oxidation or protein degradation and anabolitic e.g. gluconeogeneis, lipogenesis or protein synthesis, depending on body energy status.

Some of the key proteins involved in the **nutrient partitioning between metabolic pathways** are the ones responsible for the mobilization and transport of nutrients between different body systems and cellular compartments and in their metabolism, as well as regulatory enzymes that can activate and deactivate these proteins in that way switching on and off catabolic i.e. anabolic pathways depending on

body energy levels. Some of the examples are: lipoprotein lipase (LPL, hydrolyzes TAG in lipoproteins and is also involved in promoting cellular uptake of chylomicron remnants, cholesterol-rich lipoproteins, and fatty acids) (Yasuda et al., 2010), hormone sensitive lipase (HSL, hydrolyses TAG, DAG, and MAG as well as cholesteryl and retinyl esters), adipose triglyceride lipase (ATGL, desnutrin, hydrolases only TAG), perilipin A (lipid droplet-associated protein that controlls the magnitude of lipolysis) (Jaworski et al., 2007), glucose transporter type 4 (GLUT4, mediates insulin-stimulated glucose entry into adipocytes and muscle cells) (Wood and Trayhurn, 2003), acetyl-CoA carboxylase (ACC, controls fatty acid synthesis and oxidation through the inhibition of carnitine palmitoyltransferase (CPT1) mediated transport of FA to mitochondria) (Turkish and Sturley, 2007; Turkish and Sturley, 2007). Additionaly, activity of abovementioned enzymes is greatly controlled by the activity of protein kinase A (PKA, modifies other proteins activity by phosphorylating them, its activity is dependent on cellular levels of cyclic AMP, cAMP) (Jaworski et al., 2007) and AMP-kinase (AMPK, controls energy metabolism by switching between anabolic and catabolic pathways depending on the ATP/ADP ratio) (Long and Zierath, 2006). Thus, by controlling the activity of this and many other proteins and enzymes involved in different metabolic reactions anabolic pathways prevail when building blocks and energy are abundant, i.e. after the meals. On the other hand, catabolic pathways in tissues acutely increase during starvation, when degradation of energetic molecules is activated, and hence provision of nutrients and energy to tissues is increased.

Key transcriptional factors and cofactors involved in fat mobilization i.e. expression of genes encoding proteins related to cellular FA oxidation are: peroxisome proliferator activated receptors (PPARs) and peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1). **PPARs** (PPARα in the liver, PPARδ in the mucle and PPARγ in the adipose tissue) are nuclear receptors that modulate target gene transcription after they become activated through agonist ligand binding (Berger and Moller, 2002). The endogenous agonist ligands of PPARs are certain fatty acids (mainly polyunsaturated fatty acids, PUFAs) and fatty acid derivatives. They bind as heterodimers with RXR (retinoid X receptors) to particular DNA response elements in the promoter of target genes and activate transcription of genes encoding proteins related to fatty acid catabolism. **PGC-1** is a transcriptional coactivator highly expressed in BAT that coactivates transcriptional factors (such as PPARs) that are involved in the

expression of genes encoding proteins related to cellular fatty acid oxidation, transcription factors related to mitochondrial biogenesis and function in BAT and mitochondrial genes that contribute to the program of adaptive thermogenesis such as UCP1 (Finck and Kelly, 2006).

1.2 Effects of nutrients on physiological processes involved in body weight regulatory system

Beside effects of nutrients in general on food intake and energy expenditure food components exert some specific effects on the systems regulating energy balance and nutrient-gene interactions. Nutrient availability conditions metabolic fluxes i.e. activity of metabolic pathways but nutrients also condition hormonal control of metabolism and affect the expression and/or activity of proteins, including proteins that regulate gene expression (Marshall, 2006).

1.2.1 Effects of nutrients on food intake

It is already known that macronutrients have different **satiating capacity** with the proteins being the most and fat being the least satiating (Halton and Hu, 2004). In termes of GI effects proteins induce the highest CCK release, carbohydrates induce GLP-1 and amylin release and fat induces higher PYY release. The intake of carbohydrates induces a greater increase of circulating leptin than the isoenergetic intake of fat (Romon et al., 1999). Also, ghrelin decrease produced after food intake is longer lasting after intake of carbohydrates then in the case of fat where soon after the intake it begins to increase again leading to the sooner appearance of hunger (Sanchez et al., 2004a). In addition, dietary fat (as well as fructose) do not stimulate insulin secretion and leptin production. Therefore, attenuated production of insulin and leptin could lead to increased energy intake and contribute to weight gain and obesity during long-term consumption of diets high in fat and/or fructose (Havel, 2001). It is already well known that chronic consumption of high-fat diets impairs body negative feedback inhibition on food intake leading to overconsumption and obesity. Chronic increase in dietary fat is associated with an attenuation of the feedback signals with a consequent relative acceleration of gastric emptying, modulation of GI hormone secretion, and attenuation of the suppression of energy intake (Little et al., 2007).

However, inside this big groups of macronutrients there are differences depending on the physicochemical properties of the particular type of nutrient. For example, different carbohydrates and lipids are differently digested in the gastrointestinal system and further on differently metabolized. This affects their bioavailability ultimately leading to different effects in the organism.

Concretely, carbohydrates differ in their **glicemic index**, a grading system for carbohydrates based on their immediate effect on the levels of glucose in the blood (Venn and Green, 2007). Carbohydrates that break down rapidly during digestion releasing glucose rapidly into the bloodstream have a high glicemic index (white bread, white rice, glucose, potato); carbohydrates that break down slowly, releasing glucose gradually into the bloodstream, have a low glicemic index (whole intact grains, beans, most fruits and vegetables, fructose). Consumption of carbohydrates with high glicemic index provokes higher secretion of insulin and in that condition because of insulin anabolic effects fat deposition is promoted. Increased fat content in turn leads to increased insulin resistance which again leads to consumption of carbohydrates with high glicemic index and in that way vicious circle keeps going on. On the other hand, foods with low glicemic index have significant health benefits. A lower glycemic response is often thought to equate to a lower insulin demand, better long-term blood glucose control and a reduction in blood lipids. Thus, glicemic index of carbohydrates has an effect on energy balance and can be taken into consideration when planning a diet.

Similarly, induction of satiety by fat i.e. triacylglycerols ingestion depends on the particular composition of fatty acids i.e. the length and saturation degree of their chain and the rate of fat digestion. For example there is a considerable difference in the way of digestion between **diacylglycerols (DAG)** and triacylglycerols (TAG) which in turn affects their satiating capacity (Kovacs and Mela, 2006). DAG have glycerol esterified with 2 fatty acids, unlike TAG that have glycerol esterified on all three positions. DAG are absorbed similarly to TAG but are differently metabolized. They are more oxidized and less stored in the adipose tissue, which makes them a good substitute for fat in a diet. Both TAG and DAG are hydrolised to 2 fatty acids and monoacylglycerol, MAG. Dietary TAG is hydrolysed by 1,3-specific lipase to form 1,2-DAG, which is further hydrolysed to 2-monoglyceride (2-MAG). The 2-MAG and their hydrolysed fatty acids are absorbed and rapidly re-esterified to TAG in the small

intestine epithelial cells, and packed into chylomicrons. During circulation, the diet-derived TAG are largely taken up by the adipose tissue, where they accumulate as body fat, unless consumed as energy. Small amounts are taken up by the liver for metabolism or recirculation in other lipoprotein particles. In contrast, dietary 1,3-DAG is hydrolysed to form 1-MAG in the intestinal lumen. In comparison to 2-MAG, re-esterification of 1-MAG to TAG proceeds through a relatively less efficient route, apparently leaving a surfeit of FA in the intestinal epithelial cells. A proportion of these fatty acids will bypass the re-esterification step, instead entering the portal vein and will be transported to the liver where they are mostly oxidized. The relative amounts of fatty acids derived from 1,3-DAG that are re-esterified within the enterocyte vs. delivered directly to the liver are not clear. Several enzymes involved in fatty acid oxidation have been found to increase with diacylglycerol consumption for 14 days, whereas those involved in synthesis decreased (St-Onge, 2005).

Furthermore there is a difference in satiating capacity between TAG depending on the type and distribution of its constituting FA. TAG rich in medium-chain fatty acids (MCFA, fatty acids that contain between 6-12 carbon atoms), also called **medium-chain triacylglycerols (MCT)**, differ from long-chain triacylglycerols (LCT, rich in long-chain fatty acids, LCFA) not only in their chemical composition but also in the manner in which they are absorbed and transported from the GI tract to the organs (Kovacs and Mela, 2006). Both MCT and LCT are digested to their respective MCFA and LCFA in the GI tract, and they are absorbed inside epithelial cells of the intestine. There LCFA are repackaged as LCT into chylomicrons for transport through the peripheral circulation to peripheral tissues where they accumulate as body fat, unless consumed as energy. Some part of LCFA also end up in the liver where they are mostly used to form complex lipids and to a lesser extent oxidized. MCFA differ from LCFA in the chemical composition, circulating metabolism and mitochondrial transport. MCFA mitochondrial transport, unlike the one for LCFA, does not require enzyme carnitine palmitoyltransferase (CPT) that mediates the transport of LCFA to mitochondria. This fact probably accelerates their oxidation, and limits storage of MCT within tissues. MCFA also have less caloric density (8.3 kcal/g compared to 9 kcal/g) and are more satiating and induce higher postprandial thermogenesis then LCFA. Furthermore, because of their shorter chain lengths, MCFA do not require chylomicron formation for their absorption and transport and as a result travel directly to the liver via the portal

circulation. Therefore, they bypass peripheral tissues such as adipose tissue, which makes them less susceptible to the actions of hormone-sensitive lipase and to deposition into adipose tissue stores. As a result, LCFA are mostly deposited into adipose tissue, whereas MCFA are mostly oxidized by the liver as a source of energy, and small amounts are elongated to LCFA and incorporated into complex lipids (St-Onge, 2005). Due to this properties MCT are used in functional foods to control food intake by increasing their satiating capacity, but also by increasing energy expenditure and decreasing fat storage.

However, beside these well known general effects of large groups of nutrients, there are differences depending on the particular type of nutrient. The effects of specific nutrients on energy intake are still largely unknown. This is particularly pronounced in the case of fatty acids where the wide variety of their structures and physicochemical properties makes it difficult to draw general conclusions.

1.2.1.1 Effects of Fatty acids

Fatty acids are carboxylic acids with a long aliphatic tail (chain), which is either saturated or unsaturated. They are liberated from triglycerides during their breakdown by gastrointestinal lipases and are important sources of fuel as during oxidation yield large quantities of ATP. Based on the chain lenght they are categorized as short-chain fatty acids (SCFA, less then 6 carbons), medium-chain fatty acids (MCFA, 6–12 carbons) and long-chain fatty acids (LCFA, more then 12 carbons). Due to this great variety of **fatty acids structures** and their physicochemical properties there are still many gaps in the knowledge on the effects of specific fatty acids on food intake, energy expenditure and local tissue processes.

It is already known that most effects lipids induce in the GI tract, such as slowing of gastric emptying, stimulation of GI hormone release and suppression of ghrelin secretion and energy intake are mediated by the interaction of fatty acids (FA), and not TAG, with the gut receptors (Cummings and Overduin, 2007). Thus, **fat digestion** is a critical step for fat induced appetite inhibition, stimulation of GI hormone release and slowing of gastric emptying (Feinle-Bisset et al., 2005). All gastrointestinal tract is lined with different nutrient receptors which collect information about the ingested food and carry it to the hypothalamus even before different nutrients are being

digested and absorbed. FA liberated from TAG in duodenum readily bind to the FA receptors and in that way provoke satiety and hormone release even before they are absorbed. The interaction of FA with specific receptors in the small intestine stimulates release of satiety hormones and consequently regulates pancreatic enzyme secretion, gastric emptying and energy intake (Greenberg et al., 1987). Thus at least some of the associated satiety signals emanate from the gut, rather than from postabsorptive sites i.e. effects of fat in the intestine are independent of fat metabolism and occur before digestion products of fat enter into the bloodstream (Beglinger and Degen, 2004). It is also likely that a preabsorptive satiating action of FA involves stimulation of vagal afferent fibres that transmit satiety information to the central nervous system (Berthoud et al., 1991; Berthoud et al., 1991; Phillips and Powley, 1998; Phillips and Powley, 1998).

Moreover, this preabsorptive fat action, besides intraduodenaly, starts already from the **mouth** where FA receptor **CD36** is stimulated by FA liberated from ingested triglycerides by **linguinal lipase** (Kawai and Fushiki, 2003). The fatty acid transporter CD36 besides in apical microvilli of enterocytes is also found in the apical part of the taste bud cells and appears to be involved in the oral detection of dietary lipids. Lingual lipase produces significant levels of FA from dietary TAG within 1 to 5 seconds of exposure to the gustatory epithelium. Inhibition of lingual lipase attenuates the inhibitory effect of oral fat on subsequent energy intake (O'Donovan et al., 2003) and markedly diminishes the preference for lipids (Kawai and Fushiki, 2003). A potential physiological advantage of an oral lipid detection is the functional preparation of the digestive tract to incoming lipid, leading to the optimization of its digestion and absorption. These FA liberated from TAG by lingual lipases could act directly on the endocrine cells in the stomach.

Effects of TAG on satiety and GI hormone secretion depend in part on the physicochemical properties of their constituting FA i.e. the length of the chain and the degree of fatty acid saturation. Regarding the **effects of the chain lenght** the studies are not conclusive and although some point out the greater satiating capacity of MCFA as seen before, it is mostly accepted that reduction of hunger and food intake generally increases with increasing fatty acid chain length and the saturation level (Maljaars et al., 2009). Most studies report that the suppression of food intake is mediated at the intestine by a sensory system sensitive to fatty acids longer than 10 carbons (Greenberg

et al., 1987). However, between long chain fatty acids the effect of chain length is not clear and some studies report greater satiating capacity of the fatty acids with shorter chains, as is the case between lauric (C12) and oleic (C18) acid (Beglinger and Degen, 2004; Feltrin et al., 2008).

Considering the **effect of saturation**, it was seen that TAG with unsaturated FA increase satiety and hormone release, whereas TAG with saturated FA do not (Feinle-Bisset et al., 2005). This could be explained by the fact that the micelle are more easily formed with increased degree of fat unsaturation (Wu et al., 1991) and thus unsaturated FA are more readily available for sensing and absorption, which leads to increased satiety and hormone release (Raybould et al., 2006). Moreover, it was showed that significantly more stearic acid (saturated) than oleic acid (unsaturated) was found in stool (Jones et al., 1999). This was probably due to an increased ability of saturated FA to interact with calcium, which resulted in the formation of insoluble calcium-fatty acid soaps and increased fecal excretion (Shahkhalili et al., 2001). Both mechanisms (micelle formation, calcium binding) may be involved in the differences in satiety between saturated and unsaturated FA.

As seen before most of these **gastrointestinal effects** of TAG i.e. their constituting FA are mediated by release of CCK and ghrelin and later on by PYY and GLP-1. However, almost nothing is known considering the effects of FA on leptin expression and secretion.

Beside these effects of FA through the GI system they also exert more direct **effects on the CNS**. FA cross the blood–brain barrier mainly by simple diffusion in the unbound form and some through direct uptake of lipoprotein particles mediated by lipoprotein receptors (Jordan et al., 2010). Intracerebroventricular administration of a lipid emulsion is sufficient to suppress food intake, a signal that is independent of measurable changes in plasma insulin and does not require gastrointestinal nutrient absorption (Jordan et al., 2010). Although multiple brain regions are likely to be involved in the central effects of FA, the NPY and POMC neurons of the hypothalamic arcuate nucleus (ARC) stand out as a primary site of action for FA signalling (Wang et al., 2006).

1.2.2 Effects of nutrients on energy expenditure

Unlike in the case of food intake, the effects of nutrients on energy expenditure are quite known. Beside their different effects on food intake macronutrients also differ in terms of energy expenditure upon their ingestion. Thus, because of their different metabolism the net energy intake differs among them. Thermic effect of food (TEF) is an effect of feeding that it acutely increases the basal metabolic rate (BMR) and, as mentioned before, is the energy required for the processing of ingested nutrients (Reed and Hill, 1996). TEF is higher for proteins than for other macronutrients in the diet (20-35% of the ingested energy, compared to 5-15% for carbohydrates and 0-3% for fat) because their processing (digestion, transport, metabolism, storage) requires more energy (Halton and Hu, 2004; Westerterp et al., 1999). Thus, the part of energy being used from proteins is less then the one from carbohydrates and even less then from fat. Degradation of proteins is also considered as a possible futile cycle (combinations of reactions the net outcome of which is ATP consumption without any other evident biological output) and thus a mechanism of adaptive thermogenesis. As adaptive thermogenesis is a way of energy expenditure, by raising its rate degradation of proteins raises total energy expenditure i.e. BMR.

There is some evidence that **protein-rich diets** (≥25% of the energy as protein) can favour body fat loss even under isocaloric conditions. Protein rich-diets lead to increased thermogenesis and increased satiety as compared to low-protein diets (<15% of the energy as protein). This could happen through metabolic effects such as stimulation of protein anabolism in muscle (leucine and other branched-chain amino acids) and hormonal changes that lead to increased lipolysis (Halton and Hu, 2004; Layman and Walker, 2006).

Inside these big groups of macronutrients and their general effects, there are also distinctive effects of particular nutrients. Beside protein-rich diets, it is also well known that olive oil, polyunsaturated fatty acids (PUFA), triacylglycerols rich in medium chain fatty acids (MCT), vitamin A and pro-vitamin A carotenoids stimulate UCP expression and oxidative metabolism (Rodriguez et al., 2002; Sadurskis et al., 1995a; St-Onge and Jones, 2002; Mercader et al., 2006). Similarly, there are phytochemicals that enhance the activity and/or the effects of the sympathetic nervous system in fat cells (that is, lipolysis and thermogenesis in BAT and lipolysis in WAT), such as catechin (green tee), forskoline (Coleus forskohlii), caffeine (coffee, yerba mate, guarana), ephedrine (herbal

ephedra), synephrine (bitter orange), capsaicin (red pepper, tabasco sauce, mustard and ginger) (Kovacs and Mela, 2006; Scarpace and Matheny, 1996; Preuss et al., 2002). Interestingly, calcium anti-obesity effects are exerted through the reduction of intestinal fat absorption (Christensen et al., 2009).

For polyunsaturated fatty acids (**PUFA**) there has been described a number of molecular effects that favor the activation of oxidative metabolism in different tissues such as: liver (repression of lipogenic gene expression, activation of FA oxidation, induction of UCP2), muscle (activation of FA oxidation, induction of UCP3), WAT (repression of lipogenesis, increase of mitochondria number, activation of FA oxidation) and BAT (UCP1 induction) (Flachs et al., 2005; Baillie et al., 1999; Sadurskis et al., 1995b). This effects are explained by the fact that PUFA repress the expression and activation of SREBP1 (Sterol Regulatory Element-Binding Protein) and are activating ligands of PPARα and PPARδ (Jump, 2002; Raclot et al., 1997). These effects are especially pronounced for n-3 PUFA which supplementation in rodents led to the reduction of adiposity, especially visceral fat, without changes in body weight (Buckley and Howe, 2010). This effects of n-3 PUFA also lead to the amelioration of insulin resistance.

Brown fat adaptive thermogenesis and the process of **browning** are increasingly coming in the center of investigators interest due to the possibility of their modulation as a way to combat obesity. Different physiological, pharmacological and dietary determinants have been linked to white-to-brown fat remodelling (Bonet et al., 2013). Among the endogenous signals able to induce a program of brown fat differentiation are sympathetic nervous sytems mediators and thyroid hormones. Dietary factors that are associated with browning are olive oil, conjugated linoleic acid (CLA), n-3 fatty acids, resveratrol and retinoids. Among these latest it is already well known the positive effect of ATRA on adipose tissue oxidative and thermogenic capacity (Mercader et al., 2007; Amengual et al., 2008; Amengual et al., 2010). This eventually leads to the conversion of white adipocytes into more brown like adipocytes which in part explaines the fat loosening effect of ATRA. In addition, effects of ATRA as well as other nutrients known to induce browning are being linked more and more to their effects on mitochondria. In accordance with their metabolic role as a cellular organels responsible for fuel oxidation and thermogenesis, and bearing in mind their plasticity, they seem as a highly possible target of pharmacological and diet interventions, such as in the case of

ATRA. However, until now little is known about the adaptive responses of mitochondria during these modifying processes provoked by ATRA, especially in the adipose tissue, i.e. the role of mitochondriogenesis in the ATRA induced browning.

1.2.2.1 Effects of retinoids

Retinoids are a group of nutrients that include all vitamin A metabolites. They are known as being able to modulate growth and differentiation of a wide range of cells and tissues. Vitamin A (retinol, ROL) is ingested from a diet as a preformed vitamin A from two major precursors, retinyl esters (RE) and carotenoids (von, 2010). RE are efficiently hydrolyzed to ROL and as such absorbed from the intestine. β-carotene is the most vitamin A active of the provitamin A carotenoids, capable of producing 2 molecules of ROL from 1 molecule of β-carotene (Lee et al., 1999) and constitutes the main provitamin A source (Tourniaire et al., 2009). Diet vitamin A precursors are stored as RE or intracellularly metabolized to retinoic acid (RA), the main active form of vitamin A (von, 2010). There are two isoforms of RA, all-trans-RA (ATRA) and 9-cis-RA, which exert their effects on cell processes through, both, genomic and non genomic mechanisms (Tourniaire et al., 2009). After liver, adipose tissue is a major site of vitamin A storage and metabolism, as well as a main target of ATRA action.

ATRA is required for a wide range of biological processes, including reproduction, embryonic development, cell differentiation, immunity, and metabolic control (Michael B.Sporn et al., 1994). Effects of vitamin A on food intake are scarce. However, it was demonstrated that RA is able to directly modulate leptin synthesis in adipose tissue, and that change in circulating leptin levels could directly influence the regulation of food intake and energy metabolism (Kumar et al., 1999). On the other hand, effects of ATRA on energy expenditure and adipose tissue development have been extensively studied.

In mice, treatment with ATRA reduces body weight and adiposity independently of significant changes in food intake and improves glucose tolerance and insulin sensitivity in lean and obese animals. ATRA-induced body fat loss correlates with activation of BAT i.e. increased thermogenesis and induction of UCP1 (Puigserver et al., 1996; Serra et al., 1999; Bonet et al., 2000) and reduced adipogenic/lipogenic capabilities and increased capabilities for oxidative metabolism and thermogenesis in
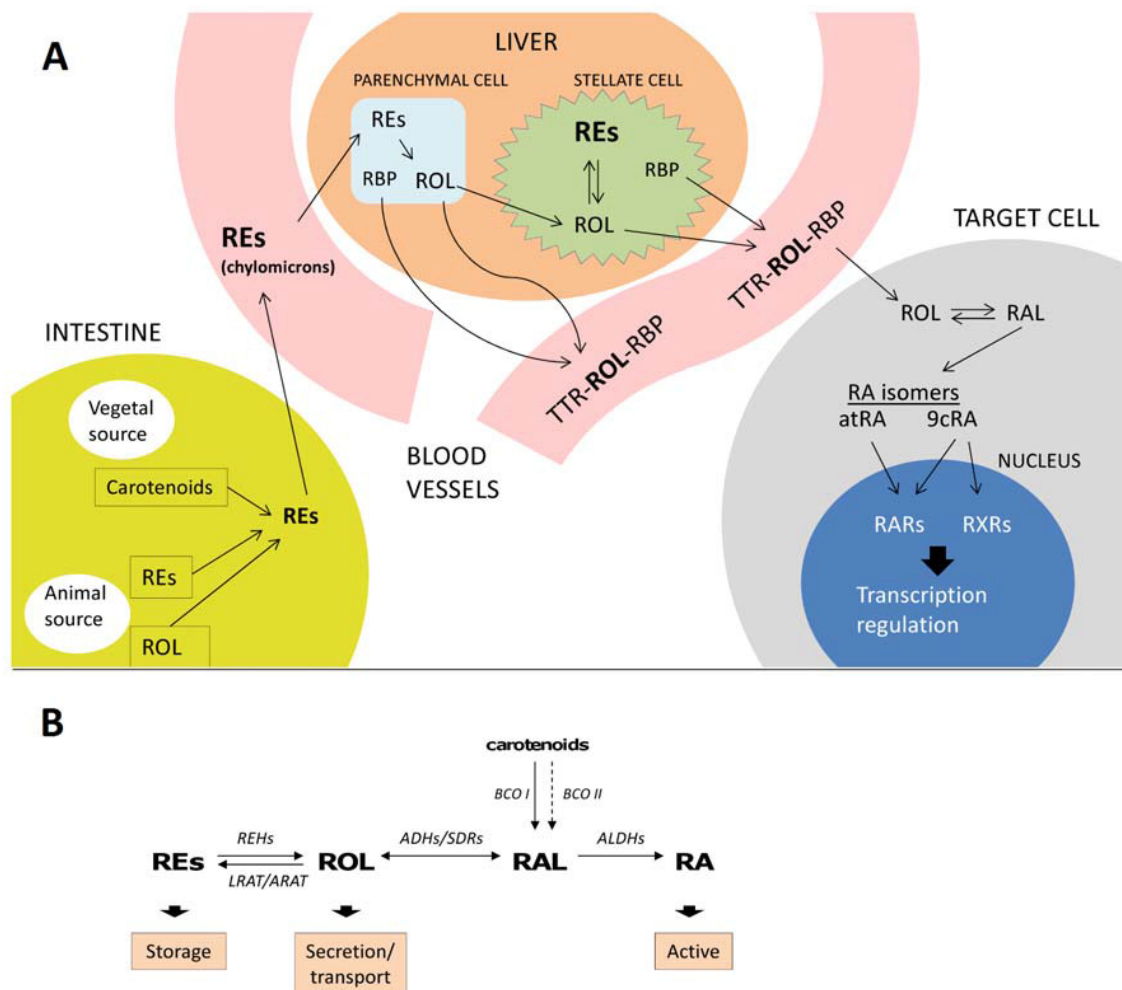
Figure 4. **Overview of the retinoid metabolism in vertebrates. A. Major pathways in retinoid metabolism.** In the intestine, retinoids and carotenoids taken in the diet (from animal and vegetable sources, respectively) are mainly converted to REs, which are incorporated into chylomicrons and taken to the liver trough the lymph and general circulation. In the liver the REs are taken up by hepatocytes and hydrolyzed to ROL which is bound to RBP for transport to storage or target cells. ROL could be stored as REs in liver stellate cells. When required, the ROL is bound to RBP and mobilized from hepatocytes or stellate cells to circulation, where is bound to TTR to avoid glomerular filtration in the kidney and to ensure delivery to target cells. Once in the target cell, free ROL is oxidized to RAL and then to RA, which enters the nucleus and activates RXRs and/or RARs, regulating gene transcription. **B: Main biochemical routes involved in the retinoid system.** (9cRA), 9-cis-retinoic acid; (ADHs) alcohol dehydrogenases; (ALDHs) aldehyde dehydrogenases; (ARAT) acyl-CoA:retinol acyltransferase; (atRA), all-trans-retinoic acid; (BCO I), β-carotene-15,15'-monooxygenase; (BCO II), β-carotene 9',10'-dioxygenase; (LRAT), lecithin:retinol acyltransferase; (RA), retinoic acid; (RAL), retinaldehyde; (RARs), retinoic acid receptors; (RBP), retinol binding protein; (REHs), retinyl ester hydrolases; (REs), retinyl esters; (ROL), retinol; (RXRs), retinoid X receptors; (SDRs) short-chain dehydrogenases/reductases; (TTR), transthyretin (Gesto et al., 2012).

WAT depots (Ribot et al., 2001; Felipe et al., 2004; Mercader et al., 2006; Mercader et al., 2007; Mercader et al., 2007; Mercader et al., 2008). This effects are unaccompanied by increased circulating nonesterifed FA (Mercader et al., 2006), suggesting that FA mobilized from fat stores undergo oxidation either within the adipocytes or in other tissues. Moreover, ATRA treatment increases lipid oxidation and thermogenic capacity also in skeletal muscle (Amengual et al., 2008) and liver (Amengual et al., 2010) in lean mice that also contribute to oxidizing FA released upon RA-induced WAT lipolysis. Similar effects were observed in ATRA treated diet induced obese mice (Berry and Noy, 2009).

Effects of retinoids are mediated through retinoid receptors that belong to the nuclear receptor superfamily of ligand-modulated transcription factors which bind to regulatory regions of target genes and activate their expression. ATRA is a hormone-like compound that regulates gene expression by binding and activating nuclear receptors **RAR** (retinoic acid receptor, responsive to both all-trans and 9-cis RA) **and RXR** (retinoid X receptor, responsive specifically to 9-cis RA). They function as homodimers and heterodimers that bind to DNA response elements in the promoters of target genes. RAR:RXR heterodimeric complexes have biological effects ranging from morphogenesis and organogenesis, to cell growth, differentiation and apoptosis (Tourniaire et al., 2009). RXR are also obligate heterodimeric partners of other class II nuclear receptors, including thyroid hormone receptor, vitamin D receptor, PPAR, farnesoid X receptor and liver X receptor. In that way through the modulation of the activity of these transcriptional factors ATRA can lead to multiple biological effects. Moreover, transcriptional activity of some RXR-containing heterodimers responds to ligands of either partner and is synergistically activated in the presence of both ligands (permissive heterodimers) (von, 2010).

An example of the later case are **PPAR:RXR** heterodimers. By the activation of RXR moiety RA influences PPAR-mediated responses. As mentioned before, PPARs are lipid-activated nuclear receptors critically involved in the transcriptional control of lipid metabolism. Thus, as PPAR and RAR, in order to bind to hormone response elements, need to form functional heterodimers with the same common partner, RXR, it appears that RA, via RXR, could interfere with the molecular action of fatty acids in adipose tissue. This indicates a crucial role of RA in the fatty acids signalling pathway and furthermore explains a synergistic effect of lipids and vitamin A on the growth of

adipose tissue in young rats i.e. their influence on the early normal development of the endogenous adipocyte precursors (Safonova et al., 1994b). An example of this is an excess of FA brought about by a high-fat diet (Redonnet et al., 2008). FA influence adipose tissue gene expression through up-regulation of a key transcriptional factor PPARγ. In this situation excess of ATRA activates RXR receptors thus making the complex PPAR:RXR even more active. In that way ATRA potentiates the effects of excess FA on preadipocyte proliferation and differentiation and facilitates the expansion of adipose tissue.

However, although much is known about general effects of RA, many details are missing considering the different doses and the time of action. It was shown that RA affects early stage of adipocyte differentiation in opposite directions depending on the dose, promoting it at low doses (Safonova et al., 1994a) and inhibiting at relatively high doses (Stone and Bernlohr, 1990; Xue et al., 1996). In the same way, despite these increasing evidence linking vitamin A and its precursors, such as β-carotene to the control of adiposity in adult animals, little is known about their effects in the early life. In particular, little is known about the effects of retinoids in the very early periods of life, especially during breastfeeding, which is particularly important as a part of the critical postnatal period of WAT development (Feve, 2005). Bearing this time frame in mind and the well known effects of ATRA on adipogenesis in adults it is to be expected the great importance of such dietary supplementation during this control period on the regulation of WAT development. This could in turn help explain the outcomes of such supplementation on the later health outcomes, in particular propensity to obesity and related disorders. Moreover, while the effects of β-carotene conversion products, especially RAL and RA, on adipocyte differentiation, adipokine secretion and lipid metabolism, have been well described, the effects of intact β-carotene remain largely unknown.

As mentioned before, mitochondria are key organels of energy expenditure, responsible for the majority of cellular ATP and heat production. Their content and activity respond and adjust to changing cellular stresses and metabolic demands. For these reasons, the process of their development i.e. **mitochondriogenesis** is increasingly coming in the center of interest for nutritional interventions possible to affect this process in that way increasing cell i.e. body oxidative capacity. That was shown for the vitamin A metabolite ATRA whose treatment induced the expression of relevant

transcription factors linked to mitochondria biogenesis in the skeletal muscle (Berry and Noy, 2009). In fact, effects of retinoids in general on body energy expenditure are especially well studied and known in many details.

1.2.3 Effects of nutrients on adipogenesis/local tissue processes

Effects of nutrients on local tissue processes are still largely unknown. Beside many intracellular mediators and hormonal signals discussed before adipogenesis is also affected by several nutrients, such as glucose, fatty acids, amino acids, vitamin A (retinoic acid), vitamin D and conjugated linoleic acid (CLA).

There are specific diets and nutrients that may affect the balance between lipogenesis and fat oxidation and thereby body composition. For example diets rich in carbohydrates and poor in lipids favor liver lipogenesis. After carbohydrate-rich meals a rise in **glucose** and insulin favor the expression of glycolytic and lipogenic genes, by stimulation of expression and/or activity of key transcription factors such as sterol regulatory element-binding protein 1 (SREBP-1), carbohydrate regulatory element-binding protein (ChREBP) and PPARs which are important for the transcriptional control of lipid metabolism (Turkish and Sturley, 2007; Marty et al., 2007; Berger and Moller, 2002). SREBP-1 activates genes for enzymes involved in fatty acid and triacylglycerol synthesis and ChREBP induces the expression of genes that encode glycolytic and lipogenic enzymes. Insulin also favors the activation of key glycolytic and lipogenic enzymes. Under these conditions, lipogenic tissues (liver, white adipose tissue) obtain all what is needed for fatty acid synthesis from insulin-stimulated glucose metabolism (ATP, acetyl-CoA and NADPH). This changes favor the conversion of excess carbohydrate in the diet into fatty acids.

Similarly, **fatty acids** in general favour adipogenesis, likely through the activation of PPARγ (Berger and Moller, 2002). n-6 PUFA, linoleic and arachidonic acid seem to be particularly proadipogenic because in preadipocytes they serve as precursors of prostacyclins, which, acting in a paracrine and autocrine manner, favor early steps of adipogenesis (Zamaria, 2004). n-3 PUFA inhibit prostacyclin production from n-6 PUFA, and in this sense are considered anti-adipogenic.

For conjugated linoleic acid (**CLA**) it was shown that it increases the percentage of lean body mass and decreases the percentage of fat mass by the inhibition of

lipoprotein lipase (LPL) i.e. adipogenesis in WAT (Gaullier et al., 2004; Gaullier et al., 2005).

There are also factors that shift the balance of protein synthesis and degradation in the muscle towards protein synthesis e.g. certain **amino acids**, in particular leucine, could favor protein anabolism in muscle through effects on the expression and activity of key components of the cellular translational machinery (Layman and Walker, 2006).

Vitamin A metabolites are another group of nutrients whose effects on different tissue processes are largely known. Retinoids exert essential effects during embryonic development and differentiation of several cell types (Michael B.Sporn et al., 1994) and have been studied as human cancer therapeutics as they inhibit cell proliferation and induce cell differentiation and apoptosis in several cancer types including neuroblastoma (Celay et al., 2013). In particular, different RA isoforms have been used for the cancer treatments exerting their anti-tumor effects by the induction of apoptosis and/or differentiation.

Moreover, as being the most active vitamin A metabolite, retinoic acid is the most studied retinoid and its effects are largely known. Especially well studied are its effects in the adipose tissue. As mentioned before, RA affects early stage of adipocyte differentiation in opposite directions depending on the dose, promoting it at low doses (Safonova et al., 1994a) and inhibiting at relatively high doses (Stone and Bernlohr, 1990; Xue et al., 1996).

Lately a large attention is being put on the remodeling of white adipocytes to aquire more BAT-like properties, a phenomena known as browning. Both in humans and rodents, some brown adipocytes are found interspersed among white adipocytes in typical WAT depots (Oberkofler et al., 1997; Bonet et al., 2013). Specific agents (including specific nutrients) induce metabolic remodeling of white adipocytes towards increased oxidative metabolism, and some of them even induce UCP1 expression. This was shown in rodents for vitamin A (Mercader et al., 2006; Mercader et al., 2007), CLA (Shen et al., 2013) and PUFA-3 (Flachs et al., 2005).

In fact, as seen before, ATRA favors the acquisition of BAT-like properties in WAT (Mercader et al., 2006). ATRA treatment triggers in WAT increased expression of the genes related to thermogenesis and fatty acid oxidation (Mercader et al., 2006; Mercader et al., 2007). These effects of ATRA on adipogenesis and energy metabolism

(thermogenesis) are already well known. However there is still many information missing considering the effects of ATRA on mitochondria biogenesis in the adipose tissue. It was shown that ATRA treatment induces the expression of relevant transcription factors linked to mitochondria biogenesis in the skeletal muscle (Berry and Noy, 2009).

2 OBJECTIVES AND EXPERIMENTAL PROCEDURE

General objectives of the presented doctoral research were to study the effects of selected nutrients of lipidic nature, namely **fatty acids (objective / line A)** and **vitamin A related compounds (objective / line B)**, on different aspects of body energy homeostasis. Special attention was given to the molecular mechanism of the observed effects on the food intake and body weight and composition, which in general included expression of the genes involved in the control of food intake, energy expenditure and tissue remodeling, including in particular gastrointestinal hormones, hipotalamic neurotransmiters and different proteins involved in the adipocytes biology.

To study the effects of different fatty acids and vitamin A related compounds on various elements of body energy homeostasis different experiments were designed both *in vivo* and *in vitro*. Most experiments were done on animals. Guidelines for the use and care of laboratory animals of the University of the Balearic Islands were followed. In all animal experiments rats and mice were kept under controlled temperature ($22 \pm \underline{1}$ ^{o}C) and a 12-h light/12-h dark cycle, with free access to water and a standard chow, unless expressly indicated otherwise. The two lines of research, each with different objectives and experimental designs, performed in our laboratory as well as during doctoral stays in the United States and France, were as follows:

1. **First line of the doctoral investigation dealt with the effects of different fatty acids (in the form of free acid) on food intake and the expression of different genes involved in the gastrointestinal short-term control of food intake**. Studies of our group and others showed that different macronutrients have different satiating capacities. It is also well known that the effects of fat depend on their constituting fatty acids. However, due to the vast variability in the structures and physicochemical properties of different fatty acids, their effects still remain largely unknown. Thus the aim of the first line of the doctoral study was to test satiating capacity of different fatty acids i.e. their effects on food intake and to study the underlying mechanism of the most responsive ones. In concrete, we were interested

in the implications of gastrointestinal peptides and hipotalamic neurotransmitters in the satiation provoked by these fatty acids. We were especially interested in the involvement of gastric leptin in the satiating effects of fatty acids, which still remain unknown. We wanted to evaluate the hypothesis that orally administered fatty acids regulate leptin expression in the stomach. Alltogether all experimments performed for this study of fatty acids satiating capacities can be grouped in three sets:

1. In the first set of experiments two-month-old male Wistar rats after 6-hour fast were treated orally with equicaloric doses (60mg/kg BW in saline at pH 8.2) of **different fatty acids** (caprylic, lauric, palmitic, stearic, oleic and linoleic) 1 hour prior the onset of the dark phase and then food intake was recorded. The control group received the vehicle alone. As rats consume most (77%) of their daily food intake at night, with a maximum during the first hour after the onset of the dark phase (Sanchez et al., 2004b) this analysis was performed **during the first hours after the onset of the dark phase**.

   Results from this first set of experiments testing effects of different fatty acids on food intake showed different behavior of the various fatty acids tested, especially palmitic and linoleic. Thus we conducted another set of experiments with these two most reponsive ones, to confirm these results and profound the underlying mechanisms responsible for observed satiating effects, including hipotalamic neuropeptides and gastrointestinal hormones expression and secretion.

2. In order to characterize on the molecular level differential effects of tested fatty acids in the short term control of food intake, subsequently a second set of experiments (developed in twice) was performed in the same model as explained above in which palmitic and linoleic acids were further evaluated for their effects on satiety. We were particularly interested in their central effects through the expression of **hipotalamic neuropeptides** involved in the food intake control and in the FA effects on gastrointestinal hormones, in concrete **leptin and ghrelin stomach expression and secretion (charpter 3.1)**. To minimise the effects of the circadian rhythms and food intake on the above parameters, adult male Wistar rats, **6 hours fasted after the onset of the light phase**, were orally treated as in the first experiment with **palmitic or linoleic acid** and were

sacrificed immediately after the treatment or 40 and 100 minutes after the treatment by swift decapitation with guillotine. Blood was collected and serum prepared by centrifugation and frozen at -20°C until biochemical analysis. Different tissues (including hipotalamus, stomach i.e. gastric mucosa, and iWAT) were rapidly removed, immediately frozen in liquid nitrogen and stored at −80 °C until RNA and protein analysis. Parts of the stomach were also collected and fixed for the immunohistochemical analysis and were posteriorly stained with anti-leptin or anti-ghrelin **antibody.**

Additionally, in an extra group of animals, after 100 minutes of fatty acid treatments, the food intake was recorded during 1h and, at indicated time, blood was collected from the tail and centrifuged at 3000 rpm for 10 min at room temperature to collect the serum which was also stored at −80 °C until analysis.

3. Effects of palmitic and linoleic acid on the expression / secretion of leptin and ghrelin in the stomach mucosa were further confirmed by the *in vitro* experiments in which **gastric mucosa explants** were cultivated and incubated with these fatty acids. In brief, two-month-old male Wistar rats were killed and stomachs were quickly removed and washed with cold PBS under sterile conditions. These minced stomach tissues were incubated with indicated doses of fatty acids for 8 h at 37 °C in humidified 95% air and 5% CO_2. After incubation, these small tissues and the cell media were collected and stored at −80 °C until analysis.

After RNA extraction from tissues and stomach explants and reverse transcription, cDNA was used for the measurement of the expression of different genes by the method of real-time qPCR amplification and using 18S rRNA as a reference gene to calculate relative expression of each mRNA. We were especially interested in the expression of orexigenic and anorexigenic neuropeptides in the hypothalamus and of gastric leptin and ghrelin as well as WAT leptin. The amount of the total suppressor of cytokine signaling 3 (SOCS3) in the hipotalamus was determined also by Western blot.

Serum and explant media leptin and ghrelin were measured by using commercially available kits of enzyme-linked immunosorbent assay (ELISA) and enzyme immunosorbent assay (EIA). Likewise, leptin and ghrelin levels after protein extraction from the stomach mucosa homogenates were also

measured in that way. The amount of gastric leptin and ghrelin in the stomach was determined also by immunohistochemistry. Serum glucose levels were measured using Accu-Chek Aviva. Serum insulin and CKK levels were measured using commercial ELISA and EIA kits, respectively. To verify the absorption of fatty acids in the circulation their levels were measured by gas chromatography.

2. **Second line of presented doctoral investigation was focused on the next group of lipid components, vitamin A related compounds.** Our group, together with other groups, has already studied the effects of all-trans retinoic acid (ATRA), the main active form of vitamin A and its precursors, such as β-carotene, on body adiposity, lipid metabolism and the control of body weight in the adult age. However, despite these increasing evidence linking vitamin A and β-carotene to the control of adiposity in adult animals, little is known about their effects in early life. Previous results of our group indicated that administration of moderate doses of vitamin A (as retinyl palmitate) during lactation induces changes in fat gain in response to a high-fat diet, developing a greater degree of obesity in the adulthood. And since breastfeeding is a critical period in WAT development, we hipothesized that early effects of these compounds on the white adipose tissue biology could help explaine its effects on later propensity to diet-induced obesity and its metabolic complications in the adulthood. Further on we were interested in the **effects of vitamin A and β-carotene during the early age** and I was involved in this line of investigation. We aimed to assess the impact of a moderate supplementation with vitamin A during this early life period on WAT development at histological and molecular level.

Inside this second general objective we were interested to profound more the role of ATRA on body adiposity in adult animals, in particular its **effects on the secretoma of the skeletal muscle**. Muscle secretoma has been described to have a regulatory role in the adipose tissue biology in different *in vitro* and *in vivo* models. Myokines are regulatory proteins secreted by muscle cells that exert their effects on many other tissues except muscle. As such they are candidate mediators of reported ATRA effects on WAT development and metabolism. We hypothesized that ATRA regulation of myokine production could contribute to ATRA biological activity.

In addition, further experiments in this line of investigation had as an objective to characterize an aspect related to the induction and maintenance of the oxidative phosphorylation / thermogenic capacities by ATRA as well as its **effect on mitochondrial biogenesis in white adipocytes** that is not yet fully characterized. This could in turn contribute to the understanding of the general anti-obesity and anti-diabetic effects of ATRA.

Alltogether, inside this second part of the doctoral study dealing with the effects of vitamin A related compounds on the biology of adipose tissue, different *in vivo* experiments were performed on rat pups and adult mice as well as *in vitro* experiments on cell systems.

1. To study the effects of early vitamin A supplementation on young rats at weaning (**charpter 3.2)** rat pups in each litter were randomly assigned into two groups which, from day 1 to day 20, were given daily orally, with the aid of a pipette, 10–15 µl of vehicle (olive oil, control rats) or an emulsion of vitamin A in the form of retinyl ester corresponding to approximately 3 times the vitamin A (retinol) ingested daily from maternal milk. The amount of extra vitamin A given daily was progressively adjusted considering the vitamin A content in rat milk and the estimated daily milk intake throughout the suckling period in rats.

2. We also studied the effects of early **β-carotene** (as a provitamin A) supplementation on young rats at weaning (**charpter 3.3**). For this experiment we have included an additional group wherein rat pups were supplemented during the suckling period as explained above with a daily oral dose of vitamin A corresponding to approximately 3 times the vitamin A ingested daily from maternal milk, but now in the form of β-carotene (pro-vitamin A), taking into account that β-carotene in oil equals approximately 2,33 times the dose of retinol.

In both experiments, during the period of lactation rats were followed for their body weight, and on the day after weaning (day 21) their body composition was measured by MRI and the rats were sacrificed by cervical dislocation. Blood was collected from the neck and serum prepared and stored at -20ºC until analysis. Intestine, liver and white adipose tissue (WAT) depots —inguinal, gonadal and retroperitoneal— were dissected, weighed, frozen in liquid nitrogen

and stored at -80ºC until analysis. A lengthways fragment of inguinal WAT was fixed by immersion in 4% paraformaldehyde for morphological and immunohistochemical analysis.

To assess if early vitamin A supplementation affected adipose tissue development in young rats the expression of different genes involved in the adipocyte differentiation (e.g. PPARγ, LPL) as well as adipose tissue proliferation (e.g. PCNA, p21) were measured by qPCR in the iWAT of the vitamin A (as retinyl palmitate and β-carotene) supplemented rat pups. Changes in the expression of some of these genes were confirmed at protein levels by Western blot or immunohistochemically. iWAT tissue sections were also analyzed for their tissue morphology i.e. cell number and size distribution. At the same time, to validate the intestinal absorption of retinoids and β-carotene as well as their delivery to the tissues crucial for their metabolism and/or storage they were extracted from the blood, liver and iWAT, separated and their levels quantified by high-performance liquid chromatography and mass spectrometry (HPLC-MS). The expression of RA-mediated response genes were also measured by qPCR in the intestine, liver and iWAT of early vitamin A supplementated young rats. Since serum, intestine, liver and adipose tissue are major sites of retinoids / carotenoids metabolism these analyzes allowed us to test the effectiveness of the treatment and to check which vitamin A metabolites could be responsible for the observed effects both in the early life and in the adulthood. HPLC-MS and Western blots analyzes were carried out during my **stay in the laboratory of Prof. Dr. Johannes von Lintig at the Case Western Reserve University in Cleveland, USA**.

3. Study of the effects of ATRA **on the secretoma of the skeletal muscle** (**charpter 3.4**) aiming to profound more the role of ATRA on body adiposity, has been addressed using ATRA-treated C2C12 myotubes and ATRA-treated adult mice as models. I was incorporated in the *in vivo* study using ATRA treated adult NMRI mice as a model.

In cell system, fully differentiated C2C12 myocytes were exposed during 24 h before harvesting to a single dose of ATRA at a final concentration of 0.1, 1 and 10 μM. ATRA was delivered dissolved in DMSO, at a final concentration of 0.1%. Subsequently cells and conditioned media were collected and stored at

-80ºC until gene analysis. For the animal study, adult NMRI male mice fed *ad libitum* regular laboratory chow received one daily subcutaneous injection of ATRA at a dose of 50 mg/kg body weight during the 4 days before they were sacrificed. Controls were injected the vehicle (100 μl olive oil). Body weight and food intake during the treatment period were followed daily on a per-cage basis (three animals per cage). The animals were sacrificed by decapitation at the start of the light cycle. Blood was collected and serum prepared by centrifugation and frozen at -20°C. Tissues, including gastrocnemius muscle, were excised in their entirety, weighted, snap-frozen in liquid nitrogen, and stored at -80°C.

In order to study the effects of ATRA on muscle cells, expression and secretion analysis of different myokines (interleukin-6 (IL-6), FNDC5/irisin and fibroblast growth factor 21 (FGF21)) was conducted. RNA was extracted from harvested cells and gastronemious muscle samples and gene expression was analysed by qPCR. Miokine concentrations in conditioned culture media and in mouse serum samples were measured using commercially available ELISA kits.

4. To characterize the **effect of ATRA on mitochondrial biogenesis,** as a component in the induction and maintenance of the oxidative metabolism / thermogenic capacities, **in mature white adipocytes (charpter 3.5)**, aspect that is not that well known, further animal and *in vitro* experimets were performed. This impact of ATRA could explain the possible remodeling of mature white adipocytes into mitochondria-rich cells with increased oxidative and thermogenic capacity.

This work started from the collaboration with the **laboratory of Dr. Jean-Francois Landrier at the University of Marseilles, France,** and involves my pre-doctoral stay in this laboratory. The laboratory of Dr. Landrier had already preliminary data from microarray analysis that clearly show an overrepresentation of the gene sets linked to mitochondria in 3T3-L1 adipocytes under ATRA treatment (2 μM of ATRA for 24 hours), as well as induction of the relevant transcription factors linked to mitochondria biogenesis. During the stay, the microarray data were validated performing new cultures of mature adipocytes treated with ATRA, and analyzing the expression of a selected group of genes coding for proteins linked to mitochondria function and biogenesis by qPCR. In addition, the induction of mitochondria quantity in cultured mature

adipocytes was also estimated by measuring mitochondrial DNA by qPCR and by staining them with Mitotracker, a specific fluorescent marker of mitochondria, and flow cytometry.

To characterize the effects of ATRA on adipose tissue mitochondria biogenesis *in vivo*, we performed an additional experiment with adult NMRI male mice that were subjected to ATRA treatment, as described above. Epididymal, inguinal and retroperitoneal WAT were excised in their entirety, weighted, snap-frozen in liquid nitrogen, and stored at -80°C until gene expression analysis. A fragment of each WAT depot was fixed by immersion in 4% paraformaldehyde for morphological and immunohistochemical analysis. Gene expression analysis of the previously selected group of genes coding for proteins linked to mitochondria function and biogenesis by qPCR in the WAT depots (epididymal, inguinal, and retroperitoneal) were performed during the **stay in the laboratory of Dr. Landrier.** Finaly, the induction of mitochondria quantity was also confirmed by the immunohystochemical staining of mitochondrial marker, CoxIV.

3   RESULTS AND DISCUSSION

Chapter 3.1

REGULATION OF GASTRIC LEPTIN SECRETION BY FREE FATTY ACIDS

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Chapter 3.1

REGULATION OF GASTRIC LEPTIN SECRETION BY FREE FATTY ACIDS

ABSTRACT

INTRODUCTION: Subsequent to meal ingestion, the interaction of nutrients with receptors in the gastrointestinal tract modulates a number of its functions, including gastric emptying, gastrointestinal hormone secretion and energy intake. It has been reported that lipids induce a cascade of events related to the regulation of appetite and satiety: slow gastric emptying; stimulate the secretion of cholecystokinin, glucagon-like peptide-1, and peptide YY; and suppress ghrelin secretion and energy intake. Most of these effects are mediated by free fatty acids. The aim of this study was to evaluate the hypothesis that orally administered fatty acids would regulate leptin expression in stomach.

METHODS: 6 hours fasted adult male Wistar rats (4-6 animals per group) were orally treated with an equicaloric load of several free fatty acids in saline. After 0, 40 and 100 minutes, rats were killed; hypothalamic neuropeptides and gastric mucosa hormones expression was determined, as well as serum hormone levels.

RESULTS: Oleic and linoleic acid showed to be more satiating than palmitic acid. Linoleic acid reduced secretion of ghrelin and induced central anorexigenic effects earlier and stronger than palmitic acid. Palmitic acid, and to a lesser extent linoleic, acutely increased leptin accumulation in stomach mucosa compared to the vehicle, and blocked itssignalling.

CONCLUSION: The results suggest that fatty acids regulate gastric leptin secretion; this could contribute to its reported effects on the regulation of appetite and satiety.

Keywords: food intake, short-term control, ghrelin, leptin

INTRODUCTION

Satiety and hunger are sensations regulated in mammals by a complex system of peripheral and central signals, which modulate food intake and energy balance (Cummings and Overduin, 2007; Jordan et al., 2010). Information on nutrient-related signals about satiety and hunger from the gastrointestinal tract are conveyed to the central nervous system through circulation by secreted hormones and through nervous system by afferent fibers of the vagus nerve and afferents passing into the spinal cord. In the central nervous system (CNS), the arcuate nucleus of the hypothalamus is crucial for feeding control and is a site of convergence and integration of these nutrient-related signals with other central and peripheral neuronal inputs as well as hormonal and metabolic signals that reflect changes in global energy status (Dowell et al., 2005; Jordan et al., 2010). Two gastrointestinal hormones vastly involved in the control of energy homeostasis are leptin and ghrelin. They have opposite effects on food intake, inhibiting or inducing, respectively (Bado et al., 1998; Cinti et al., 2000; Kojima et al., 1999; Palou and Pico, 2009).

The cascade of events in the regulation of appetite and satiety mediated by lipids include slowing gastric emptying; stimulation the secretion of several gastrointestinal satiation peptides; and suppression of the orexigenic gastric hormone ghrelin secretion and energy intake (Cummings and Overduin, 2007). Satiating signals emanate from the gut, rather than from postabsorptive sites i.e. effects of fat in the intestine are independent of fat metabolism and occur before digestion products of fat enter into the bloodstream (Beglinger and Degen, 2004). Fat-induced small-intestinal feedback inhibition is mediated by the interaction of free fatty acids (FFAs), and not triacylglycerols (TAGs), with gut receptors (Feinle-Bisset et al., 2005; Little et al., 2007). Thus, fat digestion is a critical step for fat induced appetite inhibition, stimulation of gastrointestinal hormone release and slowing of gastric emptying. The interaction of FFAs with specific receptors in the gastrointestinal tract stimulates release of satiety hormones and/or suppress the release of orexigenic ones, and consequently regulates pancreatic secretion, gastric emptying and energy intake (Greenberg et al., 1987; Lu et al., 2012). It is also likely that a preabsorptive satiating action of FFAs involves stimulation of vagal afferent fibres that transmit satiety information to the central nervous system (Berthoud et al., 1991; Phillips and Powley, 1998) but the direct

effect of individual fatty acids on the secretion of gastric satiating hormones and its relationship with hypothalamus signaling has not been previously explored.

The aim of this study was to test satiating capacity of different fatty acids on food intake and study the underlying mechanism of the most responsive ones. We were especially interested in the implication of gastric leptin in the satiating effects of fatty acids which still remain unknown.

MATERIALS AND METHODS

Animals and experimental design

Two-month-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were used. They were kept under standard conditions: 12-h light/dark cycle, temperature 20–22 °C, humidity 85%; free access to tap water and standard chow (maintenance chow; Panlab, Barcelona, Spain). Animal protocols followed in this study were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands and guidelines for the use and care of laboratory animals of the University were followed.

In the first study different fatty acids (from Sigma- Aldrich, St. Louis, MO, USA) were tested for their satiating properties. As rats consume most (77%) of their daily food intake at night, with a maximum during the 1st hour after the onset of the dark phase (Sanchez et al., 2004b) this test was performed during the first hours after the onset of the dark phase. Adult male Wistar rats (6 animals per group) were orally (by gavage) treated with an equicaloric load (60mg/kg BW in saline at pH 8.2) of different fatty acids 1 hour prior the onset of the dark phase and food intake was recorded during 2h after it. The control group received the vehicle alone.

In the second experiment (developed in twice) two most responsive fatty acids were chosen for further evaluation of their effects on the expression of hipotalamic neuropeptides and gastrointestinal hormones. To minimize the effects of the circadian rhythms (Sanchez et al., 2004b) and food intake on the above parameters, adult male Wistar rats (4-8 animals in total per group), 6 hours fasted after the onset of the light phase, were orally treated as in the first experiment with palmitic or linoleic acid. At time 0 and after 40 and 100 minutes of the treatment, the rats were sacrificed and the hipotalamus, stomach and adipose tissue were rapidly removed, immediately frozen in liquid nitrogen and stored at −80 °C until RNA and protein analysis. Blood was also

collected, centrifuged at 3000 rpm for 10 min at room temperature to collect the serum which was also stored at −80 °C until analysis.

Additionally, in an extra experiment, rats were re-fed after 100 minutes of fatty acid treatments and, food intake was recorded during 1h and, at indicated time, blood was collected from the tail and centrifuged at 3000 rpm for 10 min at room temperature to collect the serum which was also stored at −80 °C until analysis

Stomach tissue culture

Explants were prepared and cultured as described in (Sakata et al., 2006). In brief, two-month-old male Wistar rats (Charles River Laboratories, Barcelona, Spain) were killed and stomachs were quickly removed and washed with cold PBS under sterile conditions. Mucosa of the stomach was minced (approximately 1 mm^3) with a sharp razor blade in cold DMEM. These minced stomach tissues were incubated with serum-free DMEM containing indicated doses of fatty acids for 8 h at 37 °C in humidified 95% air and 5% CO_2. After incubation, these small tissues and the cell media were collected and stored at −80 °C until analysis.

RNA extraction, cDNA obtention and real-time PCR amplification

Total RNA was extracted from tissue using Tripure Reagent (Roche, Barcelona, Spain) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis. Subsequently RNA was denatured and reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamers primers according to the Applied Biosystems procedure at 42 °C for 1 h in a Perkin-Elmer 2400 Thermal Cycler (PerkinElmer, Wellesley, MA, USA). Each PCR amplification was performed from diluted (1/20) cDNA template, using specific primers (1 μM each) obtained from Sigma (Madrid, Spain; sequences are available upon request) and the Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus^{TM} Real-Time PCR System according to the Applied Biosystems procedure with the following profile: 10 min at 95 ºC, followed by a total of 40 two- temperature cycles (15 s at 95 ºC and 1 min at 60 ºC). To verify the purity of the products, a melting curve was produced after each run. The threshold cycle was calculated by the instrument's software (StepOne Software

v2.0, Applied Biosystems) and the relative expression of each mRNA was calculated according to Pfaffl (Pfaffl, 2001), using 18S rRNA as a reference gene.

Protein isolation and Western blotting analysis

Tissue proteins were extracted using Tripure Reagent (Roche, Barcelona, Spain) according to the manufacturer's instructions. Total protein content was measured by the BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific Inc. Rockford, IL, USA). For analysis, 40 μg of total protein from hypothalamus were fractionated on 14% SDS-PAGE gels using the Bio-Rad Minigel system and electrotransferred onto a nitrocellulose membrane (Bio-Rad, Madrid, Spain). Membrane was blocked with blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h and then incubated with anti-SOCS3 (1:1000 dilution) antibody (Cell Signalling, Boston, MA, USA) overnight at 4°C, followed by incubation with the infrared (IR)-dyed secondary anti-IgG antibody (LI-COR Biociences) diluted 1:10,000. For IR detection, membranes were scanned in Odyssey Infrared Imaging System (LI-COR Biociences), and the bands were quantified using the analysis software provided. β-actin was used as a reference protein (1:5000 antibody dilution; Cell Signalling).

Immunohistochemistry

Tissue specimens were fixed by immersion in 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB), pH 7.4. After washing in PBS overnight, the samples were dehydrated in a graded series of ethanol and embedded in paraffin blocks for light microscopy and immunohistochemistry. For immunohistochemistry, 5 μm sections were immunostained by means of the avidin-biotin technique (Hsu et al., 1981). Sections were incubated with primary rabbit polyclonal anti-leptin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-ghrelin antibody (Phoenix Europe GmbH, Karlsruhe, Germany), diluted 1:150 in PBS, then with the corresponding biotinylated anti-rabbit IgG secondary antibody, made in goat, diluted 1:200 (Vector Labs, Burlingame, CA, USA) and finally with ABC complex (Vectastain ABC kit; Vector Labs.). Peroxidase activity was revealed by 0.075% 3,3'-diaminobenzidine hydrochloride as chromogen (Sigma, St Louis, MO, USA) in Tris buffer 0.05M, pH 7.6. Sections were counterstained with hematoxylin and mounted in Eukitt (Kindler, Freiburg, Germany). Sections were observed with Zeiss Axioskop 2 microscope equipped with AxioCam ICc3 digital camera (Carl Zeiss Microscopy GmbH, Jena,

Germany). For morphometric analysis of ghrelin immunostaining number of ghrelin positive cells was counted per mm^2 of the section.

Quantification of gastrointestinal peptides levels in gastrointestinal mucosa, serum and cell media.

Gastrointestinal peptides levels in stomach mucosa were determinated as previously (Sanchez et al., 2004a). In brief, proteins were extracted by homogenizing mucosa in 1:3 (w/v) of PBS (PBS: 137 mm NaCl, 2.7 mm KCl, and 10 mm phosphate buffer, pH 7.4). Homogenates were centrifuged at 7000 g for 2 min at 4 °C, and the supernatant was used for leptin quantification. For ghrelin quantification, additionally, the supernatant was mixed with 10 volumes of 1M acetic acid containing 20 mM HCl, boiled for 20 min, centrifuged at at 7000 g for 2 min at 4 °C and then lyophilized and resuspended in PBS. Gastrointestinal mucosa, serum and cell media leptin levels were measured using a commercial ELISA kit (R&D Systems, Minneapolis, MN), whereas, ghrelin levels were measured using commercial EIA kits (Phoenix Europe GmbH, Karlsruhe, Germany) according to manufacturer's instructions

Fatty acid analysis

Extraction of fatty acids from a small amount of sample (10 μL of serum) and gas chromatography (GC) separation of fatty acids, as well as quantification of the peak integrals were performed as previously described (Laraichi et al., 2013). Quantification was done by standard normalization and the results expressed in relative amounts as percentages of total fatty acid analyzed in each sample. All samples were analyzed in duplicate.

Other serum quantifications

Serum glucose levels were measured using Accu-Chek Aviva (Roche, Barcelona, Spain). Serum insulin levels were measured using an Insulin rat ELISA kit (Ad Bioinstruments S.l.). Serum cholecystokinin (CKK) levels were measured using commercial EIA kit (Phoenix Europe GmbH, Karlsruhe, Germany)

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was assessed by two-tailed Student's t-test. Results were considered as statistically significant when $P < 0.05$. The analyses were performed with SPSS 19.0 for windows (Chicago, IL, USA).

RESULTS

## *Effect of fatty acid treatment on food intake.*

We have tested satiating capacity of various fatty acids that differ in their chain length and saturation level: short chain caprylic (C 8:0), medium chain lauric (C 12:0), long chain saturated palmitic (C 16:0) and stearic (C 18:0) and long chain unsaturated oleic (C18:1) and linoleic (C18:2) fatty acids. As shown in Figure 1, neither of the fatty acids tested caused significant differences in food intake, recorded during the first two hours of the dark phase, after oral treatment with an equicaloric load (60mg/kg BW in saline at pH 8.2) 1 hour prior the onset of the dark phase, compared to vehicle treated rats. However, differences in food intake were observed between fatty acid treatments. Such as rats treated with oleic or linoleic acid ingested significantly less food compared to the palmitic acid treated rats (Figure 1). These fatty acids were selected for further studies.
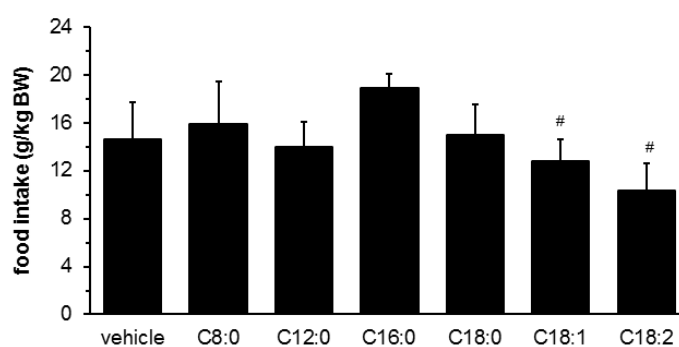
Figure 1. Effect of fatty acid treatments on food intake. Adult male Wistar rats were treated orally (by gavage) with an equicaloric load (60mg/kg BW in saline at pH 8.2) of different fatty acids 1 hour prior the onset of the dark phase and food intake was recorded during 2h after it (preliminary study). The control group received the vehicle alone. Data are means ± SEM (n = 6 each group). Statistical significance of differences was tested by two-tailed Student's t-test. Threshold of significance was set at $P < 0.05$: # fatty acid-treated versus palmitic acid-treated. C 8:0, caprylic acid; C 12:0, lauric acid; C 16:0, palmitic acid; C 18:0, stearic acid; C18:1, oleic acid; and C18:2, linoleic acid.

Effects of palmitic and linoleic acid treatments on the expression of neuropeptides and gastrointestinal hormone receptors in the hyothalamus.

To study the underlying molecular mechanisms involved in the observed different satiating capacities of fatty acids, we first analyzed gene expression of the main neuropeptides and gastrointestinal hormone receptors involved in the control of food

Results and Discussion
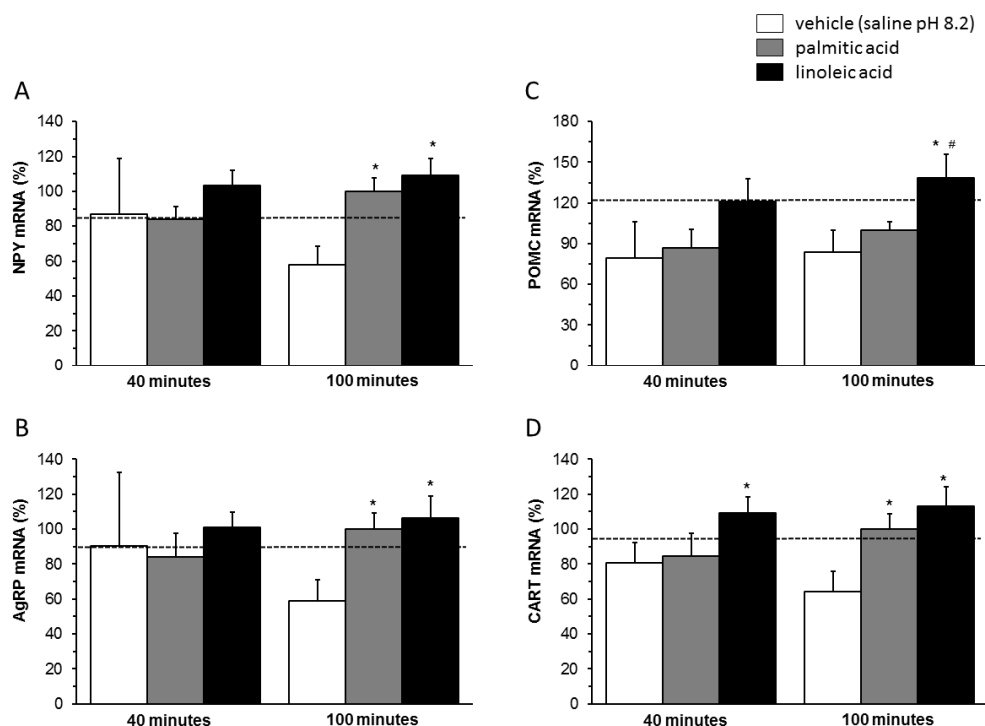
Figure 2. Effect of palmitic and linoleic acid treatment on hypothalamic neuropeptide Y (A), agouti related peptide (B), pro-opiomelanocortin (C) and cocaine and amphetamine regulated transcript peptide (D) gene expression. Adult male Wistar rats 6 hours fasted after the onset of the light phase were treated orally (by gavage) with an equicaloric load (60mg/kg BW in saline at pH 8.2) of palmitic or linoleic acid. The control group received the vehicle alone. At time 0 and 40 and 100 minutes after the treatment hypothalamic gene expression was analized by RT-qPCR. Dashed line denotes the value at time 0. Data are expressed relative to the mean value in palmitic-treated rats 100 minutes after the treatment, which was set at 100%, and are means ± SEM (n = 4-8 each group). Statistical significance of differences was tested by two-tailed Student's t-test. Threshold of significance was set at P < 0.05: * fatty acid-treated versus vehicle treated; # fatty acid-treated versus palmitic acid-treated.

intake and body energy homeostasis in the hypothalamus. Hypothalamic gene expression was analyzed, in 6 hours fasted rats, at time 0 and 40 and 100 minutes after the oral treatment with an equicaloric load (60mg/kg BW in saline at pH 8.2) of palmitic and linoleic acid (Figure 2). Expression of the orexigenic neuropeptide Y (NPY) and agouti related peptide (AgRP) was significantly increased 100 minutes after the treatments both with palmitic and linoleic acids compared to the vehicle (Figure 2A and 2B). Expression of the anorexigenic neuropeptide pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript peptide (CART) was also increased after fatty acids treatments, particularly after linoleic acid treatment compared to the vehicle and palmitic acid treatment (Figure 2C and 2D). POMC increase became significant after 100 of linoleic acid treatment compared to the vehicle and palmitic acid

treatment, (Figure 2C) and the expression of CART was significantly induced after 40 minutes of the treatment with linoleic acid compared to the vehicle and maintained after 100 minutes, whereas palmitic acid treatment induced CART expression only after 100 minutes compared to the vehicle (Figure 2D). These results indicate that the acute treatment with fatty acids induced changes at the level of hipotalamic neurotransmitter gene expression in the direction of increasing both orexigenic and anorexigenic pathways. However, while linoleic acid seemed to enhance anorexigenic signaling earlier and stronger after the treatment palmitic acid showed weaker effect. In fact, the NPY/POMC ratio suggested a prevalence of orexigenic pathways 100 minutes after palmitic acid treatment compared to the vehicle and linoleic acid treatment (palmitic acid: 1.02 ± 0.09* [#]; linoleic acid: 0.82 ± 0.06; and vehicle 0.70 ± 0.03) (4-8 animals per group, t-test).
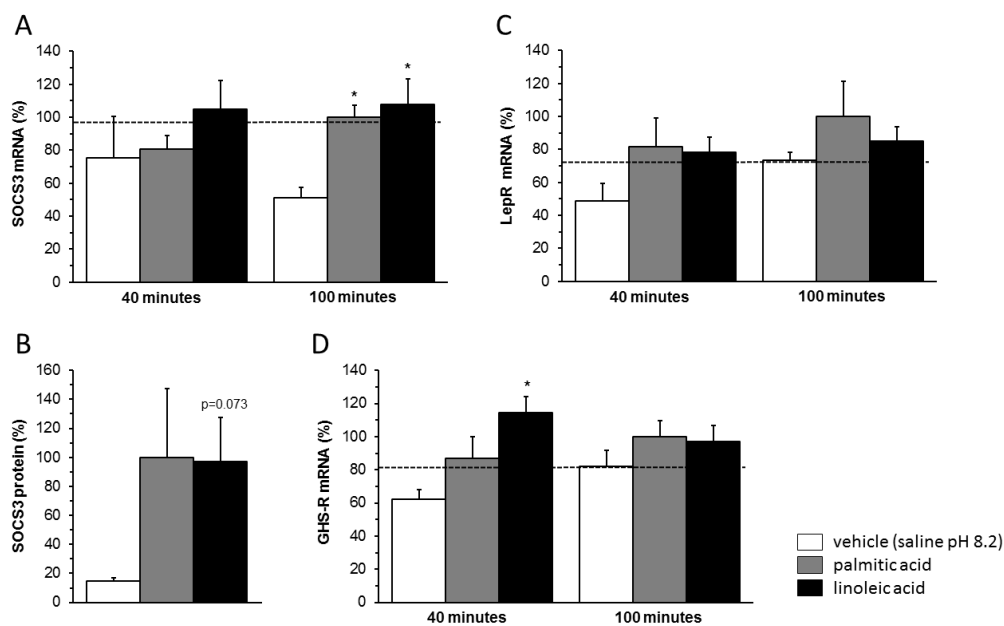
Figure 3. Effect of palmitic and linoleic acid treatment on hypothalamic suppressor of cytokine signaling 3 (A, mRNA; B, protein), leptin receptor (C) and ghrelin receptor (D) gene expression. Adult male Wistar rats 6 hours faster after the onset of the light phase were treated orally (by gavage) with an equicaloric load (60mg/kg BW in saline at pH 8.2) of palmitic or linoleic acid. The control group received the vehicle alone. At time 0 and 40 and 100 minutes after the treatment hypothalamic gene expression was analyzed by RT-qPCR and western blotting. Dashed line denotes the value at time 0. Data are expressed relative to the mean value in palmitic-treated rats after 100 minutes of treatment, which was set at 100%, and are means $\pm$ SEM (n = 4-8 each group). Statistical significance of differences was tested by two-tailed Student's t-test. Threshold of significance was set at $P < 0.05$: * fatty acid-treated versus vehicle treated.
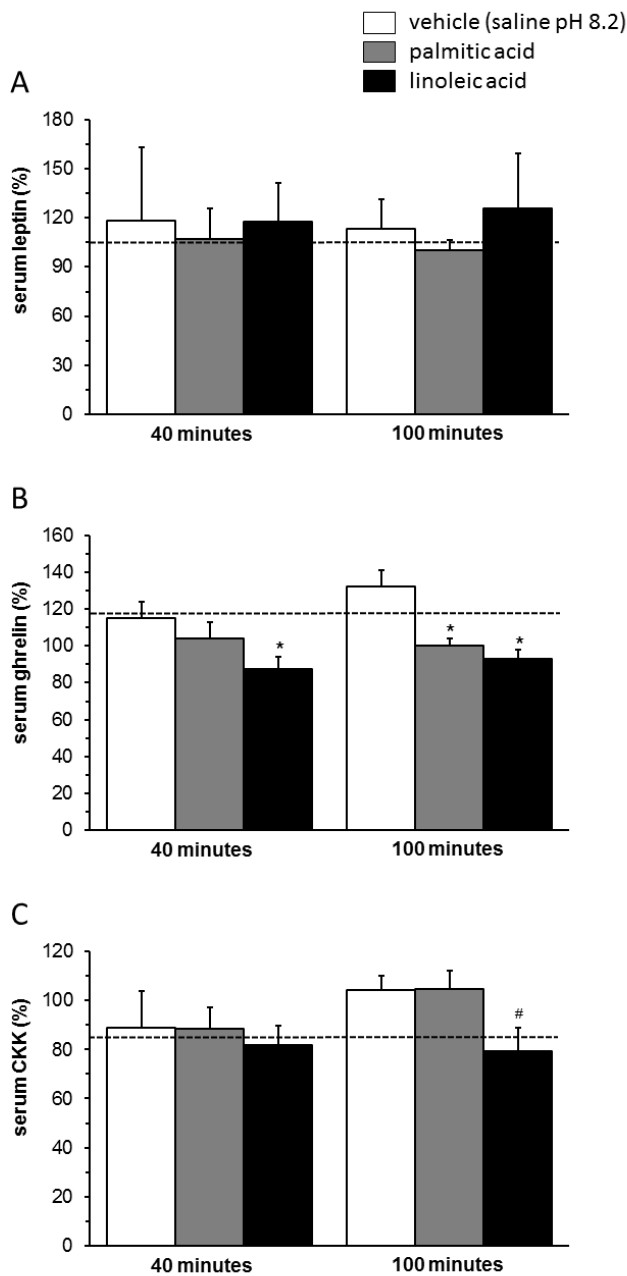
Figure 4. Effect of palmitic and linoleic acid treatment on serum leptin (A), ghrelin (B) and cholecystokinin (C) levels. Adult male Wistar rats 6 hours fasted after the onset of the light phase were treated orally (by gavage) with an equicaloric load (60mg/kg BW in saline at pH 8.2) of palmitic or linoleic acid. The control group received the vehicle alone. At time 0 and 40 and 100 minutes after the treatment serum hormone levels were analyzed by ELISA or EIA. Dashed line denotes the value at time 0. Data are expressed relative to the mean value in palmitic-treated rats after 100 minutes of treatment, which was set at 100%, and are means ± SEM (n = 4-8 each group). The hormone values at time 0 were 1.58±0.22 ng/mL for leptin, 8.34±0.28ng/mL for ghrelin and 0.69±0.06 ng/mL for CKK. Statistical significance of differences was tested by two-tailed Student's t-test. Threshold of significance was set at $P < 0.05$: * fatty acid-treated versus vehicle treated; # fatty acid-treated versus palmitic acid-treated.

Treatments with palmitic and linoleic acid increased hipotalamic expression of the suppressor of cytokine signaling 3 (SOCS3), a leptin-inducible inhibitor of leptin signaling (Howard and Flier, 2006). Linoleic acid treatment again showed the tendency to induce effect already in the first hour, while in the second hour both palmitic and linoleic acid significantly increased SOCS3 expression compared to the vehicle (Figure 3A). The levels of the SOCS3 protein showed the same tendency (Figure 3B). Expression of leptin receptor did not show any significant changes and expression of

ghrelin receptor was significantly increased 40 minutes after the linoleic acid treatment (Figure 3C and 3D).

Effects of palmitic and linoleic acid on leptin and ghrelin gene expression and stomach secretion

Interestingly, both palmitic and linoleic acid treatment did not induce any significant effect, 100 minutes after the treatment, in the relative amounts of these fatty acids in blood (data not shown), suggesting direct effects of fatty acids on the gastrointestinal mucosa. In this respect, we analyzed the serum levels and expression of gastrointestinal peptides.

Both palmitic and linoleic acid treatment did not modify the levels of leptin in the serum compared to the vehicle (Figure 4A). In addition, treatments with both palmitic and linoleic acid decreased the levels of ghrelin in the serum compared to the vehicle . However, linolecic acid showed stronger effect decreasing ghrelin levels significantly already at 40 minutes of the treatment. Later on in the second hour both palmitic and linoleic significantly decreased serum ghrelin levels (Figure 4B). CCK circulating levels were unchanged 40 minutes after the treatments but 100 minutes after LI-treatment significantly decreased it compared to the vehicle treatment (Figure 4C). No changes in circulating levels of insulin and glucose were observed (data not shown).

As previously described in rats and humans (Bado et al., 1998; Cammisotto et al., 2010; Cinti et al., 2000; Oliver et al., 2002), leptin immunoreactivity was diffuse along the whole depth of the glands in the stomach mucosa (Figure 5A). More specifically leptin was localized in the granules of chief cells but not in oxyntic and enteroendocrine cells, which showed a negative immunoreactivity for leptin (Figure 5D, enlargement). Palmitic (Figure 5B and 5E), and to a lesser extent linoleic acid (Figure 5c and 5F), acutely increased leptin immunoreactivity in the stomach mucosa after 100 minutes of the treatment compared to stomach mucosa of vehicle treated rats (Figure 5A and 5D). A similar result was observed measuring leptin concentration in stomach mucosa by ELISA (Figure 6B). However no significant changes in stomach mucosa and adipose tissue leptin mRNA levels were shown (Figure 6A and 6F, respectively). On the other hand ghrelin is produced mainly in the oxyntic glands of the stomach (Date et al., 2000). As shown in figure 6G and 6J only these cells showed a strong immunoreaction. Compared to vehicle treated rats, both palmitic and linoleic acid treatments reduced
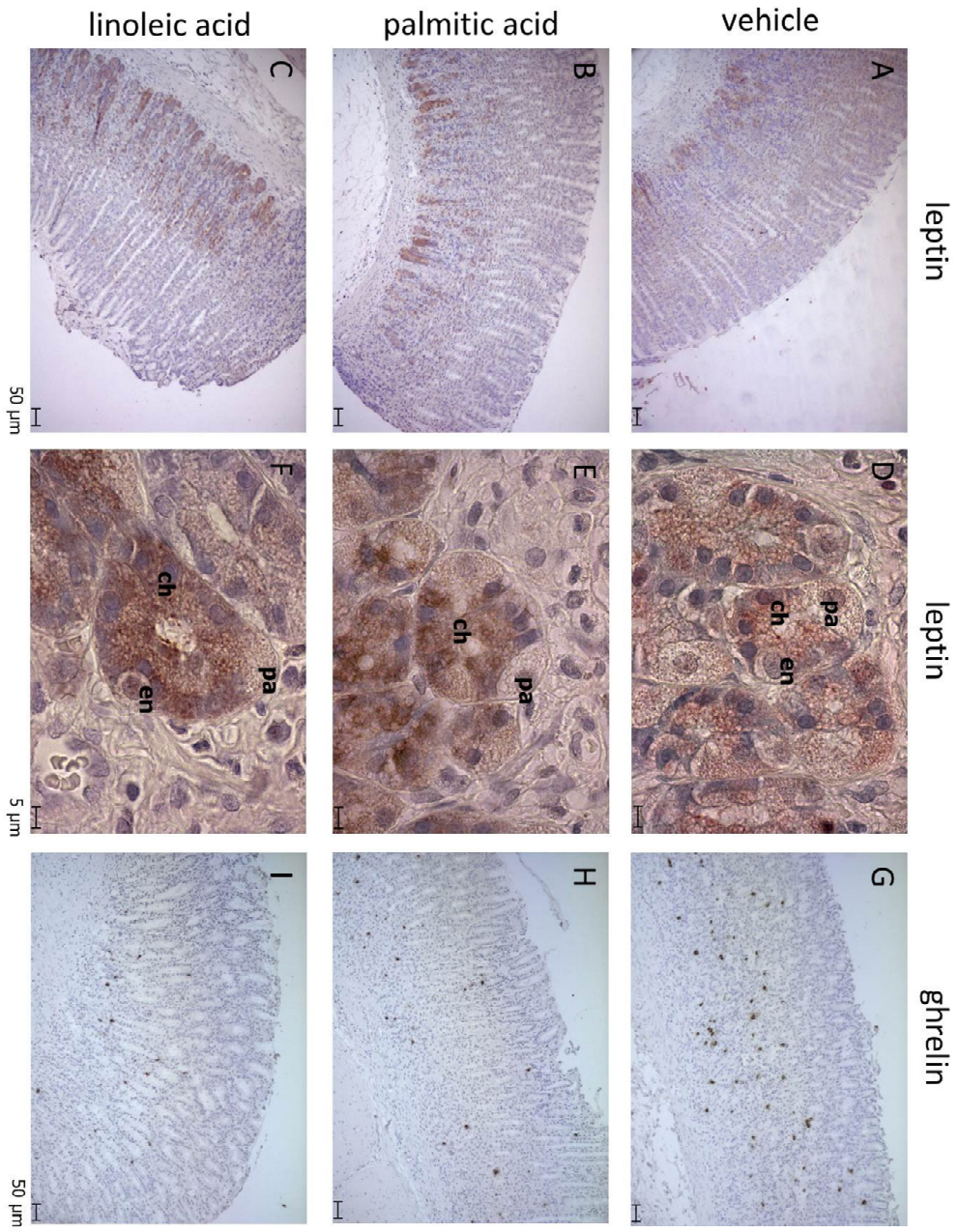
Figure 5. Effects of palmitic and linoleic acid treatments on leptin (A-F) and ghrelin (G-J) levels in stomach mucosa. Adult male Wistar rats 6 hours fasted after the onset of the light phase were treated orally (by gavage) with an equicaloric load (60mg/kg BW in saline at pH 8.2) of palmitic (B,E,H) or linoleic (C,F,I) acid. The control group received the vehicle alone (A,D,G,J). 100 minutes after the treatment leptin and ghrelin immunostainings were performed. Representative microphotographies. Scale bar: 50 μm in A-C and G-I; 5 μm in D-F and J. ch, chief cells; pa, parietal cells; and en, endocrine cells.
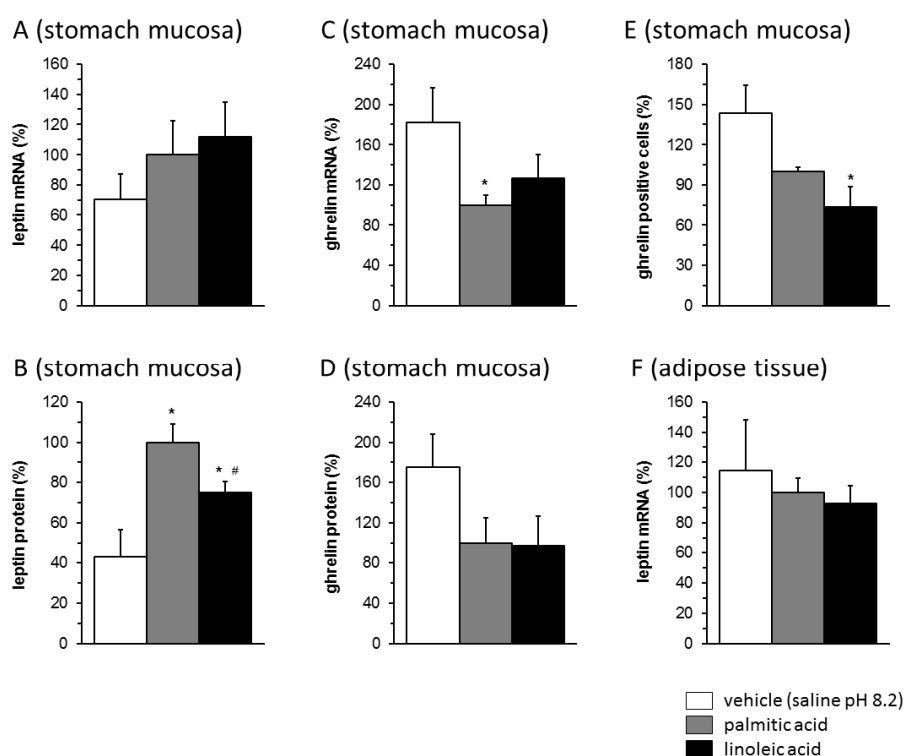
Figure 6. Effect of palmitic and linoleic acid treatment on stomach mucosa leptin (A, mRNA; B, protein), ghelin (C, mRNA; D, protein; E, immunostaining positive cells) and adipose tissue leptin (F) gene expression. Adult male Wistar rats 6 hours fasted after the onset of the light phase were treated orally (by gavage) with an equicaloric load (60mg/kg BW in saline at pH 8.2) of palmitic or linoleic acid. The control group received the vehicle alone. 100 minutes after of the treatment stomach mucosa and adipose tissue gene expression were analyzed by RT-qPCR and ELISA or EIA. For morphometric analysis of ghrelin immunostaining, number of ghrelin positive cells was counted per mm^2 of one section per animal. Data are expressed relative to the mean value in palmitic-treated rats 100 minutes after the treatment, which was set at 100%, and are means $\pm$ SEM (n = 4-8 each group). Statistical significance of differences was tested by two-tailed Student's t-test. Threshold of significance was set at $P < 0.05$: * fatty acid-treated versus vehicle treated; # fatty acid-treated versus palmitic acid-treated.

ghrelin mRNA expression (Figure 6C) and protein levels (Figure 6D and 6E) in the stomach mucosa 100 minutes after the treatment. In addition, we performed an additional experiment where, after fatty acid treatments, animals were not sacrificed but were fed a standard diet. Food administration induces leptin secretion (Bado et al., 1998; Cinti et al., 2000). Only palmitic acid treatment showed a slight but significant 11% increase in the concentration of leptin in the serum 1h after (Figure 7A) with a concomitant induction of satiety (Figure 7B) confirming our observations that leptin is accumulated in gastric mucosa. No changes were observed in serum ghrelin levels.

Results and Discussion
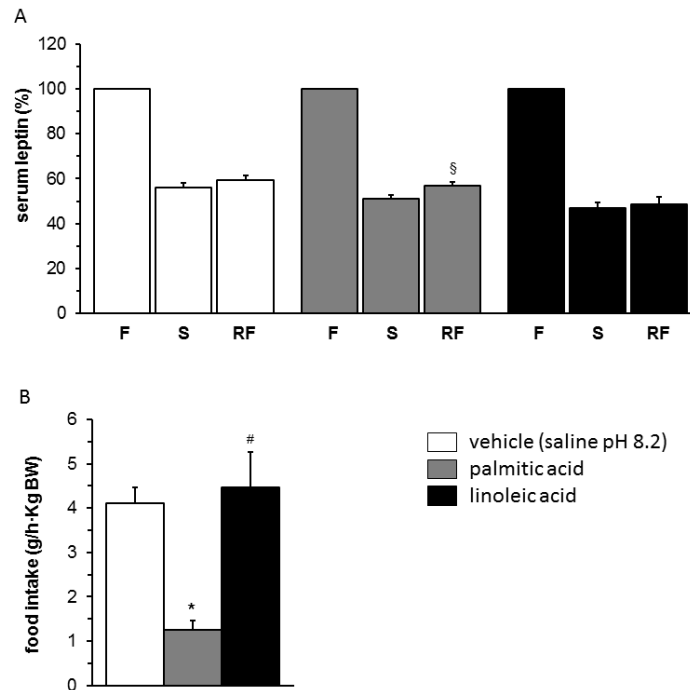
Figure 7. Effect of palmitic and linoleic acid treatment on serum leptin levels (A) and food intake (B). Adult male Wistar rats 6 hours fasted after the onset of the light phase were treated orally (by gavage) with an equicaloric load (60mg/kg BW in saline at pH 8.2) of palmitic or linoleic acid. The control group received the vehicle alone. Before fasting (F, fed), 100 minutes after the treatment (S, starved) and 1 hour after food delivery (RF, re-fed) serum leptin levels were analyzed by ELISA. Data are expressed, in each animal, relative to the value before fasting, which was set at 100%. Food intake was recorded during 1h after 100 minutes of fatty acid treatments. Data are means ± SEM ($n = 6$ each group). Statistical significance of differences was tested by two-tailed Student's t-test. Threshold of significance was set at $P < 0.05$: * fatty acid-treated versus vehicle treated; # fatty acid-treated versus palmitic acid-treated; §after versus before food delivery.

Table 1. Effect of palmitic and linoleic acid treatment on leptin and ghrelin gene expression in cultured stomach mucosa explants[a].

	vehicle	palmitic acid		linoleic acid	
		20 μM	200 μM	20 μM	200 μM
leptin					
mRNA (%)[b]	100 ± 18	52,6 ± 10*	53,7 ± 7,4*	112 ± 45	105 ± 26
secreted protein (pg/well)[c]	20,8 ± 2,7	12,3 ± 2,6*	14,8 ± 1,8	10,9 ± 1,2*	15,0 ± 0,9*
ghrelin					
mRNA (%)	100 ± 28	36,6 ± 9,0*	49,6 ± 8,8*	55,5 ± 10	25,5 ± 6,1*
secreted protein (pg/well)	0,453 ± 0,136	0,455 ± 0,152	0,170 ± 0,057*	0,193 ± 0,043	0,106 ± 0,043*

[a] Explants were prepared and cultured as described in materials and methods and were treated with the indicated doses of the different fatty acids for 8 hours. After treatment cell gene expression were analyzed by RT-qPCR and cell media protein levels by ELISA. Statistical significance of differences was tested by two-tailed Student's t-test. Threshold of significance was set at $P < 0.05$: * fatty acid-treated versus vehicle treated.

[b] Data are expressed relative to the mean value in vehicle (DMSO)-treated cells, which was set at 100%, and are means ± SEM of, at least, two independent experiments made in quadruplicated.

[c] Data are means ± SEM of two independent experiments made in quadruplicated.

To further characterize the differential effects of fatty acids on stomach mucosa we performed *in vitro* experiments. As showed in table 1, palmitic acid treatment reduced both leptin and ghrelin mRNA expression in cultured stomach explants compared to vehicle treated ones, whereas linoleic acid treatment only affected ghrelin mRNA expression. In respect to secreted proteins, both palmitic and linoleic acid treatments significantly reduced their levels.

DISCUSSION

Most of the studies about fatty acid effects on gastric emptying, gut hormones, and appetite were made via intraduodenal fat administration, avoiding the direct effects on stomach mucosa despite this is increasingly recognized as an important source of satiety signals (reviewed in (Cummings and Overduin, 2007)). Recent findings suggest that lipid meals impact gastric hormone secretion through sensing fatty acids directly (Janssen et al., 2012; Lu et al., 2012). This background and our previously found functionality of gastric leptin in humans (Cinti et al., 2001; Cinti et al., 2000) prompted us to study the effects of oral treatment with several fatty acids on the expression of the main hypothalamic neuropeptides and gastrointestinal hormones involved in the control of food intake and body energy homeostasis. Our results are first to show, to our knowledge, that gastric leptin is involved in the fatty acid satiating capacities.

Here we tested various fatty acids that differ in their chain length and saturation levels and treatments with oleic or linoleic acid were significantly different from the palmitic acid treatment showing to be more satiating probably due to their longer length and the unsaturation of their carbon chain. Related to this, it has been reported that free fatty acids are responsible for TAG effects on satiety and the secretion of gastrointestinal hormones (Feinle-Bisset et al., 2005; Little et al., 2007), and these effects likely depend (at least in part) on the physicochemical properties of their constituting FA: reduction of hunger and food intake generally increase with the increase of the chain length and the unsaturation level (Feltrin et al., 2008; Maljaars et al., 2009; Meyer et al., 1998). In accordance with our results, TAG with unsaturated fatty acids increase satiety, whereas TAG with saturated fatty acids does not (Maljaars et al., 2009) and we suggest that these effects are ready-made (at least in part) in the stomach.

At the molecular level, in our animal model, we observed that linoleic acid tends to enhance hypothalamic anorexigenic signaling earlier and stronger than after treatment with palmitic acid, which weekly effect is concomitant to an early and stronger reduction of circulating ghrelin. It is known that NPY neurones in the hypothalamic arcuate nucleus, a major brain area involved in the control of appetite, are potent stimulators of appetite and upon activation by ghrelin inhibit POMC neurones by releasing the inhibitory neurotransmitter GABA, which inhibits the release of alpha MSH, an inhibitor of appetite (Kojima et al., 1999; Korbonits et al., 2004). Thus, linoleic acid effects on circulating ghrelin levels observed here could explain, at least in part, the differential effects on food behavior compared to palmitic acid. As it is already known many of the fatty acid effects originate from preabsorptive sites (Greenberg et al., 1987). No changes in the relative amounts of individual fatty acids in the blood were seen after the treatments, suggesting direct effects of fatty acids on gastrointestinal tract. Moreover, we found that fatty acids, especially linoleic acid, directly down regulate the expression and secretion of ghrelin in cultured explants of stomach mucosa. In connection with that, it has been recently reported that serum ghrelin levels were transiently suppressed after gastric gavage of long-chain fatty acid-rich lipid meal in mice do to a direct inhibition of gastric ghrelin secretion (Lu et al., 2012). The proposed mechanism involves the long-chain fatty acid (LCFA) G protein-coupled receptor GPR120 that was highly expressed in gastric mucosa (Janssen et al., 2012; Lu et al., 2012). It can be deduced that differential effects exists depending on the individual fatty acids.

It is interesting to note that after treatments both with palmitic and linoleic acids, expression of the orexigenic neuropeptides NPY and AgRP was also increased compared to the vehicle treatment. We could speculate that the increased NPY/AgRP expression is actually the remain from the fasting phase preceding the fatty acid treatment that was not entirely vanished due to the small quantity of the fatty acids ingested (Palou et al., 2009). Nevertheless, palmitic acid, and to a lesser extent linoleic acid, increased leptin concentration in the stomach mucosa compared to the vehicle treated rats. In fact, chief cells, along the whole depth of the glands in the stomach mucosa, accumulated leptin and in that way prevented its central action. Both NPY/AgRP and POMC/CART arcuate neurons express the long form of the leptin receptor. Through its receptors in the hypothalamus, leptin activates JAK2-STAT3

signaling, subsequently increasing the synthesis and secretion of anorexigenic (POMC, CART) and decreasing the synthesis and secretion of orexigenic peptides (NPY, AgRP) in that way suppressing the food intake and inducing energy expenditure (Burgos-Ramos et al., 2010; Pico et al., 2003). Even more, treatments with palmitic and linoleic acid increased the hypothalamic expression of SOCS3, a leptin-inducible inhibitor of leptin signaling (Howard and Flier, 2006). The inhibition of gastric leptin secretion, as well as its central action, by fatty acids, especially palmitic acid, could explain the prevalence of orexigenic pathways (indicated by the NPY/POMC ratio) in palmitic acid treated rats compared to linoleic acid treated rats, and its resulting lesser satiating effect. Moreover, it has been show that leptin release into the blood after the stimulation of gastric leptin output (i.e. food intake) is absent or represents at most a ~25% increase in circulating leptin levels (Pico et al., 2003). Similarly, only gastric mucosa from palmitic treated-rats accumulated enough leptin to produce a significant increase in its circulating levels. In addition, as described in isolated rat white adipocytes (Cammisotto et al., 2003; Cammisotto et al., 2010), we found that both fatty acids repress leptin secretion in cultured explants of stomach mucosa, but only palmitic acid represses leptin mRNA expression. All in all it could be suggested that gastric leptin is involved in the satiating action of FFAs and that gastric leptin would acts as a safety mechanism to counteract anorexigenic central actions induced by FFAs via inhibition of gastric ghrelin, especially in stress situations such as fasting. Also CKK could be part of this safety mechanism, as we observed a significant reduction in its circulating levels after the linoleic acid treatment.

In conclusion, in the present study we showed a direct and specific action of luminal fatty acids in stomach secretion of hormones involved in the control of food intake, and in hypothalamic orexigenic/anorexigenic signaling. In particular, we exposed the involvement of gastric leptin in the satiating capacity of fatty acids, with different capacities for different individual fatty acids.

Conflict of interest: Authors have no competing financial interests in relation to the work described.

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Chapter 3.2

VITAMIN A SUPPLEMENTATION IN EARLY LIFE AFFECTS THE LATER RESPONSE TO AN OBESOGENIC DIET IN RATS

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Chapter 3.2

VITAMIN A SUPPLEMENTATION IN EARLY LIFE AFFECTS THE LATER RESPONSE TO AN OBESOGENIC DIET IN RATS

ABSTRACT

OBJECTIVE: To assess the influence of supplementation with a moderate dose of vitamin A in early life on adipose tissue development and the response to an obesogenic diet later in life.

METHODS: Rat pups received during the suckling period a daily oral dose of retinyl palmitate corresponding to three times the vitamin A ingested daily from maternal milk. Control rats received the vehicle (olive oil). Short-term effects of treatment on gene expression and morphology of white adipose tissue (WAT) were analyzed in animals on the day after weaning (d 21). To study long-term effects, control and vitamin A-treated rats were fed after weaning a normal fat or a high fat diet for 16 weeks.

RESULTS: WAT of vitamin A-treated young rats (d 21) was enriched in small adipocytes with a reduced expression of adipogenic markers (peroxisome proliferator-activated receptor γ and lipoprotein lipase) and an increased cell proliferation potential as indicated by increased expression of proliferating cell nuclear antigen. Increased retinoic acid-induced transcriptional responses were present in tissues of vitamin A-treated young rats (d 21) including WAT. Vitamin A-treated rats developed higher adiposity than control rats on a high fat diet as indicated by body composition analysis and increased WAT depot mass, adipocyte diameter, WAT DNA content, leptinemia and adipose leptin gene expression. Excess adiposity gain in vitamin A-treated rats developed in the absence of changes in body weight and was attributable to excess adipocyte hyperplasia. No differences in adiposity were observed between vitamin A-treated rats and control rats on a normal fat diet. Total retinol levels in WAT of vitamin A-treated rats were elevated at weaning (d 21) and normalized by d 135 of age.

CONCLUSION: Vitamin A intake in the early stages of postnatal life favors subsequent high fat diet-induced adiposity gain through mechanisms that may relate to changes in adipose tissue development, likely mediated by retinoic acid.

Keywords: vitamin A; early life nutrition; programming; adiposity; obesity; high fat diet

INTRODUCTION

Hormonal, metabolic and nutritional cues at critical periods in early life may determine the propensity to develop obesity and related disorders in adulthood (Sullivan and Grove, 2010) through mechanisms involving epigenetic changes (Godfrey et al., 2011; Palou et al., 2011) and effects on the development of anatomical structures crucial to the control of energy balance and storage, such as regulatory brain centers (Delahaye et al., 2008) and the adipose depots themselves (Garcia et al., 2011). Total energy intake, diet macronutrient composition, maternal signals present in breast milk such as leptin (Pico et al., 2007) and nutrients that participate in methyl transfer epigenetic reactions (Kalhan, 2009; Rosenberg, 2008) have been implicated in this programming. In general, however, studies dealing with the impact of specific micronutrients on the metabolic programming of obesity are scarce.

Vitamin A is a bioactive micronutrient in the control of adipose tissue biology (Bonet et al., 2003; Bonet et al., 2012). Retinoic acid (RA), its acid form, potently blocks adipogenesis of cultured pre-adipose cells when introduced at early stages of the differentiation process (Schwarz et al., 1997), although other reports indicate that RA at low doses may in fact promote adipogenesis (Bost et al., 2002; Reichert et al., 2011; Safonova et al., 1994). Studies in adult rodents treated with RA (Berry et al., 2012; Berry and Noy, 2009; Bonet et al., 2000; Felipe et al., 2004; Felipe et al., 2005; Manolescu et al., 2010; Mercader et al., 2006; Ribot et al., 2001; Strom et al., 2009) or retinaldehyde (Ziouzenkova et al., 2007) sustain an anti-adiposity action of vitamin A derivatives *in vivo*. Furthermore, results from animal studies (Felipe et al., 2003; Jeyakumar et al., 2006; Kawada et al., 1996; Kumar et al., 1999; Ribot et al., 2001) and human observational studies (Garcia et al., 2009; Zulet et al., 2008) point to an inverse relationship between vitamin A status and body fat content. The anti-adiposity action of RA has been traced to increased fatty acid oxidation and energy expenditure in adipose tissues (Berry et al., 2012; Bonet et al., 2000; Mercader et al., 2007; Mercader et al., 2006), skeletal muscle (Amengual et al., 2008; Berry and Noy, 2009) and the liver (Amengual et al., 2010), and to reduced peroxisome proliferator-activated receptor γ (PPARγ) levels and activity in white fat (Amengual et al., 2011; Lobo et al., 2010a; Ribot et al., 2001). PPARγ is a nuclear receptor transcription factor key for adipogenesis and also required for lipogenesis and survival in mature adipocytes (Lefterova and Lazar, 2009).

Despite the many studies linking vitamin A to the control of adipogenesis in cell models and adiposity in adult animals, little is known about the impact of vitamin A intake in early life on adiposity in the long-term. The objectives of this work were: first, to study the impact of supplementation with a moderate dose of vitamin A during the suckling period on adipose tissue development in young rats at weaning; and second, to study the influence of early vitamin A supplementation on later responses to a standard and an obesogenic (high fat) diet.

MATERIALS AND METHODS

Animals, diets and experimental designs

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

Analysis of vitamin A concentration in rat milk during lactation: To fix the vitamin A amount to be administered to the rat pups, we first quantified by HPLC the total vitamin A (retinol) concentration in the milk of lactating rats mated at our animal house. For this, after an acclimation period of one month, three-month-old, virgin female Wistar rats (from Charles River Laboratories, Barcelona, Spain) were caged with a male rat. After mating, the rats were individually housed. Rats were kept under controlled temperature (22ºC) and a 12-h light/dark cycle, with free access to water and a standard chow (Panlab, Barcelona, Spain; 4.5 mg vitamin A/kg). The day of delivery was defined as d0 of lactation. Milk was collected from three dams on d 7, 12 and 18 of lactation. For collection, nursing rats were separated from their pups for 6 h to guarantee that mammary glands were full of milk. The dams were then exposed to ether, and milk was obtained by manual milking and stored at -20 ºC. HPLC analysis revealed that vitamin A sources in milk were free retinol (in all-trans and 13-cis form) and retinyl ester (RE). The total vitamin A concentration (free retinol plus RE) in rat milk at d 7, 12 and 18 of lactation was, respectively, 1.15±0.23 µM, 0.93±0.25 µM and 1.32±0.13 µM. The average concentration of the three days (1.13 µM) was used in further study designs (see below).

Study of the effects of early vitamin A supplementation on young rats at weaning: Female Wistar rats were mated with male rats and housed as described above. On d1,

excess pups were removed to keep 10 pups per dam. Pups in each litter were randomly assigned into two groups which, from d1 to d20, were given daily orally, with the aid of a pipette, 10-15 μl of vehicle (olive oil, control rats) or an emulsion of vitamin A as RE (retinyl palmitate, Sigma, St. Louis, MO, USA) in olive oil supplying 3 times the daily vitamin A intake from maternal milk (RE rats). The amount of extra vitamin A given daily was progressively adjusted from 2.1 μg on d1 to 48.9 μg on d20, considering the vitamin A content in rat milk (see above) and the estimated daily milk intake throughout the suckling period in rats according to Kojima et al. (Kojima et al., 1998). On the day after weaning (d 21), control and RE rats were euthanized (16 rats from at least 4 different mothers/group; similar numbers of male and female rats were included in each group; results for both sexes were similar and were pooled). Blood was collected from the neck and serum prepared and stored at -20 ºC until analysis. Intestine, liver and white adipose tissue (WAT) depots – inguinal, gonadal and retroperitoneal –were dissected, weighted, frozen in liquid nitrogen, and stored at -70 ºC until analysis. A lengthways fragment of inguinal WAT (iWAT) was fixed by immersion in 4% paraformaldehyde for morphological and immunohistochemical analysis (see below).

Study of the effects of early vitamin A supplementation on diet-induced obesity later in life: Rats were treated as described above with vehicle or RE during the suckling period. On the day after weaning (d21), 10 control and 10 RE male rats were housed 2 or 3 animals per cage and split into two groups (5 animals per group), which were fed *ad libitum* for 16 weeks a normal fat (NF) diet (providing 15.9 kJ/g and 10% energy as fat) or a high fat (HF) diet (providing 21.8 kJ/g and 60% energy as fat), making a total of four experimental groups. Both diets were commercially available (Research Diets, Inc., New Brunswick, NJ, USA) and contained 1200 μg vitamin A/kg. Male rats were used because in our preliminary experiments were found to develop more obesity upon HF diet than female rats. Possible differences between litters were solved by ensuring the presence of pups from the same litters in each of the four experimental groups. Body weight and food intake were recorded 2-3 times a week. Rectal temperature and body composition were determined periodically using a digital thermometer and an Echo MRI-TM wall body composition analyzer, respectively. NF and HF diet-fed animals were sacrificed at d 135 of age by decapitation, under fed conditions, within the first three hours of the light period. Blood and tissues were collected and stored as described

above. This experiment was conducted twice, using two independent cohorts, each of 20 animals from 4-5 different mothers.

Oral glucose tolerance test (oGTT)

oGTT was performed to the four groups of rats in the long-term experiment at the age of 70 d and 128-130 d. In brief, a load of glucose (1.5 g/kg body weight in 1 to 1.5 ml) was orally given to the overnight fasted rats using a cannula. Tail blood samples were taken before glucose load at time zero and at 30, 60, 120, and 180 min thereafter, and used to measure glucose levels (see below). Fasting insulin and glucose concentrations at time zero were used to calculate the quantitative insulin check index of insulin sensitivity (QUICKI; $1/[\log(\text{insulin}) + \log(\text{glucose})]$) as a surrogate measure of insulin resistance (Mather, 2009).

Blood parameters

Serum insulin, leptin and resistin were measured using commercial ELISA kits (from, respectively, DRG Instruments GnbH, Marburg, Germany; R&D Systems, Minneapolis, MS, USA; and Phoenix Pharmaceutical Inc, Belmont, CA, USA). Glucose, triacylglycerol and nonesterified fatty acids (NEFA) in serum were measured enzymatically using commercial kits (from, respectively, Roche and R-Biopharm, Darmstadt, Germany; Sigma; and Wako Chemicals GmbH, Neuss, Germany) following standard procedures.

Histology and immunohistochemistry

Fixed iWAT lobules were dehydrated, cleared and paraffin-embedded so that the plane of section corresponded with the one of the wider surface. 5 μm-thick sections at the same level were obtained and stained with hematoxylin/eosin for morphometric analysis, performed by digital acquisition of adipose tissue areas (Axio Vision software and Zeiss Axioskop 2 microscope with AxioCam ICc3 digital camera). Distributions of adipocyte size were obtained from individual data of cell sizes. To avoid inter-rating variation, a single observer (E.C.) carried out morphometric analysis (coefficient of variation less than 7%). Immunohistochemistry of proliferating cell nuclear antigen (PCNA) was performed using a commercial anti-PCNA antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Results and Discussion

Extraction and analysis of retinoids

Extraction of retinoids from tissues (Amengual et al., 2011; Hessel et al., 2007) and HPLC separation of retinoids and quantification of the peak integrals (von Lintig and Vogt, 2000) was performed as previously described. Solvents for HPLC and extraction were purchased in HLPC grade from Merck (Darmstadt, Germany).

RNA isolation

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

Real-time quantitative PCR (RT-qPCR) analyses

Total RNA was denatured and reverse transcribed to cDNA, and PCRs for selected genes were performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems) as described previously (Granados et al., 2011). Primers used were obtained from Sigma (sequences are available upon request). The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated according to Pfaffl (2001) (Pfaffl, 2001), using 18S rRNA, β-actin or LRP10 as reference gene (similar results were obtained using either of them).

Other determinations

Tissue DNA content was determined by a fluorometric method that uses 3,5-diaminobenzoic acid and total lipid content was extracted using organic solvents and quantified by weight as previously described (Mercader et al., 2006). Tissue PPARγ protein levels were determined by immunoblotting as previously described (Amengual et al., 2011; Lobo et al., 2010a).

Statistical analysis

Data are presented as means±SEM. Statistical significance of differences between groups was assessed by two-way ANOVA and two-tailed Student's *t* test using SPSS 14.0 for windows (SPSS, Chicago, IL, USA). Distributions of adipocyte size were analyzed by the Quantitative Distribution method. The comparison of distributions

between the control and the RE groups was determined by Kolmogorov–Smirnov test. Threshold of significance was set at $p < 0.05$.

RESULTS

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

In this work, rat pups received a daily oral supplement of RE, the major dietary form of vitamin A from animal products, during the entire suckling period. To verify that exogenous RE was absorbed, HPLC analysis of retinoids in serum taken from 10-day-old pups between 1 and 2 h after vehicle or RE administration was performed. As expected, serum RE concentration was significantly higher in the RE-treated pups (0.619±0.152 µM) than in their control counterparts (0.242±0.030 µM) ($p < 0.05$, n= 3-4 pups/group). Similar results were obtained in 20-day-old pups. Additionally, RE-treated rats at d21 had markedly increased RE and free retinol levels in liver (Figure 1B) and total retinol (free retinol plus RE) levels in iWAT (1C). Serum retinol levels were unaffected by RE treatment, reflecting vitamin A homeostasis (1A).
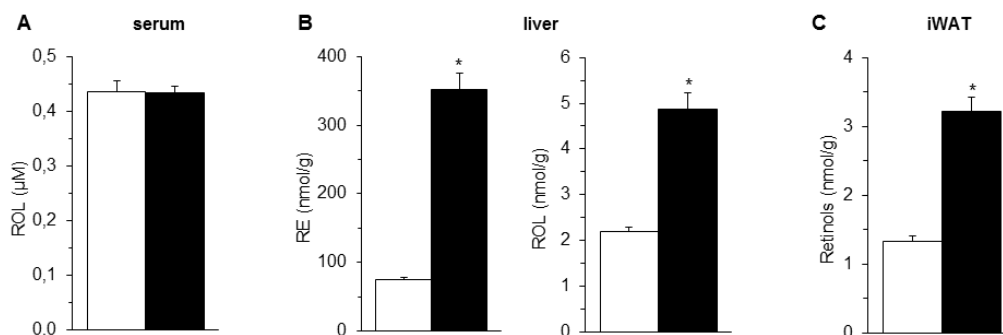
Figure 1. Retinoid levels in serum (A), liver (B) and inguinal white adipose tissue (C) of 21-day-old rats treated during the suckling period (d 1-20 of life) with vehicle (controls, white bars) or a moderate dose of vitamin A as retinyl ester (RE, black bars). Data represent the mean±SEM of 16 animals per group. [*] indicates a significant ($p < 0.05$) difference between the RE-treated group and the control group in two-tailed Student's *t* test. ROL, free retinol; RE, retinyl ester; Retinols, total retinol (free ROL plus RE).

Early vitamin A supplementation led to increased RA-mediated responses in tissues of young rats

Control and RE rats (d 21) were compared for the expression of intestine-specific homeobox (ISX) mRNA in the intestine and CYP26a1 mRNA in liver and iWAT. ISX

is a gut-specific transcription factor that is induced in intestinal cells by RA via RAR and functions to limit intestinal beta-carotene absorption and conversion to vitamin A through its effects on gene expression (Lobo et al., 2010b). CYP26a1 is a RA hydroxylase that is transcriptionally induced by RA in a RAR-dependent manner (Ozpolat et al., 2005). ISX mRNA levels in intestine and CYP26a1 mRNA levels in liver and iWAT were higher in the RE rats than in the control rats (Figure 2 A, B, C), indicating increased RA-mediated transcriptional responses in tissues of RE-treated animals. Aldehyde dehydrogenase 1a1 (Aldh 1a1), which catalyses RA production from retinaldehyde (Molotkov and Duester, 2003), was expressed in tissues of 21-day-old rats, and its mRNA levels in iWAT, but not in the liver, were down-regulated in the RE rats (Figure 2 D, E).
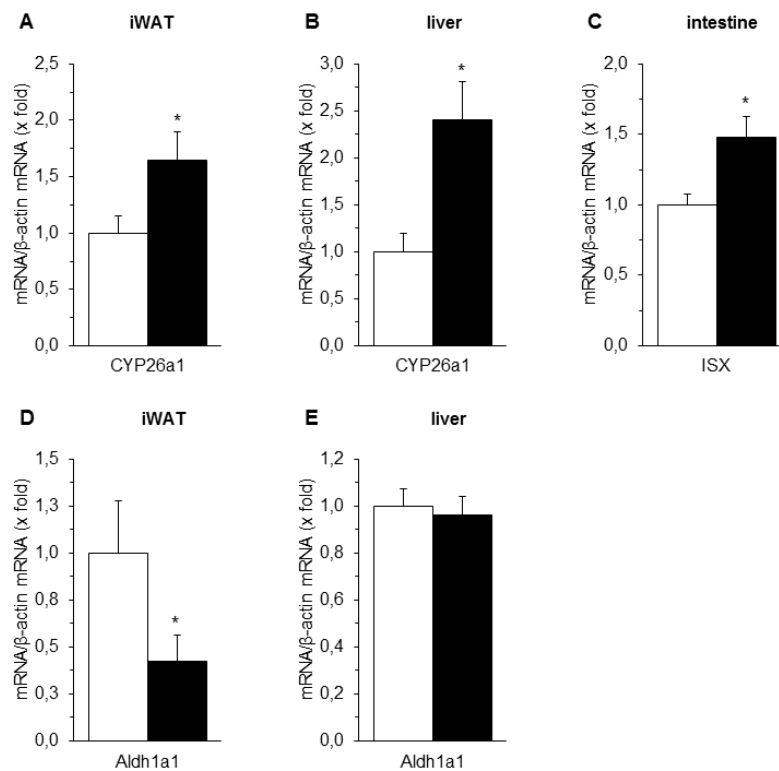
Figure 2. mRNA expression levels of retinoid-sensitive genes were analyzed in 21-day-old rats treated during the suckling period (d 1-20 of life) with vehicle (controls, white bars) or a moderate dose of vitamin A as retinyl ester (RE, black bars): CYP26a1 in inguinal white adipose tissue (iWAT, A), CYP26a1 in the liver (B), ISX mRNA in the intestine (C), and Aldh 1a1 in iWAT (D) and liver (E). Data represent the mean±SEM of at least 9 animals per group. * indicates a significant ($p<0.05$) difference between the RE-treated group and the control group in two-tailed Student's *t* test.
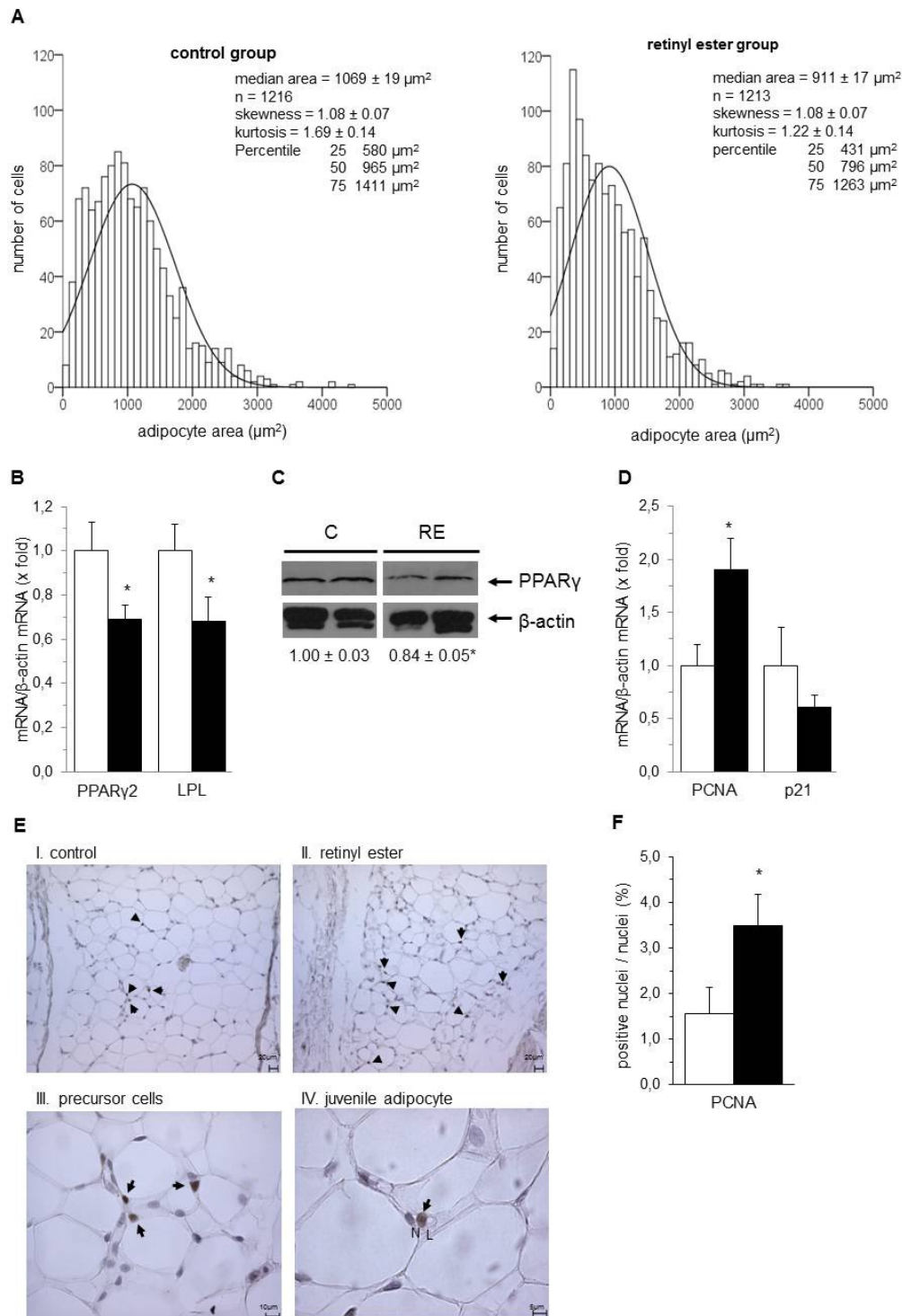
Figure 3. Inguinal white adipose tissue features in 21-day-old rats treated during the suckling period (d 1-20 of life) with vehicle (control group, white bars) or a moderate dose of vitamin A as retinyl ester (RE group, black bars). A) Distributions of adipocytes size were obtained from individual data of cell sizes and analyzed by the Quantitative Distribution Method. Six animals per group and ~200 cells per animal were included in the analysis. The area of individual adipocytes was measured using a quantitative morphometric method at 20x magnification with the assistance of Axio Vision software. The statistical comparison of distributions between the control and the retinyl ester group was determined by the Kolmogorov–Smirnov test (p < 0.0001). B) mRNA expression levels of adipogenic genes PPARγ and LPL; the means±SEM of at least 12 animals per group are represented. C)

Representative PPARγ immunoblot; numbers at the bottom are the mean±SEM PPARγ protein relative amount in the control and the RE group (n=4 per group). D) mRNA expression levels of PCNA and p21; the means±SEM of at least 8 animals per group are represented. E) Representative microphotographies of PCNA immunostaining: I, control rats; II, RE-treated rats; III, magnification of PCNA positive precursor cells; IV, magnification of PCNA positive cells presenting with small cytoplasmic lipid droplets, found in iWAT of RE-treated rats (N= nucleus; L= lipid droplet). Arrows indicate PCNA positivity. F) Percentage of PCNA positive nuclei in control and RE rats. Six animal per group and ~200 cells per animal were counted. In B, D and F, * indicates a significant (p<0.05) difference between the RE group and the control group in two-tailed Student's *t* test.

Early vitamin A supplementation affected adipose tissue development in young rats

At the end of the treatment period, on d21, there were no differences between control and RE rats in liver mass (control, 1.55±0.04 g; RE, 1.60±0.04 g), body weight (control, 41.3±0.9 g; RE, 41.3±0.6 g), fat depot mass (combined mass of WAT depots: control, 0.56±0.03 g; RE, 0.56±0.03 g), or serum leptin (control, 1233±118 pg/mL; RE, 1167±142 pg/mL) (n=13-16 animals/group). Histological and molecular analysis revealed, however, differences in iWAT between control and RE rats. A shift of adipocyte population towards smaller size was evident in iWAT of the RE rats (Figure 3A). The Kolmogorov–Smirnov test demonstrated that the difference in distributions of cell size between both groups of animals was statistically significant (p<0.0001). RE rats displayed a reduced expression of PPARγ in iWAT evidenced both at the mRNA and the protein level (Figure 3B and 3C). Gene expression of the PPARγ downstream target, lipoprotein lipase (LPL), was also reduced in iWAT of the RE rats (Figure 3B). Additionally, RE rats had 90% higher PCNA mRNA levels and a trend to reduced mRNA levels of the cell cycle break p21 in iWAT compared to the control rats (Figure 3D). Increased PCNA expression was confirmed by immunohistochemical analysis, which showed more PCNA positive nuclei in iWAT sections from the young RE rats than in those from control rats (Figure 3E, microphotographies I and II; Figure 3F). Interestingly, in the RE rats, PCNA positive staining was observed not only in typical adipocyte precursor cells (Figure 3E, III), but also in cells with small cytoplasmic lipid droplets, i.e. already engaged in terminal adipogenic differentiation (Figure 3E, IV). There were no differences between the control and the RE group in the gene expression in iWAT of the preadipocyte markers Pref-1 (control, 1.00±0.21; RE, 0.86±0.27), Sox9 (control, 1.00±0.30; RE, 1.04±0.38), and Kruppel-like factor 2 (KLF2) (control, 1.00±0.15; RE, 0.86±0.08) (numbers in brackets are the mean±SEM of 13-14 animals/group).
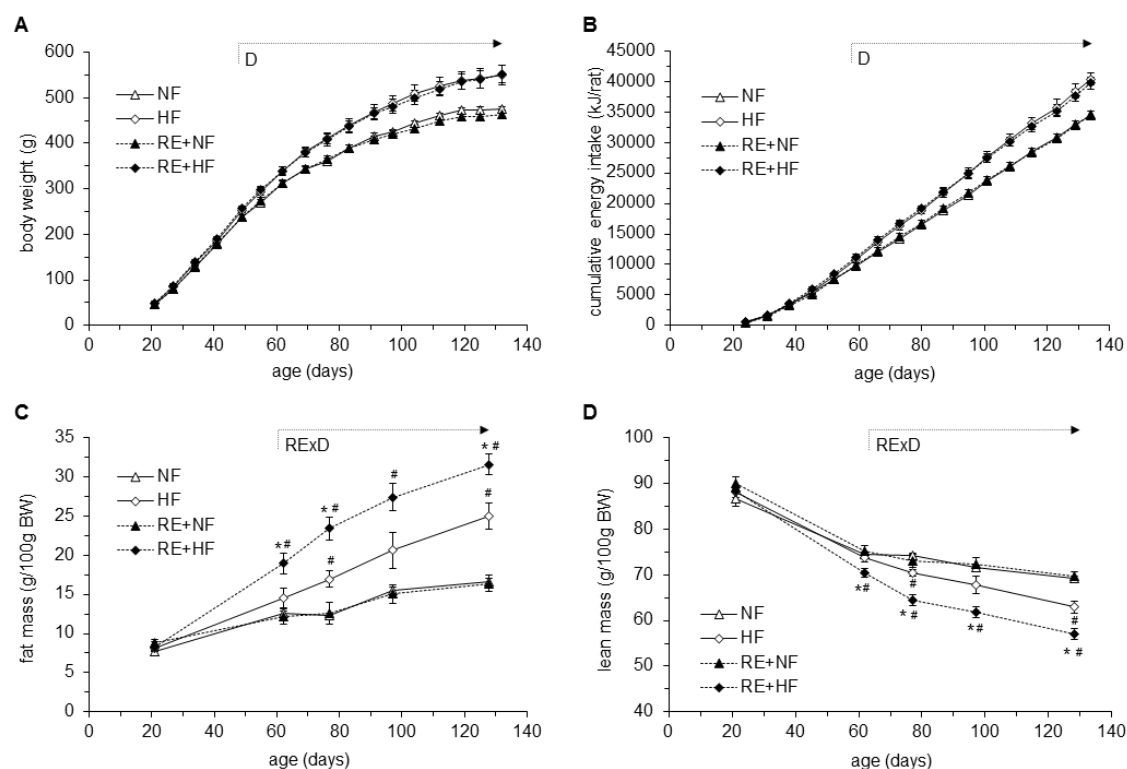
Figure 4. Growth curves (A), cumulative energy intake (B), evolution of fat body mass (C) and evolution of lean body mass (D) in rats treated with vehicle (empty symbols) or vitamin A as retinyl ester (RE, filled symbols) during the suckling period (d 1-20 of life) and fed thereafter a normal fat (NF, triangles) or a high fat diet (HF, diamonds) for 16 weeks. Fat and lean body mass were determined at the indicated ages using an Echo MRI-TM wall body composition analyzer. Body weight and body composition data represent the mean±SEM of 10 animals per group and cumulative energy intake data represent the mean±SEM of 4 cages (2-3 animals each) per group, distributed in two separate experiments. D and RExD indicate, respectively, diet effect and interaction between RE treatment and diet in two-way ANOVA analysis; * indicates a significant difference between RE-treated and control groups and # between HF and NF groups in the two-tailed Student's *t* test post hoc analysis. Threshold of significance was set at *p*<0.05.

Early vitamin A supplementation led to increased adiposity following high fat diet feeding later in life

To study the influence of vitamin A intake during the suckling period on ulterior adiposity, rats that had been treated daily with vehicle or RE from d 1 to d 20 of life were fed after weaning either a NF or a HF diet for 16 weeks. Body weight gain and cumulative energy intake were not affected by early vitamin A treatment and were higher, as expected, in the HF diet-fed groups than in the NF diet-fed groups (Figures 4A and 4B). Rectal temperature was found to be equal in the four experimental groups at different time points of the experiment (80, 91 and 123 days of age) (data not shown). Strikingly, despite no differences in body weight, the rats treated with RE during the

suckling period developed higher adiposity on the HF diet than the control rats. Increased adiposity in the RE rats was apparent by ECHO-MRI analysis already after a relatively short period of HF diet feeding (40 d) (Figure 4C), and was paralleled by reductions in the percentage of lean body mass (Figure 4D).
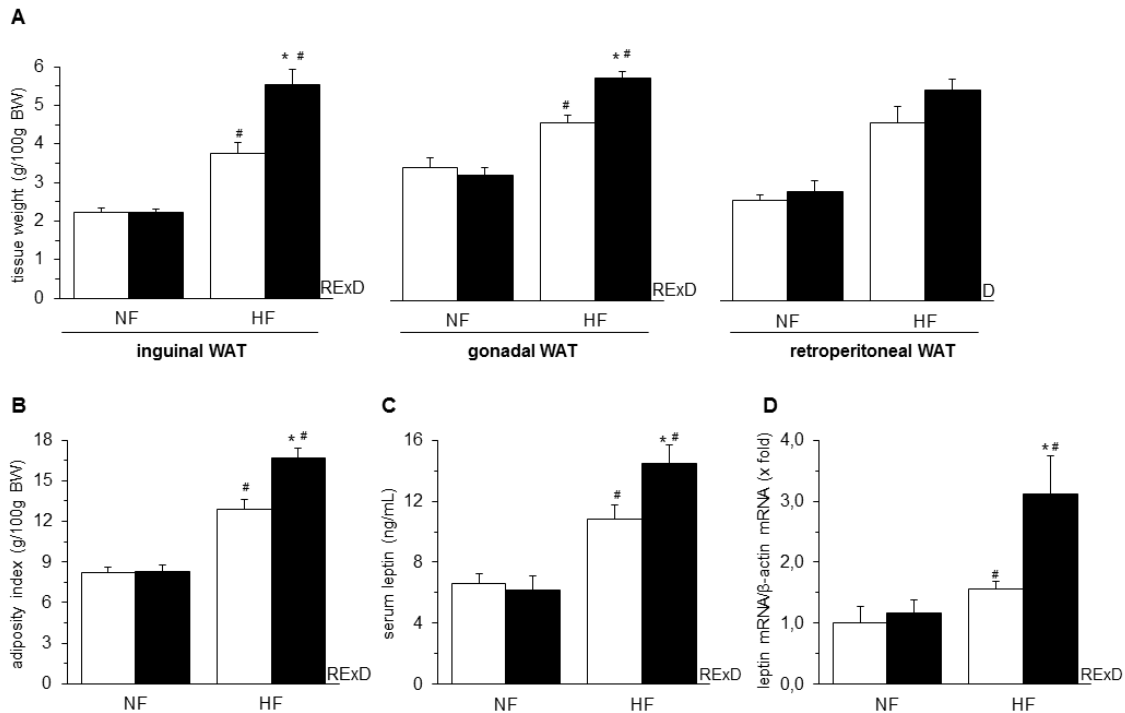
Figure 5. Vitamin A supplementation during the suckling period increases high fat diet-induced adiposity later in life. White adipose tissue (WAT) depots weight as percent of body weight (A), adiposity index (B), serum leptin levels (C) and leptin mRNA levels in inguinal WAT (D) in male rats treated with vehicle (controls, white bars) or vitamin A as retinyl ester (RE, black bars) during the suckling period (d 1-20 of life) and fed thereafter a normal fat (NF) or a high fat diet (HF) for 16 weeks. Data represent the mean±SEM of 10 animals per group, distributed in two separate experiments. D and RExD indicate, respectively, diet effect and interaction between RE treatment and diet in two-way ANOVA analysis; * indicates a significant difference between RE-treated and control groups and # between HF and NF groups in the two-tailed Student's t test post hoc analysis. Threshold of significance was set at $p<0.05$.

In accordance with the ECHO-MRI results, after 16 weeks of HF diet the mass of all WAT depots dissected was higher in the RE rats than the control rats (Figure 5A). Increased fat depot expansion was particularly pronounced for the subcutaneous inguinal depot (48% excess vs 25% and 19% excess for, respectively, the gonadal and retroperitoneal depots). Overall, the adiposity index following HF diet was 30% higher in the RE rats (Figure 5B). Importantly, no differences in body composition or adiposity were observed between the RE and the control rats on the NF diet (Figures 4C, 4D, 5A

and 5B). Leptin levels in serum (Figure 5C) and leptin mRNA levels in iWAT (Figure 5D) reflected changes in body adiposity: they were increased following HF diet, and to a greater extent in the RE rats than the control rats.

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

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

Mean adipocyte diameter data are the mean ± SEM of 3 animals per group, and for each animal at least 100 adipocytes were analyzed. Other data are the mean ± SEM of 5 animals per group. RE, D, and RExD indicate, respectively, RE effect, diet effect, and interaction between RE and diet in two-way ANOVA analysis; [*] indicates a significant difference between RE and control groups and [#] between high fat and normal fat groups in the two-tailed Student's *t* test post hoc analysis. Threshold of statistical significance was set at $p<0.05$.

In both control and RE rats, iWAT expansion following HF diet feeding was accompanied by increases in adipocyte diameter and total tissue DNA content, indicating both a hypertrophic and a hyperplasic component (Table 1). Remarkably, it was the hyperplasic component of HF diet-induced adiposity gain that was enhanced in the vitamin A-treated rats. Thus, 16 weeks of HF diet feeding associated with an increase in total iWAT DNA content by a factor of 5 in the RE rats and of 2 in the control rats, while adipocyte size increased by a factor of ~1.5 in both groups (Table 1). Adipocyte size was higher in the RE rats than the control rats under both NF and HF diet (Table 1), and additional analysis revealed higher expression levels of genes for proteins involved in lipid uptake in iWAT of the RE rats (Supplementary Figure1).

Fat depots might be involved in preventing deleterious lipid accumulation in other tissues (Virtue and Vidal-Puig, 2010). We therefore examined whether the increased adiposity in RE rats vs control rats following HF diet associated with a better metabolic profile (Supplementary Table 1). Liver fat content, circulating levels of glucose, NEFA, triacylglycerol and resistin in the fed state, and QUICKI were all negatively affected by HF diet feeding similarly irrespective of treatment with vehicle or RE in early life. Glucose tolerance by the end of the HF diet challenge was somewhat more impaired in the fattier HF diet-fed RE rats (Supplementary Table 1). Thus,

increased adipose tissue expansion after HF diet in the vitamin A-treated rats did not correlate with improvements in obesity related complications, although no gross aggravation was either evident.

Retinoid levels in serum and iWAT of adult rats (d 135) were unaffected by RE treatment in early life or HF diet feeding (Supplementary Figure 2). RE levels in liver remained slightly higher in the adult rats that had been treated with RE in early life (Supplementary Figure 2).

DISCUSSION

This study shows that a moderate (x3) excess of vitamin A intake as RE during the suckling period leads in rats to changes in WAT at the time of weaning and to increased adiposity gain following a HF diet later in life. To our knowledge, this is the first study specifically addressing the influence of early vitamin A supplementation on later adiposity. The pro-obesogenic effect of early vitamin A supplementation was evidenced in two independent experiments using different cohorts of animals and, importantly, only upon a HF challenge, i.e., under environmental conditions favoring adipose tissue expansion. The effect was strong (30% excess body fat mass in the HF diet-fed RE-treated rats relative to their control, vehicle-treated littermates) and was paralleled by increased leptinemia and adipose leptin gene expression. Higher adiposity in RE rats vs control rats after HF diet developed in the absence of changes in body weight, energy intake or a proxy measurement of basal energy expenditure (rectal temperature), thus pointing to differences in nutrient partitioning, with a greater drive to WAT in the RE rats. Excess adiposity gain in RE rats after HF diet was attributable to excess adipocyte hyperplasia, rather than excess adipocyte hyperthrophy and, interestingly, an increased cell proliferation potential was found in WAT of young RE rats at weaning. Importantly, total retinol levels in WAT of RE-treated rats were elevated by the time of weaning (d 21) but normalized by d 135 of age, supporting the concept that early effects conditioned the adipose tissue phenotype exhibited later following a dietary HF challenge.

WAT development in the rat takes place within the first 30 days of postnatal life (Cryer and Jones, 1979), and excess vitamin A within this period elicited changes in the developing tissue. In particular, iWAT of RE-treated rats at weaning was enriched in

small adipocytes and in PCNA, a classical marker of proliferative status, while displaying a reduced expression of the master adipogenic transcription factor PPARγ and its downstream target, LPL. These results are in keeping with the general assumption that cell proliferation and differentiation are mutually exclusive phenomena. However, the expression of known preadipocyte markers assayed (Pref-1, Sox-9, KLF2) was not increased in the iWAT of young RE rats. We interpret these results as indicating that a moderate excess of vitamin A at key developmental stages favors the development of immature adipocytes, with less PPARγ and more PCNA, that retain a competent proliferative status. This would in turn favor the hyperplasic component of fat expansion upon a subsequent stimulus in the form of a HF diet. Importantly, several studies have shown that partially differentiated adipocytes remain capable of replication (reviewed in (Hausman et al., 2001)).

Orally administered RE was efficiently absorbed and likely fueled RA production in tissues of young rats (d21) including WAT, which expressed Aldh 1a1, the principal postnatal Aldh isoform involved in RA production from retinaldehyde (Molotkov and Duester, 2003). Known RA-mediated transcriptional responses were enhanced in tissues of RE rats at weaning (d21), including down-regulation of Aldh 1a1 expression in iWAT (Elizondo et al., 2009; Reichert et al., 2011). Down-regulation of Aldh 1a1 in the face of up-regulation of the RA-degrading enzyme CYP26a1 is suggestive of a homeostatic mechanism to keep constant intracellular RA levels in WAT. The increased cell proliferation potential found in iWAT of young RE-treated rats is in line with repressive effects of RA on the expression of cell cycle breaks (p21 and retinoblastoma protein) previously reported in adipocyte cell models (Ribot et al., 2005), and with increased thymidine incorporation in cultured adipose precursor cells exposed to RA (Gupta et al., 2007). Reduced PPARγ expression in iWAT of vitamin A-treated young rats is also in line with previous reports (Amengual et al., 2011; Lobo et al., 2010a; Ribot et al., 2001). All in all, the data are consistent with RA being involved in mediating the effects of early RE treatment on WAT of young rats.

Many studies have addressed the impact of vitamin A derivatives on body adiposity in adult animals, pointing to an anti-obesity action (Berry et al., 2012; Berry and Noy, 2009; Bonet et al., 2000; Felipe et al., 2004; Felipe et al., 2005; Manolescu et al., 2010; Mercader et al., 2006; Ribot et al., 2001; Strom et al., 2009; Ziouzenkova et al., 2007). On the opposite, in the present study a three-fold excess of vitamin A intake

in early life (d1 to d20 of life) exacerbated WAT expansion in response to a HF diet later in life. The reason(s) of this apparent discrepancy is unknown but could relate to concentration-dependent effects, because studies in adult animals typically used relatively high retinoid concentrations (reviewed in (Bonet et al., 2012)). Developmental stage-dependent effects may also be involved. Interestingly, short-term exposure of young rats (50-70 g, age 3 weeks) to a HF (cafeteria) diet resulted in higher adiposity when the diet was enriched with a four-fold excess retinol (Redonnet et al., 2008). In the later study, the synergic effect of vitamin A and HF diet in WAT development was concomitant with a higher proliferation competence of precursor cells isolated from the animals' fat depots (Redonnet et al., 2008). In a study in lambs, supplementation with a thirty-fold excess retinyl palmitate from birth and during the whole period of growth (until d101 of age, under standard feed) did not influence adiposity, but resulted in a 30% increase in the number of adipocytes in the perirenal depot, and in smaller adipocytes in this and other fat depots (Arana et al., 2008). Therefore, our study and these previous studies point to a pro-obesogenic effect of excess vitamin A intake in early life, and to an influence of vitamin A intake in early life specifically on adipocyte number in fat depots. Studies have highlighted adipose tissue cellularity as an important determinant of fat mass (Hausman et al., 2001; Spalding et al., 2008), and suggested that increased adipocyte number might *per se* lead to obesity (Naaz et al., 2004).

In summary, this work shows that a moderate (three-fold) excess vitamin A intake in early life associates with an increased proliferative capacity in WAT at weaning, and with higher adiposity gain and WAT hyperplasia in response to an obesogenic diet afterwards. The results might have implications for the obesity pandemics and particularly for childhood obesity, as the vitamin A content of human milk is related to maternal vitamin A status and intake during lactation (Haskell and Brown, 1999).

Conflict of interest: Authors have no competing financial interests in relation to the work described.

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SUPPLEMENTARY MATERIAL
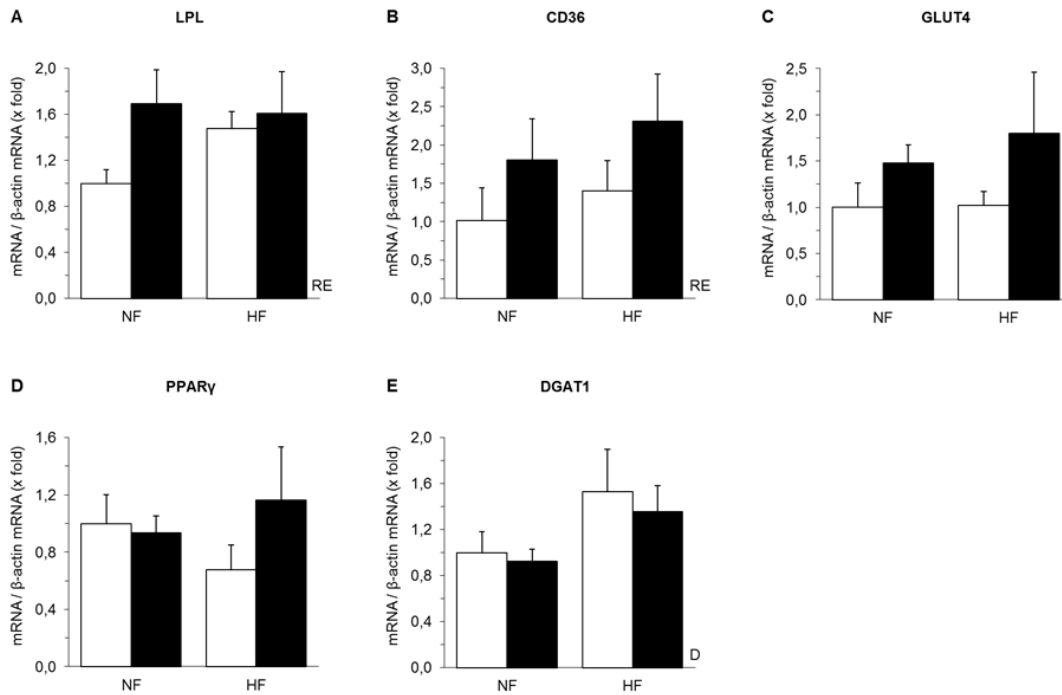
Supplementary Figure 1. Gene expression of selected genes in inguinal white adipose tissue of 135-day-old male rats treated with vehicle (controls, white bars) or vitamin A as retinyl ester (RE, black bars) during the suckling period (d 1-20 of life) and fed thereafter a normal fat (NF) or a high fat diet (HF) for 16 weeks. Data represent the mean±SEM of at least 5 animals per group and are expressed relative to the mean value in the control (vehicle-treated) NF diet-fed group, which was set to 1. The expression levels of the denoted genes were analyzed by qPCR and normalized to the expression of β-actin mRNA. RE and D indicate, respectively, retinyl ester effect and diet effect in two-way ANOVA analysis (p<0.05).
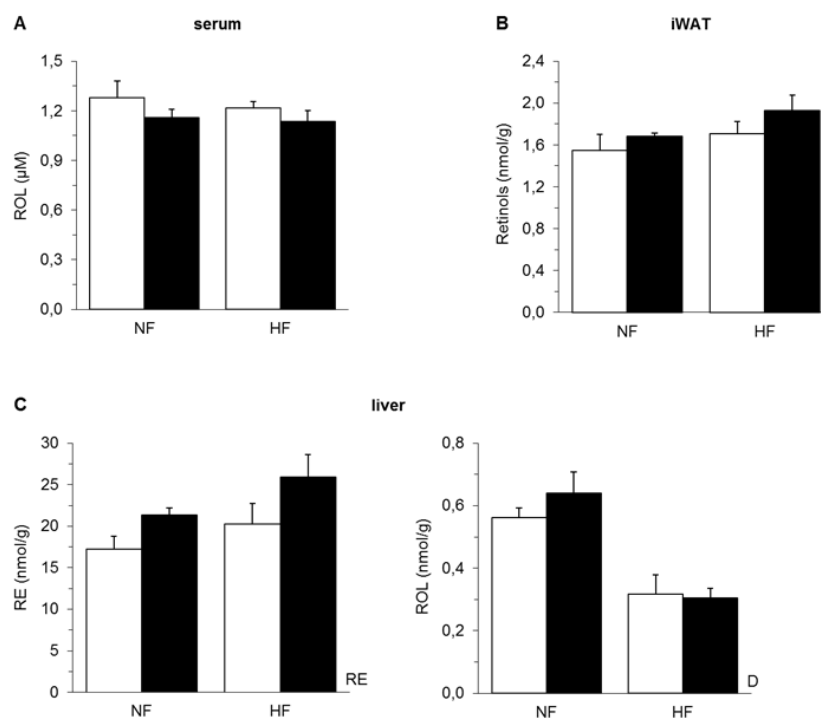
Supplementary Figure 2. Retinoid levels in serum (A), inguinal white adipose tissue (B) and liver (C) of 135-day-old rats treated with vehicle (controls, white bars) or vitamin A as retinyl ester (RE, black bars) during the suckling period (d 1-20 of life) and fed thereafter a normal fat (NF) or a high fat (HF) diet for 16 weeks. Data are the mean±SEM of 5 animals per group. RE and D indicate, respectively, retinyl ester effect and diet effect in two-way ANOVA analysis ($p<0.05$). ROL, free retinol; RE, retinyl ester; Retinols, total retinol (RE plus free ROL).

Results and Discussion

Supplementary Table 1. Parameters related to obesity-associated metabolic complications in male rats treated with vehicle (Control) or vitamin A as retinyl ester (RE) during the suckling period (d 1-20 of life) and fed thereafter a normal fat or a high fat diet for 16 weeks.

| | Normal fat | | High fat | | |
	Control	RE	Control	RE	ANOVA
hepatic lipids *(mg/g wet tissue)*	41.3 ± 4.2	41.3 ± 1.5	85.6 ± 9.8	87.1 ± 11.3	D
glucose *(mM)*	4.86 ± 0.14	4.82 ± 0.21	5.58 ± 0.17	5.74 ± 0.24	D
NEFA *(mM)*	2.86 ± 0.39	2.43 ± 0.27	4.27 ± 0.29	3.76 ± 0.50	D
triacylglycerols *(mM)*	0.316 ± 0.024	0.332 ± 0.053	0.255 ± 0.022	0.233 ± 0.020	D
resistin *(ng/mL)*	44.1 ± 4.5	53.8 ± 5.2	46.7 ± 4.7	44.8 ± 3.7	
QUICKI					
age 70 d	0.399 ± 0.003	0.402 ± 0.005	0.357 ± 0.020	0.345 ± 0.016	D
age 128 d	0.371 ± 0.015	0.379 ± 0.015	0.340 ± 0.015	0.336 ± 0.009	D
oGTT *(area under the curve, a.u.)*					
age 70 d	15660 ± 785	15825 ± 472	18690 ± 662	19145 ± 500	D
age 128 d	17919 ± 518	18227 ± 519	19428 ± 250	22247 ± 1214 *,#	RExD

Data are the mean ± SEM of at least 5 animals per group. D indicates diet effect in two-way ANOVA analysis; RExD indicates interaction between RE and diet in two-way ANOVA analysis; * indicates a significant difference between RE and control groups and # between high fat and normal fat groups in the two-tailed Student's t test post hoc analysis. Threshold of statistical significance was set at $p < 0.05$.

Chapter 3.3

β-CAROTENE DURING THE SUCKLING PERIOD IS ABSORBED INTACT AND POORLY AFFECTS ADIPOSE TISSUE DEVELOPMENT IN YOUNG RATS

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Manuscript to be submitted

Chapter 3.3

β-CAROTENE DURING THE SUCKLING PERIOD IS ABSORBED INTACT AND POORLY AFFECTS ADIPOSE TISSUE DEVELOPMENT IN YOUNG RATS

ABSTRACT

Increasing evidence is linking β-carotene (BC) to the control of adiposity in adult animals. We aimed to assess the impact of supplementation with a moderate dose of vitamin A as BC during the suckling period on white adipose tissue (WAT) development in young rats at weaning. We also aimed to compare BC effects with those brought about by an equivalent dose of vitamin A given as retynil esters (RE).

Rat pups received during the suckling period a daily oral dose of vitamin A, corresponding to approx. three times the vitamin A ingested daily from maternal milk, in the form of BC or RE (retinyl palmitate). Control rats received the vehicle (olive oil). After weaning, vehicle-, BC- and RE-treated rats were sacrificed and parameters related to gene expression and morphology of inguinal (i) WAT were analyzed.

Orally given BC was readily absorbed intact and partially metabolized by the suckling rats. This is of interest given that adult rats have been discarded as an animal model to study the toxicological and nutritional effects of BC, because, contrary to what happens in humans, adult rats do not practically absorb BC. In our study, serum and liver levels of BC were significantly increased in the pups treated with the moderate dose of BC. In contrast to RE treatment, BC treatment did not affect adipose tissue development in young rats. In particular, iWAT of BC-treated rats at weaning, like iWAT of control rats, was enriched in larger adipocytes that expressed higher levels of adipogenic markers and reduced levels of a classical marker of proliferative status, compared to iWAT of RE-treated rats.

Studies dealing with the impact of specific micronutrients at critical periods in early life may help in the understanding of the programming mechanisms of future health/disease balance. Furthermore, our study establishes a new model for the experimental evaluation of nutritional and toxicological effects of BC, including early programing and epigenetic effects.

Keywords: vitamin A; early life nutrition; animal models; adiposity

INTRODUCTION

Vitamin A is an essential nutrient that participates in the control of many crucial biological functions including adipocyte physiology and the regulation of body fat reserves, reviewed in (Bonet et al., 2003; Bonet et al., 2012). Retinoids in animals are derived from two distinct dietary sources: preformed vitamin A, mainly retinyl esters (REs), from animal products, and provitamin A carotenoids, mainly β-carotene (BC), from vegetables and fruits (von Lintig, 2010). In the intestine, REs are hydrolyzed to retinol and as such absorbed by the enterocytes, whereas BC is absorbed and upon absorption readily converted to vitamin A, mainly through symmetric cleavage by the enzyme BC 15-15' monooxygenases (BCMO1), yielding two molecules of retinaldehyde that are converted to retinol by retinol deshydrogenases. Within the enterocytes, all retinol regardless of its dietary origin is re-esterified, and as RE incorporated into nascent chylomicrons together with other dietary lipids and secreted into the circulation via the mesenteric lymph. REs, as a component of chylomicrom remnants, are mainly taken up by the liver, the major site of vitamin A storage and metabolism, which also controls retinol distribution to other tissues (Tourniaire et al., 2009; von Lintig, 2010). The efficiency of intestinal BC cleavage varies greatly across species: rat and mice cleave almost all absorbed BC, whereas humans and ferrets incorporate some of it into nascent chylomicrons and deliver it to tissues in an intact form (Lee et al., 1999). Only when BC is provided in the diet at supraphysiologic levels rodents absorb it intact (Lee et al., 1999). There is no information on the capacity of young rats to absorb BC. Retinoic acid (RA) is a major active cellular retinoid and an important regulator of gene expression. RA is synthesized intracellularly primarily from retinaldehyde, which itself can be produced from retinol or BC (Blomhoff and Blomhoff, 2006).

Results from animal studies (Jeyakumar et al., 2006; Kawada et al., 1996; Kumar et al., 1999; Ribot et al., 2001) and human observational studies (Garcia et al., 2009; Zulet et al., 2008) point to an inverse relationship between vitamin A status and body fat content. Studies in cell models and adult animals indicate that regulation of fat reserves by dietary vitamin A can be largely explained by its metabolism to biologically active retinoid derivatives which impact the differentiation and function of adipose tissues (reviewed in (Bonet et al., 2003; Bonet et al., 2012)). In particular, a connection between enhanced retinoid and carotenoid metabolism and reduced peroxisome

proliferator-activated receptor (PPAR) γ levels and activity has been evidenced in white fat cells (Amengual et al., 2011; Lobo et al., 2010a; Mercader et al., 2007; Ribot et al., 2001; Ziouzenkova et al., 2007). PPARγ is a nuclear receptor transcription factor key for adipogenesis and also required for lipogenesis and survival in mature adipocytes (Lefterova and Lazar, 2009). In line with an anti-adipogenic action of retinoids, we recently reported that treatment with a moderate dose of retinyl palmitate during the suckling period − a critical period in the development of anatomical structures of adipose tissue in the rat (Cryer and Jones, 1979) − favours the development of immature adipocytes, with less PPARγ and more proliferating cell nuclear antigen (PCNA), that retain a competent proliferative status(Granados et al., 2013). The impact on adipocyte proliferative status appeared to affect the hyperplasic component of fat expansion upon a subsequent obesigenic stimulus in adulthood (Granados et al., 2013).

Despite the evidence linking BC to the control of adipogenesis in cell models (Lobo et al., 2010a) and adiposity in adult animals(Amengual et al., 2011), little is known about BC effects in early life. The aim of this study was thus to assess the impact of supplementation with a moderate dose of vitamin A as BC during the suckling period on adipose tissue development in young rats at weaning. To this end, we first studied the absorption of BC and then performed molecular and histological analysis of inguinal white adipose tissue (iWAT) of BC-treated rats as compared to iWAT of rats given an equivalent dose of vitamin A as RE.

MATERIALS AND METHODS

Animals and experimental design

To perform the experiment, after an acclimation period, 3-month-old, virgin female Wistar rats (from Charles River Laboratories, Barcelona, Spain) were caged with male rats. After mating, the rats were individually housed. Rats were kept under controlled temperature (22 °C) and a 12-h light/dark cycle, with free access to water and a standard chow (Panlab, Barcelona, Spain; 4.5mg vitamin A kg^{-1}). The day of delivery was defined as day 0 of lactation. On day 1, excess pups were removed to keep 10 pups per dam. Pups in each litter were randomly assigned into three groups which, from day 1 to day 20 of the suckling period, were orally given daily, with the aid of a pipette, 10–15 μl of vehicle (olive oil; control rats) or an emulsion of vitamin A as retinyl palmitate

(Sigma, St. Louis, MO, USA; RE rats) or as β-carotene (Sigma, St. Louis, MO, USA; BC rats) in olive oil supplying 3 times the daily vitamin A intake from maternal milk. The amount of extra vitamin A given daily was progressively adjusted, considering the vitamin A content in rat milk (Granados et al., 2013), the estimated daily milk intake throughout the suckling period in rats (Kojima et al., 1998) and the vitamin A activity equivalence of purified BC in oil (Otten et al., 2006): from 2.1 µg of RE and 5.1 µg of BC on day 1 to 48.9 µg of RE and 122.3 µg of BC on day 20. On the day after weaning (day 21), control, RE and BC rats were euthanized (16 rats from at least four different mothers/group; similar numbers of male and female rats were included in each group; results for both sexes were similar and were pooled). Just before sacrifice the animals were weighed and body composition was determined using an Echo MRI-900t wall body composition analyzer (EchoMRI, Houston, TX, USA). Blood was collected from the neck and serum prepared and stored at -20 $^{\circ}$C until analysis. Intestine, liver and white adipose tissue (WAT) depots -inguinal, gonadal and retroperitoneal- were dissected, weighed, frozen in liquid nitrogen and stored at -80 $^{\circ}$C until analysis. A lengthways fragment of inguinal WAT (iWAT) was fixed by immersion in 4% paraformaldehyde for morphological and immunohistochemical analysis (see below). Animal protocols followed in this study were reviewed and approved by the Bioethical Committee of the University of the Balearic Islands and guidelines for the use and care of laboratory animals of the University were followed.

Extraction and analysis of retinoids and carotenoids

Extraction of retinoids and carotenoids from serum and tissues (Amengual et al., 2011; Hessel et al., 2007) and HPLC separation of retinoids and carotenoids, as well as quantification of the peak integrals (von Lintig and Vogt, 2000) were performed as previously described. Solvents for HPLC and extraction were purchased in HPLC grade from Merck (Darmstadt, Germany).

RNA isolation, cDNA obtention and real-time PCR amplification

Total RNA was extracted from tissue using Tripure Reagent (Roche, Barcelona, Spain) according to the manufacturer's instructions. Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, DE, USA) and its integrity confirmed using agarose gel electrophoresis. Subsequently RNA was denatured and reverse transcribed to cDNA using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA, USA) and random hexamers primers according

to the Applied Biosystems procedure at 42 °C for 1 h in a Perkin-Elmer 2400 Thermal Cycler (PerkinElmer, Wellesley, MA, USA). cDNA was used for PCR amplification of selected genes, using specific primers obtained from Sigma (Madrid, Spain; sequences are available upon request) and the Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus^{TM} Real-Time PCR System according to the Applied Biosystems procedure. The threshold cycle was calculated by the instrument's software (StepOne Software v2.0, Applied Biosystems) and the relative expression of each mRNA was calculated according to Pfaffl (Pfaffl, 2001), using 18S rRNA as a reference gene.

Western blotting analysis

Total protein from animal tissue was isolated by using the M-PER mammalian protein extraction reagent with protease inhibitors (Roche, Barcelona, Spain) and quantificated with the BCA protein assay kit according to manufacturer's instructions (Pierce; Thermo Fisher Scientific Inc. Rockford, IL, USA). Protein levels of ISX in intestine and PPARγ in iWAT were determined by immunoblotting as previously described (Amengual et al., 2011; Lobo et al., 2010b). In brief, proteins (50–100 μg) were fractionated on 10–12% SDS-PAGE gels using the Bio-Rad Minigel system and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). Membranes were blocked in fat-free milk (5%) dissolved in Tris buffered saline-Tween, washed and incubated overnight at 4°C with anti-ISX (C-16; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:1000 or anti-mouse PPARγ (Abcam, Cambridge, MA, USA) diluted 1:500. As a protein loading control, β-actin (Cell Signalling, Boston, MA, USA) diluted 1:1000 was used. Secondary antibody (1:5000) was horseradish peroxidase-conjugated anti-rabbit IgG (Promega, Madison, WI). Immunoblots were developed with the ECL system (Amersham Biosciences, Buckinghamshire, UK) and scanned. Quantification of bands was performed by the ImageJ.

Measuring blood parameters

Serum glucose levels were measured using Accu-Chek Aviva (Roche, Barcelona, Spain). Serum insulin, leptin and adiponectin were measured using commercial ELISA kits according to manufacturer's instructions (from, respectively, Mercodia AB, Uppsala, Sweden; R&D Systems, Minneapolis, MS, USA; and Phoenix Europe GmbH, Karlsruhe, Germany).

Immunohistochemistry

Specimens of iWAT lobules were fixed by immersion in 4% paraformaldehyde in 0.1M sodium phosphate buffer (PB), pH 7.4. After washing in PB overnight, the samples were dehydrated in a graded series of ethanol and embedded in paraffin blocks for light microscopy and immunohistochemistry. For immunohistochemistry of proliferating cell nuclear antigen (PCNA), 5 μm sections were immunostained by means of the avidin-biotin technique (Hsu et al., 1981) using a commercial anti-PCNA primary antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA), counterstained with hematoxylin and mounted in Eukitt (Kindler, Freiburg, Germany). Analysis was performed by digital acquisition of adipose tissue areas using Axioskop 2 microscope equipped with AxioCam ICc3 digital camera and cell counting with the assistance of Axio Vision software (Carl Zeiss Microscopy GmbH, Jena, Germany).

Statistical analysis

Data are expressed as means ± SEM. Statistical significance was assessed by two-tailed Student's *t*-test. Results were considered as statistically significant when $P < 0.05$. The analyses were performed with SPSS 19.0 for windows (Chicago, IL, USA).

RESULTS

Orally given BC is readily absorbed intact by suckling rats

We first studied whether a moderate dose of exogenous BC could be readily absorbed and metabolized by the suckling rat pups by performing HPLC analysis of retinoids and carotenoids in blood and tissues. Analyses were conducted in serum, liver and iWAT of 21-day-old rats treated from day 1 to day 20 of life with BC, vehicle or RE. Serum and liver levels of BC were significantly increased in the BC-treated group compared to the control and RE-treated groups, in which BC could not be detected (or, if detected, could not be quantified owing to low levels) (Figure 1A-B). In the iWAT, BC was not detectable even in the BC-treated group (Figure 1C). Serum retinol levels were unaffected by vitamin A treatments reflecting vitamin A homeostasis (Figure 1A). Additionally, vitamin A treated 21-day-old pups, especially those treated with RE, had significantly increased RE and free retinol levels in the liver (Figure 1B), as well as total retinol (free retinol plus RE) levels in iWAT (Figure 1C). These data suggest that suckling rats are able to absorb exogenous BC but only able to partially metabolize it to

retinoids, so that a significant part is stored as intact BC in the liver, and perhaps other tissues but not in WAT.
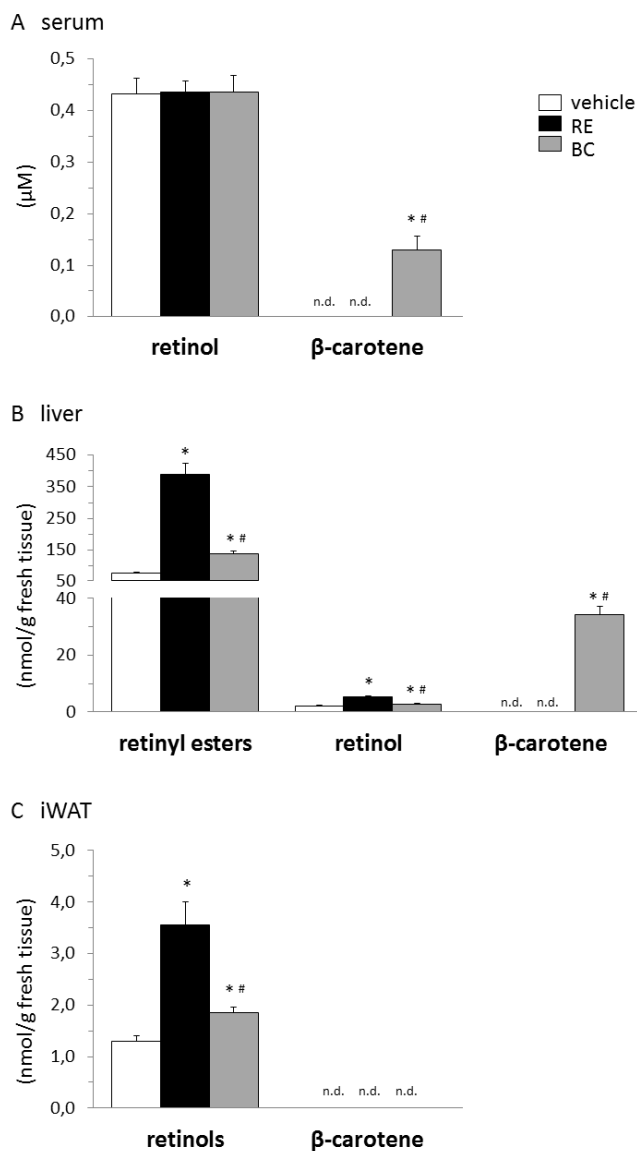
Figure 1. Retinoid and carotenoid levels in serum **(A)**, liver **(B)** and inguinal white adipose tissue **(iWAT, C)** of 21-day-old male rats treated during the suckling period (days 1–20 of life) with vehicle (controls, white bars) or a moderate dose of vitamin A as retinyl palmitate (RE, black bars) or as β-carotene (BC, grey bars). Data represent the mean ± SEM of 8 animals per group. *, indicates a significant ($p<0.05$) difference between vitamin A-treated groups and the control group and #, indicates a significant ($p<0.05$) difference between the BC-treated group and the RE-treated group in two-tailed Student's t test. Retinols, total retinol (retinyl esters plus free retinol); n.d., not detected.

BC supplementation during the suckling period does not affect adipose tissue development in young rats, in contrast to RE supplementation

Molecular and histological analysis of iWAT performed at the end of the supplementation period, on day 21, revealed differences dependent on the source of vitamin A received. Thus, a vitamin A activity equivalent supplementation with BC did

Results and Discussion
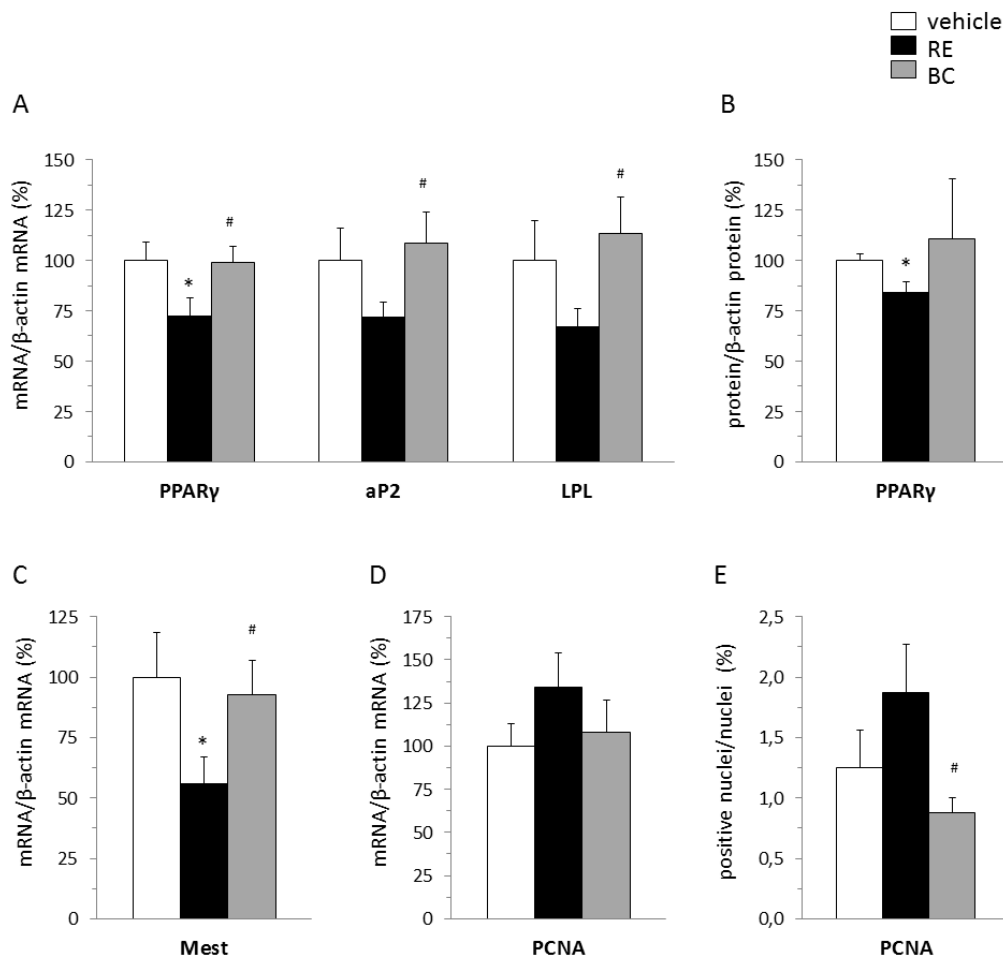
Figure 2. Expression levels of adipogenic and proliferative status-related genes in iWAT of 21-day-old rats treated during the suckling period (days 1–20 of life) with vehicle (controls, white bars) or a moderate dose of vitamin A as retinyl palmitate (RE, black bars) or as β-carotene (BC, grey bars): **(A)** mRNA expression levels of adipogenic genes PPARγ, aP2 and LPL; **(B)** protein levels of PPARγ; **(C)** mRNA expression levels of Mest; **(D)** mRNA expression levels of PCNA; and **(E)** percentage of PCNA positive nuclei. Gene expression data (A to D) are expressed relative to the mean value in vehicle-treated rats, which was set at 100%, and are the mean ± SEM of 10-16 (male and female) animals per group for mRNA data and 4 (male) animals per group for protein data. For PCNA immunostaining, iWAT sections of 8 animal per group (male and female) were used and ~200 cells per animal were counted. *, indicates a significant (p<0.05) difference between vitamin A-treated groups and the control group and #, indicates a significant (p<0.05) difference between the BC-treated group and the RE-treated group in two-tailed Student's t test.

not reproduce the previously observed effects of RE supplementation during the suckling period in iWAT of young rats (Granados et al., 2013). BC-treated rats displayed a similar expression of PPARγ in iWAT to control rats and higher than RE-treated rats, evident at both the mRNA and the protein level (Figure 2A-B). Gene expression in iWAT of other adipogenic, PPARγ downstream targets, such as adipocyte

Protein 2 (aP2) and lipoprotein lipase (LPL) was also similar in control and BC rats, and higher in the latter than in RE rats (Figure 2A). Moreover, immunohistochemical analysis of iWAT sections showed less PCNA positive nuclei in the BC-treated rats than in the RE-treated rats, and a similar percentage of PCNA positive nuclei in the BC and control animals (Figure 2E and 3, representative microphotographies), and a similar tendency was observed for iWAT PCNA mRNA levels (Figure 2D). In addition, BC-treated rats, like control rats, had larger adipocytes in iWAT compared with RE-treated rats (Figure 3). In keeping with the histological observations, expression in iWAT of mesoderm-specific transcript (Mest), a proposed marker of adipocyte size (Takahashi et al., 2005), was similar in control and BC rats and higher in BC rats as compared to RE rats (Figure 2C). As shown in Table 1, there were no differences between BC rats and either control or RE rats in body weight, adiposity (measured by ECHO-MRI analysis or direct weight of WAT depots dissected), and serum insulin, leptin and adiponectin.

Table 1. Body weight, fat and lean mass, WAT depots and liver weights and serum parameters of 21-day-old rats treated during the suckling period (days 1–20 of life) with vehicle (controls) or a moderate dose of vitamin A as retinyl palmitate (RE) or as β-carotene (BC).

	Control			RE			BC		
body weight (g) [a]	46.0	±	0.7	45.4	±	0.7	46.4	±	0.9
fat mass (g/100g BW) [b]	10.3	±	0.3	10.3	±	0.2	10.5	±	0.3
lean mass (g/100g BW) [b]	86.3	±	0.4	86.3	±	0.3	85.8	±	0.4
iWAT weight (mg)	578	±	23	574	±	24	641	±	36
rWAT weight (mg)	69	±	6	67	±	5	69	±	3
gWAT weight (mg)	122	±	8	118	±	7	125	±	9
liver weight (g)	1.80	±	0.05	1.69	±	0.04	1.74	±	0.04
serum glucose (mg/dL)	120	±	3.5	118	±	3.8	115	±	3.1
serum insulin (ng/mL)	0.297	±	0.005	0.296	±	0.007	0.299	±	0.006
serum leptin (ng/mL)	1.115	±	0.120	0.932	±	0.077	0.910	±	0.071
serum adiponectin (µg/mL)	9.32	±	1.19	9.74	±	1.08	11.42	±	1.78

[a], Data represent the mean ± SEM of 10-16 (male and female) animals per group.

[b], Fat and lean body mass were determined just before sacrifice using an Echo MRI-TM wall body composition analyzer. Data are expressed relative to the body weight (BW).
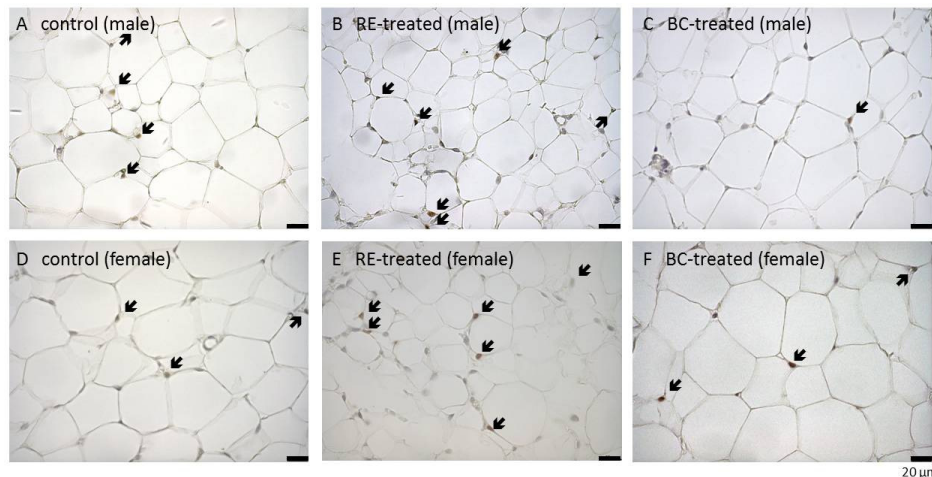
Figure 3. Representative microphotographies illustrating adipocyte size and PCNA immunostaining in iWAT of 21-day-old rats treated during the suckling period (days 1–20 of life) with vehicle **(A, D)** or a moderate dose of vitamin A as retinyl palmitate **(B, E)** or as β-carotene **(C, F)**. Male (A-C) and female (D-F) animals were analysed. Arrows indicate PCNA positivity.

BC supplementation during the suckling period leads to increased RA-mediated responses in intestine and liver, but not in iWAT of young rats, in contrast to RE supplementation

Control and vitamin A (as RE or BC)-treated rats were compared for the expression in selected tissues of known RA target genes, in particular intestine specific homeobox (ISX), carnitine palmitoyltransferase 1-liver isoform (CPT1-L), BCMO1, and CYP26a1 (Figure 4). ISX is an intestine-specific transcription factor induced by RA via its nuclear receptors that participates in vitamin A homeostasis by limiting intestinal BC absorption and conversion to vitamin A through its effects on gene expression, such as the repression of the BCMO1 gene (Lobo et al., 2010b; Seino et al., 2008). CPT1-L is the rate-limiting enzyme in mitochondrial fatty acid oxidation, shown to be induced by RA in hepatic cells through retinoid X receptor (RXR)-mediated mechanisms (Amengual et al., 2012). CYP26a1 is a RA hydroxylase that is transcriptionally induced by RA in a retinoic acid receptor (RAR)-dependent manner (Ozpolat et al., 2005). Intestinal ISX mRNA and protein levels were significantly higher in the two vitamin A-treated groups compared to the control group (Figure 4A-B). In accordance, the expression of the ISX target gene BCMO1 was found significantly reduced in the intestine of both vitamin A-treated groups (RE and BC) (Figure 4A). These results strongly suggest that the diet-responsive regulatory network that controls intestinal BC absorption and metabolism is
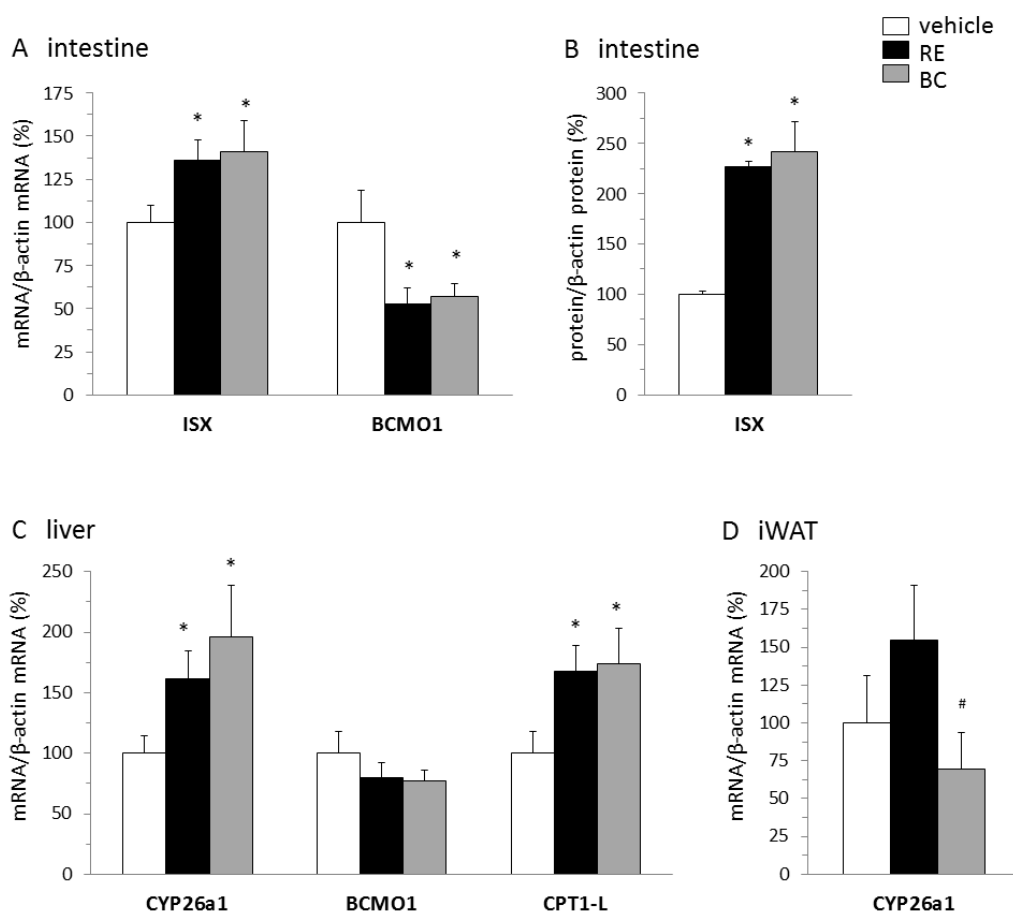
Figure 4. Expression levels of retinoid-sensitive genes in 21-day-old rats treated during the suckling period (days 1–20 of life) with vehicle (controls, white bars) or a moderate dose of vitamin A as retinyl palmitate (RE, black bars) or as β-carotene (BC, grey bars): **(A)** mRNA expression levels of ISX and BCMO1 in intestine; **(B)** protein levels of ISX in intestine; **(C)** mRNA expression levels of CYP26a1, BCMO1, and CPT1-L in the liver; and **(D)** mRNA expression levels of CYP26a1 in the iWAT. Gene expression data are expressed relative to the mean value in vehicle-treated rats, which was set at 100%, and are the mean ± SEM of 10-16 (male and female) animals per group for mRNA data and 4 (male) animals per group for protein data. *, indicates a significant ($p<0.05$) difference between vitamin A-treated groups and the control group and #, indicates a significant ($p<0.05$) difference between the BC-treated group and the RE-treated group in two-tailed Student's t test.

functional, at least in part, in 21-day-old rats. CYP26a1 and CPT1-L mRNA levels in liver were also significantly higher in the two vitamin A-treated groups compared to the control group (Figure 4C). In contrast, CYP26a1 mRNA levels in iWAT showed a differential response depending on the source of vitamin A: they were unaffected in the BC rats and tended to be increased in the RE rats. In fact, CYP26a1 mRNA levels were significantly lower in iWAT of BC-treated rats compared to RE-treated rats (Figure

4D). No consistent BCMO1 expression could be detected in iWAT of our animals. Gene expression of scavenger receptor B type 1 (SR-B1) and cluster determinant 36 (CD36), two proteins involved in the cellular uptake of provitamin A carotenoids (Reboul and Borel, 2011), was also measured in liver and iWAT. The results showed that there were no differences between the control and the two vitamin A groups and, in accordance to (Sy et al., 2012), that CD36 was more expressed than SR-B1 in iWAT and SR-B1 was more expressed than CD36 in the liver (data not shown). Together with results in Figure 2, the data indicate that, under the conditions used, vitamin A treatment in the form of BC triggered increased RA-mediated transcriptional responses in intestine and liver but not in iWAT of young (day 21) rats.

DISCUSSION

Nutritional conditions at critical stages in early life affect the susceptibility to chronic diseases including obesity in adulthood through, among others, mechanisms involving effects on the development of anatomical structures crucial to the control of energy balance and storage, such as adipose depots (Palou et al., 2013; Pico and Palou, 2013). Recent findings suggest that a moderate dose of vitamin A as RE can programme adipose tissue features in early life and condition fat expansion in the long-term (Granados et al., 2013). Furthermore, the provitamin A BC has been implicated as a dietary regulator of body fat reserves in adult animals including rodents and ferrets (Amengual et al., 2011; Lobo et al., 2010a; Murano et al., 2005). This background prompted us to study the effects of oral treatment with a moderate dose of vitamin A as BC during the suckling period on adipose tissue development in young rats at weaning. Our results are first to show, to our knowledge, that oral BC at a moderate dose can be readily absorbed and partially metabolized by suckling rats. In contrast to RE treatment, treatment with a vitamin A activity equivalent BC dose during the suckling period did not affect adipose tissue development in young rats. This allows suggesting that young rodents can be used as experimental models to study the effects of oral intake of BC, including moderate intakes of this provitamin A, and is in contrast with the lack of utility of adult rat/mice models that, contrary to what happens in humans, are unable to absorb intact BC (Keijer et al., 2005; Lee et al., 1999).

Rats cleave almost all ingested BC in the intestine producing REs that are incorporated into nascent chylomicrons and distributed to tissues (Lee et al., 1999). In accordance, we found increased levels of retinoids in liver and adipose tissue of BC-treated rats. In addition, our results provide evidence that the ISX gatekeeper that controls intestinal BC absorption and vitamin A production from BC works at the time of weaning (day 21). Interestingly, not only retinoid levels in tissues but also serum and liver levels of BC were significantly increased in rat pups treated with the moderate dose of BC used in our experiment (~2.5 mg/kg body weight). This result is in contrast with early reports that rodents accumulate carotenoids in tissues only when provided in the diet at supraphysiologic levels (Lee et al., 1999; Shapiro et al., 1984). Rodents are born at an intestinal immature stage, so that at least some of the brush border membrane enzymatic maturation occurs after birth (Drozdowski et al., 2010). This maturation process changes intestinal morphology and function and serves to prepare the pups for early feeding on high-fat milk, and then weaning onto lower fat- and higher carbohydrate-containing solid foods (Drozdowski et al., 2010). In particular, it has been described that the activity and gene expression of BCMO1 is induced in rat small intestine during the suckling-weaning transition period, between days 13 and 27 after birth (Mochizuki et al., 2012), probably to prepare the animals for BC absorption and metabolization from solid food, since rat milk contained no measurable levels of carotene (Houston and Kon, 1939). Thus, lack of the full complement in BCMO1 intestinal activity could explain that 21-day-old pups were able to metabolize only a fraction of the absorbed BC and stored the rest of it in intact form in tissues such as the liver (but not iWAT).

Early RE treatment affected adipose tissue development in young rats likely through RA-dependent effects (Granados et al., 2013). Moreover, several studies have addressed the impact of BC as a critical physiological precursor for RA production on body adiposity in adult animals, pointing to an antiobesity action (Amengual et al., 2011; Lobo et al., 2010a). In contrast, in the present study a three-fold excess vitamin A intake as BC during early life (day 1 to day 20 of life) did not elicit changes in the developing iWAT tissue. In particular, iWAT of BC-treated rats at weaning resembled that of control rats and was made out of larger adipocytes with an increased expression of PPARγ and PPARγ target adipocyte marker genes and a reduced expression of PCNA compared to iWAT of RE-treated rats. The reason(s) for this difference could

relate to concentration-dependent effects, as suggested by several observations. First, while early vitamin A treatment as RE led to an important (~3-fold) increase in total retinol in iWAT, only a moderate (40%) increase in this parameter was observed in iWAT of BC-treated rats, which probably was not high enough to sustain a noticeable effect (e.g., on RA production). Second, no measurable levels of BC were found in iWAT of BC-fed rats. This is in line with previous findings in older rats given supraphysiological doses of BC which showed lack of BC deposition in perirenal fat (Shapiro et al., 1984) or preferential accumulation of BC in the liver rather than in WAT (Sy et al., 2012). Preferential liver accumulation was attributed to BC incorporation into blood vesicles that have affinity for SR-B1, which was more strongly expressed in the liver that in WAT (Sy et al., 2012). Finally, expression of BCMO1 could not be detected by RT-qPCR in iWAT of our young rats (day 21). Considering that BCMO1 expression is induced during adipogenesis (Lobo et al., 2010a) and that WAT maturation is post-natal in rodents (Cryer and Jones, 1979), it is likely that the adipocytes of pups, like small intestine cells (Mochizuki et al., 2012), still had not developed the full enzymatic machinery for the usage of BC. All in all, the data are consistent with the absence of known RA-mediated transcriptional responses in iWAT of BC rats at weaning (day 21), including the up-regulation of CYP26a1.

In summary, this work shows that, in suckling rats, a moderate (three-fold) excess vitamin A intake as BC was readily absorbed and partially metabolized and, unlike an equivalent excess vitamin A intake as RE, did not affect adipose tissue development. Studies dealing with the impact of specific micronutrients at critical periods in early life may serve to understand mechanisms of health/disease programming. In this context, our study establishes a new potential model for the experimental evaluation of early effects including epigenetics of moderate doses of BC, both as a retinoid precursor and *per se*, in tissues where BC effectively concentrates, such as the liver. The long term consequences of this efficient absorption of BC during lactation, in particular the effects in other tissues, as lungs have not been studied here, are not known but the model appears useful to approach the unexpected observations in large-scale intervention studies (Albanes et al., 1996; Omenn et al., 1996) showing that moderate intake of BC may enhance lung cancer in smokers or asbestos exposed humans.

Conflict of interest: Authors have no competing financial interests in relation to the work described.

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Chapter 3.4

RETINOIC ACID INCREASES FATTY ACID OXIDATION AND FNDC5/IRISIN EXPRESSION IN SKELETAL MUSCLE CELLS

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Manuscript to be submitted

Chapter 3.4

RETINOIC ACID INCREASES FATTY ACID OXIDATION AND FNDC5/IRISIN EXPRESSION IN SKELETAL MUSCLE CELLS

ABSTRACT

All-trans retinoic acid (ATRA), the carboxylic form of vitamin A, is a gene expression modulator that impacts on adipokine production, and on fatty acid oxidation and energy metabolism in tissues including adipose tissues and skeletal muscle. The objective of this study was to investigate the direct effects of ATRA on lipid and glucose metabolism and the production of selected myokines in skeletal muscle cells. In fully differentiated C2C12 myocytes, exposure to ATRA increased fatty acid oxidation rate, reduced intracellular triacylglycerol content and enhanced the capabilities for mitochondrial fatty acid uptake, oxidation and intracellular mobilization. Together with these results, ATRA also induced FNDC5/irisin, a novel myokine involved in the browning of white adipose tissue, at both the mRNA and secreted protein level. Finally, and in accordance with the in vitro results, wild-type mice treated with ATRA showed higher FNDC5/irisin serum levels when compared to vehicle-treated mice. These results support the hypothesis that effects in skeletal muscle contribute to the anti-obesity and anti-diabetic action of ATRA. Knowledge of dual agents capable of enhancing oxidative metabolism in skeletal muscle and modulating myokine production may contribute to new avenues in the management of obesity, insulin resistance and related disorders.

Keywords: vitamin A; body weight; adiposity; intramyocellular lipids; myokines.

INTRODUCTION

Skeletal muscle accounts for ~40% of total body weight and 50% of total energy expenditure in mammals, and it is a highly adaptable tissue that plays a key role in whole body glucose and lipid metabolism (Matsakas and Patel, 2009). Alterations in skeletal muscle metabolism are involved in insulin resistance, the metabolic syndrome, and type 2 diabetes (Perseghin, 2005). Moreover, skeletal muscle and other metabolic tissues dynamically secrete signaling factors depending on metabolic state which allow communication between them and the orchestration of systemic functions, adaptation and homeostasis (Yoon et al., 2012). Signaling proteins produced and released by muscle fibers, collectively named myokines, exert their effects locally within the muscle as well as in a hormone-like fashion in distant organs of the body, such as adipose tissue, liver, pancreas, bones and brain, playing important roles in biological processes such as energy metabolism, angiogenesis and myogenesis (Pedersen and Febbraio, 2012).

Retinoic acid, the carboxylic acid form of vitamin A, is a nutrient derivative with many remarkable effects on lipid and energy metabolism (Bonet et al., 2003; Bonet et al., 2012; Brun et al., 2013). Previous studies have shown that treatment with all-trans retinoic acid (ATRA) reduces body weight and adiposity independently of changes in food intake and improves insulin sensitivity and glucose tolerance in lean and obese mice (Berry and Noy, 2009; Bonet et al., 2000; Felipe et al., 2004; Felipe et al., 2005; Mercader et al., 2008; Mercader et al., 2006; Puigserver et al., 1996; Ribot et al., 2001). ATRA-induced body fat loss correlates with activation of brown adipose tissue (BAT) (Bonet et al., 2000; Puigserver et al., 1996), and increased capabilities for fatty acid oxidation, oxidative metabolism and thermogenesis in tissues including white adipose tissue (WAT) (Berry and Noy, 2009; Mercader et al., 2006), liver (Amengual et al., 2010) and skeletal muscle (Amengual et al., 2008; Berry and Noy, 2009). Many biological activities of ATRA are mediated by the ligand-activated transcription factors termed retinoic acid receptors (RARs). ATRA can also bind to and activate the nuclear receptor peroxisome proliferator-activated receptors β/δ (PPARβ/δ), be converted into 9-*cis* retinoic acid activating various nuclear receptor heterodimers containing the retinoid X receptor (RXR), and exert non-genomic actions (Bonet et al., 2012).

The above background, together with the demonstration of direct action of ATRA in white adipocytes (Mercader et al., 2007) and hepatic cells (Amengual et al.,

2012) in culture, led us to hypothesize that ATRA could also exert cell-autonomous effects favoring fatty acid oxidation in skeletal muscle cells. We hypothesized that interactions of ATRA with myokine production could contribute to ATRA biological effects on weight loss and systemic functions. These aspects have been addressed herein using ATRA-treated C2C12 myocytes and intact mice as models.

MATERIALS AND METHODS

Cell culture, differentiation and ATRA treatment.

Mouse C2C12 myoblasts (ATCC-LGC Promochem, Barcelona, Spain) were cultured in Dulbecco's modified Eagle's medium with 25 mM glucose (DMEM) supplemented with 10% w/v fetal bovine serum, antibiotics (50 IU penicillin/mL and 50 µg streptomycin/mL) and 3 mM glutamine; referred to as growth media (GM). Cells were maintained at 37°C in a saturated humidity atmosphere containing 5% CO_2. To induce differentiation, C2C12 myoblasts were platted at an initial density of 1.2×10^5 cells/well in 12-well culture dishes, which allowed reaching 80% confluency after 24 h; then, GM was replaced by DMEM supplemented with antibiotics and 2% w/v horse serum; referred to as differentiation media (DM). DM was replaced every two days until complete differentiation (10 days), when cells were fused into differentiated myocytes (myotubes). Unless otherwise indicated, differentiated myocytes were exposed during the 24 h before harvesting to a single dose of ATRA (Sigma, Madrid, Spain) at a final concentration of 0.1, 1 and 10 µM. ATRA was delivered dissolved in DMSO, at a final concentration of 0.1%. Control cells received the vehicle (DMSO) only. All experiments were performed at least twice in triplicate.

Animal study.

Twelve-week-old NMRI male mice (CRIFFA, Barcelona, Spain) fed *ad libitum* regular laboratory chow (Panlab, Barcelona, Spain; 73.4% carbohydrate-, 18.7% protein-, and 7.9% lipid-derived energy; 5 UI vitamin A/kcal) received one daily subcutaneous injection of ATRA at a dose of 50 mg/kg body weight during the 4 days before they were sacrificed (six animals per group). Controls were injected the vehicle (100 µl olive oil). The animals were kept at 22°C under 12-h light/12-h dark cycles (lights on at 08:00). Body weight and food intake during the treatment period were followed daily on a per-cage basis (three animals per cage). The ATRA-treated animals did not show any

external signs of vitamin A toxicity or significant liver enlargement at sacrifice, as in previous work using similar experimental design (Amengual et al., 2008; Felipe et al., 2004; Mercader et al., 2006). The animals were sacrificed by decapitation at the start of the light cycle. Blood was collected and serum prepared by centrifugation and frozen at -20°C. Tissues including gastrocnemius muscle and epididymal, inguinal and retroperitoneal WAT were excised in their entirety, weighted, snap-frozen in liquid nitrogen, and stored at -80°C. The sum of the weight of the individual WAT depots as percentage of body weight was used as adiposity index. All animal experiments were performed following the guidelines for the use and care of laboratory animals of the University of the Balearic Islands (UIB) and the protocols were submitted to, and approved by, the UIB institutional review board.

RNA isolation.

Total RNA was extracted from C2C12 myotubes and animal gastrocnemius muscle biopsies using Tripure Reagent (Roche, Barcelona, Spain) or EaZy Nucleic Acid Isolation Kit E.Z.N.A^{TM} (Omega Bio-Tek, Vermont, USA). Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (NadroDrop Technologies Inc., Wilmington, Delaware, USA) and its integrity confirmed using agarose gel electrophoresis.

Real-time quantitative PCR (RT-qPCR).

Real-time polymerase chain reaction was used to measure mRNA expression levels of lipoprotein lipase (LPL), fatty acid translocase (FAT/CD36), hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), PPAR β/δ, peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), uncoupling protein 3 (UCP3), muscle-type carnitine palmitoyltransferase 1 (CPT1-m), acyl CoA oxidase (ACOX1), pyruvate dehydrogenase kinase 4 (PDK4), insulin receptor (InsR), hexokinase, glucose transporter 4 (GLUT4), interleukin 6 (IL-6), fibroblast growth factor 21 (FGF21), fibronectin type III domain-containing 5 (FNDC5/Irisin), and ribosomic RNA 18S, the latter used as internal control. For retrotranscription to cDNA, 0.25 µg of total RNA (in a final volume of 5 µl) was denatured at 65°C for 10 min and then incubated with MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20ºC for 15 min, followed by 30 min at 42°C, and finally 5 min at 95°C, in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from

diluted (1/5) cDNA template, forward and reverse primers (1 μM each), and Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). Primers used for the different genes are detailed in Supplementary Table 1 and were obtained from Sigma (Madrid, Spain). Real time PCR was performed using the Applied Biosystems StepOnePlusTM Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95ºC, followed by a total of 40 two-temperature cycles (15 s at 95ºC and 1 min at 60ºC). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated according to (Pfaffl, 2001), based on the efficiency of each reaction and the crossing point deviation of each sample *versus* a control and expressed in comparison with a reference gene (18S rRNA or β-actin).

Fatty acid oxidation in intact cells.

Oxidation of uniformly (U) ^{14}C-labeled palmitate to CO_2 and acid-soluble products (ASPs) in cultured cells was measured as previously described (Amengual et al., 2012; Mercader et al., 2007), with minor modifications. Briefly, C2C12 myoblasts were differentiated as described above. Differentiated myocytes were treated for 22.5 h with 10 μM ATRA or vehicle (DMSO), after which the medium was removed and cells were further incubated at 37°C for 1.5 h in fresh medium containing 0.2 mM L-carnitine (Sigma, St. Louis, MO) and 200 μM [^{14}C(U)] palmitate (0.1 μCi/mL, from Perkin Elmer, Boston, MA), in the continued presence of ATRA or vehicle. Prior the 1.5 h incubation, each well was covered with a piece of Whatman paper and the multiwell plate was sealed with parafilm. Following the 1.5 h incubation, the Whatman paper was soaked with 0.1 mL of methylbenzylamine:methanol (1:1) to trap the CO_2 produced, and 0.2 mL of HCl 6 M was injected into the wells to release the CO_2 present in the liquid phase to the gaseous phase. After 1 h of CO_2 capturing at room temperature, the pieces of Whatman paper were removed and transferred to scintillation vials for radioactivity counting. ASPs in 1 mL of the culture medium were extracted from intact [^{14}C(U)] palmitate present in the media by addition of 0.5 mL cold 1.5 M $HClO_4$. After centrifugation (15 min, 1800×*g*), radioactivity in the supernatant was measured by scintillation counting. Protein concentration in cell lysates of extra parallel culture wells was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA).

2-deoxyglucose uptake.

Cellular uptake of 2-deoxy-D-glucose (2-DOG) was evaluated in C2C12 myocytes pre-exposed for 24 h to ATRA or vehicle (DMSO), essentially as described previously (Kumar and Dey, 2003). In brief, the cells were first washed with Krebs–Ringer phosphate buffer (10 mM-KH_2PO_4, pH 7.4, containing 136 mM-NaCl, 4.7 mM-KCl, 1.25 mM-$CaCl_2$, 1.25 mM-$MgSO_4$ and 0.05 % bovine serum albumin) and then incubated for 15 min at 37°C in Krebs–Ringer phosphate buffer without insulin or with 100 nM insulin. The transport assay was then initiated by the addition of ^{3}H-2-DOG (0.1 μCi/mL in 1 μM unlabelled 2-DOG), and the cells were further incubated for 10 min with or without 100 nM insulin at 37°C. Cells were then washed three times with ice-cold PBS and lysed with 0.1 M NaOH. Radioactivity in cell lysates was counted in Hi-safe 3 scintillant (Perkin Elmer, Shelton, CT, USA) using a Beckman Coulter LS 6500 multi-purpose liquid scintillation counter (Beckman Coulter, Brea, CA, USA). Protein concentration in the same cell lysates was measured using the BCA protein assay kit.

Quantification of myokines.

Irisin, IL-6 and FGF21 concentrations in conditioned culture media and mouse serum samples were measured using ELISA kits from Pierce (Rockford, IL, USA), R&D Systems Inc. (Minneapolis, MN, USA), and Phoenix Pharmaceuticcals Inc. (Burlingame, CA, USA), respectively. All procedures were performed following the manufacturer's instructions.

Other parameters analyzed.

Cell lysates were obtained in PBS and aliquots used to measure intracellular triacylglycerol content by means of an enzymatic colorimetric kit (Trygliceride INT20, Sigma, St. Louis, MO, USA). Commercial enzymatic colorimetric kits were used for the determination of serum nonesterified fatty acid (NEFA) (Wako Chemicals GmbH, Neuss, Germany) and 3-hydroxybutyrate (BEN Srl, Milan, Italy), according to the supplier's instructions.

Statistical analysis.

Data are expressed as means±SEM. Statistical significance was assessed by Student´s t-test or one-way ANOVA followed by least-significance difference (LSD) post-hoc comparison. Results were considered statistically significant when $P<0.05$. In the animal experiment, Pearson correlation analyses between selected parameters were

performed with pooled ATRA-treated and vehicle-treated animals. The analyses were performed with SPSS 19.0 for windows (Chicago, IL, USA).

RESULTS

ATRA activates fatty acid oxidation in differentiated C2C12 myocytes.

ATRA treatment increases lipid oxidation capacity in skeletal muscle of mice (Amengual et al., 2008; Berry and Noy, 2009). We used differentiated C2C12 myoblasts in culture as a model to ascertain whether this results from direct action of ATRA on skeletal muscle cells. For that, we analyzed the mRNA levels of key genes in fatty acid handling and oxidation in C2C12 differentiated myocytes exposed to varying ATRA doses (Figure 1). Exposure to ATRA resulted in a 1.4-fold increase in the mRNA levels of the muscle isoform of CPT1, already evidenced at the lowest dose tested (0.1 μM), and in dose-dependent increases in the mRNA levels of UCP3 and PDK4, by 3- and 3.5-fold, respectively, at the highest dose tested (10 μM). CPT1 is the rate-limiting enzyme for mitochondrial long-chain fatty acid (LCFA) uptake and β-oxidation (Zammit, 2008); the activity of UCP3 may facilitate fatty acid oxidation and protect skeletal muscle mitochondria against lipid-induced oxidative damage (Schrauwen et al., 2006); and the activity of PDK4 influences cellular fuel selection favoring switching from carbohydrate to lipid catabolism (Rodriguez et al., 2010). Exposure to ATRA also induced the gene expression of FAT/CD36 (by 1.8-fold, already at the lowest dose tested) – a membrane fatty acid translocase involved in the uptake of LCFA into the cell and the mitochondria (Holloway et al., 2008) – and of ATGL (up to 3-fold) – a lipase involved in the breakdown of intracellular triacylglycerols (Watt and Spriet, 2010). Both FAT/CD36 and ATGL have been implicated in the positive regulation of muscle fatty acid oxidation, through the provision of fatty acids serving as fuels and as PPAR-activating ligands (Holloway et al., 2008; Watt and Spriet, 2010). Gene expression of LPL, which is involved in the breakdown of extracellular triacylglycerol-rich lipoproteins, was decreased by 2.5-fold by ATRA treatment, already at the lowest dose tested. No significant changes were observed in HSL, PPARβ/δ, PGC1α and ACOX1 gene expression levels in the ATRA-exposed C2C12 myocytes relative to the control cells.
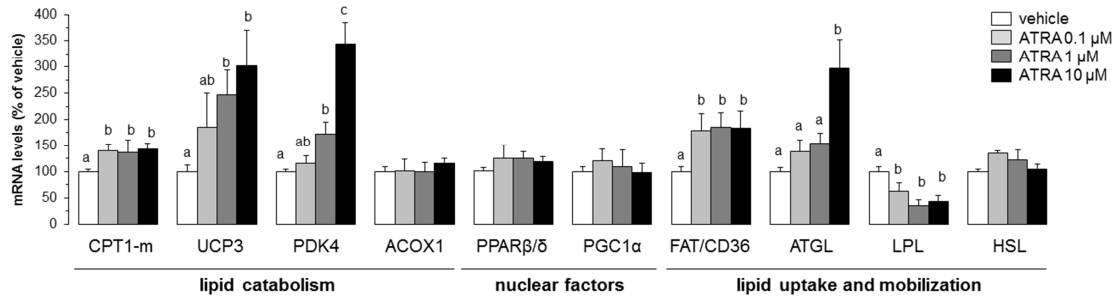
Figure 1. Effect of ATRA on the expression of lipid metabolism related genes in C2C12 myocytes. C2C12 myocytes on day 9 of differentiation were treated for 24 h with vehicle (DMSO) or ATRA at the indicated concentrations before harvesting for total RNA extraction. The expression of the indicated genes was analyzed by real-time PCR, and normalized to the expression of 18S rRNA. Data are expressed relative to the mean value in vehicle-treated cells, which was set to 100%, and are the mean±SEM of at least two independent cultures made in triplicate. Statistical significance was assessed by one-way ANOVA and LSD *post hoc* comparisons. For each gene, values not sharing a common letter were considered statistically different (*P*<0.05).
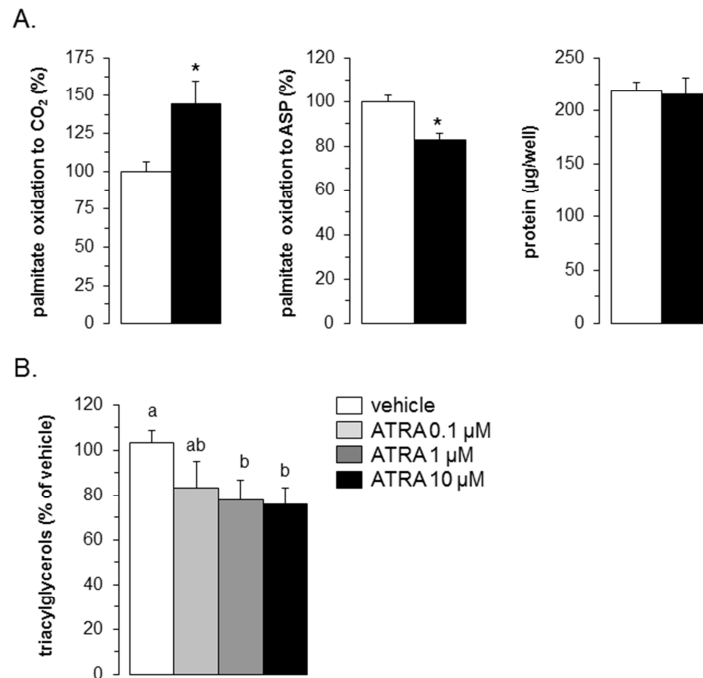
Figure 2. Effect of ATRA on fatty acid oxidation (A) and triacylglycerol content (B) in C2C12 myocytes. C2C12 myocytes on day 9 of differentiation were treated for 24 h with vehicle (DMSO) or ATRA as indicated. Palmitate oxidation, protein content and triacylglycerol content were measured as described in Materials and Methods. Data on palmitate oxidation to CO2 and ASPs (dpm/well) and on triacylglycerol content (µg/µg protein) are expressed relative to the mean value of the vehicle-treated cells, which was set to 100%, and are the mean±SEM of three (A) or two (B) independent cultures made in triplicate. Statistical significance was assessed by Student's t-test (A) or one-way ANOVA and LSD post hoc comparisons (B): *, P<0.05, ATRA-treated vs vehicle-treated cells; values in B not sharing a common letter were considered statistically different (P<0.05).

A.

B.
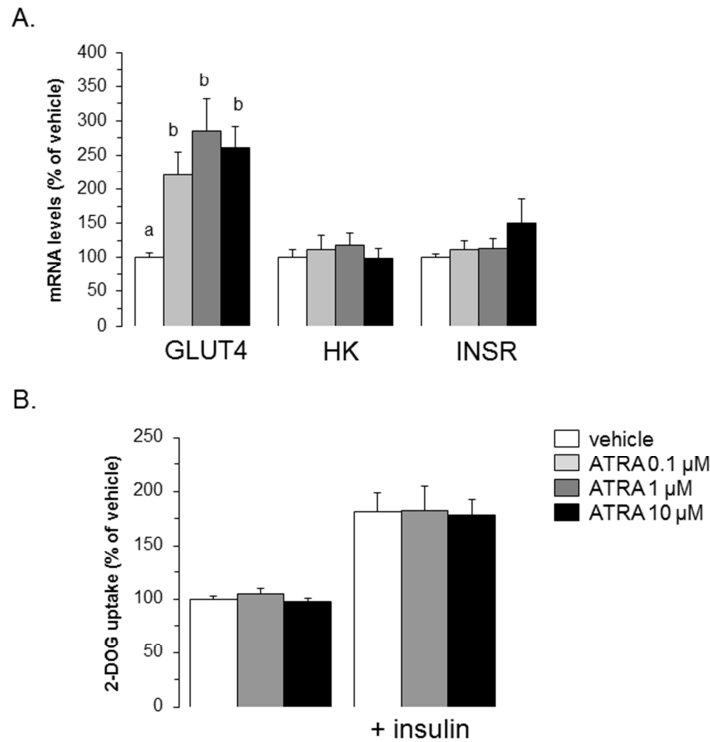
Figure 3. Effect of ATRA on the expression of glucose metabolism related genes (A) and glucose uptake (B) in C2C12 myocytes. C2C12 myocytes on day 9 of differentiation were treated for 24 h with vehicle (DMSO) or ATRA at the indicated concentrations. The expression of the indicated genes normalized to the expression of 18S rRNA and 2-deoxyglucose (2-DOG) uptake under basal and insulin-stimulated conditions were analyzed as described in Materials and Methods. Separate cultures were used for the two type of analysis. Data on 2-deoxyglucose uptake (dpm/µg protein) are expressed relative to the mean value in the vehicle-treated cells under basal conditions, which was set to 100%, and are the mean±SEM of three independent cultures made in triplicate. Gene expression data are expressed relative to the mean value in vehicle-treated cells and are the mean±SEM of at least two independent cultures made in triplicate. Statistical significance was assessed by one-way ANOVA and LSD *post hoc* comparisons: values not sharing a common letter were considered statistically different ($P<0.05$).

We next decided to analyze the functional consequences of the aforementioned changes in gene expression. For this purpose, we first studied the effects of ATRA on exogenous fatty acid oxidation rate (Figure 2A). Compared with control cells, C2C12 cells pre-exposed to 10 µM ATRA displayed a significant 44% increase in palmitate oxidation to CO_2 together with a significant 17% decrease in palmitate oxidation to ASPs, i.e. acyl-carnitines, Krebs cycle intermediates and acetyl-CoA (Veerkamp et al., 1986) (Figure 2A). Consequently, the ratio of incomplete (to ASPs) to complete (to CO_2) palmitate oxidation was lower in ATRA-treated cells than in control cells (0.57±0.04 *vs* 1.04±0.11, respectively, Student's *t*-test, $P<0.05$), indicating a more efficient mitochondrial fatty acid oxidation in the ATRA-treated cells. These changes in

palmitate oxidation took place in the absence of changes in the total amount of protein recovered per culture.

We also investigated whether ATRA interaction with lipid metabolism affected intramyocellular lipid accumulation by analyzing triacylglycerol content in ATRA-treated C2C12 myocytes. After 24h incubation, cells incubated with 1 and 10 µM ATRA showed a ~20% reduction of the intracellular triacylglycerol content when compared with vehicle-treated cells (Figure 2B).

ATRA induces GLUT4 gene expression and does not affect 2-deoxyglucose uptake in differentiated C2C12 myocytes.

We studied the interaction of ATRA with glucose metabolism by analyzing and comparing selected gene expression and glucose uptake in vehicle- and ATRA-treated C2C12 myocytes. Exposure to ATRA did not affect the gene expression of InsR and hexokinase, used as indicators of the cell's capabilities for insulin sensing and glycolysis, respectively, but resulted in a 3-fold up-regulation of GLUT4 mRNA levels (Figure 3A). Basal and insulin-stimulated 2-DOG uptake was, however, not affected by ATRA treatment under the conditions used (Figure 3B).

ATRA induces FNDC5/irisin in C2C12 myocytes and intact mice.

ATRA treatment reduces adiposity in mice through mechanisms that include the promotion of the acquisition of BAT-like features in WAT depots (the so-called *browning* of white fat) (Mercader et al., 2006) (see also the Introduction). Irisin (encoded by the FNDC5 gene) has recently been described as a metabolic regulator secreted by skeletal muscle capable of inducing WAT browning (Bostrom et al., 2012). This scenario prompted us to asses ATRA modulation of FNDC5/irisin as a myokine, in both cultured skeletal muscle cells and *in vivo*. Exposure of C2C12 myocytes to ATRA led to a dose-dependent up-regulation of FNDC5/irisin expression, which was evident both at the mRNA level (1.5-fold increase at 10 µM ATRA) and at the level of the irisin accumulated in the cell conditioned culture medium (Figure 4A). In mice, ATRA treatment triggered as expected a significant decrease in body weight that correlated with trends to reduced adiposity index, increased gastrocnemius muscle to body weight ratio, and increased serum levels of ketone bodies (Supplementary Figure 1), in keeping with a slimming action and an enhancement of whole body fatty acid catabolism. Remarkably, while the mRNA levels of FNDC5/irisin in the gastrocnemius muscle

were unaffected, serum irisin levels were 5-fold higher in the ATRA-treated mice than in the vehicle-treated mice (Figure 4B). A negative correlation was found between serum irisin levels and serum NEFA levels ($r = -0.801$, $P = 0.017$) (Supplementary Figure 2), in keeping with a role for irisin in promoting fatty acid catabolism in tissues.
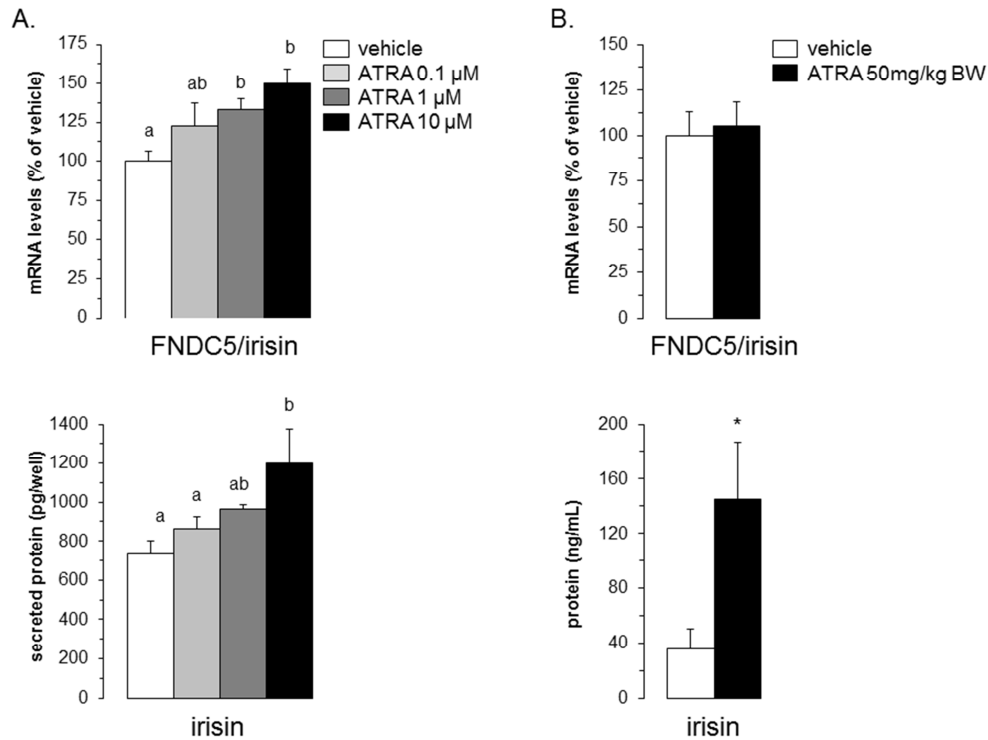
Figure 4. Effects of ATRA treatment on FNDC5/irisin expression in C2C12 myocytes (A) and intact mice (B). Gene expression of FNDC5/irisin (A, top panel) and irisin levels in the cell's conditioned culture medium (A, bottom panel) were analyzed in C2C12 myocytes on day 9 of differentiation following 24 h treatment with vehicle (DMSO) or ATRA at the indicated concentrations. Expression of FNDC5/irisin in gastrocnemius muscle (B, top panel) and serum irisin levels (B, bottom panel) were analyzed in twelve-week-old NMRI male mice following treatment with ATRA (50 mg/kg body weight per day during 4 days) or vehicle (olive oil). See Materials and Methods for further details on treatments and analysis. Data in A are the mean±SEM of at least two independent cultures made in triplicate; data in B are the mean±SEM of at least 5 animals per group. Gene expression data are expressed relative to the mean value in vehicle-treated cells/mice, which was set to 100%. Statistical significance was assessed by Student's *t*-test or one-way ANOVA followed by LSD *post hoc* comparisons; *, $P < 0.05$, ATRA-treated *vs* vehicle-treated; values not sharing a common letter were considered statistically different ($P < 0.05$).

Similar to irisin, muscle-derived FGF21 can induce WAT *browning* (Kim et al., 2013). Moreover, RAR-mediated induction of FGF21 gene expression has been recently demonstrated in liver cells (Li et al., 2013), prompting us to study the impact of ATRA on the production of FGF21 as a myokine. FGF21 mRNA levels were equal in C2C12 myocytes treated with vehicle or 10 μM ATRA, but higher in the 10 μM ATRA-

treated cells than in the cells treated with lower ATRA doses (Figure 5A, top). FGF21 protein levels in the cell's conditioned culture medium were higher for 10 µM ATRA-treated cells than for cells treated with either vehicle or lower ATRA doses (Figure 5A, bottom). Additionally, a 4-fold increase in FGF21 mRNA levels relative to vehicle-treated cells was found in C2C12 myocytes treated with 100 µM ATRA (a dose not routinely used in our experiments) (Student's *t*-test, $P<0.005$, n=3 cultures per treatment condition). However, serum levels of FGF21 remained unaffected following ATRA treatment of mice, and a tendency towards higher FGF21 mRNA levels in the gastrocnemius muscle of the ATRA-treated mice did not reach statistical significance (P=0.089)(Figure 5B).
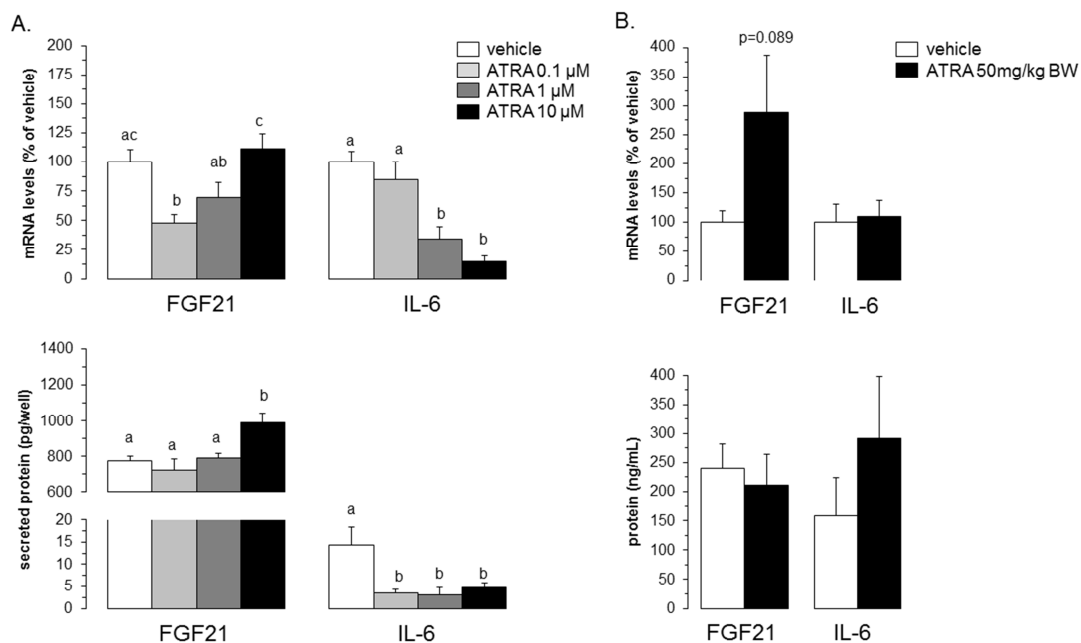
Figure 5. Effects of ATRA on FGF21 and IL-6 expression in C2C12 myocytes (A) and intact mice (B). Gene expression of FGF21 and IL-6 (A, top panel) and FGF21 and IL-6 levels in the cell's conditioned culture medium (A, bottom panel) were analyzed in C2C12 myocytes on day 9 of differentiation following 24 h treatment with vehicle (DMSO) or ATRA at the indicated concentrations. Expression of FGF21 and IL-6 in gastrocnemius muscle (B, top panel) and serum FGF21 and IL-6 levels (B, bottom panel) were analyzed in twelve-week-old NMRI male mice following treatment with ATRA (50 mg/kg body weight per day during 4 days) or vehicle (olive oil). See Materials and Methods for further details on treatments and analysis. Data in A are the mean±SEM of at least two independent cultures made in triplicate; data in B are the mean±SEM of at least 5 animals per group. Gene expression data are expressed relative to the mean value in vehicle-treated cells/mice, which was set to 100%. Statistical significance was assessed by Student's *t*-test or one-way ANOVA followed by LSD *post hoc* comparisons; *, $P<0.05$, ATRA-treated *vs* vehicle-treated; values not sharing a common letter were considered statistically different ($P<0.05$).

Finally, we analyzed ATRA effects on the production of IL-6, one of the most studied myokines involved in the control of insulin sensitivity, fat metabolism and

energy balance (Hoene and Weigert, 2008; Pedersen and Febbraio, 2008). Our results revealed a dramatic down-regulation of IL-6 gene expression and secreted protein levels in ATRA-treated C2C12 myocytes (Figure 5A, top), but no effects of ATRA treatment *in vivo* on gene expression of IL-6 in skeletal muscle or serum levels of IL-6 (Figure 5B, bottom).

DISCUSSION

Here we analyzed the effects of ATRA, the main transcriptionally active form of vitamin A, in skeletal muscle using cell culture and *in vivo* models. We show in cell culture that this compound induces the mRNA expression of many genes involved in lipid oxidation. These changes on gene expression are accompanied by a decrease of intracellular lipid content and an increase of exogenous fatty acid consumption. Finally we demonstrate for the first time that this nutrient derivative increases both the mRNA and protein level expression of FNDC5/irisin, a novel myokine for which there is evidence of anti-obesity and anti-diabetic action (Bostrom et al., 2012). The implications of these findings are discussed below.

ATRA induces fatty acid oxidation in C2C12 myocytes– Vitamin A is a vital nutrient involved in the control of many biological processes including, but not limited to vision, embryo development, immune system, adipose tissue differentiation and metabolism (Brun et al., 2013; von Lintig, 2012). Vitamin A impacts on gene expression in its carboxylic form ATRA, affecting mRNA levels of numerous genes. Previous results in our laboratory and others showed that ATRA treatment in mice reduces body weight and body fat by favouring fatty acid oxidation in brown and white adipose tissues (Berry and Noy, 2009; Bonet et al., 2000; Mercader et al., 2006; Puigserver et al., 1996). These results were fully supported by cell culture studies in adipocytes using either ATRA or its naturally occurring precursor β-carotene (Lobo et al., 2010; Mercader et al., 2007; Serra et al., 1999). Besides its effects on adipose tissue, ATRA treatment induced similar changes in other metabolically important tissues such as the liver and the skeletal muscle *in vivo* (Amengual et al., 2008, 2010; Berry and Noy, 2009), most likely also through direct effects, since ATRA was shown to enhance fatty acid catabolism in hepatic cells in culture (Amengual et al., 2012). However, evidence for direct, cell-autonomous effects of ATRA favouring fatty acid catabolism in skeletal

muscle cells was lacking. This evidence is provided here, supported by both gene expression results revealing the induction of a wide battery of genes involved in fatty acid uptake, intracellular mobilization, and oxidation, and functional studies indicating a decrease of intracellular triacylglycerol content and an increase of exogenously administrated palmitate consumption in C2C12 myocytes following exposure to ATRA.

ATRA does not impair glucose metabolism in C2C12 myocytes– The control of lipid and glucose metabolism is closely linked, and, moreover, previous studies in muscle cell models indicated that ATRA can affect glucose uptake and metabolism (Lee et al., 2008; Montessuit et al., 2006; Montessuit et al., 2008). Therefore, we measured mRNA levels of proteins involved in insulin-mediated glucose uptake and handling in ATRA-exposed C2C12 myocytes. In concordance with previous results in ATRA-treated cardiac myocytes (Montessuit et al., 2006), we found a strong induction (up to 3-fold) of the insulin-regulated glucose transporter GLUT4 gene, which however was not accompanied by an increase in either basal or insulin-stimulated uptake of 2-deoxyglucose, a non-metabolizable glucose analogue widely used in measurements of cellular glucose uptake. Differences between our results and those of Lee et al. (2008) (Lee et al., 2008) – who reported increased 2-deoxyglucose uptake in C2C12 myocytes following short exposure to ATRA – may relate to differences in the duration of the ATRA treatment, much longer in our case (24 h *vs* 1 h), and are suggestive of time-course, sequential effects of ATRA on glucose and lipid metabolism in skeletal muscle cells. In any case, the fact is to be highlighted that ATRA-induced increases in lipid catabolism in skeletal myocytes appear dissociated from impairments in cellular glucose uptake and metabolism.

ATRA affects myokine production– Along with its effects on lipid and energy metabolism, previous studies demonstrated that ATRA can influence the secretion of different adipokines involved in the control of energy balance and insulin sensitivity such as leptin, resistin, and retinol binding protein (Felipe et al., 2004; Felipe et al., 2005; Hollung et al., 2004; Mercader et al., 2008). Hence, we aimed to study the impact of ATRA on the expression and secretion of specific myokines selected on the basis of their anti-adiposity and insulin-sensitizing potential. We describe for the first time a stimulatory effect of ATRA on FNDC5/irisin expression, both in cell culture and *in vivo* systems. Induction of irisin as an endocrine factor could contribute to the insulin sensitizing effect and the promotion of mitochondrial oxidative metabolism in WAT

brought about by ATRA treatment *in vivo* (Mercader et al., 2006), since irisin has been characterized as a potent inducer of WAT browning with anti-diabetic potential (Bostrom et al., 2012; Moreno-Navarrete et al., 2013). Irisin was originally discovered as a PGC1α-dependent myokine (Bostrom et al., 2012), yet induction of FNDC5/irisin in our models was dissociated from changes in PGC1α gene expression (this work and (Amengual et al., 2008)), although ATRA-dependent increases in PGC1α activity cannot be discarded. Remarkably, the ATRA-dependent induction of FNDC5/irisin was more evident (in C2C12 myocytes) or evident only (in intact mice) at the secreted protein than the gene expression level, suggesting a post-transcriptional control.

Similar to FNDC5/irisin, muscle-derived FGF21 can induce WAT *browning* (Kim et al., 2013). Moreover, a putative retinoic acid-responsive element in the 5'-flanking region of the *Fgf21* gene has been recently described and been shown to be transactivated by RAR in liver cells (Li et al., 2013). From this, we expected circulating FGF21 levels to be elevated in ATRA-treated mice. However, this was not the case, ruling out changes in FGF21 as a systemic signal mediating the effects of ATRA treatment. On the other hand, our cell and *in vivo* results might be compatible with ATRA inducing muscle FGF21 as a local (autocrine/paracrine) signal. Interestingly in this context, recent evidence supports an effect of systemic and possibly locally generated FGF21 in promoting fatty acid oxidation in the heart (Planavila et al., 2013).

We also aimed to analyze changes in IL-6 expression and secretion. Divergent roles for this cytokine have been proposed; as a pro-inflammatory factor elevated in obesity and in insulin resistant states, and as an anti-obesity signal and an insulin sensitizing factor in skeletal muscle (Hoene and Weigert, 2008; Pedersen and Febbraio, 2008). It has been reported that the expression of IL-6 is decreased upon ATRA treatment in certain cell culture models (e.g. lung fibroblasts and B cells) (Scheffel et al., 2005; Tabata et al., 2006). Indeed, ATRA induced a pronounced decrease in IL-6 expression and secretion in C2C12 myocytes, but it did not affect expression in gastrocnemious or circulating levels in mice treated with ATRA. The down-regulation only observed in C2C12 myocytes could be related to ATRA effects on cell differentiation, since ATRA has been shown to promote myogenic differentiation of myoblastic progenitor C2C12 cells (Zhu et al., 2009), a process which involves the repression of IL-6 expression (Ramakrishnan et al., 2005).

In summary, results in this work reveal direct effects of the vitamin A derivative ATRA on skeletal muscle cell metabolism and secretome that may contribute to the anti-adiposity and insulin sensitizing action of this compound. Knowledge of dual agents capable of both enhancing oxidative metabolism in skeletal muscle and modulating myokine production may contribute to new avenues in the management of obesity, insulin resistance and related disorders.

Conflict of interest: Authors have no competing financial interests in relation to the work described.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Primers used in the PCR reactions

gene	primer sequence		Accession number
LPL	5´-CCTGATGACGCTGATTTTGT-3´	5´-TATGCTTTGCTGGGGTTTTC-3´	NM_008509
FAT/CD36	5´-GCTCAAAGATGGCTCCATTG-3´	5´-GTCCTGGCTGTGTTTGGA-3´	NM_007643
HSL	5´-TCACGCTACATAAAGGCTGCT-3´	5´-CCACCCTAAAGAGGGAACT-3´	NM_010719.5
ATGL	5´-TGTGGCCTCATTCCTCCTAC-3´	5´-AGCCCTGTTTGCACATCTCT-3´	NM_025802
PPARβ/δ	5´-GCCTCGGGCTTCCACTAC-3´	5´-TCCGTCCAAAGCGGATAG-3´	NM_011145
PGC1α	5´-AGGAGGGTCATCGTTTGTGG-3´	5´-GGAGGCAGAAGAGCCGTC-3´	NM_008904
UCP3	5´-GGAGGAGAGAGGAAATACAGAGG-3´	5´-CCAAAGGCAGAGACAAAGTGA-3´	NM_009464
CPT1-m	5´-AAGGGTAGAGTGGGCAGAGG-3´	5´-GCAGGAGATAAGGGTGAAAGA-3´	NM_009948
ACOX1	5´-TGGTGAAGAAGATGAGGGAGT-3´	5´-AGCAAGGTGGGCAGGAAC-3´	NM_025729
PDK4	5´-TCCTTCACACCTTCACCACA-3´	5´-AAAGAGGCGGTCAGTAATCC-3´	NM_013743
InsR	5´-GTCCGGCGTTCATCAGAG-3´	5´-CTCCTGGGATTCATGCTGTT-3´	NM_010568
HK	5´-CAGCCTAGACCAGAGCATCC-3´	5´-CGCATCTCTTCCATGTAGCA-3´	NM_013820
GLUT4	5´-GGCATGCGTTTCCAGTATGT-3´	5´-GCCCCTCAGTCATTCTCATC-3´	NM_009204
IL-6	5´-TGGGAAATCGTGGAAATGAG-3´	5´-GAAGGACTCTGGCTTTGTCTT-3´	NM_031168
Fndc5	5´-ATGAAGGAGATGGGGAGGAA-3´	5´-GCGGCAGAAGAGAGCTATAACA-3´	NM_027402
FGF21	5'- ACAGATGACGACCAGGACAC -3'	5'- AGGCTTTGACACCCAGGATT -3'	NM_020013
β-actin	5'- TACAGCTTCACCACCACAGCT -3'	5'- TCTCCAGGGAGGAAGAGGAT -3'	NM_007393.3
18S rRNA	5´-CGCGGTTCTATTTTGTTGGT-3´	5´-AGTCGGCATCGTTTATGGTC-3´	
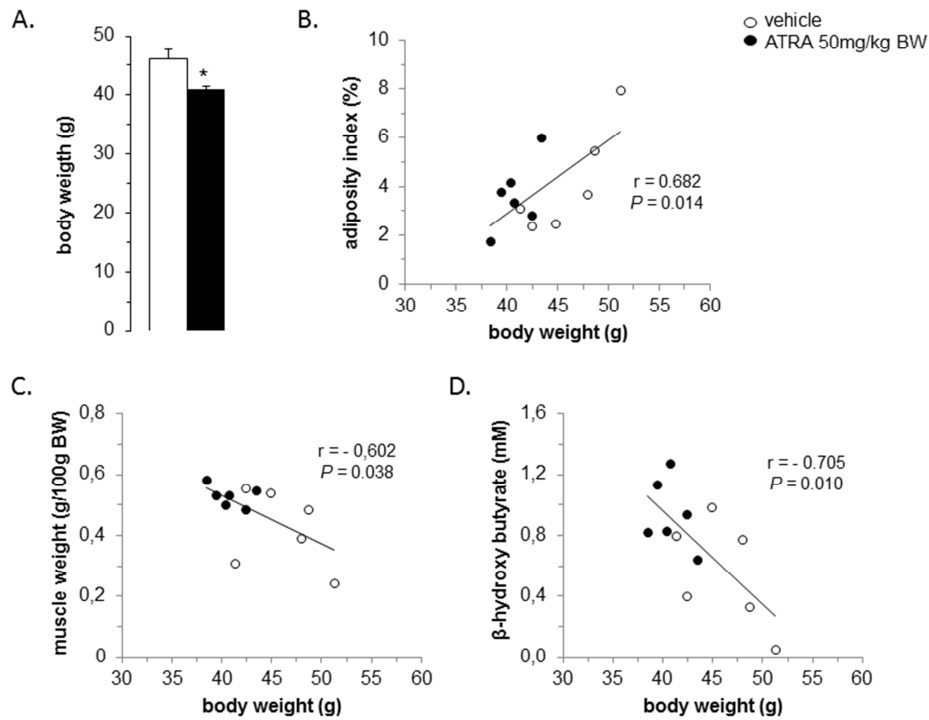
Supplementary Figure 1. ATRA effect on body weight (A) and correlation of body weight with adiposity index (B), gastrocnemius muscle to body weight ratio (C) and serum β-hydroxybutyrate levels (D) in pooled vehicle- and ATRA-treated mice. Twelve-week-old NMRI male mice received one daily subcutaneous injection of ATRA at a dose of 50 mg/kg body weight during the 4 days before euthanization. Control mice were injected the vehicle (100 μl olive oil). On the day of euthanization, the mice were weighted, tissues were collected and weighted and serum was prepared from blood. β-hydroxybutyrate levels in serum were determined using an enzymatic kit. Correlation analyses between the studied parameters were performed with pooled ATRA-treated and control animals. Pearson's correlation indexes and *P* values (bilateral) are indicated.

Results and Discussion
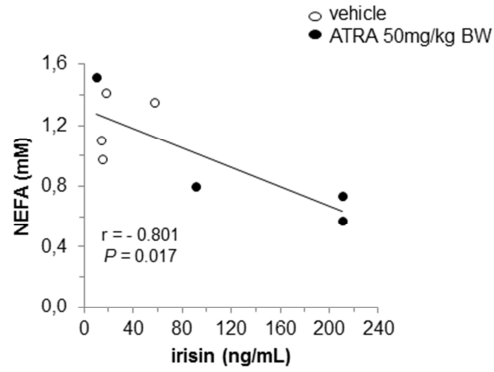
Supplementary Figure 2. Correlation of serum irisin levels with serum NEFA levels. Twelve-week-old NMRI male mice received one daily subcutaneous injection of ATRA at a dose of 50 mg/kg body weight during the 4 days before euthanization. Control mice were injected the vehicle (100 μl olive oil). On the day of euthanization, serum was prepared from blood. Serum parameters were determined by ELISA (irisin) and an enzymatic kit (NEFA). Correlation analysis was performed with pooled ATRA-treated and control animals. The Pearson's correlation index and *P* value (bilateral) is indicated.

Chapter 3.5

ALL-TRANS RETINOIC ACID INDUCES OXIDATIVE PHOSPHORYLATION AND MITOCHONDRIA BIOGENESIS IN ADIPOCYTES

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Manuscript to be submitted

Chapter 3.5

ALL-TRANS RETINOIC ACID INDUCES OXIDATIVE PHOSPHORYLATION AND MITOCHONDRIA BIOGENESIS IN ADIPOCYTES

ABSTRACT

A positive effect of all-trans retinoic acid (ATRA) on white adipose tissue (WAT) oxidative and thermogenic capacity has been described and linked to an *in vivo* fat loosening effect of ATRA in mice. However, so far little is known about the effects of ATRA on mitochondria in white fat. Our objective has been to characterize the effect of ATRA on mitochondria biogenesis and oxidative phosphorylation (OXPHOS) capacity in mature white adipocytes. Transcriptome analysis, analysis of mitochondrial DNA (mtDNA) and flow cytometry-based analysis of mitochondria density was performed in mature 3T3-L1 adipocytes after 24 h incubation with ATRA (2 μM) or vehicle. Selected genes linked to mitochondria biogenesis and function and mitochondria immunostaining were also performed in WAT tissues of ATRA-treated as compared to vehicle-treated mice. ATRA up-regulated the expression of a large set of genes linked to mtDNA replication and transcription, mitochondrial biogenesis and OXPHOS in adipocytes, as indicated by transcriptome analysis. mtDNA content and staining of mitochondria were also increased in the ATRA-treated adipocytes. Similar results were obtained in WAT depots of ATRA-treated mice. We conclude that ATRA impacts mitochondria in white adipocytes, leading to increased OXPHOS capacity and probably mitochondrial content in these cells.

Keywords: vitamin A; body weight; adiposity; white adipose tissue.

INTRODUCTION

Typical white adipocytes are relatively poor in mitochondria and have a low oxidative capacity. Because of this, the contribution of white adipose tissue (WAT) to whole body energy expenditure is considered relatively small. However, there are studies in both human and rodents documenting negative association between mitochondrial content in WAT and obesity, as well as examples of nutritional and pharmacological interventions in animals resulting in obesity resistance that associate with increased oxidative capacity in WAT (reviewed in (Flachs et al., 2013)). Stimulation of mitochondrial biogenesis and oxidative capacity in white adipocytes, when linked to increased energy expenditure in these cells through increased energy uncoupling and/or waste (e.g., futile cycles), emerges therefore as a potential novel target in the control of obesity and its related medical complications (Flachs et al., 2013).

Vitamin A metabolites, i.e. retinoids, can modulate growth and differentiation of a wide range of cells and tissues. Dietary vitamin A and pro-vitamin A are stored as retinyl esters or intracellularly metabolized to retinoic acid (RA), the main active form of vitamin A (Blomhoff and Blomhoff, 2006). There are two isoforms of RA, all-trans-RA (ATRA) and 9-cis-RA, which exert their effects on cell processes through, both, genomic and non-genomic mechanisms (Theodosiou et al., 2010). After the liver, adipose tissue is a major site of vitamin A storage and metabolism, as well as a main target of ATRA action (Tourniaire et al., 2009).

Previous studies have shown that, in mice, treatment with ATRA reduces body weight by decreasing adiposity independently of changes in food intake and improves glucose tolerance and insulin sensitivity in lean and obese animals (Berry and Noy, 2009; Bonet et al., 2000; Felipe et al., 2004; Felipe et al., 2005; Mercader et al., 2006; Puigserver et al., 1996; Ribot et al., 2001). ATRA-induced body fat loss is unaccompanied by increased circulating non-esterified fatty acids (Mercader et al., 2006), suggesting that fatty acids mobilized from fat stores are efficiently oxidized in cells. The latter may include the white adipocytes themselves. In fact, ATRA treatment has been shown to increase the expression of genes related to oxidative metabolism, fatty acid oxidation and thermogenesis in WAT depots (Berry and Noy, 2009; Mercader et al., 2006), besides activating brown adipose tissue (BAT) (Bonet et al., 2000; Puigserver et al., 1996) and increasing lipid oxidation capacity in additional tissues such as skeletal muscle (Amengual et al., 2008; Berry and Noy, 2009) and liver (Amengual

et al., 2010). Moreover, cell-autonomous effects of ATRA in mature adipocytes reducing intracellular lipids, enhancing fatty acid oxidation and triggering gene expression changes consistent with increased lipid turnover and oxidation have been described (Mercader et al., 2007).

Whether the above effects are linked to an ATRA-induced increase in mitochondria in white adipocytes has, however, not been addressed. Likewise, a systematic analysis of the impact of ATRA on oxidative phosphorylation (OXPHOS) capacities in mature adipocytes was lacking. The objective of the present work has been to characterize the effect of ATRA on mitochondria biogenesis and function, particularly OXPHOS capacity, in mature white adipocytes.

MATERIAL AND METHODS

Cell culture.

3T3-L1 preadipocytes (ATCC, Manassas, VA) were seeded in 3.5-cm diameter dishes at a density of 15×10^4 cells/well. Cells were grown in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 humidified atmosphere, as previously reported (Landrier et al., 2009). To induce differentiation, two-day postconfluent 3T3-L1 preadipocytes (day 0) were stimulated for 48 h with 0.5 mM isobutylmethylxanthine, 0.25 µmol/l dexamethasone, and 1 µg/ml insulin in DMEM supplemented with 10% FBS. Cells were then maintained in DMEM supplemented with 10% FBS and 1 µg/ml insulin. To examine the effect of ATRA on gene expression, 3T3-L1 adipocytes were incubated with 2 µM of this molecule for 24 hours, as previously reported (Gouranton et al., 2011). The data are the mean of three independent experiments each performed in triplicate.

Animal experiments.

The care and use of mice were in accordance with the guidelines of the laboratory animals of the University of the Balearic Islands (UIB) and the protocols were submitted to, and approved by, the UIB institutional review board. Twelve adult NMRI male mice (CRIFFA, Barcelona, Spain) fed *ad libitum* regular laboratory chow (Panlab, Barcelona, Spain; 73.4% carbohydrate-, 18.7% protein-, and 7.9% lipid-derived energy; 5 UI vitamin A/kcal) and kept at 22°C under 12-h light/12-h dark cycles (lights on at 08:00) were used. Six animals received one daily subcutaneous injection of ATRA at a

dose of 50 mg/kg body weight during the 4 days before they were sacrificed, as previously described (Amengual et al., 2008, 2010; Mercader et al., 2006). The other six animals were used as controls and injected the vehicle (100 μl olive oil). The animals were sacrificed by decapitation at the start of the light cycle. Inguinal, retroperitoneal and epididymal WAT were excised in their entirety, weighted, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. A fragment of each WAT depot was fixed for morphological and immunohistochemical analysis (see below).

RNA isolation and qPCR

Total cellular RNA was extracted from 3T3-L1 cells and mice fat pads using TRIzol reagent according to the manufacturer's instructions. The cDNA was synthesized from 1 μg of total RNA in 20 μl using random primers and Moloney murine leukemia virus reverse transcriptase. Real Time Quantitative RT-PCR analyses for the genes were performed using the Mx3005P Real-Time PCR System (Stratagene, La Jolla, CA, USA) as previously described (Landrier et al., 2008). For each sample, expression was quantified in duplicate, and 18S rRNA was used as the endogenous control in the comparative cycle threshold (C_T) method.

Mitochondrial DNA quantification

Total DNA was extracted from cells using DNAzol (Euromedex, France). The content of mtDNA was calculated using real-time quantitative PCR by measuring the threshold cycle ratio (ΔCt) of a mitochondrial-encoded gene Cox1 versus a nuclear-encoded gene cyclophilin A.

Hybridization Arrays and microarray data analysis

RNA quality control was performed on an Agilent 2100 Bioanalyzer (Massy, France) with 6000 Nano Chips, according to the manufacturer's instructions and as previously reported (Landrier et al., 2010). RNA from 3 independent experiments were hybridized to Agilent Whole Human Genome (4x44k; Massy, France). All labeling, hybridization, washing and scanning were performed as described in the manufacter's protocol. Arrays were scanned with an Agilent Scanner (Massy, France). Data were extracted with Agilent Feature Extraction v9.5.3 and analyzed with Agilent GeneSpring GX v11 (Massy, France). Data were normalized according to the Lowess method, and multiple correction test false discovery rate was applied, then data were filtered on the p-value ($P<0.05$) for further analyses. Pathway analyses were performed with Metacore

(http://www.genego.com/metacore.php), and Gene Set enrichment analysis were performed with GSEA software (http://www.broadinstitute.org/gsea).

Mitochondria labeling

3T3-L1 adipocytes (after 8 days of differentiation, in 12 well-plates) treated or not with ATRA (2 µM for 24h) were incubated with 50 nM MitoTracker® Green FM (Life technologies) in complete adipose medium (DMEM 4,5g/L glucose, 17 µM biotin, 33 µM panthonetate, 170 nM insulin) for 30 min at 37°C. Cells were then washed with PBS, trypsinized and resuspended in 400 µL PBS+2% FBS (PAA). MitoTracker® Green FM fluorescent intensity was measured in 10 000 cells (Supplemental figure 1) using a C6 Accuri flow cytometer.

Histology and immunohistochemistry

Tissue specimens of inguinal, retroperitoneal and epididymal WAT were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. After washing in PB overnight, the samples were dehydrated in a graded series of ethanol and embedded in paraffin blocks for light microscopy and immunohistochemistry. For the latter, 5 µm sections were immunostained by means of the avidin-biotin technique (Hsu and Raine, 1981). Briefly, sections were incubated with primary polyclonal anti-CoxIV antibody (Cell Signalling Technology, Danvers, MA, USA) raised in rabbits diluted 1:100 dilution in PBS, then with the corresponding biotinylated anti-rabbit IgG secondary antibody, raised in goat, diluted 1:200 (Vector Laboratories, Burlingame, CA, USA), and finally with ABC complex (Vectastain ABC kit; Vector Laboratories). Peroxidase activity was revealed using 0.075% 3,3'-diaminobenzidine hydrochloride as chromogen (Sigma, St Louis, MO, USA) in Tris buffer 0.05M, pH 7.6. Sections were counterstained with hematoxylin and mounted in Eukitt (Kindler, Freiburg, Germany). Sections were observed with Zeiss Axioskop 2 microscope equipped with AxioCam Icc3 digital camera (Carl Zeiss, S.A., Barcelona, Spain). Appropriate positive controls were used to check antibody specificity.

Statistical analysis

Data are expressed as means±SEM. Significant differences between control and treated cells/groups were determined by unpaired Student's *t* test or ANOVA following by Tukey Kramer post-hoc test using Statview software (SAS Institute, Cary, NC). Values of $P<0.05$ were considered significant.

RESULTS

ATRA up regulates expression of genes linked to mitochondria and oxidative phosphorylation in adipocytes.

To study in detail the impact of ATRA, in terms of gene expression, on adipocytes, and notably its putative impact on mitochondrial function, we performed microarray experiments. Mature 3T3-L1 adipocytes were incubated with ATRA (2 µM for 24 h). It is noteworthy that this treatment regulated the expression of genes previously shown to be modulated by ATRA in adipocytes and/or adipose tissue (Table 1), which validated our microarray experiments. Indeed, upon ATRA treatment classical target genes such as Rarb, Rarres1, Ucp1 and 2, Ppargc1a, Ppara or Cyp26b1 were strongly induced, whereas genes encoding leptin, resistin, CEBPα, adiponectin and RXRα were down-regulated significantly (Felipe et al., 2003, 2004; Gouranton et al., 2011; Han and Sidell, 2002; Hollung et al., 2004; Mercader et al., 2007; Tourniaire et al., 2009; Zhang et al., 2002).

Table 1: Known ATRA target genes identified as such by microarray analysis in 3T3-L1 adipocytes exposed to ATRA (2 µM, 24 h) as compared to vehicle

Probe Number	Fold change	Refseq Number	Gene name
A_51_P202440	1.35	NM_011243	Rarb
A_51_P401184	2.43	XM_130987	Rarres1
A_51_P501844	46.29	NM_175475	Cyp26b1
A_52_P374882	-24.74	NM_008493	Lep
A_51_P233597	-1.87	NM_022984	Retn
A_52_P168567	-3.00	NM_007678	Cebpa
A_51_P458451	-1.42	NM_009605	Adipoq
A_52_P188139	-1.66	NM_011305	Rxra
A_52_P499675	3.33	NM_011671	Ucp2
A_51_P426353	32.54	NM_009463	Ucp1
A_52_P5945	1.32	AK032149	Ppargc1a
A_51_P348334	3.56	NM_011144	Ppara

Table 2: Top 20 gene sets impacted by ATRA (2 µM, 24 h) in 3T3-L1 adipocytes.

Gene Set	NES	NOM p-val	FDR q-val
GLUCOSE_METABOLIC_PROCESS	2.54	0.004	0.384
LIPID_TRANSPORTER_ACTIVITY	2.45	0.004	0.348
MITOCHONDRIAL_PART	2.33	0.002	0.403
MITOCHONDRIAL_MEMBRANE_PART	2.22	0.004	0.431
CATION_TRANSPORT	2.21	0.011	0.355
MONOVALENT_INORGANIC_CATION_TRANSPORT	2.20	0.020	0.302
MITOCHONDRIAL_INNER_MEMBRANE	2.16	0.007	0.289
ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY	2.10	0.007	0.303
MITOCHONDRIAL_MATRIX	2.06	0.018	0.309
MITOCHONDRIAL_LUMEN	2.05	0.011	0.282
MITOCHONDRIAL_ENVELOPE	2.02	0.002	0.281
ORGANELLE_INNER_MEMBRANE	1.95	0.022	0.313
SUBSTRATE_SPECIFIC_TRANSPORTER_ACTIVITY	1.94	0.004	0.299
MITOCHONDRION	1.93	0.002	0.289
TRANSCRIPTION_REPRESSOR_ACTIVITY	1.88	0.017	0.316
MITOCHONDRIAL_MEMBRANE	1.86	0.014	0.317
MITOCHONDRIAL_RESPIRATORY_CHAIN	1.86	0.023	0.302
VOLTAGE_GATED_CHANNEL_ACTIVITY	1.85	0.041	0.290
ION_TRANSPORT	1.81	0.023	0.317
ION_CHANNEL_ACTIVITY	1.79	0.027	0.323

Then, transcriptomic data were analyzed by Gene Set Enrichment Analysis (GSEA). Among the top 20 gene sets positively regulated by ATRA, 9 genes sets contained the term "mitochondria" in their title (Table 2). Notably, the gene set called "Mitochondrion", which regroups 343 genes related to mitochondria was significantly enriched (NES 1.93, p-value 0.002; FDR q-value 0.289). In addition, several others gene sets related to organelle membranes or transporters were also indirectly linked to mitochondria, suggesting a global positive impact of ATRA treatment on mitochondria

function. To complete this analysis, we conducted a Metacore pathway analysis (data not shown). Interestingly, of the pathways identified, the one called "oxidative phosphorylation" (Metacore nomenclature) was deeply affected: of the 105 genes involved in this pathway, 62 were upregulated by ATRA (P<2.73e-10). These genes code for subunits of the complexes I through IV, involved in the electron transport chain and found in the inner membrane of the mitochondrion (Supplemental Table 1). Among regulated genes, Cycs, encoding cytochrome c, appeared as one of the most upregulated (3.6 fold induction). Together these data support that ATRA strongly impacted mitochondria function, notably through an induction of OXPHOS capacity.

Table 3: List of transcription factors involved in mitochondria biogenesis regulated by ATRA in 3T3-L1 adipocytes.

Probe Number	Fold change	Refseq Number	Gene name
A_52_P45724	1.52	NM_172135	Mterf
A_51_P493720	1.22	NM_009360	Tfam
A_51_P374499	1.55	NM_008249	Tfb2m
A_52_P526724	2.54	NM_133249	Ppargc1b
A_51_P279038	1.21	NM_008904	Ppargc1a
A_52_P396436	1.63	NM_010902	Nrf2
A_51_P456320	1.31	NM_009537	Yy1
A_51_P248580	1.35	NM_007953	Esrra
A_51_P348334	3.56	NM_011144	Ppara

ATRA up regulates several transcription factors and coactivators linked to mitochondrial DNA transcription, biogenesis and function in adipocytes.

To go further in detail in the analysis of genes regulated by ATRA in adipocytes, a deep analysis of genes present within the enriched genes sets was conducted. We identified several genes coding for transcription factors strongly involved in mitochondrial biogenesis and function. Indeed, gene expression of classical transcription factors controlling the transcription as well as the replication of mitochondrial DNA, i.e. Tfam, Mterf and Tfb2m was significantly upregulated (Table 3). In addition, transcription

factors governing respiratory gene expression, i.e. Nrf2, Esrra, Yy1 or Ppara were also induced by ATRA in adipocytes, as well as nuclear coactivators of mitochondrial biogenesis PGC1α and PGC1β (encoded by Ppargc1a and Ppargc1b, respectively), the latter being strongly upregulated (x 2.5 in our conditions).
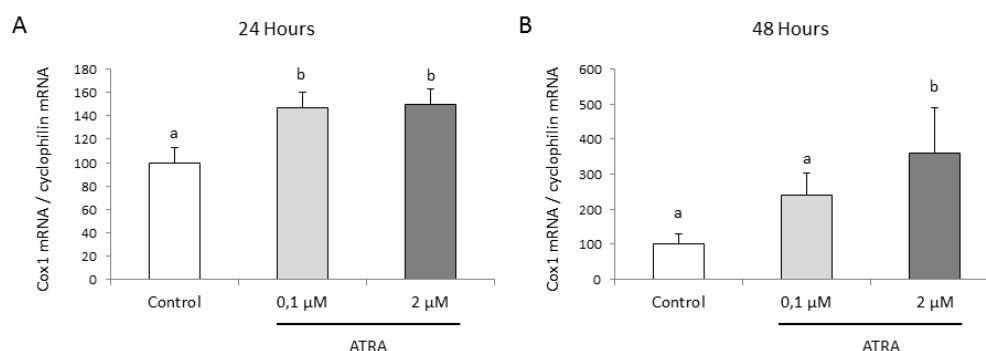
Figure 1. mtDNA to nuclear DNA ratio in 3T3-L1 adipocytes exposed to the indicated ATRA doses for the indicated times. Control refers to control cells, which received the vehicle (DMSO). Data are the mean ± SEM of 3 independants cultures per treatment condition.
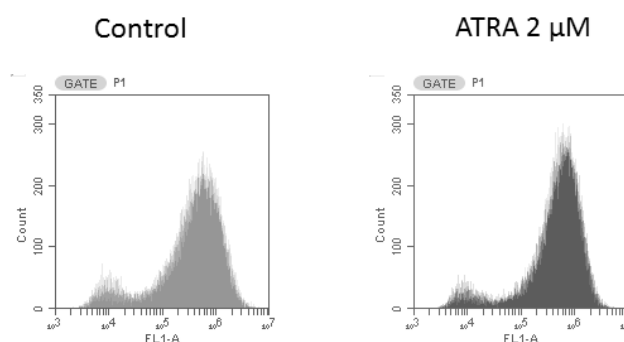
Figure 2. MitoTracker® Green FM staining of 3T3-L1 adipocytes after treatment for 24 h with vehicle (Control) or 2 μM ATRA. Incubation with the dye and flow cytometry sorting of the stained cells were conducted as described in Methods.

Interestingly, results on gene expression were extended by quantification of mitochondrial DNA. In agreement with induction of transcription factors linked to mitochondrial DNA transcription and replication, we observed a significant increase in mtDNA quantified by the ratio Cox1 DNA/cyclophilin DNA (Figure 1). The induction was significant 24 h after the beginning of incubation of cells with ATRA and was maintained after 48 h for the 2 μM condition.

In addition, consistently with the up-regulation of transcription factors for mitochondria biogenesis, the labeling of mitochondria, determined by cytometry after Mitotracker incubation, was increased by ATRA (Figure 2).

ATRA treatment in mice induces the expression of genes linked to mitochondria and increases the number of mitochondria in white adipose tissue

To investigate the impact of ATRA *in vivo*, NMRI mice received ATRA (50 mg/kg of body weight/day) subcutaneously for 4 days. Gene expression analysis of inguinal, retroperitoneal and epididymal fat pads was conducted by qPCR. The effectiveness of the treatment was confirmed by the up-regulation of classical RAR target genes such as Cyp26a1 and Ucp1, which were strongly induced in the three adipose tissue depots (Table 4). Since Ppargc1, Ppargc1b, Nrf2, and Tfam appeared as the most relevant up-regulated genes *in vitro*, we quantified the expression of these mRNAs *in vivo*. As expected, all mRNAs were significantly up-regulated with some variations between adipose fat pads (Table 4). In some depots, the induction did not reach the significance but a clear tendency towards increased expression was observed. The origin of such adipose pads specific response will require further investigations.

Table 4: Gene expression in mice adipose tissues.

Gene Name	Inguinal AT	Retroperitoneal AT	Epididymal AT
PGC1α	1.24 ± 0.24	2.35 ± 0.31 *	1.52 ± 0.19 *
PGC1β	2.87 ± 0.48 *	2.70 ± 0.52 *	2.67 ± 0.57 *
NRF2	1.76 ± 0.32	1.13 ± 0.14	1.63 ± 0.27 *
Tfam	2.51 ± 0.58 *	1.29 ± 0.18	1.29 ± 0.18
PPARα	3.10 ± 0.69 *	5.48 ± 1.04 *	2.92 ± 0.6 *
Ucp1	3.35 ± 0.81 *	3.89 ± 0.74 *	2.14 ± 0.43 *
Cyp26b1	47.51 ± 8.98 *	62.65 ± 9.26 *	43.66 ± 7.32 *
Aco2	0.96 ± 0.2 *	1.99 ± 0.32 *	1.51 ± 0.34

Values of gene expression in ATRA-treated mice have been normalized to those in control mice (which were set to 1). Expression values in control and ATRA-treated mice were compared by Student t-test; p-value < 0.05 (mentioned by an asterisk in the table) were considered as significant.
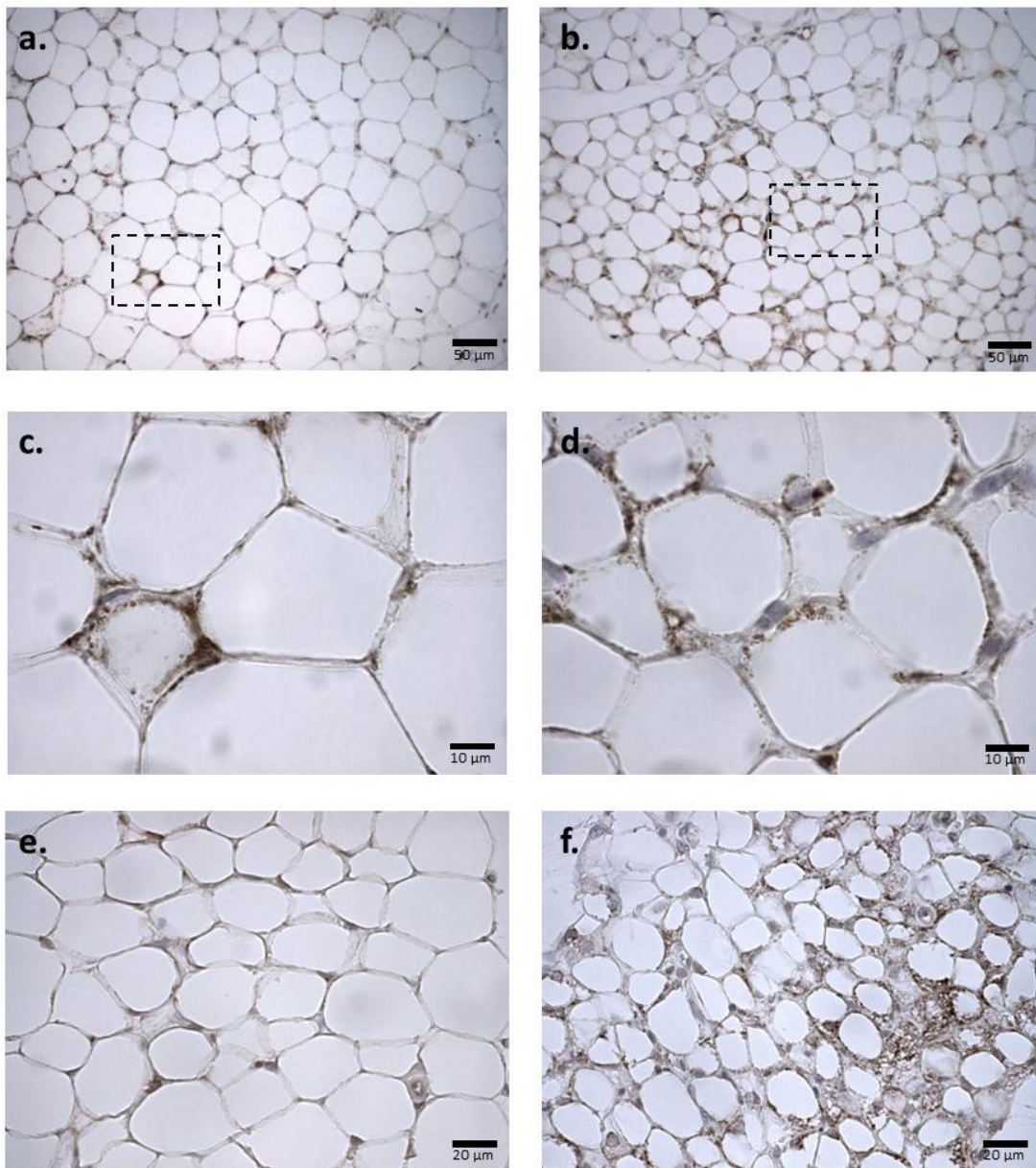
Figure 3. Representative light microscopy microphotographies and magnification of CoxIV immunostaining in the retroperitoneal white adipose depot (a-d) and in the in the inguinal white adipose depot (e,f) of control (a,c,e) and ATRA-treated (b,d,f) animals. Twelve-week-old NMRI male mice were sc. injected vehicle (olive oil, control) or 50 mg ATRA per kilogram of body weight per day during 4 consecutive days before they were sacrificed. Control animals present few, sparse COX IV positivity in the cytoplasm of the cells as compared with ATRA-treated mice. Scale bar 50 μm (a,b), 10 μm (c,d), and 20 μm (e,f).

To go further into the analysis of white adipose tissue (WAT), immunohistochemical staining was performed with COX IV antibodies. This staining revealed, for all WAT depots studied, an increased COX IV positivity in the peripheral cytoplasm of unilocular adipocytes in ATRA-treated mice as compared to control mice,

especially in visceral depots (pictures from retroperitoneal WAT samples are presented in Figure 3a-d). Additionally, in all WAT depots studied, adipocytes of ATRA-treated mice were smaller than that of the control mice (illustrated also in Figure 3). An increased number of multilocular adipocytes, highly positive for COX IV (Figure 3e-f), was seen in (3 out of 6) subcutaneous (inguinal) WAT samples of ATRA-treated mice, in concordance with a previous report (Mercader et al., 2006). In the visceral depots (retroperitoneal and epididymal) ATRA treatment increased COX IV positivity in unilocular adipocytes without inducing the appearance of multilocular cells.

DISCUSSION

White adipocytes in WAT depots are relatively poor in mitochondria and have a low oxidative and thermogenic capacity that suits their energy storage function, while the opposite is true for brown adipocytes in BAT depots, which are specialized in regulated energy dissipation as heat (adaptive thermogenesis) through UCP1 activity. A positive effect of ATRA on WAT oxidative and thermogenic capacity which eventually leads to the conversion of white adipocytes into more brown-like cells has been described and linked to the fat loss effect of ATRA (Berry and Noy, 2009; Mercader et al., 2007; Mercader et al., 2006). However, until now, little is known about the effects of ATRA on mitochondria in adipose tissue. Being the center of both oxidative metabolism and thermogenesis, mitochondria are the master organelles of cell fuel utilization and as such represent an obvious potential target of ATRA action. In this work, we show that ATRA strongly impacts mitochondria biogenesis and function leading to increased mitochondrial content and OXPHOS capacity in white adipocytes. This is revealed by transcriptome analysis of mature white adipocytes exposed to ATRA in vitro, and confirmed by additional lines of evidence and in ATRA-treated mice.

Changes in gene expression in mature 3T3-L1 adipocytes induced by ATRA included the up-regulation of genes coding for classical transcription factors governing mitochondrial DNA replication and transcription (TFAM, MTERF, TFB2M), and nuclear coactivators (PGC-1α and PGC-1β) and interacting transcription factors (NRF2, ERRα, YY1, PPARα) that control mitochondrial biogenesis and respiratory gene expression. In fact, about 60% of the genes in the OXPHOS pathway were up-regulated at the mRNA level in the ATRA-treated adipocytes. Genes encoding mitochondrial proteins capable to uncouple OXPHOS to generate heat, namely UCP1 − the molecular

marker of brown adipocytes (Cannon and Nedergaard, 2004)– and UCP2, were also up-regulated. Importantly, gene expression analysis on the adipose tissues from ATRA-treated mice revealed a similar picture, with an increased expression of genes linked to mitochondria.

Both in ATRA-exposed 3T3-L1 adipocytes and in WAT depots from ATRA-treated mice, the PGC-1β gene was up-regulated to a greater extent and more consistently than the PGC-1α gene. The two PGC-1 isoforms coactivate transcription factors controlling genes required for mitochondria biogenesis and energy metabolism, located in both the mitochondrial and nuclear genomes (Finck and Kelly, 2006). Mitochondrial biogenesis, in particular, is stimulated by the PGC-1-NRF1/2-TFAM pathway, where PGC-1 co-activates the NRF1/2 transcription factor to increase gene expression of TFAM, a final effector that enters the mitochondria to stimulate transcription and replication of mitochondrial DNA molecules (Viña et al., 2009). The best known member of the PGC-1 family is PGC-1α, which participates in the control of multiple processes, including the activation of brown fat thermogenesis (Puigserver et al., 1998; Uldry et al., 2006). PGC-1β, in its turn, does not seem to be required for adrenergically-stimulated thermogenesis in brown adipocytes (Uldry et al., 2006), but appears to play a dominant role in the control of general mitochondrial gene expression in differentiating and differentiated white adipocytes (Ji et al., 2011; Pardo et al., 2011). The number of mitochondria markedly increases during in vitro white adipogenesis, and this is accompanied by an increase in the expression of PGC-1β but not PGC-1α (Lu et al., 2010). Silencing of PGC-1β inhibits the expression of mitochondrial genes, ATP synthesis and adipogenesis in primary rat differentiating preadipocytes (Ji et al., 2011). Likewise, PGC-1β has been shown to be required for the induction of mitochondrial activity in 3T3-L1 cells in response to PPARγ -agonist treatment (Deng et al., 2011). Reciprocally, PGC-1β overexpression up-regulates mitochondrial biosynthesis and adipogenesis marker genes during differentiation of 3T3-L1 preadipocyte cells (Ji et al., 2011). PGC-1β overexpression also enhances mitochondrial biogenesis and function in fully differentiated 3T3-L1 adipocytes, together with improved insulin sensitivity (Gao et al., 2012).

Another gene that was strongly and consistently up-regulated in white adipocytes after ATRA treatment was the one encoding PPARα, which is the main transcriptional regulator of the lipid moxidation pathway (Mandard et al., 2004). This

nuclear receptor transactivates many nuclear genes involved in cellular fatty acid uptake and oxidation and, similar to PGC-1, plays a major role in tissues that normally use fatty acids for ATP or heat production, including BAT (Mandard et al., 2004). In fact, PPARα is considered a distinctive marker of the BAT with respect to WAT phenotype (Villarroya et al., 2007). Importantly, overexpression or pharmacological activation PPARα promotes mitochondrial biogenesis, fatty acid oxidation and a BAT-like pattern of gene expression in white adipocytes (Cabrero et al., 2001; Hondares et al., 2011; Li et al., 2005; Ribet et al., 2010).

In accordance with the higher expression of transcription factors and coactivators linked to mitochondria biogenesis, mitochondrial content appeared increased in ATRA-treated 3T3-L1 adipocytes, as judged by the results of mitochondrial DNA content and Mitotracker staining analysis performed. Similarly, the density of mitochondria was increased in the adipose tissue depots of the ATRA-treated mice as indicated by the stronger COX IV positivity after the immunohistochemical staining. The histochemical analysis also revealed a smaller size of the adipocytes in all examined WAT depots of ATRA-treated mice, and an increased number of adipocytes with a multilocular distribution of intracellular lipids – which is typical of brown adipocytes as opposed to unilocular white adipocytes – in the subcutaneous (inguinal) WAT samples, but not the visceral WAT samples, of these animals. This is in keeping with previous findings (Mercader et al., 2006) that multilocular, UCP1-expressing brown adipocyte-like cells (i.e. *beige* adipocytes) are readily induced in vivo upon ATRA treatment in the subcutaneous WAT of mice, and with reports that subcutaneous WAT is more prone than visceral depots to acquire brown adipocyte features in response to genetic manipulations (Seale et al., 2011) and reversibly upon cold exposure (Rosenwald et al., 2013). Altogether, our results are in concordance with previous reports showing ATRA-induced remodeling towards increased oxidative metabolism in WAT depots (Berry and Noy, 2009; Mercader et al., 2006) and cultured 3T3-L1 adipocytes (Mercader et al., 2007). Similar effects were seen in skeletal muscles of ATRA-treated obese mice, where oxidative metabolism capacities and mitochondrial content were found to be elevated (Berry and Noy, 2009).

Enhancement of energy expenditure in WAT is a potential anti-obesity strategy that can be achieved by inducing, in the white adipocytes, UCP1 expression and UCP1-mediated thermogenesis and/or a futile substrate cycle consisting in lipolysis of

intracellular triacylglycerols and fatty acid re-esterification (TAG/FA cycle) (Flachs et al., 2013). We have shown previously that ATRA induces UCP1 in mouse embryo fibroblasts-derived adipocytes (Mercader et al., 2010), and report here a clear induction of UCP1 gene transcription by ATRA in 3T3-L1 adipocytes and (in keeping with previous results (Mercader et al., 2006)) in WAT depots of mice. Additionally, evidence has been provided that ATRA may stimulate a TAG/FA cycle in association with enhanced β-oxidation in 3T3-L1 adipocytes (Mercader et al., 2007). Importantly, activation of either UCP1 or futile cycles should go hand in hand with the induction of mitochondrial β-oxidation of fatty acids and OXPHOS capacity for a meaningful effect on adipocyte lipid content and whole body energy expenditure to be achieved. It has been previously shown that RA enhances OXPHOS and oxygen consumption in SH-SY5Y human neuroblastoma cells, even without any increase in the number of mitochondria or measurable changes in the composition of the electron transport chain (Xun et al., 2012). Here, we present evidence of increased OXPHOS capacity and mitochondrial content in 3T3-L1 adipocytes and WAT following ATRA exposure, necessary for UCP1-dependent and UCP1-independent energy dissipation mechanisms.

In summary, this work shows that ATRA impacts mitochondria in white adipocytes, leading to increased OXPHOS capacity and probably mitochondrial content in these cells. This knowledge might be of interest in the context of obesity management, as modulation of WAT metabolism could contribute to reduction of adiposity. Beyond obesity, knowledge of factors capable of modulating mitochondria might find application in the treatment of disorders caused by mitochondria malfunctioning.

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SUPPLEMENTARY MATERIAL
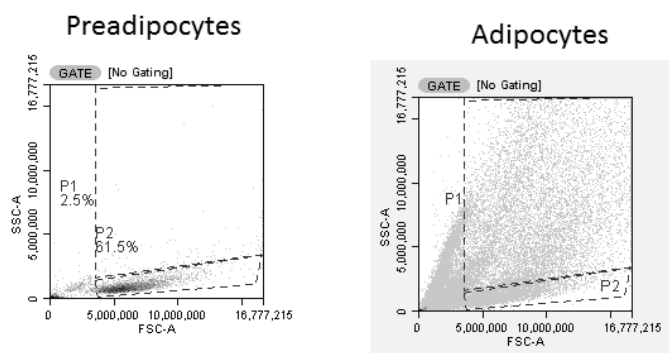
Supplemental figure 1. MitoTracker® Green FM staining of 3T3-L1 preadipocytes defined the P2 gate, which allowed to identify in P1 gate adipocytes, without contamination by preadipocytes. Incubation with the dye and flow cytometry sorting of the stained cells were conducted as described in Methods.

Supplemental Table 1: OXPHOS pathway gene list impacted by ATRA.

Probe Name	Gene Symbol	Protein name	Fold change
A_51_P387334	1110020P15Rik	Cytochrome b-c1 complex subunit 9	1,1938297
A_52_P674489	Atp5a1	ATP synthase subunit alpha, mitochondrial	1,355403
A_51_P207636	Atp5b	ATP synthase subunit beta, mitochondrial	1,4500307
A_51_P378087	Atp5c1	ATP synthase subunit gamma, mitochondrial	1,23986
A_51_P302588	Atp5d	ATP synthase subunit delta, mitochondrial	1,313622
A_52_P631514	Atp5f1	ATP synthase subunit b, mitochondrial	2,5132961
A_51_P294849	Atp5g3	ATP synthase lipid-binding protein, mitochondrial	1,5800416
A_51_P264186	Atp5h	ATP synthase subunit d, mitochondrial	1,5511075
A_51_P100866	Atp5j	ATP synthase-coupling factor 6, mitochondrial	1,4452333
A_51_P224216	Atp5j2	ATP synthase subunit f, mitochondrial	1,6204484
A_51_P301289	Atp5k	ATP synthase subunit e, mitochondrial	1,7186072
A_52_P75415	Atp5l	ATP synthase subunit g, mitochondrial	1,360508
A_51_P365521	Atp5o	ATP synthase subunit O, mitochondrial	1,3261123
A_51_P323880	COX2	Cytochrome c oxidase subunit 2	1,4124748
A_51_P335900	Cox5a	Cytochrome c oxidase subunit 5A, mitochondrial	1,5681988
A_51_P141123	Cox5b	Cytochrome c oxidase subunit 5B, mitochondrial	1,2682877
A_51_P311540	Cox6a1	Cytochrome c oxidase subunit 6A1, mitochondrial	1,808267
A_51_P509997	Cox6a2	Cytochrome c oxidase subunit 6A2, mitochondrial	1,6871346
A_51_P448032	Cox6b1	Cytochrome c oxidase subunit 6B1	1,2926496
A_51_P343323	Cox6c	Cytochrome c oxidase subunit 6C	1,3634577

(Continue)

A_51_P148612	Cox7a1	Cytochrome c oxidase polypeptide 7A1, mitochondrial	1,5492195
A_52_P37894	Cox7a2	Cytochrome c oxidase polypeptide 7A2, mitochondrial	1,425935
A_51_P160664	Cox7b	Cytochrome c oxidase subunit 7B, mitochondrial	1,6324534
A_52_P136153	Cox7c	Cytochrome c oxidase subunit 7C, mitochondrial	1,5998583
A_52_P528726	Cox8a	Cytochrome c oxidase subunit 8A, mitochondrial	1,2890536
A_52_P423814	Cox8b	Cytochrome c oxidase subunit 8B, mitochondrial	2,8493543
A_51_P295610	Cyc1	Cytochrome c1, heme protein, mitochondrial	1,4867831
A_51_P163587	Cycs	Cytochrome c, somatic	3,633
A_51_P245525	ND4L	NADH dehydrogenase 4L, mitochondrial	1,4038746
A_51_P472405	Ndufa1	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1	1,6634898
A_51_P272123	Ndufa10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	1,3065811
A_51_P279854	Ndufa11	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	1,6870229
A_51_P458540	Ndufa12	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	1,8469862
A_51_P384946	Ndufa2	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 2	1,6035903
A_51_P431772	Ndufa3	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	2,2180607

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A_52_P552832	Ndufa4	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	2,0496109
A_51_P170156	Ndufa5	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5	1,4645371
A_52_P217474	Ndufa6	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	1,751568
A_51_P263756	Ndufa7	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	1,3238703
A_51_P475502	Ndufa8	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 8	1,3135426
A_51_P280492	Ndufa9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	1,5582352
A_51_P208801	Ndufab1	Acyl carrier protein, mitochondrial	1,4270644
A_51_P516615	Ndufb10	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	1,7149581
A_51_P205573	Ndufb11	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, mitochondrial	1,2816713
A_51_P361184	Ndufb2	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 2, mitochondrial	1,2073313
A_51_P160744	Ndufb3	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3	1,5244333
A_51_P434269	Ndufb4	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 4	1,3386449
A_51_P201904	Ndufb5	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	1,21985
A_52_P210338	Ndufb6	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 6	1,5648524

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A_51_P519276	Ndufb7	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	1,2852098
A_51_P247873	Ndufb8	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8, mitochondrial	1,6368709
A_51_P177552	Ndufb9	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9	1,4429231
A_52_P254795	Ndufc1	NADH dehydrogenase [ubiquinone] 1 subunit C1, mitochondrial	1,5657411
A_51_P357459	Ndufc2	NADH dehydrogenase [ubiquinone] 1 subunit C2	2,4691007
A_51_P379597	Ndufs1	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial	1,1445307
A_51_P393761	Ndufs2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 2, mitochondrial	1,2195985
A_51_P395014	Ndufs3	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3, mitochondrial	1,2715056
A_51_P388696	Ndufs4	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	1,4504716
A_51_P214916	Ndufs5	NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	1,2480884
A_51_P335077	Ndufs6	NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial	1,5010782
A_51_P364671	Ndufs7	NADH dehydrogenase [ubiquinone] iron-sulfur protein 7, mitochondrial	1,908938
A_51_P247441	Ndufs8	NADH dehydrogenase [ubiquinone] iron-sulfur protein 8, mitochondrial	1,7849194

(Continue)

A_51_P261470	Ndufv2	NADH dehydrogenase [ubiquinone] flavoprotein 2, mitochondrial	1,7228209
A_52_P170054	Ndufv3	NADH dehydrogenase [ubiquinone] flavoprotein 3, mitochondrial	1,931101
A_52_P458708	Sdha	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	1,4217126
A_51_P234853	Sdhb	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	1,2034264
A_51_P260871	Sdhd	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit, mitochondrial	1,4204954
A_51_P300143	Uqcr	Cytochrome b-c1 complex subunit 10	1,2579782
A_51_P109828	Uqcrc1	Cytochrome b-c1 complex subunit 1, mitochondrial	1,3391999
A_51_P128648	Uqcrc2	Cytochrome b-c1 complex subunit 2, mitochondrial	1,3698565
A_51_P361951	Uqcrfs1	Cytochrome b-c1 complex subunit Rieske, mitochondrial	1,6834244
A_52_P541875	Uqcrh	Cytochrome b-c1 complex subunit 6, mitochondrial	1,8870504
A_52_P370484	Uqcrq	Cytochrome b-c1 complex subunit 8	1,8390315

Results and Discussion

4   SUMMARY

Presented thesis shows newly discovered effect of selected fatty acids and retinoids on various elements of body energy regulatory system. This is a complex system that includes numerous metabolic, hormonal and genetic elements which can be grouped in three main cathegories that include mechanisms involved either in the control of energy intake, energy expenditure or adipogenesis. Different nutrients differently affect each of these elements and there are still many gaps in the knowledge considering effects of specific nutrients on concrete mechanisms of body energy homeostasis.

First line of doctoral investigation dealt with the effects of different fatty acids (in the form of free acid) on food intake and the expression of different genes involved in the CNS and gastrointestinal short-term control of food intake. It is already known that most effects lipids induce in the GI tract are mediated by the interaction of FA, and not TAG, with the gut receptors. General effects of FA on energy expenditure and adipogenesis are quite known. However, due to the great variety of FA structures and physicochemical properties there is still much unknown considering the effects of specific fatty acids on these processes. In addition, in relation to their effects on food intake some general results have been described considering their length and saturation level but are still rather inconsistent. Thus the focus of the study on fatty acids was to profound understanding of their different satiating capacities and investigate underlying mechanisms. We were especially interested in the implication of gastric leptin in the satiating effects of fatty acids which still remain unknown.

For this purpose different experiments in rats were designed measuring their food intake after oral treatment with different fatty acids. Later on upon their sacrification different tissues involved in the body satiating response were obtained and their gene expression and protein levels were measured. In the first set of experiments where rats were treated orally with equicaloric doses of different fatty acids 1 hour prior the onset of the dark phase, neither of fatty acids tested caused significant differences on food intake compared to vehicle treated rats. However, differences on food intake were observed between fatty acid treatments such as rats treated with oleic or linoleic acid

ingested significantly less food compared to the palmitic acid treated rats. This is probably due to their longer length and the unsaturation of their carbon chain.

Accordingly, in order to characterize on the molecular level differential effects of tested fatty acids in the short term control of food intake, a second set of *in vivo* experiments as well as *in vitro* experiment were performed in which palmitic and linoleic acids were further evaluated for their effects on satiety and expression and secretion of hipotalamic neuropeptides and gastrointestinal hormones. We were especially interested in the expression of orexigenic and anorexigenic neuropeptides in the hypothalamus as well as the expression and secretion of gastric leptin and ghrelin.

Results from these FA experiments suggest that different satiating capacities of palmitic and linoleic acid can be in part explained by their differential central effect as well as distinctive gastrointestinal hormone expression and release. Palmitic and linoleic acid provoked direct effects in the CNS affecting the expression of hypothalamic neuropeptides included in the control of food intake (**charpter 3.1**). In concrete, results indicate that the acute treatment with fatty acids induced changes at the level of hipotalamic neuropeptide gene expression in the direction of increasing both orexigenic and anorexigenic pathways. However, while linoleic acid seemed to enhance anorexigenic signaling earlier and stronger after the treatment, palmitic acid showed weaker effect. This effect could explaine greater satiating capacity of linoleic acid compared to the palmitic.

In addition, beside these CNS effects both fatty acids showed distinct gastrointestinal effects. Concomitant to the stronger anorexigenic central effect linoleic acid also showed earlier and stronger reduction of circulating ghrelin levels contributing to its stronger satiating capacity in comparison to the palmitic acid. However the inhibition of gastric leptin secretion, as well as its central action (by the increase of its suppressor SOCS3), by fatty acids, especially palmitic acid, could explain the prevalence of orexigenic pathways in palmitic acid treated rats compared to linoleic acid treated rats, and its lesser satiating effect. Our results are first to show, to our knowledge, that gastric leptin is involved in the fatty acid satiating capacities and could act as a safety mechanism to counteract anorexigenic central actions induced by FFAs via inhibition of gastric ghrelin, especially in stress situations such as fasting.

Second part of the doctoral study dealt with the effects of vitamin A on the biology of adipose tissue with a special focus on the early age. Retinoids are a group of nutrients that include all vitamin A metabolites. They are known as being able to modulate growth and differentiation of a wide range of cells and tissues. The most active retinoid, all-trans retinoic acid, ATRA, is required for a wide range of biological processes and its effects have been extensively studied. Effects of vitamin A on food intake are scarce. On the other hand, its effects on energy expenditure and adipogenesis are known in many details. However, although much is known about general effects of RA, many details are still missing considering the different doses and the time of action. It was shown that RA affects early stage of adipocyte differentiation in opposite directions depending on the dose, promoting it at low doses and inhibiting at relatively high doses. In the same way, despite these increasing evidence linking vitamin A and its precursors, such as β-carotene to the control of adiposity in adult animals, little is known about their effects in early life. Thus, for the second part of the study on vitamin A *in vivo* experiment on rat pups as well as different *in vitro* experiments were performed.

Vitamin A supplementation during the suckling period (in the form of retynil ester, RE), increased known RA-mediated transcriptional responses in tissues of RE rats at weaning. We observed increased mRNA levels of intestine-specific homeobox (ISX, gut-specific TF induced in intestinal cells by RA that limits intestinal β-carotene absorption and conversion to vitamin A) in the intestine and increased mRNA levels of CYP26a1 (RA hydroxylase that is transcriptionally induced by RA) in the liver and iWAT, indicating increased RA-mediated transcriptional responses in tissues of RE-treated animals. Moreover, expression of aldehyde dehydrogenase 1a1 (Aldh 1a1), the principal postnatal Aldh isoform which catalyzes RA production from retinaldehyde, was downregulated in iWAT, but not in the liver, of RE treated rats. Downregulation of Aldh1a1 in the face of upregulation of the RA-degrading enzyme CYP26a1 is suggestive of a homeostatic mechanism to keep constant intracellular RA levels in WAT. HPLC-MS analysis of the retinoids (and carotenoids) extracted from the tissues enabled us to validate their intestinal absorption as well as delivery to the tissues crucial for their metabolism and/or storage. Vitamin A given orally as RE, was efficiently absorbed by suckling rats and likely fueled RA production in tissues of young rats.

Increased levels of retinoids were found in the blood (as RE), liver (as RE and free retinol) and iWAT (as total retinol, free retinol plus RE).

This animal experiment involving early vitamin A treatment showed that this supplementation affects adipose tissue development in young rats (**charpter 3.2**). Although RE treatment during suckling period did not affect liver mass, body weight, fat depot mass or serum leptin levels, histological and molecular analysis revealed that excess vitamin A during early postnatal life when iWAT development takes place, elicits changes in iWAT. In particular, iWAT of RE-treated rats at weaning was enriched in small adipocytes. Moreover, iWAT of RE rats displayed reduced expression and protein levels of the master adipogenic transcription factor PPARγ and its downstream target, lipoprotein lipase (LPL) and higher expression of PCNA, a classical marker of proliferative status. PCNA positive staining was observed not only in typical adipocyte precursor cells, but also in cells with small cytoplasmic lipid droplets, i.e. already engaged in terminal adipogenic differentiation. These effects of moderate excess of vitamin A at key developmental stages to favor the development of immature adipocytes that retain increased proliferative capacity can explain the favouring of the hyperplasic component of fat expansion and increased adiposity gain upon a subsequent stimulus in the form of a high fat diet later in life. Taking into account the early postnatal development of WAT this results point again the great importance of early life events for the later propensity to obesity and associated disorders.

Among the diverse group of retinoids related compounds, β-carotene is the most vitamin A active of the provitamin A carotenoids and constitutes the main provitamin A source. However, while the effects of β-carotene conversion products, especially RAL and RA, on adipocyte differentiation, adipokine secretion and lipid metabolism, have been well described, the effects of intact β-carotene remain largely unknown. Despite the evidence linking BC to the control of adipogenesis in cell models and adiposity in adult animals, little is known about BC effects in early life. We aimed to assess the impact of early supplementation with a moderate dose of BC on WAT development in young rats. We also aimed to compare BC effects with those brought about by an equivalent dose of vitamin A given as retynil esters.

For this reason we also studied the effects of β-carotene early supplementation on these same metabolic targets as in the case of RE (**charpter 3.3**). Significantly increased serum and liver β-carotene levels after the treatment indicated that β-carotene

was absorbed by lactating rats that received supplementation. In accordance with this was also the increased expression of the RA responsive genes in the liver and intestine that demonstrate that orally given BC was also partially metabolized by the suckling rats. This is of interest given that adult rats have been discarded as an animal model to study the toxicological and nutritional effects of BC because, contrary to what happens in humans, adult rats do not practically absorb BC.

However, we did not detect β-carotene in the iWAT of these animals and did not see changes in iWAT gene expression or the morphological features that would point to the increased proliferative capacity of WAT adipocytes observed before upon RE treatment. In particular, iWAT of BC-treated rats at weaning, like iWAT of control rats, was enriched in larger adipocytes that expressed higher levels of adipogenic markers and reduced levels of a classical marker of proliferative status, compared to iWAT of RE-treated rats. This results suggest that β-carotene supplementation, unlike the one with RE, during the suckling period does not affect adipose tissue development in young rats.

To further characterize effects of ATRA on fat reserves and body weight control another *in vivo* experiment was conducted aiming to study its effects in the skeletal muscle (**charpter 3.4**). The results were also confirmed in C2C12 myotubes in the cell culture. As explained before muscles produce and secrete a number of bioactive proteins called myokines, that have regulatory functions in metabolism and some of them act on the adipose tissue. Lately much attention has been put on the study of irisin (encoded by the FNDC5 gene) that has been described as a metabolic regulator secreted by skeletal muscle capable of inducing WAT browning (Bostrom et al., 2012). Similar to irisin, muscle-derived FGF21 can induce WAT *browning* (Kim et al., 2013). Moreover, RAR-mediated induction of FGF21 gene expression has been recently demonstrated in liver cells (Li et al., 2013). In addition, IL-6 is one of the most studied myokines involved in the control of insulin sensitivity, fat metabolism and energy balance (Hoene and Weigert, 2008; Pedersen and Febbraio, 2008). Together this myokine effects prompted us to asses ATRA modulation of their production and secretion, in both cultured skeletal muscle cells and *in vivo*.

Our results indicated that ATRA affects myokine production in general. Particularly, it induced expression of FNDC5/irisin in C2C12 myocytes and intact mice. Exposure of C2C12 myocytes to ATRA led to a dose-dependent up-regulation of

Summary

FNDC5/irisin expression, evident both at the mRNA and protein level of the irisin accumulated in the cell conditioned culture medium. In ATRA treated mice the most pronounced effect was significant 5-fold increase in the irisin serum levels, together with the expected decrease in body weight and adiposity. Remarkably, mRNA levels of FNDC5/irisin in the gastrocnemius muscle were unaffected. With respect to FGF21 and IL-6, ATRA treatment in cultured C2C12 myotubes up-regulated FGF21 and down-regulated IL-6, both on the mRNA and protein level, but showed no significant effects on their serum levels or their expression in the gastrocnemius muscle of the ATRA-treated mice.

These results indicate that skeletal muscle, beside its already known increased lypolysis upon ATRA treatment, may as well contribute to anti-obesity and anti-diabetic action of ATRA through the secretion of its myokines. Giving the role of myokines in the induction of WAT lipid catabolism and fat oxidation and especially the newly discovered role of irisin and FGF21 in the browning phenomena, this effects of ATRA on muscle secretoma help to understand its indirect effects on WAT. Together, these results contribute to the understanding of ATRA multiple effects on adipose tissue and help to explaine anti-obesity action of ATRA.

In addition, aiming to profound the understanding of ATRA induced oxidative and thermogenis capacities in the adipose tissue, another *in vivo* experiment was designed together with *in vitro* experiments on cultured adipocytes. We were especially interested in the effects ATRA exerts in the mitochondrias, being the central organels of cell fuel utilization both through the oxidative metabolism as well as adaptive thermogenesis, and as such represent an obvious potential target of ATRA action. ATRA treatment in mice increased expression of the genes linked to mitochondrial DNA transcription, biogenesis and function in adipocytes, in particular several transcription factors, coactivators and other proteins controling mitochondria biogenesis and respiratory gene expression (Tfam, Nrf2, Ppara, PGC-1α and PGC-1β) as well as the ones involved in the mitochondrial oxidative phosphorylation / thermogenic capacities (Cyp26b1, ACO, Ucp1) (**charpter 3.5**). These results were confirmed in the cell cultures as well. In agreement with induction of transcription factors linked to mitochondrial DNA transcription and replication, we observed a significant increase in mitochondrial DNA quantified by the ratio Cox1 DNA/cyclophilin DNA. In addition, consistently with the up-regulation of transcription factors for mitochondria biogenesis,

the labeling of mitochondria, determined by cytometry after Mitotracker incubation, was increased by ATRA.

The induction of mitochondria quantity was also confirmed by increased immonohystochemical staining of mitochondrial marker, CoxIV, in the peripheral cytoplasm of unilocular adipocytes in ATRA-treated mice. Additionally, in all WAT depots studied, adipocytes of ATRA-treated mice were smaller than that of the control mice. An increased number of multilocular adipocytes, highly positive for CoxIV, was seen in subcutaneous (inguinal) WAT samples of ATRA-treated mice, in concordance with a previous report (Mercader et al., 2006). In the visceral depots (retroperitoneal and epididymal) ATRA treatment increased CoxIV positivity in unilocular adipocytes without inducing the appearance of multilocular cells.

Altogether these results confirm microarray outputs showing the activation of several gene sets involved in the induction of mitochondria biogenesis as well as the ones related to organelle membranes or transporters indirectly linked to mitochondria, suggesting a global positive impact of ATRA treatment on mitochondria function, notably through the induction of their oxidative phosphorilation and thermogenic capacity. This impact of ATRA could explain the remodeling of mature white adipocytes into more brown-fat like cells and the associated decrease in fat mass (Berry and Noy, 2009; Mercader et al., 2006; Mercader et al., 2007).

Summary

5 CONCLUSIONS / CONCLUSIONES

Conclusions

1. **Palmitic and linoleic fatty acids show different satiating capacities which can be explained by their central effects affecting the expression of hypothalamic neuropeptides included in the control of food intake as well as distinct gastrointestinal effects, including gastrointestinal hormone expression and release.** Linoleic acid shows greater satiating capacity which could be explained by the earlier and stronger induction of the anorexigenic signalling in the hipotalamus as well as earlier and stronger decrease in circulating ghrelin levels.

2. **Gastric leptin is also involved in the fatty acid satiating action and could act as a safety mechanism to counteract anorexigenic central actions induced by FFAs via inhibition of gastric ghrelin, especially in stress situations such as fasting**. Inhibition of gastric leptin secretion, as well as its central action (by the increase of its suppressor SOCS3), by fatty acids, especially seen by palmitic acid, could explain the prevalence of orexigenic pathways in palmitic acid treated rats compared to linoleic acid treated rats, and consequently its lesser satiating effect.

3. **Vitamin A given orally as retinyl palmitate (RE) during suckling period is readily absorbed by suckling rats and provokes increased RA-mediated responses in tissues of young rats.** Increased levels of retinoids can be found in the blood, liver and iWAT. We observed increased levels of intestine-specific homeobox (ISX) mRNA in the intestine and CYP26a1 mRNA levels in the liver and iWAT, indicating increased retinoic acid (RA)-mediated transcriptional responses in tissues of RE-treated animals. Expression of aldehyde dehydrogenase 1a1 (Aldh 1a1) was downregulated in iWAT, but not in the liver, of RE treated rats.

4. **Early vitamin A supplementation affects adipose tissue development in young rats.** RE treated pups at weaning display significantly higher percentage of smaller cells in the iWAT and gene expression features consistent with an increased cell

proliferation potential correlated with a reduced expression of adipogenic markers, concretely reduced expression of PPARγ and higher expression of PCNA. This effects of moderate excess of vitamin A at key developmental stages to favor the development of immature adipocytes that retain increased proliferation potential favors the hyperplasic component of fat expansion and increased adiposity gain upon a subsequent stimulus in the form of a high fat diet later in life.

5. **Orally given β-carotene (BC) is readily absorbed intact by suckling rats.** HPLC analysis of retinoids and carotenoids revealed significantly increased BC serum and liver levels in the BC-treated group compared to the control and RE-treated group, in which BC could not be detected. In accordance with this is the increased expression of the RA responsive genes in liver and intestine. However, BC was not detectable in the iWAT and a lack of increase of RA-mediated responses in iWAT of BC-treated group was observed. All toguether points out those young rats are able only to partially metabolize BC to retinoids, so that a significant part is stored as intact BC in the liver, and perhaps other tissues but not in WAT.

6. **Unlike RE, BC supplementation during the suckling period does not affect adipose tissue development in young rats.** Upon vitamin A activity equivalent supplementation with BC we did not see changes in iWAT gene expression or the morphological features that would point to the increased proliferative capacity of WAT adipocytes observed upon RE treatment. BC-treated rats displayed a similar expression of PPARγ in iWAT to control rats and higher than RE-treated rats. Moreover, immunohistochemical analysis of iWAT sections showed less PCNA positive nuclei and larger adipocytes in the BC-treated rats than in the RE-treated rats.

7. **ATRA affects myokine production, inducing expression of FNDC5/irisin and FGF21 in skeletal muscle cells.** ATRA treatment in C2C12 myotubes showed down-regulation of IL-6 mRNA levels and up-regulation of FNDC5/irisin and FGF21 mRNA levels, in parallel with the protein levels. Similar was observed in ATRA treated mice where the most pronounced effect was significant increase in the irisin serum levels. These results reinforce the concept that changes in skeletal muscle contribute to the anti-obesity and anti-diabetic action of ATRA, and extend this concept to the muscle secretome. They contribute to the explanation of ATRA slimming action and the enhancement of whole body fatty acid catabolism.

8. **ATRA impacts mitochondria in white adipocytes, inducing the expression of genes linked to their biogenesis and function, leading to the increased mitochondrial content in these cells.** Both in cultured mature white adipocytes and in mice ATRA treatment increased expression of the genes and transcription factors (e.g. PGC-1α and β) involved in the mitochondria biogenesis and induced mitochondrial oxidative phosphorylation / thermogenic capacities. This impact of ATRA could explain the possible remodeling of mature white adipocytes into mitochondria-rich more brown-fat like cells and the associated decrease in fat mass.

Conclusiones

1. **Los ácidos grasos palmítico y linoleico muestran diferentes capacidades saciantes que se puede explicar por sus efectos a nivel central sobre la expresión de los neuropéptidos hipotalámicos implicados en el control de la ingesta de alimentos, así como por sus efectos gastrointestinales, incluyendo la expresión y la liberación de las hormonas gastrointestinales.** El ácido linoleico muestra mayor capacidad saciante que podría explicarse por la inducción más temprana y más pronunciada de la señalización anorexígenica en el hipotálamo, así como la disminución más temprana y más pronunciada en los niveles circulantes de grelina.

2. **Leptina gástrica también está implicado en la acción saciante de los ácidos grasos y podría actuar como un mecanismo de seguridad para contrarrestar las acciones centrales anorexígenicas inducidas por ácidos grasos libres a través de la inhibición de la grelina gástrica, especialmente en situaciones de estrés tales como el ayuno.** La inhibición de la secreción de leptina gástrica, así como de su acción central (por el aumento de su supresor de SOCS3), por los ácidos grasos, en especial por el ácido palmítico, podría explicar la prevalencia de las vías orexígenicas en ratas tratadas con ácido palmítico en comparación con ratas tratadas con ácido linoleico, y por consiguiente su menor efecto saciante.

3. **La vitamina A administrada por vía oral como retinil palmitato (RE) en período de lactancia es absorbida por las ratas lactantes y provoca un aumento de las respuestas mediadas por el ácido retinoico (RA) en los tejidos de las ratas jóvenes.** El aumento de los niveles de los retinoides se pueden encontrar en la sangre, el hígado y el tejido adiposo (TAB). Se observó aumento de los niveles de ARNm para el homeobox intestino-específico (ISX) en el intestino y de los niveles de ARNm para el CYP26a1 en el hígado y TAB inguinal, lo que indica el aumento de las respuestas transcripcionales mediadas por RA en los tejidos de los animales

tratados con RE. La expresión de aldehído deshidrogenasa 1a1 (ALDH 1a1) se disminuyo en TAB inguinal, pero no en el hígado, de ratas tratadas RE.

4. **Suplementacion con vitamina A en la edad temprana afecta el desarrollo del tejido adiposo en ratas jóvenes.** Crías tratadas con RE, al destete muestran un porcentaje significativamente más alto de células pequeñas en el TAB inguinal y una expresión genetica en consonancia, con un aumento del potencial de proliferación celular en correlacion con una reducción de expresión de marcadores adipogénicos, concretamente la reducción de expresión de PPARγ y la mayor expresión de PCNA. Estos efectos de exceso moderado de vitamina A en las etapas clave de desarrollo para favorecer el desarrollo de los adipocitos inmaduros que conservan un mayor potencial de proliferación que parece favorecer el componente hiperplásico en la expansión de grasa y aumento de la ganancia de la adiposidad tras la exposición a un estímulo posterior en forma de una dieta alta en grasa más tarde en la vida.

5. **El β-caroteno (BC) dado por vía oral es fácilmente absorbido intacto por las ratas lactantes.** El análisis por HPLC de los retinoides y carotenoides reveló un aumento significativo de los niveles de BC en el suero y el hígado en el grupo de BC en comparación con el control y el grupo de RE, en el que no podía ser detectado. De acuerdo con esto, es el aumento de la expresión de los genes de respuesta a RA en el hígado y el intestino. Sin embargo, el BC no era detectable en el TAB inguinal ni tampoco el aumento de las respuestas transcipcionales mediadas por RA en TAB inguinal del grupo BC. Todo lo anterior señala que ratas jóvenes son capaces de metabolizar el BC a retinoides sólo parcialmente, de modo que una parte significativa se almacena como BC intacta en el hígado, y tal vez otros tejidos pero no en TAB.

6. **A diferencia de RE, la suplementación con BC durante el período de lactanccia no afecta el desarrollo del tejido adiposo en ratas jóvenes.** Tras la suplementacion con BC, en una dosis equivalente a la actividad de vitamina A, no habia cambios en la expresión génica del TAB inguinal, ni en las características morfológicas que apuntarían a la mayor capacidad de proliferación de adipocitos del TAB, observados tras el tratamiento con RE. Ratas tratadas con BC muestran una similar expresión de PPARγ en el TAB a las ratas control pero mas alta en

comparación con las ratas tratadas con RE. Por otra parte, el análisis inmunohistoquímico de secciones TAB inguinal mostraron menos núcleos positivos para PCNA y adipocitos más grandes en las ratas tratadas con BC que en las ratas tratadas con RE.

7. **El ATRA afecta la producción de miokinas, induciendo la expresión de FGF21 y FNDC5/irisin en las células del músculo esquelético.** El tratamiento con ATRA en miotubos C2C12 mostró disminución de los niveles de ARNm de IL-6 y el aumento de los niveles de ARNm de FNDC5/irisin y FGF21, en paralelo con los niveles de proteínas. Similarmente se observó en los ratones tratados con ATRA, donde el efecto más pronunciado fue el aumento significativo en los niveles séricos de irisina. Estos resultados refuerzan el concepto de que los cambios en el músculo esquelético contribuyen a la lucha contra la obesidad y la acción anti-diabética del ATRA, y se extiende este concepto al secretoma muscular. Todo ello contribuye a la explicación de la acción adelgazante de ATRA y la mejora del catabolismo de ácidos grasos del todo el cuerpo.

8. **El ATRA afecta las mitocondrias en los adipocitos blancos, induciendo la expresión de los genes relacionados con su biogénesis y su función, dirigiendo al aumento en el contenido mitocondrial en estas células.** Tanto en los adipocitos blancos maduros cultivadas como en los ratones, el tratamiento con ATRA incremento la expresión de los genes y factores de transcripción (por ejemplo, PGC - 1α y β) implicados en la biogénesis de las mitocondrias y su capacidades de fosforilación oxidativa / termogénica. Este impacto del ATRA podría explicar la posible remodelación de los adipocitos blancos maduros en las células de grasa ricas en mitocondrias similares a las células marrón y la asociada disminución de la masa grasa.

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