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DIETARY PROANTHOCYANIDINS: THEIR EFFECTIVENESS IN DYSLIPIDEMIC NUTRITIONAL MODELS AND THE ROLE OF LIVER AND INTESTINE IN THEIR HYPOTRIGLYCERIDEMIC ACTION

Helena Quesada Vázquez

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Dietary proanthocyanidins: Their effectiveness in dyslipidemic nutritional models and the role of liver and intestine in their hypotriglyceridemic action

PhD DOCTORAL THESIS

Directed by Prof. Mª Cinta Bladé Segarra

Departament de Bioquímica i Biotecnologia

UNIVERSITAT ROVIRA I VIRGILI

Tarragona 2010

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FAIG CONSTAR que aquest treball, titulat "**Dietary proanthocyanidins: Their effectiveness in dyslipidemic nutritional models and the role of liver and intestine in their hypotriglyceridemic action**", que presenta Helena Quesada Vázquez per a l'obtenció del títol de Doctor, ha estat realitzat sota la meva direcció al Departament de Bioquímica I Biotecnologia d'aquesta universitat i que acompleix els requeriments per poder optar a Menció Europea.

Tarragona, 30 d'agost de 2010

La directora de la tesi doctoral

Dra. M. Cinta Bladé Segarra

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"És la teva tesis, fes el que vulguis"

Doctor. Josep Mª Del Bas Prior

Recuerdo la primera vez que oí estas palabras, era mi primer año de doctorado y le estaba pidiendo consejo a mi antecesor. Recuerdo como me sentí al oírlo, el corazón se me aceleró y pensé: "Esto va en serio, me he hecho mayor y me toca decidir por mi misma". Ahora después de cuatro años me doy cuenta que esa sensación te acompaña toda la vida cuando decides empezar una etapa, pero que es el primer sentimiento, luego se diluye y te hace más fuerte; maduras.

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A mis padres

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ABBREVIATION LIST

ABCA1: ATP-binding cassette transporter A1

ACC: Acetyl-CoA carboxylase

ACS: Acyl-CoA synthetase

ACSL: Long-chain acyl-CoA synthetase

ApoA-I: Apolipoprotein A-I

ApoA-II: Apolipoprotein A-II

ApoA-IV: Apolipoprotein A-IV

Apo A-V: Apolipoprotein A-V

ApoB-48: Apolipoprotein B-48

ApoB-100: Apolipoprotein B-100

Ap C: Apolipoprotein C

Apo CII: Apolipoprotein CII

Apo CIII: Apolipoprotein CIII

ApoE: Apolipoprotein E

BA: Bile acid

CE: Cholesterol ester

CETP: Cholesteryl ester transfer protein

CM: Chylomicrons

CPT1: Carnitine palmitoyl transferase 1

CVD: Cardiovascular disease

DAG: sn-1,2-diacylglycerol

DGAT: Diacylglycerol acyltransferase

EGF: Epidermal growth factor

ER: Endoplasmatic reticulum

FA: Fatty acid

FFA: Free fatty acid

FABP: Fatty acid binding protein

FABPpm: Plasma membrane fatty acid-binding protein

FAS: Fatty acid synthase

FAT/CD36: Fatty acid transporter

FATP4: Fatty acid transport protein 4

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FXR: Farnesoid X receptor

G-3-P: Glycerol-3-phosphate

HDL: High Density Lipoprotein

I-BABP: Ileal bile acid binding protein

I-FABP: Intestinal fatty acid binding protein

LCAT: Lecithin cholesterol acyl transferase

LCFA: Long-chain fatty acid

LDL: Low Density Lipoprotein

L-FABP: Liver fatty acid binding protein

LPL: Lipoprotein Lipase

LXR: Liver X receptors

LXRα: Liver X receptors alpha

LXRβ: Liver X receptors beta

MAG: sn-2-monoacylglycerol

mAspAT: Mitochondrial aspartate aminotransferase

MGAT: Monoacylglycerol acyltransferase

MTP: Microsomal triglyceride transfer protein

PA: Proanthocyanidin

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

PPAR: Peroxisome Proliferator-Acvtivated Receptor

PPARα: Peroxisome Proliferator-Acvtivated Receptor alpha

PPARγ: Peroxisome Proliferator-Acvtivated Receptor gamma

PPAR-δ: Peroxisome Proliferator-Acvtivated Receptor omega

PL: Phospholipids

PYY: Hormone peptide YY

RLP-C: Serum remnant lipoprotein cholesterol

ROR: Retinoic related orphan receptor

RXR: Retinoid-X-receptor

SCD: Stearoyl-CoA desaturase

SREBP: Sterol regulatory element-binding protein

TG: Triacylglycerides

TRL: TG-rich lipoproteins

VLCFA: Very long chain fatty acid

VLDL: Very Low Density Lipoprotein

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I. INTRODUCTION

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1. Lipid metabolism

Lipids are an important energy source out of food in the Western world. These represent a 30-40% of the daily caloric intake, equivalent to the adults' daily consumption of 60-120 g [1]. Lipids are important macronutrients for growth and development of all organisms. In fact, they are essential for energy homeostasis, reproductive and organ physiology, and numerous aspects of cellular biology [2]. In addition, they are also linked to many pathological processes, such as obesity, diabetes, heart diseases and inflammation [2]. In ligh of the evidence implicating lipids in the onset or development of chronic diseases, understanding the molecular basis of their action is crucial in order to understand their role in human health.

1.1 Lipid absorption

The intestine is the organ responsible for the absorption of lipids. The absorption process is very complex and involves many enzymes and transport proteins [1]. Lipid absorption is traditionally divided into three processes: Absorption into the enterocyte, intracellular processing and export to the mesenteric lymph.

1.1.1 Absorption into the enterocyte and the involved transport proteins

Prior to the absorption of dietary lipids, mostly triacylglycerides (TG) are hydrolysed within the lumen of the small intestine to long-chain fatty acid (LCFA) and sn-2-monoacylglycerol (MAG) by the pancreatic TG lipase [3].

Unlikely other cells that use LCFAs, such as adipocytes, myocites and hepatocytes, enterocytes own a particular microenvironment at the brush border membrane. In fact, enterocytes contain proton pumps in the apical membrane that generates a pH gradient [4]. When the local pH is lower than the LCFA pKa, the pumps donate their protons, causing the micelles dissociation. Thus, the enterocytes internalise the protonated LCFAs by simple diffusion [4]. In paralell with the simple diffusion, the protein-mediated transport takes place, a process in which different proteins, that share a high affinity with LCFA, are involved. These proteins are *plasma membrane fatty acid-binding protein* (FABPpm), *fatty acid transport protein*4 (FATP4) and *fatty acid transporter* (FAT/CD36) [4].

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Berk and his collaborators [5] isolated the first putative fatty acid transporter (FABPpm) from solubilized rat liver plasma and jejunal microvillous membranes. FABPpm, a 43 kDa protein, is expressed in the intestine, the plasma membrane of liver, the adipose tissue, the cardiac muscle and the vascular endothelium, as well as in internal membranes [5].

In the gut, immunofluorescence studies show that this protein is located both in the apical and the basolateral membrane. It has high specification to bind itself not only to LCFA but also to lisophospholipids, MAG and cholesterol. Besides, a study performed with antibodies anti FABPpm suggests its role as an LCFA transporter since it shows a partial inhibition of the oleic acid capture [4]. Currently, it seems less probable that FABPpm transports efficiently LCFA at an intestinal level since this protein appears at the intestinal crypt level, which is not involved in the nutrient absorption [4]. In addition, partial FABPpm protein sequencing revealed a striking similarity with mitochondrial aspartate aminotransferase (mAspAT) [5]. Further comparison demonstrated identical molecular mass, isoelectric point, electrophoretic and chromatographic behaviour, absorption spectra, enzymatic activity, affinity for fatty acid, subcellular distribution, and immunoligical cross-reactivity using respective antibodies [5]. Antibodies to mAspAT were able to inhibit the uptake of oleate into 3T3-L1 adipocytes, a fact that further supports the conclusion that these proteins were, in fact, identical [5]. Proteins' immunofluorescence showed that these are located both in the mitochondria and the plasmatic membrane [4]. All these features could explain that mAspAT/ FABPpm could work like an LCFA transporter.

The fatty acid translocase (FAT/CD36) is an 88 kDa glycoprotein cloned from the rat adipose tissue. It has a structure that can accept numerous molecules, such as 3 LCFA, oxidized LDL, phospholipids (PL) and thrombospondin. This protein appears in tissues that are involved in lipid metabolism, like the adipose tissue, the skeletal and cardiac muscle, the mammary gland and the intestine. Its function is the intraenterocyte transport of the LCFA [4]. In order to examine the role of the protein in LCFA uptake and adipose conversion, Sfeir et al. [6] used antisense expression of FAT/CD36 in 3T3-F442A preadipocyte cells. Expression of antisense mRNA correlated with a reduction in FAT/CD36 protein expression and reduced uptake of radiolabeled oleate. After a treatment with insulin and triidothyronine, antisense-expressing cell lines showed marked reduction in differentiation, as determined by little or no lipid accumulation and

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reduced expression of marker genes [6]. In addition, the treatment of Ob1771 preadipocytes with the LCFA palmitate, linolenate or oleate or the non-matabolizable fatty acid, 2-bromopalmitate, induced FAT/CD36 mRNA expression [7,8]. By contrast, the short chain fatty acid bromooctanoate had no effect [8].

More recent studies using FAT/CD36-null mice emphasised the role of the protein in fatty acid metabolism. FAT/CD36-null mice are phenotypically normal and experience normal fertility; However, these present several abnormalities in fatty acid metabolism and plasma profile [5]. Adipocytes from FAT/CD36-null mice showed a decrease in oleate uptake. Furthermore, the incorporation of radiolabeled fatty acid into TG decreased in the null adipocyte compared to the wildtype adipocyte; Nevertheless, the level of incorporation to diacylglycerol (DAG) increased. These changes in cellular lipid metabolism were associated with abnormal levels of blood lipids. The plasma of fasted null mice revealed higher significant levels of free fatty acids (FFA), TG and cholesterol than in wildtype mice. The increased cholesterol could be largely attributed to an increase of high density lipoprotein (HDL) particles, which were larger and experienced a bigger increase of PL content in null animals. The very low density lipoprotein (VLDL) particles were also altered since these showed an increase of associated TG [9].

In contrast to the FAT/CD36-null mice, Ibrahimi et al. [10] generated transgenic mice, which overexpress FAT/CD36 selectivelly in muscle tissue. These mice have virtually complementary abnormalities compared to the FAT/CD36-null mice, according to their plasma profiles. Trangenic mice presented a 30% less of plasma TG. The most pronounced difference appeared in the VLDL fraction, wich lost a 40% of its TG content. These also showed less plasma cholesteron and FFA [10].

At the intestinal level, FAT/CD36 is mostly present at the duojejunal villi, where lipid absorption is carried out. Unlikely observations in muscle and adipocyte cells, the invalidation of the FAT/CD36 gene did not prevent the FFA uptake by the enterocytes of FAT/CD36-null mice [4]. In addition, FAT/CD36-null mice who are treated with a lipid gavage suffer from disturbances in the FFA intraenterocyte metabolism resulting in a retention of the citoplasmatic TG. These alterations might slow down the TG secretion through the lymph and might decrease the chylomicrons (CM) size. These results suggest that, in the intestine, FAT/CD36 is involved in the lipid absorption and not in an efficiently membrane transport [4].

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The fatty acid transport protein 4 (FATP4) is a 63 kDa membrane protein implied in the capture of LCFA. It belongs to the brush border of the jejunum and ileum enterocytes. *In vitro,* the overexpression of this protein contributes to the capture of LCFA. On the contrary, the dowregulation of the gene FATP4 is followed by a proportional decrease of the LCFA capture [4]. *In vivo*, mice FAT (+/-) that have a 48% of the protein limited showed a 40% of reduction in the LCFA capture by their enterocytes [4]. Jointly, these results show that the FATP4 participates in the intestinal uptake of the LCFA. However, many arguments suggest that this is an indirect effect [4]. Firstly, predictive FATP structure indicates that the protein is mostly citosolic and does not have a short transmembrane sequence with an LCFA affinity [4]. These structural features seem hardly compatible with the function of the membrane transporter. On the other hand, there is a sequence homology between FATP4 and the acyl-CoA synthetases (ACS), which are proteins that catalyses the transformation of the FA to acyl-CoA [4]. Finally, the transfection of a FATP4 expression vector to the COS-1 cells is accompanied by the activation of ACS producing the acylation of the LCFA and the very long chain fatty acid (VLCFA) [4]. Thus, it is likely that changes in FATP4 gene expression could influence on the uptake and the acylation of LCFA [4].

In conclusion, current data indicate that the FABPpm, FAT/CD36 and FATP4 are not efficiently LCFA transporters in the small intestine. However, FAT/CD36 and FATP4 facilitate the lipid absorption mediating the maintenance of the pH gradient to stimulate the simple diffusion [4].

1.1.2 Intracellular transport of LCFA: the role of FABPs

Once the products of the lipid digestion enter into the enterocyte, they must go through the cytoplasm to the endoplasmatic reticulum (ER), where the resynthesis of clomplex lipids occurs [3]. The fatty acid binding protein (FABP) superfamily is constituted by 14-15 kDa soluble proteins which bind with a high affinity to either LCFAs, bile acids (BAs) or retinoids. In the small intestine, three different FABP isoforms that show a high affinity with LCFAs and/or BAs are expressed: the intestinal and liver-type (I-FABP and L-FABP) and the ileal bile acid binding protein (I-BABP) [11]. The respective localisations along the small intestine and the binding properties of I-FABP, L-FABP and I-BABP are the origin of a cellular specification. The ileum, which plays a

critical role in the enterohepatic circulation of BA, is the only intestinal segment where the I-BABP gene is expressed. On the other hand, I-FABP mRNA levels increase from the duodenum to reach the highest levels in the proximal ileum [11], while the highest L-FABP expression occurs in the proximal jejunum. This correlation of expression with the site of maximal lipid absorption has long reinforced the hypothesis that L-FABP function carries out the intestinal lipid assimilation [12]. Immunocytochemical localisation in the intestine of rats demonstrated that L-FABP appeared in the intestinal apical side of fasted animals; However, after performing fat feeding, L-FABP was found to be distributed in the entire cytoplasm [12]. Experiments evaluating the rate of diffussion of fluorescent probe N-(7-nitro-2,1,3-benzoxadiazol-4-yl)-stearate in the liver and HepG2 cells also provide strong support for explaining the role of L-FABP in intracellular transport of FAs [12].

Similarly, the comparison of the binding properties reveals strong differences between these 3 FABPs. In fact, I-FABP only binds LCFA with a ratio of one FA for one protein. By contrast, L-FABP can bind 2 LCFA as well as large number of bulky hydrophobic molecules, including BAs, various xenobiotics and carcinogens, but generally it binds with a ratio of 1 for 1 [11]. Although L-FABP and I-FABP present a similar affinity to saturated LCFA, I-FABP shows a lower affinity with unsaturated FA than L-FABP [11]. Besides differences in the location and their binding properties, they are also different at the gene expression level. L-FABP and I-BABP are specifically up-regulated by their main ligands, in contrast with I-FABP.

L-FABP gene expression is transcriptionally up-regulated by LCFA in both the liver and the small intestine. This regulation is mediated by a familiy of nuclear receptors known as Peroxisome Proliferator-Activated Receptors (PPARs). After being activated by LCFA, PPAR binds as an heterodimer with the retinoid-X-receptor (RXR) so as to activate specific responsive element (PPRE), generally located in the promoter of target genes [11]. However, different PPARs regulate the transcription of the L-FABP gene in the liver and the intestine since the PPAR-α/RXR heterodimer functions in the liver whereas PPAR-δ/RXR induces L-FABP in the intestine. On the other hand, different regulatory systems seem involved in the control of the I-FABP expression. The expression of the I-FABP is induced by the hormone peptide YY (PYY), which is secreted by ileal endocrine cells when the dietary lipid reaches the distal part of the

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small intestine [12]. Furthermore, the I-FABP promoter has not been reported to contain a peroxisome proliferator response element [12].

The roles of soluble FABPs seem more complex than those that are generally assigned to itself, i.e. ligand desorption from plasma membrane and facilitation of their intracellular diffusion. On the one hand, I-FABP is involved in the TG synthesis and secretion while L-FABP might be preferentially implicated in PL synthesis, membrane protection and gene regulation [11]. The study of a human polymorphism in the gene encoding for I-FABP has greatly contributed to a better understanding of its physiological importance in the TG-rich lipoprotein (TRL) synthesis. In addition, Pima indians has a substitution of an Ala by a Thr in the codon 54 of I-FABP. This substitution is associated with a high TG plasma level, an insulin resistance, and an increase in the body mass index [11]. As a consequence of this assumption, a dramatic rise in LCFA transport and TG secretion is found in Thr^{54}-I-FABP-transfected Caco-2 cells compared with cells transfected the wild isoform [13]. Taking these data altogether strongly suggest that I-FABP is involved in targeting dietary LCFA towards the endoplasmatic reticulum, where they participate in the synthesis of TRL. Nevertheless, LCFA are mainly bounded to L-FABP in the intestinal cells. Thus, it is likely to happen that L-FABP can act as a buffer protein protecting the cell against the harmful effect of an excess of FFA [11]. Furthermore, as it is previously explained, the lipid content of the diet modulates L-FABP gene expression; This regulation might be essential to the maintenance of a functional integrity of the intestinal mucosa. Such buffering action is also likely to be shared by I-BABP,which might greatly contribute to maintain an efficient FA absorption and enterohepatic circulation of BAs [11].

In conclusion, I-FABP, whose the expression is strictly restricted to the small intestine, seems preferentially involved in the supply of dietary lipids to the organism. On the other hand, L-FABP would rather have a buffering action through the control of the intracellular un-esterified FA, which might not only protect the cell against the detergent effects of FFA, but also plays an indirect, but crucial role, in gene regulation [11].

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1.1.3 Resynthesis of TG from dietary substances

Once the FA and MAG are translocated to the ER by FABP, the acyl-CoAs are preferentially oriented to the esterification of TG, predominantly through the progressive acylation of MAG (Figure 1). TG can also be synthesised by a separate route that starts acylating glycerol-3-phosphate (G-3-P) to form phosphatidic acid (Figure 1); it continues dephosphorylating the phospholipid to sn-1,2-diacylglycerol (DAG), and it ends acylating the DAG to TG (Figure 1).

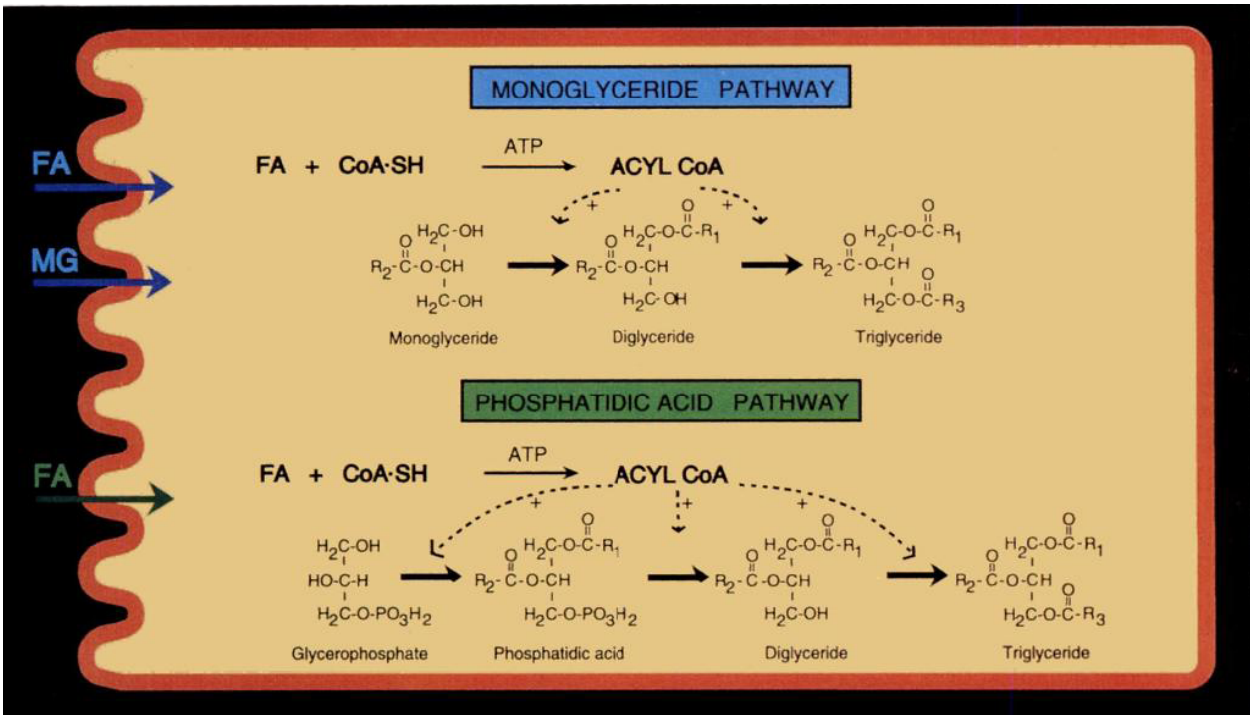

Fig. 1. Overall of TG biosynthesis in the intestinal mucosa and Caco cells [19].

It is important to note that the DAG synthesised from MAG and G-3-P are metabollicaly inequivalent: The DAG synthesised from MAG is focused on TG synthesis only whereas the DAG derived from G-3-P may be used to synthesise either TG or PL [3].

MAG pathway would predominate in the postprandial period, while the G-3-P pathway is the main one in the interprandial and fasted period (Figure 2) [4]. For example, oleate entering from the apical membrane is shunted preferentially into the MAG pathway to form TG whereas oleate entering from the basolateral membrane from the circulation is

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shunted into G-3-P acylation pathway (Figure 2). The required enzyme activating the FA prior to its incorporation into MAG or G-3-P may account for the delivery of FA to separate TG synthetic pathway (Figure 2). This enzyme is one of the five members of the acyl-CoA synthetase long chain family (ACSL) (Figure 2) [3]. Only ACSL3 and 5 are significantly expressed in the intestine (Figure 2) [14]. Within this suggested scenario, for example, oleate-CoA delivered by ACSL5 would be directed to the MAG pathway and by the ACSL3 to the G-3-P pathway (Figure 2) [3].

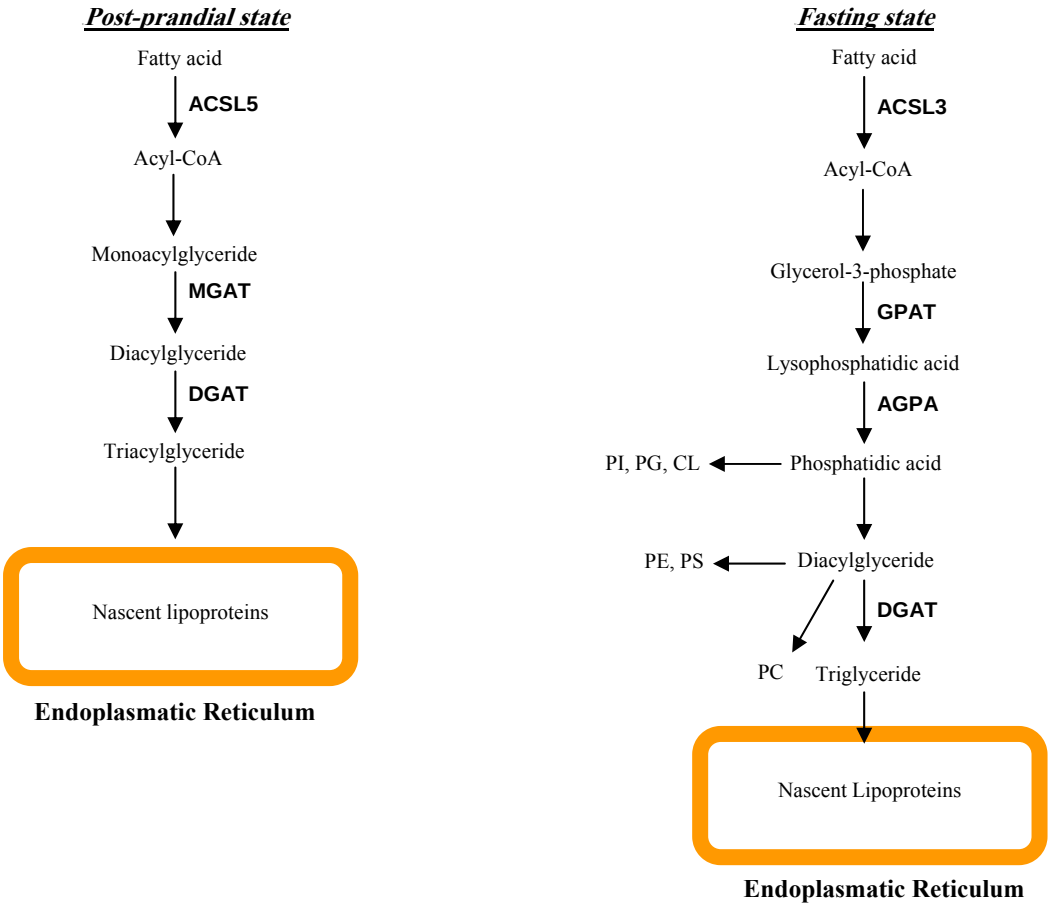

Figure 2. Liporotein synthesised pathway in the post-prandial and the fasting state [3]**.**

Several monoacylglycerol acyltransferase (MGAT) isoforms may participate in MAG acylation. Of the three isoforms MGAT (1-3), only MGAT2 and 3 are expressed in the intestine [3]. MGAT2 correlates better with lipid absorption than MGAT3 [15]. This conclusion is based on the findings that supports that MAGT2 is an ER-localised enzyme that is maximally expressed in the proximal intestine. Furthermore, MGAT2

protein expression and activity increase in response to lipid feeding. In contrast, MGAT3 is expressed at the very end of the distal intestine and it does not respond to lipid feeding [3]. MGAT preferentially acylates sn-2-MAG producing sn-1,2-DAG.

The sn-1,2-DAG produced is acylated to TG by diacylglycerol acyltransferase (DGAT) [3]. DGAT is an integral membrane protein that catalyses the final enzymatic step in the production of TG in mammals [16]. DGAT is found in most of the tissues of the body and has a high expression in the adipose tissue, the liver, and the small intestine. Two DGATs that are encoded by two different gene families have been identified: DGAT1 and DGAT2. Several studies indicate that both DGAT1 and DGAT2 play important roles in the TG synthesis [17]. Several lines of evidence clearly demonstrated that both DGAT1 and DGAT2 function as DGAT enzymes. Firstly, the overexpression of either DGAT1 or DGAT2 in mammalian cell lines increased *in vitro* DGAT activity, generating TG from a variety of FA and DAG substrates [18]. Secondly, the overexpression of each enzyme increased the de novo synthesis and the accumulation of TG in intact cells [18]. In addition, tissues out of deficient mice in each enzyme presented a decreased DGAT activity and TG levels [18]. On the other hand, several competition assays have studied the preference of each enzyme for FA substrates of specific length and desaturation; DGAT1 preferred a monounsaturated substrate, oleoyl-CoA (18:1), instead of saturated palmitoyl-CoA (16:0). DGAT2 did not show such a preference even though the DGAT2 enzyme purified from *M. ramanniana* presented an enhanced DGAT activity towards medium-chain fatty acyl-CoAs (12:0) instead of long-chain fatty acyl-CoAs (18:1) [18]. DGAT1 has acyltransferase activities beyond that of esterifing DAG *in vitro* whereas DGAT2 does not. For instance, DGAT1 is a potent acyl-CoA:retinol acyltransferase, which catalyses the synthesis of retinyl esters from retinol and fatty acyl-CoA substrates. Furthermore, DGAT1 accounts for the majority of acyl-CoA:retinol acyltransferase activity in differentiated Caco2 cells, a model of enterocytes [18].

In summary, two major pathways for TG biosynthesis are known: The glycerol phosphate pathway and the monoacylglycerol pathway. In the final reaction of both pathways DGAT forms TG. The newly synthesised TGs are thought to be released into the associated lipid bilayer, where they are channeled into cytosolic lipid dropets or, in cells that secrete TG, nascent lipoproteins [18].

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1.1.4 Intestinal lipoproteins in fed and fasted states

Newly formed TGs are packed into lipoproteins in the enterocyte. These lipoproteins are stable for transport in the aqueous environment [20]. The small intestine is an important source of plasma lipoproteins, just after the liver. The intestine secretes several different lipoproteins; CM and VLDLs are the major ones [20]. VLDL assembly occurs constitutively. VLDLs are the predominant lipoproteins during the fasting state [21]. VLDL may serve to transport lipids derived from the bile and fatty acids derived from the plasma [21]. In the postprandial state, CM secretion is induced after a fat ingestion and is impaired in the absence of bile acids [21]. For instance, during the infusion of micelles containing linoleic acid and monoolein, increases in exogenous TG levels of whole lymph are entirely accounted for by increases in exogenous CM TGs [20]. Infusion of palmitic acid, however, resulted in increases of exogenous lymph TG levels in both CM and VLDLs [20], where the major contributor was CM. Rat intestine also secretes characteristic forms of HDL in the mesenteric lymph. Two types of HDL were found in intestinal lymph, a discoid nascent particle deficient in cholesterol ester and rich in apolipoprotein A-I (ApoA-I) and one spherical HDL derived from plasma [20].

Nevertheless, we will limit our explanation to CM and VLDL in this introduction since they are the major lipoproteins in the intestine and the main transporters of TG.

CMs are spherical TRL particles synthesised by intestinal epithelial cells and they also are the major lipoproteins secreted in the small intestine to transport lipids after a meal [20]. The major lipid components of CM are TG, cholesterol ester (CE), free cholesterol and PL and their structural protein is apolipoprotein (apo) B-48. The process of CM and VLDL formation takes place in the Golgi apparatus (Figure 3), and the movement of TG from the ER to the Golgi apparatus (Figure 3) appers to be the rate-limiting step in intestinal TG transports [22]. However, the assembly of intestinal CM and VLDL may occur by two independent pathways (Figure 3).

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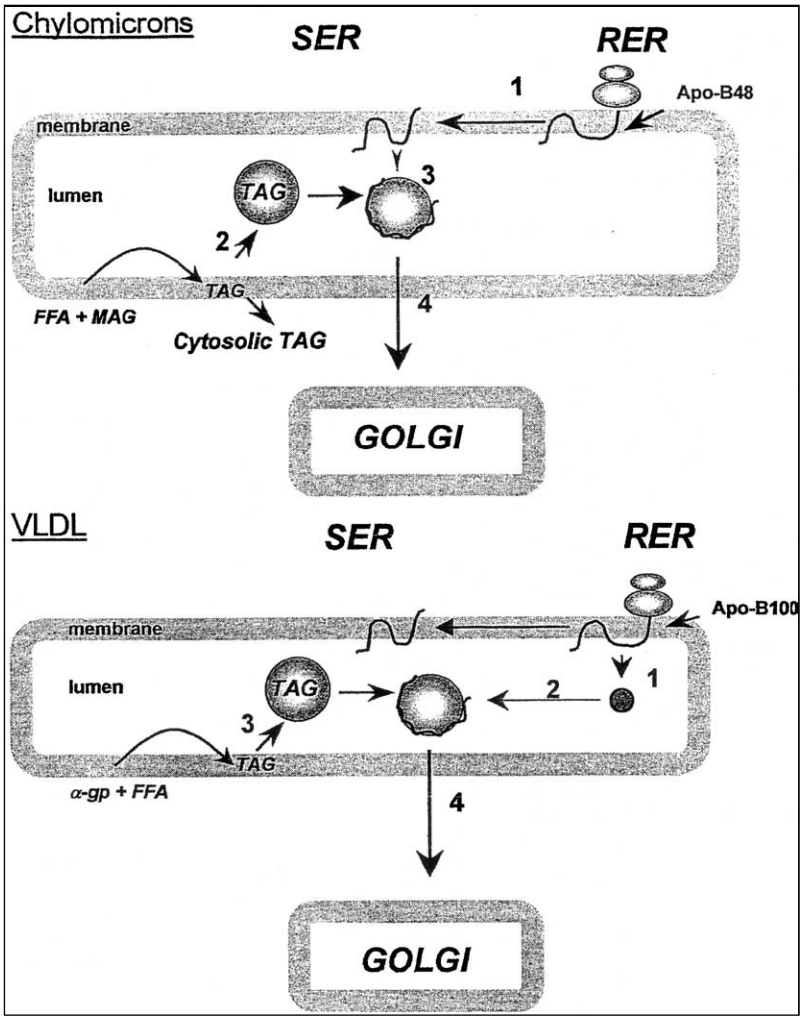

Figure 3. Comparison of models for CM and VLDL assembly [20].

On the one hand, the union of TG with apo B-48, essential for CM formation, is a complex physicochemical process in which microsomal triglyceride transfer protein (MTP) plays a pivotal role (Table 1) [1]. Some studies have been confirmed that MTP is required for the secretion of apo B (Table 1). Abetalipoproteinemia, the generalized fat malabsorption occurring in a rare autosomal recessive disease, is characterized by the inability of intestinal and hepatic cells to secrete apo B, and caused by mutations in the MTP gene that determine the absence of the protein [23][24]. In addition, MTP plays a comparable role in the assembly of TG with apo B-100 required for the formation and secretion of VLDL (Table 1) [25].

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Table 1. Microsomal Triacylglycerol transfer protein (MTP): Function, structure and expression

Function
Facilities assembly and secretion of apo B-containing lipoproteins (CM and VLDL) by transfer of TG, cholesteryl ester and phospholipid to nascent apoprotein.
Structure
Heterodimer.
59 kDa protein disulfide isomerase (*EC* 5.3.4.1)
97 kDa unique large subunit, with extensive homology to amphibian *Xenopus laevis* lipovitellin, a lipid-binding protein synthesized in the liver and found in the egg.
Expression of MTP large subunit
Gene of 55-60 kb, eighteen exons, seventeen introns, on human chromosome 4(q24).
Expressed mainly in the endoplasmic reticulum of intestine and liver.
Expression of MTP increased by:
Dietary saturated fatty acid
Dietary cholesterol
Expression of MTP decreased by:
Insulin
Ethanol consumption

On the other hand, VLDL-size particles are formed when the level of lipids is too low to drive the formation of CMs. VLDL differs from CM in its density, size, lipid content and composition, as well as in its protein content. In a study of intestinal absorptive cells from fasted rats, VLDL-sized particles were found in the ER and the Golgi apparatus [20]. It provides further evidence for the production of VLDLs in absorptive cells of fasted rat and human intestine and it also supports the assumption that the small intestine is a source of endogenous plasma VLDLs.

Luchoomun and Hussain [26] studied the assembly and secretion of CMs by differentiated Caco-2 cells. The apical media of the cells were supplemented with oleic acid together with either albumin or taurocholate. They demonstrated that CM secretion was similarly inhibited by the hydrophobic surfactant Pluronic L-81 in cell cultures as in animal studies [27][28], suggesting that differentiated Caco-2 cells are similar to enterocyte *in vivo*. They also found that apoB was secreted as VLDL- and LDL-size particles in the absence of oleic acid, supporting the evidence that VLDLs are the major

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lipoproteins during fasting [20]. The addition of oleic acid (≥0.8 mM) with taurocholate to the cells resulted in the secretion of one-third of apoB as CM, suggesting the CMs are the major lipoproteins to transport lipids [20]. In order to identify the source of lipids used for lipoprotein assembly, [^{14}C]glycerol, oleic acid and taurocholate were supplied to the cells labeled previosly with [^{3}H]glycerol to induce CM assembly and to radiolalabel nascent lipids. All the lipoproteins contained higher amounts of preformed PL compared with nascent PL [26]. VLDL contained equal amounts of nascent and preformed TG, whereas CM contained higher amounts of nascent TG even when nascent TG constituted a small fraction of the total cellular pool [20]. These studies indicate that nascent TGs and preformed PL are preferentially used for CM assembly and provide a molecular explanation for the *in vivo* observations that suggest that the fatty acid composition of TGs, but not PL, of secreted CMs reflects the composition of dietary lipids [20].

1.1.5 Apolipoproteins in the intestinal lipoproteins

Apolipoproteins play an important role in lipoprotein clearance and metabolism even though CM and VLDL contain a low quantity of them. CMs and VLDLs synthesised in the intestine contain several different apolipoproteins, such as apoB-48, apoB-100, apoA-I, apoA-IV, apoA-II, apoC and apoE. ApoA-IV comprises a 10-13% of CM apoproteins and a 24-30% of intestinal VLDLs [20].

ApoBs play a particularly critical role in the assembly of the TRL (Figure 3). mRNA editing is an important process for the generation of apoB. As a result of these process, the co-existence of two species of apoB, apoB-100 (550 KDa) and apoB-48 (260 KDa), occurs. Human enterocytes have the potential to secrete both apoB-48 and apoB-100 [20]. It is generally believed that the main difference between CM and VLDL is that CM contains apoB-48 whereas VLDL contains apoB-100 [20]. However, a study on the differention of the Caco-2 cells into enterocyte-like cells showed that both apoB-100 and apoB-48 can form large CM when these cells are treated with high concentrations of FA [26]. Davidson et al [29] studied the apoB synthesis in the rat enterocyte *in vivo* and found that the intestinal apoB synthesis was not affected by TG flux. However, biliary lipid flux plays an important role in the regulation of apoB metabolism; External bile diversion resulted in a fall of apoB synthesis of a 43 and a 70% in jejunal and ileal,

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respectively. Their results suggest that intestinal aposynthesis depend on bile salt but not on the regulation of the TG flux.

1.2 The liver in the metabolism of plasma lipoproteins

The liver synthesises daily lipids that are released as VLDL into the blood flow by exocytosis. The mechanism for synthesis and secretion of VLDL from liver is well known (Adeli et al., 2001). ApoB100 (and apoB48 in a few species) is the key component whose rate of synthesis in the rough endoplasmatic reticulum (RER) controls the overall rate of VLDL production [30]. Lipid components that are synthesised in the smooth endoplasmatic reticulum (SER) are added to apoB by MTP [30] as it moves to the junction of the two compartments. Apoproteins are glycosylated after being carried to the Golgi apparatus by means of transport vesicles. Secretory vesicles bud off the Golgi membrane, migrate to the sinusoïdal membrane of the hepatocyte and release the VLDL into the blood [30].

Animal models have shown that the availability of FA is not the only or the main determinant of the rate of the VLDL production [30]. The inhibition of MTP blocks the assembly and secretion of VLDL and CM, but leads to steatosis, at least in mice [30]. Besides, there are important differences among species as for the ability to export TG as VLDL from the liver, despite similar rates of esterification of FA to TG. It has been suggested that among different species, the rate of export of TG from the liver is proportional to the capacity of *De novo* FA synthesis [30]. In addition, the sterol regulatory element-binding protein (SREBP) family has been established as a physiological regulator of lipid synthesis [31]. Mice that overexpress SREBP1a and SREBP1c display enhanced TG synthesis in the liver [32]. Once activated, SREBP1 modulates several genes from the TG synthesis pathway, such as Fatty acid synthase (FAS), Acetyl-CoA carboxylase (ACC) and Stearoyl-CoA desaturase (SCD), among others [32]. Besides, the control of lipogenesis, SREBPs are implicated in the control of VLDL synthesis and secretion [32]. Thus, the levels of mature SREBP1 were directly correlated with of the secretion rate of the ApoB in McArdle rat hepatoma cells, while in transgenic mice that overexpress SREBP1 and lack LDLR, plasma TG levels pointed to a severe hypertriglyceridemia, ascribed to an increase of the secretion of VLDL [32].

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These findings suggest that SREBP1 could be a limitation in VLDL synthesis and secretion.

On the other hand, the origin of the FAs that incorporate into TG can affect the rate of VLDL export. In the case of obese mice, *De novo* lipogenesis in the liver does not stimulate VLDL output [30]. As for rats, high carbohydrate diets enhance the hepatic output of TG-VLDL, but this increase of TG secretion is accomplished by enhanced formation of TG-VLDL from exogenous non-esterified free fatty acid (NEFA) rather from synthesised *De novo* FA in the liver [30]. In fact, plasma NEFAs are mainly transported to the liver, where these are used for the esterification in TG or β-oxidation [33]. Plasma NEFA, therefore, seems to play an important role in the enhancement of the hepatic esterification and the stimulation of the VLDL production [30].

Additionally, the relationship between FA oxidation and esterification has been described as a key factor in regulating VLDL synthesis [34]. The importance of β-oxidation is illustrated by the severe phenotype of humans and mice with impaired β-oxidation. Deficiency of hepatic β-oxidation enzymes results in the plasma hypoketosis and the fatty liver [35]. As for the β-oxidation, the rate-limiting enzyme is the carnitine palmitoyl transferase 1 (CPT1), which couples long-chain FAs to carnitine in order to transport them into the mitochondria [35]. Transcriptional regulation of CPT1 involves several other transcriptional factors, including the peroxisomal proliferator-activated receptor gamma coactivator-1 (PGC-1). PGC-1α is able to stimulate CPT1 expression both in the liver and the heart [36]. PGC-1 acts via hepatocyte nuclear recepetor 4, PPARα and glucocorticoid receptor. PPARα also regulates CPT1 gene expression though these regulation does not reach significant levels. Besides, a peroxisome proliferator responsive element was identified in a conserved region of mammalian CPT1 [36]. Other data suggest CPT1 regulation is due to PPARα-independent pathways, at least in rodents. Therefore, the transcriptional regulation of CPT1 involves several independent process [36].

It is unknown where the limitation of VLDL synthesis or secretion resides, but it mainly depends on apoB synthesis, MTP activity, and lipid availability.

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1.3 Plasma lipoprotein metabolism

Table 2 summarises the classification and composition of lipoproteins, molecules that lead the transport of the lipids throughout the organism.

	Chylomicrons	*VLDL*	*LDL*	*HDL*
Density (g/ml)	< 0,95	0,95-1,006	1,006-1,063	1,062-1,21
Diameter (nm)	>70	30-90	18-22	5-12
Lipids (%)	98	92	78	50
Triglycerides (%)	86	55	6	4
Phospholipides (%)	7	18	22	22
Cholesterol free (%)	2	7	8	4
Cholesterol ester (%)	3	12	42	20
Proteins (%)	A-I, A-II, A-IV	B-100	B-100	A-I, A-II
	B48	C-I, C-II		C-I, C-II
	C-I, C-II	C-III		C-III
	C-III, E	E		D, E

Table 2. Classification and composition of lipoproteins

The intestinal lipoproteins do not enter the blood flow directly; These are secreted to the lymph and reach the liver for their own distribution around the body, instead. Once the intestinal lipoproteins are in contact with other plasma lipoproteins, a rapid transfer of proteins occurs. Thus, apo A-I and apoA-II enter the circulation of CM but are rapidly transferred to nascent HDL which come from intestinal [37]. Although traces of apo E, apo CII and apo CIII, important for the dislipemia of triglyceride-rich lipoproteins in peripheral tissues, are also associated with CM. These apos are mostly added on the surface of the particles after their interaction with other plasma lipoproteins [1]. The TG of CM are hydrolyzed extracellularly by the action of lipoprotein lipase (LPL) in peripheral tissues, especially in the adipose tissue, leading to a pronounced decrease of TG content of these lipoproteins [38].

LPL is a multifunctional enzyme produced by many tissues, including the adipose tissue, the cardiac and skeletal muscle, islets and macrophages. LPL is the rate-limiting enzyme for the hydrolysis of the TG core of circulating TRL, CM and VLDL [39].

Besides its hydrolytic activity, LPL can interact with lipoproteins to anchor them to the vessel wall and facilitate lipoprotein particle uptake. LPL has also been shown to promote the exchange of lipids among lipoproteins, playing an important role in the kinetics of the majority of plasma lipoprotein particles [39]. Furthermore, LPL can act as ligands for lipoprotein receptors to facilitate lipoprotein uptake [39].

The interaction of LPL and the TG of lipoproteins highly depends on apolipoproteins, such as apoCs (apo CI, apo CII and apo CIII), apo E and apo A-V [32]. Different studies have been focused on determining the role of these apolipoproteins in the modulation of the plasma lipid levels, mainly TG. It has been shown that these proteins modulate the activity of LPL in different ways, and consequently affect the hydrolysis of TG from the lipoproteins [32]. Thus, apo CII and apo A-V have been described as activators of LPL. In turn, apo CIII and apo E are known inhibitors of the activity of this lipase [32]. In addition, not only the type of apolipoprotein is a critical factor in the rate of TG hydrolisis and release from lipoproteins, but also, the interactions between them play a key role in modulation of LPL activity [32]. Thus, in patients with hypertriglyceridemia, apo A-V levels were paradoxically high while this apolipoprotein had emerged as an activator of LPL activity. The explanation was found in the elevated levels of apo CIII, a known repressor of LPL activity. The correlation of plasma TG, apo A-V and apo CIII showed that those complex interactions between both apolipoproteins are even more important than the activity of these proteins on their own [32].

The metabolization of CM by LPL results in chylomicron remnants (CMr), which are smaller, own a higher density and are proportionally more enriched in cholesterol and apo E. This configuration allows them to be recognised by specific hepatic receptors for apo E (called LRP or receptor-related protein of LDL) and be internalised [40].

In plasma, VLDLs experience a series of changes similar to those suffered by CM. They exchange esterified cholesterol to free cholesterol with the HDLs and receive more apo C and E from them; Moreover, their TGs are hydrolysed by the LPL so as to provide essential fatty acids to the underlying tissues. Thus, VLDL become Intermediate Density Lipoproteins (IDLs), which are smaller, denser and are proportionally enriched in cholesterol esters and apo E [41]. The IDLs may follow different paths: Those with higher content of apo E, are recognised by receptors LRP and the LDL recceptor (LDL-

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R), both present in the liver, and these are internalised; Another proportion of IDL is transformed in Low Density Lipoprotein (LDL) [42].

The LDL has a plasmatic origin as a result of the action of the LPL on VLDL and the exchange of components with other plasma lipoproteins. The formed LDLs are smaller, contain a higher density than VLDL. Besides, LDLs contains only one molecule of apo B-100, and the most important lipid fraction is esterified cholesterol. Its function is to transport cholesterol to peripheral tissues and the liver. The liver is the most important organ in terms of LDL uptake, in a quantitatively way, followed by the intestine, the steroidogenic glands and macrophages. The LDL is taken up by the LDL-R, which recognises apo B-100, in a process of endocytosis. The LDL-R is a transmembrane glycoprotein wich internalised the LDL forming a vesicle. This vesicle will turn into an endosome, which merges with a lysosome. This hybrid is hydrolysed by lysosomal enzyme forming cholesterol esters and leaving them ready to be used by the cells [43].

Besides, the liver plays an important role in the elimination of peripheral cholesterol, controlling the synthesis and secretion of HDL. HDL lipoproteins are the smallest and densest; They have an intestinal and hepatic origin and are rich in PL and apo AI. Initially, apo AI is secreted from liver associated with PL forming the nascent HDL in plasma, which then removes cholesterol from the peripheral tissues [32]. The delivery of cholesterol from extrahepatic cells to HDL particles is mediated through the ATP-binding cassette transporter 1 (ABCA1) [32]. Subsequently, cholesterol is esterified by Lecithin cholesterol acyl transferase (LCAT), a esterase synthesised by the liver and located in the surface of HDL [32]. Thus, cholesteryl esters remain in the nuclei of HDL while phospholipids and free cholesterol form the surface. Moreover, HDL exchange cholesterol and TG with VLDL particles by mediation of cholesteryl ester transfer protein (CETP). The lipid exchange between these lipoproteins is important in that it moves peripheral cholesterol excess into metabolic disposal or recycling process [32]. In parallel, PL and cholesterol from VLDL can be transferred to HDL by action of phospholipid transfer protein (PLTP) [32]. Large HDLs are then internalised by the liver through a process that involves TG hydrolisis by Hepatic Lipase (HL) and cholesteryl esters uptake by liver through SR-BI [32]. Finally, apo AI is internalised for recycling. Therefore, HDL are responsible for the reverse cholesterol transport.

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1.4 Nuclear receptors in the control of lipid metabolism

Several studies have implicated nuclear receptors in the control of lipid homeostasis, establishing a co-ordinated net of metabolic sensors, such as lipid metabolism, inflammation, drug metabolism, bile acid synthesis and glucose homeostasis among other processes [32]. The structure of these proteins contains, ideally, a ligand-binding domain that allows the binding of one or more ligands; A DNA binding domain to recognise conserved sequences in the promoter of different genes; And different interaction domains to allow the modulation of their activity by co-activators, co-repressors, phsophorylation/dephosphorylation and other nuclear receptors [32]. This structure provides the ability of acting as metabolite sensors to nuclear receptors, being activated by endogenous and exogenous molecules and subsequently triggering or repressing gene expression in a co-ordinated way [32]. Some of these receptors, such as Farnesoid X receptor (FXR), Liver X receptors (LXR) or PPARs, heterodimerise with other nuclear receptors, usually Retinoic X receptor (RXR), in order to bind DNA [32]. In general, inactivated nuclear receptors form a complex with co-repressors that inhibit their transcriptional activity, often through the recruitment of histone deacetylases [45]. The activation of the receptor by ligand binding and/or phosphorilation induces a conformational change, resulting in the dissociation of the co-repressor and the recruitment of a co-activator complex that facilites target gene transcription [45].

Thus, polyunsaturated fatty acids, ecosanoids, and various synthetic ligands are the main ligands of PPAR, which activates the genes for fatty acid catabolism [45]. There are three members of the PPAR family: PPAR-α, PPAR-γ and PPAR-δ and these are conserved in a 60-80% within their DNA- and ligand-binding domains [46]. PPAR-α is most prominently expressed in the liver, kidney, heart, skeletal muscle and brown adipose tissue. The role of PPAR-α in lipoprotein metabolism has been elucidated through the use of several natural and synthetic ligands [46]. Eicosanoids, FAs and drugs within the fibrate class can activate PPAR-α, resulting in the upregulation of genes involved in the uptake and β-oxidation of FAs [46]. This increase of β-oxidation decreases the pool of fatty acyl-coenzyme A needed for the TG biosyntesis and results in a reduction of serum TG levels [46]. In the liver, FAs can be oxidised in both peroxisomes and mitochondria. The contribution of hepatic peroxisomal oxidation in humans is unclear but it is thought that the mitochondrial β-oxidation is the major pathway [47]. PPAR-α is ten times more abundant in rodent livers than in human ones,

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and peroxisome proliferation appears to be a rodent-specific phenomenon [46]. In addition to the effects on FA oxidation, activation of PPAR-α decreases the expression of hepatic apo CIII and increases the expression of both LPL and ApoA-V [48]. The net effect of these changes consists on increasing the rate of TG hydrolysis and lowers the levels CM and VLDL in the plasma [46]. The TG content of LDL particles also lowers and results in a shift in LDL particle size towards less atherogenic and larger particles. HDL cholesterol levels can also be increased by PPAR-α activation through upregulation of apo-AI and apo-AII expression, the major lipoproteins of HDL. Finally, PPAR-α upregulation of ATP-binding cassette transporter A1 (ABCA1) can further raise HDL levels by promoting cholesterol efflux from macrophages, and macrophages TG levels can be lowered by means of the activation of macrophages LPL [49].

Another member of the family is PPAR-γ, which has three isoforms (PPAR-γ1, PPAR-γ2 and PPAR-γ3). PPAR-γ2 is expressed predominantly in the adipose tissue whereas PPAR-γ3 is expressed in the adipocytes and macrophages. PPAR-γ1 is more widely expressed than the other two isoforms [46]. In contrast to PPAR-α, PPAR-γ preferentially binds itself to polyunsaturated FAs. Several synthetic agonists have been developed, including the TZDs, which represent the knownest studied class of PPAR-γ agonists. TZDs are clinically used to treat insulin resistance and diabetes [50]. The activation of PPAR-γ by TZDs induces the expression of a set of genes involved in the adipocyte differentiation and lipogenesis, and these mechanisms are thought to be responsible for the insulin-sensitizing actions of these drugs. PPAR-γ activation results in the increase of FAs uptake by means of the subcutaneous adipose tissue. This, in turn, lowers circulating FFAs, thereby improving insulin resistance in the liver and skeletal muscle [46].

Up to know, little is known about the function of the last member of this family: PPAR-δ. Unlike the other members, PPAR-δ is ubiquitously expressed, overall in the brain, macrophages, lung, adipose tissue and skeletal muscle [46]. Prostanoids are the natural ligands for PPAR-δ. On the other hand, a potent selective synthetic agonist, GW501516, has been reported [51]. Recent data obtained from transgenic mouse models have implicated PPAR-δ as an important regulator of energy expenditure as well as glucose and lipid metabolism, highlighting the potential use of PPAR-δ modulators as therapeutic agents for type 2 diabetes, obesity and atherosclerosis [46]. The role of skeletal muscle as for mediating the effects of PPAR-δ activation is supported by

different studies, demonstrating that PPAR-δ activation induces the expression of several genes involved in lipid catabolism and energy expenditure in muscle cells [52]. Furthermore, the administration of GW501516 resulted in the increase of the FA oxidation and oxygen consumption in skeletal muscle, which correlated with a similar pattern of gene expression seen in muscle cells [53]. Moreover, muscle-specific overexpression of an activated PPAR-δ form resulted in a resistance to the diet-induced obesity, an increase of the metabolic rate and the TG utilisation. The resistance to body weight gain was caused by a decrease of both visceral and subcutaneous adipose depots [46]. Thus, the adipose tissue might also be a target for PPAR-δ. Mice overexpressing activated PPAR-δ in the adipose tissue reduced white adipose tissue depots and increased energy expenditure and lipid utilisation [54]. These changes correlated with the induction of genes involved in fatty acid oxidation and energy expenditure in brown and white adipose tissue. Moreover, these animals became resistant to diet-induced obesity and hyperlipidemia [54].

To sum up, the development of the combination of PPAR-α, PPAR-γ and PPAR-δ agonists have the potential to improve insulin resistance and dyslipidemia without causing weigth gain.

On the other hand, oxysterol is the natural ligand of LXR. This nuclear receptor controls a wide battery of genes related to cholesterol and bile acid metabolism, leading to a conversion of cholesterol to bile acid. There are two isoforms of LXR: LXRα and LXRβ. LXRα expression is highest in the liver and the intestine but it is also detected in macrophages, adipose tissue, kidney, lung and spleen, whereas LXRβ is ubiquitously expressed [46]. The role of LXR in the enterohepatic system and in macrophages is well-known and it is discussed below [46]. LXRs are involved in cholesterol homeostasis. Mice deficient in LXRα develop hepatomegaly and accumulate large quantities of cholesterol esters in their livers [55]. In rodents, LXRs control the catabolism of cholesterol by regulating the expression of the gene that encodes cytochrome P450 7 α-hydrolase, the rate-limiting enzyme in the conversion of cholesterol to bile acids [46]. LXR also enhaces reverse cholesterol transport by inducing the transcription of the gene that encodes ABCA1, involved in the efflux of cholesterol from lipid-laden macrophages to the liver. In addition, LXR upregulates ABCG1 in macrophages, which mediates cholesterol efflux to HDL particles, particularly HDL2 and HDL-3. LXR activation also leads to upregulation of ABCG5

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and ABCG8 in the intestine [46]. These two transporters mediate the reduction of the intestinal sterol absorption while these increase the sterol secretion from the liver into the bile. Mutations in these genes are associated with sitosterolemia and early-onset atherosclerosis [56]. In addition, LXR also regulates the expression of genes encoding apolipoproteins and enzymes involved in the reverse cholesterol pathway, including apo A-V, apo E, phospholipid transfer protein and LPL.

On the other hand, LXR also plays an important role in hepatic fat metabolism. The LXR effect on fat metabolism is mediated by a regulation of SREBP1c, which coordinately regulates the genes involved in FA and TG biosyntesis [57]. The administration of LXR agonists to mice increased SREBP1c expression and FA biosynthesis, and these effects were blocked in LXR-deficient mice [58]. Furthermore, the administration of an LXR agonist to diabetic mice (db/db) improved glycemic control but led to an increase of the lipogenesis, resulting in sever lipogenic pathology [59]. Conversely, LXR-deficient mice are protected from diet-induced obesity and have improved glycemic control, owing to decreasing hepatic FA synthesis and increasing energy expenditure [60]. It is now clear that the unwanted effects of LXR agonists on lipid response, including an increase of hepatic steatosis and the TG synthesis, are caused by the activation of SREBP1c, and that the beneficial effects result from the activation of the genes involved in the reverse cholesterol transport.

Similarly to the LXRs, FXR is overall expressed in the liver and the intestine, and serves as a bile acid sensor. Its natural ligands are bile acids, including cholic acid and chenodeoxycholic acid. Bile acids are important for the digestion and absorption of lipids, fat-soluble vitamins and cholesterol from the intestinal tract. Elevated bile acid levels are toxic, and thus their synthesis and enterohepatic circulation is tightly controlled [46]. FXR upregulates the expression of cytochrome P450 7 α-hydrolase 1 and 12α-hydrolase, an enzyme required for modulating bile acid hydrophobicity, by means of the recruitment of the small heterodimer partner (SHP). SHP is an orphan nuclear receptor (i.e. with unknown ligand), wich lacks a DNA binding domain that acts as co-repressor of conventional nuclear receptors [32]. It is expressed in the liver, small intestine, spleen, heart, pancreas, adrenal glands, ovary and testis [46]. SHP interacts with other nuclear receptors in order to inhibit their binding to the promoter of bile acid synthesis genes. As a result, the synthesis of bile acid decreases [32].

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In addition, FXR controls enterohepatic circulation of bile acids by regulating several transporters involved in efflux of bile acids from the liver, absorption by means of enterocytes and hepatic reuptake [46]. FXR has also been implicated in the regulation of lipid metabolism. Serum and hepatic TG, cholesterol levels and serum bile acids have increased in FXR-deficient mice [61,62]. Conversely, the administration of FXR agonists lowers TG levels by means of the inhibition of hepatic SREBP1c expression through a mechanism that might involve inhibition of LXR activation of SREBP1c [46]. Additional studies suggest that the increase of FA oxidation might also contribute to TG lowering. In fact, FXR can increase the expression of PPAR-α [63] and pyruvate deshydrogenase kinase 4 [64], and this fact promotes hepatic FA oxidation and the utilisation of fat. Recent data show FXR implication in the glucose metabolism [65]. The administration of bile acids to mice can prevent the induction of gluconeogenetic genes after fasting [66]. However, the treatment of non-fasted mice with an FXR agonist increased phosphoenolpyruvate carboxykinase expression and stimulated hepatic glucose output [67]. These seemingly discordant results probably result from different effects of bile acids under fasting and refeeding conditions. In fact, studies carried out with FXR-deficient animals demonstrate that FXR regulates the changes in the gene expression, which are responsible for the shift from hepatic glucose output to glucose utilisation that occurs after feeding [68]. Furthermore, a potent hypoglycemic and hypoinsulinemic effect was observed in obese, insulin-resistant mice since these overexpressed and activated FXR. Besides, the administration of an FXR agonist produced a similar effect, which was abrogated in FXR-deficient mice [46].

Thus, nuclear receptors represent novel targets on the development of therapeutic agents for treating numerous diseases, including type 2 diabetes, obesity, dyslipidemia, atherosclerosis and the metabolic syndrome.

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The table 3. Summary the PPAR, LXR and FXR effects on insulin sensitivity, lipid levels and cholesterol efflux [a].

	PPAR-α	***PPAR-γ***	***PPAR-δ***	***LXR***	***FXR***
Obesity and Lipids	↑ HDL ↓ TG ↓ FAs	↓Insulin Resistance ↑ Fat	↑↑ HDL, ↓ LDL ↓ TG ↓Insulin Resistance ↓ Fat	↑↑ HDL, ↓ LDL ↓ TG ↓Insulin Resistance ↑ Fat	↑↑ HDL, ↓ LDL ↓ TG ↓Insulin Resistance
Cholesterol efflux	↑Reverse cholesterol transport	↑Reverse cholesterol transport		↑Reverse cholesterol transport	↑Reverse cholesterol transport

Table 3. Nuclear receptors and their effects on different diseases [46]. [a] Directional arrows indicate an increase/decrease of the respective parameters.

1.5 Hypertriglyceridemia and diseases

As far as the Western diet is concerned, lipids represent more than a 40% of the daily caloric intake, while the nutritional advice is a 10% lower. This high fat supply associated with a qualitative imbalance (either an excess of plasma TRL, an excess of saturated fatty acids and cholesterol or the absence of LPL) greatly contributes not only to the increase of obesity prevalence among population, but also to the appearance of a plethora of diseases, such as atherosclerosis, non insulin-dependent diabetes, breast and colon carcers [11].

Hypercolesterolemia, especially when a high concentration of serum cholesterol in LDL occurs, is strongly related to the development of cardiovascular diseases (CVD). Recent epidemiologic studies have revealed that hypertriglyceridemia is also associated with atherosclerosis [69]. Atherosclerotic diseases with high TG levels can be found in patients with familiar hyperlipidemia combined with diabetes mellitus and metabolic syndrome, in which TRL, especially CMr and VLDL remnants, accumulate in the blood flow[69]. The remnant lipoproteins are, as well as the oxidized LDL, easily taken into the macrophage in the arterial wall, promoting foam cell formation of macrophages and forming the atherosclerotic lesion [70].

On the one hand, there are many reports about the evaluation of fasting and postprandial lipid levels in postprandial hyperlipidemic patients [69]. Tanaka et al.[70] demonstrated that serum remnant lipoprotein cholesterol (RLP-C) levels increased significantly in the postprandial state of patients with CVD or with insulin resistance. Additionally, high concentrations of RLP-C in the fasting state were also associated with the presence of CVD in dyslipidemia [71]. Kugiyama et al. [72] reported that higher levels of remnant lipoproteins in fasting serum predicted future coronary events in patients with CVD. On the other hand, an increase of postprandial TG levels may not only occur because of intestine-originated TRL (apoB-48-containing TRL), but also may be caused by liver-originated TRL (apoB-100-containing TRL) [69]. Cohn et al. [73] reported that the postprandial increase of TRL triglyceride level was predominantly (approximately 80%) due to an increase of apoB-48-containing TRL in normolipidemic male subjects with fat load-containing retinyl esters. Nevertheless, Karpe et al. [74] reported that VLDL were continuously secreted by the liver during the fasting state, and that the delipidation process was halted in the potprandial state, causing prolonged residence of VLDL remenants, which resulted from the competition by CM for the removal of TG by LPL [69].

LPL is an important marker for adipocyte differentiation, and LPL expression increases together with cellular TG accumulation in parallel as differentiated preadipocytes [39]. Although the adipose tissue can synthesise FFA *De novo*, FFAs for lipid storage are preferentially provided by LPL-mediated hydrolysis of plasma TRL. Different studies have been focused on determining the role of LPL in lipid metabolism and energy balance [39]. It has been shown that mice with a generalised deletion of LPL (LPL-/-) have three-fold higher plasma TG and seven-fold higher VLDL cholesterol levels at birth [39]. On the other hand, transgenic mice with a generalised overexpression of human LPL have a 5-fold higher LPL activity in the adipose tissue and 1.7-fold higher post-heparin plasma LPL activity with a 75% reduction in plasma TG [39]. Overexpression of LPL protects against diet-induced hypertriglyceridemia and hypercholesterolemia in these mice. It is interesting to note that overexpression of a catalytically inactive LPL also seems to improve the high-fat-diet-induced systemic insulin resistance and hypertriglyceridemia of these mice [39]. In addition, studies carried out in LPL+/- mice indicated that inactive LPL can act *in vivo* to mediate VLDL removal from plasma and uptake into the tissues in which it is expressed. LPL is, thus,

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considered a gatekeeper enzyme to play an important role in the initiation and/or development of obesity and hypertriglyceridemia [39].

Another link between obesity and metabolic complications is the excessive adipose tissue lipolysis. Under normal conditions, FFA released from adipose tissue is well regulated, allowing an appropriate availability of FFA to meet the energy requirements of the tissues [75]. An increase of adiposity can result in a relative excess of FFA release depending on the needs of the tissue. The resultant higher FFA concentrations can induce muscle and hepatic insulin resistance, endothelial and pancreatic β-cells dysfunction and increase VLDL-TG production [75]. Thus, although the adipose tissue is an excellent site for the storage of energy and can provide FFAs whenever lipid fuel is needed, an appropriate regulation of its function is necessary for them optimal health of the animals.

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2. Proanthocyanidins

Several epidemiological studies strongly suggest that proanthocyanidins, the most abundant polyphenols in human diet, protect against cardiovascular diseases (CVD). Despite the antioxidant and anti-inflammatory properties of these compounds, one of the mechanisms by which proanthocyanidins exert their cardiovascular protection is improving lipid homeostasis [76]. Therefore, the interest arisen by the protective properties of these polyphenolic compounds has placed them in the focus of the nutrition research [32].

2.1 Chemistry, human intake and metabolism

Proanthocyanidins (PA) are a class of phenolic compounds found in vegetables and derived foods such as beans, nuts, cocoa, tea and red wine [76]. These are the most structurally complex subclass of flavonoids and are represented by a large number of chemical structures. Nevertheless, all these structures are based on a benzenic ring condensed with a heterocyclic pyran that carries a phenyl benzene ring (Figure 4) [32]. PAs are the oligomeric and polymeric forms of flavan-3-ol or flavanols (Figure 4). These are condensed structures formed by polymerization of (+) catechin, (-) epicatechin and (-) epicatechin gallate [32]. The different oligomers ranging between 2 and 10 units are considered proanthocyanidins, while further polymerized structures are named tanins [76].

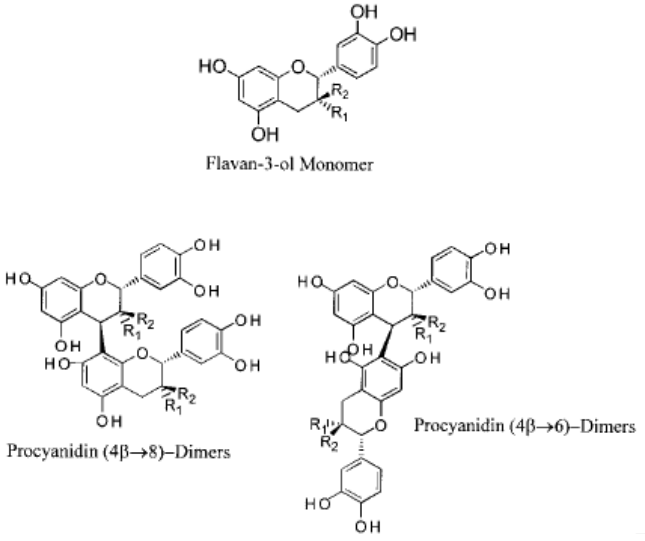

Figure 4. Chemistry structure of flavonoids

The average intake of flavonoids by humans remains unclear. Different works report flavonoid intake values ranging from 3 mg to 2 g per day [77][78]. However, the phenolic content of fruit, vegetables and other plant foods varies considerably, not only between different types but also between cultivars of the same type and can even depend on growing conditions and the time of harvest [76]. In addition, food processing can significantly influence the total content of PA and the profile of monomers and oligomers [76].

PAs are believed to exert their biological effects in different ways: As unabsorbable, complex structures with binding properties that can have local effects in the gastrointestinal tract; As absorbable PAs (probably low molecular-weight) and as absorbable metabolites from the colonic fermentation of PAs that have systemic effects on various organs [79]. Although long PAs are absorbed less efficiently than short ones in the small intestine, these may have important local functions in the gut [80], such as neutralizing oxidants and carcinogenic compounds [81], exhibiting immunomodulatory, and anti-inflammatory properties [82][83], and showing antibacterial activity towards pathogens [84]. During the small intestine digestion, higher PAs can form complexes with starch and proteins, resulting in the formation of less digestible complexes [79], and PAs can inhibit gastrointestinal lipase activity [76].

As far as experimental animals are concerned, various combinations of methylated, glucoronidated and sulfated derivates of flavan-3-ols, as well as native monomers, dimers and trimers, have been detected in body fluids and tissues after the ingestion of PA [85,86]. Recently, an improved liquid chromatography-tandem mass spectrometry method [87] has been used to analyse the rat plasma obtained 2 h after the ingestion of 1 g of grape seed procyanidin extract *per* kilogram of body weight. Conjugated forms were identified and quantified, founding different concentrations of catechin and epicatechin glucuronide, epicatechin methyl glucuronide and epicatechin methyl-sulfate [76]. Moreover, monomers, dimers and trimers in their native form also were detected and quantified in plasma samples. Thus, flavan-3-ols predominantly exist in their modified form in plasma even though the intact molecules have been found at a micromolar level [76]. A study have demonstrated that dimeric and trimeric forms of PA can be found within rat urine after administrating grape seed extract orally, while catechin metabolites can be found in the kidney and the liver [32]. Two studies have reported the detection of dimer B1 and B2 in plasma of humans, in which the volunteers

consumed approximately 2 g of PAs [76]. In another study, PA B2 has been detected in human plasma and urine after consuming cocoa [76]. These evidences point out that PAs are rapidly absorbed and quickly reach the liver and other tissues.

2.2 Effects of proanthocyanidins on lipid absorption and chylomicron production by the intestine

Lipid and lipoprotein levels within blood are the consequence of many biochemical and physiological processes, each of which is finely regulated. One of the responsible mechanisms for the hypolipidemic effect of PAs could be the delay on fat and cholesterol absorption, and a reduction of chylomicron secretion. There is clear evidence to suggest that atherosclerosis is a consequence of disordered chylomicron metabolism, and that this is probably the most common etiology [76]. As humans remain in the postpandrial state for much of the day, the reduction of CM production induced by PAs seems to be crucial for their protection against CVD [76].

Food rich in PA, such as red wine, could down-regulate CM secretion in humans. ApoB, lipid availability and MTP are known to be central to the efficient assembly and secretion of CM [76]. Postprandial apo B-48 was reduced by an acute alcoholic and non-alcoholic red wine consumption in dyslipidemic postmenopausal woman 6h after eating [76]. However, a subsequent study on 17 dyslipidemic postmenopausal women performed by the same authors [88] shows no effects of acute alcoholized or dealcoholized red wine consumption on apo B-48 measured as the area under the curve for 6h after eating, even though there was a significant reduction in apo B48 levels after an hour.

In human intestinal Caco-2 cells, the secretion of apo B-48 is significant reduced by alcoholic red wine [76] and apple polyphenol extract [89], whereas a wine polyphenolic extract has not so much effectiveness [89]. This difference in the effect of foodstuffs and extracts upon CM secretion could depend on PAs, as each food/extract has a characteristic composition and concentration. On the other hand, impaired lipid availability in enterocytes could be the first cause of the reduction of CM secretion by PAs. Before fat can be absorbed, TGs from food must be hydrolyzed, and the pancreatic lipase plays a key role in the efficient digestion of TGs [76]. Grape seed extracts [90] and apple PAs [91] inhibit the activity of pancreatic lipase, suggesting limited dietary

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TG absorption. Little information is available about changes induced by PA on MTP activity in enterocytes even though the effects seem to be less important than those induced on TG availability.

2.3 Effects of proanthocyanidins on lipogenesis and VLDL production in the liver

Although PAs exert some of their hypolipidemic effect by inhibiting the absorption of dietary lipids and disminishing CM secretion by enterocytes, the repression of VLDL secretion by the liver also plays an important role on reducing plasma lipid. Insulin resistance, type 2 diabetes and metabolic syndrome are characterised by dyslipidemia due to an overproduction of VLDL particles [76]. Thus, the reduction of VLDL secretion by the liver caused by PA could involve a minor risk of suffering from CVD.

Pure PA or an extract of it, basically from grape and wine, inhibits TGs and Apo B (a marker of VLDL) production and secretion into the media, using human transformed HepG2 [76]. Working with cell models makes it easier to identify which PAs are responsible for this phenomenon. Montagut et al. [76] concluded that trimer C1 and other oligomeric forms of PA may be largely responsible for inhibiting TG and Apo B secretion by hepatic cells.

Del Bas et al. [94] studied the effect of an acute oral grape seed proanthocyanidins extract (GSPE) treatment on healthy rats noticing a fall on the levels of plasma TG and Apo B. Moreover, GSPE enhanced the expression of SHP in the liver. This nuclear receptor has recently emerged as an important regulator of several genes involved in lipid and lipoprotein metabolism in the liver [32]. Therefore, they concluded that the liver orchestrated, at least partly, the hypotriglyceridemic action of GSPE, and SHP could be mediating these effects [94]. In order to elucidate whether SHP is the mediator of the lipid lowering activity of GSPE, the same authors used two different systems to block SHP: Human hepatoma (HepG2) cells transfected with SHP-specific siRNA, and transgenic SHP knockout mice [93]. They noticed that the hypotriglyceridemic effect of GSPE is cancelled in both SHP deficient models thus revealing this nuclear receptor as a key mediator of the hypotriglyceridemic response triggered by proanthocyanidins [93]. Gene silencing of SHP in HepG2 cells has allowed identifying two different pathways for GSPE actions upon VLDL secretion: a SHP-dependent mechanism

leading to a decrease of TG secretion and a SHP-independent pathway responsible for MTP downregulation and, subsequently, a reduction of ApoB secretion [93].

A microarray based on the comparison of the liver gene expression profile in wild-type and $SHP^{-/-}$ mice has revealed that GSPE downregulates many genes involved on lipid and lipoprotein synthesis in a SHP-dependent manner [93]. They concluded that SHP is a key mediator for the TG lowering action of GSPE.

Many works have described that the expression of SHP is subjected to the control of different nuclear receptors such as FXR [32]. Previous studies using transgenic mice lacking functional FXR have revealed that this nuclear receptor is a major controller of lipid and glucose metabolism [32]. Those mice presented impaired insulin sensitivity, and elevated levels of plasma and liver TG and cholesterol [32]. Therefore, Del Bas et al. [32] studied whether FXR could mediate the hypotriglyceridemic GSPE actions upstream SHP. *In vitro* studies based on luciferase have revealed that GSPE enhances FXR activity in the presence of bile acids, a situation that imitates the *in vivo* physiological condition of hepatocytes. Thus, $FXR^{-/-}$ mice were used to assess the role of FXR in the mediation of GSPE hypotriglyceridemic actions. In this model, GSPE was not able to lower plasma TG. Moreover, several genes of the lipid synthesis program were downregulated by GSPE in the liver of wild type mice, but not in $FXR^{-/-}$ mice. Therefore, FXR has been revealed as an essential mediator of the hypotriglyceridemic response triggered by GSPE *in vivo* [32].

The comparison between changes induced by GSPE at the liver gene expression level in $SHP^{-/-}$ mice with those in $FXR^{-/-}$ mice, reveals that all genes that were changed in a SHP-dependent manner are included into the FXR-dependent changes [32]. This evidence reinforces the role of FXR as a mediator upstream SHP of GSPE hypotriglyceridemic actions, highlighting that GSPE can act via a sequential pathway involving FXR and SHP. Moreover, it points out that GSPE-activated FXR, as SHP, could modulate the expression of other FXR target genes, which could also mediate GSPE actions [32].

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To sum up, GSPE exerts lipid-lowering effects in three different systems: rats, mice and HepG2 cells. The mechanism of action of proanthocyanidins involves FXR and SHP, a pathway leading to lowered lipogenesis and the secretion of VLDL in the liver. Therefore, the proanthocyanidins are powerful agents that ameliorate plasma lipoprotein profile and can be considered as powerful bioactive agents for improving the quality of life.

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II. OBJECTIVES

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The research work carried out in this Ph. D. Thesis is part of a more general research project developed by Nutrigenomics Research Group of the Universitat Rovira i Virgili, which deals with the potentially beneficial effects of dietary proanthocyanidins. Proanthocyanidins have been shown to exert advantageous actions on several metabolic disorders that are risk factors of cardiovascular diseases, such as atherosclerosis, inflammatory processes, obesity and diabetes. Lipoprotein metabolism play an important role in these altered lipid states. Several works have described the beneficial effects of different flavonoids and proanthocyanidins in lipoprotein metabolism improving the lipid homeostasis. Nevertheless, the molecular mechanisms underlying this effect are only partially known. Previous studies from our group have demonstrated the beneficial effects of red wine and grape seed proanthocyanidins extract on lipid metabolism in the liver and have established the molecular mechanisms by which grape seed proanthocyanidins extract modulates lipid and lipoprotein metabolism in the liver. However, the actual role of the intestine and the liver, the two main organs producing lipoproteins, in the hypolipidemic action of proanthocyanidins is still an unsolved subject.

In addition, previous studies of the Nutrigenomics Research Group dealt with the hypolipidemic action of dietary proanthocyanidins in normolipidemic models. Inadequate nutrition is an important environmental factor that contributes to the development of diseases around the world. Excessive intake of some nutrients, especially saturated fat and simple sugars, and some micronutrient deficiencies can cause serious health problems. In the past century, nutrition research objectives have focused on the identification of nutrients and their deficiencies, which cause negative effects on intermediary metabolism, growth maintenance and development of cells and tissues.

Dyslipidemia is usually related to several diseases, such as atherosclerosis, obesity and diabetes. Therefore, it is essential to assess the effectiveness of these compounds in dyslipidemic nutritional models in order to establish the potentially beneficial effects of dietary proanthocyanidins in preventing and ameliorating dyslipidemia associated with the Metabolic Syndrome.

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The purpose of this Thesis, then, has been to characterise and understand how effective dietary proanthocyanidins are in dyslipidemic nutritional models and the role of the liver and the intestine in their hypotriglyceridemic action. For this aim, two objectives were sequentially proposed:

1. To assess the contribution of the liver and the intestine in the hypolipidemic response triggered by proanthocyanidins.

In order to assess the implication of the liver and the intestine within the hypotriglyceridemic response triggered by proanthocyanidins, two experimental approaches were undertaken: On the one hand, the actual contribution of CM and VLDL in the hypotriglyceridemic action of proanthocyanidins in the postprandial state has been determined and the mechanisms by which proanthocyanidins treatment reduce TG-rich lipoproteins *in vivo* has been characterised. Lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) was orally administrated to male Wistar rats (manuscript 1). This study revealed that CM and VLDL contributed to the hypotriglyceridemic action of proanthocyanidins, but their influence depended on time (manuscript 1). Besides, the hypotriglyceridemic effect of proanthocyanidins was not due to a bigger lipid clearance by the extrahepatic tissues, but a reduction of VLDL secretion by the liver (manuscript 1).

On the other hand, the effect of proanthocyanidins on TG-CM secretion and the changes induced by proanthocyanidins in the intestine gene expression were evaluated *in vitro* by using human colonic adenocarcinoma Caco2 cells during the fasted or post-prandial state. This study revealed that the effectiveness of proanthocyanidins repressing the TG secretion depended on the physiological state (manuscript 2) and provided the first *in vitro* evidence of the fact that ACSL3 and ACSL5 are target genes of proanthocyanidins (manuscript 2). Altogether, it is suggested that proanthocyanidins may act through different pathways to achieve their hypotriglyceridemic effects.

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2. To evaluate the short-term effect of an oral intake of proanthocyanidins in dyslipidemic nutritional models.

In order to evaluate the ability of dietary proanthocyanidins to prevent or correct dyslipidemia, two experimental models have been used. On the one hand, mice were fed on a diet that contained proanthocyanidins for four weeks (manuscript 3) so as to evaluate whether the effect of proanthocyanidins counteract body weigth gain and energy intake induced by a high carbohydrate high fat diet. On the other hand, rats were fed on a cafeteria diet for 3 moths supplemented with proanthocyanidins thereafter (manuscript 4) in order to study the ability of dietary proanthocyanidins on the correction of dyslipidemia and obesity induced by a high-fat diet. Proanthocyanidins did not counteract the gain of body weight and the energy intake during a short-term of metabolic syndrome. Proanthocyanidins had a bimodal effect on energy intake, since these increased it at early-term and decreased it thereafter (manuscript 3), suggesting that proanthocyanidins-rich food must be consumed habitually and at long-term in order to be effective and improve the excess of body weight associated to metabolic syndrome. Besides, the proanthocyanidins treatment performed on dyslipidemic rats for 10 days did not counteract the gain of body weight though it repressed several genes that control lipogenesis and VLDL assembling in the liver (manuscript 4). Proanthocyanidins decreased plasmatic TG both in syndrome metabolic mice and dyslipidemic rats (manuscript 3 and 4), demonstrating that proanthocyanidins are potent hypotrigliceridemic agents (manuscript 1, 2, 3 and 4) even when acting within altered lipid metabolic models.

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III. RESULTS AND DISCUSSION

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1. Contribution of chylomicron and VLDL to postprandial hypotriglyceridemia induced by dietary proanthocyanidins in rats

(Submitted)

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Contribution of chylomicron and VLDL to postprandial hypotriglyceridemia induced by dietary proanthocyanidins in rats

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Abbreviations: GSPE, grape seed proanthocyanidin extract; TG, triglyceride; LPL, lipoprotein lipase; CM, chylomicron; NEFA, non-esterified fatty acids; FA, fatty acid

Key words: carnitine palmitoyltransferase 1a; fatty acids; flavonoids; lipoprotein lipase; triglyceride

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ABSTRACT

Scope: Proanthocyanidins, the most abundant flavonoids in human diets, reduce postprandial triglyceridemia. Plasma triglyceride (TG) levels are the result of the balance between the TG-rich lipoprotein secretion by the intestine and the liver and their uptake by the extrahepatic tissues through the lipoprotein lipase (LPL). The aim of this study was to determine the actual contribution of Chylomicron (CM) and VLDL in the hypotriglyceridemic action of GSPE in the postprandial state and to characterize the mechanisms by which the GSPE treatment reduces TG-rich lipoproteins *in vivo*.

Methods and results: Rats fasted for fourteen hours were orally loaded with lard oil containing grape seed proanthocyanidin extract (GSPE) or not. CM and VLDL contributed to the hypotriglyceridemic action of GSPE but its influence depended on time. CM was the main contributor 3 hours after providing the treatment, whereas VLDL was important at 1 and 7 hours mark. TG clearance by extrahepatic tissues was not affected by GSPE whereas VLDL-TG secretion was significantly repressed. Lipid unavailability for TG synthesis is the probable cause of the reduction of VLDL-TG secretion by the liver.

Conclusion: Besides the intestine, the VLDL and the liver are important targets of dietary proanthocyanidins *in vivo*.

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INTRODUCTION

Dyslipidemia is one of the major determinants of the development of cardiovascular diseases [1, 2]. Postprandial lipaemia has emerged as a key contributor to the risk and progression of cardiovascular disease. Elevated levels of nonfasting triglycerides are strongly associated with the increase of the risk of myocardial infarction, ischemic stroke, and early death [3].

Proanthocyanidins (PAs), the most abundant polyphenols in human diets, have been shown to improve postprandial hypertriglyceridemia in animal models [4] and the ingestion of PA-rich food, such as red wine, to decrease plasma lipids in humans [5]. The hypolipidemic action of grape seed PA extract (GSPE) is attributable to a reduction of plasma levels of TG-rich lipoproteins and to an improvement of serum cholesterol profile, both in normolipidemic [6] and dyslipidemic rats [7]. Besides, the simultaneous ingestion of apple PAs with fat inhibits the increase of plasma TGs induced by a fat ingestion just in mice and humans [8].

Plasma triglyceride (TG) levels are the result of the balance between the TG-rich lipoprotein secretion by the intestine and the liver and their uptake by the extrahepatic tissues through the lipoprotein lipase (LPL). TG-rich lipoproteins in plasma originate either in the liver (very-low density lipoprotein, VLDL) or the intestine (chylomicron, CM). The hypotriglyceridemic effect of PAs has been attributed to an inhibition of dietary lipid absorption associated with a reduction of CM secretion by enterocytes [8, 9] as well as with a decrease of VLDL secretion by the liver [7, 10-12] in a range of different studies. Nevertheless, the real contribution of CM and VLDL secretion and LPL activity to the hypotriglyceridemic action of GSPE has never been studied. Thus, the aim of this study was to determine the actual contribution of CM and VLDL in the hypotriglyceridemic action of GSPE in the postprandial state and to characterise the principal mechanisms by which the GSPE treatment reduces TG-rich lipoproteins.

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MATERIALS AND METHODS

Proanthocyanidin extract

The proanthocyanidin extract (GSPE) contained monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5-13 units) (31.7%) proanthocyanidins, essentially.

Animals

Male Wistar rats that weighed 350 g were purchased from Charles River (Barcelona, Spain). The research was conducted in conformity with the Public Health Service Policy on Humane Care and Use of Laboratory Animals policy and the studies were approved by the Animal Ethics Committee of our University. The animals were housed in animal quarters at 22ºC with a 12-h light/dark cycle (light from 8 a.m. to 8 p.m.) and were fed on standard chow and water *ad libitum*.

Plasma lipid tolerance test and measurement of plasma lipid levels

For the lipid tolerance test, rats were deprived of food for 14 h before the experiment. A lard oil (2.5 mL/Kg of body weight) with or without GSPE (250mg/Kg of body weight) was administered orally. For blood chemical analyses, aliquots from the tail vein were collected into capillary tubes with EDTA after 1, 3, 5 and 7 hours of treatment. TG and cholesterol were determined by using an enzymatic colorimetric kit (QCA, Barcelona, Spain). NEFA were determined by using the Wako assay kit (Wako chemicals GmbH). 3-hydroxy-butyrate was analysed by using an enzymatic kit (Ben srl., Italy).

Lipoprotein fractionation

Lipoprotein fractions were prepared from plasma samples by means of density gradient ultracentrifugation [13, 14]. A total volume of 2 ml was put in the bottom of a 6.5 ml polyallomer ultracentrifuge tube (Beckman, U.S.A). Then, 3 ml of 1.006 g/ml density solution was added and it was centrifuged in a 45.6 Kontor rotor. CM was subfractionated by a 30-minute centrifugation at 16,000 r.p.m and VLDL by 16-hour centrifugation at 37,000 r.p.m. After each centrifugation step, the 2ml of the top of the

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gradient that contained the respective lipoprotein subclass was aspired, and the 2ml of density solution was used to refill the tube before the next run.

The TG content was determined by using an enzymatic colorimetric kit (QCA, Barcelona, Spain) in both lipoprotein fractions.

VLDL-TG secretion assay

Rats were fasted for 3 hours and then fed on an oral gavage of GSPE (250mg/Kg of body weight) in aqueous solution (treated group) or an oral gavage with the vehicle (tap water). These rats formed the control group. The duration of the treatment was 2 hours long. Rats were injected 500 mg/kg Triton WR 1339 (Sigma-Aldrich, Louis, MO) into the tail vein to inhibit plasma VLDL-TG hydrolysis and clearance [15]. Aliquots of saphenous blood were collected at 0, 10, 20, 30, 40, 50, 60 min after Triton WR 1339 injection. Plasma TG levels were determined and plotted as a function of time.

Lipoprotein lipase assay

Rats were deprived of food for 14 h before the experiment. A lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg of body weight) was administered orally. One hour after the administration, rats were injected 300 IU heparin/kg body weight intravenously, and saphenous vein blood was sampled 10 minutes after heparin infusion. Heparinized plasma was prepared for the determination of LPL activity using the LPL activity kit (Roar Biochemical, Inc.), as described by the manufacturer.

CPT-1 assay

Rats were treated in the same way as in the plasma lipid tolerance test section; Later on, rats were anesthetised using ketamine/xylazine and sacrificed by exsanguination. The liver was excised, immediately frozen in liquid nitrogen and stored at -80ºC until the activity assay was performed.

Mitochondria were isolated from the livers which were homogenated at 4ºC in 1:3 (w:v) of a buffer containing 250mM sucrose, 1mM EDTA and 10 mM Tris-HCl, pH 7.4, by using a Teflon/glass homogenizer, and then centrifuged at 700g for 10 min at 4ºC. The

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supernatants were centrifuged at 12,000g for 10 min at 4ºC. The resulting pellet containing the purified mitochondria was resuspended in 500µL of a buffer with 70 mM sucrose, 220 mM mannitol, 2 mM HEPES and 1 mM EDTA,[16] and used to carry out the CPT-1 assay. The Bradford reagent (Sigma-Aldrich, Louis, MO) was used to determine the mitochondrial protein content.

CPT-1 activity was assessed as described by Bieber et al.[17]. Assays were performed in triplicate; 13 µL of the mitochondrial protein solution was transferred into the assay buffer (116 mM Tris-HCl, 0,09% Triton X-100, 1,1 mM EDTA, 0,035 mM Palmitoyl-CoA, 0,12 mM DTNB, pH 8.0) and incubated 3 min at 20ºC, and its absorbance was measured at 412 nm. The results were expressed in nmol CoA formed/mg protein/min.

Gene expression analysis

Rats were treated in the same way as in the plasma lipid tolerance test section; Later on, rats were anesthetised using ketamine/xylazine and sacrificed by exsanguination. The first 30 cm of the intestinal mucosa, the liver, the leg muscle and the visceral white adipose tissue were excised and immediately frozen until RNA extraction. Total RNA was isolated using an RNeasy Mini kit (Quiagen, UK), following the manufacturer's instructions. cDNA was synthesised from 2µg of total RNA using High Capacity cDNA Reverse Transcription Kit (Applied Biossystems).

Changes in mRNA expression of LPL in the muscle and the white adipose tissue were measured by quantitative PCR. Quantitative PCR amplification and detection were performed by using a specific TaqManR Assay-On-Demand probe (Applied Biosystems Rn00561482_m1), the TaqMan PCR Core Reagent Kit and the GeneAmpR 5700 Sequence Detection System, as recommended by the manufacturers. Quadruplicated quantifications, performed in singleplex assays, were performed in each cDNA. PPIA was used as the reference gene in quantitative PCR (Applied Biosystems TaqManR Assay-On-Demand probe Rn00690933_m1).

Each cDNA sample of the liver and the intestine (100µl) was added to an equal volume of 2X TaqMan Universal PCR Master Mix (Applied Biosystems). After gentle mixing and centrifugation, the mixture was then transferred into a loading port on a low-density array card (TLDA) (Applied Biosystems). Each low-density array card has eight separated loading ports that feed into 48-gene sets. Each 2 µL well contains specific

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user-defined probes, capable of detecting a single gene. Each set of 48 genes also contains three housekeeping genes, RPLP2, Ppia and 18s. In this study, however, Ppia was used.

The array was centrifuged twice for a minute each at 1,200 rpm to distribute the samples from the loading port into each well. The card was then sealed and PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system. Thermal cycling conditions were as follows: 2 minutes at 50ºC, 10 minutes at 94.5ºC, 30 seconds at 97ºC and a minute at 59.7ºC for 40 cycles [18].

Statistical analysis

Results are reported as the mean ± s.e.m of five animals. Group means were compared with an independent-samples Student's t-test except for those of the gene expression that were compared with one way ANOVA ($P \leq 0.05$), using SPSS software.

RESULTS

GSPE treatment blocked the increase of plasma total TG by mainly reducing VLDL-TG or Chylomicron-TG depending on time

In control animals the plasma TG reached its maximum level 3h after the oral administration of 2.5 mL/Kg of lard oil and then it decreased (Figure 1a). The administration of GSPE (250mg/Kg body weight) markedly blocked the increase of plasma TG, with a statistically significant reduction of 22% in the area under the curve (AUC) of plasma TG (Figure 1b). Thus, GSPE treatment improved the tolerance to oral TG. The hypotriglyceridemic effect of GSPE was very fast (it could be noted after 1.5 hours) and it was observable until 7 hours after the administration.

In order to understand the contribution of CM and VLDL to the hypotriglyceridemic effect of GSPE better, we measured the TG content of the CM and VLDL fractions (Figure 1c). GSPE treatment slightly reduced CM-TG in all the studied times, showing its highest effect after 3 hours of treatment, when CM-TG reached a maximum level. GSPE treatment reduced VLDL-TG very quickly (an hour after the administration) and the reduction became significant at 7 hours mark. Altogether, these results indicate that both CM and VLDL contributed to the hypotriglyceridemic action of GSPE but its

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influence depended on time. CM was the major contributor after 3 hours of treatment, whereas VLDL was after 1 and 7 hours. 5 hours after treatment, CM and VLDL showed a similar influence.

On the other hand, plasma cholesterol levels almost showed the same pattern as plasma TG in animals only treated with lard oil (Figure 2b). Nevertheless, GSPE had no effect on plasma cholesterol.

GSPE administration significantly reduced the NEFA levels (Figure 2b) 5 hours after its administration. 3-hydroxy-butyrate was significantly reduced 7 hours after the administration of GSPE (Figure 2c).

GSPE treatment did not affect TG clearance

Plasma TG levels could be reduced by repressing TG-rich lipoprotein secretion and/or by increasing their uptake by the extrahepatic tissues. Thus, we quantified TG clearance. As GSPE affected plasma TG very quickly, we measured plasma post-heparin LPL activity after an hour of gavaging lard oil or lard oil + GSPE. As shown in Figure 3b, GSPE had no effect on plasma LPL activity. Moreover, no differential expression of LPL was observed as a result of carring out the GSPE treatment in white adipose and muscle 1 and 5 hours after administration (Figures 3c and 3d). Thus, these results strongly suggest that the blockage of the increase of plasma TG induced by GSPE was not attributable to the increase of TG clearance.

GSPE administration decreased VLDL-TG secretion

In order to assess the VLDL-TG secretion, we injected Triton WR1339 to rats and thus inhibited the VLDL-TG hydrolysis and clearance. VLDL-TG secretion was measured 2 hours after GSPE administration in rats that had fasted for 5 hours to avoid CM secretion (Figure 3a). GSPE treatment significantly repressed (30%) the secretion of VLDL-TG. As VLDL are mainly secreted by the liver, these results strongly suggest that the decrease of plasma VLDL-TG induced by GSPE was the consequence of its action in the liver, which in turn represses VLDL-TG secretion.

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GSPE treatment modulated the expression of some lipid related genes in the liver but not in the intestine

In order to determine the molecular mechanisms underlying TG-rich lipoprotein secretion , we used a low-density array card (TLDA) to analyse the differential expression of key genes that control TG and cholesterol metabolism in the intestine and liver at 0 (before treatment, basal state), 1 and 5 hours after lard oil or lard oil plus GSPE administration. We chose genes that encoded key proteins in the lipid pathways. For cholesterol metabolism: The intestinal uptake transporter Niemann-Pick C1-like protein 1(Npc1l1), the ATP-binding cassettes implicated in the intestinal and biliary excretion of sterols (Abcg5 and Abcg8), the ATP-binding cassette sub-family A member 1 (Abca1), the enzyme controlling cholesterol synthesis 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (Hmgcr) and the enzyme Acetyl-Coenzyme A acetyltransferase 2 (Acat2) that catalyses the conversion of free cholesterol to cholesteryl esters; For fatty acid metabolism: The fatty acid transporter CD36 (Cd36) and fatty acid binding protein 1 (Fabp1), the enzyme controlling β-oxidation carnitine palmitoyltransferase 1a (Cpt1a), the enzyme fatty acid synthase (Fasn), the enzyme controlling the synthesis of unsaturated fatty acids stearoyl-Coenzyme A desaturase 1 (Scd1) and the enzyme acyl-CoA synthetase long-chain family member 1 (Acsl1) that catalyses the ligation of long chain fatty acids with coenzyme A to produce long chain acyl-CoAs; For triglyceride synthesis: The Mg2+-dependent PA phosphatase Lpin1 and the diacylglycerol O-acyltransferase homolog 1 and 2 (Dgat1 and Dgat2) that catalyse the final step of triglyceride synthesis; For lipoprotein assembling and composition: The microsomal triglyceride transfer protein (Mttp), the apolipoprotein A-V (Apoa5) and apolipoprotein C-III (Apoc3). We also selected the nuclear receptors FXR and SHP and the transcription factor SREBP-1c because they govern the expression of key lipid metabolism genes in the intestine and the liver, which are also involved in the molecular mechanism used by GSPE in the liver [10, 11].

In the intestine (Table 1), the ingestion of lard induced a generalized repression of genes related to lipid metabolism, except from Acat2 which was overexpressed. GSPE did not induce different effects from those induced by lard oil alone on the genes studied at both times, except from Cpt1a, which was repressed after 5 hours of its administration.

Table 2 shows the mRNA levels of genes related to lipid metabolism in the liver, except from those of Cpt1a, which have been included in Table 3. 1 hour after the

administration of the lard oil, the livers showed a significant overexpression of Lpin1 in conjunction with a significant repression of Acsl1 and Dgat1, in compararison with the basal group. The livers of rats treated with GSPE presented the same expression pattern for Lpin1 and Dgat1 at one hour mark. On the other hand, GSPE treatment induced different effects from those induced by the lard oil treatment on the expression Acsl1, Apoc3 and Hmgcr. Acsl1, then, was not repressed, Apoc3 was repressed and Hmgcr was overexpressed when compared to the basal state.

Five hours after the administration of the lard oil, however, the livers showed a significant repression of Dgat1, Acsl1, Mttp and Shp in conjunction with a significant overexpression of Cd36, in comparison with the basal group. The livers of rats treated with GSPE presented the same expression pattern for Dgat1, Acsl1, Mttp and Shp at five hours mark. Nevertheless, GSPE treatment induced different effects from those induced by lard oil treatment on the expression of Cd36, which was not overexpressed in comparison with the basal state.

GSPE treatment channelled fatty acid into ß-oxidation in the liver at the initial time (1 hour)

The relationship between FA oxidation and esterification in the liver has been described as a key factor in the regulation of VLDL synthesis [19]. As enzymes related to FA esterification (Dgat1 and 2) were not differentially expressed by GSPE, we made an in-depth study of the effects of lard oil with or without GSPE on FA oxidation. So as to do so, both mRNA and the activity of Cpt1a, which is the rate-limiting enzyme in ß-oxidation, were determined at different times after the administration (Table 3). Lard oil administration significantly repressed the expression of Cpt1a and decreased its activity by a 60% an hour after its ingestion. In contrast, when lard oil was administered with GSPE, there was no repression of Cpt1a and its activity remained high. Five hours after the administration, the situation was just the opposite that shown after an hour. The liver of rats only treated with lard oil recovered Cpt1a expression and activity whereas the liver of rats treated with lard oil plus GSPE repressed this enzyme expression and activity. These results suggest that GSPE treatment channelled fatty acid into ß-oxidation shortly after the treatment had initiated.

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DISCUSSION

Several authors have reported the hypotriglyceridemic effects of PAs in humans and animals (reviewed in [4]). Plasma TG levels are the result of the balance between the TG-rich lipoprotein secretion by the intestine and the liver and their uptake by the extrahepatic tissues through the LPL. Therefore, GSPE may reduce plasma TG levels by acting on the intestine, the liver and/or the peripheral tissues. However, the exact role of each organ remains unknown. For this reason, this study intended to quantify the contribution of CM and VLDL production and LPL activity to the hypotriglyceridemic action of PAs *in vivo*. With this purpose, we analysed the effects of GSPE on plasma lipids and lipoprotein kinetics during a fed state by using a lipid tolerance test [20]. As far as we are concerned, this is the first study carried out to measure the contribution of CM and VLDL to hypotriglyceridemia induced by PAs or other flavonoids.

Our results show that the oral intake of GSPE significantly blocked the increase of plasma TG induced by lard oil ingestion in the control animals. According to our results, this blockade of plasma TG has also been described with PAs from apples [8], using mice loaded with corn oil, in which the blockade is so fast as in our experiment (1 hour). In our study, the reduction of plasma TG was similar to the reductions observed in male normolipidemic rats [6] and mice [10, 11] after performing an acute GSPE treatment, and in dyslipidemic rats [7] after carring out a chronic GSPE treatment. Thus, the improvement of plasma TG is a generalized effect of PAs, as it is observed in different situations and experimental approaches. As a consequence, PA-rich foods may reduce the hypertriglyceridemia associated with the postprandial state and, therefore, improve the tolerance to dietary lipids.

Both CM and VLDL contributed to the hypotriglyceridemic action of GSPE but its influence depended on time. CM was the main contributor 3 hours after providing the treatment, whereas VLDL was important at 1 and 7 hours mark. As GSPE did not increase TG clearance by extra-hepatic tissues, the reduction of CM-TG and VLDL-TG by GSPE could be ascribed to a repressed lipoprotein secretion. It is generally assumed that CM predominantly transport ingested exogenously TG derived from dietary sources, whereas VLDL transport synthesised endogenously lipids [21]. PAs, then, repress the secretion of both synthesised endogenous TG and TG absorbed from the diet.

Several authors have considered CM and lipid absorption as the cause of the hypolipidemic actions of PAs, but just on the basis of indirect evidence or *in vitro* experiments [8] [9]. The expression of the selected genes related to FA and TG metabolism as well as those of the CM assembly were not modified in the intestinal mucosa 1 or 5 hours after GSPE administration. Therefore, other genes and/or molecular mechanisms could be implied in this repression, so future studies are warranted.

We did not determine the secretion of CM-TG. However, TG secretion associated to VLDL was repressed a 30% by GSPE treatment. Thus, the repression of VLDL secretion seems to be an important contributor to the hypotriglyceridemic effect of PAs. The liver is the main organ that secretes VLDL [22, 23]. Consequently, regarding the hypotriglyceridemia induced by PAs in the postprandial state, the liver is a significant target for GSPE.

The regulation of hepatic VLDL secretion mainly depends on apoB synthesis, MTTP activity and lipid availability [19]. Our results suggest that lipid unavailability is the cause of the reduction of VLDL-TG secretion by the liver. Shortly after GSPE administration (1 hour), CPT-1a activity and expression remained high, suggesting an elevated FA oxidation. The same pattern for CPT-1a was observed for Acsl1 mRNA. Specific ACSL isoforms provided acyl-CoAs for particular metabolic pathways [24] and Acsl1 has been reported to have a role in mitochondrial beta-oxidation in knock-out models of ACSL1. The relationship between FA oxidation and esterification has been described as a key factor in the regulation of VLDL synthesis [19]. Therefore, the increase of the oxidation of FA induced by GSPE treatment is a potential mechanism by which PAs reduce VLDL-TG secretion shortly after GSPE administration (1 hour).

On the other hand, five hours after GSPE administration, the levels of plasma NEFA decreased considerably. As plasma NEFA is one source of TG synthesis in the liver and plays an important role in stimulating hepatic VLDL production [19, 25], the reduction of plasma NEFA levels could be the mechanism hidden behind the repression of VLDL-TG secretion induced by GSPE several hours after administration. Moreover, concomitant to the decreased NEFA levels in plasma, GSPE treatment changed the expression pattern of Cd36 induced only by lard. Hepatic Cd36 protein expression is a regulatory mechanism that controls fatty acid uptake by the liver and its elevation directly affect hepatic fatty acid uptake, triglyceride storage, and VLDL-triglyceride

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secretion [26]. Therefore, a reduction of NEFA levels in plasma together with low Cd36 expression may decrease FA availability for TG synthesis in the liver 5 hours after GSPE administration, thus reducing VLDL-TG secretion.

The described molecular mechanism by which GSPE represses hepatic TG secretion involves transcriptional activation of FXR [11], overexpression of the nuclear receptor SHP [10] and repression of SREBP1 [7, 10, 11]. We have found no significant difference in the expression of SHP or SREBP1 after performing the treatment with lard only or lard plus GSPE. Nevertheless, mRNA levels of SREBP1 were always lower with GSPE treatment and SHP mRNA levels were higher after 5 hours of GSPE administration. Diet and hormones, mainly insulin, regulate the transcription of SREBP-1 [27]. In the present experiment, the animals received only a TG overload whereas in the experiments that describe the molecular mechanism of GSPE, the animals received a mixed diet. Thus, the differences in the diets, which in our case reduced the expression of SREBP1 in control animals (without GSPE), minimized the differences between animals treated with GSPE and those that were not.

In conclusion, our results show that the oral intake of GSPE significantly blocked the increase of plasma TG induced by the lard oil ingestion. Both CM and VLDL contributed to the hypotriglyceridemic action of GSPE but its influence depended on time. GSPE ingestion repressed VLDL-TG secretion, but it did not increase TG clearance. GSPE may block VLDL-TG secretion because of the unavailability of FA for TG synthesis, as a consequence an increase of FA oxidation shortly after administration and a reduction of plasma NEFA levels much later. Overproduction of TG-rich lipoproteins is characteristic of dyslipidemia in the metabolic syndrome and type 2 diabetes [28, 29]. Therefore, the blockage of TG-rich lipoprotein secretion induced by PAs may explain the reduced risk of death due to a coronary heart disease and a cardiovascular disease associated with dietary intake of flavonoids and certain foods rich on flavonoids [30].

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Conflict of interest

The authors disclose no conflicts of interest

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Table 1. mRNA levels of genes related to lipid metabolism in the intestinal mucosa of rats fed on lard oil with or without proanthocyanidins (GSPE) after 1 and 5 hours.

	Basal (0 h)	Lard 1 h	Lard + GSPE 1 h	Lard 5 h	Lard + GSPE 5 h
Npc1l1	1.20 ± 0.40	0.40 ± 0.17	0.45 ± 0.20	0.21 ± 0.20 ↓	0.13 ± 0.04 ↓
Abcg5	1.20 ± 0.35	0.27 ± 0.09 ↓	0.29 ± 0.12 ↓	0.11 ± 0.02 ↓	0.10 ± 0.05 ↓
Abcg8	1.20 ± 0,39	0.20 ± 0,05 ↓	0.22 ± 0.08 ↓	0.10 ± 0.03 ↓	0.08 ± 0.04 ↓
Abca1	1.21 ± 0.34	0.39 ± 0.10 ↓	0.47 ± 0.02 ↓	0.21 ± 0.01 ↓	0.34 ± 0.12 ↓
Acat2	1.00 ± 0,04	1.23 ± 0,08 ↑	1.20 ± 0,04 ↑	1.51 ± 0,15 ↑	1.62 ± 0,30 ↑
Hmgcr	1.10 ± 0,25	1.14 ± 0,52	1.39 ± 0.22	0.65 ± 0.21	0.71 ± 0.17
Cd36	1.12 ± 0.30	0.21 ± 0.09 ↓	0.25 ± 0.10 ↓	0.10 ± 0.02 ↓	0.07 ± 0.03 ↓
Fabp1	1.20 ± 0.35	0.51 ± 0.16	0.50 ± 0.17	0.35 ± 0.10 ↓	0.22 ± 0.05 ↓
Cpt1a	1.03 ± 0.13	0.71 ± 0.21	0.60 ± 0.14	0.57 ± 0.22	0.38 ± 0.14 ↓*
Fasn	1.11 ± 0.28	0.34 ± 0.11	0.45 ± 0.26	0.16 ± 0.05 ↓	0.32 ± 0.12 ↓
Acsl1	1.13 ± 0.31	0.30 ± 0.08 ↓	0.40 ± 0.12 ↓	0.26 ± 0.03 ↓	0.24 ± 0.01 ↓
Dgat1	1.12 ± 0.29	0.35 ± 0.13	0.40 ± 0.15	0.18 ± 0.06 ↓	0.13 ± 0.04 ↓
Dgat2	1.27 ± 0.46	0.30 ± 0.09	0.30 ± 0.11	0.44 ± 0.20	0.28 ± 0.15
Lpin1	1.22 ± 0.40	0.50 ± 0.07	0.54 ± 0.23	0.30 ± 0.07	0.50 ± 0.21
Mttp	1.10 ± 0.26	0.55 ± 0.17	0.62 ± 0.25	0.55 ± 0.17	0.33 ± 0.11
Apoa5	1.05 ± 0.20	0.79 ± 0.50	1.31 ± 0.89	0.24 ± 0.07 ↓	0.20 ± 0.13 ↓

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Apoc3	1.10 ± 0.25	0.31 ± 0.12	0.38 ± 0.17	0.16 ± 0.06 ↓	0.11 ± 0.04 ↓
Nr1h4 (Fxr)	1.02 ± 0.10	1.40 ± 0.25	1.43 ±0.33	0.63 ±0.16 ↓	0.46 ±0.04 ↓
Rxra	1.13 ± 0.30	0.39 ± 0.15	0.53 ± 0.18	0.22 ± 0.05 ↓	0.20 ± 0.03 ↓
Nr0b2 (Shp)	1.10 ± 0.22	0.42 ± 0.12 ↓	0.40 ± 0.14 ↓	0.28 ± 0.13 ↓	0.12 ± 0.05 ↓
Srebf1	1.26 ± 0.43	0.36 ± 0.12	0.30 ± 0.11	0.54 ± 0.23	0.25 ± 0.15

Rats fasted for 14 h were administered lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) orally and were killed 1 or 5 hours after the administration. The basal state (0 h) corresponds to mRNA values before the oral administration. The values are expressed as fold change using PPIA expression as the endogenous control. Each value is the mean ± s.e.m of three animals. ↓ or ↑ indicates significant differences ($p< 0.05$) versus basal values and * indicates significant differences ($p< 0.05$) between the lard group and the lard + GSPE group at the same time by ANOVA .

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Table 2. mRNA levels of genes related to lipid metabolism in the liver of rats fed on lard oil with or without proanthocyanidins (GSPE) after 1 and 5 hours.

	Basal (0 h)	Lard 1 hour	Lard + GSPE 1 h	Lard 5 h	Lard + GSPE 5 h
Abcg5	1.02 ± 0.11	1.08 ± 0.05	0.88 ± 0.10	0.94 ± 0.18	1.09 ± 0.02
Abcg8	1.08 ± 0.25	1.04 ± 0.05	0.74 ± 0.10	0.95 ± 0.18	1.01 ± 0.08
Abca1	1.02 ± 0.12	0.94 ± 0.22	0.84 ± 0.08	0.71 ± 0.08	0.86 ± 0.17
Acat2	1.00 ± 0.04	0.95 ± 0.10	1.02 ± 0.08	0.87 ± 0.09	0.70 ± 0.07
Hmgcr	1.03 ± 0.15	1.73 ± 0.32	2.96 ± 0.21 ↑	0.83 ± 0.06	1.00 ± 0.19
Cd36	1.01 ± 0.09	1.27 ± 0.15	1.28 ± 0.18	1.34 ± 0.06 ↑	1.05 ± 0.10
Fabp1	1.00 ± 0.05	1.12 ± 0.10	1.18 ± 0.04	0.83 ± 0.07	0.88 ± 0.08
Cpt1a	1.03 ± 0.14	0.66 ± 0.0 ↓	0.75 ± 0.04	0.74 ± 0.02	0.67 ± 0.05 ↓
Fasn	1.09 ± 0.26	2.65 ± 1.43	0.47 ± 0.21	0.42 ± 0.05	0.26 ± 0.01 ↓
Scd1	1.09 ± 0.28	1.95 ± 0.44	1.68 ± 0.61	1.12 ± 0.04	1.43 ± 0.28
Acsl1	1.01 ± 0.08	0.64 ± 0.05 ↓	0.86 ± 0.03	0.74 ± 0.07 ↓	0.69 ± 0.05 ↓
Dgat1	1.01 ± 0.06	0.62 ± 0.12 ↓	0.71 ± 0.08 ↓	0.51 ± 0.05 ↓	0.55 ± 0.06 ↓
Dgat2	1.01 ± 0.06	0.88 ± 0.05	1.00 ± 0.07	1.00 ± 0.11	1.15 ± 0.04
Lpin1	1.04 ± 0.18	3.02 ± 0.44 ↑	2.74 ± 0.26 ↑	0.82 ± 0.12	0.65 ± 0.02
Mttp	1.00 ± 0.05	0.95 ± 0.10	0.98 ± 0.13	0.60 ± 0.04 ↓	0.64 ± 0.04 ↓
Apoa5	1.00 ± 0.03	0.89 ± 0.08	1.03 ± 0.01	1.06 ± 0.13	0.93 ± 0.06

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Apoc3	1.01 ± 0.07	0.80 ± 0.08	0.75 ± 0.06 ↓	0.87 ± 0.09	0.81 ± 0.04
Nr1h4 (Fxr)	1.05 ± 0.17	0.71 ± 0.08	0.89 ± 0.03	1.00 ± 0.11	1.00 ± 0.15
Rxra	1.00 ± 0.01	0.93 ± 0.01	0.96 ± 0.01	0.96 ± 0.03	1.10 ± 0.16
Nr0b2 (Shp)	1.07 ± 0.20	1.85 ± 0.53	1.79 ± 0.56	0.27 ± 0.09 ↓	0.41 ± 0.07 ↓
Srebf1	1.02 ± 0.13	0.85 ± 0.28	0.75 ± 0.17	0.97 ± 0.17	0.67 ± 0.19

Rats fasted for 14 h were administered lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) orally and were killed 1 or 5 hours after the administration. The basal state (0 h) corresponds to mRNA values before the oral administration. The values are expressed as fold change using PPIA expression as the endogenous control. Each value is the mean ± s.e.m of three animals. ↓ or ↑ indicates significant differences ($p< 0.05$) versus basal values and * indicates significant differences ($p< 0.05$) between the lard group and the lard + GSPE group at the same time by ANOVA .

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Table 3: Carnitine palmitoyl transferase 1 expression and activity in the liver of rats fed on lard oil with or without proanthocyanidins (GSPE)

	Basal (0 h)	Lard 1 hour	Lard + GSPE 1 h	Lard 5 h	Lard + GSPE 5 h
CPT1 activity (nmol CoA/mg protein.min)	4.00 ± 0.80	1.6 ± 0.5	3.2 ± 0.1*	3.6 ± 0.9	1.4 ± 0.3*
CPT1 mRNA (fold change)	1.03 ± 0.14	0.66 ± 0.05 ↓	0.75 ± 0.04	0.74 ± 0.02	0.67 ± 0.05 ↓

Rats fasted for 14 h were administered lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) orally and were killed 1 or 5 hours after the administration. The basal state (0 h) corresponds to mRNA values before the oral administration.. Each value is the mean ± s.e.m of 5 animals for Cpt1 activity and of 3 animals for mRNA Cpt1. ↓ or ↑ indicates significant differences ($p < 0.05$) versus basal values and * indicates significant differences ($p < 0.05$) between the lard group and the lard + GSPE group at the same time by t-Student test.

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FIGURE LEGENDS

Figure 1: Triglyceride levels in plasma and triglyceride content in Chylomicron and VLDL of rats fed on lard oil with or without proanthocyanidins (GSPE)

Rats fasted for 14 h were administered lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) orally. Plasma triglycerides (a) were quantified in blood samples of the tail vein before (0 hours) and after 1.5, 3, 4, 5, 6, 7 and 8 hours of the administration. The figure on the right (b) represents the values of the area under the curve. Triglyceride content in Chylomicron and VLDL (c) were quantified in plasma from rats killed 1, 3, 5 and 7 hours after the administration. Each value is the mean ± s.e.m of 10 animals for plasma triglycerides and of 5 animals for Chylomicron and VLDL triglycerides. * indicates significant differences ($p < 0.05$) between the lard group and the lard + GSPE group at the same time by t-Student.

Figure 2: Plasma levels of non-esterified fatty acids, cholesterol and 3-hydroxy-butyrate of rats fed on lard oil with or without proanthocyanidins (GSPE)

Rats fasted for 14 h were administered lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) orally. Plasma cholesterol (a), non-esterified fatty acids (b) and 3-hydroxy-butyrate (c) were quantified from the plasma of rats killed 1, 3, 5 and 7 hours after the administration. Each value is the mean ± s.e.m of 5 animals. * indicates significant differences ($p < 0.05$) between the lard group and the lard + GSPE group at the same time by t-Student.

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Figure 3: VLDL-triglyceride secretion, post-heparin plasma lipoprotein lipase activity and mRNA levels of lipoprotein lipase in white adipose tissue and muscle of rats fed on lard oil with or without proanthocyanidins (GSPE)

VLDL-triglyceride secretion (a) was evaluated in rats fasted for 5 hours. Rats were administered GSPE (250mg/Kg) or water orally and injected 500 mg/kg of Triton WR 1339 through the tail vein to inhibit plasma triglyceride clearance. Aliquots of saphenous blood were collected at 0, 10, 20, 30, 40, 50, 60 min after Triton WR 1339 injection. Post-heparin plasma lipoprotein lipase activity (b) was quantified an hour after the administration of lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) to rats fasted for 14 h and injected 300 IU heparin/kg body weight intravenously. mRNA levels of lipoprotein lipase in white adipose tissue (c) and muscle (d) were determined 1 and 5 hours after the administration of lard oil (2.5 mL/Kg) with or without GSPE (250mg/Kg) to rats fasted for 14 h. The basal state (0 h) corresponds to mRNA values before the oral administration. The gene expression values are expressed as fold changes using PPIA expression as the endogenous control. Each value is the mean ± s.e.m of 3 animals for mRNA LPL and 5 animals for the other parameters. * indicates significant differences ($p < 0.05$) between the lard group and the lard + GSPE group at the same time by t-Student.

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Figure 1

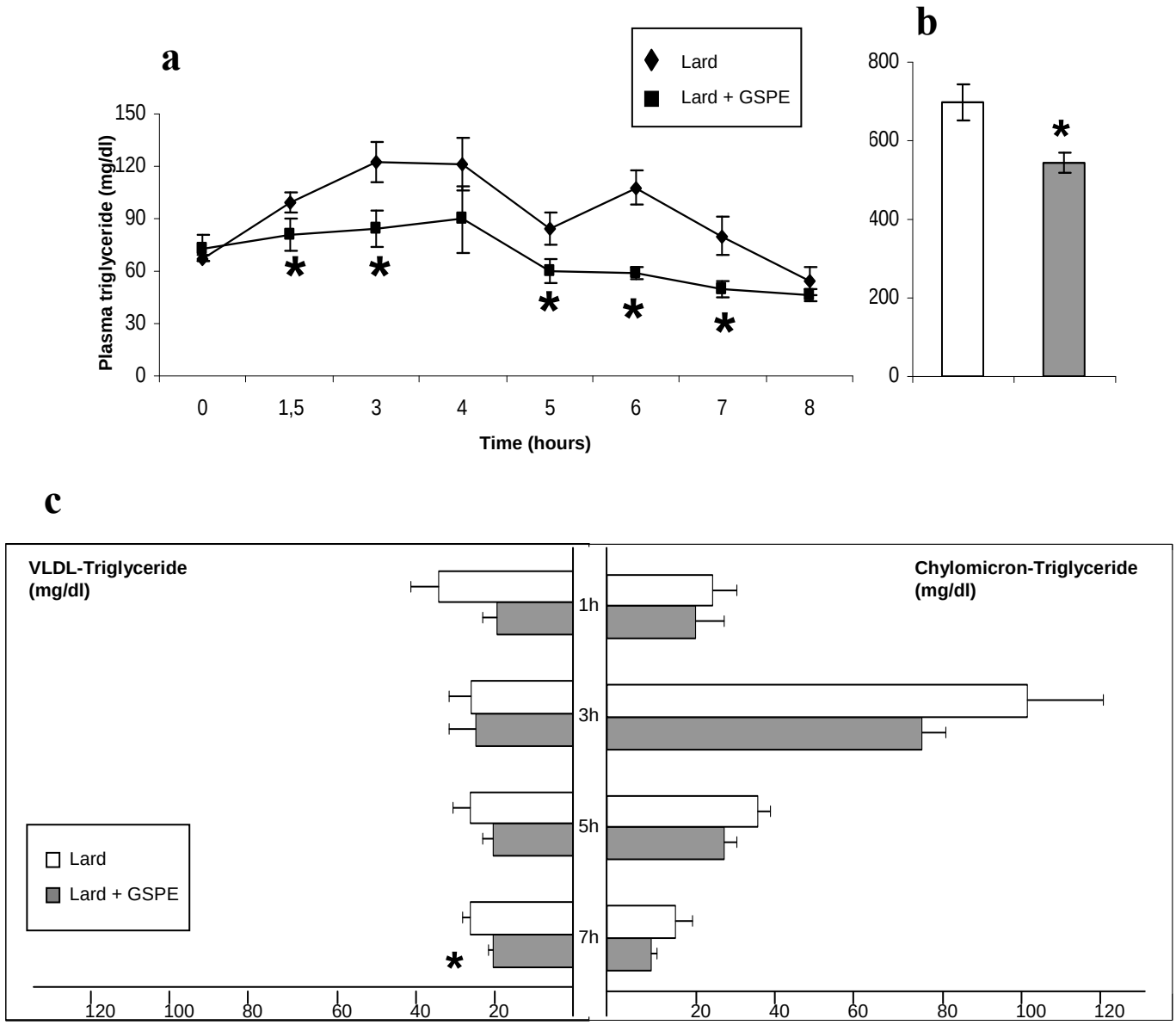

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Figure 2

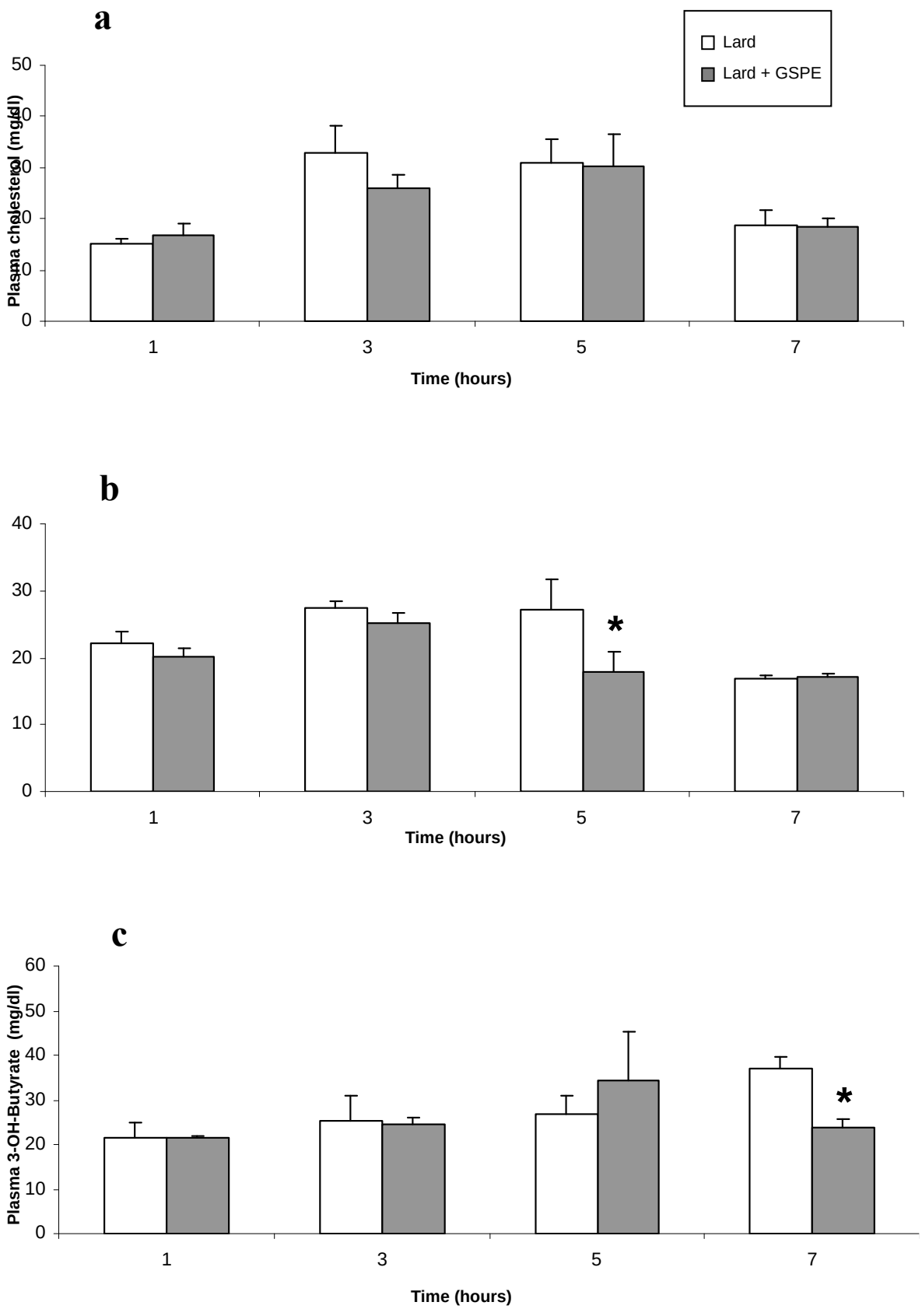

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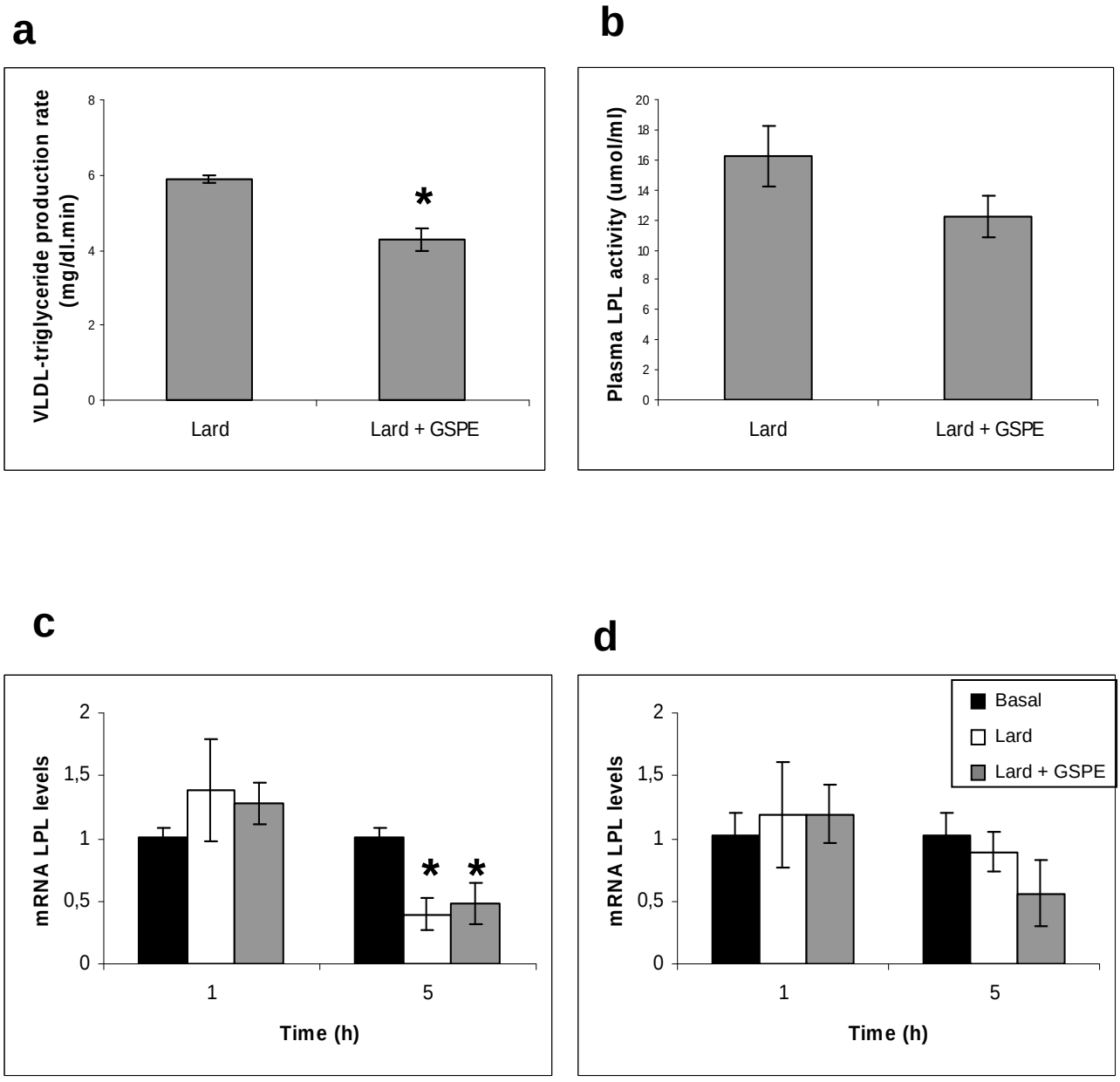
Figure 3



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2. Grape seed proanthocyanidins repress the expression of long chain acyl-CoA synthetase 5 and 3 in Caco2 intestinal cells

(Submitted)

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Grape seed proanthocyanidins repress the expression of long chain acyl-CoA synthetase 5 and 3 in Caco2 intestinal cells

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ABSTRACT

Background: Proanthocyanidins, a group of flavonoids fairly abundant in drinks and foods of vegetal origin, improve the hypertriglyceridemia associated to dietary obesity in rats. The small intestine is an important source of plasma triglyceride (TG)-rich lipoproteins (TRL). Intestinal cells can use two different pathways to carry out the TG synthesis, the monoacylglycerol (MAG) pathway in the post-prandial state and the glycerol-3-phosphate (G-3-P) pathway in the fasting state. The objective of this work is to determine the ability of proanthocyanidins to modulate TRL secretion by the enterocytes in post-prandial and fasting states

Methods: We evaluated *in vitro* the changes in the TG secretion and in the gene expression profile induced by a grape seed proanthocyanidins extract (GSPE), by using the Caco2 cell line cultured in transwells. We have emulated the post-prandial state by supplementing the apical medium with oleic acid, monoglyceride and taurocholate and the fasted state by supplementing the basolateral medium with oleic acid.

Results: GSPE did not modify the TG secretion in the post-prandial state although it repressed, significantly, the long chain acyl-CoA synthetase (ACSL) 5 and the carnitine-palmitoyl-transferase-1a gene expression. On the contrary, GSPE tended to decrease the TG secretion in the fasting state repressing, significantly, ACSL3, ACSL5, I-FABP and PPARalpha gene expression. Fatty acids (FA) delivered by ACSL5 would be directed towards the MAG pathway and the one channeled by the ACSL3 towards the G-3-P pathway. Thus, these results indicated that proanthocyanidns repress the supply of FA towards the MAG pathway in the post-prandial state whereas they repress the supply of FAs towards both the MAG and the G-3-P pathways in the fasted state.

Conclusion: The different sensitivity to GSPE between the two feeding states suggests that the feeding state is a key factor regarding the effectiveness of proanthocyanidins to reduce the triglyceridemia.

Keywords: flavonoids, triglyceride, intestine, ACSL, PPARalpha

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INTRODUCTION

The disturbances of the triglycerides (TG) metabolism are considered to be a substantial risk factor to suffer from obesity, atherosclerosis, insulin-dependent diabetes, breast and colon cancers (1). Elevated levels of TG-rich lipoproteins (TRL), especially chylomicrons (CM) remnants and very low density lipoproteins (VLDL) remnants, accumulate in the blood flow, and are strongly associated with an increased of the risk of myocardial infarction, ischemic stroke and early death (2).

The small intestine is as important source of plasma TRL, the second one just after the liver (3). The intestine secretes several different lipoproteins, CM and VLDLs are the major ones (4). VLDLs are the predominant lipoproteins during the fasting state and their assembly occurs constitutively (5). VLDLs may serve to transport lipids derived from the bile and fatty acids of the plasma (5). In the postprandial state, CM secretion is induced after a fat ingestion and is impaired by the absence of bile acids (5).

Most dietary TG are absorbed by the enterocytes as fatty acids (FA) and monoacylglycerides (MAG). FA and MAG require to reassembly to produce TG on the endoplasmatic reticulum by, predominantly, the progressive acylation of MAG via the monoacylglycerol pathway (3, 6). Additionally, TG can also be synthesised by a separated route by means of the acylation of the glycerol-3-phosphate (G-3-P) (6). The MAG pathway would predominate in the postprandial period while the G-3-P pathway is the main one in the interprandial and fasted period (7). Oleate entering from the apical membrane is preferentially shunted to the MAG pathway to form TG whereas oleate entering from the basolateral membrane comes from the circulation is shunted to G-3-P acylation pathway. The required enzyme activating the FA prior to its incorporation into MAG or G-3-P is one of the five members of the acyl-CoA synthetase long chain family (ACSL) (6). Of these ACSLs, only ACSL3 and 5 are significantly expressed in the intestine (6). In this proposed scenario, oleate-CoA delivered by ACSL5 would be directed towards the MAG pathway and the one channeled by the ACSL3 towards the G-3-P pathway (6).

Proanthocyanidins (PA), the most abundant polyphenols in grapes, apples, red grape juice, red wine and chocolate (8, 9) have been shown to reduce postprandrial hipertriglyceridemia in animal models (10) and improve plasma lipids in humans (11). The hypolipidemic action of proanthocyanidins is attributable to a reduction of plasma

levels TRL both in normolipidemic (12) and dyslipidemic rats (13). Plasma TG levels are the result of the balance between the TRL secretion by the intestine and the liver and their uptake by the extrahepatic tissues through the lipoprotein lipase (LPL). Several studies demonstrated the implication of the liver in the hypotriglyceridemic response triggered by PA (14) and established the molecular mechanisms by which PA modulated lipid and lipoprotein metabolism in the liver (15). However, the molecular mechanism by which PA modulates lipid and lipoprotein metabolism in the intestine is largely unknown. Thus, the aim of this study was to gain further insights into the role that the intestine plays in the hypotriglyceride action of PA. Intestinal cells can use two different pathways to carry out the TG synthesis, the MAG pathway in the post-prandial state and the G-3-P in the fasting state. Thus, we evaluated the effects of grape seed proanthocyanidins extract (GSPE) on the TG secretion and the gene expression by using the Caco2 cell line cultured in specific mediums simulating the two different feeding states.

MATERIALS AND METHODS

Proanthocyanidin extract

GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France).

This proanthocyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5-13 units) (31.7%) proanthocyanidins.

Materials

Taurocholate acid sodium salt (TC), fatty acid free bovine serum albumin (BSA), monoolein were obtained from Sigma. Oleic acid (OA) was purchased from Merck.

Cell Cultures

Caco2 cells (ATCC , Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) supplemented with 100U/ml penicillin, 100 µg/ml streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker) and 20% fetal bovine

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serum (BioWhittaker) in a 95% air, 5% CO_2 atmosphere at 37º C. The growth medium was replenished every 2 or 3 days. For gene expression analysis, cells at passages 58-61 were seed in 12-well plates at 2.5 $\times 10^5$ cells per well. For experiments on the secretion of TG from Caco2 cells, the cells at passages 60-63 were seed in 12 well Millicell Hanging Cell Culture Inserts (Millipore) at 1 $\times$ 10^5 per insert. The experiments were performed at 18-21 days post-seeding.

Preparation of feeding and fasted state

In order to perform a post-prandial state, the volume of the culture medium was 0.4 ml of DMEM (BioWhittaker) supplemented with 100U/ml penicillin, 100 µg/ml streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker), 0.8 mM OA, 0.5 mM TC and 0.4 Monoolein on the apical side, and 1 ml of DMEM (BioWhittaker) supplemented with 100U/ml penicillin, 100 µg/ml streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker) and 0.4 mM BSA on the basolateral side.

In order to perform a fasting state, the volume the culture medium was 0.4 ml of DMEM (BioWhittaker) supplemented with 100U/ml penicillin, 100 µg/ml streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker) supplemented with 0.4 mM BSA on the apical side, and 1 ml of DMEM without PhenolRed (BioWhittaker) supplemented with 100U/ml penicillin, 100 µg/ml streptomycin (BioWhittaker), 2 mM L-Glutamine (BioWhittaker), 0.8 mM OA and 0.4 mM BSA on the basolateral side.

Transepithelial electrical resistance (TEER) was measured just after removing the growth medium by using the Millicell-ERS system (Millipore). The TEER value of Caco2 cultured in each transwells chamber was around 308 ± 3.5 $\Omega \times cm^2$ in the post-pandrial state and 319 ± 5.9 $\Omega \times cm^2$ in the fasted state indicating the formation of tight monolayers (16).

Triglyceride secretion

Cells were cultured for 24 hours with the medium described above and treated with different concentrations of GSPE diluted in ethanol. In all the experiments GSPE was added into the apical side. Medium and cells were harvested after the treatments. The medium was ultracentrifugated with Amicon Ultra-4 centrifugal filter (Millipore) to

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concentrate it and the amount of TG secreted by the Caco2 cells was measured by using an enzymatic colorimetric kit (QCA, Spain). Values were corrected per mg cell protein determined by colorimetric assay (Bradford, Sigma).

Gene expression analyses

Caco2 cells were cultured for an hour with the post-prandial or fasting medium; all of them were treated with different concentrations of GSPE diluted in ethanol. Total RNA was isolated by using an RNeasy Mini kit (Quiagen, UK) following the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). A total 20 ng of cDNA was subjected to quantitative RT-PCR amplification using Taqman Master Mix (Applied Biosystems). Specific Taqman probes (Applied Biosystems) were used for different genes: Microsomal triglyceride transfer protein (MTP: Hs00165177_m1), Long-chain acyl-CoA synthetase 3 (ACSL3: Hs00244853_m1), Long-chain acyl-CoA synthetase 5 (ACSL5: Hs00212106_m1), Diacylglycerol acyltransferase 1 (DGAT1: Hs00201385_m1), Apolipoprotein A4 (ApoA4: Hs00166636_m1), Carnitine palmitoyl transferase 1 (CPT-1a: Hs00157079_m1), Peroxisome Proliferator-Acvtivated Receptor alpha (PPARα: Hs00223686_m1), Intestinal-fatty acid binding protein (I-FABP: Hs00164552_m1). Cyclophilin (Ppia: Hs99999904_m1) was used as an endogenous control. Real-time quantitative PCR reactions were performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems).

Statistical analysis

The results are reported as the mean ± S.E.M of three independent experiments for the TG secretion and two independent experiments for the gene expression analyses. Group means were compared with an independent-samples Student's t-test ($p \leq 0.05$) using SPSS software.

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RESULTS

GSPE treatment repressed the TG secretion in a fasted state but not in a post-prandial state in Caco2 intestinal cells

Caco2 cells 21 days post-confluence were cultured for 24 hours with a post-prandial or a fasted medium in 12 well Millicell Hanging Cell Culture Inserts (Millipore) and treated with 25-100 mg/L GSPE always added into the apical side. As shown in Figure 1, only 100mg/L of GSPE tended to decrease the TG secretion in the fasted state while in a post-prandial condition there were no changes. Thus, GSPE repressed TG secretion differently depending on the feeding state and it was more effective in the fasted state.

GSPE treatment repressed ACSL5 and ACSL3 in a fasted state but only ACSL5 in a post-prandial state

In order to assess whether GSPE could modulate the expression of genes related to lipid and lipoprotein metabolism depending on the feeding state, we measured the expression of key proteins in Caco2 intestinal cells (Tables 1 and 2). We have chosen proteins involved in the intracellular transport of the long chain fatty acids (I-FABP), TG synthesis (ACSL3, ACSL5, DGAT1), the fatty acid oxidation (CPT-1a) and CM and VLDL assembly (MTTP, ApoA4). Furthermore, the nuclear receptor PPARalpha was also selected because proteins like CPT-1a, ACSLs and FABPs are its targets (17, 18).

Caco2 cells cultured in post-prandial medium treated with 25 and 100 mg/L of GSPE showed a significant repression of ACSL5 and a significant overexpression of CPT-1a when compared with the control group (Table 1.). This expression profile suggests an increase of the channeling of fatty acid towards oxidation.

In contrast, Caco2 cells cultured in fasting medium treated with 100 mg/L of GSPE (Table 2) showed a significant repression of ACSL5, ACSL3, I-FABP and PPARalpha when compared to the control group. Thus, Caco2 cells cultured in a medium that simulates the fasted condition were more sensitive to GSPE in accordance with their sensitivity to TG secretion (Figure 1). Altogether, these results suggest that, depending on the feeding state, proanthocyanidins may act through different pathways to get the beneficial effects that have been described.

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DISCUSSION

In the Western diet, lipids represent more than a 40% of the daily caloric intake while the nutritional advice is a 10% lower (19). This high fat supply associated with a qualitative imbalance (excess of plasma TRL, saturated fatty acids and cholesterol and the absence of LPL) greatly contributes not only to the increase of obesity prevalence among the population, but also to the appearance of a plethora of diseases such as atherosclerosis, non insulin-dependent diabetes, breast and colon cancers (20). Therefore, dietary components that could reduce TRL production by the intestine would be decisive to ameliorate the appearance of obesity and these other pathologies. PA, a group of flavonoids that can be found in common foodstuffs (8, 9), actively reduces plasma TG and ApoB in normolipidemic rats (12), dyslipidemic rats (13), hamsters fed on a hypercholesterolemic diet (21) and humans (22). In previous studies we showed the implication of the liver in the hypotriglyceridemic response triggered by PA (14) and established the molecular mechanisms by which PA modulated lipid and lipoprotein metabolism in the liver (15). This study, then, intended to determine the role that plays the intestine in the hypotriglyceridemic action of GSPE. Some studies have indicated that intestines can supply a 20% or more of the total plasma TG in the absence of dietary fat (23) and a 40% in fasted rats (24). Thus, we study the effectiveness of GSPE in both post-prandial and fasted states.

We have chosen Caco2 cells because these represent the sole enterocyte model capable of differentiate spontaneously under standard cell culture conditions and allow the study of lipoprotein processing (3). Caco2 cells secrete apoB-containing particles that have similar flotation properties to those of plasma LDL (5). However, supplementation of Caco2 cells with OA has generally been shown to result in the secretion of more VLDL-sized particles and fewer LDL size particles (5). Since growing Caco2 cells in cell culture transwells, we have emulated the post-prandial state by supplementing the apical medium with OA, MAG and TC, and the fasted state by supplementing the basolateral medium with OA. The results of this study showed that Caco2 cells were more sensitive to a GSPE treatment in the fasted state. GSPE levels up to 100 mg/L did not modify TG secretion in post-prandial state but repressed TG secretion in fasted conditions. In the post-prandial state GSPE treatment repressed ACSL5 and overexpressed CPT-1a significantly. Nevertheless, in the fasted state GSPE treatment repressed ACSL5, ACSL3, I-FABP and PPARalpha significantly.

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ACSL are essential for *de novo* lipid synthesis, fatty acid catabolism and remodeling of membranes (25). The ACSL isoforms differ from their substrate preferences, enzyme kinetics, intracellular locations and the direction of their acyl-CoA products towards independent downstream pathways (26, 27). Only ACSL3 and ACSL5 are significantly expressed in the intestine (6, 26). ACSL5 is the only isoform found in both mitochondria membranes and endoplasmatic reticulum (28) although it was detected also in the plasma membrane (25). The overexpression of ACSL5 in rat hepatoma cell lines increases fatty acid (exogenous, not endogenous ones) incorporation into TG and without changes in β-oxidation or phospholipid synthesis (26, 29). Moreover, hepatic ACSL5 expression increases after applying an insulin treatment or a sterol regulatory element-binding protein (SREBP-1c) overexpression (26). Nevertheless, ACSL3 was detected in lipase-activated lipid droplets but not in un-induced droplets (25). Hepatic ACSL3 expression is upregulated in hyperlipidemic hamsters (30). Additionally, Yao H. et al (31) showed that ACSL3 played a crucial role in secretion of VLDL in human hepatoma Huh7 cells. Knockdown of ACSL3 in human hepatocytes decreases [1-^{14}C] oleic acid incorporation to phospholipids for VLDL synthesis (29). Despite there is not experimental evidence of the ACSL5 and ACSL3 roles in intestine, it has been postulated that FA delivered by ACSL5 would be directed towards the MAG pathway and by ACSL3 towards the G-3-P pathway (6). Thus, at the light of the results, it can be suggested that GSPE represses only the supply of FA towards the MAG pathway in the post-prandial state (ACSL5) whereas represses FA delivered towards both pathways in fasted state (ACSL5 and ACSL3). Therefore, GSPE could be more effective reducing TG secretion in the fasted than in the post-prandial state.

In the intestinal cells two fatty acid binding proteins (FABPs) are largely and equally expressed: Liver-FABP (L-FABP), which is also expressed in the liver and intestinal-FABP (I-FABP), which is specially expressed in fully differentiated proximal absorptive enterocytes (20). It has been suggested that there is a potential function of I-FABP directing long chain FA to specific subcellular sites of utilisation, such as β-oxidation and esterification into phospholipids and TG (32). Thus, the repression of I-FABP by GSPE in the fasted state could work together with ACSL5 and ACSL3 reducing TG synthesis and secretion.

PPARalpha, which is abundantly expressed in enterocytes, is an important nuclear receptor that mediates the effects of dietary lipids on gene expression (33). Natural

agonists of PPARalpha normally found in diet are oleic acid, eicosapentaenoic acid and docosahexaenoic acid (34). Cytoplasmatic FABP transfers and channels the FA and its CoA metabolites into nuclei, binds PPARalpha and activates the transcription of genes involved in fatty acid and glucose metabolism (35). PPARalpha controls the expression of several genes involved in fatty acid metabolism such as those involved in the transport across the cell membrane, the intracellular binding (I-FABP), the formation of acyl-CoA (ACSL) and the mitochondrial and peroxisomal β-oxidation (CPT-1a) (18). Therefore, the repression of ACSL and I-FABP by GSPE in the fasted state may be secondary for the reduction of PPARalpha expression.

In conclusion, Caco2 cells were more sensitive to GSPE treatment in the fasted state than in the post-prandial one. The repression of ACSL5, ACSL3, I-FABP and PPARalpha expressions in the fasted state may account for the lower levels of TG in the medium. Furthermore, the different modulation of gene expression by GSPE between the two feeding states suggests that the feeding state is an important condition in order that GSPE can carry out its hypotriglyceridemic action in the intestine.

These results provide the first *in vitro* evidence of the fact that ACSL3 and ACSL5 are target genes of PA, supporting the idea that they are powerful agents for preventing and treating lipid altered metabolic states.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. mRNA of lipid-related genes in Caco2 cells grown in a medium simulating the post-prandial state and treated with 25 and 100 mg/L of grape seed proanthocyanidin extract (GSPE).

	Control	GSPE 25 mg/L	GSPE 100 mg/L
ACSL5	1.01 ± 0.07	0.68 ± 0.01 *****	0.72 ± 0.06 *****
ACSL3	1.00 ± 0.04	0.92 ± 0.01	0.94 ± 0.01
DGAT1	1.02 ± 0.09	1.11 ± 0.05	0.87 ± 0.07
ApoA4	1.01 ± 0.08	1.29 ± 0.16	1.05 ± 0.23
MTTP	1.05 ± 0.14	1.23 ± 0.16	1.06 ± 0.13
CPT-1a	1.03 ± 0.12	1.73 ± 0.17 *****	1.41 ± 0.04 *****
I-FABP	1.03 ± 0.10	0.86 ± 0.08	0.81 ± 0.09
PPARalpha	1.02 ± 0.06	1.06 ± 0.05	0.94 ± 0.08

The culture medium on the apical side was supplemented with 0.8 mM oleic acid, 0.5 mM Taurocholate and 0.4mM Monoolein. GSPE was added to the apical side. mRNA levels were measured after an hour of culture. Each value is the mean ± s.e.m of two independent experiments. * indicates significant differences ($p \leq 0.05$) between control cells and GSPE treated cells by Student's t-test.

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Table 2. mRNA of lipid-related genes in Caco2 cells grown in a medium simulating the fasted state and treated with 100 mg/L of grape seed proanthocyanidin extract (GSPE).

	Control group	GSPE 100 mg/L
ACSL5	1.01 ± 0.06	0.75 ± 0.06 *****
ACSL3	1.01 ± 0.03	0.82 ± 0.04 *****
DGAT1	1.00 ± 0.03	0.94 ± 0.04
ApoA4	0.97 ± 0.05	0.78 ± 0.14
MTTP	1.05 ± 0.14	0.97 ± 0.03
I-FABP	1.01 ± 0.06	0.65 ± 0.04******
PPARalpha	1.00 ± 0.04	0.88 ± 0.02 *****
CPT1a	1.01 ± 0.04	0.95 ± 0.04

The culture medium on the basolateral side was supplemented with 0.8 mM oleic acid and 0.4 mM BSA. GSPE was added to the apical side. mRNA levels were measured after an hour of culture. Each value is the mean ± s.e.m of two independent experiments. * indicates significant differences ($p \leq 0.05$) between control cells and GSPE treated cells by Student's t-test

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FIGURE LEGENDS

Figure 1: Triglyceride secretion by Caco2 cells grown in a medium simulating post-prandial or fasted states and treated with a grape seed proanthocyanidin extract (GSPE).

In the post-prandial state, the culture medium on the apical side was supplemented with 0.8 mM oleic acid, 0.5 mM Taurocholate and 0.4 Monoolein. In the fasting state, the culture medium on the basolateral side was supplemented with 0.8 mM oleic acid and 0.4 mM BSA. GSPE (25, 50 or 100 mg/L) was always added to the apical side. Triglycerides were measured after 24 hours of culture. Each value is the mean ± s.e.m of three independent experiments. # indicates significant differences ($p = 0.08$) between control cells and GSPE treated cells by Student's t-test.

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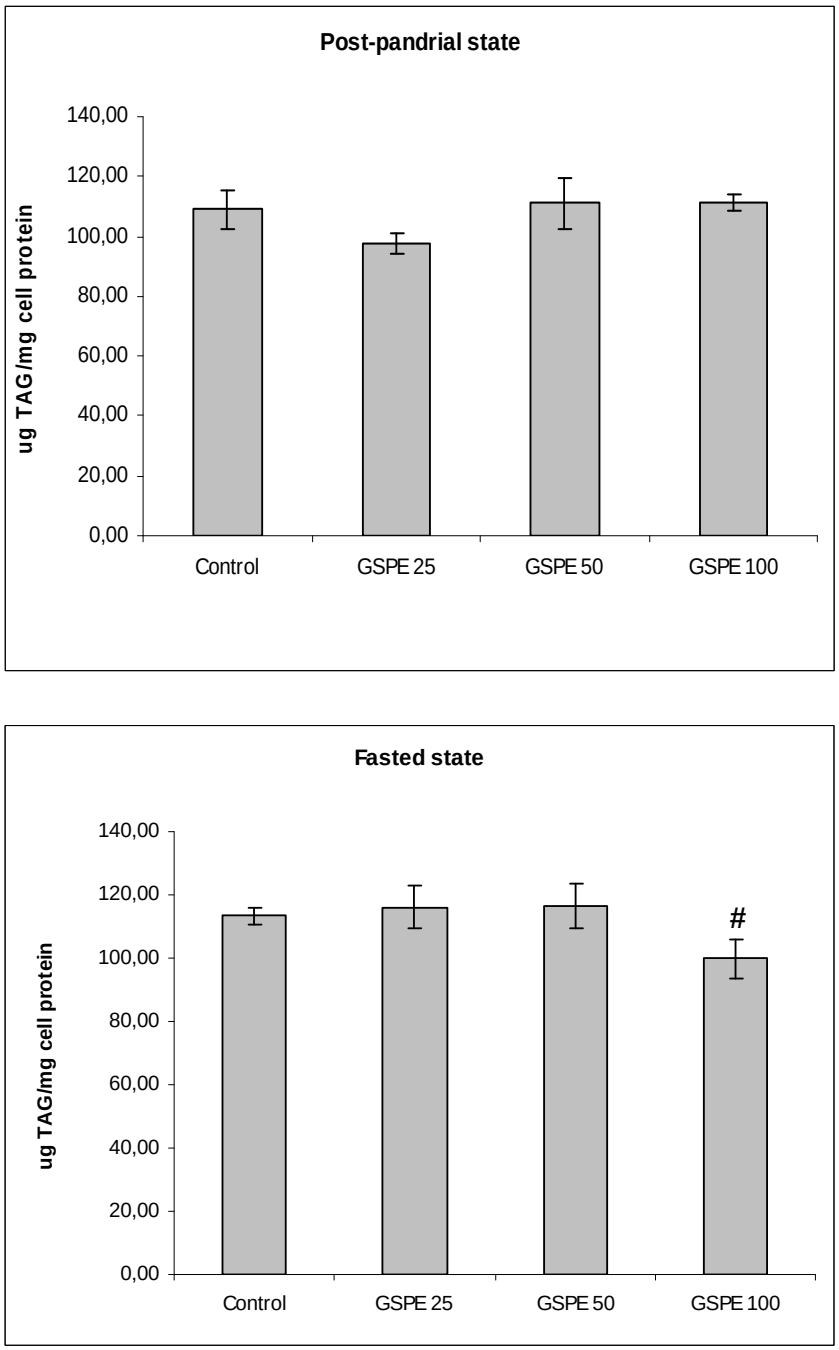
Figure 1



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3. High and Chronic doses of proanthocyanidins do not revert weight gain in mice fed with high-fat high-carbohydrate diet at short-term.

(Submitted)

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High and Chronic doses of proanthocyanidins do not revert weight gain in mice fed with high-fat high-carbohydrate diet at short-term.

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ABSTRACT

Flavonoids are benefitial compounds against risk factors for metabolic syndrome. Proanthocyanidins, the most abundant flavonoids in human diet, counteract atherogenic dyslipidemia associated with obesity and metabolic syndrome. Diet composition is know to influence energy intake and body-weight changes in rats and humans. A high carbohydrate high fat diet induce a metabolic syndrome model in rats leading them to hyperlipidemia and hypercholeterolemia, and increases the risk of cardiovascular diseases (CVD) raising triglyceride (TG) and lowering high density lipoprotein-cholesterol (HDL-C) levels. The objective of this paper was to evaluate the effect of proanthocyanidins on body weigth and energy intake at the early period of a cronic study. To this end, we have used male C57BL6 mice fed on a high carbohydrate high fat (HCHF) diet supplemented with low (0.03%), medium (0.15%) or high (0.3%) of grape seed proanthocyanidins extract (GSPE) for 4 weeks. After 4 weeks administiring the treatment, medium- and high-GSPE treatment tended to reduce plasmatic TG and cholesterol, and high-GSPE treatment significantly reduced free fatty acid levels in plasma. By contrast, GSPE treatment did not counteract the weight gain induced by HCHF, even induced a low increase in body fat deposition. Moreover, GSPE delayed palmitate oxidation at two weeks. Medium- and high-GSPE treatment tended to increase energy retention in mice at second week (39% and 18%, respectively), whereas the three doses of GSPE decreased energy retention at week fourth (36%, 14% and 34% for low-, medium- and high-GSPE, respectively). GSPE modified energy retention by changing energy intake but not energy lost in feces. These results indicate that proanthocyanidins have a bimodal effect on energy intake, increasing it at early-term and decreasing it thereafter. Thus, proanthocyanidins initially exacerbated the effect of high-fat diet by further increasing energy intake and decreasing palmitate oxidation. Therefore, it can be suggested that proanthocyanidins-rich foods must be consumed habitually and at long-term in order to be effective improving the excess of body weight associated to metabolic syndrome.

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INTRODUCTION

Diet composition is know to influence energy intake and body-weight changes in rats and humans (1). Therefore, multiple and complex mechanisms have evolved to regulate energy intake and expenditure to maintain body weight (2). For weight maintenance, not only does energy intake have to mach energy expenditure, but also macronutrient intake must balance macronutrient oxidation (2). It is suggested that macronutrients imbalance may be responsible of not only to the increase of obesity prevalence in the population, but also to the appearance of a plethora of diseases such as atherosclerosis, non insulin-dependent diabetes, breast and colon cancers (3). Thus, a high carbohydrate high fat diet induce a metabolic syndrome model in rats, (4) in fact, it is well known that a diet high in saturated fat and refined-carbohydrate (sucrose) leads to hyperlipidemia and hypercholeterolemia, and increases the risk of cardiovascular diseases (CVD) (5) raising triglyceride (TG) and lowering high density lipoprotein-cholesterol (HDL-C) levels (6). Hyperlipidemia is the result of increased plasma concentration of very low density lipoprotein (VLDL)(7). This increse is consequence of overproduction of VLDL by the liver and the possible delay catabolism of these lipoproteins (7). Thus, excess carbohydrate and fat uptake by the liver has a deleterious effect on liver metabolism, causing increased liver fat, increased VLDL-TG, and apolipoprotein B-100 (apoB-100) secretion as well as increased secretion of inflammatory markers C reactive protein, fibrinogen and serum amyloid A by the liver into the plasma (6).

Proanthocyanidins (PA) are the most abundant polyphenols in grapes, apples, red grape juice, red wine and chocolate (7, 8). The health benefits of PA have been most studied in cocoa and grape seed, each of which has a characteristic and specific oligomeric composition that conditions its biological activity. Different mechanisms have been proposed for weight loss and weight maintenance induced by flavonoids. It has been proposed that flavonoids may increase energy expenditure, fat oxidation and counteract the decrease in metabolic rate that is present during weight loss (9). Some studies have focused on the influence of PA reducing body weight. The administration of grape seed extract (36 mg /kg body weight), enriched with oligomeric PA, reduces body weight gain and abdominal fat accumulation induced by high-fat diet in hamsters after 12 weeks of treatment (10). Our group has demonstrated that a moderate red-wine consumption (equivalent to 3.4 mg PA/day per animal for 8 weeks) partially prevents body weight gain in rats fed with a hiperlipidic diet (11). However, in another study we

have demonstrated that grape seed proanthocyanidins extract (25 mg/Kg animal) administered for 10 days to rats fed with high-fat diet, corrected dyslipidemia and repressed genes controlling lipogenesis and VLDL assembling in liver but did not reduce body weight (7). Thus, it seems that the effect of PA on body weight is not immediate. Altogether suggest that the benefitial effects of PA depend on the doses, the way of administration as well as the duration of the treatment.

The purpose of this study was to examine the effect of three different doses (low, medium and high) of grape seed PA extract (GSPE) on body weight gain and development of obesity at short-term. To this end, we have used mice fed on a high carbohydrate high fat diet (HCHF) as a model of metabolic syndrome and we have assessed the effect of GSPE on body weight, food intake, substrate oxidation, plasma profile and adiposity.

MATERIALS AND METHODS

Proanthocyanidin extract

GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). This proanthocyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5-13 units) (31.7%) proanthocyanidins.

Animals, diets and experimental setup

Male 9-10 week old C57BL6 mice weighing 26 g were purchased from Charles River (Suzfeld, Germany). The experimental protocol was approved by the Ethical Committee on the Use of Animals as Experimental Subjects of the Ministry of Agriculture, Nutrition and Forestry (State Brandenburg, Germany). The animals were housed individually in a climate-controlled room with a 12-h light-dark cycle (light of 8 h am to 20 pm) and were fed with standard chow and water ad libitum for 1 week. After that week, the animals were randomly assigned to experimental diet (n= 8 per group) with or witout GSPE (Table 2), which were consumed ad libitum for 4 weeks. The experimental diet (High Carbohydrate High Fat Diet, HCHFD) (Table 1) have adequate protein content (about 20% calories) and high dietary fat content (about 40% calories). Mice

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were fed on an HCHF diet containing three concentrations of GSPE: 0.3 g/Kg (0.03% wt/wt)(low-GSPE group), 1.5 g/Kg (0.15% wt/wt)(medium-GSPE group) or 3 g/Kg (0.3% wt/wt) (high-GSPE group). Mice fed on an HCHF diet without GSPE were used as control group. Water was provided ad libitum.

Food intake, body weight and body composition were monitored weekly. After 1 week of feeding, a substrate oxidation test of orally applied ^{13}C-labelled palmitate was determined as described below. The energy intake, the energy retained and the energy lost in the feces were measured by an adiabatic bomb calorimeter (IKA-Calorimeter C5000, IKA-Werke GmbH & Co.KG, Germany) at the second and fourth week after feeding. Finally, mice were sedated by isofluorane inhalation and killed by cervical dislocation in the postabsorptive state. Blood samples were collected from heart and plasma was obtained by centrifugation.

Substrate Oxidation Test

Substrate oxidation of orally applied ^{13}C-labelled palmitate was determined during the experiment. ^{13}C- labeled potassium palmitate (1-^{13}C, 99%, MW 294.5 g/mol, Cambridge Isotope Lab., Inc. Andover, MA, USA) was emulsified by vortexing at 60°C in 5% lecithine (Emultop, Cargill Texturizing Solutions Deutschland GmbH & Co. KG, Hamburg, Germany), dissolved in sterilized water and 60 µmol/kg were applied by oral gavage in a volume of 50 µl/20 g body weight. Breath samples were taken at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270 and 300 min[3]. ^{13}C was measured by gas chromatography–combustion isotope ratio mass spectrometry (GC-C-IRMS).

Plasma parameters assay

Plasma free fatty acids (FFAs) were determined using NEFA C kit (Wako Chemicals GmbH, Neuss, Germany). Triglicerides, cholesterol, hidroxybutirate, insulin and leptin were determined using colorimetric and enzymatic standard methods (Cobas Mira, Hoffmann La Roche AG, Grenzach-Whylen, Germany).

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Statistical analysis

Data are reported as means ± SEM. Differences between mean values were determined by independent-samples Student's t-test ($p \leq 0.05$) using SPSS software.

RESULTS

GSPE treatment did not counteract the weight gain induced by the HCHF even induced a low increase in body fat deposition

Mice ingested a HCHF diet containing low (0.3 g GSPE/kg chow), medium (1.5 g GSPE/kg chow) or high (3 g GSPE/kg chow) GSPE levels. Mean values of GSPE intake by mice during the four weeks were 38 ± 1.62 mg of GSPE /kg body weight for the low-GSPE group, 230 ± 15.37 mg of GSPE /kg body weight for the medium-GSPE group and 420 ± 17.61 mg of GSPE / kg of body weight for the high-GSPE group. These intakes of GSPE are below of the no-observed-adverse effect level (NOAEL) defined for this extract in sub-chronic studies with rats (1400 to 1700 mg /kg body weight for males (12, 13).

Control mice fed four weeks with HCHF diet increased 19% their body weight (Table 2). The addition of GSPE to the diet did not influence body weight gain induced by the HCHF diet (19%, 21% and 23% increase in body weight for low-, medium- and high-GSPE groups respectively).

HCHF diet consumption induced body fat deposition (Table 2) in control mice in which body fat rose from 5% of body weight in basal state to 17% after four weeks of HCHF diet. Mice treated with medium- and high-GSPE treatment increased slight fat deposition and fat reached to 20% of body weight.

The addition of GSPE to the diet did not significantly increase liver, visceral white adipose tissue (WAT), epididymal WAT, subcutaneous WAT and BAT weights. However, mice treated with medium- and high-GSPE treatment shown 10% increase of visceral WAT and epididymal WAT compared with the control mice fed four weeks with HCHF. These results are in accordance with the tendency to increase body fat deposition in mice treated with medium- and high-GSPE.

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GSPE treatment altered the energy retained by mice and delayed palmitate oxidation.

The mean of chow intake by mice through the four weeks (Table 4) was not affected by GSPE added to foodstuff comparing to control mice. However, GSPE treatment modified energy intake (Table 4) when was calculated at two and four weeks. Medium- and high-GSPE treatment tended to increase energy intake in mice at two weeks (36% and 15% of control, respectively), whereas the three doses of GSPE decreased energy intake at four weeks (30%, 10% and 20% for low-, medium- and high-GSPE, respectively). Thus energy intake at week fourth was significantly decreased by GSPE treatment respect to the second week. These results indicate that GSPE had a bimodal effect on energy intake, increasing it until two weeks and decreasing thereafter.

After two weeks of HCHF diet, the energy retained (Table 5) by mice treated with low-GSPE was equal to those of control mice, whereas was increased by 51% and 14% in mice treated with medium- and high-GSPE respectively. The bigger energy retention in medium- and high-GSPE mice was largely due to increased energy intake and not to energy lost in feces.

On the other hand, lost of energy in feces was not affected by GSPE treatment, neither at two and four weeks.

At two weeks of HCHF diet, the energy retained (Table 4) by mice treated with low-GSPE was equal to those of control mice, whereas was increased by 51% and 14% in mice treated with medium- and high-GSPE respectively. The bigger energy retention in medium- and high-GSPE mice was largely due to increased energy intake and not to energy lost in feces.

At four weeks of HCHF diet, the energy retained by mice treated with medium-GSPE was equal to those of control mice, whereas was reduced by 35% and 25% in mice treated with low- and high-GSPE. Like at two weeks, the mainly component that caused the reduction in energy retention was a decreased energy intake. Thus, GSPE modified energy retention by changing energy intake but not energy lost in feces.

There were not significant differences in palmitate oxidation by GSPE treatment (Figure 1), but the maximum of palmitate oxidation was delayed by GSPE treatment (a delay of 15 minutes in medium- and high-GSPE treated mice and 30 minutes in low-GSPE treated mice).

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GSPE treatment tended to improve plasma lipid profile

GSPE treatment tended to decrease plasma TG levels (Table 5) in mice treated with medium- and high-GSPE treatment compared with the control group. Furthermore, the mice treated with the medium-GSPE treatment tended to decrease plasma cholesterol levels (Table 5) compared with the control group. Additionally, the mice treated with the high-GSPE treatment decreased significantly plasma FFA concentration (Table 5) compared with the control group. Neither β-hidroxibutirate, insulin and leptin plasma levels (Table 5) were affected by GSPE treatment. Thus, medium and high doses of GSPE tended to ameliorate the dyslipidemia induced by the HCHF diet.

DISCUSSION

There is a strong positive association between obesity and type II diabetes, cardiovascular disease and hypertension (14). These associations describe the metabolic syndrome, a clustering of risk factors including abdominal obesity, insulin resistance, and dyslipidemia (14). PA, a group of flavonoids that can be found in common foodstuffs (15, 16), improve risk factors associated to metabolic syndrome. PAs actively reduce plasma TG and ApoB in normolipidemic rats (17), hamsters fed a hypercholesterolemic diet (18) and humans (19). In previous studies we have demonstrated that PA correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver (7). Additionally, we showed the implication of the liver in the hypotriglyceridemic response triggered by PA (20) and established the molecular mechanisms by which PA modulated lipid and lipoprotein metabolism in the liver (8). However, the effect of Pas improving obesity is not clear. This study, then, intended to evaluate the effect of proanthocyanidins on body weigth and energy intake at short-term.

Short-term GSPE treatment did not counteract the weight gain induced by the HCHF even induced a low increase in body fat deposition resulted in a slight increase of visceral WAT and epididymal WAT in mice treated with medium- and high-GSPE treatment. This slight increase of body fat deposition induced by GSPE may be consequence of the biggest energy retention in medium- and high-GSPE group mice at two weeks. Moreover, at this time, GSPE treatment delays palmitate oxidation. Thus, this excess of energy may be transformed in fat and deposited in the body.

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Interestingly, GSPE had a bimodal effect on energy retention. GSPE increased initially the energy retention and further decreased it. The mainly component responsible of these changes was the energy intake and not the energy lost in the feces. GSPE increased energy intake until two weeks and reduced it at four weeks. In contrast to our results, a grape seed extract do not modify food and energy intake in high-fat fed hamsters in which PA reduce body weight gain and abdominal fat accumulation after 12 weeks (9). Therefore, it can be suggested that PA modify food and energy intake depending on the length of the treatment. Other flavonoids, like isoflavones, reduce obesity by decreasing food intake, and increasing cholecystokinin and peptide YY levels, which can induce satiety (21, 22). So, further studies of the effects of PA at different length on fluctuations of oroxigenic and anoroxigenic peptides are warranted.

Furthermore, PA regulate adipocyte biology, modifying lipid synthesis, lipid degradation,glucosa uptake and differentiation (23). Perhaps, also the effect of GSPE on adipocyte functionality *in vivo* is not constant and depends on the length of treatment.

By contrast to the effects of GSPE on body weight and fat deposition, GSPE treatment tended to decrease plasma TG in medium and high doses. Besides, the medium-GSPE treatment tended to decrease plasma cholesterol and the high-GSPE treatment decreased significantly plasma FFA. Thus, it can be suggested that PA modify lipid metabolism and ameliorates plasma lipoid profiles at short-term treatment.

In conclusion, proanthocyanidins are powerful agents for preventing and treating lipid altered metabolic states although they do not counteract the gain of body weight and the energy intake for a short-term metabolic syndrome. Proanthocyanidins have a bimodal effect on energy intake, increasing it at early-term and decreasing it thereafter. Thus, proanthocyanidins initially exacerbate the effect of high-fat diet by further increasing energy intake and decreasing palmitate oxidation. According to our results, it has been described that quercetin worsens hepatic insulin resistance induced by high-fat diet in mice at early-term (14). Therefore, it can be suggested that proanthocyanidins-rich foods must be consumed habitually and at long-term in order to be effective improving the excess of body weight associated to metabolic syndrome.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. Composition of the high-carbohydrate high-fat diet

Casein[a] (g/100g)	18
Wheat starch[b] (g/100g)	43
Saccharose[c] (g/100g)	5
Coconut oil[d] (g/100g)	18
Safflower oil[e] (g/100g)	1
Linseed oil[f] (g/100g)	1
Cellulose[g] (g/100g)	7
Mineral mixture[h] (g/100g)	5
Vitamin mixture[i] (g/100g)	2

[a] Dauermilchwerk Peiting GmbH, Landshut, Germany, contained 86% crude proteína (% N x 6.38); [b] Heller u. Strauß, Berlin, Germany; [c] Nordzucker GmbH, Uelzen, Germany; [d, e, f] Kunella-Feinkost GmbH, Cottbus, Germany; [g] Rettenmeier, Ellwangen, Germany; [h] Mineral mixture per 100g diet: Ca, 930 mg; P, 730 mg; Mg, 80 mg; Na, 440 mg; K, 710 mg; S, 170 mg; Cl, 360 mg; Fe, 20 mg; Mn, 10 mg; Zn, 3 mg; Cu, 800 mg; J, 40 mg; F, 400 mg; Se, 20 mg; Co, 10 mg (Altromin GmbH, Lage, Germany); [i] Vitamin mixture containing 17.5 g/100g DL-methionine; vitamina content in 100g diet: A, 0.45 mg; D_3, 1.3 mg; K_3, 1 mg; B_1, 2 mg; B_2, 2 mg; B_6, 1.5 mg; B_{12}, 3 mg; niacin, 5 mg; pantothenate, 5 mg; folic acid, 1 mg; biotin, 20 mg; choline chloride, 100 mg; p-aminobenzoic acid, 10 mg; inositol, 10 mg; E, 16.4 mg (Altromin GmbH, Lage, Germany)

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Table 2. Body weight and body fat of mice fed with a high-carbohydrate high-fat diet supplemented with several concentrations of grape seed proanthocyanidin extract (GSPE) for 4 weeks.

	Control	low-GSPE	medium-GSPE	high-GSPE
Initial body weight (g)	27.0 ± 0.3	26.4 ± 0.7	27.2 ± 0.4	26.3 ±0.5
Final body weight (g)	32.1 ± 1.2	31.3 ± 0.3	32.9 ± 1.2	32.5 ± 0.7
Body weight gain (g)	5.7 ± 0.8	4.7 ± 0.8	6.2 ± 0.8	6.2 ± 0.8
Initial body fat (g)	1.5 ± 0.1	1.5 ± 0.1	1.3 ± 0.2	1.8 ± 0.2
Final body fat (g)	5.6 ± 0.9	5.4 ± 0.8	6.5 ± 1.0	6.6 ± 0.8
Body fat gain (g)	4.2 ± 0.9	3.8 ± 0.7	5.1 ± 0.9	4.8 ± 0.7
Body fat (% body weight)	16.9 ± 2.1	17.2 ± 2.2	19.2 ± 2.5	20.0 ± 2.3
Mean food intake (g/wk)	4.6 ± 0.4	4.0 ± 0.4	4.8 ± 0.3	4.5 ± 0.4

Mice were fed on an HCHF diet containing three concentrations of GSPE: 0.3 g/Kg (0.03% wt/wt)(low-GSPE group), 1.5 g/Kg (0.15% wt/wt)(medium-GSPE group) or 3 g/Kg (0.3% wt/wt) (high-GSPE group). Mice fed on an HCHF diet without GSPE were used as control group. Food intake, body weight and body composition were monitored weekly. Finally, mice were sedated by isofluorane inhalation and killed by cervical dislocation in the postabsorptive state. Each value is the mean ± s.e.m of eight animals.

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Table 3. Tissues weight of mice fed with a high-carbohydrate high-fat diet supplemented with several concentrations of grape seed proanthocyanidin extract (GSPE) for 4 weeks.

	Control	low-GSPE	medium-GSPE	high-GSPE
Liver (g)	1.24 ± 0.04	1.31 ± 0.06	1.25 ± 0.05	1.31 ± 0.05
Visceral WAT (g)	0.55 ± 0.10	0.52 ± 0.07	0.65 ± 0.07	0.51 ± 0.06
Epididymal WAT (g)	0.79 ± 0.14	0.76 ± 0.09	0.89 ± 0.14	0.94 ± 0.11
Subcutaneous WAT (g)	0.46 ± 0.05	0.44 ± 0.07	0.44 ± 0.07	0.46 ± 0.05
BAT (g)	0.17 ± 0.02	0.18 ± 0.02	0.15 ± 0.02	0.16 ± 0.01

Mice were fed on an HCHF diet containing three concentrations of GSPE: 0.3 g/Kg (0.03% wt/wt)(low-GSPE group), 1.5 g/Kg (0.15% wt/wt)(medium-GSPE group) or 3 g/Kg (0.3% wt/wt) (high-GSPE group). Mice fed on an HCHF diet without GSPE were used as control group. Mice were sedated by isofluorane inhalation and killed by cervical dislocation in the postabsorptive state. Each value is the mean ± s.e.m of eight animals.

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Table 4. Plasma levels of triglyceride, cholesterol, free fatty acids, hydroxybutyrate, leptin and insulin of mice fed with a high-carbohydrate high-fat diet supplemented with several concentrations of grape seed proanthocyanidin extract (GSPE) for 4 weeks.

	Control	low-GSPE	medium-GSPE	high-GSPE
Triglycerides (mg/ml)	6.9 ± 0.9	7.0 ± 0.7	5.1 ± 0.3#	5.2 ± 0.4#
Cholesterol (mg/dL)	110.6 ± 5.9	101.1 ± 5.9	96.3 ± 4.8#	107.6 ± 5.1
FFA (mmol/L)	1.28 ± 0.03	1.31 ± 0.07	1.27 ± 0.05	1.14 ± 0.04*
Hidroxybutirate (mg/dL)	0.61 ± 0.08	0,58 ± 0.07	0.67 ± 0.07	0.79 ± 0.08
Leptin (pg/ml)	7548 ± 2290	6280 ± 1554	3714 ± 624	6340 ± 1978
Insulin (pg/ml)	966 ± 258	804 ± 137	916 ± 387	1172 ± 356

Mice were fed on an HCHF diet containing three concentrations of GSPE: 0.3 g/Kg (0.03% wt/wt)(low-GSPE group), 1.5 g/Kg (0.15% wt/wt)(medium-GSPE group) or 3 g/Kg (0.3% wt/wt) (high-GSPE group). Mice fed on an HCHF diet without GSPE were used as control group. Mice were sedated by isofluorane inhalation and killed by cervical dislocation in the postabsorptive state. Blood samples were collected from heart and plasma was obtained by centrifugation. Each value is the mean ± s.e.m of eight animals. Leptin and insulin is the mean ± s.e.m of four animals. # indicates significant differences ($p \leq 0.1$) versus control values and * indicates significant differences ($p \leq 0.05$) versus control values by T-student.

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Table 5. Food intake. Percentage of the increase in the energy in the faeces, the energy intake and energy retained.

	Week	Control	low-GSPE	medium-GSPE	high-GSPE
Mean food intake (g/wk)		4.6 ± 0.4	4.0 ± 0.4	4.8 ± 0.3	4.5 ± 0.4
Energy intake (KJ/mouse) *(% of control value)*	2 w	75.2 ± 4.2 *(100)*	75.4 ± 5.4 *(100)*	102.0 ± 3.3 *(136)*	86.7 ± 6.4 *(115)*
	4 w	99.9 ± 14.4 *(100)*	69.1 ± 4.5 *(69)*	89.4 ± 4.5 *(89)*	79.8 ± 3.2 *(80)*
Energy intake at week 4 respect to week 2 (%)		16.44 ± 11.2	-10.62 ± 5.6#	-14.02 ± 5.7*****	-11.04 ± 7.2#
Energy in feces (KJ/mouse) *(% of control value)*	2 w	8.6 ± 0.4 *(100)*	9.5 ± 0.4 *(110)*	9.3 ± 0.5 *(108)*	8.3 ± 0.4 *(97)*
	4 w	11.4 ± 0.8 *(100)*	12.3 ± 0.7 *(108)*	12.2 ± 0.5 *(107)*	12.1 ± 0.3 *(106)*
Energy in feces at week 4 respect to week 2 (%)		26.5 ± 4.5	23.4 ± 4.2	23.8 ± 4.2	33.3 ± 2.7
Energy retained (KJ/mouse) *(% of control value)*	2 w	66.6 ± 4.4 *(100)*	66.1 ± 5.3 *(99)*	92.8 ± 3.3 *(139)*	78.3 ± 6.1 *(118)*
	4 w	88.5 ± 14.1 *(100)*	57.0 ± 4.0 *(64)*	77.5 ± 4.5 *(86)*	67.6 ± 2.9 *(76)*
Energy retained at week 4 respect to week 2 (%)		14.55 ± 5.5	-18.08 ± 6.8#	-20.40 ± 7.7*****	-18.47 ± 7.0#

Mice were fed on an HCHF diet containing three concentrations of GSPE: 0.3 g/Kg (0.03% wt/wt)(low-GSPE group), 1.5 g/Kg (0.15% wt/wt)(medium-GSPE group) or 3 g/Kg (0.3% wt/wt) (high-GSPE group). Mice fed on an HCHF diet without GSPE were used as control group. The energy intake, the energy retained and the energy lost in the feces were measured by an adiabatic bomb calorimeter (IKA-Calorimeter C5000, IKA-Werke GmbH & Co.KG, Germany) at the second and fourth week after feeding. Each value is the mean ± s.e.m of eight animals. # indicates significant differences ($p \leq 0.1$) versus control values and * indicates significant differences ($p \leq 0.05$) versus control values by T-student.

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FIGURE LEGENDS

Mice were fed with a HCHF diet containing three levels of GSPE, 0.3 g/Kg (0.03% wt/wt)(low-GSPE group), 1.5 g/Kg (0.15% wt/wt)(medium-GSPE group) or 3 g/Kg (0.3% wt/wt) (high-GSPE group). Mice fed with HCHF diet without GSPE were used as control group. Substrate oxidation of orally applied ^{13}C-labelled palmitate (a) was determined during the experiment. Breath samples were taken at 15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 210, 240, 270 and 300 min. The figure below (b) represents the values of the area under the curve. # indicates significant differences ($p \leq 0.1$) versus control values by T-student.

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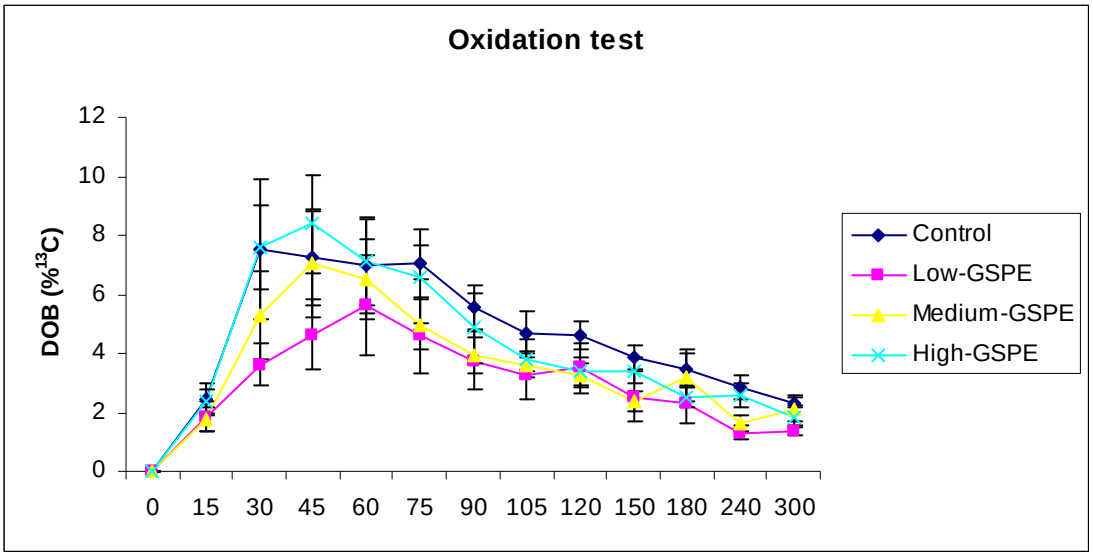
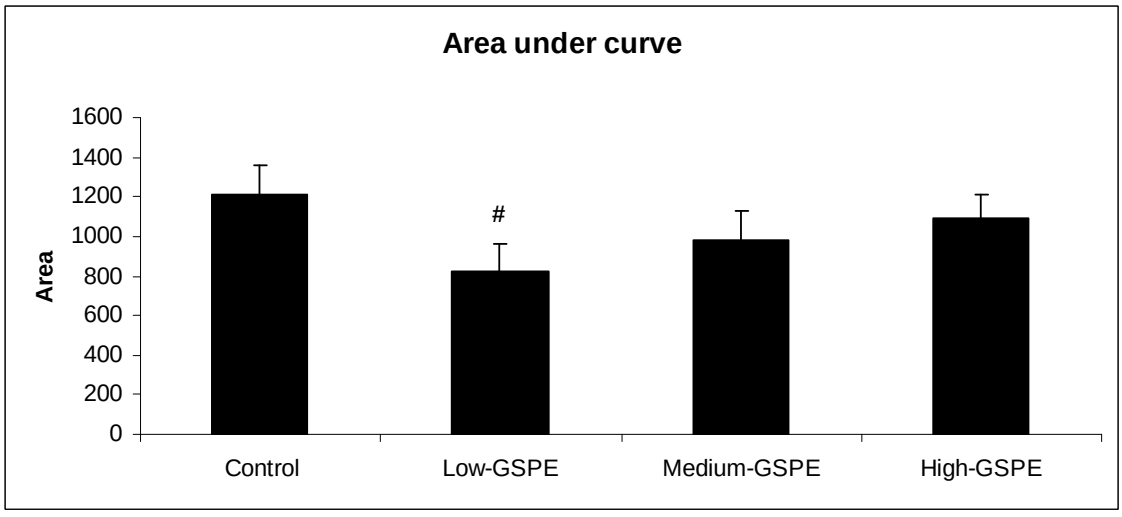
Figure 1

a)



b)



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4. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver

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ORIGINAL ARTICLE

Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver

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Objective: To determine whether proanthocyanidins can protect against dyslipidemia induced by a high-fat diet (HFD) and to address the mechanisms that underlie this hypolipidemic effect.
Design and measurements: Female Wistar rats were fed on a HFD for 13 weeks. They were divided into two groups, one of which was treated with a grape seed proanthocyanidin extract (25 mg kg^{-1} of body weight) for 10 days. Plasma and liver lipids were measured by colorimetric and gravimetric analysis. Liver, muscle and adipose tissue were used to study the expression of genes involved in the synthesis and oxidation of fatty acids and lipoprotein homeostasis by real-time RT-PCR.
Results: The administration of proanthocyanidins normalized plasma triglyceride and LDL-cholesterol (both parameters significantly increased with the HFD) but tended to decrease hypercholesterolemia and fatty liver. Gene expression analyses revealed that proanthocyanidins repressed both the expression of hepatic key regulators of lipogenesis and very low density lipoprotein (VLDL) assembling such as SREBP1, MTP and DGAT2, all of which were overexpressed by the HFD.
Conclusion: These findings indicate that natural proanthocyanidins improve dyslipidemia associated with HFDs, mainly by repressing lipogenesis and VLDL assembly in the liver, and support the idea that they are powerful agents for preventing and treating lipid altered metabolic states.
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Keywords: high-fat diet; liver; proanthocyanidins; triglycerides; SREBP1; MTP

Introduction

Hypertriglyceridemia is a strong predictor of atherogenic cardiovascular disease (CVD).[1] In both the metabolic syndrome and type 2 diabetes, hypertriglyceridemia is the result of increased plasma concentration of very low density lipoprotein (VLDL).[2] This increase is the consequence of overproduction of VLDL by the liver, and the possible delayed catabolism of these lipoproteins, caused by insulin resistance.[3] In turn, elevated VLDL and hypertriglyceridemia reduce the high-density lipoprotein (HDL) level and generate small, dense low-density lipoprotein (LDL) due to lipid exchange.[4] High serum triglyceride (TG) levels, low serum HDL-cholesterol (HDL-C) levels and a preponderance of small, dense LDL particles are the 'atherogenic lipid triada' characteristic of the dyslipidemia that commonly occurs in the metabolic syndrome.[5] It has also been suggested that the fact that cardiovascular risk indexes are lower in obese patients who lose weight may be closely connected to a reduction in VLDL secretion by the liver.[6]

Flavan-3-ols and their oligomeric condensation products, proanthocyanidins (PA), are the most common group of flavonoids in the American diet.[7] PA can be found in such common foodstuffs as cereals, legumes, fruits, vegetables and beverages (red wine and tea, in particular).[8] The health benefits of PA have been most studied in tea, cocoa and grape seed, each of which has a characteristic and specific oligomeric composition that conditions its biological activity. Grape seed proanthocyanidin extracts (GSPE) reduce foam cells,[9] prevent aortic atherosclerosis[10] from developing

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in a hamster atherosclerosis model, decrease oxidized LDL in hypercholesterolemic humans[9] and improve endothelial function by modifying NO production.[11] Consequently, they protect against atherosclerosis and CVD. Besides their antioxidant activity and their effects on the vascular endothelium, the antiatherogenic properties of PA are also related to an improved serum lipid profile. Plasma TG and apolipoprotein B (apoB) levels are reduced by GSPE in normolipidemic rats,[12] and by lyophilized grape powder in postmenopausal women.[13] The lipoprotein profile is also improved in both healthy subjects and hemodialysis patients by concentrated red grape juice.[14]

Although PA can exert part of their hypolipidemic effect by inhibiting the absorption of dietary lipids and diminishing chylomicron secretion by enterocytes, the liver has an important role in reducing plasma TG through GSPE. We have determined that GSPE limit VLDL secretion by repressing lipogenic genes and the microsomal transfer protein (MTP; the key controller of VLDL assembling)[12] and by overexpressing the carnitine palmitoiltransferase-1 (CPT1; the key controller of free fatty acid (FFA) oxidation)[15] in mouse liver. GSPE exert some of these effects by a pathway that involves overexpressing the nuclear receptor small heterodimer partner (SHP) and repressing the transcription factor sterol regulatory element-binding protein 1 (SREBP1).[15]

As overproduction of VLDL and hypertriglyceridemia are at the basis of atherogenic dyslipidemia, and GSPE can inhibit VLDL secretion in a healthy situation, the objectives of this study were to determine whether proanthocyanidins could prevent rats from developing atherogenic dyslipidemia and to establish the mechanism underlying this hypolipidemic effect. To this end, we have used rats fed on a high-fat diet (HFD) as a model of atherogenic dyslipidemia and we have determined the role of liver and extrahepatic tissue in normalizing the lipid profile.

Methods

Proanthocyanidin extract

GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). This proanthocyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5–13 units; 31.7%) proanthocyanidins.

Animals

Female Wistar rats weighing 150 g were purchased from Charles River (Barcelona, Spain). The Animal Ethics Committee of our University approved all procedures. The animals were housed in animal quarters at 22 °C with a 12-h light/dark cycle (light from 0800 hours to 2000 hours) and were fed *ad libitum* with standard chow diet (Panlab, Barcelona, Spain). After 5 days, 12 rats were fed *ad libitum* with standard chow plus a cafeteria diet as an HFD model which had 13.6% fats, 21% carbohydrates and 9% protein.[16] The cafeteria diet consisted of the following foods: cookies with foie-gras and cheese triangles, sweets, bacon, biscuits, chocolate, croissants, carrots and sugary milk. Six rats were kept on the standard chow diet (control group).

After 13 weeks, rats feeding on the HFD were trained to lick condensed milk (1 ml) and were divided into two groups. One group was supplemented every day, at 0900 hours, with 25 mg of GSPE per kg body weight dissolved in condensed milk (HFD-GSPE group). The other group received the same volume of condensed milk (HFD group).

On day 10 of the GSPE treatment, all the rats were killed at 1400 hours by beheading and the blood was collected using heparin as the anticoagulant. Plasma was obtained by centrifugation and stored at −80 °C until analysis. Liver, leg muscle and mesenteric adipose tissue were excised and frozen immediately in liquid nitrogen and stored at −80 °C until RNA and lipid extraction.

Plasma and liver lipid analysis

Plasma total cholesterol (TC) was measured with an enzymatic colorimetric kit (QCA, Barcelona, Spain). HDL-C was measured, using the same kit, after treating the plasma with phosphotungstic acid to precipitate the non-HDL lipoproteins.[17] The LDL-cholesterol (LDL-C) was measured after plasma treatment with polyvinyl sulfate and polyethylene glycol monomethyl ether to precipitate LDL lipoproteins. LDL-C was calculated as TC minus cholesterol in plasma after LDL precipitation.[18] TGs were assayed using an enzymatic colorimetric kit (QCA). FFA were measured using an enzymatic colorimetric kit (Wako chemicals GmbH, Madrid, Spain).

Liver lipids (0.5 g) were extracted using the Folch method.[19] An aliquot of extract was used to measure the total lipids by gravimetry. The rest of the extract was evaporated to dryness and redissolved in 2% triton X-100 to determine the TG and TC using the same kits that were used for plasma quantification.

Gene expression analyses

Total RNA from the liver was obtained using a NucleoSpin RNA2 kit (Macherie-Naegel, Germany) and total RNA from muscle and adipose tissue was obtained using Trizol reagent (Invitrogen, Barcelona, Spain) following the manufacturer's protocol. Additional purification and DNAse treatment was performed using a NucleoSpin RNA2 kit (Macherie-Naegel, Germany). cDNA was synthesized from 2 μg of total RNA using the Taqman Reverse transcription reagent kit (Applied Biosystems). A total of 20 ng of cDNA was subjected to quantitative RT-PCR amplification using Taqman Master Mix (Applied Biosystems). Specific Taqman probes (Applied Biosystems) were used for different genes. Ppia was used as an endogenous control. Real-time quantitative PCR

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reactions were performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems).

Statistical analysis

Results are reported as mean ± s.e.m. of six animals. Group means were compared with an independent-samples Student's *t*-test ($P \leqslant 0.05$) using SPSS software.

Results

GSPE treatment prevents dyslipidemia induced by high-fat diet

The body weight of rats fed with an HFD was significantly higher (approximately 40%) than those in the control group (Table 1). After 13 weeks of HFD administration, initial body mass of the rats increased by 126%, whereas that of rats fed with the standard chow diet only increased by 73%. The body weight of HFD-fed rats reduced slightly when treated with GSPE for 10 days (Table 1).

HFD also significantly increased liver mass, hepatic lipids, TG and TC levels (Table 1). GSPE treatment slightly lowered TG and TC content in liver, although the total lipids remained as elevated as in nontreated HFD rats.

HFD-fed rats were normoglucemic (results not shown) but presented hypertriglyceridemia and hypercholesterolemia and increased HDL-C and LDL-C (Table 2). Moreover, the HDL-C/LDL-C ratio, calculated to evaluate the atherosclerosis risk, was reduced in HFD-fed rats, which indicated a greater risk of atherosclerosis.

Treatment with GSPE reversed the dyslipidemia induced by the HFD. The plasma levels of TG and LDL-C decreased to the same values observed in the control group of normolipidemic rats (Table 2). Consequently, the atherogenic risk index HDL-C/ LDL-C improved in GSPE-treated animals. Additionally, FFA levels, which were not affected by the HFD, were significantly reduced by GSPE treatment (Table 2). Plasma TC and HDL-C, however, were only slightly reduced (Table 2).

Table 1 Body weight and liver lipids of rats fed with a standard diet or high-fat diet, either with or without proanthocyanidin treatment

	Control group	*HFD group*	*HFD-GSPE group*
Body weight (g)	282 ± 9.0	392 ± 27*	380 ± 21*
Liver weight (g)	8.99 ± 0.23	11.73 ± 0.88*	10.85 ± 0.64
Liver lipids (mg g^{-1} liver)	48.7 ± 5.5	60.9 ± 5.87	60.3 ± 0.6
Liver cholesterol (mg g^{-1} liver)	2.24 ± 0.15	3.66 ± 0.51*	2.7 ± 0.16
Liver triglyceride (mg g^{-1} liver)	4.53 ± 0.48	7.25 ± 1.01*	6.55 ± 0.55

Abbreviations: GSPE, grape seed proanthocyanidin extracts; HFD, high-fat diet. Each value is the mean ± s.e.m. of six rats. Rats were fed with a standard chow diet (control group) or high-fat diet (standard chow plus cafeteria diet) for 13 weeks. After 13 weeks, rats fed with a high-fat diet were orally treated with 25 mg of grape seed procyanidin extract per kg body weight (HFD-GSPE group) or vehicle (HFD group) for 10 days. * Indicates a significant difference ($P \leqslant 0.05$) versus control group; ♦ indicates that the *t*-test found a significant difference between the HFD and HFD-GSPE groups.

GSPE treatment counteracts hepatic overexpression of SREBP1, MTP and DGAT2 induced by a high-fat diet

The liver governs the homeostasis of circulating lipids and lipoproteins. Thus, we used reverse transcription–PCR to analyze the differential expression of key genes controlling TG and cholesterol metabolism in the liver (Table 3) and gain further insight into how GSPE improves the plasma lipid profile. We have chosen genes that encode key proteins in cholesterol pathways (LDL-receptor, CYP7A1 and HMG-CoA reductase), in fatty acid oxidation (CPT1-a), in TG synthesis (DGAT2) and in VLDL assembling (apoB and MTP). We have also selected two nuclear receptors, namely FXR and SHP, and the transcription factor SREBP1 because they govern the expression of key lipid metabolism genes in the liver and are involved in the molecular mechanism used by GSPE in the liver.

The liver of HFD rats showed a significant repression of SHP and CPT1-a in concert with a significant overexpression of DGAT2 when compared to the control group. SREBP1 and MTP also showed a slight overexpression. This expression profile in the liver of HFD rats suggests active TG synthesis and VLDL assembling as well as impaired fatty acid oxidation, which is consistent with the fatty liver,

Table 2 Plasma lipid levels of rats fed with a standard diet or high-fat diet, either with or without proanthocyanidin treatment

	Control group	*HFD group*	*HFD-GSPE group*
Triglycerides (mg per 100 ml)	107.3 ± 10.6	204.0 ± 2.3*	129.4 ± 12.3♦
Total cholesterol (mg per 100 ml)	57.9 ± 2.8	95.9 ± 5.7*	83.5 ± 4.5*
HDL cholesterol (mg per 100 ml)	35.6 ± 7.9	60.6 ± 4.1*	51.0 ± 4.9
LDL cholesterol (mg per 100 ml)	3.5 ± 0.1	15.2 ± 2.0*	6.6 ± 1.0♦
HDL-C/LDL-C ratio	8.7 ± 2.2	4.0 ± 0.6	7.0 ± 0.2
Total C/HDL-C ratio	1.4 ± 0.03	1.6 ± 0.15	1.7 ± 0.11
Free fatty acids (mg per 100 ml)	20.5 ± 2.1	22.9 ± 2.0	14.3 ± 1.1*,♦

Abbreviations: GSPE, grape seed proanthocyanidin extracts; HFD, high-fat diet. Experimental details and symbols as in Table 1.

Table 3 mRNA levels of lipid-related genes in the liver of rats fed with a standard diet or a high-fat diet, either with or without proanthocyanidin treatment

	Control group	*HFD group*	*HFD-GSPE group*
FXR	1.05 ± 0.16	0.89 ± 0.13	1.38 ± 0.24
SHP	1.13 ± 0.20	0.29 ± 0.10*	0.46 ± 0.13*
SREBP1	0.98 ± 0.07	1.54 ± 0.42	0.58 ± 0.11♦
ApoB	1.43 ± 0.32	1.62 ± 0.53	1.48 ± 0.36
MTP	1.08 ± 0.17	1.49 ± 0.06	0.99 ± 0.05♦
CPT1-a	1.03 ± 0.02	0.50 ± 0.09*	0.50 ± 0.09*
DGAT2	1.05 ± 0.05	1.80 ± 0.11*	1.19 ± 0.10♦
CYP7A1	1.19 ± 0.29	2.63 ± 0.63	1.55 ± 0.40
HMG-CoA reductase	1.13 ± 0.21	0.72 ± 0.04	1.08 ± 0.17
LDL receptor	0.91 ± 0.16	1.05 ± 0.38	0.75 ± 0.17

Abbreviations: GSPE, grape seed proanthocyanidin extracts; HFD, high-fat diet. The values are expressed as fold change, using PPIA expression as the endogenous control. Experimental details and symbols as in Table 1.

hypercholesterolemia and hypertriglyceridemia present in these animals.

In contrast, the liver of HFD-fed rats treated with GSPE for 10 days showed a significant repression of SREBP1, MTP and DGAT2 versus the HFD group. GSPE treatment also increased SHP, although the expression was lower than that of the control group. GSPE treatment did not affect the expression of CPT1-a, which remained repressed as in the HFD group. The changes induced by GSPE treatment in HFD-fed rats strongly suggest that proanthocyanidins repress TG synthesis and VLDL assembling, processes that are exacerbated by an HFD diet. We observed, however, that neither GSPE nor HFD affected the expression of the genes involved in cholesterol metabolism, which suggests that the effects of both the HFD diet and GSPE on plasma cholesterol are not linked to changes in hepatic gene expression.

Given that plasma TG levels heavily depend on extrahepatic uptake, we quantified lipoprotein lipase (LPL) and CPT1 expression in adipose tissue and muscle (results not shown). Neither an HFD diet nor GSPE treatment induced any significant change in the mRNA levels of the enzymes controlling the uptake of TG and fatty acid oxidation.

Discussion

Metabolic syndrome and obesity are associated with an increased risk of CVD, in part, due to their association with atherogenic dyslipidemia.[20] Overproduction of VLDL and hypertriglyceridemia are the basis of atherogenic dyslipidemia,[4] so managing VLDL secretion and hypertriglyceridemia could be a good strategy for reducing the risk of CVD associated with these pathologies. PA, a group of flavonoids that can be found in common foodstuffs,[8] actively reduce plasma TG and apoB in normolipidemic rats,[12] in hamsters fed a hypercholesterolemic diet[10] and in humans.[11] In a previous study we showed that an acute dose of GSPE decreases VLDL secretion by repressing MTP and lipogenic genes in normolipidemic rats.[12] This study, then, intended to determine, first, the ability of PA to correct atherogenic dyslipidemia associated with obesity and, second, to gain insight into the mechanisms that underlie the improvement of plasma TG levels, the effects of PA on the lipid metabolism in liver and in extrahepatic tissues.

With this purpose, we chose a cafeteria diet as an HFD model to induce obesity in rats. The cafeteria diet is a feeding regime in which animals are offered a choice of several palatable food items of varied composition, appearance and texture in addition to their normal chow diet.[21] This diet induces obesity due to hyperphagia,[21] and mimics human behavior when the control system for food intake is overwhelmed by psychological or social influences. Rats fed with this diet for 15 weeks showed obesity, hypertriglyceridemia, hypercholesterolemia, elevated plasma LDL-C and fatty liver. Ten days of oral intake of GSPE normalized plasma TG and LDL-C. Therefore, the reduction in TG levels and LDL-C, in association with the increased HDL-C/LDL-C ratio, determined an improvement in the atherosclerotic risk after GSPE intake. However, GSPE did not correct obesity, and only slightly reduced hypercholesterolemia and fatty liver.

In a cafeteria diet approach, it is very difficult to exactly quantify the ingestion of each food, so we cannot be sure that GSPE treatment changed the food pattern. However, although GSPE treatment can induce changes in food preferences, which can indirectly lead to a plasma lipid reduction, a direct effect of GSPE could not be discounted because in previous studies we found a considerable reduction in plasma TGs and LDL-C in other experimental models that consumed no food after GSPE treatment. In this study with female hyperlipidemic rats, the effects of chronic GSPE treatment on plasma lipids are similar to those observed in a previous study on the effects of acute GSPE treatment with male normolipidemic rats.[12] In this study, GSPE treatment reduced plasma TG, TC and LDL-C levels by about 40, 13 and 40% with respect to animals fed the HFD, respectively. In the previous study, GSPE treatment reduced plasma TG, TC and LDL-C levels by about 50, 12 and 43% with respect to the untreated animals, respectively. GSPE, then, is also a powerful agent for reducing plasma TG and LDL-C in dyslipidemia associated to HFD. GSPE treatment was also effective in both male and female rats, so it seems that sex does not affect GSPE efficiency on plasma lipids.

The liver gene expression of rats fed an HFD indicated active lipogenesis and impaired fatty acid oxidation together with increased assembling of VLDL, which points to the liver as an important contributor to HFD-induced atherogenic dyslipidemia. Oral GSPE treatment repressed SREBP1, DGAT2 and MTP. These three proteins are key regulators of VLDL synthesis and secretion. SREBP1 activates the expression of several genes involved in FFA and TG synthesis, as well as other components of the regulatory machinery of lipid metabolism. Although it can be regulated at different levels, its transcriptional repression has been linked to lower FFA and TG synthesis and release from the liver, leading to hypolipidemic states.[22] Therefore, the repressive effects of GSPE on SREBP1 may account for the lower plasma levels of TG. Together with SREBP1, DGAT2 is a key enzyme in the FFA reesterification process that delivers TG to the nascent VLDL.[23] As the availability of lipids is a key factor that drives the synthesis and secretion of VLDL,[23] the repression of DGAT-2 and SREBP1 suggests that lipoprotein release may be blocked at the primary level, that is, fewer lipids are available for lipoprotein assembly. The repression of MTP also suggests a decreased assembly of VLDL, because MTP is responsible for the association of apoB with lipids and the intracellular trafficking of the newly synthesized VLDL. Altogether, the repression of MTP, SREBP1 and DGAT2 by GSPE suggests a VLDL synthesis blockage at two different levels: first, lipid availability is decreased and then lipoprotein assembly. Moreover, GSPE did not modify the expression of the LDL-receptor, indicating that the decrease in LDL-C by GSPE was

not due to an increased uptake of LDL by the liver. Altogether, these results strongly suggest that GSPE improved dyslipidemia in HFD rats by reducing VLDL secretion, which, in turn, led to lower levels of TG and lower LDL production.

In contrast, GSPE did not increase CPT1-a expression, suggesting that PA did not normalize FFA oxidation in liver. Nevertheless, increased FFA oxidation by GSPE cannot be ruled out because CPT1-a activity is tightly regulated by its physiological inhibitor malonyl-CoA, which physiologically regulates β-oxidation depending on the availability of fatty acids and glucose.[24] SREBP1 activates the expression of acetyl-CoA synthetase, the synthesizing enzyme of malonyl-CoA,[25] so repression of SREBP1 by GSPE could result in a lower concentration of malonyl-CoA in hepatocytes and, therefore, a lower repression of CPT-1a. For this reason, the effect of GSPE on FFA oxidation still requires further study.

SHP is a nuclear receptor that acts as an inducible repressor of other nuclear receptors and transcription factors. With this mechanism, it controls lipogenesis and cholesterol metabolism in liver.[26] Thus, the low levels of SHP expression in HFD-fed rats could be behind the exacerbated expression of SREBP1, which, in turn, induces overexpression of lipogenic genes, fatty liver and hypertriglyceridemia.[25] In a previous study we showed that GSPE uses an SHP-dependent molecular mechanism to reduce TG secretion in HepG2,[15] so the slight increase in SHP expression induced by GSPE could be sufficient to reduce TG secretion, but not enough to counteract fatty liver.

To assess whether lipoprotein uptake and metabolization can account for the hypolipidemic effects of GSPE, LPL expression was analyzed in both muscle and adipose tissue. HFD and GSPE were found to have no effect, suggesting that uptake of TG by these tissues does not affect TG levels in plasma as much as the production of TG by the liver.

Although GSPE affects the target genes of lipid metabolism in the liver of HFD rats in almost the same way as it does in normolipidemic rats,[12] there are some differences, mainly in MTP and CYP7A1 expression. MTP expression is not modified by GSPE in normolipidemic rats but it is strongly repressed in HFD rats. On the other hand, CYP7A1 expression is increased by GSPE in normolipidemic rats but it is not affected in HFD rats. These differences can be explained in a variety of ways. In normolipidemic rats GSPE was administered acutely whereas in the present experiment it was administered chronically. The effects of acute administration may be a little different from the effects of chronic administration, where the organism is better adapted to receiving GSPE. Moreover, the metabolic stress induced by the HFD, which exacerbates VLDL assembly, hyperlipidemia and fatty liver, may modify the response to GSPE.

In conclusion, oral intake of GSPE improves dyslipidemia induced by HFD in rats, mainly by repressing lipogenesis and VLDL assembly in the liver, which are overexpressed as a result of the HFD. Therefore, increasing the intake of PA-rich foods can be a strategy for counteracting atherogenic dyslipidemia associated with obesity and metabolic syndrome.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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IV. GENERAL DISCUSSION

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Several authors have reported the PAs hypotriglyceridemic effects on humans and animals (review in [1]). Plasma TG levels are the result of the balance between the TRL secretion by the intestine and the liver and their uptake by the extrahepatic tissues through the LPL. Therefore, PA may reduce plasma TG levels by acting on the intestine, the liver and/or the peripheral tissues. However, the precise participation of each organ remains unknown. Besides, the liver presides over the metabolism and homeostasis of plasma lipids, becoming, as a consequence, a target for the study of PAs actions [2]. Thus, many studies have been addressed to reveal the mechanism by which polyphenolic compounds could modulate lipid and lipoprotein metabolism in the liver [3-5]. Nevertheless, little is known about the real contribution of CM and VLDL secretion and LPL activity to the hypotriglyceridemic action of PA.

On the other hand, diet composition is known to influence energy intake and body-weight changes on rats and humans [6]. For weight maintenance, not only energy intake does have to match energy expenditure, but also macronutrient intake must balance macronutrient oxidation [7]. It is suggested that macronutrients imbalance may not only be responsible for the increase of obesity prevalence in the population, but also for the appearance of a plethora of diseases such as atherosclerosis, non insulin-dependent diabetes, breast and colon cancers [8]. Therefore, a high saturated-fat and refined-carbohydrated diet leads to contract hyperlipidemia and hypercholesterolemia, and increases the risk of suffering from cardiovascular diseases (CVD) [9] by raising TG and lowering high density lipoprotein-cholesterol (HDL-C) levels [10]. Thus, it would be interesting to study the proanthocyanidins hypolipidemic effect on altered metabolic states. For this reason, the aim of this Ph.D. Thesis were i) to assess the contribution of the liver and the intestine in the hypolipidemic response triggered by proanthocyanidins and ii) to evaluate the short-term effect of an oral intake proanthocyanidins in dyslipidemic nutritional models. For these purposes, three experimental models have been used: Rats, mice and human colonic adenocarcinoma Caco2 cells. In these models, the bioactivity of Grape Seed Proanthocyanidins Extract (GSPE) together with the involved molecular mechanisms have been studied by using acute chronic and no-toxic doses.

Liver and intestine's contribution to the hypolipidemic response triggered by proanthocyanidins

In order to find out the role of the liver and the intestine in the hypotriglyceridemia induced by GSPE, we carried out a fat tolerance test on rats. A single oral administration of lard plus GSPE significantly blocked the increase of plasma total TG induced only by the lard at all the studied times mark. In our study, the reduction of plasma TG was similar to the reductions observed in male normolipidemic rats [11] and mice [4,5] after providing an acute GSPE treatment. As a consequence, PA-rich foodstuffs may reduce the hypertriglyceridemia associated with the postprandial state and, therefore, improve the tolerance to dietary lipids. Both CM-TG and VLDL-TG contributed to the hypotriglyceridemic action of GSPE but their influence depended on time. CM was the major contributor after 3 hours of administrating the treatment, whereas VLDL became important at 1 and 7 hours mark. Plasma CM-TG and VLDL-TG levels are the result of the balance between their secretion by the intestine and the liver and their uptake by extrahepatic tissues through the LPL. Therefore, PA may reduce plasma TG levels by acting on the intestine, the liver and/or the peripheral tissues. Post-heparin LPL activity in plasma and LPL gene expression in muscles and the adipose tissue were not affected by GSPE, indicating that PAs did not increase TG clearance by extra-hepatic tissues. Therefore, the reduction of CM-TG and VLDL-TG by GSPE could be ascribed to a repressed lipoprotein secretion by the intestine and the liver. It is generally assumed that CM predominantly transports exogenously ingested TG derived from dietary sources, whereas VLDL transports endogenously synthesised lipids [12]. PAs, then, repress the secretion of both endogenous synthesised TG and TG absorbed from the diet. Next, we studied the effects of GSPE on the secretion of TG by the liver and the intestine. In previous studies, we have described that GSPE represses TG in an HepG2 *in vitro* model. As for our study, we have analised the effect of GSPE *in vivo* and we have observed that the TG secretion associated with VLDL was repressed a 30% by the GSPE treatment. Thus, the repression of VLDL secretion is an important contributor to the hypotriglyceridemic effect of PAs. The liver is the main organ secreting VLDL [13,14]. Consequently, the liver is a significant target for GSPE as for the hypotriglyceridemia induced by PAs *in vivo* in the postprandial state.

PAs effect in the intestine has been studied to a lesser extent than in the liver. Therefore, in order to assess the implication of intestine in the hypotriglyceridemic effect of PAs,

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we have used the human colonic adenocarcinoma Caco2 cells since these form a simpler model than an *in vivo* one [15]. The intestine secretes TG in both the fasting and the fed states [15]. Since growing Caco2 cells in cell culture transwells, we have emulated the post-prandial state by supplementing the apical medium with OA, MAG and TC, and the fasted state by supplementing the basolateral medium with OA. The results of this study showed that the supplementation of GSPE on the apical side repressed TG secretion in Caco2 cells more efficiently in the fasted state than in the postprandial state, where doses of GSPE up to 100 mg/dL did not repress TG secretion. These results diverged from those obtained *in vivo*, where the reduction of CM-TG significantly contributed to the hypotriglyceridemia induced by GSPE at 3 hours mark. Nevertheless, other authors have described a repression of TG secretion in Caco2 cells when high concentrations of apple PAs are used (equal or higher to 200 mg/dL) [16]. Thus, the enterocytes need to be treated with high PAs concentrations to attain the hypotriglyceridemic effect showed *in vivo*.

At the light of the results, PAs repressed TG secretion in the liver and the intestine by different molecular mechanisms: *In vivo*, GSPE treatment induced different effects to those induced only by the lard oil on the expression of ACSL1 and CPT1a at an hour mark and Cd36 at five hours mark. On the contrary, neither these genes nor other genes related to FA, TG and CM assembly were modified in the intestinal mucosa 1 or 5 hours after the GSPE administration.

Our results suggest that lipid unavailability is the cause of reduced VLDL-TG secretion by the liver *in vivo*.

As we did not identify any different change in the gene expression induced by GSPE in the intestinal mucosa *in vivo*, we studied the expression of genes related to TG metabolism in Caco2 cells in depth. It has been described that enterocytes synthesise TG by two different pathways: The monoacylglycerol (MAG) pathway and the glycerol-3-phosphate (G-3-P) pathway [15,17]. The MAG pathway would predominate during the postprandial period, while the G-3-P is the main pathway during the interprandial and the fasted periods [17]. Oleate entering from the apical membrane is preferentially shunted towards the MAG pathway to form TG whereas oleate entering from the basolateral membrane comes from the circulation is shunted towards G-3-P acylation pathway. The required enzyme to activate FA prior to its incorporation into MAG or G-3-P is one of the five members of the acyl-CoA synthetase long chain family

(ACSL) [17]. Of these ACSLs, only ACSL3 and 5 are significantly expressed in the intestine [17]. In this proposed scenario, oleate-CoA delivered by ACSL5 would be directed towards the MAG pathway and by the ACSL3 towards the G-3-P pathway [17]. Our results have showed that GSPE treatment repressed ACSL5 and overexpressed CPT-1a in the post-prandial state, significantly (Figure 5). Nevertheless, GSPE treatment repressed ACSL5, ACSL3, I-FABP and PPARalpha in the fasted state, significantly (Figure 6).

Thus, at the light of the results, it can be suggested that GSPE repress only the supply of FA towards the MAG pathway (Figure 5) within the post-prandial state (ACSL5) whereas it represses FA delivered towards both pathways within the fasted state (ACSL5 and ACSL3) (Figure 6). Therefore, GSPE could be more effective reducing TG secretion during the fasted than the post-prandial state.

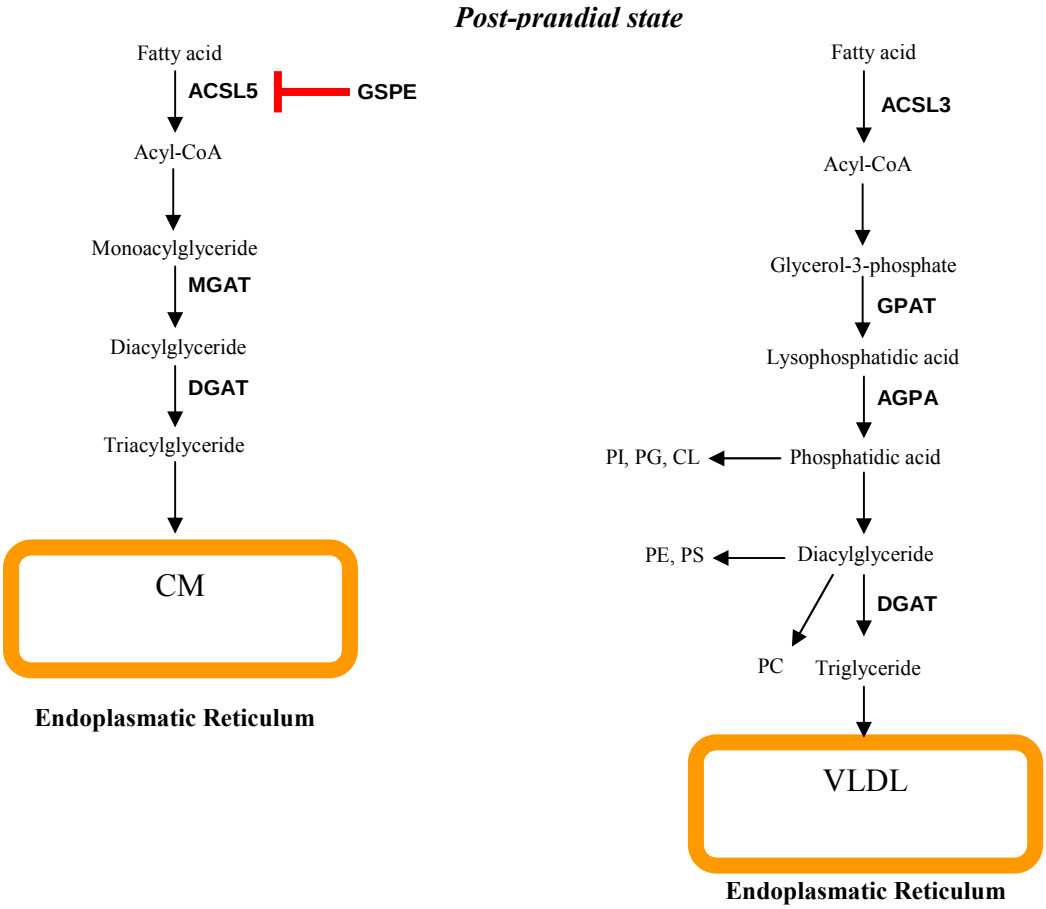

Figure 5. Proposed pathway used by proanthocynidins to repress the suply of FA into the enterocytes.

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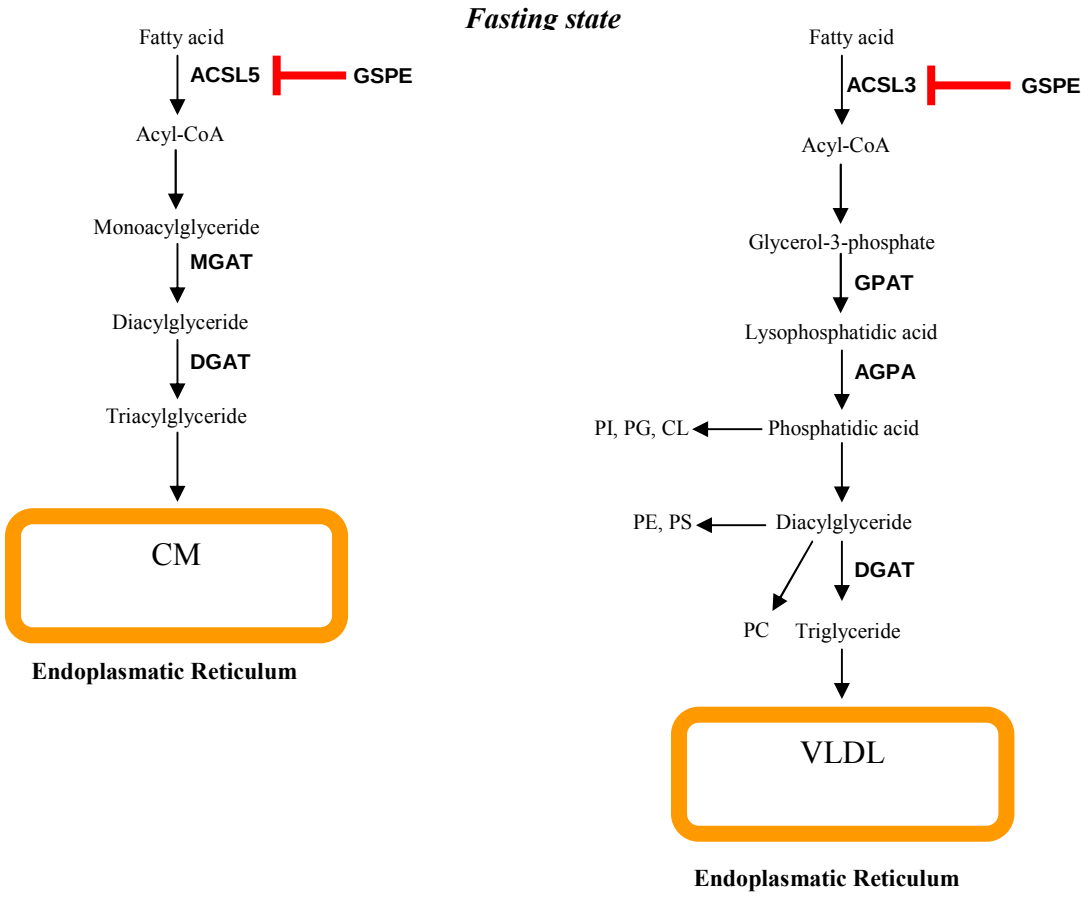

Figure 6. Proposed pathway used by proanthocynidins to repress the suply of FA into the enterocytes.

Short-term effect of an oral intake proanthocyanidins in dyslipidemic nutritional models

As it has been described above, an imbalance in the diet composition could be the responsible for the increase of obesity and the metabolic syndrome. Therefore, we evaluated the short-term effect of an oral intake PAs in dyslipidemic nutritional models. In order to evaluate the ability of dietary PAs to prevent or correct dyslipidemia, two experimental models have been used: On the one hand, mice fed on a diet containing proanthocyanidins for four weeks so as to evaluate the effect of proanthocyanidins on body weigth and energy intake in a situation of dyslipidemia and obesty induced by a high carbohydrate high fat diet; On the other hand, rats fed on a cafeteria diet for 3 moths supplemented with proanthocyanidins thereafter (manuscript 4) in order to study the ability of dietary proanthocyanidins to correct dyslipidemia and obesity induced by

a high-fat diet. In the first case, we used a high-carbohydrated and high-fat diet because it has been shown that it is good to induce a metabolic syndrome model on rats [18]. As for the second case, we chose a cafeteria diet as a high fat diet (HFD) model to induce obesity on rats.

Short-term treatment with GSPE did not correct the excess of body weight induced by the cafeteria diet in rats. In the same way, short-term treatment with GSPE did not counteract the wheight gain induced by the high-carbohydrated and high-fat diet in mice, even though induced a low increase in body fat deposition. GSPE tended to increase energy retention in mice at the second week mark, whereas it decreased energy retention at the fourth week mark. GSPE modified energy retention by changing energy intake but not energy lost in feces. These results indicate that PAs have a bimodal effect on energy intake, increasing it at early-term and decreasing it thereafter. Thus, PAs initially exacerbated the effect of high-fat diet by further increasing energy intake and decreasing palmitate oxidation. Therefore, it can be suggested that PA-rich foodstuffs must be consumed habitually and at long-term in order to be effective on improving the excess of body weight associated to metabolic syndrome.

By contrast to the effects of GSPE on body weight and fat deposition, the oral intake of GSPE improved plasma TG levels at short-term in both models. Thus, it can be suggested that PAs modify lipid metabolism and ameliorate plasma lipid profiles earlier than body weight.

In order to gain insights on the molecular mechanisms related to the hypotriglyceridemic action of GSPE in the metabolic syndrome, we have studied the different expressions of key genes that control TG and cholesterol metabolism in the liver of dyslipidemic rats. The oral GSPE treatment repressed SREBP-1, DGAT-2 and MTP. These three proteins are key regulators of VLDL synthesis and secretion. SREBP-1 activates the expression of several genes involved in FA and TG synthesis, as well as other components of the regulatory machinery of lipid metabolism. Although it can be regulated at different levels, its transcriptional repression has been linked to lower FA and TG synthesis and release from the liver, leading to hypolipidemic states [19]. Together with SREBP-1, DGAT-2 is a key enzyme in the FFA reesterification process that delivers TG to the nascent VLDL [20], the repression of DGAT-2 and SREBP-1, which means that there are fewer lipids available for lipoprotein assembly. The repression of MTP also suggests a decrease of the VLDL assembly because MTP is

responsible for the association of apoB with lipids and the intracellular trafficking of the newly synthesized VLDL. Altogether, the repression of MTP, SREBP1 and DGAT2 by GSPE suggests a VLDL synthesis blockage at two different levels: On the one hand, the decrease of lipid availability and, on the other hand, the reduction of the lipoprotein assembly. In contrast, GSPE did not increase CPT1-a expression, suggesting that PA did not normalise FFA oxidation in the liver.

In conclusion, GSPE exerts lipid-lowering effects in three different models in different feeding states: Rats, mice and Caco2 cells. Therefore, proanthocyanidins are powerful agents to prevent and treat altered lipid metabolic states.

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V. CONCLUSIONS

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1) In a fat tolerance test, both CM and VLDL contribute to the hypotriglyceridemic action of proanthocyanidins but their influence depends on time.

CM was the major contributor after 3 hours of the administration of lard oil plus proanthocyanidins, whereas VLDL was important after 1 and 7 hours after that administration. As GSPE did not increase TG clearance by extra-hepatic tissues, the reduction of CM-TG and VLDL-TG by GSPE could be ascribed to a repressed lipoprotein secretion. Proanthocyanidins, then, repress the secretion of both endogenous synthesised TG and TG absorbed from the diet.

2) Proanthocyanidins repress significantly VLDL-TG secretion *in vivo*.

As the liver is the main organ secreting VLDL, it can be suggested that the liver is a significant target for proanthocynidins in the postprandial state. Lipid unavailability for TG synthesis is the main cause of reduced VLDL-TG secretion by the liver, shortly after GSPE administration by increasing oxidation of FA in liver and thereafter as a result of reduced free fatty acids in plasma.

3) Proanthocyanidins repress TG secretion by enterocyte depending on the feeding state *in vitro*.

The intestine secretes TG in both fasting and fed state. In Caco2 culture cells, emulating the post-prandial state and the fasted state, GSPE are more effective to repress the TG secretion in a fasted state. This fact strongly suggests that, *in vitro*, the feeding state is important in order that proanthocyanidins could perform their beneficial effects in the intestine.

4) Long Chain Acyl-CoA Synthetase is a target of proanthocyanidins in intestinal cells.

Despite there is not experimental evidence of the ACSL5 and ACSL3 roles in intestine, it has been postulated that FA delivered by ACSL5 would be directed towards the MAG pathway and by ACSL3 towards the G-3-P pathway. In Caco2 culture cells, GSPE

repress ACSL5 in the post-prandial state and ACSL5 and ACSL3 in the fasted state. Thus, it can be suggest that proanthocyanidins represses only the supply of FA towards the MAG pathway in the post-prandial state (ACSL5) whereas represses FA delivered towards both pathways in fasted state (ACSL5 and ACSL3).

5) Short-term treatment with GSPE neither corrected the excess of body weight induced by a cafeteria diet in rats nor counteracted the weight gain induced by a high-carbohydrate high-fat diet in mice.

Therefore, it can be suggested that proanthocyanidins must be consumed habitually and at long-term in order to be effective improving the excess of body weight associated to metabolic syndrome

6) Proanthocyanidins have a bimodal effect on energy retention in mice fed with high-carbohydrate high-fat diet.

GSPE tended to increase energy retention in mice at the second week, whereas decreased energy retention at week fourth. GSPE modified energy retention by changing energy intake. Thus, proanthocyanidins initially exacerbate the effect of high-fat diet by further increasing energy intake and decreasing palmitate oxidation..

7) Short-term treatment with GSPE correct dyslipidemia associated with a high-fat diet in rats.

Ten days of oral intake of GSPE normalise the plasma TG and LDL-cholesterol in dyslipidemic rats. Furthermore, GSPE repress the expression of genes related to the VLDL synthesis and secretion. Thus, increasing the intake of proanthocyanidins can be a strategy for counteracting atherogenic dyslipidemia associated with obesity and metabolic syndrome.

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