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UNIVERSITAT ROVIRA I VIRGILI
Departament de Bioquímica i Biotecnologia

Modulation of hepatic lipoprotein metabolism by dietary procyanidins

Memòria presentada per optar al Grau de
Doctor per la Universitat Rovira i Virgili
Amb menció de Doctorat Europeu
Tarragona, Abril de 2007

Vist i plau dels directors de Tesis

M. Cinta Bladé Segarra Juan B. Fernández Larrea

Departament de Bioquímica i Biotecnología

L'interessat

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"Intentar escalar l'Everest és un acte intrínsicament irracional, un triomf del desig sobre la sensatesa. Cap persona que ho considerés seriosament es trobaria, casi per definició, sota l'influx d'un argument raonable."

Jon Krakauer. Into thin air. 1997

Jo no he escalat mai l'Everest, però puc sentir aquestes paraules. No en va porto quatre anys treballant sabent l'hora a la que començo, però ignorant quan acabaré. He hagut d'ignorar les vacances fins al punt d'oblidar-les. He deixat de banda família i amics massa vegades. En ocasions, més de les que qualsevol recomanaria, he treballat set dies a la setmana. I sovint he perdut els nervis davant un miserable gràfic preguntant-me què dimonis he fet malament. Tot això per menys de mil euros al mes. Ser estudiant de doctorat no és un acte racional, sinó un triomf del desig sobre la sensatesa. I es que si faig la vista enrere, recordo els companys que s'han convertit en família. Recordo com vaig viure Milà, que no a Milà. Aquell concert a la catedral de Budapest. El verd de les Highlands Escoceses. Recordo els focs artificials del 4 de juliol esclatant sota els meus peus mentre volava cap a Houston. I sobretot, recordo aquell dia que el miserable gràfic va decidir que havia arribat el moment de donar-me una alegria. Potser no és un argument raonable, però poder fer el que realment vols fer, sempre serà un triomf.

No voldria esplaiar-me massa, però entén que aquest apartat de la tesis és l'únic en el que puc opinar i oblidar-me de resultats, discussions i abstracts. Però sobretot és l'únic moment en el que puc agrair-te sincerament el temps que m'has dedicat.

Mai deixaré d'agrair haver tingut a la Dra Cinta Bladé com a directora de tesis. Sé que sóc afortunat per haver pogut decidir el rumb d'aquesta tesis. Agraeixo igualment la direcció del Dr Juan Fernández-Larrea, un torrent d'idees que van arribar al moment just. Sóc oblidadís, però sempre recordaré els bons moments que hem gaudit tot intentant treure endavant aquest treball. Tots dos heu fet que els moments crítics restin al meu record com experiències inoblidables. Si portés barret, me'l trauria. Ha estat un autèntic luxe.

Vull fer especial menció de la Dra Marie-Louise Ricketts i del Dr. David D Moore, del Baylor College of Medicine. Many thanks for your implication in this project, your help and initiative have been vital for boosting this thesis.

Res del que aquí podràs llegir seria possible de no haver treballat al grup de Nutrigenòmica de la URV. Per tant, vull agrair també l'aportació a aquesta tesis i el recolzament dels Dr/es Gerard Pujadas, Anna Ardèvol, Pepa Salvadó, Lluís Arola i Mayte Blay. Així com als companys de laboratori, la nostra segona llar, Ximena, Isa Quesada, Isa Baiges, Sabina, Mario, Pajuelo, Niurka, Esther, i en especial a la Gemma i a la Montse, que sou qui més temps m'heu patit, i a la Helena, per la paciència que tens amb mi, sou tos/es uns/es cracks.

L'agraïment és extensible als companys de Bioinformàtica, Pere, Pep, Safae, Gerard i, com no, Albert, que a lo tonto a lo tonto, a saber els anys que portem fent animalades. I a la Lídia, jo sempre he sabut que

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vols ser bioquímica. També agraeixo als companys d'Enologia per l'ajut brindat en els moments que l'he necessitat, especialment a l'Àngel, gràcies pels riures.

Com no pot ser d'una altra manera, tothom te uns inicis. I els meus van ser immillorables, en aquell laboratori rònic de la facultat vella, on érem quatre gats, Cesc, Nino, Montse i Vanessa. Gràcies pels bons moments que hem passat junts, i sobretot pels que passarem.

Anche ringraziare l'aiuto e collaborazione del professore Maurizzio Crestani, della Università degli studi di Milano. E la pazienza della Cri con me ed il ChIP assay. Ed anche voglio ringraziare la simpatia e bravura degli altri compagni a Milano, Andrea (Phenomenon), Elda, Germana, Mario, Federica, Esther. Ma che bella esperienza!.

I a tots els que han patit i viscut aquesta tesis des de Granollers, Girona, Tarragona, Mòra. A tu, gràcies.

Mama, si debo a alguien el estar aquí y ahora, esos sois vosotros. En aquellos días en los que todo cuanto aprendía lo olvidaba, vosotros, y sólo vosotros, luchasteis para desbloquear mi neurona. Ha llegado el momento de revelarte que, en realidad, continúo igual, pero ¿A que lo disimulo mejor?.

Noemí. Ningú ha hagut d'aguantar més que tu. I segur que cap altre ho hagués fet millor. Sé que saps quant t'admiro i com omples la meva vida. I per molt que escrivís no podria apropar-me en absolut al que em fas sentir cada dia. Dir-te gràcies és massa poc.

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Al meu pare

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ABBREVIATION LIST

ABC: ATP-binding cassette transporter

ACAT: Acetyl-CoA acetyltransferase

ACC: Acetyl-CoA carboxylase

Apo: Apolipoprotein

BSEP: bile salt export pump

CA: Cholic acid

CAD: coronary artery disease

CDCA: Chenodeoxycholic acid

CE: Cholesterol ester

CETP: Cholesteryl ester transfer protein

CVD: cardiovascular diseases

CYP: Cytochrome P450

DGAT: Diacylglycerol acyltransferase

EGCG: Epigallocatechin gallate

EGF: epidermal growth factor

EGFR: epidermal growth factor Receptor

ERK: Extracellular signal-regulated kinase

ET-1: Endothelin-1

FAS: Fatty acid synthase

FFA: Free fatty acids

FXR: Farnesoid X receptor

GST: Gluthathione S-transferase

HDL: High Density Lipoprotein

HL: Hepatic Lipase

HNF4: Hepatocyte nuclear factor 4

ICAM: Intracellular adhesion molecule

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IDL: Intermediate Density Lipoprotein

IL: Interleukin

JNK: c-Jun kinase

LCAT: Lecithin cholesterol acyl transferase

LDL: Low Density Lipoprotein

LDL-C: LDL-Cholesterol

LDLR: LDL receptor (LDLR)

LFA-1: lymphocyte function-associated antigen-1

Lp(a): Lipoprotein (a)

LPL: Lipoprotein Lipase

LRH-1: Liver receptor homolog 1

LRP: LDL receptor related protein

LXR: Liver X receptor

MAC-1: integrin alpha-M or Complement receptor type 3

MAPK: Mitogen-activated protein kinase

MCP-1: Monocyte chemotactic protein

MDR: Multidrug resistance

MTP: microsomal triglyceride transfer protein

N-CoR: Nuclear receptor co-repressor

NF-kB: Nuclear factor kappa beta

NTCP: Sodium/taurocholate cotransporting polypeptide

PDGF: platelet-derived growth factor receptor

PI3K: Phosphoinositide 3-kinase

PK: Protein kinase

PLC: Phospholipase C

PLTP: Phospholipid transfer protein

PPAR: Peroxisome proliferator activated receptor

PXR: Pregnane X receptor

RasGAP: Ras GTPase activating protein

RCT: Reverse cholesterol transport

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RER: rough endoplasmic reticulum

RGJ: Red grape juice

ROR: Retinoic related orphan receptor

RXR: Retinoic X receptor

SCD: Stearoyl-CoA desaturase

SHP: small heterodimer partner

SMART: Silencing mediator of retinoic acid and thyroid hormone receptor

SR-BI: Scavenger receptor class B type I

SREBP: sterol regulatory element binding protein

TG: triglycerides

TNF: Tumour necrosis factor

TRL: triglyceride-rich lipoproteins

VCAM: Vascular cell adhesion molecule

VLA: Very late activation protein

VLDL: Very Low Density Lipoproteins

VLDLR: VLDL receptor

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I. INTRODUCTION

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1. Lipoproteins

In mammals, the need for transporting hydrophobic lipids through the tissues is solved by means of lipoproteins. Lipoproteins are composed by a core of hydrophobic lipids, i.e. cholesterol esters and triglycerides, surrounded by amphipathic lipids to guarantee the solubility of these particles in plasma, a hydrophilic media (1-3). Thus, cholesterol and phospholipids compose an external monolayer, exposing their hydrophilic terminus to the aqueous media (3). In lipoproteins, lipids are assembled with a proteic fraction, known as apolipoproteins. Lipoproteins are highly dynamic systems, which can exchange lipids and apolipoproteins between them and are exposed to modification or endocytosis by tissues. Apolipoproteins play essential roles in lipoprotein structure and function. First, they guarantee the interactions between different lipoprotein particles. Second, they are responsible for lipoprotein metabolization by tissues. Finally, apolipoproteins stabilize the structure of the lipoprotein particle (2-6).

1.1 *Function and metabolism of plasma lipoproteins*

The lipoproteins responsible for exogenous and endogenous lipid distribution among tissues are known as triglyceride-rich lipoproteins (TRLs), which comprise two main classes, chylomicrons and Very Low Density Lipoproteins (VLDL) (7-9). Both TRLs classes differ in their origin and composition. Chylomicrons are assembled in enterocytes, contain a large amount of triglycerides (TG) and the characteristic apolipoprotein ApoB-48 (10). VLDL are secreted by the liver, and ideally contain the complete isoform of apolipoprotein B, ApoB100 (11). Both ApoB isoforms are obtained from the same primary transcript, which can be subjected to post-transcriptional edition. This editing process leads to the achievement of the ApoB-48 isoform instead of the full length protein, ApoB-100 (8). In humans, the liver synthesizes ApoB-100 whereas the intestine secretes mainly apoB-48, despite the fact that the assembling and secretion of ApoB100-containing lipoproteins by human enterocytes have been described as well (12). In addition, while human liver secretes apoB-100, murine hepatocytes can secrete both isoforms assembled into VLDL particles (13). The physiological function of chylomicrons and VLDL are slightly different. Whereas the secretion of chylomicrons by the intestine responds to the necessity of distributing the newly absorbed dietary lipids, the secretion of VLDL by the liver depends on the metabolic state of the organism, an is the major endogenous source of TG for extrahepatic cells (14, 15). Once

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in plasma, TG of TRLs are hydrolyzed by lipoprotein lipase (LPL) releasing fatty acids which are then available for the peripheral tissues. By this process, TRLs lose TG and, therefore, gain density (16, 17). The interaction of TRLs with LPL is highly dependent on apolipoproteins, such as apoCs (apoCI, apoCII and apoCIII), ApoE and ApoAV (18-22). Different studies have been addressed to determine the role of these apolipoproteins in the modulation of plasma lipid levels, mainly TG (6, 19, 23, 24). It has been shown that these proteins modulate the activity of LPL in different manners, and consequently affect the hydrolysis of TG from TRLs. Thus, ApoCII and ApoAV have been described as activators of LPL (18, 25, 26). In turn, ApoCIII and ApoE are known inhibitors of the activity of this lipase (26-28). Therefore, apolipoproteins are critical factors in the rate of TG hydrolysis and release from TRLs. In addition, not only the type of apolipoprotein, but the interactions between them play a key role in the modulation of LPL activity (5). Thus, in patients with hypertriglyceridemia, apoAV levels were paradoxically elevated while this apolipoprotein had emerged as an activator of LPL activity. The explanation was found in the elevated levels of apoCIII, a known repressor of LPL. The correlation between plasma triglycerides, apoAV and apoCIII showed that complex interactions between both apolipoproteins are even more important than the activity of these proteins alone (20).

The TRLs metabolization by LPL and the interaction with other lipoproteins result in TRLs remnants. Thus, the continuous metabolization of chylomicrons results in chylomicron remnants, while the hydrolisis of TG from VLDL results in Intermediate Density Lipoproteins (IDLs) (12, 29-31). Next, IDLs are submitted to further metabolization and also lose their apolipoprotein content, with the exception of ApoB (8). The resulting lipoproteins, known as Low Density Lipoproteins (LDLs), have a cholesterol-enriched nucleus and a low TG content (32). Different studies have shown a wide heterogeneity among lipoproteins, and proposes a more accurate sub-speciation based in their density and their lipid content (32), as shown in Table 1. Thereby, VLDLs have been divided into larger (VLDL1) and smaller ones (VLDL2), being the later similar to IDLs (8). IDLs can be subdivided into IDL1 and IDL2, while LDLs have been subclassified in LDL-I, LDL-II, LDL-III and LDL-IV (32).

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	Density	Diameter (Å)	%PR	%CE	%UC	%TG	%PL
VLDL1	<1.006	330-700	11	8	6	58	17
VLDL2	<1.006-1.010	300-330	18	24	9	29	22
IDL1	1.008-1.022	285-300	17	35	10	16	21
IDL2	1.013-1.019	272-285	17	37	11	13	21
LDL-I	1.019-1.023	272-285	18	43	9	7	22
LDL-II	1.023-1.034	247-265	19-21	45	10	3-4	22-23
LDL-III	1.034-1.044	242-247	22-24	44-46	8-9	3	21
LDL-IV	1.044-1.06	220-242	26-29	40-42	7	5-6	18-19

PR, protein; TG, triglycerides; CE, cholesterol esters; PL, phospholipids; UC, unsterified cholesterol

Table1. Classification of lipoproteins. From Berneis, KK and Krauss, RM. 2002. J.Lipid Res.

The clearance of plasma LDL by liver and extrahepatic tissues is mediated by LDL receptor (LDLR), VLDL receptor (VLDLR) and LDL receptor related protein (LRP) (33). Once more, the presence of specific apolipoproteins is required. Thus, while these receptors recognize apoE and ApoB in order to bind and internalize the lipoproteins (34, 35), apoCs inhibit the binding of LDL to the receptors (27, 28, 36, 37). The internalization process requires two main steps. Firstly, lipoproteins are bound to matrix proteoglycans, a process facilitated by LPL and Hepatic Lipase (HL). The second step is the receptor mediated internalization of LDL (34).

The liver plays a key role in the elimination of peripheral cholesterol by means of the synthesis and secretion of High Density Lipoproteins (HDL) (38, 39). Initially, ApoAI is secreted from liver associated with phospholipids forming the nascent HDL in plasma (pre β-HDL) (39) which then removes cholesterol from the peripheral tissues. The delivery of cholesterol from extrahepatic cells to HDL particles is mediated through the ATP-binding cassette transporter 1 (ABCA1) (40). Subsequently, cholesterol is esterified by Lecithin cholesterol acyl transferase (LCAT), a esterase synthesized by the liver and located in the surface of HDL (39). Thus, cholesteryl esters remain in the nuclei of HDL while the surface is formed by phospholipids and free cholesterol. From this stage, namely HDL_3, HDL can accept cholesterol from cells by passive diffusion or by mediation of SR-BI, another cholesterol transporter, becoming larger HDL particles (HDL_2) (41). HDL_2 exchange cholesterol and TG with VLDL particles by mediation of

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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 processes (39). In parallel, phospholipids and cholesterol from VLDL can be transferred to HDL by action of phospholipid transfer protein (PLTP) (32, 42). Large HDL are then internalized by the liver through a process which involves TG hydrolysis by Hepatic Lipase (HL) and cholesteryl esters uptake by liver through SR-BI (38). Finally, ApoAI is internalized for recycling. Therefore, HDL are responsible for the reverse cholesterol transport (RCT).

In summary, lipoprotein metabolism is a finely tuned network of interactions that leads the transport of lipids through the whole organism (summarized in Figure 1). Together with the intestine, liver orchestrates the synthesis and secretion of lipoproteins, playing a major role in the reverse cholesterol transport and clearance of lipids. Thus, liver is a key organ in the maintenance of lipid homeostasis.

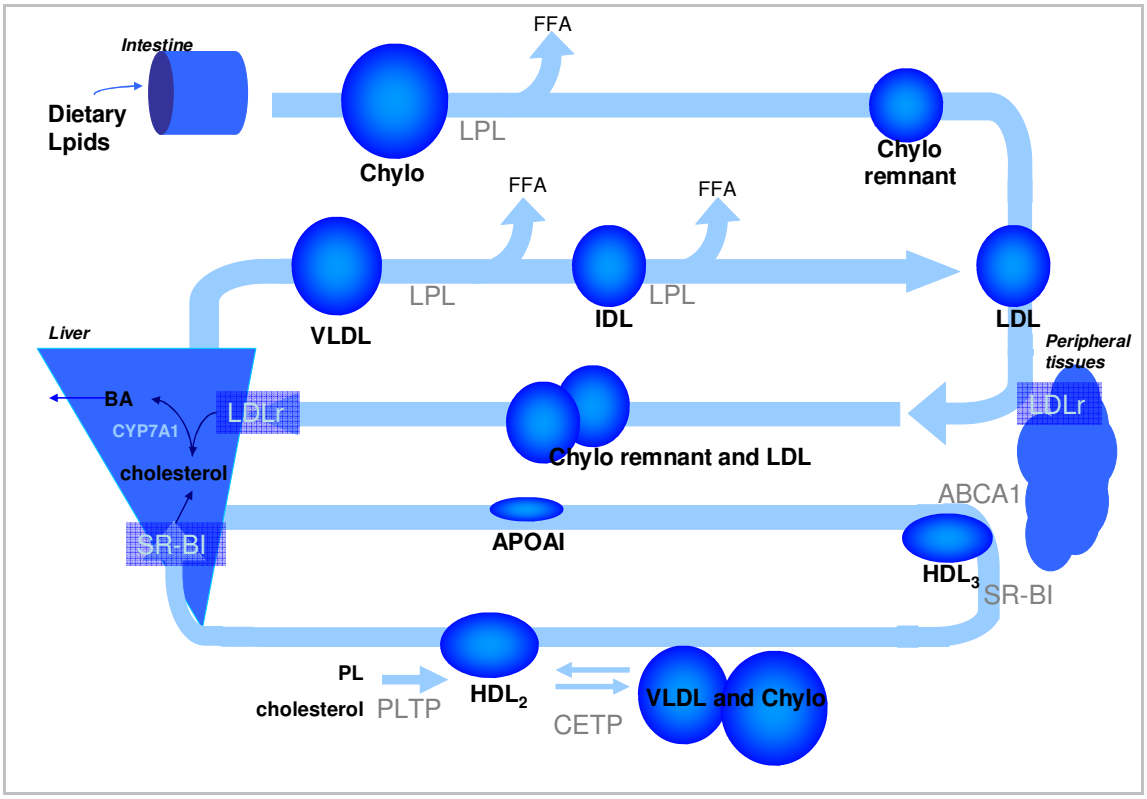

Figure 1. Lipoprotein metabolism.

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1.2 Dyslipemia and diseases

Plasma lipids and lipoproteins are in the focus of a vast number of studies addressed to clarify the origin of cardiovascular diseases (CVD) (12, 32, 43-45). Although lipoproteins are vital in distributing hydrophobic lipids through the organism, the dysregulation of these plasmatic particles is the consequence or the origin of many altered states (9, 38, 41, 42, 44, 45). Thus, the modulation of lipoprotein homeostasis has been revealed as a key target for the enhancement of the quality of life in developed countries, where dyslipemia in general, and concretely hyperlipidemia, are achieving the status of epidemics (15, 46).

The large half-life of LDL and the presence of ApoB, with a high susceptibility to be oxidized, points to these lipoproteins as important factors for atherogenesis. Once oxidized, apoB can be recognized by scavenger receptors of macrophages, being internalized (47). Large amounts of internalized lipoproteins cause the evolution of macrophages to foam cells and its deposition in the arterial wall, starting an immune response which results in atherosclerotic lesions (48). Angiographic studies revealed that lowering LDL cholesterol (LDL-C) has a great impact in severe lesions, close to a 50%. Thus, traditionally, antiatherogenic therapies have been focused on reducing LDL-C.

Nevertheless, TRL remnants have been gaining relevance as atherogenic agents due to their high content in TG, which enhances the susceptibility of macrophages for becoming foam cells (15, 49). TRL have been identified as a key factor in the progression of mild to moderate lesions in 50% of cases (49-51). Quantitative coronary angiography studies have demonstrated that progression of mild to moderate lesions is a significant predictor of clinical coronary events, and that lowering TGs reduces progression of CAD to the same degree as the lowering of LDL-C (49-51). Additionally, TRL remnants have been strongly associated with CAD, since particles isolated from human atherosclerotic plaques have been shown to be structurally and compositionally similar to remnants of TRL (49). Postprandial lipoproteins are such an important factor in atherosclerosis development that, nowadays, hypertriglyceridemia itself is considered as an independent risk factor for cardiovascular disease (52). Thus,

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not only LDL, but apoB-containing lipoproteins are considered responsible of atherogenesis, being the quantization of ApoB a better CVD risk index than LDL-C (4).

A 40% to 50% of patients with coronary artery disease (CAD) have an atherogenic lipoprotein profile characterized by elevated IDL, TG, dense LDL, and dense VLDL, and by low levels of HDL2 (49). Furthermore, 20% of patients with premature CAD have elevated Lipoprotein (a). Lp(a) is a lipoprotein that resembles LDL in composition with an abnormal protein, termed [a], attached. Lp(a) is abnormal among the lipoproteins in that it gains density with an increase in particle diameter (53). Abnormal lipoprotein concentrations can result from changes in the production, conversion or catabolism of lipoprotein particles. Patients with familial hypercholesterolemia, are characterized by a decreased clearance of LDL, concomitant with LDLR defects (54). Nevertheless, further studies have revealed an abnormal increased production of VLDL in addition to receptor dysfunction (55, 56). A relevant example of lipoprotein metabolism dysregulation are the metabolic syndrome and type 2 diabetes, which implicate increased secretion rates of VLDL and reduced rates of TG, VLDL and LDL conversion (30, 31). In subjects presenting this type 2 diabetes, angiographically evaluated CAD, positively correlated with plasma TRL levels, independently of HDL and LDL (57). Visceral fat, hepatic fat, insulin resistance and plasma adiponectin seem to be the primary responsible of abnormal apoB and TG kinetics under this situation (31, 58). Increased amounts of peritoneal fat lead to alterations of portal free fatty acids flux (59), altered pattern of adipocytokines, and perturbed ratio of proinflamatory to anti-inflamatory processes (60). Despite the fact that each factor may increase VLDL secretion by itself, it is unclear which one has the greatest impact on driving hepatic TRL overproduction.

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1.3 The liver in the metabolism of plasma lipoproteins

As has been shown, liver presides over the control of lipoprotein homeostasis. Therefore, modulation of the mechanisms that control lipoprotein synthesis, secretion and uptake in the liver have gained great interest for the treatment of dyslipemias.

1.3.1. VLDL synthesis and secretion

The exact mechanisms of VLDL assembly remain unclear despite the fact that evidences point to a multi-step process (61). The present working model for VLDL synthesis proposes that TG become associated to ApoB in different steps through the assembly pathway. ApoB is synthesised in the rough endoplasmic reticulum (RER) membrane. While the newly synthesised ApoB translocates into the RER lumen, it is lipidated by action of the microsomal triglyceride transfer protein (MTP), forming the pre-VLDL complex (11, 61). Thus, ApoB can be folded on a core of neutral lipids. Further lipidation of pre-VLDL particles results in larger and more buoyant particles, namely VLDL2. Alternatively, if no lipids are available, pre-VLDL are sorted to degradation (61). A second stage in the VLDL synthesis is the maturation of the small precursor, which fuses to a larger TG droplet, obtaining the VLDL1 or mature VLDL. It is not clear whether this maturation occurs at the RER or in the Golgi apparatus prior to being secreted (8, 11).

The mechanisms controlling the synthesis of VLDL in the liver have been widely studied in different animal and cell models (8, 13, 61, 62). Synthesis and secretion of VLDL is controlled at several levels and coordinated by MTP (10, 63). MTP shuttles lipids to the newly synthesized apoB, promoting the assembly of the nascent VLDL (10, 61). Therefore, three main points of control are involved, (i) the availability of lipids, (ii) the rate of ApoB synthesis and degradation, and (iii) the MTP activity.

The assembly of VLDL is highly dependent of lipid availability. The activities of different lipogenic key enzymes and regulatory proteins have been related with the synthesis and secretion of VLDL (1, 11, 64-66). That is the case of sterol regulatory element binding proteins (SREBPs) 1 and 2, master regulators of the TG and cholesterol biogenesis programs, respectively. Mice overexpressing SREBP1a and SREBP1c display enhanced TG synthesis in the liver (64, 65, 67-70). Once activated, SREBP1

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modulates several genes from the TG synthesis program, such as Fatty acid synthase (FAS), Acetyl-CoA carboxylase (ACC) and stearoyl-CoA desaturase (SCD) among others (67, 71). Along with the control of lipogenesis, SREBPs are implicated in the control of VLDL synthesis and secretion (1, 65). Thus, in McArdle rat hepatoma cells, the levels of mature SREBP1were directly correlated with the secretion rate of ApoB (65), while in transgenic mice overexpressing SREBP1 and lacking LDLR, plasma TG levels pointed to a severe hypertriglyceridemia, ascribed to an increased secretion of VLDL (67). Additionally, SREBPs have been shown to bind the promoter of the MTP gene, inhibiting its expression *in vitro* (72). Therefore, SREBPs are major controllers of lipid synthesis and lipoprotein synthesis and secretion in the liver. Apart from the vast number of lipogenic factors, several authors have demonstrated that the fatty acids used for synthesising VLDL are recruited from cytosolic TG pools (73-75). The lipolysis of cytosolic TG is carried out by arylacetamide deacetylase (AADA) and triacylglycerol hydrolase (TGH) (61). The released fatty acids reach the RER where they are re-esterificated to TG by diacylglycerol acyltransferase 2 (DGAT2) (61). These new TG are then assembled into pre-VLDL particles by MTP (61). Consequently, the enzymes involved in the intracellular trafficking of these fatty acids also modulate the formation of VLDL (76-78). Therefore, along with lipogenesis, intracellular lipolysis correlates with VLDL synthesis (61).

ApoB is another key factor in the synthesis of VLDL, since it presides over the assembling and stabilizes these lipoproteins (8, 62). The lack of functional or the presence of truncated ApoB is the cause for hypobetalipoproteinemia, a dysregulation that results in extremely low levels of plasma apoB-containing lipoproteins (79). Nevertheless, while low levels of ApoB critically affect the synthesis of VLDL, excess of ApoB is rapidly overcome by hepatocytes. Thus, it is generally believed that ApoB secretion is regulated post-transcriptionally by co-translational and post-translational degradation in at least three different pathways (3, 11, 61, 80): (i) By retraction from the RER lumen while it is synthesised, being translocated into the cytoplasm, ubiquitinated and subsequently degraded via proteosoma. (ii) Post-translationally, by a mechanism that have been observed in cultured cells exposed to polyunsaturated fatty acids (80). (iii) By immediate reuptake of the newly secreted VLDL, by means of the LDLR (37, 67). It has been shown that LDLR can bind VLDL with degradation purposes even before those lipoproteins have been secreted (37). Therefore, while ApoB is essential

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for VLDL synthesis, an excess is easily compensated by the cell through these three mechanisms.

The third critical factor modulating VLDL synthesis is the MTP activity. The lack of a functional MTP is the cause for Abetalipoproteinemia, a complete loss of circulating apoB-containing lipoproteins (81). The vital role of this enzyme has been widely demonstrated in different in vivo and in vitro models (81-84). Beyond its lipid transferring activity, it is thought that MTP stabilizes ApoB folding until the complete maturation of VLDL, otherwise ApoB would be degraded (3). It has been shown that the FAO cell line, which stably expresses MTP, is able to secrete ApoB while in L35 cells, derived from FAO, the secretion of VLDL is abolished because L35 cells cannot express MTP (84). Nevertheless, TG secretion is still functional in L35 cells, despite being a 25 % lower than in the case of the FAO cell line (84). Although the mechanism underlying this last observation remains unknown, it is thought that TG can be secreted into nascent ApoE and ApoA-IV-containing lipoproteins (84). Therefore, while MTP activity and ApoB levels are tightly linked factors in the synthesis of VLDL, lipid availability is an independent rate-limiting factor. In other words, while the lack of MTP or ApoB results in the absence of apoB-containing lipoproteins, liver is still able to secrete TG.

1.3.2 Cholesterol clearance.

Liver is responsible for cholesterol elimination of the whole organism. First by controlling RCT via HDL (38), and second by secreting the recruited cholesterol to bile canaliculi, both directely and after its conversion to bile acids (14, 85). The pathway leading to bile acids is subjected to a complex control. Thus, bile acid synthesis is coordinated to other metabolisms, such as cholesterol biosynthesis, and subjected to the control of signalling pathways, such as insulin or c-jun terminal kinase (86, 87). The biosynthesis of bile acids is mediated by sequential action of different enzymes from the P450 cytochrome family (CYPs). The classical pathway consist on a cascade of 15 reactions (86), where CYP7A1 is considered the rate-limiting enzyme in the synthesis of two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA) (86). A second key enzyme in this pathway is CYP8B1, which controls the ratio between CA and CDCA production (86, 88). It has been shown that bile acid synthesis is under the

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control of redundant pathways, allowing negative and positive feedback regulation (89-91). The action of different nuclear receptors, along with the activation of signalling pathways by bile acids, are vital in these mechanisms and guarantee the control of both intracellular and plasma bile acids content (87, 89, 92, 93). Thus, liver is responsible for bile acid synthesis, but also for plasma bile acids uptake via Sodium/taurocholate cotransporting polypeptide (NTCP) (94) and its secretion into bile canaliculus mediated by ATP-binding cassette transporters such as ABCG5, ABCG8, ABCB11 (known as bile salt export pump; BSEP), ABCB4 (MDR3) or ABCC2 (MRP2) (95, 96). Together with bile acids, liver is able to secrete sterols to the bile acid canaliculus by mediation of the sterol transporters ABCG5 and ABCG8 (97). Therefore, liver presides over the clearance of cholesterol: The reverse transport of cholesterol is started with the secretion of ApoAI lipoproteins by the liver and is completed by the synthesis of bile acids and its secretion into the bile.

1.3.3 Nuclear receptors in the control of lipid metabolism in liver.

Several studies have implicated nuclear receptors in the control of lipid homeostasis, establishing a coordinated net of metabolic sensors which integrates lipid metabolism, inflammation, drug metabolism, bile acid synthesis and glucose homeostasis among other processes (87, 97-101). The structure of these proteins contains, ideally, a Ligand binding domain that allow 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 coactivators, corepressors, phosphorylation/dephosphorylation and other nuclear receptors (102, 103). This structure provides to nuclear receptors the ability of acting as metabolite sensors, being activated by endogenous or exogenous molecules and subsequently triggering or repressing gene expression in a coordinate manner (103). Some of these receptors, such as Farnesoid X receptor (FXR), Liver X receptors (LXRs) or peroxisome proliferator activated receptors (PPARs), heterodimerize with other nuclear receptors, usually Retinoic X receptor (RXR), in order to bind DNA. Other nuclear receptors, such as Retinoic related orphan receptors (RORs), can act as homodimers (104). It is generally believed that nuclear receptors are bound to their target sequences in an inactive form, associated with co-repressors such as nuclear receptor co-repressor (N-CoR) and silencing mediator of retinoic acid and thyroid hormone receptor (SMART). Ligand binding to either RXR or its partner, leads to the release of

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corepressors and recruitment of coactivators (e.g., SRC-1, p300, ACS-2, TRRAP, or PGC-1α). By means of these coactivators, other mediators are recruited, such as histone deacetylases or methylases, which subsequently enhance the transcription of the gene (87, 97, 104, 105). Thus, despite the fact that the actions of nuclear receptors are coordinated through complex mechanisms, the ability of these proteins to modulate a wide battery of genes has placed them in the focus of different studies addressed to identify the cause of different altered metabolic states, such as diabetes or dyslipemia (101, 102, 104, 106).

As mentioned above, nuclear receptors play a key role in RCT. Thus, LXRs, an oxysterol-binding nuclear receptors subfamily, control a wide battery of genes related to cholesterol and bile acid metabolism, leading to the conversion of cholesterol to bile acids in response to high intracellular oxysterol levels (94, 106). On the other hand, FXR is a nuclear receptor which binds to, and is activated by, different bile acids, promoting the repression of bile acids synthesis and triggering their secretion when they are in excess (94, 106). The activated FXR enhances the transcription of small heterodimer partner (SHP). SHP is an orphan (i.e., with no known ligand) nuclear receptor lacking a DNA binding domain that acts as a corepressor of conventional nuclear receptors (107). Subsequently, SHP interacts with other nuclear receptors, such as HNF4 and LRH-1, inhibiting their binding to the promoter of bile acid synthesis genes. As a result, the synthesis of bile acids is decreased (86, 88, 89, 107, 108).

In addition to its implication in bile acids metabolism, FXR plays a key role in the control of cholesterol and TG metabolism, since different studies have shown that mice lacking this nuclear receptor present elevated cholesterol and TG levels in plasma and liver (109-111). This hyperlipidemia has been associated to the modulation exerted by FXR on a battery of genes related with TRL clearance, such as apoCIII (112), apoCII (113) and VLDLR (114). Furthermore, FXR can also control different genes involved in lipid synthesis, as demonstrated by treatment of mice with bile acids (115). The hypotriglyceridemic actions of bile acids can be attributed, at least in part, to the activation of FXR and subsequent induction of transcription of its target gene SHP (116). Thereafter, SHP represses the expression of different genes by inhibiting the action of other nuclear receptors and transcription factors (87, 92, 116, 117). SREBP1 is one of these genes repressed by SHP (115). It has been shown that the activity of

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SREBP1 can be downregulated by transient activation of SHP (115). Nevertheless, continuous overexpression of SHP in transgenic SHP-tagged mice leads to increased expression of SREBP1 (117). This last observation was ascribed to a diminished bile acid pool, and subsequent activation of LXR in the liver of those mice. Therefore, prolonged overexpression of SHP triggers SREBP1 expression by indirect mechanisms derived from the altered physiological state of the mouse (117). In contrast, in a normal situation, transient induction of SHP negatively regulates SREBP1 (115, 117). Thus, the regulation of gene expression by FXR and SHP could be extended to the lipid synthesis program, which is a key factor in the synthesis and secretion of TRLs.

Together with FXR, other nuclear receptors play major roles in the control of lipid and lipoprotein metabolism, such as LXR, PPARs, Pregnane X receptor (PXR) and RORs. LXR is strictly coordinated with FXR. Both are known to play antagonistic roles in the control of bile acid and lipid metabolism (97). Thus, while LXR promotes the activation of the fatty acid and TG synthesis program, FXR is known as an effective repressor of lipogenesis (97, 98, 106). Moreover, their antagonistic roles are also involved in the control of VLDL secretion, which is activated by LXR and repressed by FXR (Figure 2) (97). Once more, SHP plays a key role in the modulation between FXR and LXR (117, 118). The activation of FXR leads to the induction of SHP expression. Among other targets, SHP can repress FXR and LXR transcriptional activity, establishing a negative feedback mechanism that allows SHP to control its own expression along with that of many other genes (92, 108, 117). Thus, SHP, which was initially described as an important controller of bile acid synthesis, is gaining relevance in different metabolisms such as inflammation or lipid homeostasis (92, 115, 119).

In summary, nuclear receptors are key regulators of lipid and lipoprotein metabolism in the liver, playing central roles in the hepatocyte efflux of bile acids, fatty acids and cholesterol. The role as major controllers of lipid homeostasis places nuclear receptors as important pharmacological targets for the modulation of dislypemias.

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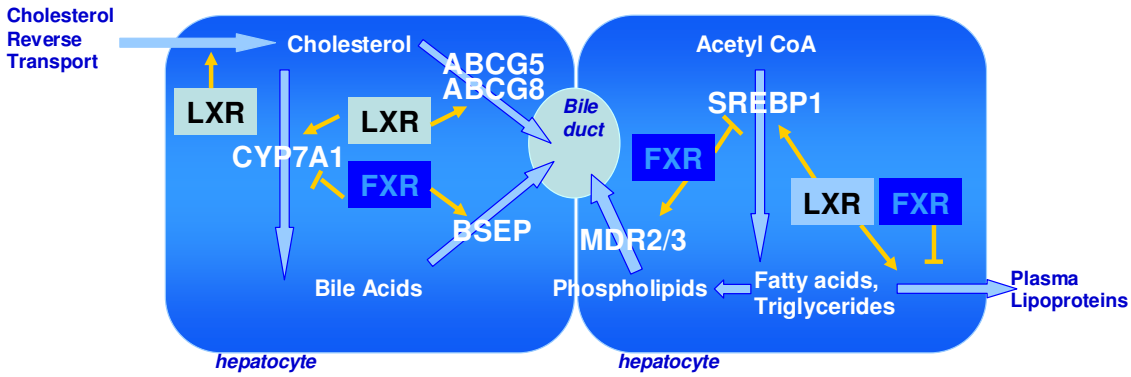

Figure 2. Control of Bile acid, cholesterol an fatty acids efflux by nuclear receptors LXR and FXR in hepatocytes.. Adapted from Kalaany and Mangelsdorf. 2006. Annu Rev Physiol.

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2. Flavonoids and procyanidins.

Several studies have reported a wide range of beneficial effects of dietary procyanidins, and flavonoids in general, in human and animal models, protecting against CVD and oxidative damage or ameliorating insulin resistance states (62, 120-124). Therefore, the interest arisen by the protective properties of these polyphenolic compounds has placed them in the focus of nutrition research, in order to understand the processes and molecular mechanisms responsible for their effects (62, 125, 126).

2.1 Chemistry, human intake and metabolism

Procyanidins are a group of flavonoids found in vegetables and derived foods such as tea, cocoa, red wine or fruit juices (127-130). Flavonoids are widely distributed among vegetables, and are represented by a large number of chemical structures. Nevertheless, all these structures are based in a benzenic ring condensed with a heterocyclic pyran that carries a phenyl benzene ring (Figure 3) (120). From this basic structure, a plethora of different compounds arise depending on the additionally bound functional groups. The main subgroups into the flavonoids family are anthocyanidins, flavonols, flavones, flavanones, isoflavonoids and flavanols (64, 120). Procyanidins are included in the group of flavanols, also named flavan-3-ols or catechins, and are condensed structures formed by polymerization of (+) catechin, (-) epicatechin and (-) epicatechin gallate (Figure 3). The different oligomers ranging between 2 and 10 units are considered procyanidins, while further polymerized structures are named tanins (64, 120).

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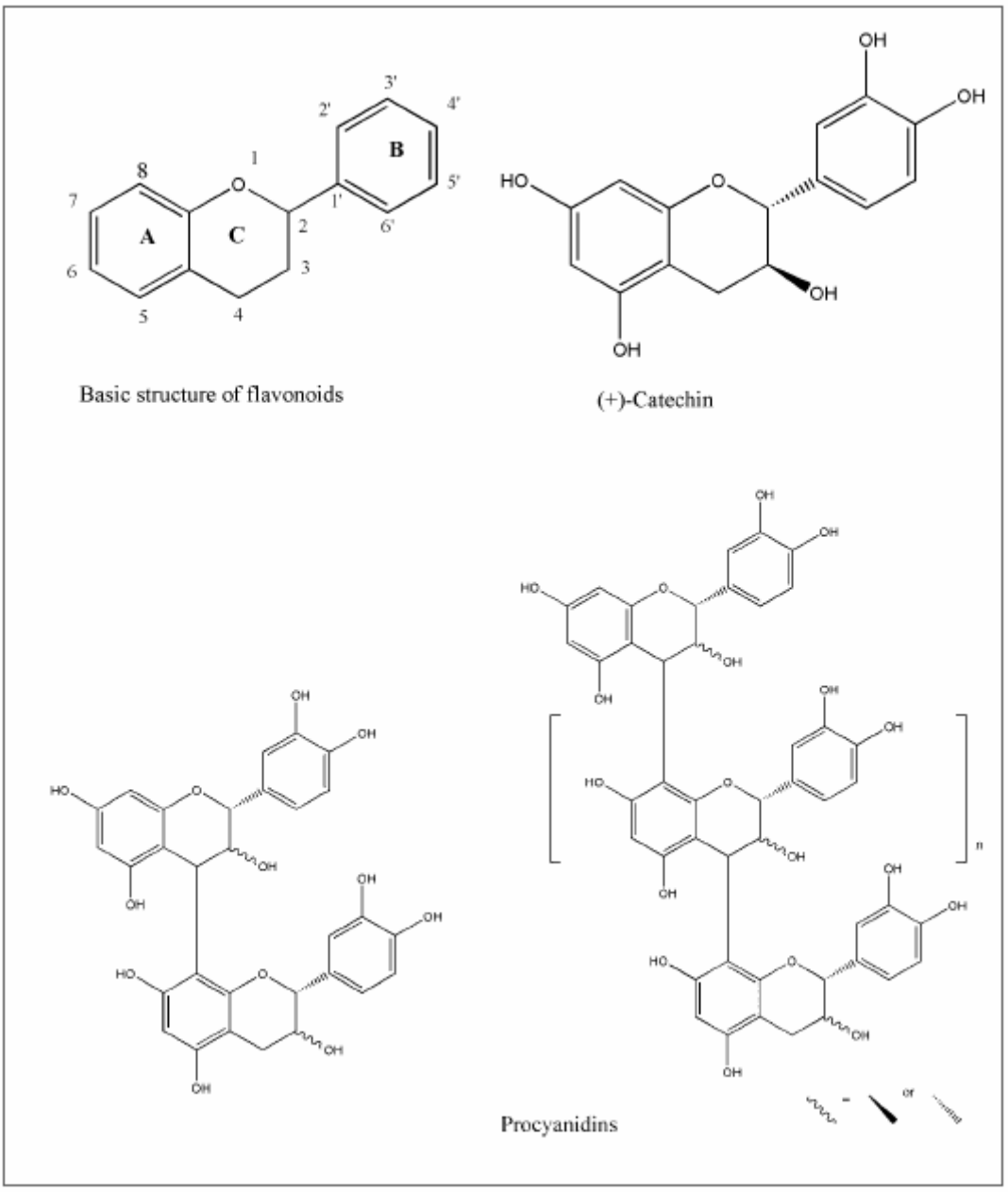

Figure 3. Chemical structure of flavonoids.

The average intake of flavonoids by humans remains unclear. Different works report flavonoid intake values ranging from 3 mg to 2 g per day (120, 131). Nevertheless, these studies were mainly focused in a limited number of flavonoids and foods. On the other hand, the bioavailability of these phenolic compounds has not been yet precisely elucidated (120). The appraisal of the precise active forms of these compounds is subjected to technical limitations on their analysis. Thus, the mechanisms of absorption, modifications in their structure or possible depolymerisation remain unclear (64, 131). Nevertheless, it has been shown that the bioactive form of flavonoids differ from those

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found in vegetables. Thus, aglycones (i.e., non-glycosilated forms) can be metabolized during intestinal transit by colonic microflora (132, 133). In enterocytes and hepatocytes, flavonoids are metabolized by the oxidative metabolism, P450 related metabolism, and/or glucuronidated, sulphated, methylated and/or conjugated with thiols such as gluthatione (132-134). Despite the fact that those modifications were observed in monomers, such as quercetin, hesperetin, naringenin and epicatechin, oligomeric procyanidins may be metabolized in a similar manner (132). Evidence suggest that oligomeric procyanidins can be modified after absorption, being glucuronidated, methylated or sulfated, and that procyanidins are not necessarily depolymerised (135, 136). Tsang et al (2005) demonstrated that dimeric and trimeric forms of procyanidins can be found in rat urine after oral administration of grape seed extract, while catechin metabolites can be found in kidney and liver (135). Moreover, Garcia-Ramirez et al (2006) reported the presence of tetramethylated dimeric procyanidins in the liver of rats fed a single dose of synthetic procyanidins (136). Despite procyanidins or procyanidins monomers are able to reach different tissues, it has been demonstrated that flavan-3-ols can not pass through the blood-cerebrospinal fluid barrier (137). Another key aspect in the study of polyphenolic compounds is the rate of absorption. It has been shown that the maximum concentration of plasma quercetin in humans is achieved 3 hours after intake of red grape juice (RGJ) (138). In agreement, in rats fed with synthetic oligomeric procyanidins, tetramethylated dimeric forms were detected in plasma as soon as 1 hour after administration (136). This evidence and the wide diversity of effects exerted by these compounds in *in vivo* models (125, 126, 131) point out that procyanidins are rapidly absorbed and readily reach the liver and other tissues.

2.2.*Interactions of flavonoids with intracellular signalling pathways*

The antioxidant properties of flavonoids were initially postulated as the main explanation for their beneficial effects. Their structure, rich in double carbon bonds, facilitates the stabilization of reactive oxygen species (64). Thus, green tea polyphenols were shown to have significant antioxidant activity both in *in vivo* and *in vitro* systems, acting by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metal ions, while modestly ameliorated *ex-vivo* oxidation of lipoproteins (139). On the other hand, red wine polyphenols exert a protective effect *in vitro* and *in vivo* against LDL free radical–mediated oxidation (140, 141). Therefore, flavonoids

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may be important in preventing CVD by reducing the susceptibility of LDL to oxidation *in vivo*.

Nevertheless, accumulating evidence suggest that beneficial effects of flavonoids may be mediated by interactions with cellular components of protein kinase and lipid kinase signalling cascades such as phosphoinositide 3-kinase (PI3K), protein kinase C (PKC) and MAP kinases (142-145). Thus, genistenin, an isoflavone, modulates the expression of antioxidant enzymes by enhancing the phosphorilation of extracellular regulated kinase (ERK1/2) (146). Procyanidins derived from grape seed inhibited the phosphorilation of JNK, p38 and ERK1/2, leading to a protection against UV induced oxidative damage in keratinocytes (147). Red wine flavonoids inhibits ligand binding to platelet-derived growth factor beta receptor (βPDGFR) and subsequent ligand-induced recruitment of signalling molecules such as RasGAP, PI3K or PLCγ, resulting in the prevention of downstream events in vascular smooth muscle cells (148). In HepG2 cells, naringenin, another flavonona, decreased apoB secretion via activation of PI3K (14). The interaction of flavonoids with these signalling pathways leads to the transcriptional modulation of different genes. Thus, in HepG2 cells, grape seed procyanidins trigger the expression of gluthathione S-transferase (GST) (121). Naringenin repress LDLR expression in HepG2 cells and wild type hepatocytes (14), and decrease MTP and ACAT mRNA levels in HepG2 (149).

Additionally, different in vitro experiments have demonstrated that tea flavonoids are able to inhibit fatty acid synthase (FAS), subsequently inhibiting TG biosynthesis by interfering the epidermal growth factor (EGF) signalling pathway (Figure 4) (150, 151). Therefore, the modulation of signalling pathways and gene expression exerted by flavonoids provides a wide base for the physiological effects ascribed to these compounds.

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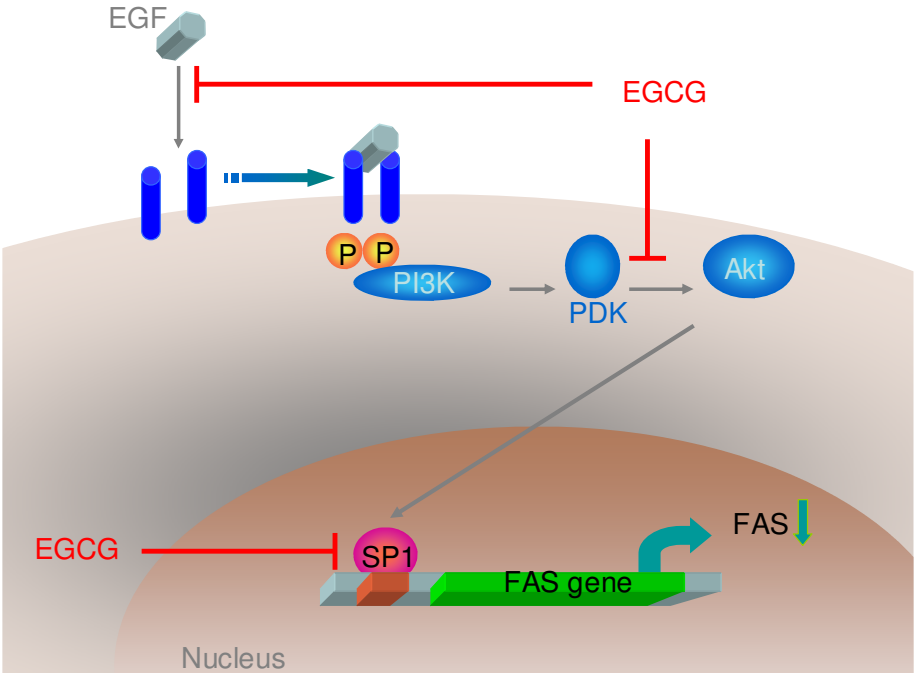

Figure 4. Proposed mechanism for FAS downregulation by Epigallocathechin gallate via EGF signalling pathway. Adapted from Jen-Kun Lin and Shoei-Yn Lin-Shiau. 2006. Mol Nutr Food Res

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2.3. Effects of flavonoids and procyanidins on lipid metabolism and cardiovascular diseases

The beneficial effects ascribed to flavonoid consumption are supported by several studies performed in different models (72, 126, 131, 152). During the past decade, Artaud-Wild et al. (1993) described that, in 40 different countries, mortality by cardiovascular heart disease correlated with cholesterol and saturated fat intake. Intriguingly, the low mortality by CVD of the French population contrasted with their high consumption of cholesterol and saturated fats (Figure 5A) (153). Nowadays, this is known as the French paradox, and can be extended to other European southern countries.

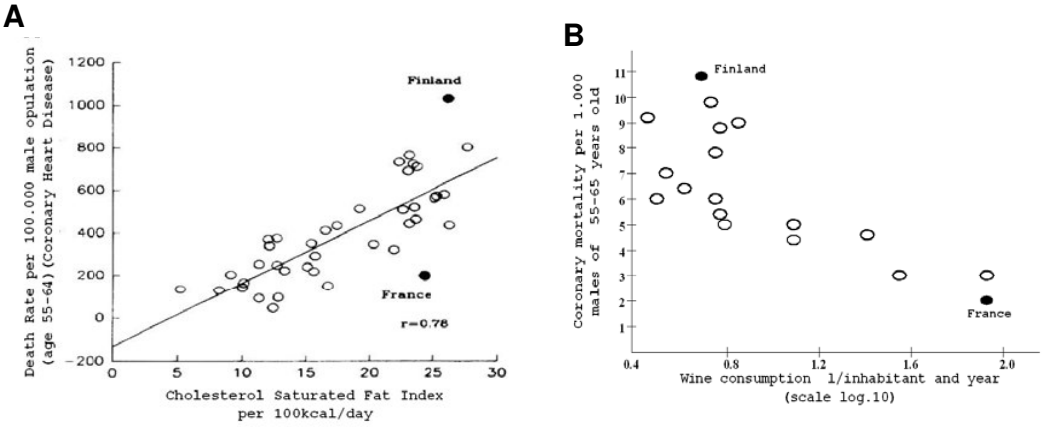

Figure 5. The French Paradox

A. From Artaud-Wild et al. 1993. Circulation.
B. From Renaud M et al. 1992. The Lancet.

The explanation for this paradox can be found in the nutrition habits associated to the Mediterranean diet (154). Among other factors, red wine consumption has been postulated as an explanation for the paradox, since France fits in the correlation when wine intake is plotted against coronary mortality (Figure 5B) (155). The observation that non-alcoholic components in red wine were responsible, at least in part, for the beneficial actions against CVD, led to the identification of flavonoids as potent agents for preventing and ameliorating different pathologies (120, 123, 131). From that starting point, evidence demonstrating the beneficial effects of flavonoids consumption are supported by many *in vitro* and *in vivo* studies (123, 152, 156-158). Nowadays, flavonoids are in the focus of nutrition research, and many studies have been addressed

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to identify the molecular mechanisms underlying the beneficial effects of these polyphenolic compounds (64, 72, 123, 126, 131).

Several studies have been addressed to determine the potential applications of flavonoids in the prevention and treatment of different pathologies which implicate oxidative metabolism, insulin resistance or dyslipemia, such as atherosclerosis or metabolic syndrome (124, 159). Thus, it has been shown that procyanidins elicit protective effects against oxidative damage in neurons and fibroblasts (132). Once more, the mediation of signalling cascades is involved, such as caspase-3 and pro-apoptotic MAPK (160). In addition, different studies have revealed that flavonoids are able to affect growth-related signal transduction pathways involved in the progression of cancer (161-165). Their actions could be divided into two main categories (i) the ability to induce apoptosis of cancer but not normal cells (161, 163, 166), and (ii) the inhibition of signalling pathways involved in the progression of carcinogenesis (167). On the other hand, the ability of flavonoids to ameliorate insulin resistance has been described by different authors (124, 159). Thus, grape seed procyanidins have shown an antihyperglycemic effect in diabetic rats and insulin sensitive cell lines such as 3T3-L1 adipocytes and L6E9 myotubes (124), while naringenin showed insulin like effects regarding ApoB secretion in HepG2 cells (168).

The effects of flavonoids against CVD have been widely described, and can be extended to hepatic cholesterol metabolism and inflammation, being protective at several levels (126). First, the suggested actions of polyphenols reducing cholesterol absorption have been related with their ability to interact with ATP steroid binding cassettes found in enterocyte cholesterol transporters (169). In rats, the absorption of cholesterol was inhibited by tea procyanidins (170). In addition, when Caco-2, a human intestinal cell line, were treated with dealcoholized wine, the secretion of apoB48 was importantly decreased (171). This effect was ascribed to a lack of lipids for assembling into the nascent chylomicrons as a result of a diminished lipid absorption (126). Studies in dyslipemic post-menopausal women showed that, after intake of dealcoholized red wine, apoB48 levels were markedly reduced, pointing out a decrease in circulating postprandial chylomicrons caused by a decrease of fat absorption (172). Therefore, a lowered lipid absorption at the intestine level has been suggested as a primary

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mechanism for reducing plasma lipid content by flavonoids (126). Nevertheless, the main part of studies addressed to identify the actions of polyphenols in the lipoprotein assembling and secretion processes have been focused on hepatic production of VLDLs. Thus, naringenin and hesperitin decreased apoB secretion in hepatocytes (14, 149). These results were associated with decreased cholesteryl ester (CE) mass, inhibition of ACAT2 expression and lowered MTP activity and mRNA levels. The actions of flavonoids decreasing CE pool have been attributed to their binding with plasma membrane transport P-glycoprotein, decreasing CE incorporation into newly synthesized lipoproteins (126). Additionally, tea flavonoids are able to inhibit fatty acid synthase (FAS), consequently inhibiting TG biosynthesis, by interfering the epidermal growth factor (EGF) signalling pathway in HepG2 and MCF-7 cells (150, 151). The suppression of FAS was accompanied by reduced levels of cholesterol and fatty acid biosynthesis (150). Thus, the hypolipidemic activity ascribed to flavonoids can result from the inhibition of lipid biosynthesis and reduced assembling of lipoproteins.

It has been described that alcohol-stripped red wine, in addition to lowering ApoB100 secretion in HepG2 cells, increases the binding activity of LDLR (173). Thus, dietary flavonoids actions can be extended to an enhanced clearance of plasma lipoproteins by the liver. In agreement with this idea, red grape juice (RGJ) polyphenols increased the activity and expression of LDLR in HepG2 and HL-60 cells in the presence and absence of LDL (174). Similar effects have been ascribed to Epigallocatechin Gallate (EGCG), which induced LDLR expression and its binding activity in HepG2 cells, lowering as well the concentration of intracellular cholesterol (175). In summary, flavonoids are able to lower plasma lipid content by interfering with lipoprotein metabolism at several levels, including intestinal absorption of lipids, TRL synthesis by intenstine and liver and hepatic LDL clearance.

The actions of flavonoids lowering lipoprotein synthesis and secretion in liver and intestine, and enhancing the hepatic clearance of plasma cholesterol are translated into ameliorated levels of pro-atherogenic lipoproteins. It has been shown that, in healthy humans and hemodyalisis patients, RGJ exerted antioxidant, hypolipidemic and antiinflamatory effects. RGJ consumption increased the antioxidant capacity of plasma without affecting concentrations of uric acid or ascorbic acid and reduced the concentration of oxidized LDL. Additionally, RGJ supplementation caused a significant

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increase in HDL-cholesterol and apoA-I plasma content and a decrease in LDL-cholesterol and apoB-100 concentrations (138).

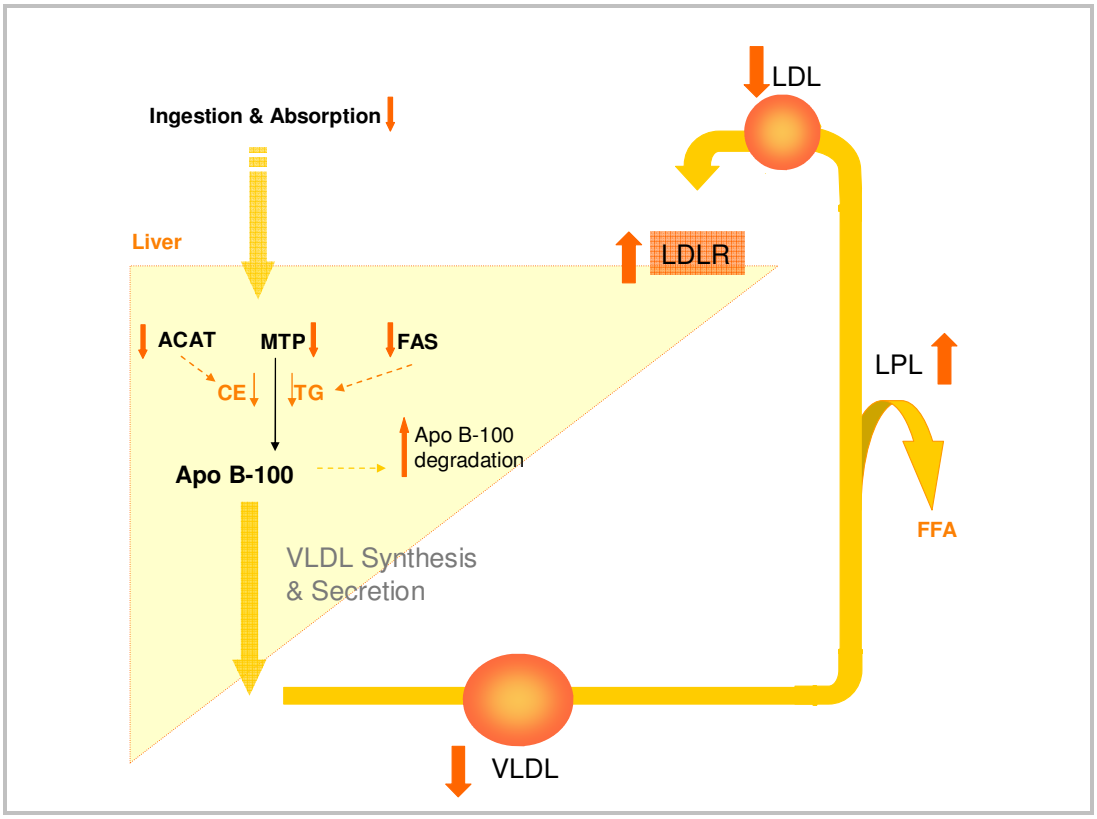

Figure 6. Effects of dietary flavonoids at different levels in the lipoprotein metabolism. Adapted with modifications from Zern and Fernandez. 2005. The Journal of Nutrition

Grape polyphenols have also been shown to exert hypolipidemic effects in pre- and post-menopausal women (176). Those women showed a significant decrease of plasma TG and apoB after the consumption of lyophilized grape powder during 4 weeks. Concomitantly, a reduction in ApoE levels was observed. ApoE can act by displacing ApoCII from VLDL, inhibiting LPL activity and, thus, TG hydrolysis and VLDL catabolism (26). Therefore, not only the synthesis and secretion of lipoproteins is involved in the beneficial effects of flavonoids, but also metabolization of TRL may account, leading to a reduction in circulating TG by decreasing ApoE, which result in enhanced LPL activity. Accordingly, hawthorn flavonoids treated mice did not show increased levels of plasmatic LPL, but the expression of this protein increased in muscle, while decreased in adipose tissue (177). In another work, Rutin, a citrus flavonoid, ameliorated different plasmatic parameters, such as VLDL-cholesterol, LDL-

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cholesterol and HDL-cholesterol in streptozotocin-induced diabetic rats, while significantly triggered the activation of plasma LPL activity (178). Therefore, flavonoids can act at many levels in order to lower plasma lipid content (Figure 6).

Along with their actions in lipoprotein metabolism, flavonoids can act against CVD by other mechanisms. It has been shown that red wine oligomeric procyanidins are directly able to modulate vasoconstriction by reducing the synthesis of endothelin-1 (ET-1). ET-1 inhibition was concomitant with the content of oligomeric procyanidins when comparing different wines (179). Additionally, flavonoids have been shown to lower inflammation, which is another key factor in the development of atherosclerosis. Thus, grape procyanidins are able to decrease plasma levels of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α and Interleukin (IL-6) in both pre- and post-menopausal women (176). The comparison of red wine and gin consumption revealed that, in healthy men, red wine intake significantly triggered the downregulation of adhesion molecules such as VLA-4, LFA-1, Mac1 and MCP-1 on monocytes and T-limphocytes (180). In addition, the soluble adhesion molecules ICAM-1 and VCAM-1 were significantly reduced in the same subjects. Also, in another work, a downregulation of monocytes surface adhesion molecules promoted by red wine was observed (181). It has been suggested that the suppression of NF-kB signalling could be underlying these effects (126). NF-kB is a transcription factor responsible for enhanced expression of cytokines, adhesion molecules and pro-coagulant proteins (182). Thus, the incidence in its signalling pathway could provide an explanation for the anti-inflammatory effects of flavonoids. In this sense, when healthy subjects were given a fat rich meal with or without red wine, the consumption of red wine significantly decreased the activity of NF-kB in monocytes (183), indicating that flavonoids are able to ameliorate inflammation.

In summary, flavonoids are powerful agents ameliorating plasma lipoprotein profile. Along with these actions, flavonoids are able to inhibit different mechanisms leading to inflammation. The beneficial effects of flavonoid consumption have been widely demonstrated in different models, in many altered states, but also in healthy conditions. Thus, dietary flavonoids can be considered as powerful bioactive agents for ameliorating the quality of live.

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I. Introduction

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II OBJECTIVES

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In the past decade, nutrition has gained great interest in industrialized countries as a natural way to improve the quality of life and ameliorate different pathological states. The health benefits of a wide variety of nutrients have prompted a shift in nutrition research from epidemiology and physiology to molecular biology and genetics, with a clear objective: to understand the basis of nutrients actions. The new technologies, allowing the analysis of complex systems and nutrient-protein, nutrient-gene interactions, have leaded to the apparition of the new, –omics disciplines: Nutrigenomics, Proteomics and Metabolomics. These emerging disciplines are opening a wide window of possibilities and are rapidly increasing the knowledge regarding the molecular mechanisms that underlie the bioactivity of nutrients, with the final goal of translating the data into an accurate prediction of the effects of dietary components.

The study of red wine is not avoiding this shift of focus. From the initial studies, which related moderate consumption of red wine with the French Paradox - i.e., the low mortality rate in the French population despite having an elevated intake of saturated fats- until nowadays, different research groups have been investigating the beneficial actions of red wine. As a result, procyanidins, a group of flavonoids, have emerged as the main responsible components exerting the protective qualities of this beverage. Along with its antioxidant properties, procyanidins have shown other beneficial effects related with the ability of this polyphenolic compounds to interact with different signalling pathways, thus modulating gene expression. Nowadays, Nutrigenomics is providing the tools for studying the molecular mechanisms underneath the effects of procyanidins and for understanding the molecular basis of the French Paradox.

Procyanidins have been shown to exert beneficial actions on a broad array of metabolic disorders that are risk factors for cardiovascular diseases, such as atherosclerosis, inflammatory processes, obesity and diabetes. In these altered states, lipoprotein metabolism plays an important role. Different works have described the beneficial effects of different flavonoids and procyanidins in lipoprotein metabolism, decreasing triglyceride rich lipoproteins and boosting the reverse transport of cholesterol. Nevertheless, the molecular mechanisms underlying these effects are only partially known. How these compounds can alter the composition, metabolism and clearance of plasma lipoproteins by interacting with intracellular signalling pathways is still an unsolved subject. Previous studies from our group have demonstrated the beneficial

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effects of red wine and a grape seed procyanidin extracts on lipid metabolism in the liver, a key organ in the control of lipid homeostasis. Liver leads two major processes in the lipoprotein metabolism, namely VLDL production and reverse cholesterol transport. Therefore, the activity of grape seed procyanidins in this organ becomes a key target of study in order to improve our knowledge of how these polyphenolic compounds can modulate lipoprotein metabolism.

The research work carried out in this Ph. D. Thesis is part of a more general research project developed by the Nutrigenomics Research Group of the Universitat Rovira i Virgili, which deals with the potential beneficial effects of dietary procyanidins in preventing and ameliorating the metabolic disorders associated with the so called Metabolic Syndrome. The purpose of this Thesis has been to characterize and understand how dietary procyanidins modulate lipid and lipoprotein metabolism in hepatic cells. With this aim, three objectives were sequentially proposed:

1. **To evaluate the effects of oral intake of procyanidins on postprandial plasma lipoprotein profile.**

   The effect of procyanidins in the plasma lipoprotein profile has been evaluated in healthy rats in the postprandial phase. A single high and non-toxic dose of a grape seed procyanidin extract was administered orally. This experimental design was intended to assess the short-term effects of procyanidins, in order to evaluate the primary changes leading to the long-term beneficial effects ascribed to these compounds. This study revealed that procyanidins are potent hypotriglyceridemic agents (manuscript 1). This effect has been also observed in wild type mice (manuscripts 3 and 4).

2. **To assess the role of liver in the response triggered by procyanidins on plasma lipid profile.**

   To assess the implication of liver in the hypotriglyceridemic response triggered by procyanidis two experimental approaches were undertaken. The effect of procyanidins on VLDL secretion was evaluated *in vitro* using human hepatoma HepG2 cells, showing that procyanidins repress VLDL secretion (manuscript 3). *In vivo*, the changes induced by procyanidins in liver global gene expression profile were analyzed by microarray hybridization in order to identify procyanidin target genes and putative mediators of the hypotriglyceridemic response (manuscripts 1

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and 2). These analyses revealed several key regulatory factors of inflammation and lipid metabolism as targets of procyanidins. Among them, the nuclear receptor Small Heterodimer Partner (SHP) stands out as a putative mediator of the hypolipidemic action of procyanidins in the liver.

3. To establish the molecular mechanisms by which procyanidins modulate lipid and lipoprotein metabolism in the liver.

To achieve this objective, two models have been used, namely HepG2 cells and mice. The activity of SHP was blocked in HepG2 by means of the silencing RNA technology. To achieve a similar in vivo model, $SHP^{-/-}$ mice were used. The actions of procyanidins were assayed in both models lacking or underexpressing SHP. Results have revealed that SHP is a key mediator of the hypotriglyceridemic actions of procyanidins in the liver (manuscript 3).

Farnesoid X receptor (FXR) is a nuclear receptor controlling SHP expression. This prompted the study of FXR as a putative mediator of the hypotriglyceridemic action of procyanidins upstream SHP. *In vivo* studies using $FXR^{-/-}$ mice and *in vitro* luciferase based assays confirmed FXR as a key component of the signalling pathway used by procyanidins to elicit the triglyceride lowering effect (manuscript 4).

The research work carried out in this Ph. D. Thesis has been supported by a grant from the Spanish government and performed mainly in the Nutrigenomics Research Group laboratory, of the Universitat Rovira i Virgili,. Two international stages have been done. Firstly, in the Giovanni Galli laboratory of the Department of Pharmacological Sciences from the Università degli studi in Milan, Italy, supported by a grant from the Marie Curie research training network of the European comission. The second international stage took place in the David D. Moore laboratory, in the Department of Molecular and Cell Biology from the Baylor College of Medicine in Houston, Texas, and was supported by a grant from the Spanish government.

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III RESULTS AND DISCUSSION

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GRAPE SEED PROCYANIDINS IMPROVE ATHEROSCLEROTIC RISK INDEX AND INDUCE LIVER CYP7A1 AND SHP EXPRESSION IN HEALTHY RATS

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ABBREVIATIONS:

Apo, apolipoprotein. CA, chenocholic acid. CDCA, chenodeoxycholic acid. CM, chylomicrons. CMR, chylomicron remnant. CVD, cardiovascular disease. CYP27A, cytochrome P450 27A. CYP7A1, cytochrome P450 7A1. CYP8b1, cytochrome P450 8b1. ERα, estrogen receptor α. FFA, free fatty acid. FXR, farnesoid X receptor. GSPE, grape seed procyanidin extract. HDL, high density lipoprotein. HDL-C, HDL-cholesterol. HMG-CoA, 3-Hydroxy-3-methylglutaryl CoA. LDL, low density lipoprotein. LDL-C, LDL-cholesterol. LPL, lipoprotein lipase. LXR, liver X receptor. MAPK, mitogen-activated protein kinase . nonHDL:nonLDL-C, nonHDL:nonLDL colesterol. RAR retinoic acid receptor. RWPs, red wine polyphenols. RXR, retinoid X receptor. SHP, small heterodimer partner. SREBP, sterol regulatory element binding protein. TC, total colesterol. TG, triglyceride. VLDL, very low density lipoprotein.

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ABSTRACT

Moderate consumption of red wine reduces risk of death from cardiovascular disease. The polyphenols in red wine are ultimately responsible for this effect, exerting antiatherogenic actions through their antioxidant capacities and by modulating intracellular signaling pathways and transcriptional activities. Lipoprotein metabolism is crucial in atherogenesis, and liver is the principal organ controlling lipoprotein homeostasis. This study was intended to identify the primary effects of procyanidins, the most abundant polyphenols in red wine, on both plasma lipoprotein profile and the expression of genes controlling lipoprotein homeostasis in the liver. We show that procyanidins lowered plasma triglyceride, free fatty acids, apolipoprotein B (apoB), LDL-cholesterol and nonHDL:nonLDL-cholesterol levels and slightly increased HDL-cholesterol. Liver mRNA levels of small heterodimer partner (SHP), cholesterol 7alpha-hydroxylase (CYP7A1) and cholesterol biosynthetic enzymes increased, whereas those of apoAII, apoCI and apoCIII decreased. Lipoprotein lipase (LPL) mRNA levels increased in muscle and decreased in adipose tissue. In conclusion, procyanidins improve the atherosclerotic risk index in the postprandial state, inducing, in the liver, the overexpression of CYP7A1 (suggesting an increase of cholesterol elimination via bile acids), and SHP, a nuclear receptor emerging as a key regulator of lipid homeostasis at the transcriptional level. These results could explain, at least in part, the beneficial long-term effects associated with moderate red wine consumption.

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INTRODUCTION

Many epidemiologic studies have demonstrated that moderate consumption of alcoholic beverages is associated with reduced mortality and risk of cardiovascular disease (CVD) (1-3). The greatest degree of cardioprotection is related to ingestion of red wine rather than white wine, beer or spirits(4, 5). The consumption of red wine is a primary cause for the "French paradox," i.e., a low mortality rate from CVD despite a high consumption of saturated fat and cholesterol (6, 7). Wine contains phenolic compounds, which have been reported to have a number of antioxidant properties(8), and thus may contribute to a reduced risk of CVD in wine drinkers. Chronic moderate consumption of red wine protects rats from oxidative stress *in vivo* (9). Also, there is evidence that oxidized LDL play a crucial role in atherogenesis (10, 11), and red wine polyphenols (RWPs) protect in vitro and in vivo from LDL free radical–mediated oxidation (12, 13). Thus, RWPs may be important in preventing CVD by reducing the susceptibility of LDL to oxidation in vivo(14-16).

Increasing evidence shows that RWPs, and particularly flavonoids, contribute to cardioprotection through mechanisms that are independent of their antioxidant capacities. These mechanisms comprises alterations in cell membrane receptors, intracellular signaling pathway proteins and modulation of gene expression(16-19). Thus, RWPs induce the synthesis and release of nitric oxide by the vascular endothelium, which in turns, promotes vasorelaxation, reduces platelet aggregation, and limits the flux of atherogenic lipoproteins into the artery wall (17, 19). In addition, RWPs inhibit proliferation and migration of vascular smooth muscle cells, by interfering on platelet-derived growth factor (PDGF) receptor signaling through the phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. A plethora of transcriptional changes underlay and follow these actions of RWPs on the components of the vascular system(17, 19) (20-22). Much less is known about the mechanisms that underlay antiatherogenic actions of RWPs on other tissues. Liver presides over the homeostasis of circulating lipids and lipoproteins adjusting its metabolic fluxes to the supply of nutrients and the requirements of all other tissues(23). RWPs have been reported to reduce plasma lipids and atherogenic lipoproteins (mainly

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LDL, and chylomicron remnants) in different animal models. Thus, moderate and chronic consumption of red wine, but not of alcohol, reduce LDL cholesterol (LDL-C) in normocholesterolemic rats (24). In hyperlipidemic hamsters, prolonged ingestion of dealcoholized red wine, or of RWPs, produces a significant reduction in plasma LDL concentrations, apolipoprotein B (apoB), triglycerides (TG) and cholesterol preventing early aortic atherosclerosis (25, 26). In cultured human liver cells HepG2, dealcoholized red wine decreases production of apoB100 (a marker of VLDL and LDL in humans), while increases mRNA expression of the 3-hydroxy-3-methylgluteryl coenzyme A (HMG-CoA reductase) (a key cholesterol biosynthetic enzyme) and the LDL receptor gene. In this regard, RWPs resemble statins, potent lipid-lowering antiatherogenic drugs that inhibit HMG-CoA reductase activity (27). It has recently been found that acute consumption of red wine (alcoholic and nonalcoholic) in dyslipidemic postmenopausal women produces a decrease in postprandial levels of apoB48 (marker of CM and CMR in humans) whereas total cholesterol (TC), LDL-cholesterol (LDL-C) and HDL-cholesterol (HDL-C) as well as TG plasma levels are unaffected (28). Catechin has already been shown to reduce cholesterol absorption in the intestine (29). All together, these results suggest that RWPs, when consumed during meal, can reduce the amount of circulating proatherogenic lipoproteins, by decreasing their production in intestine and liver, while increasing their clearance by the liver.

Again, modifications of enzymatic and transcriptional activities lie beneath the effects of RWPs on liver metabolism. More studies are needed to elucidate the effects that RWPs exert on metabolic fluxes of cholesterol, bile acids, fatty acids (FA), TG and lipoproteins in the liver, which are, in most cases, ultimately controlled in a coordinated manner at the transcriptional level. The mechanisms underlying of this coordination are not fully understood due their complexity and the implication of a large number of different transcription factors. Some of them are well known but partially understood, like hepatocyte nuclear factor-4 (HNF-4), peroxysome proliferators activated receptors (PPARs), retinoid X receptors (RXRs), retinoic acid receptors (RARs), farnesoid X receptor (FXR), sterol regulatory element binding proteins (SREBPs) (30) or small heterodimer partner (SHP), an orphan nuclear receptor which was initially described as a corepressor involved in feedback regulation of bile acid synthesis(30, 31), and at present is emerging as a key factor in the control of lipid homeostasis(32-35).

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In the present study, we have investigated the short term effects of procyanidins, the most abundant polyphenols present in red wine, *in vivo* and in healthy (normolipidemic) animals, in order to gain insight on the primary mechanisms that underlie the long-term antiatherogenic and cardioprotector effects ascribed to RWPs. To do that, we orally administered a single, high and non-toxic(36) dose of grape seed procyanidin extract (GSPE) to chow-fed male rats, and analyzed plasma lipid and lipoprotein profile after 5 hours. Changes in the gene expression pattern in the liver of GSPE treated animals were analyzed using microarray hybridizations, in order to identify procyanidin target genes involved in lipoprotein metabolism.

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METHODS

Chemical

Grape seed procyanidin extracts (GSPE) were kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract contained essentially monomeric (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%), oligomeric procyanidins (5-13 units) (35,7%) and phenolic acids (4.22%).

Animals

Male Wistar rats, 2 month old and weighing 250 g, were purchased from Charles River (Barcelona, Spain). The Animal Ethics Committee of University Rovira I Virgili approved all procedures. The animals were housed in animal quarters at 22°C with a 12-h light/dark cycle (light from 8 h a.m. to 20 h p.m.) and were fed *ad libitum*. At 11 a.m. on experimental day, the rats (6 animals per group) were fed an oral gavage of grape seed procyanidins extract in aqueous solution (250 mg/Kg body weight) (GSPE group) or were fed an oral gavage with vehicle (tap water) (Control group). The used procyanidin dose is one-fifth of the NOAEL (no-observed-adverse-effect level) described for GSPE and male rats(36), and we have previously shown that this dose is effective in reducing glycemia in streptozotocin-induced diabetic rats (37). 5 hours after treatment, the rats were sacrificed by beheading and blood was collected using heparin as anticoagulant. Plasma was obtained by centrifugation and stored at -80ºC until analysis. Liver, muscle and adipose tissue were excised and froze immediately in liquid nitrogen and stored at -80ºC until RNA and lipid extraction.

Lipid analysis in plasma and liver.

Plasma TC was measured with an enzymatic colorimetric kit (QCA, Barcelona, Spain). HDL-C was measured, by the same kit, after the treatment of plasma with phosphotungstic acid to precipitate the non-HDL lipoproteins(38). For LDL-C quantification, cholesterol 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(39). Triglycerides were assayed using an enzymatic colorimetric kit (QCA, Barcelona, Spain). Lipids of liver were extracted by the Folch method (40). An aliquot of the lipid extract was used

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to measure total lipids by gravimetry (in duplicate). The rest of the extract was evaporated to dryness and re-dissolved in 2% triton X-100 to determine TG, TC, free cholesterol and esterified cholesterol. TG and TC were assayed as described above for plasma determinations. Free cholesterol was measured by the same method used for TC analysis except that cholesterol esterase was not included. Esterified cholesterol was calculated as TC minus free cholesterol.

Plasma fed state indicators analysis

Plasma β-hydroxybutyrate was analyzed using enzymatic kits (Ben srl. Italy). FFA and glucose were measured using enzymatic colorimetric kits (Wako chemicals GmbH and QCA, Spain respectively).

ApoB SDS-PAGE and Immunoblotting

Plasma samples and purified apoB-100 (Calbiochem, Merck KGaA, Darmstadt, Germany) were separated by SDS PAGE in a 4% polyacrylamide gel (0.5M Tris-HCl, 10% glycerol, 2% SDS, β-mercaptoethanol and 0.01 % Bromophenol blue for the sample buffer) in a Bio-Rad Mini-Protean electrophoresis cell. Separated proteins were electrotransferred onto a nitrocellulose transfer membrane (Schleider & Schuell, Keene, NH, USA). Membranes were blocked overnight and incubated with a goat anti-rat apo B antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) as a primary antibody. The antybody used for this purpose was raised against the amino terminus of apoB so both isoforms, apoB-100 and apoB-48, could be detected and quantified simultaneously in a single plasma sample.. As a secondary antibody a Horse raddish peroxidase conjugated anti-Goat IgG antibody (Santa Cruz Biotechnology) was used. Band detection was performed with Amersham Biosciences (Freiburg, Germany) ECL western detection reagents and Hyperfilm ECL. Bands were quantitated using Quantity One software from BioRad after background subtraction. Purified apoB 100 was used as an internal standard for normalizing apoB bands.

Statistical analysis

Results are reported as means ± sem of 6 animals. Comparison among groups mean was done by independent-samples t test ($p \leq 0.05$) by SPSS software .

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RNA methods

Total RNA was purified from each frozen liver, muscle and adipose tissue by using the NucleoSpinR RNA II kit (Macherey-Nagel, Düren, Germany) following the instructions of the manufacturers. Equal aliquots of total RNA from three rat livers, muscle and adipose tissue in each group, were pooled and used for oligonucleotide array hybridization and quantitative PCR analysis. Integrity of pooled RNAs was assessed by using the Agilent (Madrid, Spain) 2100 Bioanalyzer and the RNA 6000 LabChipR. For microarray hybridization, Cy3- or Cy5-labeled cRNA was obtained from each RNA pool by using the Agilent Low RNA Input Fluorescent Linear Amplification Kit as described in the Agilent manual (Part Number 5185-5818). Fluorescent probes containing 500 ng of each labeled cRNA were pooled and hybridized against Agilent Rat Oligo Microarrays (Part Number G4130A) following the Agilent 60-mer oligo microarray processing protocol (Part Number G4140-90030). Images of hybridized microarrays were acquired with the Agilent G2565BA scanner, and data from the microarray images were obtained and analyzed with the Agilent Feature Extraction software. For each pair of RNA samples being compared, duplicate hybridizations with a dye-swap labeling was performed.

Changes in mRNA expression of selected genes were verified by quantitative PCR. cDNA corresponding to each RNA pool was generated using TaqMan^R Reverse Transcription Reagents (Applied Biosystem, Madrid, Spain) and quantitative PCR amplification and detection were performed by using specific TaqMan^R Assay-On-Demand probes (Applied Biosystems, Rn00589173_m1 for SHP, Rn00564065_m1 for CYP7A1, Rn00565598_m1for HMG-CoA Reductase, Rn00561482_m1 for LPL), the TaqMan^R PCR Core Reagent Kit and the GeneAmpR 5700 Sequence Detection System, as recommended by the manufacturers. Quadruplicated quantifications, performed in singleplex assays, were performed for each gene in each cDNA pool. Actine B was used as the reference gene in quantitative PCR (Applied Biosystems TaqMan^R Assay-On-Demand probe Rn00667869_m1).

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RESULTS

Plasma and liver parameters.

TG levels in GSPE group were reduced to 50% versus the control group 5 hours after treatment (table1). In addition, TC levels and its distribution among lipoproteins was analyzed (table 1). Whereas we found no statistical differences in plasma TC between control and GSPE-treated group, the cholesterol distribution among different lipoproteins was altered significantly. While LDL-C and nonHDL:nonLDL-C levels were significantly lowered in GSPE group, HDL-C levels were slightly increased. When these values were referred to the TC (fig. 1), the cholesterol percentage in HDL fraction was increased while the cholesterol in LDL and nonHDL:nonLDL fractions decreased in the GSPE-treated rats. Also, HDL-C/LDL-C and TC/HDL-C ratios were calculated to evaluate the atherosclerosis risk(41). While HDL-C/LDL-C was increased, TC/HDL-C decreased in the GSPE-treated group (table 1).

To find a link between the nonLDL:nonHDL-C, LDL-C and TG decreasing, the content of both apoB isoforms in plasma was analyzed by immunoblotting (fig 2). Total plasma apoB decreased to a 60 %. The observed decrease resulted from a 50% decrease of apoB-48 isoform and a 10% increase of the apoB-100 isoform.

β-hydroxybutirate and glucose were measured as fed state indicators. Whereas no differences were observed between control and GSPE groups respecting glucose and β-hydroxybutirate levels, FFA levels were reduced significantly in the GSPE group (table 1), thus indicating that rats were under a normal fed situation.

To study the effect of GSPE over liver lipids; triglyceride, total cholesterol, free cholesterol and esterified cholesterol were quantified (Table 2). No changes were observed in rats after the 5 hours GSPE treatment versus the control group

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Liver gene expression.

Relative changes in expression level of genes related to lipid metabolism in liver were quantified by microarray and RT(q)PCR analysis. Table 3 shows a list of selected genes related to lipid metabolism present in the Agilent rat Oligo (Part Number G4130A) and the changes observed in its expression by the GSPE treatment.

No changes were found in the expression of fatty acid synthesis enzymes and fatty acid oxidation. Despite the expression of cholesterol synthesis pathway key enzymes was increased in the GSPE-treated rats, cholesterol estherification enzymes mRNA levels were not affected. Bile acid pathway controlling enzymes Cyp7A1, Cyp8b1, Cyp27A gene expression was affected in a different manner. While CYP7A1 was increased 2.4 fold, CYP8B1 expression slightly decreased 0.8 fold and CYP27A remained unaffected.

Of all the analyzed apolipoproteins, apoC-I, apoC-III and apoA-II decreased their expression over a threshold of 20% of change. apoA-II showed the most important decrease concerning apolipoprotein gene expression, with a fold change of 0.67. Lipoprotein related proteins and receptors did not show considerable changes, excepting a 1.2 fold change in the case of apobec-1 complementation factor. Concerning lipid related nuclear transcription factors expression, only SHP (Nr0b2) was changed, showing a drastic increase (close to 3 fold change).

Given that the plasma triglyceride levels markedly depend upon the activity of extrahepatic lipases, we quantified lipoprotein lipase expression in adipose tissue and muscle. As shown in table 4, muscle LPL mRNA was increased 1.57 fold while adipose tissue LPL mRNA was decreased 0.57 fold.

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DISCUSSION

Many studies have been addressed to asses long term effects of chronic intake of flavonoids on lipid homeostasis in dyslipidemic human and hypercholesterolemia animal models, and have shown their beneficial hypolipidemic effects(24, 42-44). This study was intended to identify the primary, short term effects of grape seed procyanidins on lipid metabolism, in a postprandial situation, in order to gain insight into the mechanisms that underlie their long term effects. With this purpose, we have given a single, high non-toxic (36) oral dose of procyanidins to chow-fed, healthy rats, and have analyzed their plasma lipid profile and hepatic gene expression 5 hours after the treatment. Our results show that oral intake of procyanidins significantly affect the postprandial lipidemic profile by drastically lowering plasma triglyceride, FFAs and apoB48 levels. In addition, GSPE treatment slightly increased HDL-C, and significantly lowered LDL-C and nonHDL:nonLDL-C, without affecting plasma TC levels. Therefore, the ratio TC/HDL-C was decreased and HDL-C/LDL-C was increased, thus determining an improvement in the atherosclerotic risk index(41). These changes in plasma lipid profile were paralleled by changes in liver expression of genes involved in the control of lipid homeostasis: the mRNA levels of SHP, CYP7A1 and cholesterol biosynthetic enzymes increased, whereas those of apoAII, apoCI and apoCIII decreased.

The observed 50% reduction in plasma triglyceride and plasma apoB content, together with the lowered nonHDL:nonLDL-C level, indicates that the number of apoB-containing triglyceride-rich lipoproteins has decreased in GSPE treated rats. We have shown that total apoB decreased mainly due to a reduction of the apoB48 isoform. In rats, apoB48 is secreted by both liver and intestine (45). In livers of adult rats, apoB48 is the predominant synthesized and secreted isoform (45). Therefore, the amount of circulating apoB48 in plasma is determined by the balance between synthesis of VLDL by the liver and of CM by the intestine (46), on one side, and the utilization of VLDL and CM by peripheral tissues (mainly muscle and adipose tissue) on the other side(23).

Concerning the production of VLDL, we have found that the level of the precursor mRNA for both apoB100 and apoB48 was not changed in the liver. On the other hand, neither the total lipid content nor the mRNA expression of triglyceride and fatty acid

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biosynthetic enzymes, were modified en the liver of GSPE treated rats. Nevertheless, our results do not rule out the possibility that the livers of GSPE treated rats were producing less VLDL since the secretion by the liver of apoB is not controlled at the transcriptional level but mainly by posttranscriptional mechanisms that include mRNA stability, apoB translation, translocation and proteasomal degradation (45-47). In addition, it has been shown (48) that apoB secretion is inhibited by epicatechin, present in the GSPE used here, and this inhibition is independent of lipid biosynthesis in human, liver derived HepG2 cells. Although we have not data concerning the production of apoB48 by the intestine, this factor is also expected to contribute to the observed reduction of apoB48 levels in the plasma of GSPE treated rats, since wine polyphenols are known to attenuate postprandial CM and their remnants, thus lowering plasma apoB48, in dyslipidemic women (28).

On the other hand, liver mRNA levels of apoAII, apoCI and apoCIII have decreased notably, implying relevant changes in lipoprotein composition and subsequent metabolism since, in contrast to apoB, the secretion of these apolipoproteins by the liver is directly controlled at the transcriptional level (49, 50). As explained below, these transcriptional changes could functionally explain, at least in part, the observed plasma triglyceride and FFA reduction as well as the increase in HDL-C concentration.

Little is known about the mechanism of apoAII function, but the correlation between apoAII and TG, FFA, VLDL and HDL is firmly established. Recent studies have shown that apoAII levels are controlled mainly by its rate of synthesis in the liver rather than by its catabolism (49, 50), and a decrease in apoA-II transcription has been associated with low plasma apoA-II levels (51). It has also been shown that the overproduction of human apoAII in transgenic mice results in a large decrease of HDL levels, associated with very high postprandial levels of VLDL (52, 53). Conversely, ApoA-II knockout mice display low plasma levels of FFA (52, 54). Also the role of ApoCI - which resides on CM, VLDL and HDL - in lipid metabolism remains unclear. Nevertheless, it is known that mice overexpressing ApoCI have elevated levels of plasma FFA (55), and strongly elevated levels of TC and TG due to the inhibitory action of ApoCI on VLDL uptake via hepatic receptors, in particular the LDL receptor-related protein (55, 56)

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Apo C-III is a key player in plasma triglyceride metabolism. In humans, apoCIII is synthesized in the liver and, to a much lesser extent, in the intestine. The expression of apoCIII is strongly regulated at the transcriptional level (57). It is well established that the plasma concentration and synthesis rate of apoCIII are positively correlated with plasma triglycerides, both in normal and hypertriglyceridemic subjects (58-60). In fact, apoCIII deficiency in humans results in increased catabolism of VLDL particles (61), whereas increased apoCIII synthesis is associated with hypertriglyceridemia (62). Overexpression of human apoCIII in mice results in severe hypertriglyceridemia (63), whereas disruption of the endogenous apoCIII gene protects the mice from postprandial hypertriglyceridemia (64). ApoCIII acts by delaying the catabolism of triglyceride-rich particles by several mechanisms (62, 65, 66), including inhibition of lipoprotein binding to the cell surface glycosaminoglycan matrix (61, 66) and lipolysis by lipoprotein lipase (LPL) (61).

Concomitantly with the decrement in apoCIII expression in liver, we found that the mRNA expression of muscle LPL was increased whereas that of adipose LPL was decreased in the GSPE treated rats. LPL plays a pivotal role in the metabolism of lipids and of lipoproteins. Major functions of LPL include the hydrolysis of TG-rich lipoproteins and the release of FFA, which are taken up and used for production of energy in peripheral tissues such as muscle, or are re-esterified into TG and stored in adipose tissue (23). These transcriptional changes found in the GSPE treated rats, strongly suggest the plasma TG utilization in these animals is directed preferentially to energy production by the muscle instead of to energy storage by the adipose tissue. Thus, these short term effects of GSPE on LPL expression could lead, on the long term, to a reduced rate of weight gain, as has been described for animals consuming flavonoids in the diet (27, 44).

The reduction of plasma LDL-C and nonHDL:nonLDL-C found in GSPE-treated animals, together with the increment in HDL-cholesterol, and the slight decrease in TC, points out to an increment in reverse cholesterol transport for its elimination as bile salts by the liver (67). In agreement with this view, we found a concomitant three fold increase in liver mRNA level of CYP7A1, the rate-limiting enzyme in bile acid synthesis, whose production is tightly controlled at the transcriptional level (68-70).

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Simultaneously, the GSPE treated rats showed increased expression of cholesterol biosynthetic enzymes in liver and this effect was not accompanied by an increase in cellular cholesterol levels. This could indicate that cholesterol synthesized de novo by the liver is being channeled to maintain the increased flux of bile acids pathway. Alternatively, it is possible that this increased expression does not result in a net cholesterol synthesis. It has been described that red wine polyphenols simultaneously decrease cholesterol content and increase the mRNA of HMG-CoA reductase in HepG2 cells and has been suggested that they may act as competitive HMG-CoA reductase inhibitors, in a similar way to statins (27).

Whereas total bile acid synthesis is expected to be elevated in the liver of GSPE treated animals due to the high activation of CYP7A1 expression, the slight reduction found in CYP8B1 expression, required for the synthesis of cholic acid, is expected to determine an increase in the chenodeoxycholate to cholate ratio in the bile acid pool (69). In addition, in murine, chenodeoxycholic acid is converted to muricholic acids that are more soluble and less cytotoxic (71). The hydrophilic-hydrophobic balance of bile acids modifies cholesterol absorption in the intestine, being cholic acid more efficient in facilitating absorption of cholesterol and muricholic the most powerful inhibitor (72). Therefore, intestinal cholesterol absorption could be reduced in GSPE treated animals. In addition, it has been described that the overexpression of CYP7A1 in transgenic mice reduces serum cholesterol and prevents atherosclerosis (73, 74).

Our microarray analysis has revealed SHP as a major target gene of procyanidin treatment in the liver. SHP is a promiscuous nuclear orphan receptor able to interact with, and modulate the transcriptional activity of, many other nuclear receptors, including, among others, peroxisome proliferator-activated receptors (PPAR) γ and α (33, 75), hepatocyte nuclear factor-4 (HNF-4) (76), α-fetoprotein/LRH-1 (77), retinoid X receptor (RXR) (76), and liver X receptor (LXR) (78), all of them involved in the control of lipid homeostasis. Therefore, SHP has the potential to influence a wide array of cellular processes, and has emerged as a key regulator of lipid metabolism. In humans, mutations in the SHP gene are associated with mild hyperinsulinemia and the development of insulin resistance and mild obesity (34, 79). Here, we found that GSPE treated rats displayed a lipid profile opposed to that associated to those pathologies,

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suggesting that the increased liver expression of SHP and the beneficial changes in the lipid profile triggered by procyanidins treatment could be functionally correlated.

Our results showed that the three-fold increase in liver SHP mRNA levels occurs concomitantly with a 50% reduction in plasma triglyceride levels in GSPE treated rats. Several studies have previously described a similar inverse correlation between SHP gene expression and plasma TG levels (32), although the mechanisms underlying this reciprocal relationship are not clear at present. Dietary chenodeoxycholic acid (CDCA) reduces plasma TG in hypertriglyceridemic humans (80, 81) and so does cholic acid (CA) in hyperlipidemic animals, where this effect is accompanied by the activation of SHP transcription (32). CA and CDCA acts as ligands of FXR, which then binds to the promoter of the SHP gene activating its transcription. SHP, in turn, binds to LRH1, an orphan nuclear receptor that regulates CYP7A1 expression positively, thereby inhibiting its activity (31). This is a well characterized mechanism of negative feedback regulation of bile acid synthesis by its end product (31, 70). The ability of CDCA to lower plasma TAG levels has been attributed, at least in part, to a direct stimulation of ApoCII gene transcription by CDCA-activated FXR (82). Since we have found that in GSPE treated rats the expression of both SHP and CYP7A1 is upregulated simultaneously, whilst ApoCII expression remains unchanged, our results could seem paradoxical at first glance. However, other authors have already described situations in which SHP expression is induced, whilst CYP7A1 expression is not repressed. This is the case for the induction of SHP expression by guggulsterone, another FXR ligand, that inhibits FXR activation by CDCA, induces SHP expression and fails to downregulate CYP7A1 transcription (83-85). Dietary guggulsterone, as GSPE, triggers a reduction of plasma TG, and increases HDL-C while decreasing LDL-C (84, 85). A similar situation is described for estrogens. These hormones induce the expression of the SHP in mouse, rat and HepG2 cells promoting the binding of estrogen receptor ERα to the ERE present in the SHP gene promoter, which overlaps with the FXR binding (86); the elevated SHP expression induced by ERα agonists does not result in an inhibition of CYP7A1 transcription (86). Again, estrogens are known to reduce LDL-C and increase HDL-C and some of them, such as 17β-estradiol, also lower total serum cholesterol and TG(87). Thus, it seems that the repression of CYP7A1 by SHP is only functional when bile acids

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act as FXR agonist. It has been recently shown that FXR controls gene expression in a ligand- and promoter-selective fashion (88).

Since activated transcription of SHP is under the control of FXR/RXR and ERα, it might be possible that procyanidins act as ligands of FXR, RXR or ERα. Ligands that target these nuclear receptors are emerging as potentially powerful therapeutic agents for treatment of diabetes, hypercholesterolemia, atherosclerosis and cancer (89-92).

In conclusion, a single, high and non-toxic dose of grape seed procyanidins, administered orally, drastically improved plasma lipidic profile in healthy, chow-fed rats in a postprandial situation. The expression of the key enzyme controlling bile acid synthesis, CYP7A1, was increased, suggesting an increased cholesterol elimination via bile acids. The upregulation of SHP expression in the liver could be fundamental in mediating the procyanidins actions by controlling the activity of other transcription factors involved in the maintenance of lipid homeostasis. If the observed improvement in lipemia induced by oral administration of procyanidins in rats were functional in humans, in which postprandial lipemia increases the risk of atherogenesis and coronary artery disease, the consumption of red wine associated with meals could be fundamental to explain the long-term beneficial effects described by the “French Paradox”.

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Table 1. Plasma lipids and fasted state markers analysis in control rats and Grape seed procyanidin extract (GSPE) - treated rats.

Rats were treated with an oral gavage of GSPE (250 mg/Kg body weight) and sacrificed after 5 h. Plasma lipids were analyzed as described in materials and methods. Results are shown as mean ± sem (n=6). *Significant difference ($P<0.05$) versus the control value using independent samples t-test.

	Control	GSPE
tryclyceride (mg/dL)	183 ± 18	92 ± 9 *
Total Cholesterol (mg/dL)	61 ± 5	54 ± 2
LDL cholesterol (mg/dL)	14 ± 2	8 ± 1 *
HDL cholesterol (mg/dL)	29 ± 1	32 ± 2
nonHDL:nonLDL cholesterol (mg/dL)	19.4 ± 2.8	12.8 ± 1.0 *
ratio HDL cholesterol/LDL cholesterol	1.9 ± 0.3	3.8 ± 0.2 *
Ratio total cholesterol/HDL cholesterol	2.1± 0.1	1.6 ± 0.04 *
Glucose (mg/dL)	130±4	132±3
Free fatty acid (mg/dL)	14±1	8±1 *
β-Hydroxybutirate (mg/dL)	2.2±0.4	2.6±0.6

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Table 2. Liver lipid analysis in control rats and Grape seed procyanidin extract (GSPE) treated rats. Rats were treated with an oral gavage of GSPE (250 mg/Kg body weight) and sacrificed after 5 h. Liver lipids were analyzed as described in materials and methods after chloroform/methanol extraction. Results are shown as mean ± sem (n=6). No statistical differences were found between control rats and GSPE treated rats at the $P<0.05$ level.

	Control	**GSPE**
triglyceride (mg/g tissue)	8.8 ± 0.5	9.4 ± 0.6
Total Cholesterol (mg/g tissue)	6.2 ± 0.4	6.3 ± 0.4
Free Cholesterol (mg/g tissue)	2.5 ± 0.4	2.5 ± 0.2
Esterified Cholesterol (mg/g tissue)	3.8 ± 0.3	3.7 ± 0.4

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Table 3. Changes in mRNA levels of lipid related genes in liver of rats treated with grape seed procyanidin extract (GSPE) versus control rats.

Rats were treated with an oral gavage of GSPE (250 mg/Kg body weight) and sacrificed after 5 h. Equal amounts of liver RNA from three animals were pooled and used to hybridize against Agilent Rat Oligo Microarrays (Part Number G4130A). Fold-change represents the mean of duplicate hybridizations with dye-swap labeling. Real time quantitative PCR (RT-q-PCR) of SHP and HMG-CoA reductase genes were performed to confirm microarray data (shown in bold characters). CYP7A1 mRNA fold-change was determined by RT-q-PCR (shown in italic characters) since its probe was absent from the microarray.

Genbank ID		Mean fold-change	Standard error mean
	Cholesterol synthesis pathway key regulators		
BM392175	3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase)	1.30 **1.50**	0,13
NM_017268	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (Hmgcs1)	1.46	0,20
NM_017136	Squalene epoxidase (Sqle)	1.44	0,07
NM_080886	Sterol-C4-methyl oxidase-like (Sc4mol)	1.54	0,04
	Fatty acid synthesis		
NM_017332	Fatty acid synthase (Fasn)	1.08	0,00
NM_053922	Acetyl-Coenzyme A carboxylase beta (Acacb)	1.00	0,01
NM_022193	Acetyl-coenzyme A carboxylase (Acac)	1.00	0,04
	Fatty acid beta-oxidation key regulator		
NM_012930	Carnitine palmitoyltransferase 2 (Cpt2)	1.00	0,02
NM_031559	Carnitine palmitoyltransferase 1 (Cpt1a)	1.00	0,08
	Cholesterol esther synthesis		
BF542749	Acyl-coenzyme A:cholesterol acyltransferase (ACACT)	1.00	0,01
NM_031118	Acyl-coenzyme A:cholesterol acyltransferase (Soat1)	1.00	0,00
	Apolipoproteins		

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NM_012738	Apolipoprotein A-I	1.11	0,06
	Apolipoprotein C-I	0.80	0,18
NM_013112	Apolipoprotein A-II	0.67	0,04
NM_012501	Apolipoprotein C-III	0.81	0,08
BM385272	Apolipoprotein C-II	1.00	0,11
NM_080576	Apolipoprotein A-V	1.00	0,01
NM_138828	Apolipoprotein E.	1.11	0,23
NM_019373	Apolipoprotein M	1.00	0,06
NM_012737	Apolipoprotein A-IV	1.10	0,15
NM_012777	Apolipoprotein D (Apod) (CETP)	1.15	0,08
CB547563	Apolipoprotein B precursor; apoB-100; apoB-48	1.10	0,16
	Lipoprotein related proteins		
NM_017024	Lecithin-cholesterol acyltransferase (Lcat)	1.00	0,22
CB547807	Microsomal triglyceride transfer protein (mtp)	1.16	0,15
BF553164	Apolipoprotein B mRNA editing enzyme complex-1 (apobec-1)	1.00	0,10
BF285350	Apolipoprotein B mRNA editing enzyme complex-2 (apobec-2)	0.90	0,09
NM_012907	Apolipoprotein B editing protein (Apobec1)	1.00	0,03
NM_133400	Apobec-1 complementation factor. APOBEC-1 stimulating protein (Acf)	1.17	0,22
BE329208	SREBP cleavage activating protein (SCAP)	1.01	0,22
	Lipoprotein receptors		
NM_133306	Oxidised low density lipoprotein (lectin-like) receptor 1	1.00	0,05
CB606186 CB606214	Low density lipoprotein receptor related protein	1.00	0,08
CB546853	Low density lipoprotein receptor-related protein 8. apolipoprotein E receptor	1.00	0,06
NM_053541	Low density lipoprotein receptor-related protein 3	1.00	0,11
NM_013155	Very low density lipoprotein receptor	1.00	0,01
BF548789	Low density lipoprotein receptor-related protein 6	1.10	0,17
NM_031541	CD36 antigen (collagen type I receptor, thrombospondin receptor)-like 1 (scavanger receptor class B type 1) (Cd36l1)	1.00	0,15
	Bile Acid related genes		
NM_012942	Cyp7A1	**2.4**	
NM_031241	Cyp8B1	0.8	0,03
NM_053763	Cyp27A	1.0	0,06

TABLE 3 (Continued from previous page)

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	Selected lipid related Nuclear transcription factors		
NM_012493	Alpha-fetoprotein (AFP)	0.97	0,10
NM_012669	Hepatocyte nuclear factor 1 (HNF1)	1.00	0,01
AF329936.1	Hepatocyte nuclear factor 3 alpha (HNF3a)	0.95	0,07
NM_012742	Hepatocyte nuclear factor 4 alpha (HNF4alpha)	1.07	0,08
NM_057133	Nuclear receptor subfamily 0, group B, member 2 (Nr0b2). Small Heterodimer Partner (SHP)	2.45 **3.0**	0,70
NM_052980	Nuclear receptor subfamily 1, group 1, member 2 (Nr1i2) (PXR)	1.00	0,06
NM_052980	Nuclear receptor subfamily 1, group H, member 2 (Nr1h2). Liver X Receptor beta (LXRbeta)	1.08	0,04
NM_031627	Nuclear receptor subfamily 1, group H, member 3 (Nr1h3) Liver X receptor alpha (LXRalpha)	0.94	0,04
NM_021745	Nuclear receptor subfamily 1, group H, member 4 (Nr1h4). Farnesoid X Receptor (FXR)	0.98	0,19
NM_080778	Nuclear receptor subfamily 2, group F, member 2 (Nr2F2) (ARP-1)	0.84	0,01
NM_021742	Nuclear receptor subfamily 5, group A, member 2 (Nr5a2) (LRH-1)	0.98	0,01
NM_013196	Peroxisome proliferator activated receptor alpha (PPARalpha)	1.00	0,33
NM_012805	Retinoid X receptor alpha (RXRalpha)	1.00	0,11
AF016387.1	Retinoid X receptor gamma (RXRgamma)	1.00	0,10
AW916150	Sterol regulatory element binding protein-2 (SREBP-2)	0.97	0,01

TABLE 3 (continued from previous page)

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FIGURE LEGENDS

Figure 1. Total cholesterol distribution among different lipoprotein fractions.
Experimental procedure was the same as indicated in table 1. Results are shown as percentage of total cholesterol in HDL, LDL and nonHDL:nonLDL lipoproteins. Results are shown as mean ± sem (n=6). * Significant difference (P<0.05) versus the control value using independent samples t-test.

Figure2. Plasma total apolipoprotein B, apolipoprotein B-100 and apolipoprotein B-48 in control rats and grape seed procyanidin extract (GSPE)-treated rats.
(a) Representative ApoB-48 and ApoB-100 inmunoblotting of a control (lanes 1 and 2), GSPE (lanes 4 and 5) plasma samples and apoB-100 standard (lane 3). Rats were treated with an oral gavage of GSPE (250 mg/Kg body weight) and sacrificed after 5 h. Plasma samples (120 μg of protein) and apoB-100 standard (0.2 μg) were subjected to SDS-PAGE and transferred onto PVDF membrane for inmunoblotting with goat antiapoB antibody. The antibody used with this purpose was raised against the amino terminus of apoB so both isoforms, apoB-100 and apoB-48, could be detected and quantified simultaneously in a single plasma sample. (b) Amounts of plasma total-ApoB, ApoB-48 and ApoB-100 in control group and GSPE treated group. ApoB-100 standard was used to quantify the relative ammount of apoB-100 and apoB-48 using Quantity One software from Bio-Rad. Data are expressed as mean ± sem (n=6). * Significant difference at the P< 0.05 level versus the control value.

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Figure 1

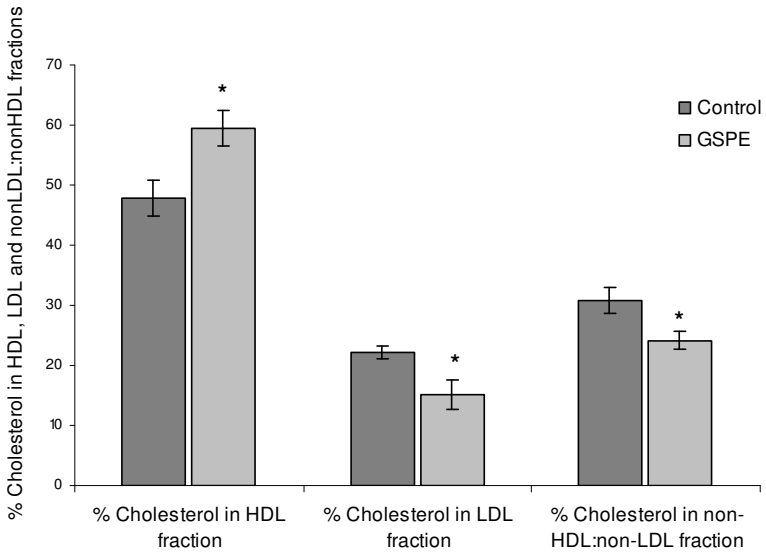

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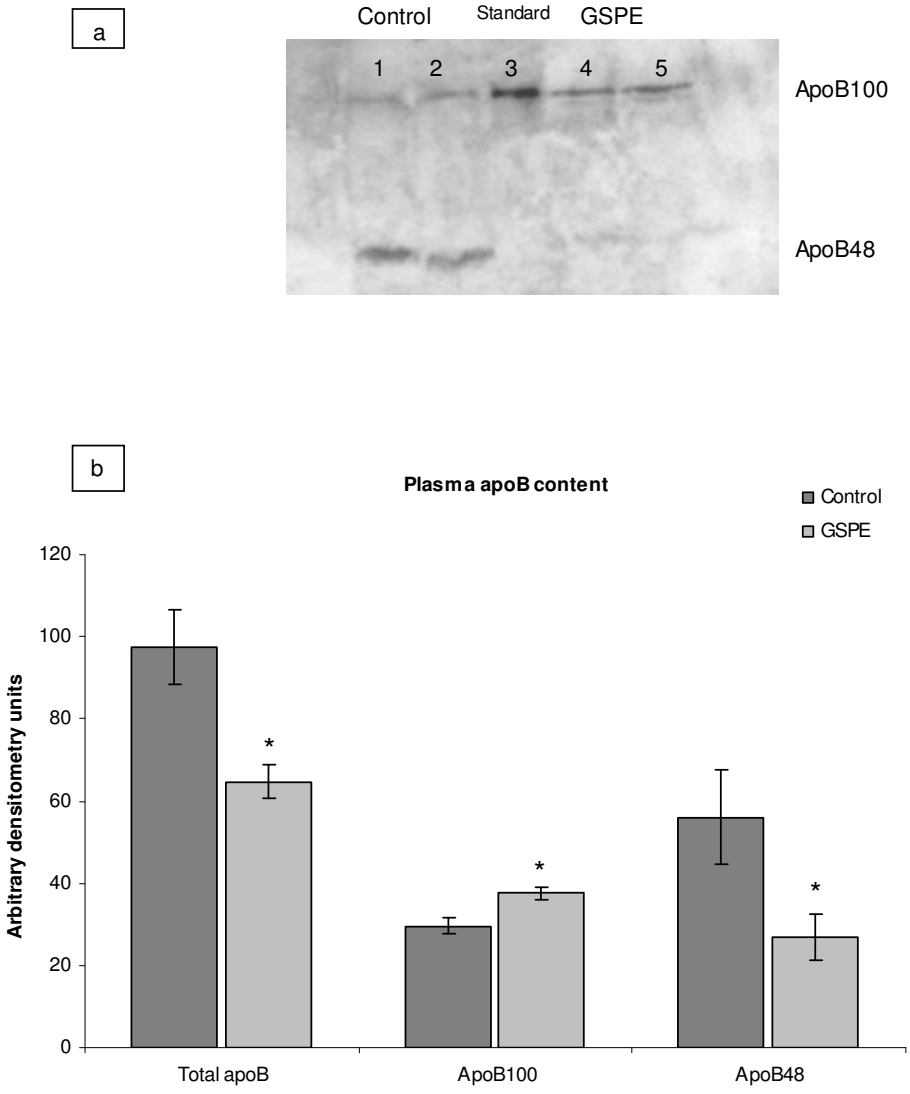
Figure 2



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2. Dietary Procyanidins Elicit An Anti-inflammatory Gene Expression Profile In Liver Of Rats In The Postprandial Phase

Submitted

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DIETARY PROCYANIDINS ELICIT AN ANTI-INFLAMMATORY GENE EXPRESSION PROFILE IN LIVER OF RATS IN THE POSTPRANDIAL PHASE.

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Key words:
Procyanidins, flavonoids, rat, liver, gene expression profile, postprandial, inflammation, SHP, Foxa2, Egr1, Nfkbia, MAPK pathways.

Running title:
procyanidin target genes in the postprandial liver

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ABSTRACT

Numerous studies in humans and animals have demonstrated that procyanidin-rich diets reduce the risk of cardiovascular diseases, diabetes, atherosclerosis, obesity, hypertension, cancer and neurodegenerative diseases. These beneficial effects have been attributed to the well-known antioxidant activity of procyanidins and to their ability to act as signalling agents, eventually modulating gene expression at the transcriptional level. In this work, we have performed microarray analysis to characterize the global gene expression profile associated to oral intake of procyanidins in the liver of healthy rats at the postprandial phase, in order to gain insight into the primary mechanisms that underlie the long-term antiatherogenic and cardioprotector effects ascribed to procyanidins. Procyanidin target genes in the liver included transcriptional regulators, and components of signal transduction pathways as well as genes involved in glucose and lipid metabolism, detoxification, apoptosis and the inflammatory response. Remarkably, procyanidin treatment upregulates the expression of nuclear receptor *SHP* and downregulates *Foxa2*, both of which are key regulators of glucose and lipid metabolism in the liver. Also, the expression of transcription factor *Egr1*, a mediator of the hepatic inflammatory response, and several genes which encode acute-phase proteins – *haptoglobin*, *fibrinogen B* and *alpha-1-antitrypsin* – are repressed by procyanidins. In addition, expression of *Dusp6*, a component of the MAPK/ERK1/2 signal cascade, is repressed by procyanidins, and *Nfkbia*, a repressor of NF-kB activity, is overexpressed. The postprandial state is a proinflammatory and proatherogenic phase due to transiently increased plasma levels of glucose, triglycerides and proinflammatory cytokines and we have previously shown that this same procyanidin treatment triggers a drastic reduction of postprandial plasma triglycerides [1]. Taken together, the changes in liver gene expression profile triggered by procyanidins suggest that these polyphenols attenuate the hepatic inflammatory response associated to the postprandial state by attenuating IL-6 and NF-kB signalling pathways, and exert an hipotrygliceridemic effect by inducing *SHP* expression and downregulating *Foxa2*. Therefore, consumption of procyanidin-rich foods should help to ameliorate inflammatory and atherogenic processes associated to the postprandial state and in the long term, prevent chronic metabolic disorders associated to inflammation and atherogenesis.

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INTRODUCTION

Procyanidins (PC) are polyphenols of flavonoid type that comprise the oligomeric forms of monomeric catechins, (+)-catechin, (-)-epichatechin, and their glycosylated and gallated derivatives. They are fairly abundant in numerous aliments and drinks of plant origin such as grapes, cocoa, different berries, apples, nuts, red wine, chocolate and tea, and are considered bioactive micronutrients that form an integral part of human diet [2-5]. Also, PC-rich extracts from different sources, such as grape seeds, pomegranate and pine bark, are commercialized as food additives and nutritional supplements [6, 7]. The interest of PC in nutrition arises from the demonstrated and potential benefits of regular consumption of flavonoids for human health [3, 8, 9]. Thus, numerous studies in humans and animal have demonstrated that PC-rich diets reduce the risk of cardiovascular diseases, diabetes, atherosclerosis, obesity, hypertension, [5, 6, 10-14], cancer [15, 16] and neurodegenerative diseases [17, 18].

The beneficial effects of PC have been largely attributed to their well-known antioxidant (and associated anti-inflammatory) activity [6, 16, 18-20]. Yet, the bioactivity of PC is not limited to their direct antioxidant actions. Flavonoids have been shown to interact with specific plasma membrane receptors, cytoplasmic signal transduction factors and nuclear receptors, serving themselves as signaling agents and eventually modulating gene expression at the transcriptional level [12, 14, 21, 22].

The postprandial state is a proinflammatory and proatherogenic phase, due to the transient increased levels of glucose, triglycerides (TG) and proinflammatory cytokines. Postprandial glycemia and trygliceridemia are better predictors of mortality from CVD than fasting parameters alone [23-25]. We have previously shown [1] that oral administration of a grape seed procyanidin extract (GSPE) to healthy rats lowers plasma TG levels to 50% in the postprandial state. Hence, in the present study, we have chosen the postprandial conditions to characterize liver gene expression profile associated to GSPE consumption. Monitorization of global transcriptional changes is a simple way to asses the bioefficacy of micronutrients and to identify early target genes of the tested substances [26, 27], and should pave the way to the characterization of the primary mechanisms that underlie the long-term antiatherogenic and cardioprotector effects ascribed to PC. Here we show that the changes in liver gene expression profile triggered by PC could explain, at least in part, their previously described hypotriglyceridemic effects, via induction of *SHP* expression and downregulation of

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Foxa2. In addition, the transcriptional changes elicited by procyanidins in the liver suggest that these polyphenols attenuate the hepatic inflammatory response associated to the postprandial state by attenuating IL-6 and NF-kB signalling pathways.

MATERIALS AND METHODS

Chemicals. Grape seed procyanidin extracts (GSPE) were kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract contained essentially monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13,3%) and oligomeric (5-13 units) (31,7%) procyanidins.

Animals. Male Wistar rats, 2 months old and weighing 250 g, were purchased from Charles River (Barcelona, Spain). The Animal Ethics Committee of University Rovira i Virgili approved all procedures. The animals were housed in animal quarters at 22°C with a 12 h light/dark cycle (light from 8 h a.m. to 8 p.m.) and were fed *ad libitum* with chow (Panlab A) and water. At 11 a.m. on experimental day, rats (6 animals per group) were fed either an oral gavage of GSPE in aqueous solution (250 mg/kg body wt.; GSPE group) or, either, an oral gavage of vehicle (tap water; Control group). The procyanidin dose used in this work is one-fifth of the no-observed-adverse-effect level (NOAEL) described for GSPE and male rats [28], and we have previously shown that this dose is effective in reducing glycemia in streptozotocin-induced diabetic rats [29] and triglycerides in normolipidemic rats [1]. Five hours after treatment, the rats were sacrificed and liver was excised, immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

RNA methods. Total RNA was purified from each frozen liver by using the NucleoSpinR RNA II kit (Macherey-Nagel, Düren, Germany) and following the instructions of the manufacturers. Equal aliquots of total RNA from six rat livers in each group were pooled and used for oligonucleotide array hybridization and quantitative PCR analysis. Integrity of pooled RNA was assessed by using the Agilent 2100 Bioanalyzer and the RNA 6000 LabChipR. For microarray hybridization, Cy3- or Cy5-labeled cRNA was obtained from each RNA pool by using the Agilent Low RNA Input Fluorescent Linear Amplification Kit as described in the Agilent manual. Fluorescent probes containing 500 ng of each labeled cRNA were pooled and hybridized against Agilent Rat Oligo Microarray following the Agilent 60-mer oligo microarray processing

protocol. Images of hybridized microarrays were acquired with the Agilent G2565BA scanner, and data from the microarray images were obtained and analyzed with the Agilent Feature Extraction software. For each pair of RNA samples being compared, duplicate hybridizations with a dye-swap labelling were performed.

Changes in mRNA expression of selected genes were verified by quantitative PCR. Complementary DNA corresponding to each RNA pool was generated using TaqMan Reverse Transcription Reagents (Applied Biosystem), and quantitative PCR amplification and detection were performed by using specific TaqMan Assay-On-Demand probes (Applied Biosystems, Rn00589173_m1 for *SHP*, Rn00565598_m1 for *3-hydroxy-3-methylglutaryl-CoA reductase*, Rn00565467_m1 for *Glucokinase regulatory protein*, Rn00565347_m1 for *Glucose-6-phosphatase*), the TaqMan PCR Core Reagent Kit, and the GeneAmpR 5700 Sequence Detection System, as recommended by the manufacturer. Quadruplicated quantifications, performed in singleplex assays, were performed for each gene in each cDNA pool. *Actine B* was used as the reference gene in quantitative PCR (Applied Biosystems TaqMan Assay-On-Demand probe Rn00667869_m1).

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RESULTS

Global gene expression changes induced by oral administration of GSPE in the liver of healthy rats in the postprandial phase were monitored by differential cRNA hybridization of oligonucleotide microarrays containing probes specific for about 20000 genes and ESTs. Table 1 displays those genes of known function which were over- or under-expressed by more than 30% in GSPE treated animals compared to controls. The genes have been grouped according to their molecular functions or the biological processes in which they participate: nuclear receptors, transcription factors, components of signalling pathways, metabolic pathways, detoxification processes, apoptosis and inflammatory response. The molecular function and biological process ascribed to each gene in this section were obtained from the *Protein* and *Gene* Databases of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/).

Procyanidin intake modulated the expression of nine genes encoding for factors that directly control the transcription of other genes. Thus, GSPE triggered a 3-fold overexpression of one nuclear receptor, namely *Small Heterodimer Partner,* which is a key controller of lipid homeostasis in the liver. The mRNA levels of five transcription factors were also altered in the liver of GSPE-treated animals: *zinc finger protein 354A* (involved in development in kidney) and *forkhead box E1* (a negative controller of thyroid-specific gene expression) were overexpressed, whereas *forkhead box A2* (whose target genes are involved in glucose and lipid metabolisms), *early growth response 1* (which triggers the expression of numerous inflammatory mediators) and *hematopoietically expressed homeobox* (essential for liver development) were downregulated. Likewise, three genes encoding components of different signal transduction pathways were modified by GSPE: *nuclear factor of kappa light chain gene enhancer in B-cells inhibitor alpha* (an inhibitor of NF-kB activity) was upregulated, whereas *regulator of G-protein signalling 3* (that inhibits G-protein coupled receptor signalling) and *dual specificity phosphatase 6* (a protein tyrosine phosphatase which regulates the activity of mitogen-activated protein kinases) were repressed.

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In addition, liver expression of numerous genes related to glucose, lipid, amino acid and energetic metabolism was differentially affected by GSPE treatment. mRNA levels of *ATPase inhibitory factor 1* (inhibitor of F1F0-ATP synthase) were reduced, whereas those of *ion transport regulator 7* (regulator of Na-K-ATPase activity) were increased. Related to glucose metabolism, the expression of *glucose-6-phosphatase* (a gluconeogenic enzyme) was increased, whereas *glucokinase regulatory protein* (an inhibitor of glucokinase activity), and *solute carrier family 2, member 5* (a transporter of fructose and glucose also known as *GLUT5*) were repressed. Related to amino acid metabolism, GSPE induced the over-expression of *tyrosine aminotransferase* (involved in tyrosine catabolism and in gluconeogenesis), *short-branched chain acyl-CoA dehydrogenase* (implied in the catabolism of branched chain amino acids), *ornithine aminotransferase* (involved in arginine and ornitine catabolism) and *5-aminolevulinate synthase* (that catalyzes the first step of heme biosynthesis and is involved in the catabolism of serine and glycine). On the other hand, *cysteine sulfinic acid decarboxylase* (involved in the conversion of cysteine to taurine), *glycine methyltransferase* and *betaine-homocysteine methyltransferase* were under-expressed in the liver of rats treated with GSPE. These last two enzymes are involved in the metabolism of sulfur-containing amino acids, serine and glycine, and in the generation of S-adenosyl-methionine, the universal donor of methyl groups in for methylation reactions of DNA and proteins. mRNA levels of *UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase*, the key enzyme of sialic acid biosynthesis, were also more abundant in the liver of GSPE-treated than in control animals.

Related to lipid metabolism, three genes encoding enzymes of the cholesterol biosynthesis pathway became overexpressed in liver upon GSPE administration: *sterol-C4-methyl oxidase*, *3-hydroxy-3-methylglutaryl-CoA synthetase* and *squalene epoxidase*. On the contrary, *phytanoyl-CoA hydroxylase* and *acyl-CoA oxidase 2, branched chain*, involved in the peroxysomal oxidation of fatty acids, became underexpressed, and so it was *apolipoprotein A-II*, a component of High Density Lipoproteins.

Administration of GSPE also modulated the expression of several genes related to detoxification processes. *Glutathione-S-transferase, alpha type* (involved in cellular detoxification by catalyzing the conjugation of glutathione with a wide range of endogenous and xenobiotic alkylating agents) and *selenium binding protein 2* (probably

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involved in mediating anti-carcinogenic effects of selenium) were upregulated by GSPE treatment. Five genes coding for *cytochrome P450* (*CYP*) enzymes, and corresponding to families *CYP3* (involved in xenobiotic and steroid metabolism) and *CYP4* (implicated in fatty acid hydroxylation) were downregulated by GSPE: *CYP3A2* (catalyzes the conversion of testosterone to 6-beta-hydroxytestosterone), *CYP3A3* (catalyzes the alpha-hydroxylation of fatty acids, hydroxylation of melatonin and mediates the detoxification of diverse xenobiotics), *CYP3A9* (catalyzes the hydroxylation of progesterone), *CYP4A3* (oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics) and *CYP4A10* (catalyzes the omega-hydroxylation of fatty acids). Also, the genes of two sulfotransferase enzymes, which catalyze the sulfate conjugation of many hormones, neurotransmitters, drugs, and xenobiotic compounds, were under-expressed in the livers of the GSPE group: *sulfotransferase family 1A, phenol-preferring, member 1* and *dopa/tyrosine sulfotransferase* (catalyzes the transfer of sulfate groups onto various tyrosine and 3,4-dihydroxyphenylalanine isomers).

Finally, GSPE administration also modulated the expression of several genes related to the immune system and the inflammatory response in the liver. Of those, only *RT1 class Ib, locus Aw2* (a class Ib gene of the rat major histocompatibility complex, involved in the presentation of foreign antigens to the immune system) was upregulated by GSPE treatment. Four genes coding for acute-phase proteins were downregulated in the liver by GSPE: *retinol-binding protein*, *haptoglobin*, *fibrinogen B* and *alpha-1-antitrypsin*. Genes for *metallothionein 1* and *2* (metal chelating proteins involved in zinc and copper homeostasis and in detoxification of heavy metals and reactive oxygen species) where also strongly downregulated in the liver of GSPE treated animals. Also, GSPE repressed the expression of *secretory leukocyte protease iInhibitor* (suppresses the inflammation and joint damage caused by bacterial cell wall-induced arthritis), *High Mobility Group Box 1* (which is both a chromatin-binding factor, and a secreted protein that mediates inflammation), *cadherin 17* (a calcium dependent cell adhesion protein), *B-cell translocation gene 1* (which encodes for an anti-proliferative protein) and *ubiquitin D* (whose human homolog is a diubiquitin protein that may function in antigen processing and presentation).

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DISCUSSION

Numerous studies have demonstrated the beneficial long-term effects of regular intake of flavonoid-rich foods on oxidative stress, inflammation, lipid and glucose metabolic disorders, which are the common backstage of diabetes, atherosclerosis and cardiovascular diseases, with great incidence in Western societies [12, 30-32].

We have previously demonstrated that a single, high non-toxic [28] oral dose of procyanidins significantly reduces trigly and apoB levels in plasma of healthy rats [1] and glycaemia in streptozotozin-induced diabetic rats [29], 5 hours after gavage. This study was intended to identify the primary target genes of procyanidins on liver, in order to gain insight into the molecular mechanisms that underlie their metabolic effects. In addition, genes whose expression level is affected by procyanidin intake have the potential to be used as a biomarker for assessing physiological functions of phytochemicals [27, 33]. Here, we show that oral intake of procyanidins significantly affects liver expression of nuclear receptors, transcription factors, components of signalling pathways and genes involved in metabolism, detoxification, apoptosis and inflammatory response. Importantly, oral intake of procyanidins significantly affects liver expression of transcription regulatory factors that control lipid and glucose metabolisms – namely *SHP* and *Foxa2* –, and inflammatory processes - *Egr1*. Procyanidin treatment also modifies the expression of components of the MAPK and the NF-kB signal transduction pathways. Changes in the expression of these genes, which directly control the expression of other genes, could be key events mediating the metabolic modifications that, in turn, result in the physiological long-term effects ascribed to procyanidins.

Expression of SHP - an orphan nuclear receptor, which acts as a transcriptional co repressor controlling steroidogenesis, lipogenesis, cholesterol and bile acid metabolism, glucose homeostasis and xenobiotic metabolism in the liver [34, 35]- is strongly up regulated by procyanidins. It is known that bile acids lower triglyceride levels and apoB secretion in hepatic cells via the upregulation of expression of SHP [36, 37], and we have previously described the putative implication of SHP in the hypotrygliceridemic effect of GSPE in a postprandial situation [1].

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This work also reveals *Foxa2* as another important procyanidin target gene, which is repressed by GSPE treatment. In the liver, some Foxa2 target genes are involved in glucose and lipid homeostasis, particularly in response to fasting [38-40]. Foxa2 is a key regulator of insulin sensitivity [41] being phosphorylated in an Akt phosphorylation site in response to insulin signaling. This phosphorylation results in inhibition of its transcriptional activity by nuclear exclusion [38, 42]. In the starved state, Foxa2 is translocated to the nucleus where activates the transcription of multiple genes, driving increased hepatic glucose production, fatty acid oxidation, and ketogenesis [38, 42]. Foxa2 under-expression in liver of GSPE treated animals could be involved in the hypotriglyceridemic effect of procyanidins [1], since activation of Foxa2 in the liver leads to increased VLDL secretion through induced expression of *microsomal triglyceride transfer protein* (MTP) [43]. The effects of decreased *Foxa2* expression upon VLDL secretion in the liver of GSPE treated rats could be magnified by the associated increase of SHP expression triggered by procyanidins, because SHP inhibits the transcriptional activity of Foxa2 [44]. Regarding glucose metabolism, rats treated with GSPE showed a simultaneous increase of *glucose-6-phosphatase* expression and a decrease of *glucokinase regulatory protein* (an inhibitor of glucokinase activity). These changes in gene expression, together with the normoglucemia observed in GSPE treated rats [1], suggest the existence of a futile cycle between glucose-6-phosphate and glucose. Also, GSPE treatment increased the expression of some genes related to cholesterol biosynthesis. Despite this over-expression, rats treated with GSPE did not show elevated plasma cholesterol levels in even display an improved atherosclerotic index, probably due to the concomitant increase in the synthesis of bile acids, as suggested by the increased expression of Cyp7A1, the rate limiting enzyme in bile acid biosynthesis [1]. Besides genes implied in lipid in glucose metabolism, Foxa2 is a positive transcriptional regulator of many other genes, including the transcription factor *Hhex* [45], *5-aminolevulinate synthase* [46], and *fibrinogen B beta chain* [47], and all of them are repressed by GSPE concomitantly with down-regulation of Foxa2.

The *early growth response 1* (*Egr1*) transcription factor has been associated with a broad range of biological functions such as cell proliferation [48], apoptosis [49], and differentiation [50] in a cell-type-dependent manner. *Egr1* is rapidly induced by many stimuli, including insulin, growth factors, cytokines, and a variety of cellular stresses [51, 52] and may function as a master switch to trigger the expression of numerous

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mediators of the inflammatory response [52]. Oral administration of GSPE, repressed the expression of *Egr1*. Repression of *Egr1* expression by (-)-epigallocatechin gallate [53] and resveratrol [54] have been already described. Down regulation of *Egr1* by GSPE occurs concomitantly with the repression of several genes encoding acute phase proteins: *retinol-binding protein*, *haptoglobin*, *fibrinogen B* and *alpha-1-antitrypsin*. Acute phase protein genes have been divided into two classes according to their response to different cytokines [55]: class 1 genes, which include *haptoglobin*, are induced by IL-1 and TNFalpha, either alone or in combination with IL-6; class 2 genes, which include *fibrinogen*, respond to IL-6 alone. Thus, it seems that GSPE blocked IL-6 signalling pathways. Reinforcing this suggestion, we found that expression of *secretory leukocyte protease inhibitor* (*Slpi*) is repressed by GSPE treatment. Expression of this gene is inducible by IL-6, but not by TNF and IL-1beta [56]. The ATPase inhibitor, whose expression was repressed by GSPE, is over-expressed in inflammated tissues, where it is involved as regulator of inflammatory processes [57] . Also, metallothionein 1 and 2 expression, wich are upregulated by pro-inflammatory stimuli [58] are strongly repressed by GSPE. Remarkably, Foxa2 is also upregulated by IL-6 [59]. Also, a Foxa2 binding site in the promoter of fibrinogen B beta chain is essential for a full response of this promoter to IL-6 [47]. Taken together, the changes in the expression pattern of inflammation-related genes triggered by GSPE strongly suggest an anti-inflammatory effect of procyanidins that is partly mediated by blocking IL-6 signalling pathways.

IL-6 exert its action via the signal transducers gp130 leading to the activation of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) and MAPK (mitogen-activated protein kinase) cascades: ERK1/2, p38 and JNK [60]. Both pathways lead to the activation of transcription factors: STAT members -activated directly by JAK kinases- and C/EBP members - activated through the MAPK pathways [60]. Foxa2 promoter contains a binding site for C/EBP proteins that mediates the induction of Foxa2 expression by cytokines Il-1 and IL-6 [59]. Also, activation of MAPK transduction pathways is essential for induced expression Egr1 by various stimuli [51] Thus, without discarding the involvement of the JAK/STAT pathway, our results suggest that procyanidins block the MAPK pathways.

The Egr-1 expression is regulated, among others factors [61], by Elk-1, which is activated via ERK and JNK subfamilies of MAPKs [62]. The flavonoid (-)-

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epigallocatechin gallate suppresses Egr1 gene expression by blocking the ERK signaling pathway [53]. In this work, we have found that repression of Egr-1 expression by GSPE is simultaneous with down-regulation of DUSP6, a MAPK phosphatase (MKPs) specific for ERKs [63]. It is also known that transcription of several MKPs, including DUSP6, is induced by the same MAPK signaling pathway they regulate, thus forming a negative feedback loop that attenuate the MAPK signal [64]. All together, the available data suggest that procyanidins interfere the ERK1/2 signalling pathway and this results in a modulation of transcription of several ERK1/2 target genes in the liver.

Previous results in our group showed insulin-like effects of GSPE in insulin-sensitive cell lines, involving PI3K and p38 MAPK [29]. It has been described an attenuation of the IL-6 mediated stimulation of acute-phase-protein synthesis by insulin activation of the PI3K/Atk signalling [65, 66]. Hence, the observed anti-inflammatory effect of GSPE might result from the insulin-mimetic actions of procyanidins and/or from a direct inhibition of IL-6 signalling pathways.
Remarkably, GSPE induced the expression of Nfkbia, which encodes IkappaB-alpha, an inhibitor of the NfkappaB cascade [67, 68]. Among other functions, NF-kB controls the expression of genes encoding pro-inflammatory inducible enzymes and cytokines (including IL-6) as well as acute phase proteins, all of which play critical roles in controlling most inflammatory processes [67, 68]. Nfkbia traps NF-kB in the cytoplasm and exposure of cells to oxidative and pro-inflammatory stimuli causes activation of a series of upstream kinases such as MAPKs, IKK, PKC, and PI3K which then phosphorilate Nfkbia targeting it to ubiquiting-mediated degradation. Free activated Nf-kB translocates to the nucleus, where it binds to kB sequences located in the promoters of target genes [67, 68]. Several polyphenols, such as resveratrol, epigallocatechin gallate, procyanidins and quercetin, have been shown to exert anti-inflammatory effect by inhibiting NF-kB activation at different levels [69, 70]. Many acute phase genes have both C/EBP as well as NF-kB binding sites in their promoters, suggesting that C/EBP proteins and NF-kB cooperatively regulate the acute phase response [71].

Inflammation and metabolic regulation are highly integrated and improper integration can lead to a cluster of chronic metabolic disorders, including obesity, type 2 diabetes and cardiovascular diseases [72, 73]. NF-kB and IL-6 signalling pathways have

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emerged as important therapeutic target for drugs to treat many inflammation-related diseases [70, 74]. Our results reveal procyanidins as potential modulators of inflammation, attenuating IL-6 and NF-kB pathways, and of lipid and glucose metabolisms, increasing SHP expression and downregulating Foxa2. These effects are especially relevant considering that they have been found in the postprandial state, which is a proinflammatory and proatherogenic phase, due to the transient increased levels of glucose, TG and proinflammatory cytokines [23-25]. Humans are in the postprandial condition most of the time in Western societies and thus, consumption of procyanidin-rich foods should help to prevent and ameliorate chronic metabolic disorders associated to inflammatory and atherogenic processes.

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Table 1. **Genes whose expression in liver is modulated by oral intake of grape sed procyanidin extract in the postprandial phase.**

GenBank ID	Full Name	Symbol	Mean fold-change
	Nuclear Receptors		
NM_057133	nuclear receptor subfamily 0. group B, member 2 (Small Heterodimer Partner)	Nr0b2 (SHP)	2.45 **(3.08)**
	Transcription Factors		
NM_052798	zinc finger protein 354A	Zfp354a	1,48
NM_138909	forkhead box E1 (thyroid transcription factor 2)	Foxe1	1,38
NM_012743	forkhead box A2 (hepatocyte nuclear factor 3, beta)	Foxa2	-1,35
NM_012551	early growth response 1	Egr1	-1,79
NM_024385	hematopoietically expressed homeobox	Hhex	-2,98
	Signalling Pathways Components		
X63594.1	nuclear factor of kappa light chain gene enhancer in B-cells inhibitor, alpha	Nfkbia	1,31
NM_019340	regulator of G-protein signalling 3	Rgs3	-1,85
NM_053883	dual specificity phosphatase 6	Dusp6	-2,18
	Metabolism		
	Energy		
NM_022008	ion transport regulator 7	Fxyd7	1,31
NM_012915	ATPase inhibitory factor 1	Atpif1	-1,67
	Glucose		
NM_013098	glucose-6-phosphatase	G6pc	1,91 **(2.78)**
NM_013120	glucokinase regulatory protein	Gckr	-1,41 **(-1.52)**
NM_031741	solute carrier family 2, member 5	Slc2a5	-1,43
	Lipids		
NM_080886	sterol-C4-methyl oxidase	Sc4mol	1.52
NM_017268	3-hydroxy-3-methylglutaryl-CoA synthetase	Hmgcs1	1,47 **(1.50)**
NM_017136	squalene epoxidase	Sqle	1,35
AI029057	phytanoil-CoA-hydroxylase	Phyh	-1,32
NM_145770	acyl-CoA oxidase 2, branched chain	Acox2	-1,34
NM_013112	apolipoprotein A-II	Apoa2	-1,5
	Amino Acids		
NM_012668	tyrosine aminotransferase	Tat	1,63
U64451	short branched chain acyl-Coa dehydrogenase	Acadsb	1,5
NM_022521	ornithine aminotransferase	Oat	1,39
NM_024484	aminolevulinic acid synthase 1	Alas-1	1,3
NM_021750	cysteine-sulfinate decarboxylase	Csad	-1,45
NM_017084	glycine methyltransferase	Gnmt	-1,43
NM_030850	betaine-homocysteine methyltransferase	Bhmt	-1,85

	Amino sugars		
NM_053765	UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase	Gne	1,38
	Detoxification		
NM_031509	glutathione-S-transferase, alpha type	Gsta1	1,32
NM_080892	selenium binding protein 2	Selenbp2	1,3
NM_031834	sulfotransferase family 1A, phenol-preferring, member 1	Sult1a1	-1,31
NM_022513	dopa/tyrosine sulfotransferase	Sult1b1	-1,33
NM_013105	cytochrome P450, subfamily IIIA, polypeptide 3	Cyp3a3	-1,34
M33936.1	cyp4a locus, encodingcytochrome P450	IVA3	-1,45
NM_031605	cytochrome P450, 4a10	Cyp4a10	-1,49
NM_153312	testosterone 6-beta-hydroxilase	CYP3A2	-1,66
NM_147206	cytochrome P450 3A9	CYP3A9	-1,95
	Apoptosis and inflamatory response		
NM_012645	RT1 class lb	RT1-Aw2	1,9
NM_053372	secretory leuKocyte protease inhibitor	Slpi	-1,36
NM_012963	high mobility group box 1	Hmgb1	-1,48
NM_017258	B-cell translocation gene 1	Btg1	-1,31
NM-053977	cadherin 17	Cdh17	-1,94
NM_053299	ubiquitin D	Ubd	-1,87
BF556648	metallothionenin2 and metallothionein-1	Mt1, Mt2	-3,23
	Acute phase proteins		
AA858962	retinol-binding protein	RBP	-1,43
NM_012582	haptoglobin	Hp	-1,47
BF418815	fibrinogen B beta chain	Fgb	-1,51
NM_022519	serine (or cysteine) proteinase inhibitor, clade A (alpha-1-antitrypsin)	Serpina1	-1,52

Genes which were over- or under-expressed more than 30% of control values upon GSPE treatment, are grouped by molecular functions and biological processes, which were obtained for each individual gene from the Protein and Gene Databases from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). Values in parenthesis and bold characters refer to the mean fold change in mRNA levels determined by quantitative PCR.

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3.The Hypotriglyceridemic Biocativity Of Dietary Procyanidins Is Mediated In The Liver Via SHP

Submitted

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THE HYPOTRIGLYCERIDEMIC BIOCATIVITY OF DIETARY PROCYANIDINS IS MEDIATED IN THE LIVER VIA SHP.

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Running title: *Procyanidins lower triglyceride levels via SHP*

Key words: flavonoids, procyanidins, triglycerides, SHP, microarray, signalling pathway, mouse, HepG2

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ABSTRACT

The regular long-term consumption of dietary flavonoids has been associated with reduced mortality and risk of cardiovascular disease (CVD), partially by reducing triglyceride-rich lipoprotein secretion from the liver. We have previously reported that an induction in the expression of liver small heterodimer partner (SHP) paralleled a significant decrease in plasma triglycerides in rats treated with a grape seed procyanidin extract (GSPE) (del Bas et al. 2005. FASEB j.). Due to the link between SHP and lipid metabolism, our objective in this study was to elucidate whether SHP is the mediator of the lipid lowering activity of GSPE. We used two different systems to block SHP activity: human hepatoma cells transfected with a SHP-siRNA and a SHP knockout mouse model. The hypotriglyceridemic effect of GSPE is abolished in both SHP deficient models thus revealing this nuclear receptor as a key mediator of the hypotriglyceridemic response triggered by procyanidins. Moreover, SHP is a direct and early target of procyanidins in HepG2 cells. Microarray based comparison of liver gene expression profiles between GSPE treated wild-type and SHP $^{-/-}$ mice identified several SHP target genes, including SREBP1, as putative down-stream effector of the TG-lowering response triggered by procyanidins in liver. In conclusion, dietary procyanidins lower TG levels signalling through SHP in vitro in human liver cells and in vivo in mouse liver. Due to the relevance of triglyceridemia in the development of atherosclerosis, SHP is emerging as an important target of anti-atherogenic phytochemicals.

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INTRODUCTION

The regular long-term consumption of dietary flavonoids has been associated with reduced mortality and risk of cardiovascular disease (CVD) [1]. Many studies have addressed the properties of dietary flavonoids, which ameliorate different altered states such as atherosclerosis. Initially, the beneficial effect of these polyphenolic compounds was ascribed to their antioxidant activity. Thus, green tea polyphenols were shown to have significant antioxidant activity both in *in vivo* and *in vitro* systems, and were shown to act by scavenging reactive oxygen and nitrogen species and chelating redox-active transition metal ions, while modestly ameliorating *ex-vivo* oxidation of lipoproteins [2]. On the other hand, red wine polyphenols exert a protective effect *in vitro* and *in vivo* by inhibiting free radical–mediated LDL oxidation [3, 4]. Therefore, flavonoids may be important in preventing CVD by reducing the susceptibility of LDL to oxidation *in vivo*. Nevertheless, increasing evidence shows that flavonoids contribute to cardioprotection through mechanisms that are independent of their antioxidant capabilities. These mechanisms comprise interactions with cell membrane receptors, intracellular signalling pathway proteins and modulation of gene expression [5-8]. In this way, for instance, polyphenols induce the synthesis and release of nitric oxide by the vascular endothelium, which in turn, promotes vasorelaxation, reduces platelet aggregation, and limits the flux of atherogenic lipoproteins into the artery wall [6, 8]. In addition, flavonoids inhibit proliferation and migration of vascular smooth muscle cells, by interfering with platelet-derived growth factor (PDGF) receptor signalling through the phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. A plethora of transcriptional changes are therefore responsible for the actions of various flavonoids on the components of the vascular system [6, 8] [9-11].

Several studies have addressed the role of post-prandial lipemia in the development of CVD, revealing the importance of triglyceride -rich lipoprotein as an important factor in the development of these pathologies [12, 13]. Furthermore, hypertriglyceridemia has been revealed as an independent risk factor for coronary heart disease [14]. Different studies have shown beneficial effects of flavonoids regarding atherosclerosis prevention, reducing apolipoprotein B (apoB), triglyceride (TG) and cholesterol levels, thereby preventing early aortic atherosclerosis in hyperlipidemic hamsters [15, 16]. In

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cultured human liver cells (HepG2) de-alcoholized red wine was shown to decrease the production of apoB100 (a marker of VLDL and LDL in humans) [17], and red grape juice polyphenols were shown to induce liver LDL receptor expression and activity [18]. Additionally, we have previously shown that, in rats fed an acute non-toxic dose of grape seed procyanidin extract (GSPE), the postprandial plasma lipid profile was ameliorated, thereby lowering different atherosclerotic risk indices [19]. The main changes ascribed to those beneficial effects were related to TG-rich, apoB containing lipoproteins, supported by a decrease in plasma TG and apoB levels in rats treated with GSPE. Thus, the beneficial effects of procyanidins, and flavonoids in general, on plasma TG have been widely demonstrated. Nevertheless, little is currently known regarding precise the molecular mechanisms underlying these effects.

The liver is a major organ involved in the control of lipid homeostasis, and together with the intestine, it modulates and orchestrates the synthesis of lipoproteins in response to the physiological state of the organism [20]. Nevertheless, the mechanisms modulating the different pathways controlling the synthesis and secretion of lipoproteins are complex, and further research is needed to completely understand precisely how the different hormones, cytokines or nutrient-activated signals affect its homeostasis by acting, ultimately, at the transcriptional level in a coordinated manner. Among a wide variety of mediators are nuclear receptors which have emerged as key modulators of lipid and lipoprotein metabolism [21, 22], including hepatocyte nuclear factor-4 (HNF-4), peroxisome proliferator activated receptors (PPARs), retinoid X receptor (RXR), retinoic acid receptor (RAR), farnesoid X receptor (FXR) and small heterodimer partner (SHP). SHP is an atypical orphan nuclear receptor initially described as a co-repressor involved in the feedback regulation of bile acid synthesis [23, 24], and is now emerging as a key factor in the control of lipid homeostasis [25-28]. Different studies have implicated a role for SHP in mediating the hypolipidemic effects ascribed to bile acids [25, 29]. Cholic acid reduces plasma TG in mice by activating FXR, the bile acid receptor. The activation of FXR is followed by the induction of SHP which, in turn, represses different genes involved in TG synthesis, including SREBP1 [25, 30]. SREBP1 is a key regulator of lipogenesis, as revealed by the enhanced TG synthesis in the liver of mice over-expressing SREBP1a and SREBP1c [26, 31]. Moreover, in HepG2 cells, CDCA lowers VLDL synthesis and production [29]. The proposed mechanism is based, in part, by the activation of FXR

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and subsequent induction of SHP which, in turn, interferes with the binding of HNF4 to the promoters of apolipoprotein B (APOB) and microsomal triglyceride transfer protein (MTP) [29], which is essential in the assembly of VLDL by shuttling lipids to the nascent apoB containing lipoprotein [32]. SHP can therefore modulate the maintenance of the lipid pool and the expression of MTP, key factors in the control of VLDL synthesis and secretion [32, 33].

We have previously reported that an induction in the expression of SHP paralleled a significant decrease in plasma TG in rats treated with GSPE [19]. Due to the link between SHP and lipid metabolism, we hypothesized that this nuclear receptor could be the mediator of the lipid lowering activity of GSPE. In order to test this hypothesiswe have used two different systems to block SHP activity: human hepatoma cells transfected with a SHP-siRNA and a SHP knockout mouse model. First we demonstrate that GSPE exerts a TG lowering effect in the HepG2 cell line as well as in the wild-type mouse. Next we show that the hypotriglyceridemic effect of GSPE is abolished in both SHP deficient models thus revealing this nuclear receptor as a key mediator of the hypotriglyceridemic response triggered by procyanidins in the liver. Finally, the microarray based comparison of liver gene expression profiles between GSPE treated wild-type and $SHP^{-/-}$ mice identified several SHP target genes, including SREBP1, as putative down-stream effector of the TG lowering response triggered by procyanidins in hepatic cells *in vivo*.

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MATERIALS AND METHODS

Chemicals

Grape seed procyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract contained essentially monomeric (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%), oligomeric (5-13 units) (35,7%) procyanidins and phenolic acids (4.22). CDCA was from SIGMA, and was diluted in DMSO (Sigma).

Cells and Cytotoxicity Assays

HepG2 cells (ATCC , Manassas, VA, USA) were grown until 70% confluent in DMEM (Cambrex) supplemented with 1% penicillin/streptomycin (Cambrex), 2 mM L-Glutamine (Cambrex), 1% non essential amino acids (Sigma) and 10% FBS (Cambrex). For experiments, 12 hours before treatment media was replaced with serum depleted media (DMEM supplemented with 2 mM L-Glutamine, 1% non essential amino acids and 0.25% Oleic acid: Albumin) (sigma). After 12 hours, GSPE or CDCA was added to reach the appropriate working concentration. Media and cells were harvested after treatments.

To study the cytotoxicity of GSPE, cells were treated for 24 hours with different concentrations of GSPE diluted in aqueous solution. To assess the cytotoxicity of GSPE, two methods were used; LDH leakage and Alamar Blue quantitaion. For LDH activity assays, media and cells were collected and assayed as previously described [34]. The Alamar Blue (Biosource, USA) assay was used to assess GSPE cytotoxicity concerning cellular RedOx activity, following the manufacturers instructions. Media containing GSPE at the assayed concentrations was used to discard interactions between procyanidins and Alamar Blue Reagents.

In vivo feeding studies

Mice were housed under standard conditions. Experimental procedures were approved by the local Committee for Care and Use of Laboratory Animals at Baylor College of Medicine. *SHP*-deficient mice were generated by gene targeting as previously described [35], and were backcrossed with C57BL6 mice to the 10th generation. The correct

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genotype was verified using previously reported primer sequences and reaction conditions [36]. Age-matched groups of 8-10 week-old male mice were used in all experiments (n=5 per experimental group). Mice were fed a standard rodent chow and water *ad libitum*. Experiments were performed for 14hrs and mice were fed either vehicle (water), or procyanidins (250 mg/L) via oral gavage. A first dose was administered at 9:00 pm and a second dose at 9:00 am. At the end of the experiment, mice were fasted for the final 2 hours, and blood was collected from the orbital plexus after mice were anaesthetized with isoflurane. Tissues were snap frozen and stored at -80^{o}C until use.

Lipid analysis.

For the *de novo* synthesis of TG, HepG2 cells were seeded in 12-well plates. Media was replaced 12 hours before treatment. GSPE (50 mg/L) and ^{14}C-acetate were added to the media of the cells and 12 hours after treatment media and cells were collected and lipids extracted using chloroform/methanol. Thin layer chromatography was performed as previously described [37] with an additional separation using a Hexane/MTBE/NH3 solvent to obtain the TG fraction. ^{14}C-labeled TG were scraped and determined by scintillation counting. Values were corrected per mg protein, determined by a colorimetric assay (BIO-RAD).

For non-radioactive measurements, cell media and plasma TG or cholesterol were assayed using enzymatic kits (QCA, Spain).

Apolipoprotein B and SHP immunoblotting

To measure the amount of apolipoprotein B protein produced in vitro, media was collected after treatment. In siRNA experiments, media was collected and concentrated using Ultrafree-4 centrifugal filter units (Millipore, USA). For apolipoprotein B immunoblotting, equal volumes of media were loaded into each lane, separated by electrophoresis and transferred to a nitrocellulose membrane (Millipore, USA). ApoB detection was performed using an anti-apoB antibody (S-14, Santa Cruz Biotechnology) as the primary antibody, an HRP-conjugated anti-IgG was used as the secondary antibody (sc-2020, Santa Cruz Biotechnology), and an immobilon chemiluminiscent HRP substrate kit (Millipore, USA) was used as the chemiluminiscent reagent. Membranes were exposed to hyperfilm ECL (Amersham, UK). Bands were quantified using Quantity One software (Bio-Rad) and values were corrected by cell number. For

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SHP analysis, cells were lyzed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA) containing a protease inhibitor cocktail (SIGMA). Protein was quantified using a protein assay reagent (Bio-Rad, Germany). Equal amounts of cellular protein were separated by electrophoresis and electrotransferred to a nitrocellulose membrane. An anti-SHP antibody (SHP Q14, Santa Cruz Biotechnology) was used against SHP, and GAPDH (V-18, Santa Cruz Biotechnology) was quantified as the loading control. An HRP-conjugated anti-IgG antibody (sc-2020, Santa Cruz Biotechnology) was used as the secondary antibody. Bands were quantified using Quantity One software.

siRNA experiments

HepG2 cells were transfected using an Ambion silencer siRNA transfection kit. siRNA sequences targeting SHP were obtained from Ambion. Scramble RNA (Ambion) was used for transfecting control cells. Briefly, siRNAs were incubated with siPort NeoFx reagent to obtain transfection complexes. 10^5 cells were incubated with 0.03 nmol of siRNA and seeded in 12 well plates. Eight hours after transfection, the media was replaced with serum free media and cells were grown for additional 24 hours. To assess interference efficiency, total RNA was extracted using a Nucleospin 2 kit (Macherey Naegel, Germay), and SHP and RPLP0 expression were determined by RTqPCR using Applied biosystems Taqman predefined assays (Hs00222677_m1 for SHP and Hs 99999902 for RPLP0) as described below.

Gene expression analysis.

After treatment, HepG2 cell total RNA was obtained using a NucleoSpin RNA2 kit (Macherie-Naegel, Germany). Reverse transcription reactions were performed using the Taqman Reverse transcription reagent kit (Applied Biosystems). For gene expression quantification, specific primer and Taqman probes (Applied Biosystems) for different genes were used: SHP (Hs00222677_m1), APOB (Hs00181142_m1), SRBEP1 (Hs01081785_m1), MTP (Hs00165177_m1), FAS (Hs00188012) and RPLP0 as the endogenous control (Hs99999902_m1). Real Time quantitative PCR reactions were performed using the ABI Prism 7300 SDS Real Time PCR system (Applied Biosystems). For *in vivo* experiments, total RNA was obtained using Trizol reagent (invitrogen) following the manufacturer's protocol. Additional purification and DNAse treatment was performed using a NucleoSpin RNA2 kit (Macherie-Naegel, Germany).

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For microarray hybridization, RNAs from individual mice (5 per group) were pooled for the 4 different groups, (1) wild-type vehicle, (2) wild-type GSPE treated, (3) $SHP^{-/-}$ vehicle and (4) $SHP^{-/-}$ GSPE treated. The integrity of the pooled RNA was assessed using the Agilent 2100 Bioanalyzer (Madrid, Spain). For microarray hybridization, Cy3- or Cy5-labelled cDNA was obtained from each RNA pool by using the Agilent Low RNA Input Fluorescent Linear Amplification Kit as described in the Agilent manual (Part Number 5185-5818). Labeled cDNAs were hybridized against Agilent Mouse Oligo Microarrays (Part Number G4122A) following the Agilent 60-mer oligo microarray processing protocol. Images of hybridized microarrays were acquired with the Agilent G2565BA scanner, and data from the microarray images were obtained and analyzed with the Agilent Feature Extraction software. For each pair of RNA samples being compared, duplicate hybridizations with a dye-swap labelling was performed. For microarray validation, CYP7A1, MTP, APOA5, MT1, MT2 and SHP gene expression were analyzed by RTqPCR, using GAPDH as the endogenous control. RNA was retrotranscribed using a Taqman Reverse transcription reagent kit (Applied Biosystems) and gene expression was evaluated using the ABI Prism 7300 SDS Real Time PCR system (Applied Biosystems) using SYBR green reagent (Applied Biosystems). Primer sequences can be provided upon request.

Microarray data processing and statistical analysis

Data was filtered to avoid aberrant values derived from fluorescent labelling or extreme gene expression values. A whole array of data was constructed matching each gene symbol with its fold-change value from the microarray analysis. Genes were clustered into different biological processes using Panther software [38]. The gene expression profile deviation of each biological process group from the whole array expression pattern was calculated using the Mann-Whitney U Test (**Wilcoxon** Rank-Sum test) as previously described [38], resulting in a p-value. P-values under 0.05 were considered significant. From the resulting data using Mann-Whitney U Test, a second approach was used, consisting of fixing a fold-change threshold value of 0.7 for down-regulation and 1.5 for up-regulation to identify genotype-dependent changes caused by GSPE. For statistical analysis in the lipid, gene expression and apoB studies, T-test and ANOVA analyses were performed using SPSS software.

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RESULTS

GSPE decreases apoB and TG synthesis and secretion in HepG2 cells.

GSPE presented no cytotoxic effects on HepG2 cells, as measured by LDH leakage and Alamar Blue quantification, in concentrations up to 150 mg/L. Concentrations of 300 mg/L decreased cell viability by 20%. Consequently, in this work GSPE was used in the range of 20 to 100 mg/L.

In order to assess whether HepG2 cells are a valid system to study the bioactivity of GSPE on TG secretion, we performed dose- and time-response experiments. ApoB levels in the culture media decreased, 24 hours after addition of GSPE, in a dose-dependent manner, as assessed by immunoblotting analysis (figure 1A). The ApoB concentration in the media, normalized by cell number, was 20% and 40% lower in cells treated with 50 and 100 mg/L GSPE, respectively, than in vehicle-treated cells. Treatment of cells with 50 mg/L GSPE, completely blocks the accumulation of ApoB in the media during the first 12 h of GSPE treatment, while during the following 12 hours, this secretion is partially restored (figure 1B). In this same set of experiments, accumulation of TG in the media was drastically reduced upon treatment with 50 mg/L GSPE for 24h (figure 1C). Therefore, GSPE triggers a hypotriglyceridemic effect in HepG2 cells and is therefore a valid model to the study of mechanisms underlying the effect of GSPE *in vitro*.

In order to assess the action of GSPE on the *de novo* synthesis and secretion of TG, HepG2 cells were treated for 12 hours with ^{14}C-acetate and either vehicle or GSPE (figure 1D). Compared to controls, cells treated with procyanidins showed a slight reduction in ^{14}C-TG synthesis, and a significant decrease in ^{14}C-TG secretion, this did not result in intracellular 14C-TG accumulation. Altogether, these results strongly suggest that GSPE lowers TG-rich lipoprotein secretion, in part by inhibiting lipogenesis.

SHP is a target of GSPE in HepG2 cells.

We recently demonstrated that SHP mRNA levels were rapidly upregulated in the liver of rats fed a single dose of GSPE [19]. To assess whether that fast up-regulation could be reproduced in the HepG2 cell system, we treated HepG2 cells with different doses of GSPE and analyzed changes in SHP gene expression by RTqPCR after 2h treatment (figure 2A). Results show a dose-dependent effect of GSPE on SHP mRNA levels. Up-regulation of SHP mRNA levels by GSPE is very rapid, reaching a 2-fold increase after

2 hours treatment. In addition to the increase in SHP gene expression there is also a concomitant increase in SHP protein as assessed by immunoblotting (figure 2B) of GSPE treated HepG2 cells during 3 hours with a dose of 100 mg/L. Longer periods of GSPE treatment, assayed with a dose of 50 mg/L, resulted in a strong reduction in SHP mRNA, reaching 15% of the control value after 10 hours of treatment (Figure 2C). To assess whether SHP repression found at 6 and 10 hours of GSPE treatment could be explained by the well known negative feed-back mechanism that allows SHP to control its own expression [30, 39], HepG2 cells were co-incubated with GSPE and CDCA, a bile acid which induces SHP [40, 41] (Figure 3). After 10 hours, CDCA had triggered a 2.5 fold increase of SHP mRNA levels, while the addition of GSPE to CDCA-exposed cells resulted in an inhibition of SHP gene expression, paralleling the results showed in figure 2C 10 hours after GSPE treatment. These results suggest that the repression of SHP, after 10 hours treatment with procyanidins, is mediated by additional factors other than SHP itself.

The rapid and drastic induction in SHP mRNA and protein triggered by GSPE in HepG2 cells reveals that SHP is a direct and early target of procyanidins in hepatic cells, reinforcing the hypothesis that SHP might mediate the hypotriglyceridemic response induced by GSPE, without the involvement of extrahepatic tissues.

GSPE modulates the expression of genes related to lipid and lipoprotein metabolism.

The increase in liver SHP expression induced by bile acids has been related to a decreased secretion of TG in both HepG2 cells [29] and mice [25]. We therefore hypothesized that the up-regulation of SHP by GSPE could be related to the observed decrease in apoB and TG release triggered by these compounds. Therefore, we analyzed the effect of GSPE on the expression of known SHP target genes involved in VLDL synthesis and secretion, during the first 10 hours of GSPE treatment (figure 4). APOB mRNA levels are unaffected by GSPE in spite of the fact that the levels of secreted apoB protein are reduced. Likewise, SREBP1 mRNA levels are unaffected by GSPE treatment. Despite the decreased production of TG when HepG2 cells are treated with GSPE (as shown in figure 1C), FAS mRNA levels are induced by GSPE. In contrast, MTP mRNA levels are significantly reduced after 6 hours of treatment and reach33% of control values after 10 hours of GSPE treatment. Altogether, these results suggest that GSPE could be decreasing VLDL accumulation in the media by lowering MTP expression.

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Silencing SHP abolishes the effect of GSPE on TG secretion in HepG2 cells.

We next used a siRNA targeting SHP to study the relevance of SHP as a mediator in the effects of GSPE on apoB and TG secretion. Transfection of HepG2 cells with SHP siRNA results in a 60% reduction in SHP mRNA levels 32 hours after transfection (figure 5A). Concomitantly, apoB and TG release into the media is increased by silencing SHP, strongly suggesting that SHP represses apoB and TG secretion under basal conditions (figure 5B). In SHP-silenced cells, the TG-lowering effect of GSPE is abolished. In contrast, the effect of GSPE lowering ApoB secretion in SHP- knocked down HepG2 cells remains unaffected. These results suggest that two different mechanisms mediate the repression triggered by GSPE on ApoB and TG secretion in HepG2 cells: GSPE action on TG secretion involves a SHP-dependent mechanism, whereas repression of apoB release follows a SHP-independent pathway. Since MTP is tightly involved in ApoB secretion [42, 43], we wondered whether the MTP repression exerted by GSPE (shown in figure 4) could be mediated by the putative SHP-independent pathway. To gain further insight into this possibility, HepG2 cells were coincubated with GSPE and CDCA, which represses MTP via a pathway involving SHP [25]. Results (Figure 6) show that while MTP is still unaffected by CDCA, the addition of GSPE triggers the repression of MTP. Hence, GSPE represses MTP through different mechanisms other than the pathway followed by CDCA, which implicates SHP up-regulation.

SHP is a key mediator of the hypotriglyceridemic effect of GSPE in mice

In order to gain further insight into the relevance of SHP as a mediator of GSPE hypotriglyceridemic actions in an *in vivo* system, we compared the effects of GSPE administration in $SHP^{-/-}$ versus wild type mice. In wild type mice, oral GSPE gavage triggers a 40% reduction in plasma TG levels, while it does not affect plasma total cholesterol levels (figure 7). This response of GSPE administration on the plasma lipid profile is identical to what we previously found in rats [19]. The hypotriglyceridemic effect of GSPE was abolished by the $SHP^{-/-}$ genotype. These results clearly show that SHP is a key mediator of the hypotriglyceridemic effects exerted by procyanidins *in vivo*.

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GSPE induces changes in genes related to lipid, fatty acid and steroid metabolism in wild type but not SHP$^{-/-}$ mice.

To gain further insight into the SHP-dependent actions of GSPE, we next analyzed the differential response in gene expression changes induced by procyanidins in the livers of wild type and SHP$^{-/-}$ mice using oligonucleotide microarray hybridization. The changes induced by GSPE treatment in the expression level of all genes, clustered by biological process, were analyzed using Panther software. In wild type mice, changes in genes clustered in the biological process "Lipid, fatty acid and steroid metabolism", including 747 genes, show a significant deviation (p-value 0.018) from the overall changes in gene expression patterns (figure 8). In contrast, in SHP$^{-/-}$ mice, this gene cluster does not significantly deviate (p-value 0.175) from the global change pattern. The significant deviation of genes in the "Lipid, fatty acid and steroid metabolism" group in the GSPE treated wild-type mice was attributed to an abnormal predominance of genes in the fold-change range from 0.5 to 0.8, indicating a down-regulation of numerous genes included in this metabolic group. Altogether, these results indicate that lipid metabolism is more strongly repressed by GSPE in wild type than in SHP$^{-/-}$ mice, thus pointing to SHP as a key mediator of the repression of lipid metabolism induced by procyanidins.

Next, in order to identify SHP-dependent genes involved in the hypotriglyceridemic effect of GSPE in wild type mouse, we selected those genes clustered into the "Lipid, fatty acid and steroid metabolism" group that were changed in wild-type mice but remained unaltered in SHP$^{-/-}$ mice, setting a fold-change threshold of 1.5 for up-regulated and 0.7 for down-regulated genes (Table 1). In total, 24 lipogenic genes were identified which show SHP-dependent repression by GSPE, including key regulators of lipid synthesis pathways such as Sterol regulatory element binding protein 1 (SREBP1), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase, acyl-CoA synthetase, stearoyl-Coenzyme A desaturase and (PPARγ)-interacting Protein. Other genes involved in lipoprotein metabolism that were not classified by Panther software in this cluster, also changed in a SHP-dependent manner, such us ApoA5, C/EBP beta, and phospholipid transfer protein (Table 1). Remarkably, numerous genes (marked with an asterisk in Table 1) which show a SHP-dependent response to GSPE, have been described as targets of SREBP1, a master regulator of lipid and lipoprotein metabolism [44-47]. Therefore, SREBP1 emerges as a putative SHP-dependent downstream effector of the hypotrycliceridemic response triggered by procyanidins in hepatic cells.

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DISCUSSION

Many studies have addressed the long term effects resulting from a chronic intake of flavonoids on lipid homeostasis in dyslipidemic human subjects and hypercholesterolemic animal models, where they have been shown to exert beneficial hypolipidemic effects [19, 48-51]. Previously [19], we studied the short term effect of an acute oral dose of GSPE in healthy rats, in order to gain insight into the initial mechanisms underlying the long-term beneficial effects on lipid related metabolic disorders associated with a chronic intake of procyanidins and other flavonoids. We found that GSPE triggered a 3-fold increase in liver SHP mRNA expression with a concomitant 50% reduction in plasma apoB and TG levels, in the postprandial state in healthy rats. SHP is an orphan nuclear receptor, which directly modulates the activities of nuclear receptors and transcription factors by acting as an inducible and tissue-specific corepressor thus modulating multiple pathways [30]. In the liver, SHP regulates cholesterol and bile acid metabolism, steroidogenesis and lipogenesis [30]. Therefore, SHP emerged as a putative mediator of the observed hypolipidemic response triggered by GSPE. To assess whether SHP is a key mediator of procyanidin activity, we tested the effect of GSPE administration on the human hepatoma HepG2 cell line transfected with SHP siRNA and on a SHP knockout mouse model.

HepG2 cells are a valid system to study lipid synthesis and secretion [52-54]. In addition, various studies have revealed that procyanidins and other flavonoids decrease VLDL secretion in HepG2 cells [17, 48, 55, 56]. In the present study we have shown that GSPE strongly blocks TG and apoB secretion in HepG2 cells. Since the rate of *de novo* TG synthesis is slightly decreased by GSPE, blocking TG secretion does not involve intracellular accumulation of TG during the first 12 hours of treatment. These results strongly suggest that GSPE decreases VLDL secretion, thus reinforcing previous reports. The results presented herein also show an early induction in SHP expression in response to GSPE treatment in a dose-dependent fashion, therefore establishing this nuclear receptor as a direct target of procyanidins. These results indicate that GSPE triggers a TG-lowering effect via the induction of SHP expression which acts directly on hepatocytes. This is consistent with the effects we previously described in rats [19] and, altogether, strongly suggest that, *in vivo*, procyanidins act directly on liver cells, without the need to invoke the intervention of extrahepatic tissues.

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In HepG2 cells, an induction in SHP expression was observed during the first hours of GSPE treatment, while its expression decreased steadily from 6 hours onward. This expression pattern could, in principle, be attributed to the negative feedback mechanism that allows SHP to repress its own expression, interfering with transcription factors that bind to the SHP promoter, such us FXR, LXR or PXR [57-59]. Nevertheless, when we co-incubated HepG2 cells with GSPE and CDCA, a bile acid which induces SHP, the repression of SHP was still active after 10 hours of treatment, while those cells exposed only to CDCA still showed increased levels of SHP mRNA. Upon observing the repression in SHP expression by GSPE treatment in the presence of CDCA we wondered whether GSPE could be acting as an inverse agonist/antagonist of FXR. However, in a transient transfection assay GSPE did not inhibit the transactivation of FXR by CDCA (del Bas, Ricketts Moore & , unpublished observation). Therefore, we postulate that procyanidins induce SHP expression during the first hours of treatment and, afterwards, repress SHP expression through mediators, other than SHP itself, that are able to over-ride the earlier induction triggered by procyanidins and by CDCA.

The expression pattern of MTP, ApoB, FAS and SREBP1 genes, which are related to SHP and VLDL secretion [25, 29, 30, 59], could explain the TG lowering actions of GSPE in HepG2 cells. In these studies we have found that the GSPE-dependent TG and apoB lowering effects were achieved during the first 12 hours of treatment. Thus, mRNA levels were monitored during this period of time. SREBP1 and FAS, key factors in the control of lipogenesis, have been described as targets of flavonoids and polyphenolic compounds [60-62]. No effects were observed for SREBP1, whereas FAS was induced by GSPE. These results indicate that the actions of GSPE in lowering lipogenesis in HepG2 cells are mediated by other genes, or by mechanisms acting at the post-transcriptional level [48, 63]. Together with the inhibition of TG synthesis, the repression of MTP expression exerted by GSPE in HepG2 cells could be the explanation for the decreased release of TG and apoB. MTP is the key enzyme regulating the synthesis of TG-rich lipoproteins, shuttling TG to apoB nascent lipoproteins [33, 64]. Together with the availability of lipids, both are the main limiting factors in the synthesis and secretion of VLDL [32, 33, 65]. In this process, APOB mRNA levels play a minor role, since it can be regulated at post-transcriptional stages and, thus, the ApoB protein that has not been assembled into the nascent lipoprotein can be degraded via proteosomes [33]. This regulatory mechanism allows a plausible

explanation for the unchanged expression of APOB gene expression while the levels of the ApoB protein were decreased in the media of the cells treated with GSPE. In summary, our results are consistent with other reports, showing that flavonoids can repress MTP, thereby decreasing apoB secretion [17, 48]. Nonetheless, control of MTP expression is subjected to numerous factors other than SHP [32, 66].

To further investigate the role of SHP in mediating the TG and ApoB lowering effects of GSPE in HepG2 cells, a siRNA system targeting SHP was established. A 60% silencing in SHP mRNA levels was concomitant with increased TG and ApoB in the media, highlighting the role of SHP in the control of apoB-containing lipoprotein synthesis and secretion in hepatic cells. Our results agree with a previous report [19] showing that bile acids control the secretion of VLDL, and that this can be mediated, in part, by SHP; SHP interferes in the binding of HNF4 to the MTP promoter in HepG2 cells, therefore linking SHP with MTP expression. Surprisingly, when the SHP knocked-down cells were treated with GSPE, the reduction in apoB secretion was similar to that seen in control cells, suggesting that the apoB lowering effect of GSPE is exerted by mediators other than SHP. In contrast, the activity of GSPE lowering TG secretion was abolished by interfering with SHP, indicating that it is the mediator of the TG lowering activity of procyanidins. Therefore, two separate mechanisms could be proposed to be responsible for the apoB and TG lowering effects of GSPE. The first one is a SHP-independent pathway which would act by inhibiting the release of apoB, and thus, decrease the number of VLDL in the media of GSPE-treated cells, since a single molecule of apoB is assembled in each apoB-containing lipoprotein [32, 33, 65]. This idea is reinforced by the results obtained when wild type HepG2 cells were co-incubated with CDCA and GSPE. CDCA has been described as an inhibitor of MTP expression by acting, in part, through a SHP-dependent mechanism [29]. Nevertheless, while those cells treated with CDCA presented no alterations in MTP mRNA levels, those treated with both CDCA and GSPE showed a down-regulation in the expression of this gene. In addition, as has been discussed above, the same treatment reported that GSPE represses SHP despite the presence of CDCA. These results suggest that GSPE could be repressing MTP expression and subsequently inhibiting apoB secretion without the need for an up-regulation in SHP. Along with this SHP-independent pathway, a SHP-dependent mechanism could lead to the inhibitory effect of GSPE on the secretion of TG. Whether this SHP-dependent mechanism would also be responsible

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for the decrease in the *de novo* synthesis of TG is clarified by the *in vivo* experiments in mice, which show that numerous lipogenic genes are down-regulated in a SHP-dependent manner by GSPE. Accordingly with these two proposed mechanisms of action of GSPE on VLDL secretion, it has previously been reported that MTP, which we propose implicated in the SHP-independent GSPE effects, is essential for hepatic secretion of apoB but not TG [67]. Previous results from our group [68] showed insulin-like effects of GSPE in insulin-sensitive cell lines, suggesting that procyanidins are able to signal through PI3K and p38 MAPK. Naringenin, another flavonoid, has been shown to decrease MTP expression and apoB secretion from HepG2 cells by signalling through a MAPK pathway [17] and PI3K [48] in rat hepatoma cells. Therefore, the SHP-independent down-regulation of MTP expression and apoB secretion might by explained, at least partially, by the insulin-like properties of GSPE. Taken together, our results suggest a convergence of two pathways, a SHP-dependent and a SHP-independent pathway, to achieve the VLDL lowering effect of GSPE in hepatic cells.

In order to verify the role of SHP as a mediator of the hypotriglyceridemic activity of GSPE, we compared the effect on plasma TG levels elicited by procyanidins in wild type and $SHP^{-/-}$ mice, fed with GSPE via oral gavage. Wild type mice treated with GSPE displayed a clear hypotriglyceridemic response, whereas, in contrast, in mice lacking functional SHP this response was blocked. SHP mRNA levels were similar in GSPE and vehicle treated wild type mice at the time of analysis (Data not shown). The experiments in HepG2 cells revealed a highly dynamic modulation of SHP by GSPE. If SHP expression pattern in the liver of mice treated with GSPE were similar to that observed in HepG2 cells, an upregulation in SHP expression prior to the moment of sample collection may be considered.

We have identified the gene cluster “lipid, fatty acid and steroid metabolism” as a SHP-dependent target of GSPE in liver, by genome-wide microarray screening. This analysis shows a down-regulation of genes involved in lipogenesis in wild type but not in SHP null mice. TG rich lipoprotein synthesis in the liver is highly dependent on lipid availability [32, 33, 65, 69]. Thus, SHP mediation in the modulation of plasma TG by GSPE could be related, at least in part, to a down regulation of lipogenic genes in the liver. From the initial 747 genes matching lipid, fatty acid and steroid metabolism, 28 lipogenic genes were identified as differentially affected by GSPE in a genotype-

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dependent manner. Among them, SREBP1 stands out; it is a master regulator of the lipid synthesis program and lipoprotein metabolism [31, 70, 71], together with a number of SREBP1 target genes including stearoyl-Coenzyme A desaturase 2, sterol-C5-desaturase, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1, acyl-CoA synthetase short-chain family member 2, 7-dehydrocholesterol reductase, farnesyl diphosphate synthetase, CCAAT/enhancer binding protein beta, phospholipid transfer protein and apolipoprotein A5. ApoA5 has recently emerged as a potent regulator of plasma TG by activating lipoprotein lipase, thus accelerating VLDL catabolism [72]. Increased expression of this apolipoprotein could be indicative of enhanced clearance of plasma TG, providing additional mechanisms for the SHP-dependent hypotriglyceridemic activity of GSPE. Remarkably, other SREBP1 target genes involved in glucose metabolism, such as glucokinase and aldehyde dehydrogenase 1A2, are downregulated by GSPE only in wild type mice (data not shown). Likewise, it is well known that bile acids, other powerful hypotriglyceridemic agents, act through a pathway involving FXR, SHP, and SREBP1 to lower TG levels [25].

In conclusion, grape seed procyanidins exert TG lowering effects in the human hepatoma HepG2 cell line and in mice. In both *in vitro* and *in vivo* systems, the hypotriglyceridemic properties of procyanidins depend on the presence of SHP. Due to the relevance of postprandial TG in the development of atherosclerosis [13, 73], the elucidation of how nutrients can modulate plasma lipid levels has emerged as an important target of nutrition research [74]. In this regard, this work provides new hints for understanding the mechanisms associated with the beneficial effects ascribed to the regular consumption of procyanidins and, more broadly, flavonoids.

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Table 1. **SHP-dependent changes induced by GSPE in lipid and lipoprotein related genes in mouse.**

Differential changes induced by GSPE in WT and SHP $^{-/-}$

Genes reported by Panther Software				
Genbank ID	**SREBP1 target**	**Name; gene symbol**	**WT**	**SHP$^{-/-}$**
NM_008903		phosphatidic acid phosphatase 2a;Ppap2a	0.7	0.9
NM_023556		mevalonate kinase;Mvk	0.7	1.2
NM_177664		DNA segment, Chr 3, Brigham & D3Bwg0562e	0.5	1.0
NM_172769	*	sterol-C5-desaturase;Sc5d	0.7	1.0
NM_145942	*	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1;Hmgcs1	0.6	1.2
NM_018784		ST3 beta-galactoside alpha-2,3-sialyltransferase 6;St3gal6	0.7	0.8
NM_028089		cytochrome P450, family 2, subfamily c, polypeptide 55;Cyp2c55	0.6	1.2
NM_146197		acyl-CoA synthetase medium-chain family member 2;Acsm2	0.6	1.0
NM_019811	*	acyl-CoA synthetase short-chain family member 2;Acss2	0.6	0.8
NM_013634		peroxisome proliferator activated receptor binding protein;Pparbp	0.6	0.8
NM_009128	*	stearoyl-Coenzyme A desaturase 2;Scd2	0.7	0.9
NM_008845		phosphatidylinositol-4-phosphate 5-kinase, type II, alpha;Pip5k2a	0.7	1.0
NM_207683		phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide;Pik3c2g	0.7	1.0
NM_010941		NAD(P) dependent steroid dehydrogenase-like;Nsdhl	0.7	1.0
NM_007856	*	7-dehydrocholesterol reductase;Dhcr7	0.7	0.9
NM_018830		N-acylsphingosine amidohydrolase 2;Asah2	0.5	1.0
NM_011480	*	sterol regulatory element binding factor 1;Srebp1	0.7	1.0
NM_134469	*	farnesyl diphosphate synthetase;Fdps	0.8	1.6
NM_175443		ethanolamine kinase 2;Etnk2	0.7	0.9

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NM_013490		choline kinase alpha;Chka	0.7	0.9
NM_019677		phospholipase C, beta 1; Plcb1	0.7	1.1
NM_028057		cytochrome b5 reductase 1;Cyb5r1	0.7	1.3
NM_026784		phosphomevalonate kinase;Pmvk	0.7	0.9
NM_007703		elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 3;Elovl3	0.7	1.0
NM_011372		ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3;St6galnac3	0.7	1.0
Other SREBP1 target genes (not included in Panther classification)				
NM_080434	*	Apolipoprotein A5; ApoA5	1.4 **1.7**	1.0 **1.0**
NM_009883	*	CCAAT/enhancer binding protein (C/EBP), beta; Cebpb	0.7	0.8
NM_011125	*	phospholipid transfer protein; Pltp	0.7	1.0

Wild type and SHP$^{-/-}$ mice were fed either vehicle or GSPE (250 mg/Kg) via oral gavage (n=5 in each group, age 8-10 week). After 14 hours treatment, liver total RNA from the 4 groups was obtained and pooled. Microarray data was obtained by comparing gene expression of WT control versus WT GSPE-treated mice and SHP$^{-/-}$ control versus SHP$^{-/-}$ GSPE-treated mice. The whole microarray fold-changes were processed using Panther software in order to identify SHP-dependent changes induced by GSPE in genes clustered in the "Lipid, fatty acid and steroid metabolism" metabolic pathway. Fold-change thresholds were fixed as 0.7 and 1.5 for down-regulation and up-regulation respectively. Real time quantitative PCR was performed with selected genes to confirm the microarray data (shown in bold characters). Known SREBP1 target genes are denoted by *****. WT column: fold-change in expression induced by GSPE in wild-type mice versus vehicle. SHP$^{-/-}$ column: fold-change in expression induced by GSPE in SHP$^{-/-}$ mice versus vehicle.

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Figure 1. Effect of GSPE on apoB release and triglyceride synthesis and secretion in HepG2 cells.

(A) Changes in media ApoB levels in response to 20, 50 and 100 mg/L GSPE doses. HepG2 cells were treated with either vehicle (control) or GSPE at the indicated concentrations. After 24 hours, media ApoB immunoblotting was performed as described in materials and methods. ApoB band intensities were corrected by cell number. The immunoblot of one from three independent experiments is shown. (B) Time-response dependent changes in media ApoB accumulation induced by GSPE. HepG2 cells were treated with 50 mg/L GSPE or vehicle (control) and media was collected after 6, 12 and 24 h. ApoB levels were analyzed as in Fig. 1A. (C) Changes in TG accumulation in media of cells treated with GSPE. HepG2 cells were treated with 50 mg/L of GSPE or vehicle (control) for 24 h. TG were assayed as indicated in materials and methods (D) Changes induced by GSPE in the *de novo* synthesis and secretion of TG. HepG2 cells were incubated with ^{14}C-labelled acetate and 50 mg/L of GSPE or vehicle (control). 12 hours after treatment, radioactivity incorporated into media and cellular TG was measured. Values were normalized to mg of cell protein. All **v**alues are the mean +/- SEM of three independent experiments. * denotes significant difference ($p<0.05$).

Figure 2. Effects of GSPE on SHP expression in HepG2 cells.

(A) Fold-change of SHP gene expression in response to 20, 50 and 100 mg/L GSPE relative to vehicle treated cells. HepG2 cells were treated with the indicated concentrations of GSPE for 2 hours. Gene expression levels of SHP were determined by RT-qPCR and normalized to RPLP0 expression (endogenous control). (B) Representative immunoblot of SHP changes protein levels in response to GSPE treatment. HepG2 cells were treated with vehicle or 100 mg/L GSPE. After 3 hours, cell lysates were obtained and SHP levels were analyzed by immunoblotting, using GAPDH as the loading control. (C) Time course of SHP gene expression in response to treatment with 50 mg/L of GSPE. HepG2 total RNA was obtained at the indicated times and analyzed as in fig 2A. All **v**alues are the mean +/- SEM of three independent experiments * denotes significant difference ($p<0.05$).

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Figure 3. Effect of CDCA and GSPE co-incubation on SHP expression in HepG2 cells.

HepG2 cells were incubated with 100 μM CDCA and various concentrations of GSPE. After 10 hours of treatment, total RNA was obtained and expression levels of SHP were determined as in figure 2. Values are the mean +/- SEM of three independent experiments. * denotes significant difference versus CDCA treatment ($p<0.05$).

Figure 4. Effect of GSPE on the expression of different lipid and lipoprotein-related genes in HepG2 cells.

HepG2 cells were incubated with 50 mg/L GSPE for the indicated times. After treatment, cells were harvested and total RNA obtained. Gene expression quantification was performed by RTqPCR and normalized to RPLP0 gene expression (endogenous control). All values are the mean +/- SEM of three independent experiments * denotes significant difference ($p<0.05$). MTP: Microsomal triglyceride transfer protein; APOB: Apolipoprotein B; SREBP1: sterol response element binding protein 1; FAS: Fatty Acid Synthase.

Figure 5. Effect of GSPE on apoB and triglyceride secretion in HepG2 cells transfected with SHP siRNA.

HepG2 cells were transfected with a siRNA coding for SHP (indicated by +) or non-coding scramble siRNA (indicated by -). 32 hours after transfection, media was replaced with serum depleted medium. After 12 hours, cells were treated with 50 mg/L GSPE (indicated by +) or vehicle (indicated by -). (A) SHP gene expression after 2h of GSPE treatment. (B) Media ApoB and TG after 24h of GSPE treatment. After treatment, media was harvested for ApoB and TG analysis, as described in figure 1. A representative ApoB immunoblot is shown. All values are the mean +/- SE of three independent experiments. a, b or c denotes significant differences between groups, as determined by ANOVA.

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Figure 6. Effect of CDCA and GSPE co-incubation on MTP expression in HepG2 cells.

HepG2 cells were incubated with 100 μM CDCA and various concentrations of GSPE. After 10 hours of treatment, total RNA was obtained and expression levels of MTP were determined as in figure 2. Values are the mean +/- SE of three independent experiments. * denotes significant difference versus CDCA treatment ($p<0.05$).

Figure 7. Effect of GSPE on plasma triglyceride and cholesterol levels in wild-type and SHP$^{-/-}$ mice.

Wild type (WT) and SHP$^{-/-}$ mice were fed with vehicle (control) or GSPE (250 mg/Kg) via oral gavage (n=5 in each group, age 8-10 week). Plasma was obtained 14 hours after treatment and TG and cholesterol were determined as described in materials and methods. * denotes significant differences versus control ($p< 0.05$).

Figure 8. Effect of GSPE on expression of genes clustered into the "Lipid, fatty acid and steroid metabolism" in wild-type versus SHP$^{-/-}$ mice.

Wild type (WT) or SHP$^{-/-}$ mice were fed either vehicle (control) or GSPE (250 mg/Kg) via oral gavage (n=5 in each group, age 8-10 week). After 14 hours treatment, liver total RNA from individual mice in the different groups was obtained and pooled. Microarray data was obtained by comparing gene expression of WT control versus WT GSPE-treated mice and SHP$^{-/-}$ control versus SHP$^{-/-}$ GSPE-treated mice. The whole microarray fold-changes were processed using Panther software. The plot of the cluster "lipid, fatty acid and steroid metabolism" against the whole microarray fold-changes plot is shown for the two genotypes. Number: number of genes matched to Lipid, fatty acid and steroid metabolism by Panther software; OverUnder: tendency of the cluster deviation from the whole array expression pattern, namely upregulation tendency (+) and downregulation tendency (-); A $p<0.05$, calculated using the Mann-Whitney U Test, is considered significant.

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Figure 1

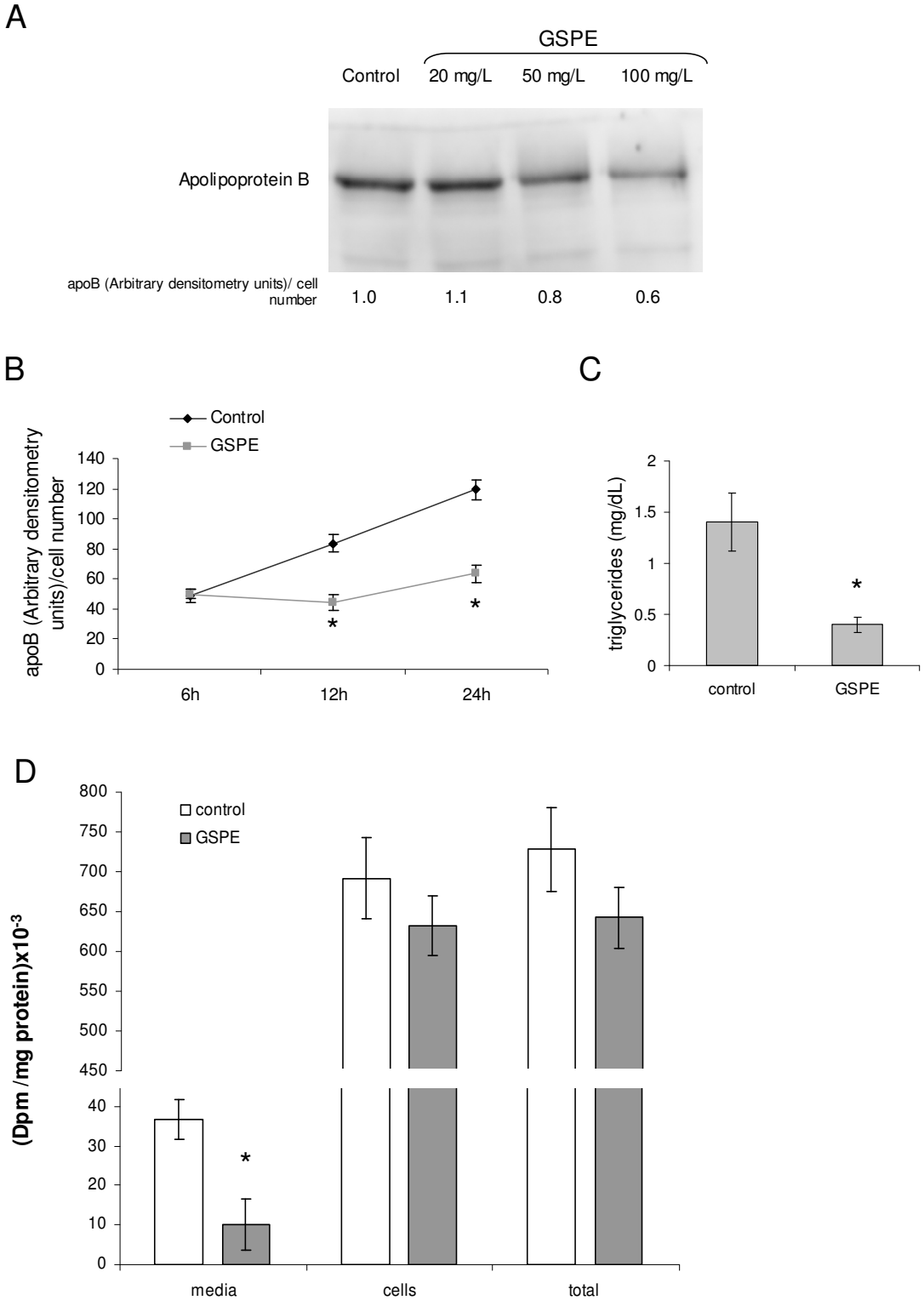

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Figure 2

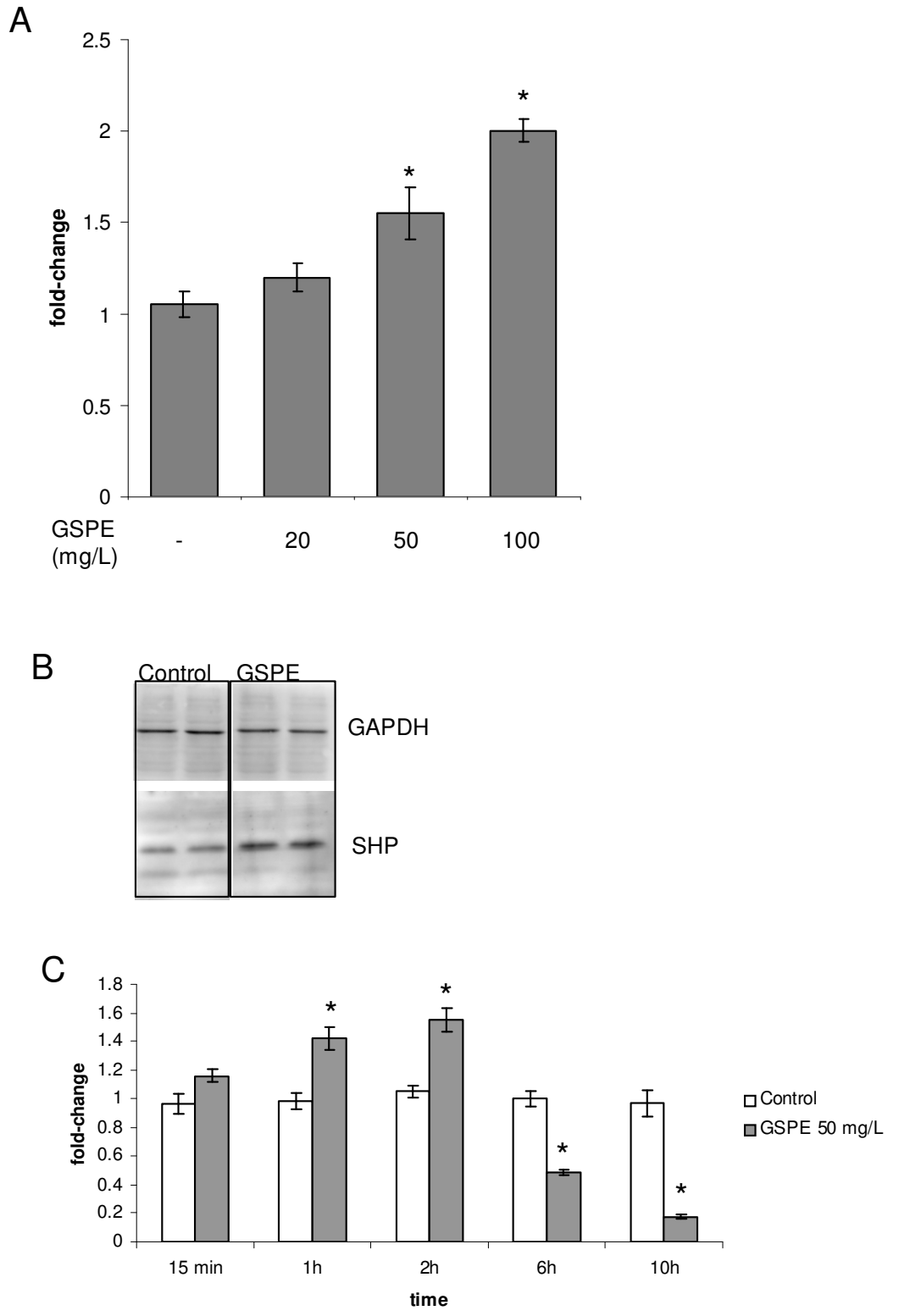

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Figure 3

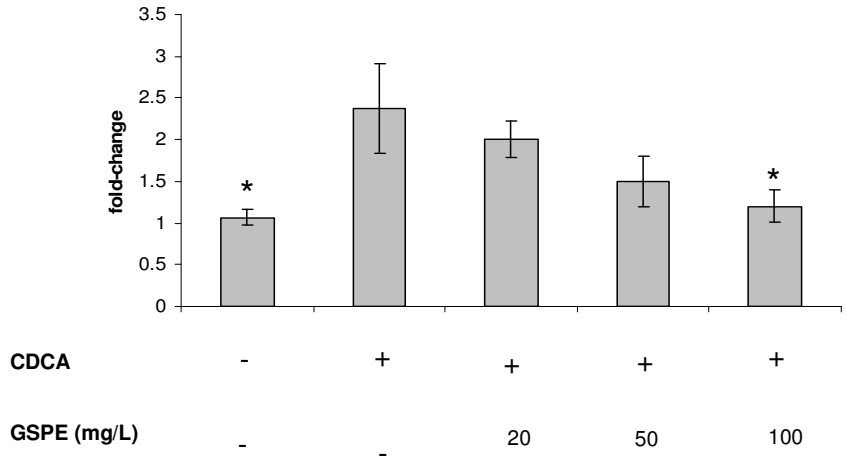

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Figure 4

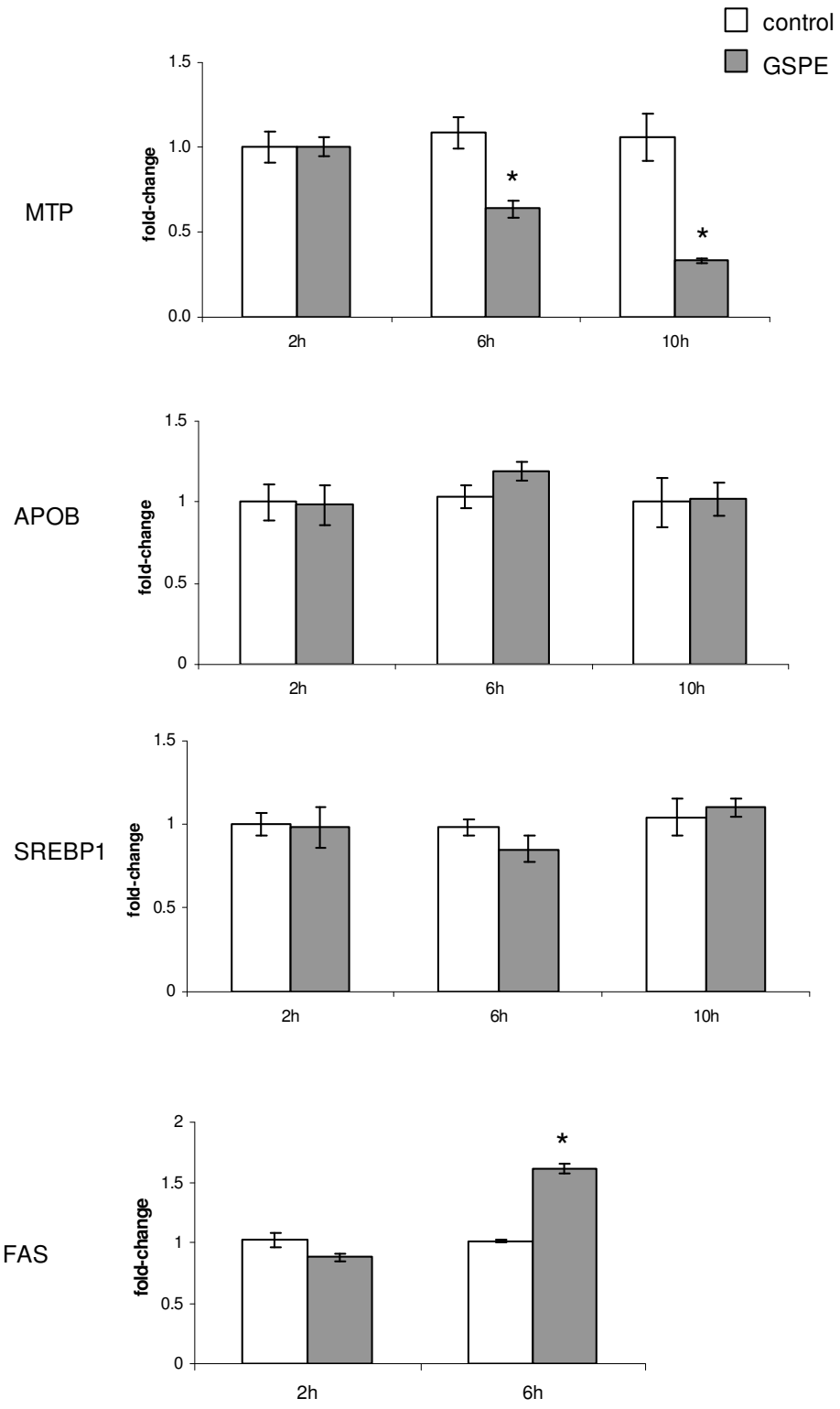

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Figure 5

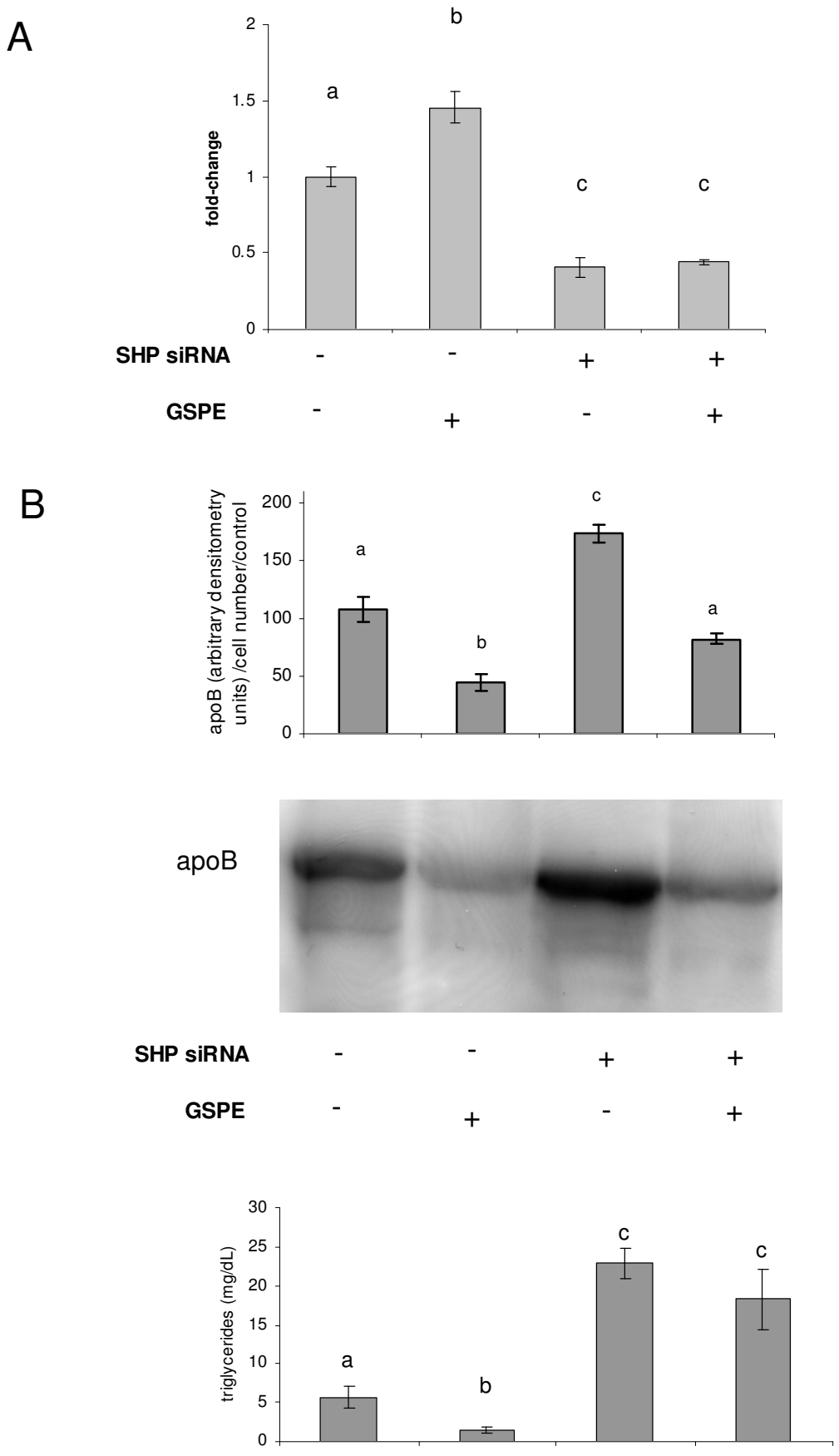

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Figure 6

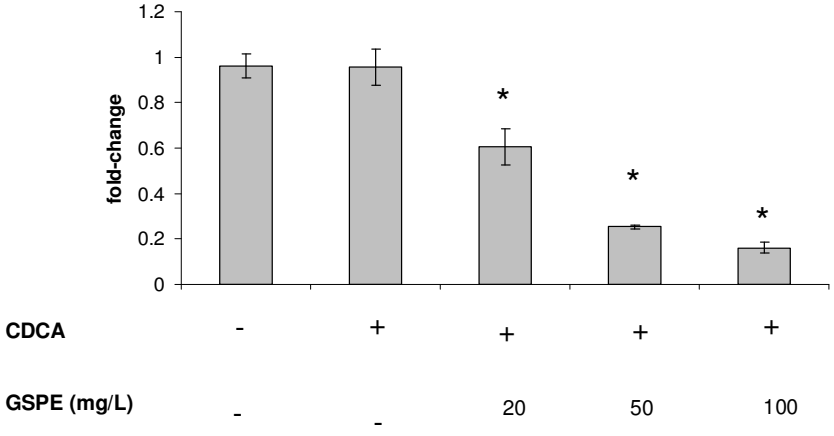

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Figure 7

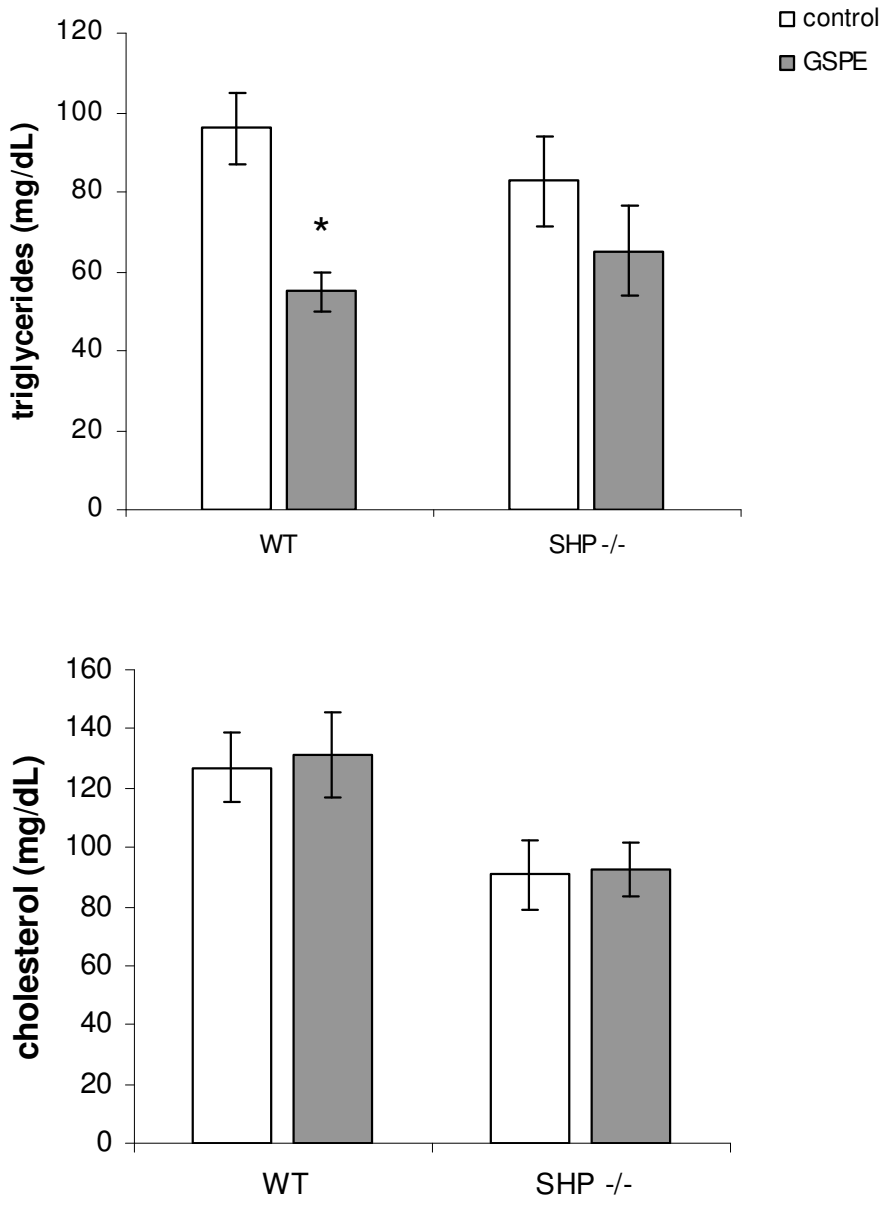

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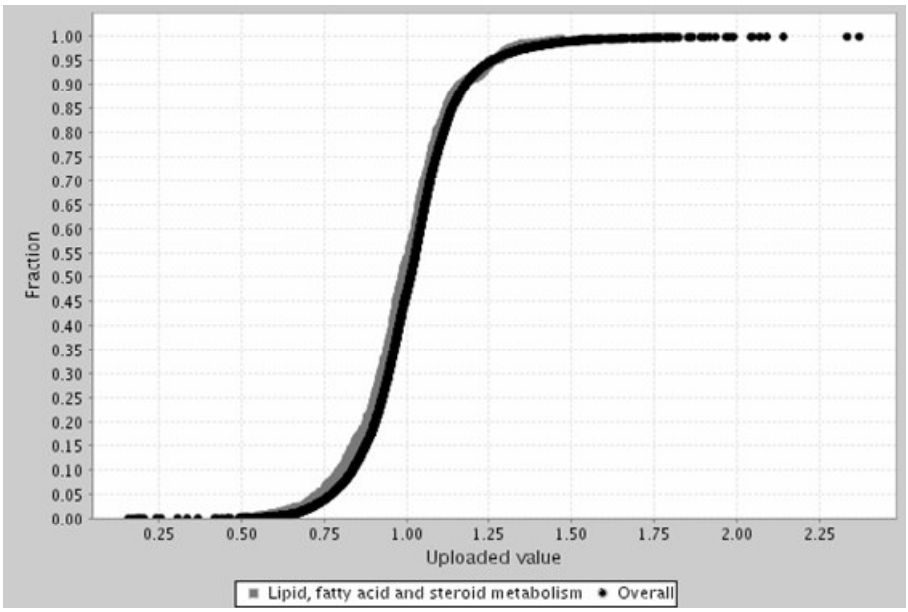
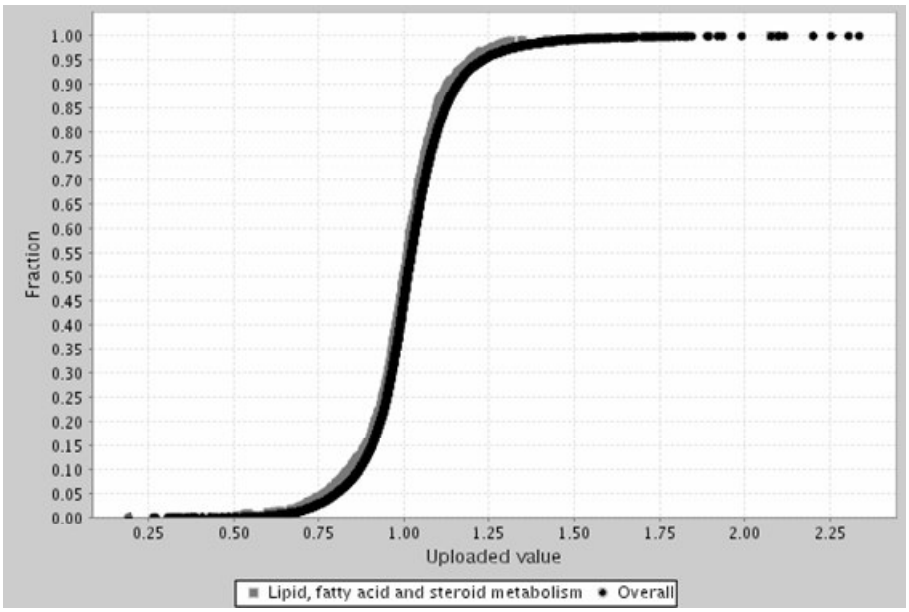
Figure 8

WT



SHP$^{-/-}$



	WT			SHP -/-		
Biological Process	number	overUnder	pvalue	number	overUnder	pvalue
Lipid, fatty acid and steroid metabolism	747	-	1.82E-02	747	-	1.75E-01

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4. Procyanidins Lower Plasma Triglyceride Levels Signalling Through FXR In Mice

Submitted.

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DIETARY PROCYANIDINS LOWER PLASMA TRIGLYCERIDE LEVELS SIGNALLING THROUGH THE NUCLEAR RECEPTOR FXR IN MICE

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Running title: *Procyanidins lower triglyceride levels via FXR*

Key words: flavonoids, procyanidins, plasma triglycerides, FXR, SREBP, microarray, liver, signalling pathway, knockout mice

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ABSTRACT

The regular long-term consumption of dietary flavonoids has been associated with reduced mortality and risk of cardiovascular disease (CVD), partially by reducing triglyceride-rich lipoprotein secretion from the liver. We have previously reported that SHP is a key mediator of the hypotriglyceridemic actions of procyanidins (del Bas et al. 2005, manuscript 3). Due to the functional link between SHP, FXR and lipid metabolism, our objective in this study was to elucidate whether FXR could mediate the lipid lowering activity of GSPE. In wild type mice GSPE drastically reduced plasma triglyceride (TG) levels, while its effect was abolished in the FXR-/- mice, revealing that FXR is a key mediator of GSPE hypotriglyceridemic actions. Moreover, liver gene expression profile analyses revealed SREBP1 and other lipogenic genes as targets of GSPE actions in the liver, indicating that liver plays an important role in the hypotriglyceridemic action of procyanidins. In order to confirm the ability of GSPE to enhance FXR transcriptional activity, this nuclear receptor was expressed in two different cell lines as a full length or a chymeric protein, along with luciferase reporter plasmids. Results show that GSPE synergistically enhances FXR activity in the presence of its natural ligands bile acids, but not in the presence of the synthetic ligand GW4064. In conclusion, dietary procyanidins enhance bile acid-bound FXR activity and are able to lower TG levels by signalling through FXR in mouse. The identification of dietary procyanidins as FXR activators reveals these phytochemicals as promising agents for the prevention and amelioration of hypertriglyceridemic states, including type 2 diabetes and the metabolic syndrome.

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INTRODUCTION

Different studies have been addressed to determine the role of postprandial lipemia in the development of cardiovascular diseases (CVD), revealing triglyceride-rich lipoproteins as important factors in the development of these pathologies(1, 2). Furthermore, hypertriglyceridemia itself has been revealed as an independent risk factor for coronary heart disease (3). Different works have shown the beneficial effects of flavonoids regarding atherosclerosis prevention, reducing apolipoprotein B (apoB), triglycerides (TG) and cholesterol, preventing early aortic atherosclerosis in hyperlipidemic hamsters (4). In cultured human liver cells HepG2, dealcoholized red wine decreases the production of apoB100 (a marker of VLDL and LDL in humans) (5) and red grape juice polyphenols induces liver LDL receptor expression and activity (6). Additionally, in a previous work (7), we showed that an acute and non-toxic dose of a grape seed procyanidin extract (GSPE) ameliorates the plasma lipid profile in the postprandial phase by drastically reducing TG and apoB levels, and thus TG-rich apoB-containing lipoproteins. Furthermore, LDL-Cholesterol was significantly reduced by this GSPE treatment, whereas HDL-Cholesterol was increased, resulting in the amelioration of different atherosclerotic risk indexes. Thus, the beneficial effects of procyanidins and other flavonoids in plasma lipid profile, have been widely demonstrated. Nevertheless, little is known regarding the molecular mechanisms underlying these effects.

Dietary polyphenols have been shown to interact with different signalling pathways in hepatic cells. For instance, the citrus flavonoid naringenin inhibits *microsomal triglyceride transfer protein* (MTP) expression and apoB secretion from HepG2 cells by acting through MAPK and PI3K pathways (8, 9). In addition, polyphenols are able to modulate both the expression and the transcriptional activity of numerous nuclear receptors, such as Pregnane X Receptor (PXR) (10), Liver X Receptor alpha (LXRα) (11) and Estrogen Receptors (ER) (12-14). These nuclear receptors play major roles in the control of lipid homeostasis, establishing a coordinated net of metabolic sensors

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which integrates lipid metabolism, inflammation, drug metabolism, bile acid synthesis and glucose homeostasis (15-20). The molecular structure of nuclear receptors provides them with the ability to act as metabolite sensors, being activated by endogenous or exogenous molecules, and subsequently modulating gene expression in a coordinate manner (21, 22). The ability of these nuclear receptors to modulate a wide battery of genes reveals them like targets for the treatment of different altered states such as diabetes or dyslipemia (20, 21, 23, 24).

We have previously shown that Small Heterodimer Partner (SHP, *Nr0b2*), an orphan nuclear receptor lacking the DNA binding domain, was upregulated in the liver of rats treated with GSPE, concomitantly with the reduction of plasma TG and apoB levels (7) and that SHP is a key mediator of the TG-lowering effect of procyanidins in HepG2 cells and mice (manuscript 3). SHP interacts with different nuclear receptors and transcription factors in order to modulate gene expression of steroidogenesis, lipogenesis, cholesterol and bile acid metabolism, glucose homeostasis and xenobiotic metabolism in liver (25). SHP gene expression is modulated by different transcriptional regulators (25). Among those, FXR has been widely studied as a classical activator of SHP gene transcription (26-28). FXR is as nuclear receptor able to enhance SHP expression when activated by bile acids. Once activated by FXR, SHP inhibits the expression of CYP7A1, the key enzyme in the biosynthesis of bile acids. This mechanism has been proposed as a negative feedback leading to the control of bile acid levels (18, 29). In addition, the use of transgenic mice lacking functional FXR, as well as other models where FXR activity is modified, has revealed that this nuclear receptor is a major controller of lipid and glucose metabolism (26, 30-32). Those mice lacking FXR presented impaired insulin sensitivity, and elevated levels of plasma and liver TG and cholesterol (31, 33-35). This abnormal control of plasma lipid content have been associated to the modulation exerted by FXR on a battery of genes related with TG-rich lipoproteins clearance, such as apolipoproteins CIII (36) and CII (37) and very low density lipoproteins receptor (VLDLr) (38). Furthermore, FXR also modulates the expression of different genes related to lipid synthesis (27). Thus, FXR activation has been proposed as a therapeutic target for ameliorating different lipid-altered states (23, 26, 31).

Since SHP is a key mediator of the hypolipidemic actions of GSPE in mouse and HepG2 cells (manuscript 3) and FXR controls lipid metabolism and SHP transcription,

this work was intended to assess the role of FXR mediating the hypotriglyceridemic response triggered by GSPE. With this aim, we have studied the TG lowering effect of GSPE in wild-type and $FXR^{-/-}$ mice, and demonstrated that GSPE hypotriglyceridemic action is abolished in mice lacking FXR. Moreover, liver whole genome expression analysis has revealed that several genes of the lipid synthesis program are downregulated by GSPE in a FXR-dependent fashion, explaining, at least in part, the FXR-dependent hypotriglyceridemic effect of GSPE. Using *in vitro* models, FXR-driven luciferase expresion studies have shown that FXR is synergistically activated by GSPE in the presence of bile acids, thus demonstrating that procyanidins signal through FXR

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MATERIALS AND METHODS

Chemicals

Grape seed procyanidin extracts (GSPE) were kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract contained essentially monomeric (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%), oligomeric (5-13 units) (35,7%) procyanidins and phenolic acids (4.22). Ligands used in the luciferase based study were from Sigma.

Cell culture

Human ephitelial cells (HeLa) and African green monkey fibroblasts (CV-1) were obtained from the American Type Culture Collection (Manassas, VA). HeLa and CV-1 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS.

In vitro interaction assays

The Gal4 DNA binding domain - receptor ligand-binding domain chimeras used in this study have been published previously (39). The full length nuclear receptor plasmids and corresponding luciferase reporter plasmids used were mouse FXR together with $(PLTP)_2$ TKluc reporter plasmid (40). For Gal4 assays 1.3 x 10^5 (HeLa) or 1 x 10^5 (CV-1) cells were plated in 24-well dishes with DMEM supplemented with 10% charcoal stripped serum. Cells were transfected using the calcium phosphate precipitation method. The next morning, cells were washed with phosphate buffered saline and ligands were added. All ligands were dissolved in DMSO while GSPE was dissolved in ethanol. Typically, transfections included 100 ng of the receptor plasmid, 200ng of the luciferase reporter plasmid, 10ng CDM-RXRα, 200ng of the β-galactosidase (β -gal) internal control plasmid with 490ng pGEM4 as carrier DNA to make a total of 1μg of plasmid DNA per well. Cells were assayed for luciferase (Promega) activities 24 hours after addition of ligands, and reporter expression was normalized to β-gal activity (Applied Biosystems, Chicago, IL) according to the manufacturers' instructions using a MLX luminometer (DYNEX technologies, Chantilly, VA, USA). Similar results were obtained from at least three independent experiments, each performed in triplicate.

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In vivo feeding studies

Mice were housed under standard conditions. Experimental procedures were approved by the local Committee for Care and Use of Laboratory Animals at Baylor College of Medicine. FXR-deficient mice were previously described (39), and were backcrossed with C57BL6 mice to the 10th generation. The correct genotype was verified using previously reported primer sequences and reaction conditions (39). Age-matched groups of 8-10 week-old male mice were used in all experiments (n=5 per experimental group). Mice were fed a standard rodent chow and water *ad libitum*. Experiments were performed for 14hrs and mice were fed either vehicle (water), or procyanidins (250 mg/L) by oral gavage. A first dose was administered at 9:00 pm and a second dose at 9:00 am. At the end of the experiment, mice were fasted for final 2 hours, and blood was collected from the orbital plexus after mice were anaesthetized with isoflurane. Tissues were snap frozen and stored at -80^{o}C until use. Plasma TG and cholesterol were assayed using enzymatic kits (QCA, Spain).

Microarray hybridization and RTqPCR.

Total RNA was obtained using Trizol reagent (invitrogen) following the manufacturer protocol. Additional purification and DNAse treatment was performed using NucleoSpin RNA2 kit (Macherie-Naegel, Germany). For microarray hybridization, RNAs from individual samples (5 per group) were pooled for the 4 different groups, wild-type vehicle, wild-type GSPE treated, FXR-/- vehicle and FXR-/- GSPE treated mice. The integrity of pooled RNAs was assessed by using the Agilent (Madrid, Spain) 2100 Bioanalyzer. For microarray hybridization, Cy3- or Cy5-labeled cDNA was obtained from each RNA pool by using the Agilent Low RNA Input Fluorescent Linear Amplification Kit as described in the Agilent manual (Part Number 5185-5818). Labeled cDNAs were hybridized against Agilent Mouse Oligo Microarrays (Part Number G4122A) following the Agilent 60-mer oligo microarray processing protocol. Images of hybridized microarrays were acquired with the Agilent G2565BA scanner, and data from the microarray images were obtained and analyzed with the Agilent Feature Extraction software. For each pair of RNA samples being compared, duplicate hybridizations with a dye-swap labeling was performed. For microarray validation, CYP7A1, MTP, APOAV, MT and SHP genes were analyzed by RTqPCR, using

GAPDH as endogenous control. RNA was retrotranscribed using Taqman Reverse transcription reagents kit (Applied Biosystems) and gene expression was evaluated in the Abi Prism 7300 SDS Real Time PCR system (Applied Biosystems) using SYBR green reagent (Applied Biosystems). Sequences can be provided upon request.

Microarray data processing and statistical analysis

A whole array of data was constructed matching each gene symbol or Genbank ID with its fold-change value from the microarray analysis. Genes were clustered into different biological processes using Panther software(41). The gene expression profile deviation of each biological process group from the whole array expression pattern was calculated using the Mann-Whitney U Test (Wilcoxon Rank-Sum test) as described (42), resulting a p-value. P-values under 0.05 were considered significant. From the resulting values using Mann-Whitney U Test, a second approach was used, consisting in fixing a fold-change threshold value of 0.7 and 1.5 for downregulation and upregulation respectively to identify genotype-dependent changes induced by GSPE. For luciferase-based studies, T-test analyses were performed using SPSS software.

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RESULTS

FXR is a key mediator of the hypotriglyceridemic effect of GSPE in mice

In order to gain insight into the relevance of FXR as a mediator of GSPE hypotriglyceridemic actions in an *in vivo* model, we compared the effects of GSPE administration in $FXR^{-/-}$ versus wild type mice. As described previously (32, 43), $FXR^{-/-}$ mice present elevated basal levels of plasma triglycerides and cholesterol when compared to wild type mice (figure 1). Oral GSPE gavage triggers a 40% reduction in plasma TG levels in wild type mice; whereas it does not modify plasma total cholesterol levels. This response to GSPE administration in plasma lipid profile is identical to that which we have previously found in rats (7). When GSPE is administered to FXR-/- mice, the hypotriglyceridemic effect is abolished. These results clearly show that FXR is a key mediator of the hypotriglyceridemic effects exerted by procyanidins *in vivo*.

GSPE induces changes in genes related to lipid, fatty acid and steroid metabolism in wild type but not $FXR^{-/-}$ mice.

To gain further insight into the FXR-dependent actions of GSPE, we next analyzed the differential response in gene expression changes induced by procyanidins in liver of wild type and $FXR^{-/-}$ mice using oligonucleotide microarray hybridization. The changes induced by GSPE treatment in the expression level of all genes, clustered by biological process, were analyzed using Panther software. In wild type mice, changes in genes clustered in the biological process "Lipid, fatty acid and steroid metabolism", including 747 genes, show a significant deviation (p-value 0.018) from the overall changes in gene expression patterns (figure 2). In contrast, in $FXR^{-/-}$ mice, this gene cluster does not significantly deviate (p-value 0.5) from the global change pattern. The significant deviation of genes in the "Lipid, fatty acid and steroid metabolism" group in the GSPE treated wild-type mice is due to an abnormal predominance of genes in the fold-change range from 0.5 to 0.8, indicating a down-regulation of numerous genes included in this metabolic group. Altogether, these results indicate that lipid metabolism is more strongly repressed by GSPE in wild type than in $FXR^{-/-}$ mice, thus pointing to FXR as a key mediator of the repression of lipid metabolism induced by procyanidins.

Next, in order to identify FXR-dependent genes involved in the hypotriglyceridemic effect of GSPE in wild type mouse, we selected those genes clustered into the "Lipid,

fatty acid and steroid metabolism" that were changed in wild-type mice but remained unaltered in $FXR^{-/-}$ mice, by setting a fold-change threshold of 1.5 for up-regulated and 0.7 for down-regulated genes (Table 1). In total, 34 lipogenic genes were identified showing FXR-dependent repression by GSPE, including key regulators of lipid synthesis pathways such as Sterol regulatory element binding protein 1 (SREBP1), 3-hydroxy-3-methylglutaryl-Coenzyme A synthase, acyl-CoA synthetase, stearoyl-Coenzyme A desaturase and (PPARγ)-interacting Protein. Other genes involved in lipoprotein metabolism, but not classified by Panther software in this cluster, were also changed in a FXR-dependent manner and were therefore included in table 1, such as ApoA5 and C/EBP beta. Remarkably, numerous genes (marked with an asterisk in table 1) which show an FXR-dependent response to GSPE, have been described as targets of SREBP1, a master regulator of lipid and lipoprotein metabolism (44-47). Therefore, SREBP1 emerges as a putative FXR-dependent effector of the hypotrycliceridemic response triggered by procyanidins.

GSPE synergistically enhances the activity of CDCA-bound FXR in CV-1 and HeLa cells.

The lack of hypotriglyceridemic effects of GSPE in FXR null mice, prompted us to test whether procyanidins can modulate FXR/RXR transcriptional activity, using CV-1 cells co-transfected with different constructs. Gal4 was used as a control, in order to discard interactions of GSPE with the DNA-binding domain of this protein (Figure 3A). GSPE displayed no significant effects on RXR activity as assayed using a RXR:LBD-Gal4:DBD chimera (figure 3B). In order to assess the interactions of GSPE with FXR, RXR expression plasmid was cotransfected with either a FXR:LBD-Gal4:DBD chimera (Figure 3C), or with full length FXR (Figure 3D). In both cases, GSPE alone was unable to activate FXR/RXR transcriptional activity. In contrast, GSPE co-incubation with CDCA, a natural ligand of FXR, showed enhanced transcriptional activity of FXR/RXR. This synergy was GSPE dose-dependent, reaching a 2-fold increase when cells are incubated with 100 mg/L of GSPE and CDCA compared with the CDCA treatment alone. These results point out that GSPE can enhance FXR activity only when the nuclear receptor is activated by CDCA. In contrast, GSPE did not increase the transcriptional activity of FXR induced by GW4064, a synthetic agonist of FXR (Figure 3D). In order to discard cell-specific actions of GSPE, the full length FXR construct

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along with RXR, were cotransfected in HeLa cells, achieving equivalent effects than those found in CV-1 (Figure 4).

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DISCUSSION

Different studies have been addressed to determine the role of post-prandial lipemia in the development of CVDs, revealing triglyceride-rich lipoproteins as important factors in the development of these pathologies(1, 2). Furthermore, hypertriglyceridemia itself has been revealed as an independent risk factor for coronary heart disease (3). In previous works, we have shown that GSPE display a potent hypotrigliceridemic effect in rats in the postprandial phase (7), and that the nuclear receptor SHP is a key mediator of these effects (manuscript 3). Expression of SHP is modulated by a wide array of transcriptional regulators, including the nuclear receptor FXR (25). The link between FXR and SHP has emerged as an important pathway in the modulation of bile acids, lipids and lipoproteins metabolisms (16, 27, 44). FXR plays a key role in the control of bile acids, glucose and plasma lipids homeostasis as has been demonstrated by the suppression of FXR activity in transgenic mouse models (16, 26, 32, 33). Consequently, FXR has been revealed as a key target in the treatment of different physiological altered states such as metabolic syndrome (48) or hyperlipidemia (33). Likewise, FXR activators are promising therapeutic agents for treatment of dyslipemias and diabetes mellitus (49, 50). Therefore, we wondered whether FXR could be implicated in the mediation of GSPE effects in addition to SHP. To gain insight into this hypothesis, the effects of procyanidins has been tested in wild-type and FXR-/- mice. Along with these *in vivo* models, *in vitro* FXR-driven reporter gene assay have been used in order to study the effects of GSPE in the modulation of FXR activity.

In basal conditions, FXR null mice display higher levels of plasma cholesterol and TG than wild type mice. These results are in agreement with previous works intended to study the lack of FXR in plasma parameters, thus highlighting the relevance of this nuclear receptor in the control of plasma lipid levels (23, 32, 50, 51). When wild type mice were treated with GSPE, plasma TG levels where drastically lowered, an equivalent effect to that we have previously described in rats (7). In contrast, the lack of FXR resulted in abolishment of the hypotriglyceridemic effects of GSPE. Therefore, the TG lowering actions of GSPE in mouse are mediated by FXR. In agreement with these results, different works have demonstrated that activation of FXR leads to the

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amelioration of dyslipemic states, by decreasing de novo lipogenesis and VLDL secretion from the liver, along with increased lipoprotein catabolism (50-52).

In addition, microarray analysis reported clear FXR-dependent effects of GSPE on the expression of lipid related genes in the liver. GSPE treatment downregulated the genes classified into the lipid, fatty acids and steroid metabolism cluster in wild-type mice but not in FXR-/- mice. These results point to a reduction of lipid synthesis exerted by GSPE in a FXR-dependent manner. Since liver lipid pool is a limiting factor in the synthesis and secretion of VLDLs by the liver (53-55), a decrease in liver lipogenesis would reduce the number of VLDLs or the triglyceride content of these lipoproteins. Thus, the results found at liver gene expression level could explain, at least in part, the FXR-dependent effects of GSPE in the plasma triglycerides of these mice.

Within the lipid, fatty acids and steroid metabolism cluster, a group of 34 genes was identified as changed by GSPE in a FXR-dependent manner. Among them, stands out SREBP1, a master regulator of the lipid synthesis program and lipoprotein metabolism (56-58), together with a number of SREBP1 target genes including stearoyl-Coenzyme A desaturase 2, stearoyl-Coenzyme A desaturase 1, sterol-C5-desaturase, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase, acyl-CoA synthetase short-chain family member 2, 7-dehydrocholesterol reductase, farnesyl diphosphate synthetase, CCAAT/enhancer binding protein beta, CCAAT/enhancer binding protein delta, phospholipid transfer protein and apolipoprotein A5. Altogether, these results suggest that FXR is essential for mediating the repression of SREBP1 and subsequent modulation of lipogenic genes, emerging as a plausible explanation for the hypotriglyceridemic effects of GSPE observed at plasma level. The FXR-dependent repression of SREBP1 expression has been described as the mechanism responsible for the TG lowering properties of bile acids in mouse (27). Also, in hyperlipidemic hamsters, activation of FXR by CDCA led to diminished de novo lipogenesis, ascribed to the repression of SREBP1 and stearoyl-Coenzyme A desaturase 1, and consequently, decreased liver lipid pool was translated into lowered plasma lipid levels (50). In addition, xanthohumol, a prenylflavonoid, has been shown to bind FXR and, once more, decrease liver fatty acid synthesis by repressing SREBP1 and its target genes (52). Therefore, our results are consistent with the different works reporting a correlation between FXR activation, SREBP1 repression and lipid synthesis

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reduction, suggesting that FXR activation by GSPE is responsible, at least in part, for the decreased plasma lipid levels.

The activation of FXR by GSPE has been studied in vitro. GSPE exerted a synergistic effect on the CDCA activated FXR. In the liver, the enterohepatic circulation and the intracellular production guarantee the presence of bile acids. Therefore, the synergistic actions of GSPE with CDCA are expected to occur in vivo. This synergistic effect of GSPE could be due to the recruitment of FXR coactivators induced by procyanidins. In this sense, FXR is thought to be prebound to DNA in a complex with corepressors. Once activated, it releases corepressors to bind coactivators such as TRRAP or PGC-1α (59, 60). Additionally, it has been shown that FXR modulation of gene transcription can be ligand-specific (61) and that FXR, broadly nuclear receptors, are able to bind coactivators in a ligand-specific fashion as well (62). With this background, it could be hypothesized that GSPE could be triggering the activation of FXR coactivators able to interact with FXR/RXR only in the presence of CDCA. This hypothesis is supported by the coincubation of cells with GSPE and GW4064, a synthetic specific FXR ligand. In the presence of this agonist, procyanidins could not synergistically enhance FXR activity. Altogether, these results suggest that the synergistic enhancement of bile acid-activated FXR activity by GSPE is conditioned to the presence of bile acids, a situation that mimics the in vivo physiological situation.

In conclusion, our in vitro experiments have shown that GSPE can synergistically enhance the activity of CDCA-activated FXR, while the studies performed in wild-type and FXR-/- mice demonstrate a key role of FXR in the hypotriglyceridemic actions of GSPE. Therefore, FXR activation could be underneath, at least in part, the beneficial actions of dietary procyanidins. The activation of FXR have been widely suggested for the treatment of hyperlipidemia and insulin resistance (26, 32, 33, 48, 51), thus placing dietary procyanidins as potential agents for preventing and ameliorating diabetes mellitus, metabolic syndrome, and cardiosvascular diseases that are associated to dyslipemic states. With this regard, this work provides new hints for understanding the molecular mechanisms underlying the cardioprotective effects of dietary procyanidins.

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TABLE 1. . **FXR-dependent changes induced by GSPE in lipid and lipoprotein related genes in mouse.**

Genebank ID	SREBP target	Gene name; symbol	WT	FXR-/-
NM_008903		phosphatidic acid phosphatase 2a;Ppap2a	0.6	0.8
NM_023556		mevalonate kinase;Mvk	0.7	0.9
NM_172769	*	sterol-C5-desaturase ; Sc5d	0.7	0.9
NM_145942	*	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1;Hmgcs1	0.7	0.9
NM_011374		ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1;St8sia1	0.7	0.9
NM_146197		acyl-CoA synthetase medium-chain family member 2;Acsm2	0.6	0.8
NM_008846		phosphatidylinositol-4-phosphate 5-kinase, type 1 alpha;Pip5k1a	0.7	0.9
NM_019811	*	acyl-CoA synthetase short-chain family member 2;Acss2	0.6	0.9
NM_207683		phosphatidylinositol 3-kinase, C2 domain containing, gamma polypeptide;Pik3c2g	0.7	1.0
NM_010941		NAD(P) dependent steroid dehydrogenase-like;Nsdhl	0.7	0.9
NM_009127	*	stearoyl-Coenzyme A desaturase 1;Scd1	0.6	1.1
NM_009605		adiponectin, C1Q and collagen domain containing;Adipoq	0.6	1.1
NM_007856	*	7-dehydrocholesterol reductase;Dhcr7	0.7	0.9
NM_028089		cytochrome P450, family 2, subfamily c, polypeptide 55;Cyp2c55	0.6	1.0
NM_018784		ST3 beta-galactoside alpha-2,3-sialyltransferase 6;St3gal6	0.7	1.3
NM_018830		N-acylsphingosine amidohydrolase 2;Asah2	0.5	1.2
NM_011480	*	sterol regulatory element binding factor 1;Srebf1	0.7	1.0
NM_134469	*	farnesyl diphosphate synthetase;Fdps	0.7	0.9
NM_013634		peroxisome proliferator activated receptor binding protein;Pparbp	0.7	1.0
NM_009128		stearoyl-Coenzyme A desaturase 2;Scd2	0.7	1.0
NM_019677		phospholipase C, beta 1;Plcb1	0.7	1.0
NM_008845		phosphatidylinositol-4-phosphate 5-kinase, type II, alpha;Pip5k2a	0.7	0.9

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NM_138656		mevalonate (diphospho) decarboxylase;Mvd	0.7	0.9
NM_008255	*	3-hydroxy-3-methylglutaryl-Coenzyme A reductase;Hmgcr	0.7	0.8
NM_153389		ATPase, Class V, type 10D;Atp10d	0.7	1.0
NM_175443		ethanolamine kinase 2;Etnk2	0.7	0.9
NM_013490		choline kinase alpha;Chka	0.7	0.8
NM_028057		cytochrome b5 reductase 1;Cyb5r1	0.7	1.5
NM_008963		prostaglandin D2 synthase (brain);Ptgds	0.7	0.9
NM_026784		phosphomevalonate kinase;Pmvk	0.7	0.9
NM_011372		ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3;St6galnac3	0.7	0.9
NM_012054		acyloxyacyl hydrolase;Aoah	0.6	1.0
Other SREBP1 target genes (not included in Panther classification)				
NM_080434	*	Apolipoprotein A5; ApoA5	1.4 **1.7**	1.0 **1.0**
NM_009883	*	CCAAT/enhancer binding protein (C/EBP), beta; Cebpb	0.7	0.8

Wild type and SHP$^{-/-}$ mice were fed either vehicle or GSPE (250 mg/Kg) via oral gavage (n=5 in each group, age 8-10 week). After 14 hours treatment, liver total RNA from the 4 groups was obtained and pooled. Microarray data was obtained by comparing gene expression of WT control versus WT GSPE-treated mice and SHP$^{-/-}$ control versus SHP$^{-/-}$ GSPE-treated mice. The whole microarray fold-changes were processed using Panther software in order to identify SHP-dependent changes induced by GSPE in genes clustered in the "Lipid, fatty acid and steroid metabolism" metabolic pathway. Fold-change thresholds were fixed as 0.7 and 1.5 for down-regulation and up-regulation respectively. Real time quantitative PCR was performed with selected genes to confirm the microarray data (shown in bold characters). Known SREBP1 target genes are denoted by *****. WT column: fold-change in expression induced by GSPE in wild-type mice versus vehicle. SHP$^{-/-}$ column: fold-change in expression induced by GSPE in SHP$^{-/-}$ mice versus vehicle.

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FIGURE LEGENDS

Figure 1. Effect of GSPE on plasma triglyceride and cholesterol levels in wild-type and FXR$^{-/-}$ mice.

Wild type (WT) and FXR$^{-/-}$ mice were fed with vehicle (control) or GSPE (250 mg/Kg) via oral gavage (n=5 in each group, age 8-10 week). Plasma was obtained 14 hours after treatment and TG and cholesterol were determined as described in materials and methods. * denotes significant differences versus control ($p< 0.05$).

Figure 2. Effect of GSPE on expression of genes clustered into the "Lipid, fatty acid and steroid metabolism" in wild-type versus FXR$^{-/-}$ mice.

Wild type (WT) or FXR$^{-/-}$ mice were fed either vehicle (control) or GSPE (250 mg/Kg) via oral gavage (n=5 in each group, age 8-10 week). After 14 hours treatment, liver total RNA from individual mice in the different groups was obtained and pooled. Microarray data was obtained by comparing gene expression of WT control versus WT GSPE-treated mice and FXR$^{-/-}$ control versus FXR$^{-/-}$ GSPE-treated mice. The whole microarray fold-changes were processed using Panther software. The plot of the cluster "lipid, fatty acid and steroid metabolism" against the whole microarray fold-changes plot is shown for the two genotypes. Number: number of genes matched to Lipid, fatty acid and steroid metabolism by Panther software; OverUnder: tendency of the cluster deviation from the whole array expression pattern, namely upregulation tendency (+) and downregulation tendency (-); A $p<0.05$, calculated using the Mann-Whitney U Test, is considered significant.

Figure 3. **Effects of GSPE in the interaction assays for FXR and RXR in CV-1 cells**. (A)CV-1 cells were transfected with Gal4 expression plasmid along with Gal4 luciferase reporter plasmid. Transfected cells were treated with the indicated concentrations of GSPE. (B) RXR interactions with GSPE were assayed using the Gal4:DBD-RXR:LBD expression vector, along with the Gal4 luciferase reporter plasmid. Transfected cells were treated with 9-cis-retinoic acid 1mM (+) and GSPE at the indicated concentrations or vehicle (-). (C) To study FXR/RXR interactions with GSPE, the Gal4:DBD-FXR:LBD expression vector and the Gal4 luciferase reporter plasmid were used. Ligands for FXR were CDCA 100μM. GSPE was added in the indicated concentrations. (D) A full length FXR expression plasmid along with the reporter construct $(PLTP)_2$ TKluc were co-transfected to study GSPE interactions with

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the FXR/RXR heterodimer. Ligands for FXR were CDCA 100μM or GW4064 1μM. GSPE was added in the indicated concentrations. All controls (-) were treated with the respective vehicles in a concentration lower than 0.1%. All transfections included the expression vector for RXR to allow the formation of heterodimers, and CMX-β-Gal as internal control. Values are represented as fold-change respect control values and the standard error mean from three independent experiments is represented. * denotes significant differences at the $p<0.05$ level versus the CDCA treatment.

Figure 4. **Effects of GSPE in the interaction assays for FXR in HeLa cells** HeLa cells were transfected with Full length FXR along with RXR expression vectors and the luciferase reporter construct $(PLTP)_2$ TKluc. All transfections included CMX-β-Gal as internal control. Transfected cells were treated with CDCA 100 μM or GW4064 1 μM and/or GSPE in the indicated concentrations or vehicle (-) at concentration lower than 0.1%. Values are represented as fold-change respect control values. All controls were treated with the respective vehicles DMSO, EtOH or both in a concentration lower than 0.1%. Standard error mean from three independent experiments is represented. * denotes significant differences at the $p<0.05$ level versus the CDCA treatment.

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Figure 1

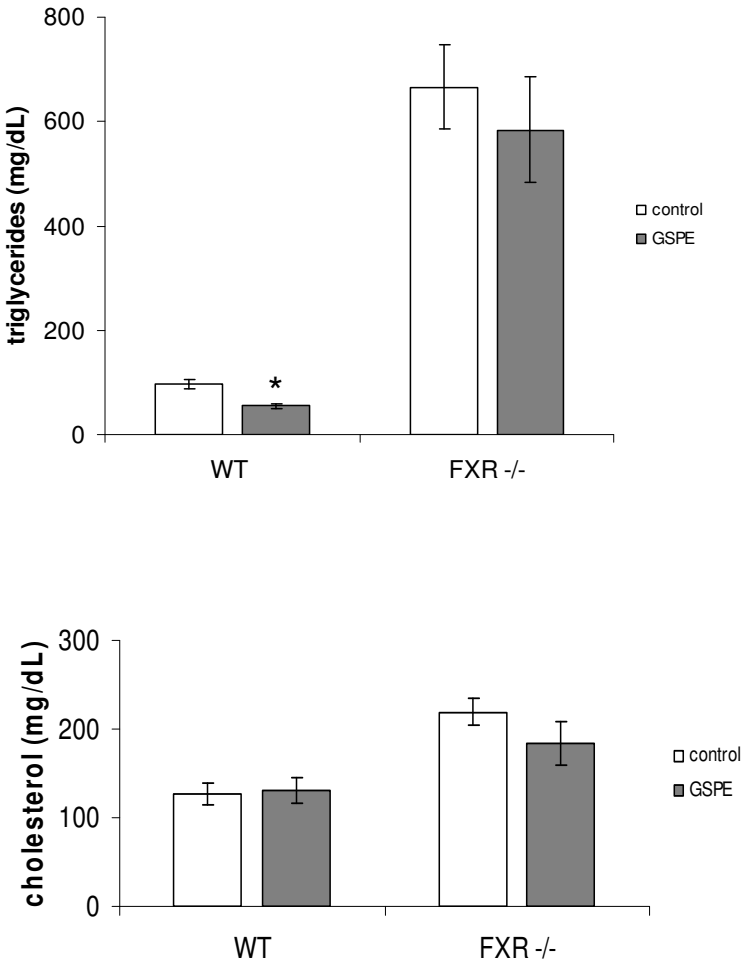

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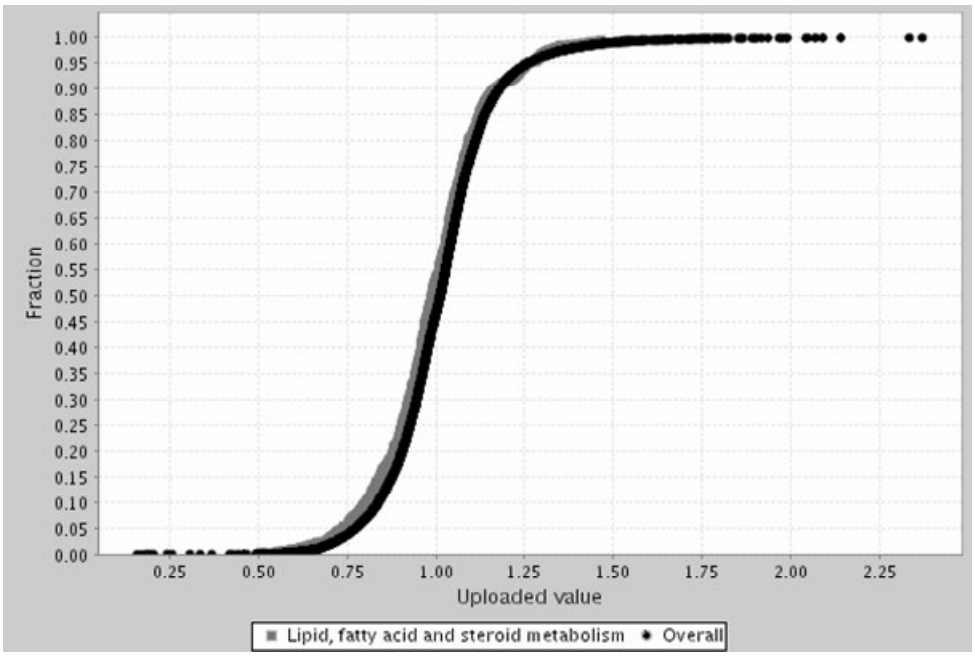
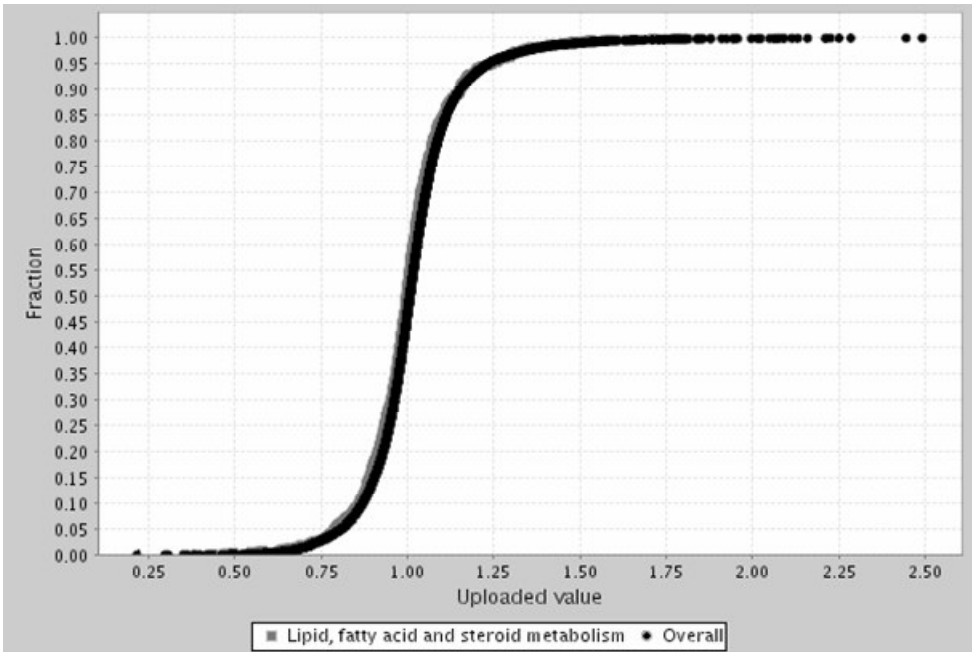
Figure 2

WT



FXR$^{-/-}$



	WT			FXR -/-		
Biological Process	number	overUnder	pvalue	number	overUnder	pvalue
Lipid, fatty acid and steroid metabolism	747	-	1.82E-02	747	-	5.20E-01

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Figure 2

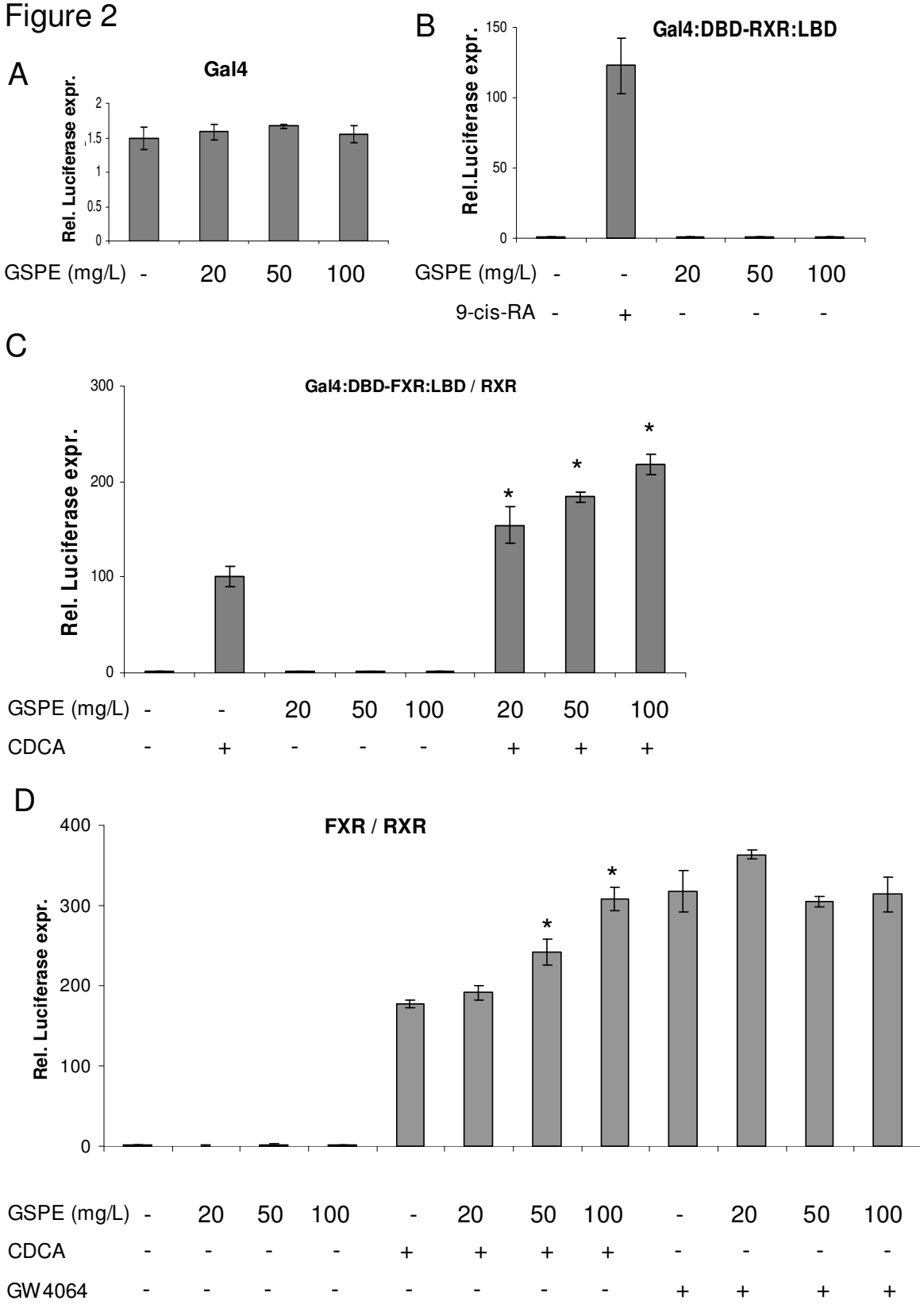

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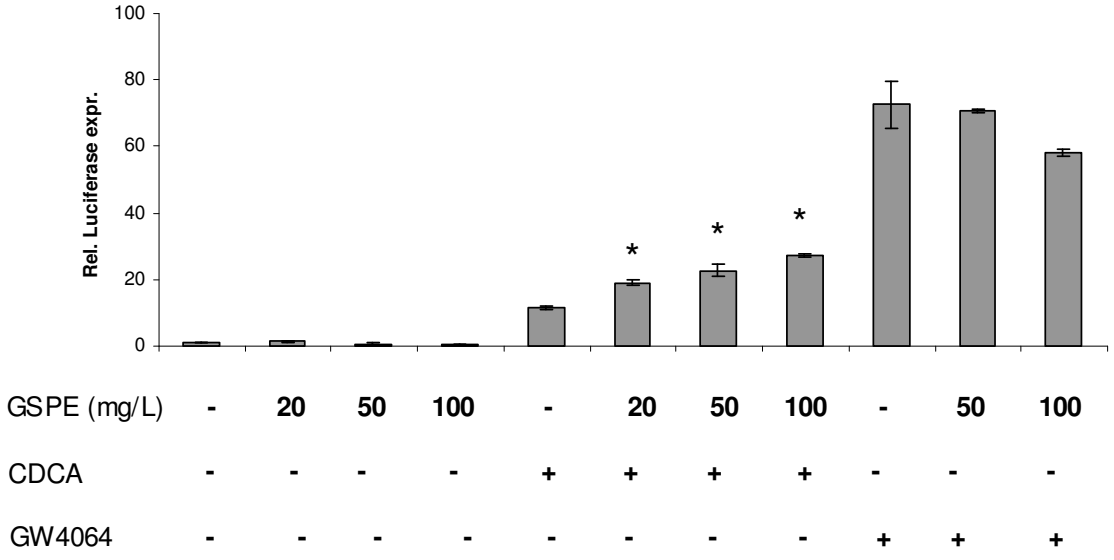
Figure 3



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IV. GENERAL DISCUSSION

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Different studies have been addressed to assess the mechanisms underlying the hypolipidemic actions of dietary procyanidins (1-7). These compounds are able to decrease plasma cholesterol and TG levels acting at different levels, inhibiting lipid absorption by the intestine (8-10) and the synthesis of VLDL in the liver (1, 11, 12), as well as enhancing the catabolism and clearance of plasma lipoproteins (3, 13). Together with the intestine, liver presides over the metabolism and homeostasis of plasma lipids, placing this organ as a target for the study of procyanidins actions. Thus, many studies have been addressed to reveal the activity of these polyphenolic compounds in the liver. As a result, evidences have emerged pointing out that flavonoids may modulate expression and activity of different genes involved in lipid synthesis and lipoprotein secretion. (1, 14-16). Nevertheless, little is known about intracellular mediators of flavonoids actions. The aim of this Ph.D. Thesis were i) to gain insight into the initial effects underlying the long-term antiatherogenic and cardioprotective effects ascribed to procyanidins and ii) to identify liver intracellular signaling pathways involved in the hypotriglyceridemic actions of procyanidins. With these purposes, three experimental models have been used: rat, mice and the human hepatocyte cell line HepG2. In these models, the bioactivity of a Grape Seed Procyanidin Extract (GSPE), and the molecular mechanisms involved, have been studied using acute and non-toxic (17) doses.

First, healthy rats fed with a standard chow diet were submitted to an acute oral GSPE treatment. Procyanidins drastically reduced triglyceride-rich-lipoprotein (TRL) levels in plasma 5 hours after treatment, as pointed out by the lowered amounts of plasma TG and ApoB. In addition, GSPE affected the distribution of cholesterol in the different lipoprotein fractions. Thus, levels of nonHDL:nonLDL-cholesterol and LDL-cholesterol decreased, while HDL-cholesterol increased. In agreement with these results, other authors have also demonstrated hypolipidemic actions of grape polyphenols (6, 7, 18). Concomitantly, changes in liver gene expression could explain, at least in part, the lipoprotein profile associated to GSPE administration, by reducing VLDL secretion and cholesterol conversion into bile acids in the liver. Importantly, expression of Small Heterodimer Partner (SHP) was enhanced by GSPE. This nuclear receptor has recently emerged as an important regulator of several genes involved in lipid and lipoprotein metabolism in the liver. Thus, bile acids repress VLDL secretion by hepatocytes via a pathway involving SHP in mice and HepG2 cells (19, 20), and humans with naturally

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occurring mutations in SHP develop mild-obesity (21). Nevertheless, whereas transient inductions of SHP result in lowered TG secretion, transgenic mice overexpressing SHP show increased levels of hepatic TG. This observation was ascribed to the inbalance in bile acid metabolism caused by an abnormal continous overexpression of SHP (22). Therefore, the role of SHP in TG metabolism is well established, but only partially understood. Altogether, our results suggested that liver could be orchestrating, at least in part, the hypotriglyceridemic actions of GSPE, and that SHP could be mediating these effects.

In addition, changes at the liver gene expression level revealed that many markers of the inflammatory process were ameliorated by GSPE treatment. Thus, acute phase proteins and components of the IL-6 and NF-kB signalling pathways emerged as targets of procyanidins, along with the transcription factor Foxa2, which has been related with inflammation, glucose and lipid homeostasis control (23-26). Therefore, beyond the lipid lowering actions, procyanidins are able to modulate inflammation, acting at two complementary levels against the factors leading to atherosclerosis. Hypotriglyceridemia and the anti-inflammatory liver gene expression profile triggered by procyanidins *in vivo* are especially relevant considering that they have been found in the postprandial phase, a proinflammatory and proatherogenic situation due to the transient increment in the levels of glucose, TG and proinflammatory cytokines (27-29). Humans in Western societies are in the postprandial condition most of the time and, thus, consumption of procyanidin-rich foods should help to prevent and ameliorate chronic metabolic disorders associated to inflammatory and atherogenic processes.

In order to assess the implication of liver in the plasma TG lowering effect of procyanidins, the human hepatoma cell line HepG2 was used, since these cells are a valid system for the study of lipid synthesis and secretion (30-32). GSPE lowered VLDL secretion in HepG2 as a consequence of both, a reduction in the *de novo* synthesis of TG and the expression of Microsomal Triglyceride-transfer Protein (MTP), which are key factors in the TRL synthesis process (33, 34). In agreement with these findings, various studies have revealed that procyanidins and other flavonoids decrease VLDL secretion in this cell line (1, 35-37). Moreover, the treatments of HepG2 cells with GSPE have placed SHP as a direct target of procyanidins, being rapidly induced in cultured hepatocytes. Altogether, these results are consistent with the effects observed

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in rats, and reinforce the idea that GSPE can act directly in hepatocytes without the need to invoke the intervention of other tissues.

In order to elucidate whether SHP is the mediator of the lipid lowering activity of GSPE, two different systems to block SHP activity were used: human hepatoma cells transfected with SHP-specific siRNA, and transgenic SHP knockout mouse. 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 procyanidins. Gene silencing of SHP in HepG2 cells has allowed us to identify two different pathways for GSPE actions upon VLDL secretion: a SHP-dependent mechanism leading to a decreased TG secretion, and a SHP-independent pathway responsible for MTP downregulation and, subsequently, diminished ApoB secretion. It has been shown that flavonoids can signal through MAPK and insulin related pathways to inhibit MTP expression and ApoB secretion (1, 11), thus providing a plausible explanation for SHP-independent GSPE actions. Both mechanisms would converge in the synthesis process of TRL, decreasing the amount of VLDL secreted by the hepatocyte. Therefore, SHP has emerged as a key mediator of the TG lowering actions of GSPE in HepG2 cells.

When wild type mice were treated with an acute dose of GSPE, plasma TG levels were drastically reduced, extending the observation made in rats. In contrast, in $SHP^{-/-}$ mice the hypotriglyceridemic actions of procyanidins were abolished, reinforcing the key role of SHP in mediating this effect of GSPE. Microarray based comparison of liver gene expression profiles in wild-type and $SHP^{-/-}$ mice has revealed that GSPE downregulates many genes involved in lipid and lipoprotein synthesis in a SHP-dependent fashion. Lipid availability is a rate limiting factor in the synthesis and secretion of TRL from the liver (38, 39). Thus, these results can explain, at least in part, the SHP-dependent hypotriglyceridemic actions of procyanidins, which would be related, among other factors, with reduced synthesis of VLDL in the liver.

Many works have described that expression of SHP is subjected to the control of different nuclear receptors such as LRH-1, ERα and FXR (40-42). Previous studies using transgenic mice lacking functional FXR, have revealed that this nuclear receptor is a major controller of lipid and glucose metabolism (43-46). Those mice lacking FXR

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presented impaired insulin sensitivity, and elevated levels of plasma and liver TG and cholesterol (43, 47-49). Therefore, we hypothesized that FXR could be mediating hypotriglyceridemic GSPE actions upstream SHP. *In vitro* luciferase based studies have revealed that GSPE enhance FXR activity in the presence of bile acids, a situation that mimics the *in vivo* physiological condition of hepatocytes. Thus, $FXR^{-/-}$ mice were used to assess the role of FXR in mediating 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*.

The comparison between changes induced by GSPE at the liver gene expression level in SHP-/- mice with those induced in FXR-/- mice, reveals that all genes that were changed in a SHP-dependent fashion are included into the FXR-dependent changes. This evidence reinforces the role of FXR as 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 could modulate the expression, in addition to SHP, of other FXR target genes, which could also mediate GSPE actions.

In summary, GSPE exert lipid-lowering effects in three different systems: rat, mice and HepG2 cells. The mechanism of action of procyanidins involves FXR and SHP, a pathway leading to lowered lipogenesis and secretion of VLDL in the liver, as shown in figure 7. The FXR/SHP pathway has been shown to have crucial roles in controlling bile acid (50), lipoprotein (43, 47, 48, 51) and glucose metabolisms (52-55). In this sense, this work provides new hints for the understanding of the mechanisms leading to the actions promoted by procyanidins, which emerge as powerful agents in the prevention and treatment of lipid altered metabolic states.

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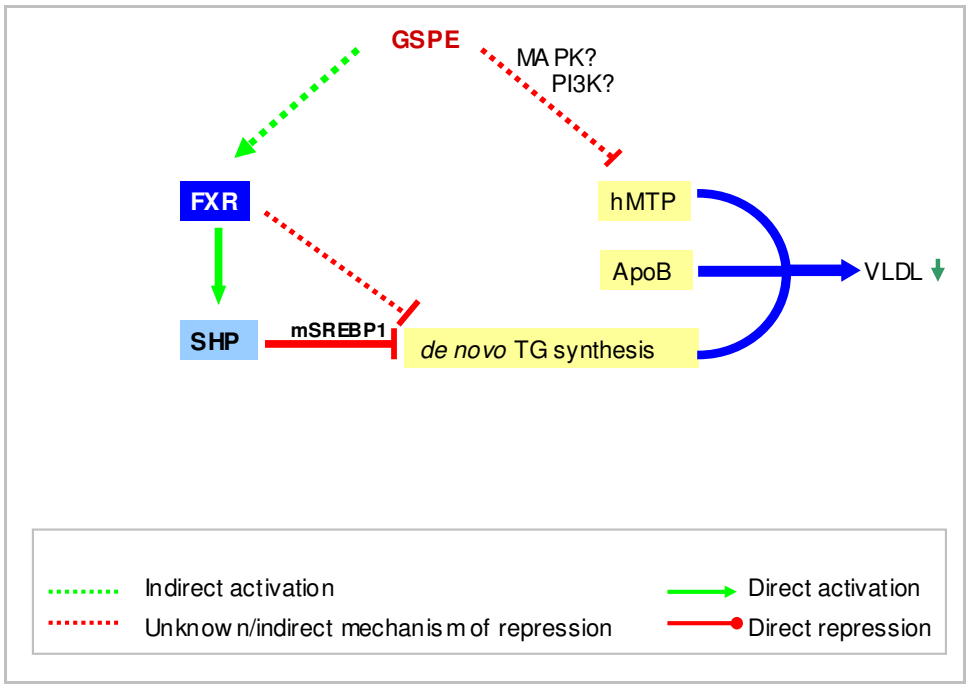

Figure 7. Proposed pathways used by procyanidins to reduce VLDL secretion by hepatocytes.

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V. CONCLUSIONS

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1. **Procyanidins improve plasma lipid profile in the postprandial phase in rats**. A single oral dose of procyanidins decreases plasma triglycerides and ApoB levels to 50% of control values. In addition LDL-Cholesterol is significantly reduced, thus improving the atherosclerotic risk index.

2. **Procyanidins display a triglyceride-lowering effect both in vivo and in vitro**. In rat and mouse, procyanidin treatment triggers a hypotriglyceridemic response. In HepG2 cultures, procyanidins down-regulate the secretion of triglycerides and ApoB, thus showing that these flavonoids act directly on hepatic cells. This fact strongly suggests that, in vivo, a direct action of procyanidins on the liver contributes to their hypotriglyceridemic response.

3. **Nuclear receptor Small Heterodimer Partner (SHP) is a target of procyanidins in hepatic cells**. Procyanidins modulate the expression of SHP, rapidly increasing its expression in rat liver as well as in HepG2 cultured cells.

4. **SHP mediates the triglyceride-lowering activity of procyanidins in vitro and in vivo.** When SHP expression is silenced in HepG2 or abolished in SHP-null mice, procyanidins lose their hypotriglyceridemic activity. In contrast, in SHP-silenced HepG2 cells, procyanidins are still able to reduce apoB secretion. Hence, procyanidins reduce triglyceride via a SHP-dependent mechanism, whereas they reduce apoB in a SHP-independent manner.

5. **Nuclear receptor Farnesoid X Receptor (FXR) is an essential mediator of the hypotriglyceridemic action of procyanidins upstream SHP**. Oral gavage of procyanidins to FXR-null mice have not a hypotriglyceridemic effect. Moreover, luciferase based *in vitro* assays showed that procyanidins increase the transcriptional activity of FXR. Thus, FXR is an essential component of the signalling pathway used by procyanidins to elicit the triglyceride lowering effect.

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6. **Key genes of the inflammation process are targets of procyanidins in liver, in the postprandial phase**. Oral administration of procyanidins to rats rapidly downregulates the expression, in liver, of transcription factor Egr1, a mediator of the hepatic inflammatory response, and several acute-phase proteins, namely haptoglobin, fibrinogen B and alpha-1 antitrypsin. In addition, expression of DUSP6, a component of the ERK1/2 subfamily of MAPK, is repressed by this treatment. Nfkbia, a repressor of NF-kB activity, is overexpressed upon procyanidin treatment. This expression pattern strongly suggests that procyanidins attenuate the pro-inflammatory state associated to the postprandial phase.

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