Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats

Authors:

Josep Maria Del Bas, Juan Fernández-Larrea, Mayte Blay, Anna Ardèvol, Maria Josepa Salvadó, Lluis Arola, and Cinta Bladé

Affiliation:

Departament de Bioquímica i Biotecnologia, CeRTA, Universitat Rovira i Virgili, 43005 Tarragona, Spain

Corresponding author: Cinta Bladé, Departament de Bioquímica i Biotecnologia, Universitat Rovira i Virgili, Plaça Imperial Tarraco, 1, 43005 Tarragona, Spain. E-mail: mcbs@astor.urv.es

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 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 7a-

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.

Key words: red wine, cholesterol, triglycerides, microarrays, lipoprotein lipase, apoB

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 LDLs 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 comprise 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 turn, 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 underlies and follows these actions of RWPs on the components of the vascular system (17, 19, 20-22). Much less is known about the mechanisms that underlie 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 LDL and chylomicron remnants) in different animal models. Thus, moderate and chronic consumption of red wine, but not of alcohol, reduces 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 it increases mRNA expression of the 3-hydroxy-3-methylgluteryl CoA (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-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). Altogether, these results suggest that RWPs, when consumed during meals, 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 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 that 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).

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, 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 nontoxic (36) dose of grape seed procyanidin extract (GSPE) to chow-fed male rats and analyzed plasma lipid and lipoprotein profile after 5 h. Changes in the gene expression pattern in the liver of GSPE-treated animals were analyzed using microarray hybridizations to identify procyanidin target genes involved in lipoprotein metabolism.

MATERIALS AND METHODS

Chemical

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 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. At 11 a.m. on experimental day, the rats (6 animals/group) were fed an oral gavage of GSPE in aqueous solution (250 mg/kg body wt.; GSPE group) or were fed an oral gavage with vehicle (tap water; Control group). The used procyanidin dose is one-fifth of the no-observed-adverse-effect level (NOAEL) 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). Five hours after treatment, the rats were killed by beheading, and blood was collected using heparin as anticoagulant. Plasma was obtained by centrifugation and stored at -80°C until analysis. Liver, muscle, and adipose tissue were excised, frozen immediately in liquid nitrogen, and stored at -80°C 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). TGs were assayed using an enzymatic colorimetric kit (QCA). Lipids of liver were extracted by the Folch method (40). An aliquot of the lipid extract was used to measure total lipids by gravimetry (in duplicate). The rest of the extract was evaporated to dryness and redissolved 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 B-hydroxybutyrate was analyzed using enzymatic kits (Ben srl.). FFA and glucose were measured using enzymatic colorimetric kits (Wako Chemicals GmbH and QCA).

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 (0.5 M Tris-HCl, 10% glycerol, 2% SDS, B-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 and Schuell, Keene, NH). Membranes were blocked overnight and incubated with a goat anti-rat apoB antibody (Santa Cruz Biotechnology, Santa Cruz, California) as a primary antibody. The antybody used for this purpose was raised against the N 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 horseraddish 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 Bio-Rad after background subtraction. Purified apoB 100 was used as an internal standard for normalizing apoB bands.

Statistical analysis

Results are reported as means ± SE of six animals. Comparison among groups mean was done by independent-samples t test ($P < 0.05$) by SPSS software.

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 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 Microarrays 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.

Changes in mRNA expression of selected genes were verified by quantitative PCR. CDNA 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, Rn00564065 ml for CYP7A1, Rn00565598_m1 for HMG-CoA reductase, Rn00561482_ml for LPL), the TaqMan PCR Core Reagent Kit, and the GeneAmpR 5700 Sequence Detection System, as recommended by the manufacturers. Quadruplicated quantifications, performed in singleplex assays, were performed 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_ml).

RESULTS

Plasma and liver parameters

TG levels in the GSPE group were reduced to 50% vs. the control group 5 h after treatment (Table 1). In addition, TC levels and TC 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. In addition, whereas 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). Whereas 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 60%. The observed decrease resulted from a 50% decrease of apoB-48 isoform and a 10% increase of the apoB-100 isoform.

B-Hydroxybutirate and glucose were measured as fed state indicators. Whereas no differences were observed between control and GSPE groups respecting glucose and B-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, TG, TC, free cholesterol, and esterified cholesterol were quantified (Table 2). No changes were observed in rats after the 5 h GSPE treatment vs. the control group.

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 microarray and the changes observed in its expression by the GSPE treatment.

No changes were found in the expression of FA synthesis enzymes and FA oxidation. Despite that the expression of cholesterol synthesis pathway key enzymes was increased in the GSPE-treated rats, mRNA levels of the cholesterol estherification enzymes were not affected. Bile acid pathway controlling the CYP7A1, CYP8b1, and CYP27A gene expression of enzymes was affected in a different manner. Whereas 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

196 expression, with a fold change of 0.67. Lipoprotein related proteins and receptors did not show
197 considerable changes, except a 1.2-fold change in the case of apobec-1 complementation factor.
198 Concerning lipid related nuclear transcription factors expression, only SHP (Nr0b2) was changed,
199 showing a drastic increase (close to 3-fold change).

200 Given that the plasma TG levels markedly depend on the activity of extrahepatic lipases, we quantified
201 LPL expression in adipose tissue and muscle. As shown in Table 4, muscle LPL mRNA was increased
202 1.57-fold, whereas adipose tissue LPL mRNA was decreased 0.57-fold.

203 DISCUSSION

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

218 The observed 50% reduction in plasma TG and plasma apoB content, together with the lowered
219 nonHDL:nonLDL-C level, indicates that the number of apoB-containing TG-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 predominantly 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 TG and FA biosynthetic enzymes was modified in 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 do not have 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 TG and FFA reduction as well as the increase in HDL-C concentration.

Little is known about the mechanism of apoAll 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 apoAll 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).

apoC-III is a key player in plasma TG 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 TGs, 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 TG-rich particles by several mechanisms (62, 65, 66), including inhibition of lipoprotein binding to the cell surface glycosaminoglycan matrix (61, 66) and lipolysis by 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, in 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-C, and the slight decrease in TC, points 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 threefold increase in liver mRNA level of CYP7A1, the rate-limiting enzyme in bile acid synthesis, the production of which is tightly controlled at the transcriptional level (68-70). 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 RWPs simultaneously decrease cholesterol content and increase the mRNA of HMG-CoA reductase in HepG2 cells, and it 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 acid 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) gamma and alpha (33, 75), hepatocyte nuclear factor-4 (HNF-4) (76), a-fetoprotein/LRH-1 (77), retinoid X receptor (RXR) (76), and liver X receptor (LXR) (78), all of which are 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, 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 threefold increase in liver SHP mRNA levels occurs concomitantly with a 50% reduction in plasma TG levels in GSPE-treated rats. Several studies have described previously 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 act 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 TG 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, while 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, while 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 ERa to the ERE present in the SHP gene promoter, which overlaps with the FXR binding (86); the elevated SHP expression induced by ERa 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 17B-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 act as a 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 ERa, it might be possible that procyanidins act as ligands of FXR, RXR, or ERa. 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 nontoxic 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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606 # FIGURE LEGENDS

607

608 Figure 1 Caption: Total cholesterol distribution among different lipoprotein fractions.
609 Experimental procedure was the same as indicated in Table 1. Results are shown as percentage of total
610 cholesterol in HDL, LDL, and nonHDL:nonLDL lipoproteins. Results are means +/- SE (n=6).
611 *Significant difference ($P < 0.05$) vs. control value using independent samples t test.
612

613 Figure 2 Caption: Plasma total apolipoprotein B, apolipoprotein B-100 and apolipoprotein B-48 in control
614 rats and grape seed procyanidin extract (GSPE)-treated rats.
615 A) Representative ApoB-48 and ApoB-100 inmunoblotting of a control (lanes 1 and 2), GSPE (lanes 4
616 and 5) plasma samples and apoB-100 standard (lane 3). Rats were treated with an oral gavage of GSPE
617 (250 mg/kg body wt) and killed after 5 h. Plasma samples (120 ug of protein) and apoB-100 standard (0.2
618 ug) were subjected to SDS-PAGE and transferred onto PVDF membrane for inmunoblotting with goat
619 antiapoB antibody. The antibody used with this purpose was raised against the N terminus of apoB so
620 both isoforms, apoB-100 and apoB-48, could be detected and quantified simultaneously in a single
621 plasma sample.
622 B) Amounts of plasma total-ApoB, ApoB-48 and ApoB-100 in control group and GSPE treated group.
623 ApoB-100 standard was used to quantify the relative amount of apoB-100 and apoB-48 using Quantity
624 One software from Bio-Rad. Results are means +/- SE (n=6). *Significant difference at the $P < 0.05$ level
625 vs. control value.

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629 # TABLES

630 **Table 1.** Plasma lipids and fasted state markers analysis in control rats and GSPE-treated rats.

	Control	**GSPE**
Triglyceride (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*
B-Hydroxybutirate (mg/dl)	2.2 ± 0.4	2.6 ± 0.6

631 Rats were treated with an oral gavage of grape seed procyanidin extract (GSPE; 250 mg/kg body wt.) and
632 killed after 5 h. Plasma lipids were analyzed as described in Materials and Methods. Results are means ±
633 SE (n=6). *Significant difference ($P < 0.05$) vs. control value using independent samples t test.

634 **Table 2.** Liver lipid analysis in control rats and GSPE-treated rats.

	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

635 Rats were treated with an oral gavage of GSPE (250 mg/kg body wt.) and killed after 5 h. Liver lipids
636 were analyzed as described in Materials and Methods after chloroform/methanol extraction. Results are
637 means ± SE (n=6). No statistical differences were found between control rats and GSPE treated rats at the
638 $P < 0.05$ level.

639 **Table 3.** Changes in mRNA levels of lipid related genes in liver of rats treated with GSPE vs. control
640 rats.

GenBank ID	Gene	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-CoA 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-CoA	1.00	0.01

	carboxylase (Acacb)		
NM_022193	Acetyl-CoA carboxylase (Acac)	1.00	0.04
Fatty acid B-oxidation key regulator			
NM_012930	Carnitine palmitoyltransferase 2 (Cpt2)	1.00	0.02
NM_031559	Carnitine palmitoyltransferase 1 (Cptla)	1.00	0.08
Cholesterol esther synthesis			
BF542749	Acyl-CoA:cholesterol acyltransferase (ACACT)	1.00	0.01
NM_031118	Acyl-CoA:cholesterol acyltransferase (Soat1)	1.00	0.00
Apolipoproteins			

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	Low density lipoprotein receptor related protein	1.00	0.08
CB606214	Low density lipoprotein receptor-related protein 8. apolipoprotein E receptor	1.00	0.06
CB546853	Low density lipoprotein receptor-related protein 3	1.00	0.11
NM_053541	Very low density lipoprotein receptor	1.00	0.01
NM_013155	Low density lipoprotein receptor-related protein 6	1.10	0.17

BF548789	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
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	0.95	0.07

	factor 3 (HNF3a)		
NM_012742	Hepatocyte nuclear factor 4 (HNF4)	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 (LXRbeta)	1.08	0.04
NM_031627	Nuclear receptor subfamily 1, group H, member 3 (Nr1h3) Liver X receptor (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 (PPARalpha)	1.00	0.33
NM_012805	Retinoid X receptor (RXRalpha)	1.00	0.11
AF016387.1	Retinoid X receptor gamma (RXRgamma)	1.00	0.10
AW916150	Sterol regulatory element binding	0.97	0.01

	protein-2 (SREBP-2)		

Rats were treated with an oral gavage of GSPE (250 mg/kg body wt.) and killed 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 is 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 italics) since its probe was absent from the microarray.

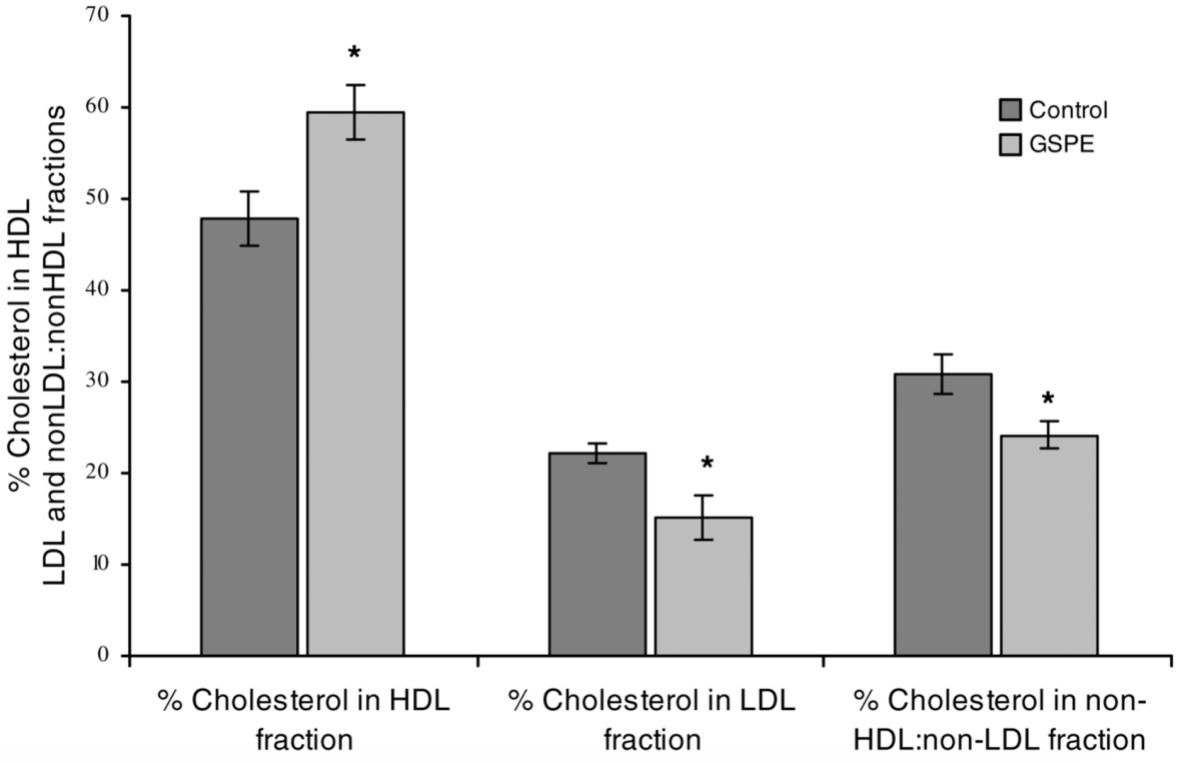
Table 4. Changes in muscle and adipose tissue mRNA levels of LPL from rats treated with GSPE vs. control rats.

Gene	**Fold Change**
Muscle LPL	1.57
Adipose tissue LPL	0.57

Rats were treated with an oral gavage of GSPE (250 mg/kg body wt.) and killed after 5 h. Equal amounts of liver RNA from 3 animals were pooled and mRNA levels of lipoprotein lipase (LPL) were determined by real time quantitative PCR.

654 Figure 1

655



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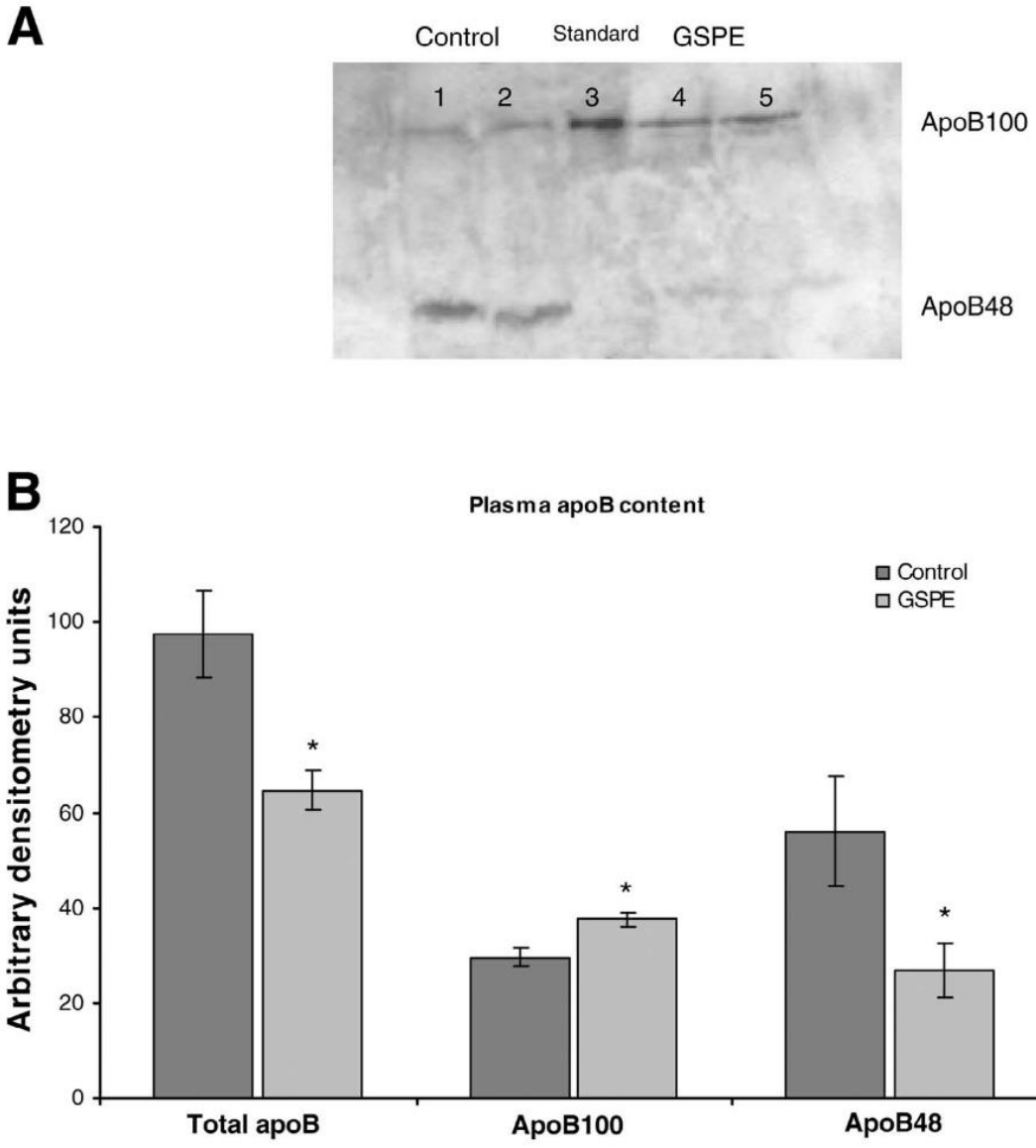
658 Figure 2


A
Control
Standard
GSPE
1
2
3
4
5
ApoB100
ApoB48
B
Plasma apoB content
Control
GSPE
Arbitrary densitometry units
120
100
80
60
40
20
0
*
*
*
Total apoB
ApoB100
ApoB48

659