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

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Abstract

Objective: To determine whether proanthocyanidins can protect against dyslipidemia induced by a high-fat diet (HFD) and to address the mechanisms that underlie this hypolipidemic effect.

Design and measurements: Female Wistar rats were fed on a HFD for 13 weeks. They were divided into two groups, one of which was treated with a grape seed proanthocyanidin extract (25 mg/kg of body weight) for 10 days. Plasma and liver lipids were measured by colorimetric and gravimetric analysis.

Liver, muscle and adipose tissue were used to study the expression of genes involved in the synthesis and oxidation of fatty acids and lipoprotein homeostasis by real-time RT-PCR.

Results: The administration of proanthocyanidins normalized plasma triglyceride and LDL-cholesterol (both parameters significantly increased with the HFD) but tended to decrease hypercholesterolemia and fatty liver. Gene expression analyses revealed that proanthocyanidins repressed both the expression of hepatic key regulators of lipogenesis and very low density lipoprotein (VLDL) assembling such as SREBP1, MTP and DGAT2, all of which were overexpressed by the HFD.

Conclusion: These findings indicate that natural proanthocyanidins improve dyslipidemia associated with HFDs, mainly by repressing lipogenesis and VLDL assembly in the liver, and support the idea that they are powerful agents for preventing and treating lipid altered metabolic states.

Keywords: high-fat diet; liver; proanthocyanidins; triglycerides; SREBP1; MTP

Introduction

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

Flavan-3-ols and their oligomeric condensation products, proanthocyanidins (PA), are the most common group of flavonoids in the American diet.7 PA can be found in such common foodstuffs as cereals, legumes, fruits, vegetables and beverages (red wine and tea, in particular).8 The health benefits of PA have been most studied in tea, cocoa and grape seed, each of which has a characteristic and specific oligomeric composition that conditions its biological activity. Grape seed proanthocyanidin extracts (GSPE) reduce foam cells,9 prevent aortic atherosclerosis10 from developing in a hamster atherosclerosis model, decrease oxidized LDL in hypercholesterolemic humans9 and improve endothelial function by modifying NO production.11 Consequently, they protect against atherosclerosis and CVD. Besides their antioxidant activity and their effects on the vascular endothelium, the antiatherogenic properties of PA are also related to an improved serum lipid profile. Plasma TG and apolipoprotein B (apoB) levels are reduced by GSPE in normolipidemic rats,12 and by lyophilized grape powder in postmenopausal women.13 The lipoprotein profile is also improved in both healthy subjects and hemodialysis patients by concentrated red grape juice.14

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

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

Methods

Proanthocyanidin extract

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

Animals

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

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

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

Plasma and liver lipid analysis

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

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

Gene expression analyses

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

7300 SDS Real-Time PCR system (Applied Biosystems).

Statistical analysis

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

Results

GSPE treatment prevents dyslipidemia induced by high-fat diet

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

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

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

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

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

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

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

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

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

Discussion

Metabolic syndrome and obesity are associated with an increased risk of CVD, in part, due to their association with atherogenic dyslipidemia.20 Overproduction of VLDL and hypertriglyceridemia are the basis of atherogenic dyslipidemia,4 so managing VLDL secretion and hypertriglyceridemia could be a good strategy for reducing the risk of CVD associated with these pathologies. PA, a group of flavonoids that can be found in common foodstuffs,8 actively reduce plasma TG and apoB in normolipidemic rats,12 in hamsters fed a hypercholesterolemic diet10 and in humans.11 In a previous study we showed that an acute dose of GSPE decreases VLDL secretion by repressing MTP and lipogenic genes in normolipidemic rats.12 This study, then, intended to determine, first, the ability of PA to correct atherogenic dyslipidemia associated with obesity and, second, to gain insight into the mechanisms that underlie the improvement of plasma TG levels, the effects of PA on the lipid metabolism in liver and in extrahepatic tissues.

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

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

The liver gene expression of rats fed an HFD indicated active lipogenesis and impaired fatty acid oxidation together with increased assembling of VLDL, which points to the liver as an important contributor to HFD-induced atherogenic dyslipidemia. Oral GSPE treatment repressed SREBP1, DGAT2 and MTP. These three proteins are key regulators of VLDL synthesis and secretion. SREBP1 activates the expression of several genes involved in FFA and TG synthesis, as well as other components of the regulatory machinery of lipid metabolism. Although it can be regulated at different levels, its transcriptional repression has been linked to lower FFA and TG synthesis and release from the liver, leading to hypolipidemic states.22 Therefore, the repressive effects of GSPE on SREBP1 may account for the lower plasma levels of TG. Together with SREBP1, DGAT2 is a key enzyme in the FFA

reesterification process that delivers TG to the nascent VLDL.23 As the availability of lipids is a key factor that drives the synthesis and secretion of VLDL,23 the repression of DGAT-2 and SREBP1 suggests that lipoprotein release may be blocked at the primary level, that is, fewer lipids are available for lipoprotein assembly. The repression of MTP also suggests a decreased assembly of VLDL, because MTP is responsible for the association of apoB with lipids and the intracellular trafficking of the newly synthesized VLDL. Altogether, the repression of MTP, SREBP1 and DGAT2 by GSPE suggests a VLDL synthesis blockage at two different levels: first, lipid availability is decreased and then lipoprotein assembly. Moreover, GSPE did not modify the expression of the LDL-receptor, indicating that the decrease in LDL-C by GSPE was not due to an increased uptake of LDL by the liver. Altogether, these results strongly suggest that GSPE improved dyslipidemia in HFD rats by reducing VLDL secretion, which, in turn, led to lower levels of TG and lower LDL production.

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

SHP is a nuclear receptor that acts as an inducible repressor of other nuclear receptors and transcription factors. With this mechanism, it controls lipogenesis and cholesterol metabolism in liver.26 Thus, the low levels of SHP expression in HFD-fed rats could be behind the exacerbated expression of SREBP1, which, in turn, induces overexpression of lipogenic genes, fatty liver and hypertriglyceridemia.25 In a previous

study we showed that GSPE uses an SHP-dependent molecular mechanism to reduce TG secretion in HepG2,15 so the slight increase in SHP expression induced by GSPE could be sufficient to reduce TG secretion, but not enough to counteract fatty liver.

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

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

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

associated with obesity and metabolic syndrome.

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Tables

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

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

*Experimental details and symbols: Values are mean ± s.e.m. of six rats. * Indicates a significant difference ($P \leq 0.05$) versus control group.*

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

Parameter	Control group	HFD group	HFD-GSPE group

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

*Abbreviations: GSPE, grape seed proanthocyanidin extracts; HFD, high-fat diet. * Indicates a significant difference ($P \leq 0.05$) versus control group; † indicates that the t-test found a significant difference between the HFD and HFD-GSPE groups.*

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

Gene	**Control group**	**HFD group**	**HFD-GSPE group**

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

339 *Abbreviations: GSPE, grape seed proanthocyanidin extracts; HFD, high-fat diet. The values are*

340 *expressed as fold change, using PPIA expression as the endogenous control.*