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Research Article

Dietary procyanidins lower triglyceride levels signaling through the nuclear receptor small heterodimer partner

Josep Maria Del Bas[1], Marie Louise Ricketts[2], Isabel Baiges[1], Helena Quesada[1], Anna Ardevol[1], Maria Josepa Salvadó[1], Gerard Pujadas[1], Mayte Blay[1], Lluis Arola[1], Cinta Bladé[1], David D. Moore[2] and Juan Fernandez-Larrea[1]

[1] Departament de Bioquímica i Biotecnología, Universitat Rovira i Virgili, Campus Sescelades, Tarragona, Spain
[2] Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, USA

Hypertriglyceridemia is an independent risk factor in the development of cardiovascular diseases, and we have previously reported that oral administration of a grape seed procyanidin extract (GSPE) drastically decreases plasma levels of triglycerides (TG) and apolipoprotein B (ApoB) in normolipidemic rats, with a concomitant induction in the hepatic expression of the nuclear receptor small heterodimer partner (NR0B2/SHP). Our objective in this study was to elucidate whether SHP is the mediator of the reduction of TG-rich ApoB-containing lipoproteins triggered by GSPE. We show that GSPE inhibited TG and ApoB secretion in human hepatocarcinoma HepG2 cells and had and hypotriglyceridemic effect in wild-type mouse. The TG-lowering action of GSPE was abolished in HepG2 cells transfected with a SHP-specific siRNA and in a SHP-null mouse. Moreover, in mouse liver, GSPE downregulated several lipogenic genes, including steroid response element binding protein 1c (SREBP-1c), and upregulated carnitine palmitoyltransferase-1A (CPT-1A) and apolipoprotein A5 (ApoA5), in a SHP-dependent manner. In HepG2 cells GSPE also inhibited ApoB secretion, but in a SHP-independent manner. In conclusion, SHP is a key mediator of the hypotriglyceridemic response triggered by GSPE. This novel signaling pathway of procyanidins through SHP may be relevant to explain the health effects ascribed to the regular consumption of dietary flavonoids.

Keywords: Liver / NR0B2/SHP / Procyanidins / SREBP-1c / Triglycerides

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1 Introduction

The regular long-term consumption of dietary flavonoids has been associated with reduced mortality and risk of cardiovascular diseases (CVD) [1–3]. Hypertriglyceridemia is an important contributing factor for atherosclerosis and represents an independent risk factor for CVD [4–8] and several studies have shown beneficial effects of flavonoids regarding atherosclerosis prevention, reducing apolipoprotein B (ApoB), and triglyceride (TG) levels *in vivo* [9, 10] and *in vitro* [11, 12]. Using *in vitro* models, it has been demonstrated that specific flavonoids decrease hepatocyte ApoB secretion acting through phosphoinositide-3 kinase (PI3K) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathways [13, 14]. In contrast, there are not studies about the mechanisms used by flavonoids to reduce TG production and secretion by hepatic cells.

We have previously shown that oral administration of procyanidins, a class of flavonoids, drastically reduce the postprandial levels of TG-rich, ApoB-containing lipoproteins, and improve different atherosclerotic risk indexes in normolipidemic rats [15]. These changes in plasma lipid profile were paralleled by a significant increase in the expression of the orphan nuclear receptor small heterodimer partner (NR0B2/SHP) in the liver. SHP is involved in

Correspondence: Dr. Cinta Bladé, Departament de Bioquímica i Biotecnología, Universitat Rovira i Virgili, Campus Sescelades s/n, 43007 Tarragona, Spain
E-mail: mariacinta.blade@urv.cat
Fax: +34977558232

Abbreviations: ApoA5, apolipoprotein A5; **ApoB,** apolipoprotein B; **CPT-1A,** carnitine palmitoyltransferase-1A; **FA,** fatty acid; **GSPE,** grape seed procyanidin extract; NR0B2/**SHP,** nuclear receptor small heterodimer partner; **SREBP-1c,** steroid response element binding protein 1c; **TG,** triglyceride

bile acid, cholesterol, TG, and glucose homeostasis [16–18] and mediates the hypotriglyceridemic effect of bile acids [19] and guggulsterone [20]. Thus, we hypothesized that SHP could be a key mediator in the hypotriglyceridemic effect triggered by procyanidins.

In this work we demonstrate that GSPE reduces TG secretion in the human hepatocarcinoma cell line HepG2 and has and hypotriglyceridemic effect in wild-type mouse. This hypotriglyceridemic effect of GSPE is abolished in two SHP deficient models: HepG2 cells transfected with a SHP-specific siRNA and a SHP knockout mouse, thus showing this nuclear receptor as a key mediator of the hypotriglyceridemic response triggered by procyanidins in the liver. Additionally, we have identified SHP target genes, including the steroid receptor element-binding protein 1c (SREBP1c), as putative downstream effectors of the TG lowering response triggered by procyanidins in hepatic cells *in vivo*.

2 Materials and methods

2.1 Chemicals

Grape seed procyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). According to the manufacturer, this procyanidin extract contains monomeric (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%), oligomeric (5–13 units) (35.7%) procyanidins, and phenolic acids (4.22%).

2.2 Cell cultures and cytotoxicity assays

HepG2 cells (ATCC code HB-8065) were grown until 70% confluence in DMEM (Cambrex) medium supplemented with 10% FBS (Cambrex). Twelve hours before GSPE treatment, media was replaced with serum-depleted media (DMEM supplemented with 0.25% oleic acid: albumin) (Sigma). Cytotoxicity of GSPE was assessed by measuring LDH leakage as described [21] and by the Alamar Blue assay (Biosource, USA), in cells treated 24 h with different concentrations of GSPE diluted in aqueous solution.

2.3 *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 [22], and were backcrossed with C57BL6 mice to the tenth generation. The correct genotype was verified using previously reported primer sequences and reaction conditions [23]. Age-matched groups of 8–10 wk-old male mice were used in all experiments ($n = 5$ *per* experimental group). Mice were fed a standard rodent chow and water *ad libitum*. On experimental day, mice were fed either vehicle (water), or procyanidins (250 mg/kg) *via* oral gavage. A first dose was administered at 9:00 pm and a second dose at 9:00 am; food was then retired and mice were sacrificed 2 h later. Blood was collected from the orbital plexus after isoflurane anesthesia. Livers were snap-frozen and stored at -80°C until use.

2.4 Lipid analysis

To study the synthesis of TG, HepG2 cells were seeded in 12-well plates and grown to 70% confluence. Growth medium was replaced by serum free medium 12 h before treatment with GSPE (50 mg/L) or vehicle. Twelve hours after the simultaneous addition of GSPE (50 mg/L) and ^{14}C-acetate (1 μCi/well), media and cells were collected and lipids extracted using chloroform/methanol. TLC was performed as previously described [24] with an additional separation using a Hexane/MTBE/NH_3 solvent to obtain the TG fraction. ^{14}C-labeled TG were scraped and determined by scintillation counting. Values were normalized *per* mg of total cellular protein, determined by a colorimetric assay (BioRad). For nonradioactive measurements, cell media and plasma TG or cholesterol were assayed using enzymatic kits (QCA).

2.5 Quantification of ApoB and SHP proteins

ApoB protein secreted by HepG2 cells into the culture media was determined by immunodetection using Western blot analysis. At the end of the treatments, media were collected and concentrated using Ultrafree-4 centrifugal filter units (Millipore). Equal volumes of media were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Millipore, USA). ApoB was immunodetected using an anti-apoB antibody (S-14, Santa Cruz Biotechnology) as the primary antibody, HRP-conjugated anti-IgG (sc-2020, Santa Cruz Biotechnology) as the secondary antibody, and immobilon chemiluminescent HRP substrate kit (Millipore, USA) as the chemiluminescent reagent. Membranes were exposed to Hyperfilm ECL (Amersham), and ApoB-specific signals quantified using Quantity One software (Bio-Rad); intensities values were always normalized *per* cell number. For quantification of SHP protein, HepG2 cells were lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing a protease-inhibitor cocktail (Sigma). Protein was quantified using a protein assay reagent (Bio-Rad). Equal amounts of cellular protein were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. SHP protein was detected using anti-SHP antibody (SHP Q14, Santa Cruz Biotechnology) and HRP-conjugated anti-IgG antibody (sc-2020, Santa Cruz Biotechnology). Signal intensities were quantified using Quantity One software (BioRad). SHP protein intensities in each sample were normalized with respect to GAPDH protein, detected

with anti-GAPDH antibody (V-18, Santa Cruz Biotechnology) and anti-IgG antibody.

2.6 siRNA experiments

HepG2 cells were transfected with either siRNA oligonucleotides targeting SHP (Ambion no. 103854) or with negative control siRNA (Ambion no. AM4636) using Ambion silencer siRNA transfection kit. Briefly, siRNAs were incubated with siPort NeoFx reagent (Ambion) to obtain transfection complexes. A total of 10^5 cells were incubated with 0.03 nmol of siRNA and seeded in 12-well plates. Eight hours after transfection, transfection media were replaced with serum free media and cells were grown for additional 24 h before extraction of lipids, protein, and RNA. To assess interference efficiency, SHP mRNA was quantified by quantitative PCR.

2.7 Gene expression analysis

HepG2 total RNA was isolated using NucleoSpin RNA2 kit (Macherie-Naegel). Liver total RNA was obtained using Trizol reagent (Invitrogen). Reverse transcription reactions were performed on an ABI Prism 7300 SDS Real Time PCR system (Applied Biosystems) using the Taqman Reverse Transcription reagent kit (Applied Biosystems) and specific primers and Taqman probes (Applied Biosystems). Primer sequences are available upon request. Results were normalized to cyclophilin or RPLP0. For microarray analysis, Cy3- or Cy5-labelled cRNA was obtained from each RNA pool sample, hybridized against Agilent Mouse Oligo Microarrays, and data from the microarray images were obtained and analyzed with the Agilent Feature Extraction software. Two differential hybridizations (dye swap) were performed in each microarray experiment.

2.8 Statistical analysis

For statistical analysis in the lipid, gene expression, immunodetection studies, *t*-test, and ANOVA analyses were performed using SPSS software.

3 Results

3.1 GSPE decreases ApoB and TG synthesis and secretion in HepG2 cells

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

In order to assess whether HepG2 cells are a valid system to study the bioactivity of GSPE on TG and ApoB secretion, we performed dose- and time-response experiments. As assessed by immunoblotting analysis, ApoB levels in the

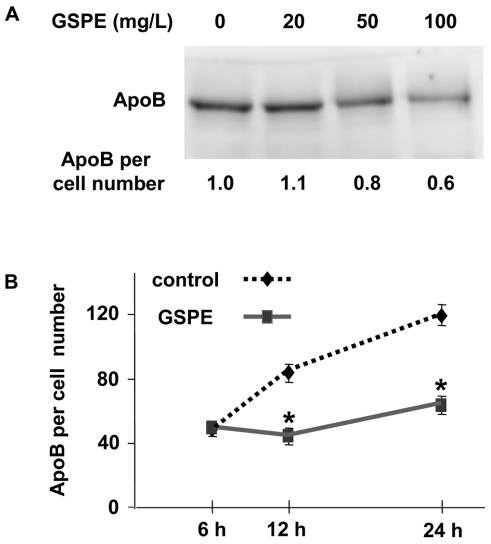

Figure 1. Effect of GSPE on ApoB release in HepG2 cells. (A) Changes in media ApoB levels in response to 20, 50, and 100 mg/L GSPE doses. HepG2 cells were treated with either vehicle (line 0) or GSPE at the indicated concentrations. After 24 h, ApoB in the media was quantified by immunoblotting as described in Section 2. Relative intensities of the ApoB bands were normalized 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 (A). All values are the mean ± SEM of three independent experiments; * on a GSPE-treated sample denotes a significant difference ($p < 0.05$) between the value of that sample and its control counterpart.

culture media, normalized by cell number, decreased dose-dependently 24 h after addition of GSPE, being 20 and 40% lower in cells treated with 50 and 100 mg/L GSPE, respectively, than in vehicle-treated cells (Fig. 1A). Treatment of cells with 50 mg/L GSPE, completely blocked the accumulation of ApoB in the media during the first 12 h of GSPE treatment, while during the following 12 h, this secretion was partially restored (Fig. 1B). In this same set of experiments, accumulation of TG in the media was drastically reduced upon treatment with 50 mg/L GSPE for 24 h, reaching 30% the value of vehicle-treated cells (Fig. 2A). In order to assess the action of GSPE on the *de novo* synthesis and secretion of TG, HepG2 cells were treated for 12 h with ^{14}C -acetate and either vehicle or GSPE (Fig. 2B). Compared to controls, cells treated with GSPE showed a reduction in total ^{14}C incorporation into TG, a significant decrease in secreted ^{14}C-TG, without significant intracellular ^{14}C-TG accumulation. Altogether, these results strongly suggest that GSPE lowers TG-rich lipoprotein secretion. Therefore, GSPE reduces TG and an ApoB secretion in HepG2 cells, which may be regarded as a valid system to

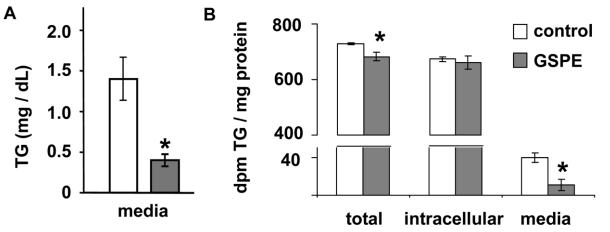

Figure 2. Effect of GSPE on TG synthesis and secretion in HepG2 cells. (A) Changes in TG accumulation in media of cells treated with GSPE. HepG2 cells were treated with 50 mg/L of GSPE or vehicle (control) for 24 h. TG were assayed as indicated in Section 2 and values were normalized to mg of cell protein. (B) Changes induced by GSPE in the *de novo* synthesis and secretion of TG. HepG2 cells were simultaneously incubated with ^{14}C-labelled acetate and 50 mg/L of GSPE or vehicle (control). After 12 h of treatment, radioactivity incorporated into media and cellular TG was measured as described in Section 2. All values are the mean ± SEM of three independent experiments; * on a GSPE-treated sample denotes a significant difference ($p < 0.05$) between the value of that sample and its control counterpart.

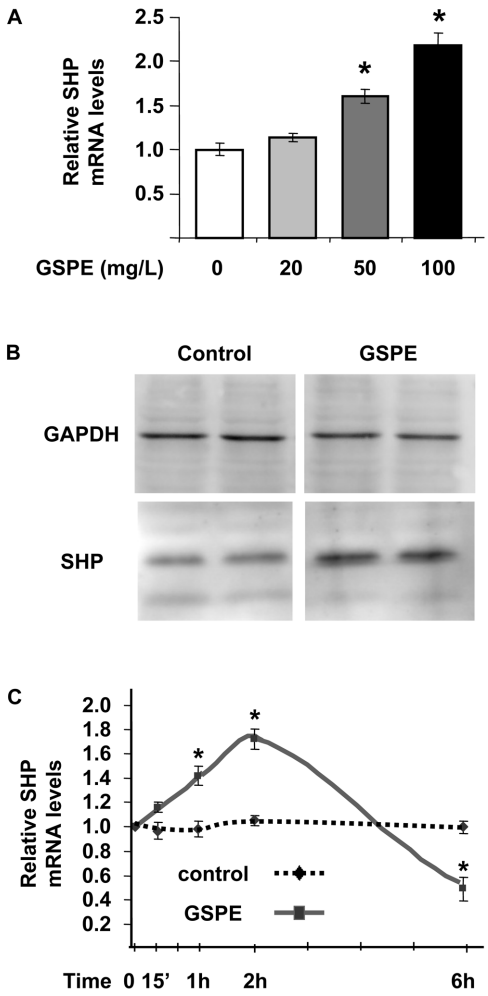

Figure 3. Effects of GSPE on SHP expression in HepG2 cells. (A) Fold-change of SHP gene expression in response to 20, 50, and 100 mg/L GSPE relative to vehicle treated cells. HepG2 cells were treated with the indicated concentrations of GSPE for 2 h. SHP mRNA levels were determined by RT-qPCR and normalized to RPLP0 mRNA levels. (B) Changes in SHP protein levels in response to GSPE treatment monitored by Western blot. HepG2 cells were treated with either vehicle (control) or 100 mg/L GSPE. After 3 h, 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 (A). All values are the mean ± SEM of three independent experiments; * on a GSPE-treated sample denotes a significant difference ($p < 0.05$) between the value of that sample and its control counterpart.

study the mechanisms employed by procyanidins to exert these effects *in vitro*.

3.2 GSPE modulates SHP expression in HepG2 cells

In healthy normolipidemic rats, the hypotriglyceridemic effect of GSPE *in vivo* occurs concomitantly with an upregulation of SHP expression in the liver, 5 h after oral administration of GSPE [15]. To assess whether SHP expression is also modulated by GSPE in HepG2 cells we first treated HepG2 cells with different doses of GSPE and analyzed changes in SHP mRNA levels by quantitative PCR after 2 h of treatment (Fig. 3A). These GSPE treatments resulted in dose-dependent upregulation of SHP mRNA levels, reaching a two-fold increase *versus* control values with a dose of 100 mg/L. The increase in SHP mRNA level was followed by an increase in the level of SHP protein, as assessed by immunoblotting, in HepG2 cells treated during 3 h with a dose of 100 mg/L (Fig. 3B). Longer periods of GSPE treatment, assayed with a dose of 50 mg/L, resulted in a reduction of SHP mRNA, which reached 50% of control values after 6 h of treatment (Fig. 3C). Therefore, GSPE modulates the expression of SHP in HepG2 cells, triggering a rapid and transient induction of SHP expression.

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

To study the relevance of SHP as a mediator of the effects of GSPE on ApoB and TG secretion in HepG2, we inhibited SHP expression by transfecting the cells with SHP-specific siRNA. Transfection of HepG2 cells with SHP-siRNA resulted in a 60% reduction in SHP mRNA levels 32 h after transfection (Fig. 4A). Concomitantly with this reduction in SHP expression, ApoB, and TG release into the media increased 1.5- and 4-fold, respectively (Figs. 4B and C), strongly suggesting that SHP activity constrains ApoB and TG secretion in HepG2 cells in basal conditions. In SHP-silenced HepG2 cells, the TG-lowering effect of GSPE was

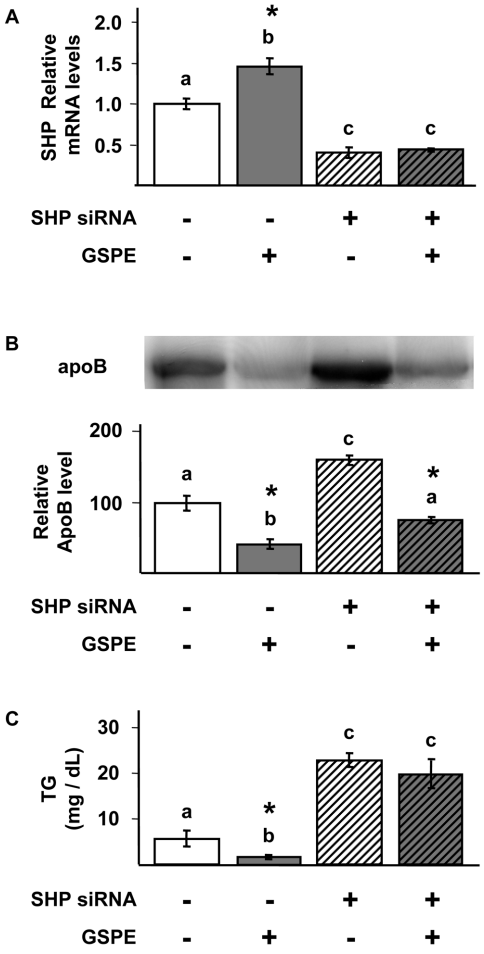

Figure 4. Effect of SHP silencing on GSPE modulation of ApoB and TG secretion in HepG2 cells. HepG2 cells were transfected with a SHP-specific siRNA (+ bars) or negative control, scramble siRNA (−bars). Thirty-two hours after transfection, media was replaced with serum depleted medium, and 12 h afterwards, cells were treated with 50 mg/L GSPE (indicated by +) or vehicle (indicated by −). After GSPE treatment, cells and media were harvested for SHP, ApoB, and TG analysis. (A) SHP gene expression, determined as in Fig. 3A, after 2 h of GSPE treatment. (B) ApoB in the cell culture media after 24 h of GSPE treatment, analyzed as in Fig. 1A. A representative ApoB immunoblot is shown. (C) TG levels in culture media after 24 h of GSPE treatment, analyzed as in Fig. 2A. All values are the mean ± SEM of three independent experiments. a, b, or c denotes significant differences between groups, as determined by ANOVA; * on a GSPE-treated sample denotes a significant difference ($p < 0.05$) between the value of that sample and its control counterpart.

completely abolished (Fig. 4C). In contrast, the effect of GSPE lowering ApoB secretion remained unaffected in SHP-knocked down HepG2 cells (Fig. 4B). These results show that the inhibition of ApoB and TG secretion by GSPE in HepG2 cells follow at least two different mechanisms: the action of GSPE on TG secretion involves a SHP-dependent mechanism, whereas repression of ApoB release follows a SHP-independent pathway.

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

In order to gain 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 wild-type *versus* $SHP^{-/-}$ mice. Mice were feed two oral doses of GSPE (250 mg/kg), with a 12 h interval and plasma TG and total cholesterol were analyzed 2 h after the second oral gavage. In wild-type mice, GSPE gavage triggered a 40% reduction in plasma TG levels (Fig. 5A), a similar response to that previously found in rats [15]. This hypotriglyceridemic effect of GSPE was abolished by the $SHP^{-/-}$ genotype. Therefore, SHP is a key mediator of the hypotriglyceridemic effects exerted by procyanidins *in vivo*. In contrast, GSPE gavage produced no effects on plasma total cholesterol levels, neither in wild-type nor in SHP-null mice (Fig. 5B).

3.5 GSPE modulates the hepatic expression of numerous genes related to lipid, fatty acid (FA), and steroid metabolism in wild-type but not in $SHP^{-/-}$ mice

To gain insight into the SHP-dependent actions of GSPE that accompany the hypotriglyceridemic effect of GSPE *in vivo*, we compared the changes in gene expression induced by GSPE in the livers of wild-type and $SHP^{-/-}$ mice, using oligonucleotide microarray hybridization. In order to identify those genes whose expression in liver is modulated by GSPE in a SHP-dependent manner and are putatively involved in the hypotriglyceridemic effect of GSPE, we selected those genes clustered into the "lipid, FA, and steroid metabolism" group by Panther software [25], whose expression was changed by GSPE treatment in wild-type mice but remained unaltered in SHP-null mice, setting a fold-change threshold of 1.4 for upregulated and 0.7 for downregulated genes (Table 1). Several genes related to FA, TG, and cholesterol synthesis were identified which show SHP-dependent repression by GSPE, including key regulators of lipid synthesis pathways such as SREBP1c, 3-hydroxy-3-methylglutaryl-coenzyme A synthase, acyl-CoA synthetase, stearoyl-coenzyme A desaturase, and phosphatidic acid phosphatase 2a. Also the expression of several genes involved in FA oxidation and in lipoprotein metabolism was modulated by GSPE in a SHP-dependent manner, including carnitine palmitoyltransferase-1a (CPT-1A), apolipoprotein A5 (ApoA5), CCAAT/enhancer binding protein β (C/EBPb), and phospholipid transfer protein (Pltp). 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

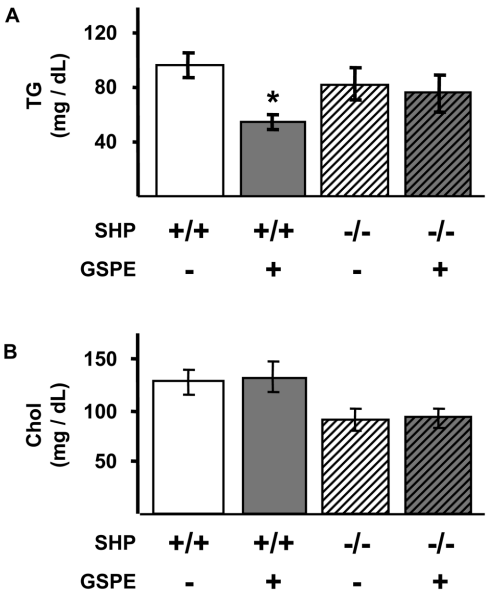

Figure 5. Effect of GSPE on plasma TG and total cholesterol levels in wild-type and SHP-null mouse. Wild-type ($SHP^{+/+}$) and SHP-null ($SHP^{-/-}$) mice were fed with either vehicle (–bars) or GSPE (250 mg/Kg) (+ bars) *via* oral gavage ($n = 5$ in each group, age 8 – 10 wks) as described in Section 2. Plasma was obtained 14 h after the first oral gavage (2 h after the second oral gavage) and TG and total cholesterol were determined as described in Section 2. All values are the mean ± SEM of three independent experiments; * on a GSPE-treated sample denotes a significant difference ($p < 0.05$) between the value of that sample and its control counterpart.

regulator of lipid and lipoprotein metabolism [26, 27]. Therefore, SREBP1 emerges as a procyanidin target gene in the liver and as a putative SHP-dependent downstream effector of the hypotriglyceridemic response triggered by procyanidins in hepatic cells.

4 Discussion

Elevated TG levels are prevalent among the western population, often occurring in persons who are overweight or obese, or who have type 2 diabetes or the metabolic syndrome [5, 6, 8]. Compelling evidence from multiple clinical studies has established hypertriglyceridemia as an independent risk factor for atherosclerosis [4 – 7]. Thus, using strategies to manage TG levels, along with low-density lipoprotein cholesterol levels, is warranted to help reduce the risk of CVD. Previously [15] we have shown that oral administration of GSPE triggered a 50% reduction in plasma ApoB and TG levels with a concomitant three-fold increase in liver SHP mRNA expression, in the postprandial state in healthy rats. In the liver, the orphan NR0B2/SHP regulates, among other processes, cholesterol and bile acid metabolism, steroidogenesis, and lipogenesis [16 – 18]. Therefore, SHP emerged as a putative mediator of the observed hypotriglyceridemic response triggered by GSPE.

In this work, we have shown that GSPE exerts hypotriglyceridemic activity when administered orally to mouse and reduces TG secretion in HepG2 cells, confirming the TG-lowering effect of GSPE previously observed in rats [15]. The similarity in the effects of GSPE *in vitro* and *in vivo* suggests that procyanidins species that exert the TG-lowering effect have a low polymerization degree, because only monomeric to pentameric procyanidins have been detected in plasma after oral consumption of procyanidin mixtures in murine models [28, 29]. The TG-lowering effect of GSPE is abolished in the *in vitro* and *in vivo* models when SHP expression is diminished or abolished: in HepG2 cells transfected with a SHP-specific siRNA and in SHP knockout mouse. Therefore, GSPE needs the activity of SHP to reduce TG secretion *in vitro* and triglyceridemia *in vivo*. In contrast, silencing of SHP expression in HepG2 cells did not abolish the ApoB-lowering effect of GSPE in this cells. Thus, repression of TG-rich ApoB-containing lipoproteins secretion by GSPE in hepatocytes involves both a SHP-dependent pathway, that affects TG secretion, and SHP-independent mechanisms, that affects ApoB release. Some flavonoids have been shown to decrease ApoB release, a marker of lipoprotein secretion, in *in vitro* hepatocyte models. Although the intracellular mediators involved in this effect are not fully elucidated, the inhibition of PI3-K and MAPK/ERK signaling pathways and repression of microsomal TG transfer protein (MTP) activity have been demonstrated [12 – 14, 30 – 32].

Comparison of the gene expression changes elicited by GSPE in the liver of wild-type mouse to those elicited in the SHP-null mouse has allowed the identification of SHP-dependent procyanidin-target genes putatively implicated in the hypotriglyceridemic effect of GSPE. Among these genes, it stands out SREBP-1c, that is downregulated by GSPE in the liver of wild-type mouse, and ApoA5 and CPT-1A, which are upregulated by GSPE.

SREBP-1c is a master regulator of FA synthesis and a mediator for insulin/glucose signaling to lipogenesis, and could be involved in the development of insulin resistance and fatty livers, being its inhibition a proposed target for lowering lipogenesis and TG levels [33 – 35]. Transgenic mouse overexpressing SREBP1c displays increased expression of FA synthase (FAS), and higher levels of hepatic FAs, and TG [36]; in contrast, SREBP-1c knockout mouse displays diminished FAS expression, lower hepatic FA amounts, and lower plasma TG levels [37]. It is well established that bile acids and synthetic FXR ligands downregulate the hepatic secretion of TG by a pathway that involves induction of SHP and subsequent inhibition of SREBP-1c expression by SHP [19]. In a similar way, SREBP-1c could be a mediator of the hypotriglyceridemic effect of GSPE acting downstream of SHP. Among the genes that we have found to be downregulated by GSPE in mouse liver in a

Table 1. SHP-dependent changes induced by GSPE in the expression of lipid-related genes in mouse liver

Genbank ID	Name; gene symbol	WT	SHP$^{-/-}$
FA synthesis			
NM_146197	Acyl-CoA synthetase medium-chain family member 2; Acsm2	0.6	1
NM_019811	Acyl-CoA synthetase short-chain family member 2; Acss2*	0.6	0.8
NM_009128	Stearoyl-coenzyme A desaturase 2; Scd2*	0.7	0.9
NM_011480	Sterol regulatory element binding factor 1; Srebp1c*	0.7	1.0
		0.5 ± 0.1	**1.0 ± 0.2**
NM_028089	Cytochrome P450, family 2, subfamily c, polypeptide 55; Cyp2c55	0.6	1.2
NM_007703	Elongation of very long chain FAs (FEN1/Elo2, SUR4/Elo3, yeast)-like 3; Elovl3	0.7	1
Glycerophospholipid, diacylglycerol, and triacylglycerol synthesis			
NM_008903	Phosphatidic acid phosphatase 2a; Ppap2a	0.7	0.9
NM_177664	DNA segment, Chr 3, Brigham and amp; D3Bwg0562e	0.5	1
NM_008845	Phosphatidylinositol-4-phosphate 5-kinase, type II, a; Pip5k2a	0.7	1
NM_175443	Ethanolamine kinase 2; Etnk2	0.7	0.9
NM_013490	Choline kinase a; Chka	0.7	0.9
Steroid and cholesterol synthesis			
NM_023556	Mevalonate kinase; Mvk	0.7	1.2
NM_172769	Sterol-C5-desaturase; Sc5d*	0.7	1
NM_145942	3-Hydroxy-3-methylglutaryl-Coenzyme A synthase 1; Hmgcs1*	0.6	1.2
NM_010941	NAD(P)-dependent steroid dehydrogenase-like; Nsdhl	0.7	1
NM_007856	7-Dehydrocholesterol reductase; Dhcr7*	0.7	1
NM_134469	Farnesyl diphosphate synthetase; Fdps*	0.8	1.6
FA oxidation			
NM_013495	Carnitine palmitoyltransferase 1a, liver; Cpt1a	1.4	1.0
		1.6 ± 0.2	**0.9 ± 0.2**
Other lipid-related genes			
NM_011125	Phospholipid transfer protein; Pltp*	0.7	1
NM_080434	Apolipoprotein A5; ApoA5*	1.4	1.0
		2.1 ± 0.2	**1.0 ± 0.2**
NM_026784	Phosphomevalonate kinase; Pmvk	0.7	0.9
NM_028057	Cytochrome b5 reductase 1; Cyb5r1	0.7	1.3
NM_207683	Phosphatidylinositol 3-kinase, C2 domain containing, g polypeptide; Pik3c2g	0.7	1
NM_018784	ST3 b-galactoside alpha-2,3-sialyltransferase 6; St3gal6	0.7	0.8
NM_009883	CCAAT/enhancer binding protein (C/EBP), b; Cebpb*	0.7	0.8

Wild-type and SHP$^{-/-}$ mice were fed either vehicle or GSPE (250 mg/Kg) *via* oral gavage (five animals in each treatment group) as described in Section 2. Total RNA was obtained from livers and pooled for each treatment group. Two different microarray hybridizations (dye swap) were performed to asses changes induced by GSPE in WT or SHP$^{-/-}$ mice. The values for each gene are the mean fold change obtained from two microarray hybridization experiments (dye swap labeling) comparing signal intensities obtained from GSPE-treated *versus* control groups. The table lists those genes which are involved in "lipid, FA, and steroid metabolism" following the classification of Panther software [25], and whose expression is changed by GSPE treatment in wild-type mice (mean fold changes in column "WT") but not in SHP-null mice (mean fold changes in column "SHP$^{-/-}$"). Bold characters denote the mean fold change values obtained by RT-qPCR using individual RNAs instead of pooled RNAs for quantification. Known SREBP-1c target genes are denoted by *.

SHP-dependent manner there are several which are known or putative targets of SREBP1c. These include genes involved in the synthesis of monounsaturated and PUFAs: a long chain FA elongase (Elovl3) [38, 39], two acyl-CoA synthetases (Acsm2 and Acss2) [26, 33, 35], and a FA desaturase, the stearoyl-coenzyme A desaturase 2 (Scd2) [40, 41]. Scd2 is a microsomal enzyme required for the biosynthesis of oleate and palmitoleate, which are the major MUFAs of membrane phospholipids, TG, and cholesterol esters. Deficiency in FA desaturase activity greatly reduces hepatic TG synthesis [42] and protects mouse against hypertriglyceridemia induced by liver X receptor (LXR) activation [43]. Other genes which we have found to be downregulated by GSPE in a SHP dependent manner, although not known targets of SREBP-1c, include genes encoding enzymes directly involved in TG synthesis such as the phosphatidic acid phosphatase 2. Altogether, this pattern of gene expression induced by GSPE in mouse liver, suggest a diminished rate of TG and FA synthesis.

Along with the downregulation of FA and TG synthetic genes, GSPE upregulated the hepatic expression of CPT-1A, the rate limiting enzyme in the β-oxidation of FA.

Enhancement of CPT-1 expression and activation of the FA oxidation pathway are potential targets for the treatment of hypertrygliceridemia, type-2 diabetes, and obesity [44, 45]. Our results suggest that SHP activity is necessary for the GSPE-induced upregulation of CPT-1A expression in mouse liver, establishing CPT-1A as a putative target of SHP activity.

Besides the effects of GSPE in reducing TG secretion by the liver, increasing plasma TG catabolism could also contribute to the hypotriglyceridemic effect of GSPE. In this regard, we have found that GSPE upregulates ApoA5 expression in mouse liver, in a SHP-dependent manner. Overexpression of ApoA5 is known to reduce plasma TG and cholesterol in hypertriglyceridemic mouse [46, 47] whereas ApoA5 deficiency results in marked hypertriglyceridemia [48]. The hypotriglyceridemic action of ApoA5 is attributed to the activation of lipase-mediated VLDL-TG hydrolysis an consequent acceleration of VLDL catabolism [48 – 50]. Again, this result suggests that ApoA5 expression may be under the control of SHP activity. It is known that ApoA5 gene expression is regulated by LXR ligands in a negative manner through upregulation of SREBP-1c [51].

In summary, we have shown here that grape procyanidins exert hypotriglyceridemic effects when orally administered to mouse, and when applied to HepG2 cells. In both models, procyanidins require the presence of SHP to reduce TG-levels. We have demonstrated the SHP-dependent hepatic downregulation of expression of SREBP-1c and upregulation of CPT-1 and ApoA5 in the liver of GSPE-treated mouse, strongly suggesting that procyanidins repress hepatic FA synthesis, enhance hepatic FA oxidation, and activate the catabolism of TG-rich ApoB-containing lipoproteins, by a mechanism that depends on the activity of SHP. In addition, we have shown in HepG2 cells that procyanidins downregulate the secretion of ApoB in a SHP-independent way. Altogether, these results show that procyanidins, using SHP-dependent and SHP-independent mechanisms, may lower the secretion of TG-rich ApoB-containing lipoproteins by hepatic cells. Both mechanisms would act in concert to reduce the number of circulating VLDLs and also their TG content providing a plausible explanation for the hypolipidemic properties of grape polyphenols present in foods, which lower TG and ApoB levels in human subjects [52, 53] when administered chronically at lower doses than those used in this study. Due to the relevance of postprandial triglyceridemia in the development of atherosclerosis and type 2 diabetes [7, 8], the elucidation of how nutrients can modulate plasma lipid levels has emerged as an important target of nutrition research [54]. In this regard, this work provides new hints for understanding the mechanisms associated with the beneficial effects ascribed to the regular consumption of dietary procyanidins.

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