Research Article

Dietary procyanidins enhance transcriptional activity of bile acid-activated FXR *in vitro* and reduce triglyceridemia *in vivo* in a FXR-dependent manner

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Consumption of dietary flavonoids has been associated with reduced mortality and risk of cardiovascular disease, partially by reducing triglyceridemia. We have previously reported that a grape seed procyanidin extract (GSPE) reduces postprandial triglyceridemia in normolipidemic animals signaling through the orphan nuclear receptor small heterodimer partner (SHP) a target of the bile acid receptor farnesoid X receptor (FXR). Our aim was to elucidate whether FXR mediates the hypotriglyceridemic effect of procyanidins. In FXR-driven luciferase expression assays GSPE dose-dependently enhanced FXR activity in the presence of chenodeoxycholic acid. GSPE gavage reduced triglyceridemia in wild type mice but not in FXR-null mice, revealing FXR as an essential mediator of the hypotriglyceridemic actions of procyanidins *in vivo*. In the liver, GSPE downregulated, in an FXR-dependent manner, the expression of the transcription factor steroid response element binding protein 1 (SREBP1) and several SREBP1 target genes involved in lipogenesis, and upregulated ApoA5 expression. Altogether, our results indicate that procyanidins lower triglyceridemia following the same pathway as bile acids: activation of FXR, transient upregulation of SHP expression and subsequent downregulation of SREBP1 expression. This study adds dietary procyanidins to the arsenal of FXR ligands with potential therapeutic use to combat hypertriglyceridemia, type 2 diabetes and metabolic syndrome.

Keywords: Bile acids / FXR / Liver / Procyanidins / SHP

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1 Introduction

Procyanidins, which are oligomers and polymers of polyhydroxyflavan-3-ol units, are the most abundant polyphenols in grapes, apples, red grape juice, red wine, and chocolate [1, 2]. These flavonoids have been shown to prevent and ameliorate atherosclerosis and other factors of cardiovascular disease, a fact that was primarily ascribed to their antioxidant activity and the modulation of diverse signaling pathways in vascular system [2, 3]. However, the antiatherogenic properties of procyanidins are also attributable to a reduction of plasma levels of apolipoprotein B (ApoB)-containing triglycerides (TG)-rich proatherogenic lipoproteins, *i.e.*, intestinal chylomicrons and hepatic VLDL and LDL, as well as to an improved serum cholesterol profile. Thus, in hamster models of diet-induced atherosclerosis, chronic

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Abbreviations: ApoA5, apolipoprotein A5; **ApoB,** apolipoprotein B; **BA,** bile acid; **CDCA,** chenodeoxycholic acid; **FA,** fatty acid; **FXR,** farnesoid X receptor (NR1H4); **GSPE,** grape seed procyanidin extract; **LBD,** ligand binding domain; **RXR,** retinoid X receptor (NR2B1); **SBARM,** selective bile acid receptor modulator; **SCD,** stearoyl-coenzyme A desaturases; **SHP,** small heterodimer partner (NR0B2); **SREBP1,** steroid response element binding protein 1; **TG,** triglycerides; **WT,** wild type

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administration of procyanidins inhibit aortic fatty streak area and progression of atherosclerosis, and lowers plasma TG, ApoB, and non-HDL-cholesterol [4, 5]. In ovariectomized Guinea pigs and in postmenopausal women, grape-seed procyanidin-rich extracts diminish plasma TG and VLDL-cholesterol, as well as cholesterol accumulation in the aorta [6]. In rats fed hypercholesterolemic diets, chronic consumption of a grape seed procyanidin extract (GSPE) reduced plasma TG, LDL-cholesterol, and VLDL concentrations, while increasing plasma HDL-cholesterol in the fasted state [7, 8]. The hypolipidemic effect of procyanidins may be exerted in part through the inhibition of the absorption of dietary lipids, and diminished chylomicron secretion by enterocytes [9, 10]. But also hepatocytes respond to red wine and red grape juice polyphenols diminishing the secretion of ApoB100 and increasing LDL-receptor activity [11–13]. Nevertheless, the molecular mechanisms that underlie the improvement of plasma lipid profile by procyanidins are largely unknown.

We have previously shown that an acute dose of GSPE reduces postprandial triglyceridemia and plasma ApoB levels in normolipidemic rats while concomitantly increasing hepatic mRNA levels of small heterodimer partner (NR0B2) (SHP) [14], an orphan nuclear receptor that regulates bile acids (BAs), cholesterol, TG, and glucose homeostasis in the liver (recently reviewed in [15]). In HepG2 cells, grape procyanidins require the activity of SHP to reduce secretion of TG whereas, in contrast, they reduce the secretion of ApoB in a SHP-independent way [16]. Also, SHP mediates the hypotriglyceridemic effect of grape procyanidins in wild type (WT) mouse in the postprandial state, which is accompanied by the downregulation of hepatic expression of transcription factor steroid response element binding protein 1 (SREBP1) [16], a master mediator for insulin/glucose signaling to lipogenesis (reviewed in [17, 18]).

It is well established that BAs are potent hypotriglyceridemic agents. These effects of BA are mediated by their binding to the BA receptor farnesoid X receptor (NR1H4) (FXR), a transcription factor that, like SHP, exerts metabolic control on BAs, lipid and glucose homeostasis (reviewed in [19–22]). FXR activation by BAs lowers plasma TG levels by repressing hepatic lipogenesis and TG secretion, and by increasing the clearance of TG-rich lipoproteins from the blood (reviewed in [22, 23]). In the liver, BA-activated FXR upregulates the expression of SHP, which in turn represses the expression of SREBP1, which is translated into a diminished hepatic fatty acid (FA) synthesis and an increased plasma TG catabolism [24].

Besides procyanidins, two other phytochemicals, guggulsterone from the guggul tree [25, 26] and xanthohumol from beer hops [27] have been described to lower triglyceridemia *in vivo* regulating the hepatic expression of a subset of FXR target genes, including SHP and SREBP1. Both xanthohumol and guggulsterone behave as selective BA receptor modulators (SBARMs) that, like BAs, enhance transcriptional activity of FXR in *in vitro* assays [25–27].

The partial similarity between activators of FXR and GSPE regarding changes elicited in liver gene expression profile and in plasma lipid parameters prompted us to hypothesize that procyanidins might enhance the transcriptional activity of FXR and, consequently, that FXR could mediate the hypotriglyceridemic effects of GSPE. To test these hypotheses we have here evaluated the effect of GSPE on plasma TG levels in WT and $FXR^{-/-}$ mice, and have assessed the effects of GSPE on a cell-based FXR-responsive luciferase expression assay. The results show that procyanidins enhance the activity of chenodeoxycholic acid (CDCA)-activated FXR, and that, like BAs, signal through FXR to lower triglyceridemia, concomitantly inhibiting hepatic expression of SREBP1 and several SREBP1 target genes involved in lipogenesis in an FXR-dependent manner.

2 Materials and methods

2.1 Chemicals

A GSPE was kindly provided by *Les Dérives Résiniques et Terpéniques* (Dax, France). This extract contains monomeric catechins (polyhydroxyflavan-3-ol) (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%), and oligomeric (5–13 units) (35.7%) procyanidins, as well as phenolic acids (4.22%). CDCA was from Sigma and GW4064 was a kind gift from Tim Willson (GlaxoSmithKline).

2.2 Cell transfections and luciferase reporter assays

Human epithelial cells (HeLa) and African Green Monkey Fibroblasts (CV-1) were obtained from the American Type Tissue Culture Collection. HeLa and CV-1 cells were maintained in DMEM supplemented with 10% FBS at 37°C and 5% CO_2. For transfections, cells were plated into 24-well plates (Becton Dickinson) (1.3×10^5 (HeLa) or 1×10^5 (CV-1)) in DMEM plus 10% charcoal stripped serum to 80% confluence, then cotransfected the next day using the calcium phosphate precipitation method. Plasmids for expression of full-length mouse FXR, for expression of Gal4 DBD fused to FXR ligand binding domain (LBD), the FXR-responsive luciferase reporter plasmid ($(PLTP)_2$ TKluc), the Gal4-driven luciferase reporter plasmid, and the plasmid expressing β-galactosidase (CMX-β-gal) have been previously described [25, 28]. The CDM-retinoid X receptor (NR2B1) (RXR)α plasmid (expressing full-length human RXRα) has also been described [29]. Cells were plated in 24-well dishes with DMEM supplemented with 10% charcoal-stripped serum. Transfections included 100 ng of the plasmid encoding full-length FXR or the

Gal4 DBD-FXR LBD fusion, 200 ng of the luciferase reporter plasmid, 10 ng of CDM-RXRα, 200 ng of CMX-β-gal (used as internal control for transfection efficiency) and 490 ng of pGEM4 (Promega), used as carrier DNA, to make a total of 1 μg of plasmid DNA *per* well. The next morning, cells were washed with PBS and FXR ligands (CDCA, GW4064) or GSPE were added as indicated. Ligands were dissolved in DMSO whereas GSPE was dissolved in ethanol. Cells were assayed for luciferase activities (Promega luciferase assay kit) 24 h after addition of vehicle, ligands or GSPE, and reporter expression was normalized to β-galactosidase activity (β-gal Assay kit, Applied Biosystems), measured with an MLX luminometer (Dynex Technologies). Results were obtained from at least three independent experiments, each performed in triplicate. For luciferase-based studies, *t*-test analyses were performed using SPSS software.

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. The FXR-deficient mice have been previously described [30], and were backcrossed with C57BL6 mice to the tenth generation. The correct genotype was verified using previously reported primers and PCR conditions [30]. Age-matched groups of 8 – 10 week-old male mice were used in all experiments ($n = 5$ *per* experimental group). Mice were fed a standard rodent chow and water *ad libitum*. 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 12 h later; food was then retired and 2 h later mice were anesthetized with isoflurane. Blood was collected from the orbital plexus and livers were snap-frozen and stored at $-80^{\circ}C$ until use. Plasma cholesterol and TG levels were assessed with enzymatic kits as described [14].

2.4 Gene expression analysis

Total RNA was obtained using Trizol reagent (Invitrogen). and further purified using NucleoSpin RNA2 kit (Macherie-Naegel). For microarray hybridizations, the five RNA samples from each of the four treatment groups were pooled and its integrity was assessed using the Agilent 2100 Bioanalyzer. Cy3- or Cy5-labeled cRNA was obtained from each RNA pool using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent manual 5185 – 5818). Labeled cRNAs were hybridized against Agilent Mouse 60-mer Oligo Microarrays (Part Number G4122A) following Agilent's instruction. Duplicate hybridizations with dye-swap labeling were performed with each pair of RNA samples being compared. 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 validation of microarray data, relative mRNA levels of SREBP1, cholesterol-7α-hydroxylase (CYP7A1), apolipoprotein A5 (ApoA5), and SHP genes were analyzed by real-time PCR, using GAPDH as the endogenous control. RNA was retrotranscribed using TaqMan Reverse Transcription Reagents kit (Applied Biosystems) and gene expression was evaluated in the ABIPrism 7300 SDS Real Time PCR system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) and gene-specific primers (sequences of the primers used in real-time PCR reactions are available upon request).

2.5 Microarray data processing

A whole array of data was constructed matching each gene symbol or Genbank ID with its fold-change value from the microarray analysis. Genes were clustered into different biological processes using Panther software [31]. The gene expression profile deviation of each biological process group from the whole array expression pattern was calculated using the Mann – Whitney U test (Wilcoxon Rank-Sum test) as described [32]. The resulting *p*-values were considered significant when smaller than 0.05.

3 Results

3.1 GSPE requires FXR activity to reduce triglyceridemia in mice

In order to assess the relevance of FXR as a mediator of GSPE hypotriglyceridemic actions in an *in vivo* model, we compared the effects of GSPE administration in $FXR^{-/-}$ *versus* WT mice. As previously described [30], $FXR^{-/-}$ mice present elevated basal levels of plasma TG and cholesterol when compared to WT mice (Fig. 1). Oral GSPE gavage triggered a 40% reduction in plasma TG levels in WT mice in the postprandial state whereas it did not modify plasma total cholesterol levels. This response to GSPE administration is identical to that previously found in rats [14]. In contrast, GSPE gavage did not cause any statistically significant reduction in plasma TG levels in $FXR^{-/-}$ animals. Therefore, FXR is a key mediator of the hypotriglyceridemic activity of procyanidins in mice.

3.2 GSPE represses the expression of SREBP1 and SREBP1-target genes in wild type but not $FXR^{-/-}$ mice

To gain further insight into the FXR-dependent actions of procyanidins, we next analyzed the differential response in gene expression changes induced by GSPE gavage in the livers of WT and $FXR^{-/-}$ mice using oligonucleotide microarray hybridization. The changes induced by GSPE

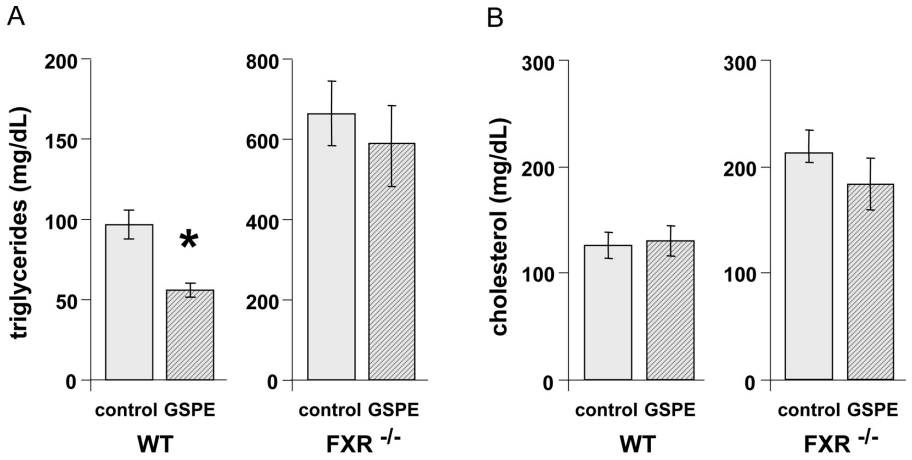

Figure 1. Effect of GSPE gavage on plasma TG and cholesterol levels in WT and FXR-null mice. WT and FXR$^{-/-}$ mice were fed with vehicle (control) or 250 mg/kg GSPE and plasma TG (A) and total cholesterol (B) were determined as described in Materials and Methods. * Denotes significant differences *versus* control ($p < 0.05$).

treatment in the expression level of all genes, clustered by biological process, were subjected to unbiased analysis using Panther software [31], evaluating the changes in each cluster of genes by means of the Mann–Whitney U test (Wilcoxon Rank-Sum test) [32]. In WT mice, changes in genes clustered in the biological process "Lipid, FA, and steroid metabolism," including 747 genes, showed a significant overall repression (*p*-value 0.018). In contrast, in FXR$^{-/-}$ mice, GSPE treatment did not significantly affect this gene cluster (*p*-value 0.5). These results could indicate that lipid metabolism is repressed to some extent by GSPE in WT but not in FXR$^{-/-}$ mice, thus placing FXR as a mediator of the repression of lipid related genes by procyanidins in liver.

Next, in order to identify FXR-dependent changes in the expression of genes putatively involved in the hypotriglyceridemic effect of GSPE in WT mice, we selected those genes clustered into the "Lipid, FA, and steroid metabolism" by Panther method [31] whose expression was altered by GSPE treatment in WT mice but remained unaltered in FXR$^{-/-}$ mice, setting a fold-change threshold of 1.5 for upregulated and 0.7 for downregulated genes (Table 1). In total, 31 lipid-related genes were identified that showed FXR-dependent repression by GSPE, including transcription factor SREBP1, a key regulator of FA and TG synthesis and lipoprotein metabolism [17, 18], acyl-CoA synthetase Acss2/Acsl1 (involved in FA synthesis) [33], the FA desaturases Scd1 and Scd2 [34], and several genes encoding cholesterol biosynthetic enzymes. Two other genes involved in lipid and lipoprotein metabolism, although not classified by Panther software in this cluster, also changed in a FXR-dependent manner, and were included in Table 1: ApoA5 (involved in VLDL catabolism) [35, 36] and the transcription factor C/EBP-β (a regulator of glucose and lipid homeostasis [37]. Remarkably, several of the genes which showed an FXR-dependent response to GSPE, have been previously described as targets of SREBP1. Also, many of the genes which have been identified in this screening, including SREBP1, have been previously characterized as SHP-dependent GSPE-responsive genes (marked with an asterisk in Table 1) [16]. Therefore, SREBP1 and SREBP1 target genes emerge as putative FXR-and SHP-dependent effectors of the hypotriglyceridemic response triggered by procyanidins *in vivo*.

3.3 GSPE enhances the transcriptional activity of CDCA-activated FXR in CV-1 and HeLa cells

The lack of hypotriglyceridemic effect of GSPE in FXR-null mice prompted us to test whether procyanidins can modulate the transcriptional activity of the FXR/RXR heterodimer in an *in vitro* system, cotransfecting CV-1 cells with different constructs expressing full-length RXR and either a Gal4 DBD-FXR LBD chimera or full-length FXR together with an FXR-responsive luciferase reporter plasmid (Fig. 2). A vector expressing full-length Gal4 was cotransfected as a control, in order to discard interactions of GSPE with the DNA-binding domain of this protein (data not shown). GSPE displayed no significant effects on RXR activity as assayed using a Gal4 DBD-RXR LBD chimera (Fig. 2B). In order to assess the interactions of GSPE with FXR, a Gal4 DBD-FXR LBD chimera (Fig. 2A) or full length FXR (Fig. 2C) was cotransfected with an RXR expression plasmid. In both cases, GSPE alone did not cause transactivation of FXR/RXR. In contrast, when GSPE was added to transfected cells together with the BA CDCA, a natural FXR ligand, it enhanced the transactivation of FXR in a dose-dependent manner, reaching a two-fold increase when cells where incubated with 100 mg/L of GSPE and 100 μM CDCA compared with the CDCA treatment alone. On the contrary, GSPE did not increase the transactivation of FXR induced by GW4064, a synthetic

Table 1. FXR-dependent changes induced by GSPE in the hepatic expression of lipid and lipoprotein related genes

Genebank ID	Gene name; symbol	Fold change upon GSPE treatment		SREBP1 target
		WT	$FXR^{-/-}$	
FA and TG synthesis and metabolism				
NM_011480	Sterol regulatory element binding factor 1;Srebf1 (SREBP1) *	0.7	1.0	[53]
		0.6	**1.0**	
NM_019811	Acyl-CoA synthetase short-chain family member 2; Acss2/Acsl1 *	0.6	0.9	[33]
NM_146197	Acyl-CoA synthetase medium-chain family member 2; Acsm2 *	0.6	0.8	
NM_009127	SCD 1; Scd1	0.6	1.1	[34]
NM_009128	SCD 2; Scd2 *	0.7	1.0	[34]
NM_028089	Cytochrome P450, family 2, subfamily c, polypeptide 55; Cyp2c55 *	0.6	1.0	
NM_175443	Ethanolamine kinase 2; Etnk2 *	0.7	0.9	
NM_008903	Phosphatidic acid phosphatase 2a; Ppap2a *	0.6	0.8	
NM_008846	Phosphatidylinositol-4-phosphate 5-kinase, type 1 α; Pip5k1a	0.7	0.9	
NM_008845	Phosphatidylinositol-4-phosphate 5-kinase, type II, α; Pip5k2a *	0.7	0.9	
NM_207683	Phosphatidylinositol 3-kinase, C2 domain containing, γ polypeptide; Pik3c2g	0.7	1.0	
NM_013490	Choline kinase α; Chka *	0.7	0.8	
NM_019677	Phospholipase C, β 1; Plcb1	0.7	1.0	
NM_080434	Apolipoprotein A5; ApoA5 *	1.4	1.0	[36]
		1.7	**1.0**	
Cholesterol Biosynthesis				
NM_145942	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1; Hmgcs1 *	0.7	0.9	[39, 42]
NM_008255	3-hydroxy-3-methylglutaryl-Coenzyme A reductase; Hmgcr *	0.7	0.8	[39, 42]
NM_023556	Mevalonate kinase; Mvk *	0.7	0.9	[39, 42]
NM_026784	Phosphomevalonate kinase; Pmvk *	0.7	0.9	[39, 42]
NM_138656	Mevalonate (diphospho) decarboxylase; Mvd	0.7	0.9	[39, 42]
NM_134469	Farnesyl diphosphate synthetase; Fdps *	0.7	0.9	[39, 42]
NM_010941	NAD(P) dependent steroid dehydrogenase-like; Nsdhl *	0.7	0.9	[39, 42]
NM_172769	Sterol-C5-desaturase; Sc5d *	0.7	0.9	[39, 42]
NM_007856	7-dehydrocholesterol reductase; Dhcr7 *	0.7	0.9	[39, 42]
Other lipid-related				
NM_009883	CCAAT/enhancer binding protein (C/EBP), β; Cebpb *	0.7	0.9	[37]
NM_013634	Peroxisome proliferator activated receptor binding protein; Pparbp *	0.7	1.0	
NM_011374	ST8 α-*N*-acetyl-neuraminide α-2,8-sialyltransferase 1; St8sia1	0.7	0.9	
NM_018784	ST3 β-galactoside α-2,3-sialyltransferase 6; St3gal6 *	0.7	1.3	
NM_011372	ST6 (α-*N*-acetyl-neuraminyl-2,3-β-galactosyl-1,3)-*N*-acetylgalactosaminide α-2,6-sialyltransferase 3; St6galnac3	0.7	0.9	
NM_018830	*N*-acylsphingosine amidohydrolase 2; Asah2	0.5	1.2	
NM_153389	ATPase, Class V, type 10D; Atp10d	0.7	1.0	
NM_028057	Cytochrome b5 reductase 1; Cyb5r1 *	0.7	1.5	
NM_012054	Acyloxyacyl hydrolase; Aoah	0.6	1.0	

WT and $FXR^{-/-}$ mice ($n = 5$ in each treatment group, 8 – 10 weeks old) were fed two doses of either vehicle or GSPE (250 mg/kg), separated by a 12 h time lapse, as described in Section 2. Two hours after the second gavage, liver total RNA from the four groups was obtained and pooled. Microarray data were obtained by comparing gene expression of WT control *versus* WT GSPE-treated mice and $FXR^{-/-}$ control *versus* $FXR^{-/-}$ GSPE-treated mice. The whole microarray fold-changes were processed using Panther software in order to identify FXR-dependent changes induced by GSPE in genes clustered in the "Lipid, FA, and steroid metabolism" metabolic pathway. ApoA5 and CEBP/β are not included in this cluster by Panther software and were added separately. Fold-change thresholds were fixed as 0.7 and 1.5 for downregulation and upregulation, respectively. Real time quantitative PCR was performed with selected genes to confirm the microarray data (shown in bold characters). Selected references are given for known SREBP1 target genes. Asterisks highlight those genes previously found to be responsive to GSPE in an SHP-dependent way [16]. WT column: fold-change in expression induced by GSPE in WT mice relative to the expression in WT mice treated with vehicle. $FXR^{-/-}$ column: fold-change in expression induced by GSPE in $FXR^{-/-}$ mice relative to the expression in $FXR^{-/-}$ mice treated with vehicle only.

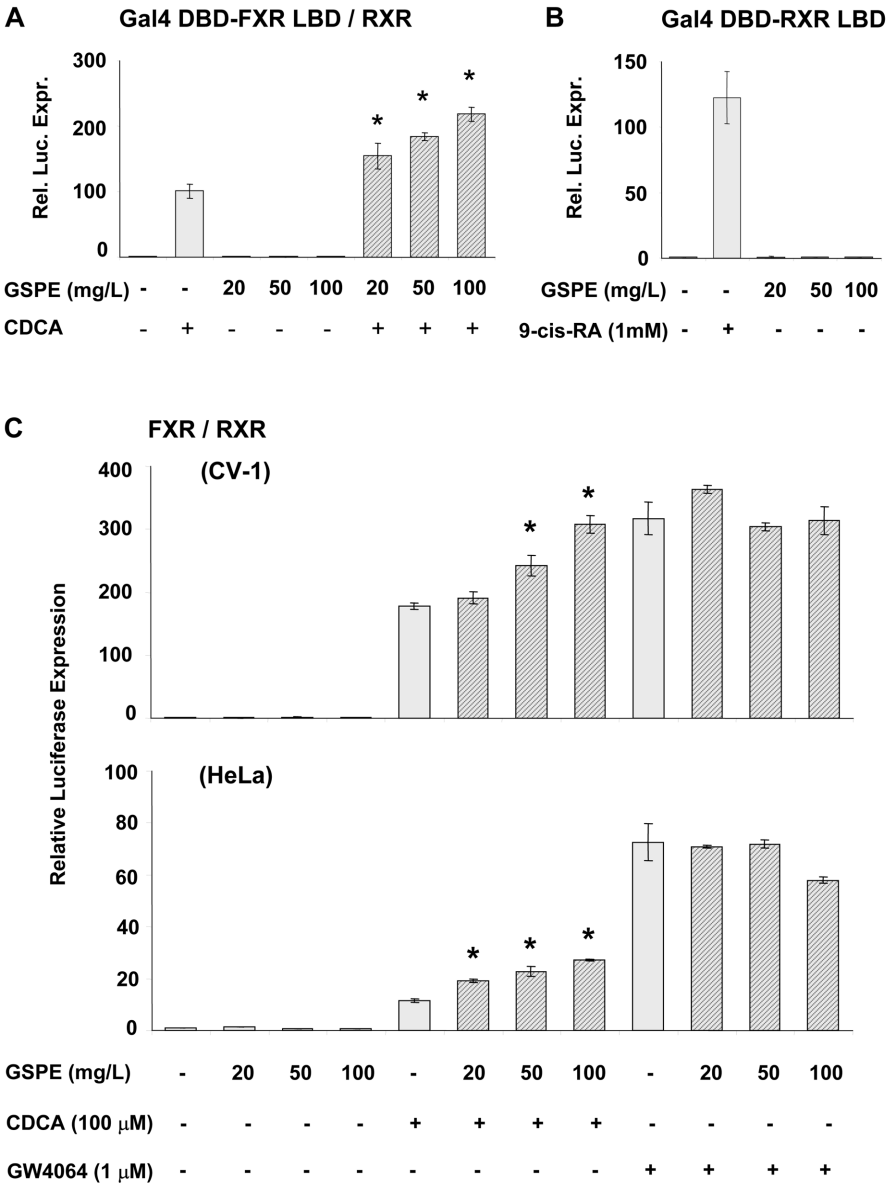

Figure 2. Cell-based assays of GSPE effects on FXR-dependent luciferase expression. (A) To study the effect of GSPE on transcriptional activity of FXR/RXR, the Gal4 DBD-FXR LBD expression vector and the Gal4 luciferase reporter plasmid were cotransfected in CV-1 cells, and these were treated with vehicle, GSPE and/or 100 μM CDCA. (B) RXR interactions with GSPE were assayed using the Gal4:DBD-RXR:LBD expression vector, along with the Gal4 luciferase reporter plasmid. Transfected cells were treated with vehicle (−) or 1 mM 9-*cis*-retinoic acid (+) and GSPE at the indicated concentrations. (C) To assess activation of full-length FXR by GSPE, a full length FXR expression plasmid along with a reporter construct containing a $(PLTP)_2$ TKluc were co-transfected in CV-1 cells (upper panel) or in HeLa cells (lower panel). Ligands for FXR were CDCA (100 μM) or GW4064 (1 μM). GSPE was added in the indicated concentrations. All controls (−) were treated with the respective vehicles in a final concentration lower than 0.1%. All transfections included the expression vector for RXR to allow the formation of FXR/RXR heterodimers, and CMX-β-Gal as internal control. All DNA constructs have been previously described [25, 28, 29]. Values represent the mean fold-change with respect to control values, obtained from three independent experiments. * Denotes significant differences at the $p < 0.05$ level respect the CDCA treatment.

nonsteroidal FXR agonist (Fig. 2C). In order to discard cell-specific actions of GSPE on FXR transactivation, the vector expressing full-length FXR was transfected, along with the vector expressing full-length RXR and the FXR-responsive luciferase reporter plasmid $(PLTP)_2$ TKluc, in HeLa cells, and equivalent transactivation to those found in CV-1 was observed (Fig. 2C). These results show that procyanidins enhance the transcriptional activity of CDCA-

activated FXR, but not that of GW4064-activated FXR, and thus they behave as activator-dependent FXR co-agonists in a cell-based FXR-driven luciferase expression assay.

4 Discussion

The essential role of FXR in mediating the hypotriglyceridemic actions of procyanidins *in vivo* has been revealed by administering GSPE to WT and FXR-null mice. In accordance with our previous results in WT rats and mice [14, 16], oral gavage of GSPE elicited a hypotriglyceridemic effect in WT mice in the postprandial state, without affecting total plasma cholesterol levels. In contrast, GSPE was ineffective in reducing plasma TG levels in FXR-null mice, which, as previously reported [30] displayed hypertriglyceridemia and hypercholesterolemia. Therefore, procyanidins need the presence and activity of FXR, *i.e.*, they act through an FXR-dependent pathway, to exert hypotriglyceridemic actions *in vivo*. Genome-wide analysis of liver gene expression profile has identified a group of genes whose expression is responsive to GSPE treatment in WT mouse but not in the FXR-null genotype, *i.e.*, FXR-dependent GSPE target genes, that provide some clues to understand the FXR-dependent mechanisms used by procyanidins to lower plasma TG levels. Remarkably, most of these FXR-dependent GSPE targets have been already identified as SHP-dependent targets of GSPE [16], including the transcription factor SREBP1 and several genes known to be regulated by it. SREBP1 is a key mediator for insulin/glucose signaling to lipogenesis; overexpression of the mature form of SREBP1a or SREBP1c leads to increased hepatic FA biosynthesis and TG levels and its inhibition has been proposed as a method for lowering triglyceridemia [17, 38, 39]. Known targets of SREBP1 found here to be regulated by GSPE include genes involved in the synthesis of monounsaturated and polyunsaturated FA: acetyl-CoA synthetase Acss2/Acsl1 [33], and two stearoyl-coenzyme A desaturases (SCD), Scd1 and Scd2 [34]. Deficiency in SCD activity, the rate-limiting enzyme for the biosynthesis of monounsaturated FA, greatly reduces hepatic TG synthesis and protects mice against hypertriglyceridemia induced by LXR activation [40]. Since the liver lipid pool is a limiting factor in the synthesis and secretion of VLDLs by the liver [41], a decrease in hepatic lipogenesis should reduce the number of VLDLs or the TG content of these lipoproteins. Also the genes encoding cholesterol biosynthetic enzymes that are downregulated by GSPE in an FXR-dependent and an SHP-dependent manner are targets of SREBP1a, which regulates their expression in concert with SREBP2 [39, 42]. Downregulation of these genes in WT mice suggests that GSPE could potentially inhibit cholesterol biosynthesis in the liver, although this was not translated into total plasma cholesterol levels in the postprandial phase. In this regard, our previous study in rats [14] showed no effects of GSPE on plasma total cholesterol, although significantly lowered the cholesterol associated with TG-rich lipoproteins. However, chronic ingestion of monomeric catechins or oligomeric procyanidins is effective in lowering not only triglyceridemia, but also cholesterolemia, VLDL- and LDL-cholesterol, while increasing HDL-cholesterol [7] suggesting that, in the long term, downregulation of cholesterol biosynthetic enzymes by GSPE may be translated into diminished plasma cholesterol levels. In addition, GSPE upregulated ApoA5 expression in mouse liver in a FXR- and SHP-dependent manner. It is known that ApoA5 gene expression is downregulated by LXR ligands through upregulation of SREBP1c and that overexpression of ApoA5 reduces plasma TG in hypertriglyceridemic mice; the hypotriglyceridemic action of ApoA5 is attributed to the inhibition of lipidation of ApoB and the activation of lipase-mediated VLDL-TG hydrolysis and consequent acceleration of VLDL catabolism [35, 36]. Taken together, this pattern of FXR-dependent changes elicited by GSPE strongly suggest that the hypotriglyceridemic effect elicited by dietary procyanidins is brought about, at least in part, through inhibition of hepatic lipogenesis and acceleration of TG-rich lipoproteins catabolism.

It is noteworthy that the changes in liver gene expression observed here in GSPE-treated mice differ from that previously observed in the liver of rats treated with GSPE [14]. Thus, SHP and CYP7A1 were found to be upregulated by GSPE in rats livers, but none of these changes have been observed here in the liver of GSPE-treated mice. However, since microarray analysis was performed at one time point in each case, it is feasible that the observed differences in the expression of known FXR targets just reflect time-dependent variations in gene expression. In support of this view, it is known that BAs and GW4064 enhance hepatic expression of SHP only transiently, and that transient inductions of SHP are sufficient to exert an hypotriglyceridemic effect [24, 43]. Upregulation of SHP expression by GSPE is also transient in HepG2 cells, were GSPE inhibits TG secretion [16]. Another possible factor to explain the observed variations between hepatic gene expression induced by GSPE in rats and mice is the fact that the strength and specificity of FXR activity is ligand and promoter-dependent, as has been shown comparing the transcriptional activity of FXR bound to the different BAs (*i.e.*, cholate, lithocholate, deoxycholate, chenodeoxycholate, and ursodeoxycholate) [44] and to synthetic FXR agonists such as GW4064 and fexaramine [45, 46]. The binding of each ligand results in a different FXR conformation, which in turn differentially regulates expression of a subset of FXR targets, and with different potency. This is related to the binding of different transcriptional coactivators, such as TRRAP [47] or PGC-1α [48], to the LBD of FXR, which occurs in a ligand and promoter-specific fashion [45]. According to the results of the FXR-responsive luciferase expression assays, procyanidins from the grape seed extract cannot by themselves

enhance the transcriptional activity of the FXR/RXR heterodimer, but instead behave as ligand-dependent co-agonists, enhancing the transcriptional strength of CDCA-activated FXR, but not that of GW4064-activated FXR. Although this result strongly suggest that procyanidin species present in GSPE, or their metabolites, directly bind to CDCA-bound FXR to enhance its transcriptional activity, it does not exclude the possibility that procyanidins might enhance FXR activity by binding through an FXR cofactor which should be commonly present in HeLa and CV-1 cells. In any case, different procyanidin/BA combinations, ultimately due to species specific differences in BAs metabolism and in the absorption and metabolization of procyanidins, should result in activation of different subsets of FXR-target genes, different strengths of activation and/or different temporal patterns of expression. The enhancement of BA-activated FXR activity by procyanidins is expected to occur *in vivo*, both in hepatic cells and enterocytes, where FXR is highly expressed and hepatic synthesis and enterohepatic circulation guarantee the presence of BAs. In this regard, monomeric catechins and dimeric to trimeric procyanidins have been detected in urine after oral gavage of GSPE to rats [49]. Likewise, catechins and dimeric to pentameric procyanidins have been detected in plasma after administration of apple procyanidin extracts to rats [50].

In summary, our results indicate that procyanidins act through an FXR-dependent pathway to exert hypotriglyceridemic actions *in vivo*. This effect is also dependent on SHP-activity [16] and is concomitant with downregulation of hepatic expression of SREBP1. FXR-responsive luciferase expression assays indicate that procyanidins act as BA dependent coactivators of FXR activity. Taken together, these results suggest that, *in vivo*, procyanidins exert hypotriglyceridemic effect following a pathway that goes with, and is dependent on, that followed by BAs, *i.e*., activation of FXR, transient upregulation of SHP expression and subsequent downregulation of SREBP1 expression, which is translated into a diminished hepatic FA synthesis and an increased plasma TG catabolism.

FXR activity plays a key role in controlling not only triglyceridemia but also cholesterol, BA and glucose homeostasis, and modulation of FXR has been proposed as a therapeutic target in the treatment of hyperlipidemia, hyperglycemia, and metabolic syndrome [21, 22, 51, 52]. Consequently, dietary procyanidins, acting as activators of FXR, emerge as promising natural agents for the treatment of these metabolic disorders. Further research is required to identify the individual procyanidins that enhance FXR activity and to define the mechanisms and metabolic consequences of this activation.

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