RESEARCH ARTICLE

Dietary procyanidins selectively modulate intestinal farnesoid X receptor-regulated gene expression to alter enterohepatic bile acid recirculation: elucidation of a novel mechanism to reduce triglyceridemia

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Received: October 8, 2015
Revised: December 9, 2015
Accepted: December 14, 2015

Scope: Understanding the molecular basis by which dietary procyanidins modulate triglyceride and cholesterol homeostasis has important implications for the use of natural products in the treatment and prevention of cardiovascular disease.

Methods: To determine whether modulation of bile acid (BA) homeostasis contributes to the hypotriglyceridemic action of grape seed procyanidin extract (GSPE) we examined the effect on genes regulating BA absorption, transport and synthesis in vitro, in Caco-2 cells, and in vivo, in wild type (C57BL/6) and farnesoid x receptor knockout (*Fxr*$^{-/-}$) mice.

Results: We provide novel evidence demonstrating that GSPE is a naturally occurring *gene-selective* bile acid receptor modulator (BARM). Mechanistically, GSPE down-regulates genes involved in intestinal BA absorption and transport in an *Fxr*-dependent manner, resulting in decreased enterohepatic BA recirculation. This correlates with increased fecal BA output, decreased serum triglyceride and cholesterol levels, increased hepatic cholesterol 7α-hydroxylase (*Cyp7a1*), and decreased intestinal fibroblast growth factor 15 (*Fgf15*) expression. GSPE also increased hepatic HmgCoA reductase (*Hmgcr*) and synthase (*Hmgcs1*) expression, while concomitantly decreasing sterol regulatory element-binding protein 1c (*Srebp1c*).

Conclusion: GSPE selectively regulates intestinal *Fxr*-target gene expression in vivo, and modulation of BA absorption and transport is a critical regulatory point for the consequential hypotriglyceridemic effects of GSPE.

Keywords:
Bile acids / Enterohepatic recirculation / Fxr / Procyanidins / Triglycerides

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1 Introduction

Cardiovascular disease (CVD) is the number one cause of death worldwide [1]. Hypercholesterolemia and mild to moderate hypertriglyceridemia are important risk factors for CVD [2]. Within the body, cholesterol homeostasis is maintained via endogenous biosynthesis (regulated by 3-hydroxy-3-methyl-glutaryl-CoA (Hmg-CoA) reductase; hmgcr); uptake (via the low-density lipoprotein receptor (ldlr)); and

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Abbreviations: **ALT**, alanine aminotransferase; **Asbt**, apical sodium-dependent bile acid transporter; **AST**, aspartate aminotransferase; **BA**, bile acid; **BARM**, bile acid receptor modulator; **BiP**, immunoglobulin heavy chain-binding protein; **Bsep**, bile salt export pump; **CAM**, complementary and alternative medicine; **CDCA**, chenodeoxycholic acid; **Cpt1a**, carnitine palmitoyltransferase 1a; **CVD**, cardiovascular disease; **Cyp7a1**, cytochrome P450 cholesterol 7α-hydroxylase; **Fgf15/19**, fibroblast growth factor 15/19; **Fxr**, farnesoid x receptor; **GSPE**, grape seed procyanidin extract; **Hmgcr**, 3-hydroxy-3-methyl-glutaryl-CoA (Hmg-CoA) reductase; **Hmgcs1**, 3-hydroxy-3-methyl-glutaryl-CoA (Hmg-CoA) synthase; **Ibabp**, ileal bile acid binding protein; **Ntcp**, Sodium-taurocholate co-transporting polypeptide; **Ost**α/β, organic solute transporters alpha/beta; **Srebp1c**, sterol regulatory element-binding protein 1c; **Shp**, small heterodimer partner; **TG**, triglyceride; **WT**, wild type

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elimination (in the form of bile acids (BAs)) [3], which represents the primary mechanism to lower serum cholesterol levels. BAs facilitate the absorption of dietary fats and fat-soluble vitamins [4], and undergo enterohepatic recirculation, with ~95% reabsorbed daily from the distal ileum, while the remaining 5% are excreted in the feces [5]. This 5% is then replenished via hepatic *de novo* biosynthesis from cholesterol [4].

Efficient enterohepatic recirculation is achieved via coordinated regulation along the gut-liver axis and is tightly controlled by nuclear receptors (NRs) [6]. Farnesoid x receptor (Fxr) is the major BA-responsive NR critical for the maintenance of BA homeostasis [6–10]. Notably, intestinal Fxr is crucial for appropriate BA signaling under normal physiological conditions [11]. Absorption of BAs occurs via active transport in the distal ileum through the apical sodium-dependent bile acid transporter (Asbt) [12]. Once inside the enterocyte BAs are bound and transported to the basolateral membrane of the enterocyte by ileal bile acid-binding protein (Ibabp) [13], and then secreted into portal circulation via the organic solute transporters alpha and beta (Ostα/β) [14]. Additionally, BAs, via Fxr, induce the expression of fibroblast growth factor 15 (Fgf15) in the intestine, a hormone which is then secreted into portal circulation [15, 16]. Fgf15 circulates to the liver to suppress BA biosynthesis [15], which is mediated via binding to, and activation of, Fgf receptor 4 (Fgfr4) complexed with β-Klotho [17], which then stimulates the c-jun N-terminal kinase (Jnk) pathway, eventually suppressing *Cyp7a1*, encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in the classical pathway for BA synthesis [15, 18, 19]. When ileal Fxr is activated by BAs, *Asbt* is down-regulated [20], while *Ibabp*, *Ostα/β* and *Fgf15/19* are induced [15, 19, 21–23]. These Fxr-mediated effects lead to reduced BA uptake at the luminal membrane of the enterocyte, increased transport into portal circulation, and reduced hepatic BA synthesis [15, 18, 19]. Fundamentally, the tight regulation exerted by these gut-liver Fxr-BA feedback mechanisms regulates the BA pool size and composition [16], ultimately maintaining cholesterol and BA homeostasis.

High cholesterol is one of the major controllable risk factors for coronary heart disease, heart attack and stroke. Dietary intervention and lifestyle modifications are often initial treatment strategies for dyslipidemia [24], but patients may subsequently be prescribed pharmaceuticals to treat these disorders. Complementary and alternative medicine (CAM) therapies, including plant derived extracts, are popular alternatives for a variety of conditions, including dyslipidemia, with 40% of adults reporting the use of CAM. Elucidating the molecular mechanisms by which natural products and bioactive dietary components exert beneficial effects against CVD risk factors is of fundamental importance.

Grape seed procyanidin extract (GSPE) is a procyanidin-rich compound isolated from the seeds of white grapes, *vitis vinifera*. Bioactive procyanidins have been reported to exert beneficial health effects with respect to metabolic syndrome, type 2 diabetes and CVD [25]. We previously showed that GSPE is a BA-dependent co-agonist ligand for Fxr [26], and that it reduces serum triglyceride (TG) levels via a pathway involving Fxr, small heterodimer partner (Shp) and sterol regulatory element-binding protein 1c (Srebp1c) in the liver [26, 27].

Since GSPE is a co-agonist ligand for Fxr [26], we would expect *Cyp7a1* to be repressed following GSPE administration. Intriguingly, however, previous studies in rats [28] and hamsters [29] reported increased *Cyp7a1* expression following administration with grape seed extracts, after 5 h and 6 wks, respectively. Consequently, we hypothesized that GSPE may selectively modulate intestinal Fxr-regulated gene expression, leading to reduced enterohepatic BA recirculation, which would then necessitate increased *Cyp7a1* expression. In order to delineate the underlying molecular mechanism, we systematically assessed the molecular regulatory effects of GSPE on Fxr-target genes important for BA homeostasis in vitro, using Caco-2 cells, and in vivo using wild-type and *Fxr*$^{-/-}$ mice, to gain further insight regarding how the induction of *Cyp7a1* may contribute to the hypotriglyceridemic actions of GSPE. Herein, we now demonstrate that GSPE acts as a *gene-selective* bile acid receptor modulator (BARM), inhibiting intestinal BA absorption, leading to decreased enterohepatic BA recirculation and increased fecal BA output. By inhibiting BA absorption, transport and enterohepatic recirculation, GSPE induces the utilization of endogenous TG and cholesterol sources to facilitate replenishment of BAs lost via the feces, thereby reducing serum TG and cholesterol levels in order to sustain metabolic homeostasis.

2 Materials and methods

2.1 Chemicals and antibodies

All chemicals were obtained from Thermo Fisher Scientific unless otherwise stated. Grape Seed Procyanidin Extract (GSPE) was obtained from *Les Dérives Résiniques et Terpéniques* (Dax, France), and contains monomeric catechins (polyhydroxyflavan-3-ol) (16.55%), dimeric (18.77%), trimeric (16%), tetrameric (9.3%), and oligomeric procyanidins (5–13 units) (35.7%), as well as phenolic acids (4.22%), as previously described [26–28]. The flavanol and phenolic acid composition of the GSPE used in this study was previously analyzed by reverse-phase high-performance liquid chromatography-mass spectrometry and is reported in reference [30]. The β-actin antibody was obtained from Sigma Aldrich (A5441), and the mouse anti-immunoglobulin heavy chain-binding protein (BiP) antibody was obtained from Enzo Life Sciences. The anti-Cyp7a1 antibody was kindly provided by Dr. D. Russell (UT Southwestern, Dallas), and the anti-Asbt antibody was kindly provided by Dr. P. Dawson (Emory University, Atlanta).

2.2 Cell culture

Caco-2 cells (HTB-37™), originally isolated from a 72 year old male Caucasian, were purchased from ATCC® and used between passage numbers 5–20 for these studies. Cells were maintained in 10 cm Corning cell culture dishes in Dulbecco's Modified Eagles medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 1% L-glutamine, and cultured at 37°C and 5% CO_2. Once the cells reached confluence, they were sub-cultured into 6 well plates, at 1×10^6 cells per well for subsequent experiments. The cells were allowed to reach confluence and grown an additional 10-days post-confluence, with replacement of fresh media every 48 h. Cells were then grown for an additional 24 h, after which the media was removed and replaced with DMEM supplemented with 1% L-glutamine and 0.5% charcoal-stripped FBS. A lower concentration of FBS was used to minimize the effect of bile salts commonly found in FBS, which may otherwise cause interference when assessing the effects of GSPE. After 24 h media was replaced, cells were treated for time points ranging from 1 to 24 h with either water or DMSO, 100 μM chenodeoxycholic acid (CDCA), GSPE (20, 50 or 100 mg/L), or a combination of both CDCA and GSPE in DMEM supplemented with 1% L-glutamine and 0.5% charcoal-stripped FBS. Similar results were obtained from at least three independent experiments, performed in triplicate.

2.3 Animal studies

Mice were housed under standard conditions and all experimental procedures were approved by the local Institutional Committee for Care and Use of Laboratory Animals (IACUC) at the University of Nevada, Reno (Protocol # 00502). Age-matched groups of 8- to 10-wk-old male mice were used in all experiments ($n = 6$ per experimental group). Animal cohort sizes were determined based on previous similar studies [26, 27]. Wild-type (C57BL/6) and *Fxr*$^{-/-}$ mice (Jackson Laboratory), have been described previously [31]. The correct genotype was verified for all mice using previously reported primer sequences and reaction conditions [31]. All animals were housed in the Laboratory of Animal Medicine at the University of Nevada, Reno and provided standard rodent chow and water *ad libitum*. Mice were orally gavaged with either vehicle (water), or GSPE (250 mg per kg) and 14 h later blood was collected from the orbital plexus under isoflurane anesthesia, as previously described [26, 27]. The dose of procyanidins used is one-fifth of the no-observed-adverse-effect level (NOAEL) described for GSPE in male rats [32], and we previously showed that this dose reduces serum TG levels in C57BL/6 mice [26, 27]. Intestines and livers were snap-frozen in liquid nitrogen and stored at –80°C until use. Gene expression changes in the liver and intestine were assessed following GSPE administration for 14 h. To assess *Fgf15* expression at additional time points, experiments were performed as detailed above, and mice were terminated at 2-, 4-, or 8 h post-administration with vehicle or GSPE (250 mg/kg). To measure Cyp7a1 protein levels, mice were gavaged with vehicle or GSPE (250 mg/kg) for 3 consecutive days and terminated on day 3 at 11:00 am, after the final gavage at 09:00 am.

2.4 RNA isolation, cDNA synthesis and real-time QPCR analysis

Total RNA was extracted from tissues and cells using TRIzol (Life Technologies) according to the manufacturer's instructions. Complimentary DNA (cDNA) was reverse transcribed using superscript III reverse transcriptase (Life Technologies), and real-time quantitative polymerase chain reaction (qPCR) was used to determine gene expression changes. The reaction mix comprised 4 μL 10x buffer, 3.6 μL 50mM $MgCl_2$, 150 μM dNTPs, 0.5 μM of each primer, 0.125 μM of probe, 1.25 U Taq polymerase and 10 μL of cDNA (10 ng/μL), and was made up to a final reaction volume of 40 μL with water. The cycling conditions used in the amplification of each gene were: step 1: 95°C for 60 sec (1 cycle); step 2: 95°C for 15 sec and step 3: 60°C for 60 sec, with steps 2 and 3 repeated for 40 cycles. qPCR was performed using a CFX96 Real-Time System (BioRad). Forward and reverse primers and probes were designed using the Oligo Architect Software (Sigma-Aldrich) and obtained from Sigma-Aldrich. Primer and probe sequences can be found in Supporting Information Table 1. Expression of *β-actin* (Applied Biosystems), *cyclophilin* and *Gapdh* were used as endogenous controls. Target gene expression was normalized to the average of the three endogenous control genes and the ΔΔCt method was used to calculate the fold change in gene expression. Each sample was analyzed in triplicate.

2.5 Western blot analysis

Frozen intestines were homogenized in modified RIPA lysis buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM EDTA) containing protease inhibitors (Roche, Indianapolis). Equal amounts of total cellular proteins (15 μg) were separated in a 10% SDS-page gel, transferred to PVDF membrane, probed using a rabbit anti-Asbt antibody [12] at a dilution of 1:1000 and detected by chemiluminescence using a Clarity ECL kit (BioRad). The blots were also probed using a mouse anti-β-actin antibody (1:80 000) as a control for protein loading. We first performed Western analysis for all 5 sections of the small intestine and found that Asbt expression was exclusively located in the fifth segment (the most distal portion), in agreement with previous reports [12]. Therefore, Western analysis using only the 5th segment of the small intestine for each sample is presented.

To assess hepatic Cyp7a1 protein expression, microsomal membranes were prepared from frozen liver samples as previously reported [33]. A rabbit-generated polyclonal antibody

that recognizes amino acids 476–490 of the murine cholesterol 7α-hydroxylase [33] was used to detect the Cyp7a1 protein, at a dilution of 1:1000. A polyclonal antibody against the immunoglobulin heavy chain-binding protein (BiP), at a dilution of 1:1000, was used as a control for protein loading, as previously reported [12, 33].

2.6 Plasma analyses

Serum triglyceride and total cholesterol levels were measured enzymatically (Infinity™ kits, Thermo Scientific) according to the manufacturers' instructions using 1.5 μL serum and 150 μL of reagent. Bile acid concentrations in serum (20 μL per sample) were measured enzymatically using the Total Bile Acids Assay kit from Diazyme Laboratories. Alanine aminotransferase (ALT: Cat. No.: A526-120) and aspartate aminotransferase (AST: Cat. No.: A561-120) were measured using colorimetric based kits from Teco Diagnostic, according to the manufacturers' instructions, using 100 μL of sample and 500 μL of reagent. All analyses were performed in triplicate using a Biotek Synergy HT microplate reader.

2.7 Fecal bile acid output measurement

To determine fecal bile acid excretion, mice were placed in clean cages prior to the start of the experiment and feces were manually collected at the end of the 14 h experiment, air-dried and weighed. A modified version of the method reported previously [34] was used to measure the bile acid content. Briefly, 0.2 g of dried feces were mixed with 2 mL of 2 mg/mL sodium borohydrate in ethanol and left at room temperature for 1 h. Hydrochloric acid and sodium hydroxide were added and samples were vortexed and left to digest for 12 h under reflux. The samples were then filtered and dried under nitrogen. Samples were re-suspended in milli-Q water and filtered through Sep-Pak C18 cartridges, washed and eluted with methanol and dried under nitrogen. Samples were re-dissolved in 1 mL methanol and bile acid concentrations were measured enzymatically using the Total Bile Acids Assay kit from Diazyme Laboratories.

2.8 Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey post-hoc analysis was employed to detect significant differences between groups. Student's *t*-test was employed when comparing differences between vehicle and GSPE treatment for Asbt and Cyp7a1 protein expression in mouse liver. Treatment differences were considered statistically significant at $p<0.05$. All statistical analyses were performed using GraphPad Prism version 6.05 for Windows, GraphPad Software (San Diego, CA). Data represent mean ± SEM, $n = 3$–4 (Caco-2 cells) or $n = 6$ mice per treatment, per group, analyzed in triplicate.

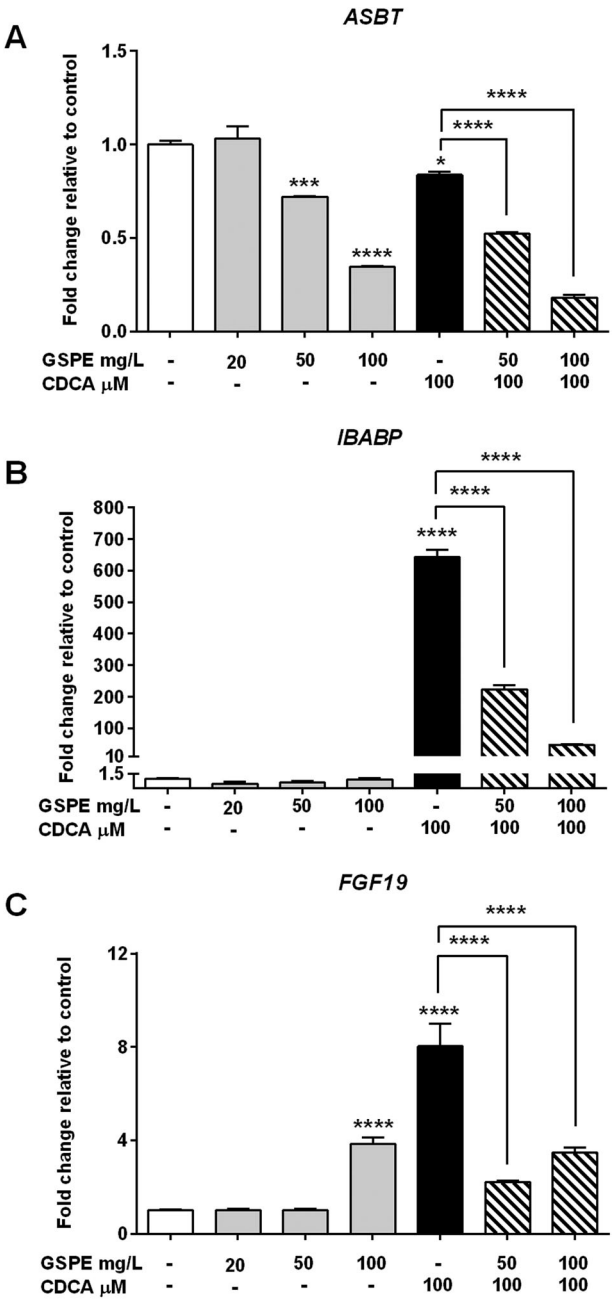


Figure 1. GSPE down-regulates FXR-target gene expression in vitro in Caco-2 cells. Caco-2 cells were treated with either a negative control (water or DMSO), GSPE, CDCA, or in combination, as indicated. Relative gene expression is shown for (A) *ASBT* and (B) *IBABP* after 24 h and (C) *FGF19* expression after 4 h. (Negative control, *open bars*; GSPE (20, 50 or 100 mg/L) (*grey bars*), CDCA (100 μM) (*black bars*), or in combination (*hatched bars*). Statistical differences are shown as: $*p<0.05$, $***p<0.001$, $****p<0.0001$. Asterisks above the bars show comparison to the control unless otherwise indicated.

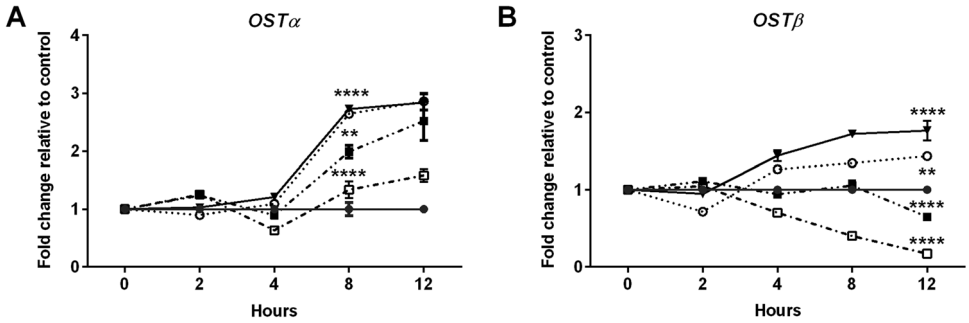


Figure 2. GSPE down-regulates basolateral BA transporters in vitro in Caco-2 cells. Time course for (A) *OST*α and (B) *OST*β gene expression over 12 h (Negative control: ●; 100 μM CDCA: ▼; 100 μM CDCA + 20 mg/L GSPE: ○; 100 μM CDCA + 50 mg/L GSPE: ■; 100 μM CDCA + 100 mg/L GSPE: □). Statistical differences are shown as: $^{**}p<0.01$, $^{****}p<0.0001$.

3 Results

3.1 GSPE selectively modulates intestinal FXR-target gene expression in vitro

The effects of GSPE on intestinal FXR-target gene expression were first determined in vitro using human colorectal Caco-2 cells. *ASBT* expression was reduced by CDCA and dose-dependently by GSPE treatment compared to control (Fig. 1A). Consistent with GSPE acting as a co-agonist ligand for FXR, co-administration with GSPE + CDCA further reduced *ASBT* expression in a dose-dependent manner compared to CDCA alone (Fig. 1A). CDCA-treatment increased *IBABP* expression compared to control, while co-treatment with GSPE dose-dependently inhibited the CDCA-induced increase (Fig. 1B). No significant differences were observed following treatment with GSPE alone. *FGF19* expression (the human homolog to murine *Fgf15*) was transiently induced by treatment with either CDCA or GSPE individually, compared to control (Fig. 1C), with a maximal effect observed at 4 h (Supporting Information Fig. 1A). In contrast, compared to CDCA alone, co-treatment with CDCA + GSPE resulted in a significant reduction in *FGF19* expression (Fig. 1C and Supporting Information Fig. 1B). Basolateral BA transporter expression (*OST*α/β) was increasingly induced over time with CDCA, compared to control (Fig. 2A and B), while co-treatment with GSPE + CDCA dose-dependently inhibited the CDCA-induced increase (Fig. 2A and B).

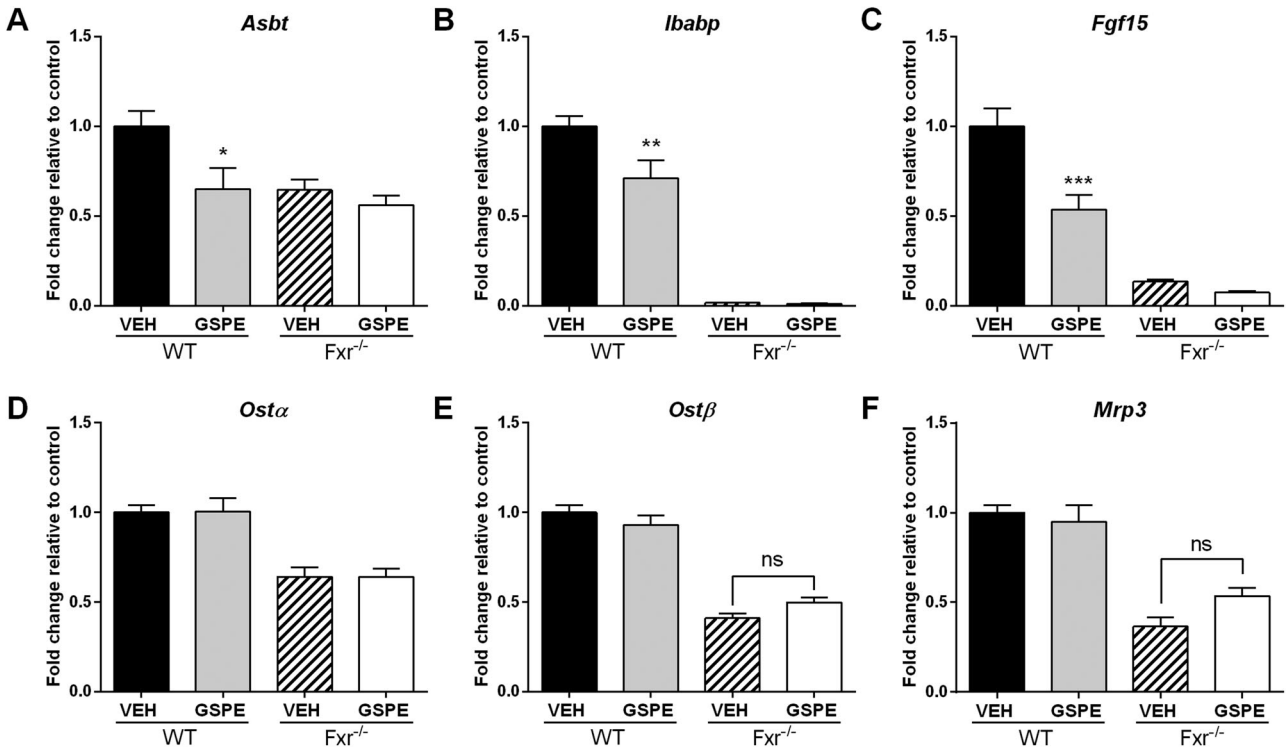


Figure 3. GSPE selectively modulates intestinal Fxr-regulated gene expression in vivo in an Fxr-dependent manner. Relative gene expression is shown for (A) *Asbt*, (B) *Ibabp*, (C) *Fgf15*, (D) *Ost*α, (E) *Ost*β, and (F) *Mrp3*. Statistical differences are shown as: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$. Asterisks above the bars show comparison to WT-VEH unless otherwise indicated.

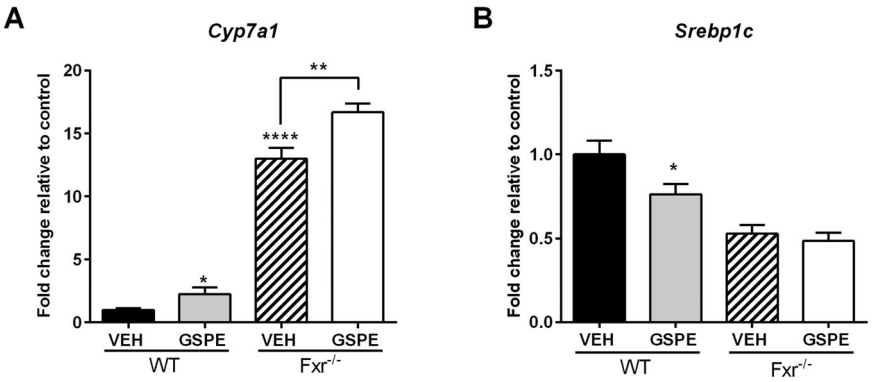


Figure 4. GSPE increases BA synthesis and represses lipogenesis in vivo. Relative gene expression is shown 14 h after administration for (A) *Cyp7a1* and (B) *Srebp1c*. Statistical differences are shown as: *$p<0.05$, **$p<0.01$, ****$p<0.0001$. Asterisks above the bars show comparison to WT-VEH unless otherwise indicated.

Consistently, GSPE treatment alone repressed *OSTα/β* expression in a dose-dependent manner, compared to control (data not shown).

3.2 GSPE alters the expression of genes involved in BA absorption, transport and synthesis in vivo

Our in vitro results indicate that GSPE selectively modulates intestinal FXR-target gene expression, as demonstrated by reduced *ASBT*, *IBABP* and *FGF19* expression, thus supporting our initial hypothesis. This prompted us to determine whether these effects also occur in vivo. To clarify the molecular mechanism by which GSPE exerts these effects, studies were conducted using C57BL/6 (wild-type, WT) and *Fxr*$^{-/-}$ mice to determine the effects on hepatic and intestinal Fxr-target gene expression following treatment via oral gavage with an acute dose of GSPE (250 mg/kg).

Consistent with our in vitro results showing that treatment with GSPE + CDCA reduced FXR-target gene expression, GSPE administration in vivo reduced intestinal *Asbt* (Fig. 3A), *Ibabp* (Fig. 3B) and *Fgf15* expression (Fig. 3C) in WT mice after 14 h, with no effect in *Fxr*$^{-/-}$. Due to the transient increase in *FGF19* expression observed in vitro at 4 h with GSPE alone (Fig. 1C and Supporting Information Fig. 1A) we wondered whether a transient induction in intestinal *Fgf15* expression in vivo could have occurred at an earlier time point. Consequently, additional experiments were conducted, whereby mice were treated with GSPE for 2-, 4- and 8 h. GSPE decreased intestinal *Fgf15* expression at each time point compared to control, with significance being reached at 4 h ($p \leq 0.001$) (data not shown). In contrast to our in vitro results, no changes in vivo in intestinal basolateral BA transporter expression, including *Ostα/β* (Fig. 3D and E) or *Mrp3* (Fig. 3F), were observed following GSPE treatment. Hepatic *Cyp7a1* expression was increased following GSPE administration (Fig. 4A), and consistent with our previous reports [26, 27], a significant decrease in hepatic *Srebp1c* expression in WT, but not *Fxr*$^{-/-}$ mice was observed (Fig. 4B), indicating reduced lipogenesis. Consistent with the observed changes in gene expression, intestinal Asbt protein levels were reduced following GSPE administration in WT mice (Fig. 5A), while hepatic Cyp7a1 protein expression was increased (Fig. 5B). Also, in agreement with our previous reports [26, 27], hepatic carnitine palmitoyltransferase 1a (*Cpt1a*) expression was increased in WT mice, indicating increased β-oxidation (data not shown). We did not observe any changes in hepatic BA transporter expression, including *Bsep* and *Ntcp*,

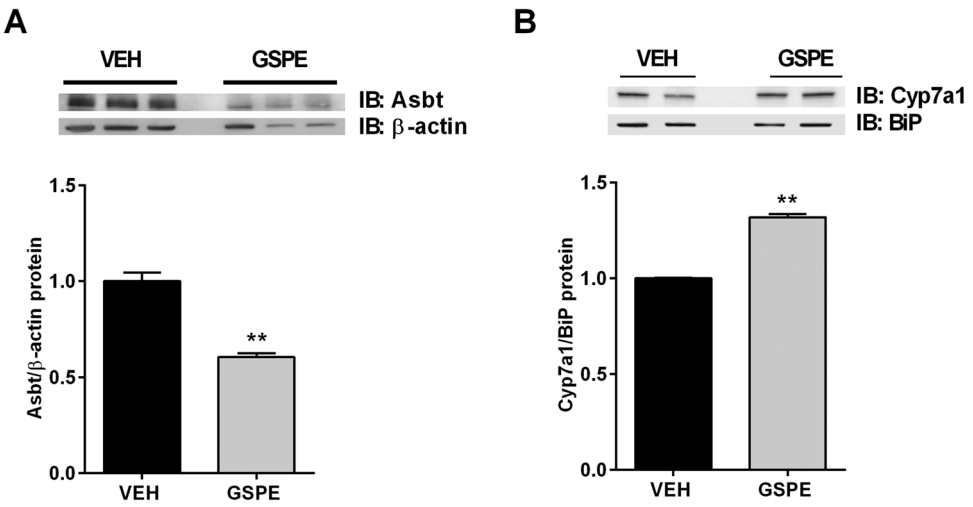
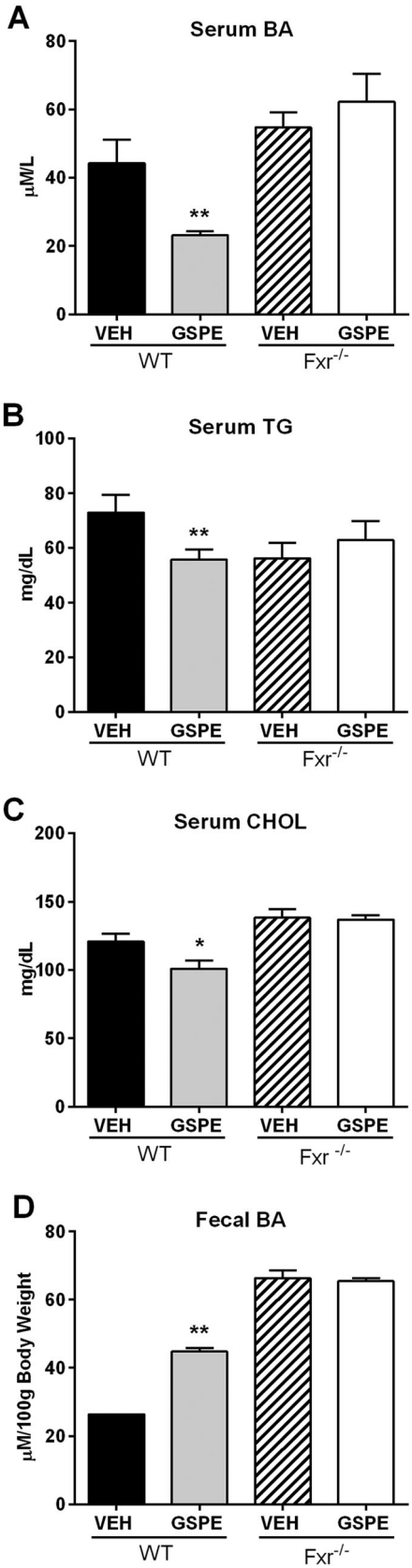


Figure 5. GSPE decreases intestinal Asbt protein expression leading to increased hepatic Cyp7a1 expression. Relative protein expression is shown for (A) Asbt, 14 h after administration and, (B) Cyp7a1, 3 days after administration. Protein quantification is represented normalized to β-actin or BiP, as indicated. Statistical differences are shown as: **$p<0.01$. Asterisks above the bars indicate comparison to VEH.



following GSPE administration (Supporting Information Fig. 2). Furthermore, serum levels of BA (Fig. 6A), TG (Fig. 6B), and cholesterol (Fig. 6C), were markedly decreased following GSPE administration in WT mice, while fecal BA output was markedly increased (Fig. 6D). Notably, these GSPE-induced changes were abrogated in GSPE-treated *Fxr*$^{-/-}$ mice, thereby establishing the Fxr-dependence of these effects. Importantly, GSPE did not alter markers for hepatocellular injury, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in this study, which remained within normal limits (Table 1).

4 Discussion

Our novel findings demonstrate, for the first time, that GSPE is a naturally occurring *gene-selective* bile acid receptor modulator (BARM), and provide evidence for a new mechanism by which it could lower serum triglyceride and cholesterol. Specifically, GSPE selectively modulates genes associated with intestinal BA absorption and transport, in an Fxr-dependent manner, resulting in decreased enterohepatic BA recirculation and increased fecal BA output. Ultimately these changes induce triglyceride catabolism, increase cholesterol and BA biosynthesis (via increased Cyp7a1), consequently leading to reduced serum cholesterol and TG levels.

Development of synthetic bile acid receptor modulators was previously described [35], and the tea catechin, epigallocatechin-3-gallate (EGCG) was shown to be a unique Fxr modulator by activating Fxr in a tissue- and gene-specific manner [36]. We now show that GSPE modulates gene expression similarly in both human-based in vitro and mouse-based in vivo studies. In particular, our in vivo studies provide further insight into the complex gene-regulatory actions of GSPE along the gut-liver axis and identify it as a novel naturally occurring BARM. We propose that the unique intestinally-mediated effects induced by GSPE, resulting in decreased BA absorption and enterohepatic recirculation, represent an additional potential mechanism underlying the already recognized hypotriglyceridemic action of this extract. Individuals with hyperlipidemia benefit from decreased BA absorption and increased BA biosynthesis [37] and, based on the results presented herein, we propose that GSPE may be a beneficial natural therapy against hypertriglyceridemia due to its ability to modulate BA absorption and homeostasis.

In the current study we used an acute dose of GSPE in order to gain insight into its' molecular targets, subsequently revealing a novel Fxr-dependent mechanism of action. Using

◀ **Figure 6.** GSPE administration reduces serum bile acid, triglyceride and cholesterol levels while increasing fecal bile acid output in vivo, in an Fxr-dependent manner. Serum was analyzed for (A) bile acid (BA) (B) triglyceride (TG), (C) cholesterol (CHOL), and (D) fecal bile acid (BA) excretion, 14 h after administration. Statistical differences are shown as: *$p<0.05$, **$p<0.01$. Asterisks above the bars indicate comparison to WT-VEH.

Table 1. Liver function biochemical parameters after GSPE administration

	WT		$Fxr^{-/-}$		
	VEH	GSPE	VEH	GSPE	Normal Reference Range (units/L)
ALT (units/L)	34.71 ± 6.66	45.38 ± 6.95	19.39 ± 3.14	28.68 ± 7.97	17–77
AST (units/L)	33.64 ± 4.46	38.88 ± 9.22	26.80 ± 3.60	34.22 ± 2.83	54–298

Data represent mean ± SEM, $n = 6$ per treatment, per group. **ALT:** Alanine aminotransferase; **AST:** Aspartate aminotransferase.

a translation of animal to human doses based on metabolic efficiency (energy expenditure) [38] and estimating the intake for a 60-kg human, the dose used herein corresponds to ~703 mg. The average proanthocyanidin intake for US adults over 19 years of age is reported to be 95 mg/d [39], while the mean total intake of polyphenols from consumption of fruits, berries, cereals, and vegetables by Finnish adults is 863±415 mg/d [40]. Therefore, increased consumption of procyanidins to reach the levels used in this study may be achieved via increased dietary intake of procyanidin-rich foods and/or supplementation.

Intestinal absorption of BAs is a critical step in the maintenance of both BA and cholesterol homeostasis [41], and appropriate function of Asbt is crucial for enterohepatic BA recirculation [42]. Asbt inhibition reduces circulating BA levels, leading to increased BA biosynthesis, and ultimately reducing cholesterol levels [41], as evidenced in $Asbt^{-/-}$ mice [12]. Inhibitors of BA absorption also reduce plasma cholesterol [12]. Therefore, the down-regulation in Asbt observed herein suggests that GSPE may lower cholesterol by directly altering intestinal BA absorption. This notion is supported by the fact that GSPE impairs intestinal BA uptake and transport, ultimately leading to a 47% decrease in serum BA levels, increased Cyp7a1 expression and decreased serum cholesterol levels.

It is well known that *Asbt* abrogation also leads to reduced serum TG levels, while simultaneously lowering hepatic *Srebp1c* [43], consistent with our observations following GSPE administration. Reduced serum BA levels and increased fecal BA excretion necessitates not only increased BA biosynthesis, consistent with the observed increase in Cyp7a1, but also increased β-oxidation, through increased *Cpt1a* expression [27, 44]. We, therefore, propose that reduced enterohepatic BA recirculation may explain, at least in part, the increased β-oxidation observed following GSPE administration.

Furthermore, GSPE decreased not only serum BA and cholesterol levels, but also induced HmgCoA-reductase and synthase expression, while simultaneously increasing fecal BA output. Taken together, these results indicate that reduced enterohepatic BA recirculation increases the conversion of cholesterol into BA, via increased Cyp7a1, while also coordinately increasing cholesterol synthesis in order to facilitate continued BA biosynthesis to replace those lost via fecal excretion.

In summary, we now show that the physiological changes consequential to GSPE administration are dependent upon Fxr and result in decreased enterohepatic BA recirculation, a property not previously associated with this natural product. The initial event triggering reduced enterohepatic BA recirculation and the subsequent metabolic events is mediated via Asbt, a key control point for BA entry into the enterocyte. These results indicate that mechanistically GSPE, via Fxr, induces a distinct coordinated metabolic response that inhibits intestinal BA absorption and transport, induces cholesterol and BA synthesis, inhibits lipogenesis and promotes TG catabolism.

Collectively our studies have uncovered GSPE as a potential new therapeutic avenue to manipulate TG and cholesterol levels through intestinally-based down-regulation of target-genes controlled by the nuclear receptor Fxr. Oral administration with the synthetic Fxr agonist, fexaramine, was recently reported to mediate beneficial metabolic changes via intestinal Fxr activation, without direct effects on hepatic Fxr-target genes [45]. The study presented herein now broadens the scope of knowledge regarding selective Fxr modulators and the potential for further advancement in alternative natural therapeutic strategies in the control of metabolic dysregulation, particularly hypertriglyceridemia and hypercholesterolemia, both significant risk factors associated with the development of cardiovascular disease.

MLR conceived and designed the study; RMH, GCC and MLR performed the experiments and analyzed the data; RMH performed statistical analysis; MLR and GCC wrote the manuscript.

We would like to thank Nicholas Davis, Kelvin Rodriguez and Brian Wong for technical assistance during the course of the study. Funding was provided by the USDA National Institute of Food and Agriculture (Hatch-NEV0738 and Multistate project W-3122: Beneficial and Adverse Effects of Natural Chemicals on Human Health and Food Safety) to MLR. GCC was the recipient of a CONICYT Bicentennial Becas-Chile Scholarship.

The authors have declared no conflict of interest.

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