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Activation of FXR by the synthetic agonist PX20350 ameliorates hepatic steatosis and enforces fecal neutral sterol loss in LXR agonist-treated mice

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Liver X Receptor (NR1H3/LXR) agonists have distinct anti-atherosclerotic properties in animal models. The concomitant induction of hepatic triglyceride (TG) accumulation and increased production of TG-rich very-low density lipoproteins (VLDL) have, however, delayed clinical development. Activation of the bile salt-activated nuclear receptor Farnesoid X Receptor (NR1H4/FXR) appears to cause opposite effects on (hepatic) TG metabolism. We have evaluated the potency of a novel FXR agonist, *i.e.*, PX20350, to overcome hepatic TG accumulation caused by LXR activation using the well-known T0901317 compound. We show that concomitant pharmacological activation of LXR and FXR in mice ameliorates hepatic TG accumulation induced by LXR agonism alone, without affecting stimulatory effects of LXR activation on expression of its lipogenic target genes. Hepatic carboxylesterase 1 (*Ces1g*) was found to be upregulated in PX20350 and T0901317/PX20350-treated mice, indicating increased TG hydrolysis upon FXR activation to contribute to reduction of hepatic steatosis. Surprisingly, PX20350 enhanced LXR-dependent stimulation of fecal neutral sterol excretion, likely explained by increased transintestinal cholesterol excretion upon FXR activation. In conclusion, FXR activation by the novel PX20350 compound ameliorates the adverse effect of LXR agonism on TG metabolism *via* a SREBF1/SREBP1C-independent route that probably involves accelerated hydrolysis of hepatic TG. Furthermore, simultaneous FXR and LXR activation additively promotes fecal neutral sterol excretion.

ABSTRACT

INTRODUCTION

Liver X Receptors (NR1H3/LXRα and NR1H2/LXRβ) are transcription factors that belong to the nuclear receptor superfamily and are activated by oxygenated metabolites of cholesterol (oxysterols) [1]. In the past decade, LXRs have emerged as important regulators of cholesterol, fatty acid and glucose homeostasis as well as of innate immunity [2]. As LXR activation leads to elevated plasma HDL levels and potentially promotes cholesterol efflux and reverse cholesterol transport to reduce atherosclerotic plaque formation in rodents, these nuclear receptors are considered as potential drug targets for anti-atherosclerosis therapies [3-5].

Unfortunately, in addition to the plethora of beneficial effects on cholesterol metabolism and inflammation, LXR agonists cause substantial hepatic triglyceride (TG) accumulation [6] and promote hepatic secretion of TG-rich very-low density lipoprotein (VLDL) particles [7]. These adverse effects, have so far precluded further clinical development of LXR agonists. Watanabe *et al.* [8] reported that treatment of mice with a high dose of bile salts (BS) prevented LXR activation-induced hepatic steatosis. This effect was attributed to activation of the BS-activated receptor FXR and involved repression of the Sterol Regulatory Element-Binding Protein-1c (SREBF1/ SREBP1C) controlled pathway of lipogenesis. Intriguingly, treatment of diabetic subjects with a pharmacological FXR agonist (obeticholate; INT-747; 6α-ethyl-chenodeoxycholate) was recently also shown to reduce hepatic steatosis [9]. Recently, PX20350 (PX), a novel pharmacological Farnesoid X receptor (NR1H4/FXR) agonist, was described as a selective synthetic FXR agonists and was found to lower plasma cholesterol and TG in C57BL/6J mice fed high-fat diet [10]. This compound is structurally related to the widely used GW4064 compound, with similar properties in *in vitro* assays but with considerably better systemic exposure, including the liver as major target organ for FXR activation [11]. The aim of the present study was to assess whether pharmacological FXR activation might specifically ameliorates the steatotic effects of an LXR agonist, while retaining the beneficial LXR-mediated stimulation of reverse cholesterol transport. We demonstrate that combined treatment with T0901317 (T09), a potent activator of LXR, and PX ameliorate the fatty liver caused by LXR agonism alone while maintaining the beneficial effects of pharmacological LXR activation on cholesterol metabolism. Surprisingly, the effect was independent of LXR-mediated effects on the classical hepatic lipogenic gene expression repertoire, while PX actually enforced the effects of LXR activation on fecal neutral sterol (FNS) excretion. This novel FXR agonist PX might therefore open new avenues

for therapeutic interventions in the treatment of atherosclerosis and cardiovascular disease (CVD).

MATERIALS AND METHODS

Animal experiments

Three month old male C57BL/6 (Charles River, L'Arbresle Cedex, France) mice were housed individually in a temperature- and light-controlled facility with 12 hours light-dark cycling. During the experiments, mice received food and water *ad libitum*. Mice either received standard laboratory chow (RMH-B, AB Diets, Woerden, The Netherlands) or, when stated, chow supplemented with either 10mg/kg/day T0901317 (T09, Cayman Chemical Company, Ann Arbor, MI, USA.), 30mg/kg/day Px20350 [10,11] (PX, Phenex Pharmaceuticals, AG, Heidelberg, Germany), or the combination of T09 and PX (dual) for 1 week. All experiments were approved by the Ethical Committee for Animal Experiments of the University of Groningen. Dietary intake was measured during the experiment and feces was collected 24 hrs prior to bile canulation and termination.

Experimental procedures

After one week of treatment regime, mice were anesthetized by intraperitoneal injection with Hypnorm (1ml/kg) (Janssen Pharmaceuticals, Tilburg, The Netherlands) and Diazepam (10mg/kg) (Actavis, Baarn, The Netherlands). After puncturing the gallbladder and disposal of its contents, hepatic bile was collected for 20 minutes from the common bile duct *via* the gallbladder. During the collection period, body temperature was stabilized in a humidified incubator at 37°C. The mice were sacrificed by cardiac puncture and the liver and the small intestine were rapidly excised. The small intestine was rinsed with cold phosphate-buffered saline containing 100µM phenylmethylsulforylfluoride (PMSF) and divided into three equal parts. Parts of both the liver and small intestine were snap-frozen in liquid nitrogen and stored at -80°C for mRNA and protein isolation and biochemical analyses. Feces were collected from individual mice 72 hours prior to termination.

Analytical procedures

Hepatic and biliary lipids were extracted according to Bligh & Dyer [12]. Plasma and liver TG, free fatty acids, total cholesterol and free cholesterol contents were determined using commercially available kits (Roche Diagnostics, Mannheim, Germany and DiaSys Diagnostic Systems, Holzheim, Germany). Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using commercially available kits (Spinreact, Santa Coloma, Spain). Biliary phospholipid content was determined according to Böttcher *et al*.[13]. Cholesterol in bile was measured according to Gamble *et al.* [14]. Pooled plasma samples were subjected to fast protein liquid chromatography (FPLC) gel filtration using a Superose 6 column (GE Healthcare, Little Chalfont, UK). Biliary BS were determined by an enzymatic fluorometric assay in 96-well black microwell plates (Greiner bio-one, Alphen aan den Rijn, The Netherlands) [15]. Biliary and fecal BS species and fecal cholesterol and its derivatives (also known as fecal neutral sterols (FNS)) were quantified using gas chromatography [16].

RNA isolation and qPCR

Tissue samples for isolation of RNA were snap frozen in liquid nitrogen and stored at -80°C. Samples were homogenized and total RNA using TRI-Reagent (Sigma, St. Louis, MO, USA). RNA concentration was determined using the Nanodrop spectrophotometer (NanoDrop 2000c, Thermo Scientific Inc., Walham, MA, USA). cDNA was obtained from total RNA using the RT procedure using Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (RT) (Invitrogen, Life Technologies, Bleiswijk, The Netherlands) with random primers according to manufacturer's protocol. Gene expression was measured with a 7900HT FAST system using FAST PCR mix, Taqman probes and MicroAmp FAST optical density 96-well plates (Applied Biosystems Europe, Nieuwekerk ad IJssel, The Netherlands). PCR results of liver and intestine were normalized to 36B4 mRNA levels. Ces1g primers were purchased from Life Technologies (Taqman Gene Expression Assays Ces1g Mm253603_m1, Cat#4351372),other primer and probe sequences can be obtained elsewhere (www.rtprimerdb.org).

Statistics

Data are shown as either means and standard deviations or Tukey's Box-and-Whiskers plots. Statistical analysis was assessed by using Kruskal-Wallis H test followed by Conover post-hoc comparisons using Brightstat [17]. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Combined T0901317 and PX20350 treatment modulates plasma cholesterol levels and ameliorates hepatic steatosis

To determine the effects of combined pharmacological activation of LXR and FXR on lipid metabolism, male C57BL/6 mice were either treated with the commonly used LXR agonist T0901317 (T09), the FXR agonist PX20350 (PX), or with both (dual) for 1 week and compared to chow-fed controls. As expected, total plasma cholesterol

Table 1. Animals characteristics of C57BL/6 wild-type mice treated with T0901317, PX20350 or both compared to untreated controls.

	Control	T0901317	PX20350	Dual
body weight (g)	24.4 ± 1.8	24.4 ± 1.4	24.4 ± 1.4	23.6 ± 1.5
liver weight (g)	1.2 ± 0.2	1.7 ± 0.2 *	1.6 ± 0.2 *	1.8 ± 0.2 *$
ratio LW/BW (%)	5.0 ± 0.6	6.9 ± 0.6 *	6.3 ± 0.4 *#	7.8 ± 0.5 *#$
cumulative food intake (g/7days)	29.2 ± 4.3	31.0 ± 4.5	32.4 ± 2.5	29.5 ± 2.5
fecal mass (g/24h)	0.99 ± 0.17	0.97 ± 0.06	1.10 ± 0.10	1.02 ± 0.11
Plasma lipids (mM)				
triglycerides	1.52 ± 0.47	1.29 ± 0.86	1.04 ± 0.16	1.24 ± 0.47
total cholesterol	2.28 ± 0.37	4.06 ± 0.53 *	1.70 ± 0.08 *#	3.51 ± 0.33 *#$
free cholesterol	0.68 ± 0.11	1.11 ± 0.17 *	0.45 ± 0.06 *#	0.92 ± 0.19 *#$
free fatty acids	0.6 ± 0.1	0.7 ± 0.2	0.5 ± 0.1	0.7 ± 0.2
Plasma liver enzymes (U/L)				
AST	56 ± 39	72 ± 30	78 ± 46	88 ± 49
ALT	53 ± 49	83 ± 37 *	88 ± 57 *	124 ± 52 *
Plasma bile salts (µM)	21.3 ± 22.9	21.7 ± 25.4	11.3 ± 2.1	13.8 ± 2.6

C57BL/6 wild-type mice were treated for one week either with T09, PX or both and compared to chow-fed controls. Mice characteristics are presented as means and standard deviations (n=8 animals per group). * $p < 0.05$ vs. control, # $p < 0.05$ vs. T09, $ $p < 0.05$ vs. PX.

levels in T09-treated mice were increased compared to control mice, while PX-treated mice show reduced levels of plasma cholesterol compared to controls (Table 1). Combined treatment reduced the total plasma cholesterol levels compared to T09 alone, yet, control levels were not reached (Table 1). FPLC-profiling revealed reduced cholesterol contents in especially the high-density lipoprotein (HDL)-sized fractions of PX-treated mice compared to controls (Fig. 1A). As observed in previous studies [5,7], a LXR-mediated induction of HDL-cholesterol was seen in the T09-treated mice. Grefhorst *et al.* [5] observed that treatment of T09 resulted in the appearance of cholesterol in FPLC fractions preceding the 'normal' HDL peak. Dual treatment

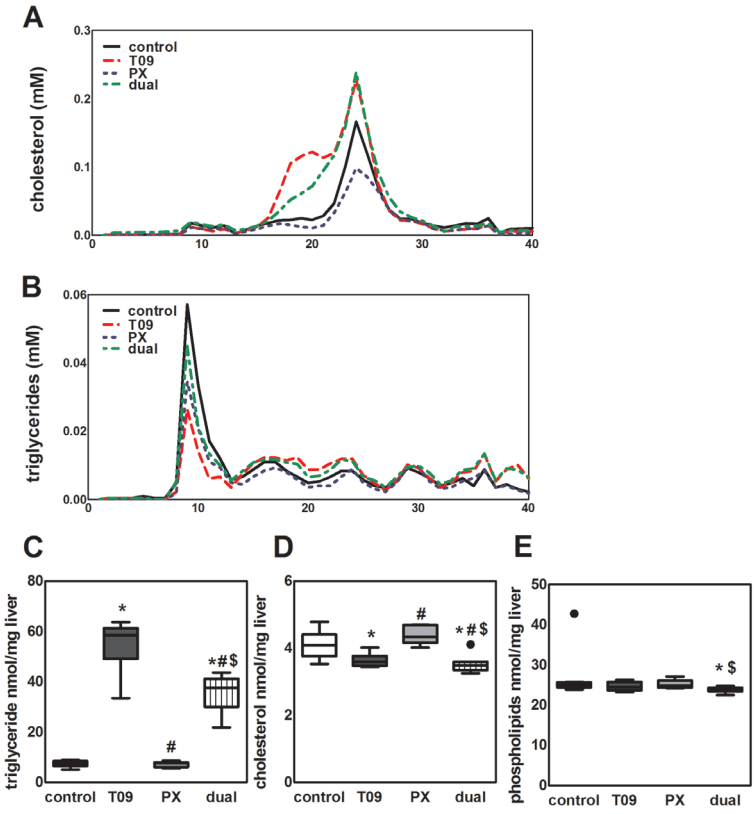

Figure 1. Plasma and hepatic lipids of C57BL/6 wild-type mice treated with T09, PX or both compared to untreated controls

C57BL/6 wild-type mice were treated for one week. Lipoprotein fractions were separated on FPLC using pooled plasma samples and cholesterol (A) and TG (B) levels were measured. Hepatic lipids were isolated from total livers and triglycerides (C), cholesterol (D) and phospholipid (E) contents were measured. (n=8 animals per group). * $p < 0.05$ vs. control, # $p < 0.05$ vs. T09, $ $p < 0.05$ vs. PX.

caused a pronounced reduction of HDL-cholesterol in this 'early' Apoa1-containing large HDL peak compared to T09-treatment (Fig. 1A). Both T09- and PX-treatment reduced TG levels in VLDL-sized fractions (Fig. 1B). Total plasma TG levels tended to be reduced in PX-treated mice, although this was not significant (Table 1).

Combined treatment reduced hepatic TG accumulation caused by pharmacological LXR activation alone, yet, control levels were not reached (Fig. 1C). This shows that simultaneous FXR activation ameliorates LXR activation-induced fat accumulation in the liver. Furthermore, liver weights and liver-to-body weight ratios increased upon pharmacological activation of LXR and FXR (Table 1). After one week of treatment, hepatic cholesterol levels were increased upon PX treatment, decreased upon T09 and even further decreased in the dual-treated group (Fig. 1D). Hepatic phospholipids levels in the dual treated mice were slightly decreased compared to the other three groups (Fig. 1E).

Amelioration of hepatic triglyceride accumulation is independent of direct LXR targeting of lipogenic genes

To determine efficiency of the treatment protocol and to better understand the molecular mechanism underlying the partial prevention of liver steatosis induced by combined treatment, we used quantitative RT-PCR to measure hepatic mRNA levels of several important genes involved in lipid and BS homeostasis (Fig. 2A-C). Expression of relevant nuclear receptors was similar between the groups (Fig. 2A). Expression of cholesterol 7 alpha-hydroxylase (*Cyp7A1*), the key regulator of BS metabolism, was significantly induced by LXR activation as expected (Fig. 2B) [18-20]. Upon PX-treatment, as also expected, *Cyp7a1* mRNA expression was suppressed. Expression of *Abcg5/8* was increased upon both T09 and PX treatment as well as the dual treatment, indicating increased ability for biliary cholesterol excretion (Fig. 2B). Furthermore, in agreement with the reduced plasma HDL-levels, expression levels of SCARB1/SRB1, involved in the HDL uptake on hepatocytes, was upregulated in PX- and dual-treated animals (Fig. 2B) [21]. The major protein component of HDL-particles, Apoal [22], was down-regulated in livers of T09- and dual-treated mice compared to controls and comparable to controls upon PX-treatment. Expression of *Srebp-1c*, the key transcription factor in lipogenesis, was induced upon T09-treatment, likely contributing to the well-established LXR-mediated activation of the lipogenic pathway (Fig. 2C). Additional treatment with PX slightly reduced *Srebp-1c* mRNA expression levels, but still showed increased levels compared to controls. Moreover,

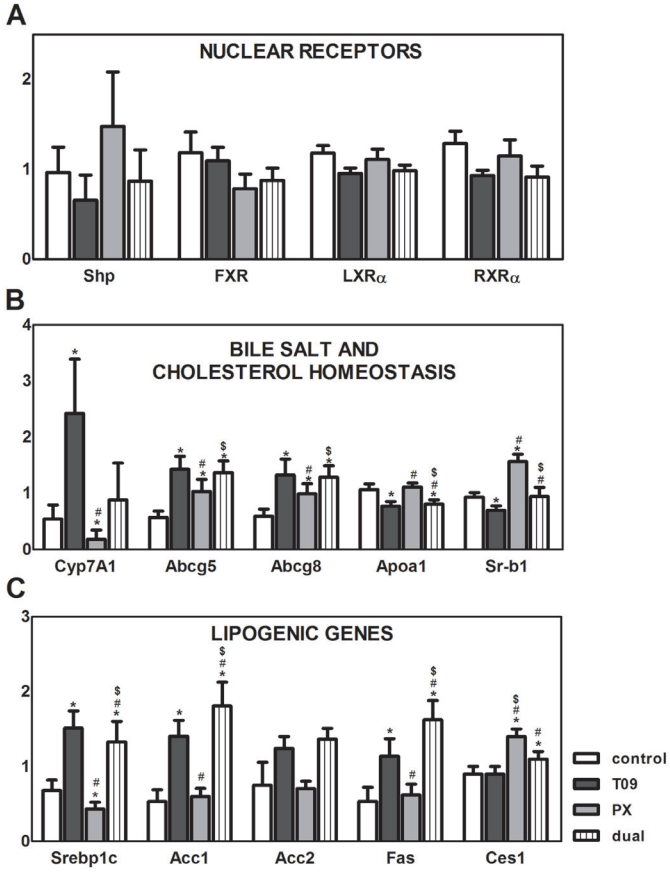

Figure 2. Relative hepatic gene expression of C57BL/6 wild-type mice treated with T09, PX or both compared to untreated controls

Quantitative real-time PCR was performed on liver tissues of C57BL/6 wild-type mice treated for one week. Nuclear receptors (A), genes involved in BS en cholesterol homeostasis (B) and lipogenic genes (C) were measured. Gene expression levels were normalized to 36B4. Values are presented as means and standard deviations (n=8 animals per group). * $p < 0.05$ vs. control, # $p < 0.05$ vs. T09, $ $p < 0.05$ vs. PX.

the lipogenic genes *Acaca /Acc1*, and *Fasn/Fas* were even further increased in the dual-treated mice compared to T09-treatment alone, while *Acacb/Acc2* did not show significant differences between the groups. These results demonstrate that the amelioration of hepatic TG seen upon dual treatment is independent of direct interference with the expression of LXR target genes. Hepatic carboxylesterase 1 (CES1 in humans and CES1G in mice), a novel FXR target [23], impacting both lipid

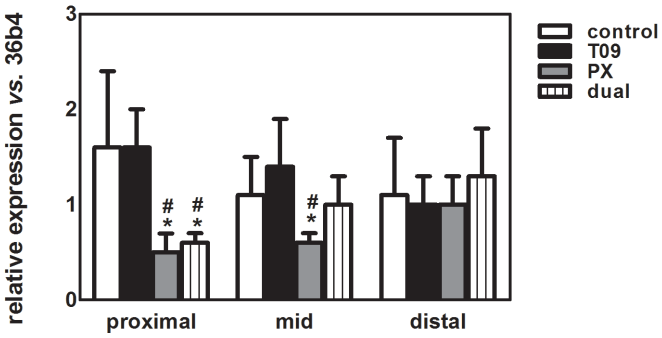

Figure 3. Relative intestinal Ces1g gene expression of C57BL/6 wild-type mice treated with either T09, PX or both compared to untreated controls
C57BL/6 wild-type mice were treated for one week either with T09, PX or both and compared to untreated controls The intestine was dived into a proximal, mid and distal part. Ces1g gene expression levels were normalized to 36b4. Data are presented as means and standard deviation. * $p < 0.05$ vs. control, # $p < 0.05$ vs. T09, $ $p < 0.05$ vs. PX.

and carbohydrate metabolism by enhancing TG- and cholesterol ester hydrolysis, was induced when treating mice with PX alone, and also slightly when treating mice with both T09 and PX (Fig. 2C), which could contribute to the reduced hepatic TG levels seen in the dual treatment compared to T09 alone. Intestinal *Ces1g* expression was reduced upon PX- and dual treatment (Fig. 3), suggestive for less lipoprotein assembly in the intestine which might contribute to the reduced plasma cholesterol levels seen upon PX-treatment.

Pharmacological FXR activation using PX20350 strongly increases fecal neutral sterol excretion

To examine the effects of T09 or/and PX on net body sterol fluxes, we calculated the cholesterol input and output in these mice (Fig. 4). The calculated dietary cholesterol intake was similar between the treatment groups (Fig. 4A). As expected, T09 treatment significantly induced biliary cholesterol excretion compared to controls (Fig. 4B). In the dual-treated mice, biliary cholesterol excretion was induced compared to PX-treatment alone. Fecal neutral sterol (FNS) loss was induced upon T09 treatment, as expected, but to a much stronger extent by PX and dual-treatment (Fig. 4C).

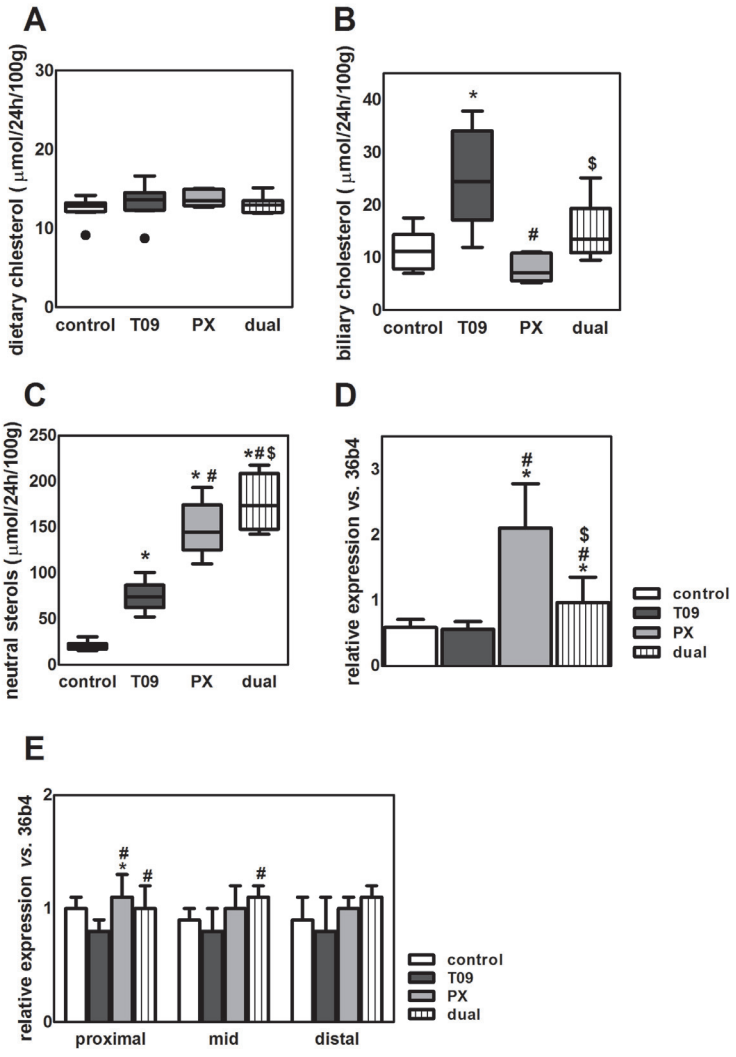

Figure 4. Biliary and fecal cholesterol and bile salt excretion and HMGCoA-reductase mRNA expression levels in liver and intestine of C57BL/6 wild-type mice treated with T09, PX or both compared to untreated controls

C57BL/6 wild-type mice were treated for one week. A cholesterol balance was calculated to calculate the contribution of dietary (A), biliary (B) to fecal neutral sterols (C). * $p < 0.05$ versus control, # $p < 0.05$ versus T09, $ $p < 0.05$ versus PX. Quantitative real-time PCR was performed on ~ 1 cm of the proximal, middle and distal ileum as well as liver tissues of C57BL/6 wild-type mice treated for one week. Hepatic and intestinal HMGCoA-reductase (Hmgcr) expression levels were measured and normalized to 36b4 (D and E). Values are presented as means and standard deviations (n=8 animals per group). * $p < 0.05$ vs. control, # $p < 0.05$ vs. T09, $ $p < 0.05$ vs. PX.

The massive FNS loss seen in PX-treated mice affects cholesterol homeostasis in these animals. For that reason, we measured hepatic and intestinal expression levels of HMGCoA-reductase, the rate-controlling enzyme in the biosynthesis of cholesterol (Fig. 4D and E). PX-treated mice showed significantly increased expression levels of hepatic *HMGCoA-reductase*, indicating increased hepatic cholesterol synthesis. The dual-treated mice showed an even further increased fecal cholesterol excretion compared to PX mice (Fig. 4C), yet, although dual-treatment showed increased *HMGCoA-reductase* expression increased levels seen upon PX-treatment alone were not reached (Fig. 4D). Also in the proximal part of the small intestine, *HMGCoA-reductase* expression was significantly upregulated in the PX-treated mice compared to controls, indicating increased cholesterol synthesis in the intestine (Fig. 4E). Yet, induction of intestinal cholesterol synthesis appeared to be much less pronounced than in the liver. Overall, PX treatment massively induced FNS excretion and expression data indicate particularly hepatic cholesterol synthesis is induced in these mice.

Pharmacological FXR activation using PX20350 alters bile and bile salt formation in C57BL/6 mice

To further explore the effects of the pharmacological activation of LXR or/and FXR on BS metabolism, the biliary and fecal BS species and total biliary and fecal BS were measured (Fig. 5C, 5D and Table 2). The increased biliary cholesterol excretion seen upon T09 treatment was accompanied by an increased total biliary BS excretion (Fig. 5C). As expected, fecal BS loss, reflecting *de novo* BS synthesis in the liver, was decreased upon pharmacological FXR activation (Fig. 5D). When looking at the specific BS species in these mice, the BS pool of PX-treated mice showed a reduced relative cholate content compared to controls and T09-treated mice and an induced relative content of muricholates (Fig. 5C, 5D and Table 2). Muricholates are more hydrophilic than cholate and will hence attract more water upon their secretion into the bile canaliculi, which likely explains the increased bile flow seen in these mice in the face of lower total BS secretion rates (Fig. 5B).

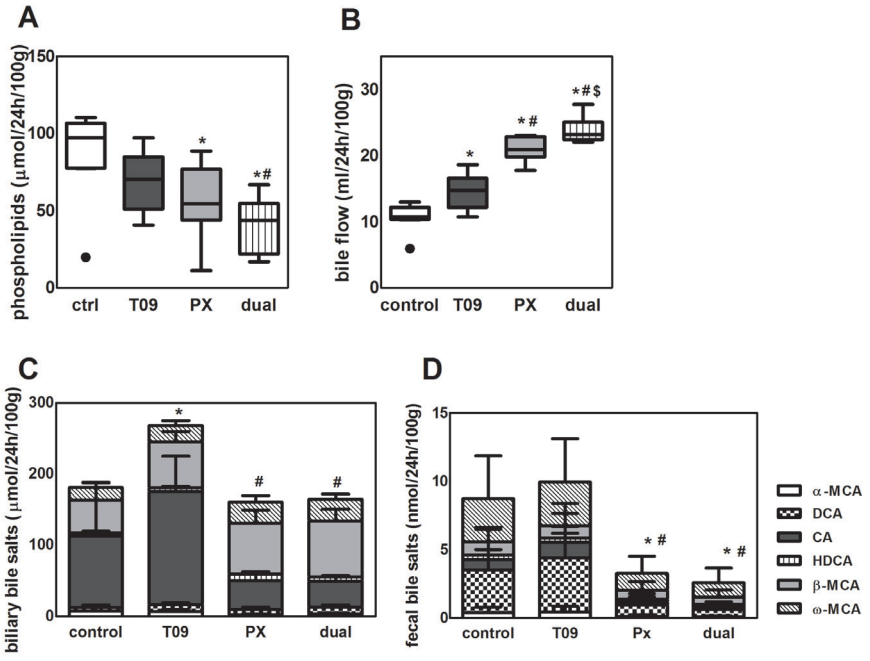

Figure 5. Hepatic bile formation and fecal bile salt excretion of C57BL/6 wild-type mice treated with T0901317, PX20350 or both compared to untreated controls

Biliary phospholipids (A), bile flow (B) and biliary (C) and fecal (D) bile salt species were determined in C57BL/6 wild-type mice treated with T09, PX or both compared to untreated controls. Mice were subjected to gallbladder canulation (n=8 animals per group). Feces was collected 24 hours prior to termination. The murine bile salt species include cholate (CA), deoxycholate (DCA), chenodeoxycholate (CDCA), α-muricholate (α-MCA), β-muricholate (β-MCA), ω-muricholate (ω-MCA), hyodeoxycholate (HDCA), lithocholate (LCA) and ursodeoxycholate (UDCA). * $p < 0.05$ vs. control, # $p < 0.05$ vs. T09, $ $p < 0.05$ vs. PX.

Table 2. Biliary and fecal bile salt profile of C57BL/6 wild-type mice treated with T0901317, PX20350 or both compared to untreated controls

	Control	T0901317	PX20350	Dual
Total biliary bile salts	**181.2 ± 114.3**	**266.8 ± 65.9***	**160.7 ± 37.0#**	**164.5 ± 31.5#**
α-muricholate	7.3 ± 7.6	6.6 ± 2.9	2.3 ± 1.1*#	3.5 ± 1.3
Deoxycholate	5.0 ± 3.8	9.9 ± 2.3*	7.3 ± 3.2	9.5 ± 3.2*
Cholate	100.6 ± 75.3	158.8 ± 49.8*	40.5 ± 11.7*#	36.3 ± 8.0*#
hyodeoxycholate	4.4 ± 2.4	5.3 ± 2.2	9.4 ± 3.6*#	6.1 ± 1.8$
β-muricholate	46.2 ± 24.5	64.5 ± 14.7	71.5 ± 18.4*	78.7 ± 16.6*
ω-muricholate	17.5 ± 6.7	23.2 ± 6.8	29.7 ± 8.9*	30.5 ± 7.4*#
Total fecal bile salts	**8.7 ± 1.3**	**9.9 ± 2.8**	**3.3 ± 0.7*#**	**2.6 ± 0.6*#**
α-muricholate	0.4 ± 0.1	0.4 ± 0.1	0.1 ± 0.0*#	1.0 ± 0.0*#
Deoxycholate	3.1 ± 0.6	4.0 ± 1.1*	0.8 ± 0.2*#	0.6 ± 0.3*#$
Cholate	0.8 ± 0.4	1.1 ± 0.6	0.2 ± 0.1*#	0.2 ± 0.0*#
hyodeoxycholate	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.0*#	0.2 ± 0.0*#
β-muricholate	0.9 ± 0.3	0.9 ± 0.5	0.6 ± 0.2	0.5 ± 0.1*#
ω-muricholate	3.1 ± 0.6	3.2 ± 1.2	1.2 ± 0.3*#	1.1± 0.3*#

C57BL/6 wild-type mice were treated for one week. Bile was canluated for 20 minutes prior termination and feces was collected 24 hrs prior termination. The specific biliary and fecal bile salts are presented as μmol/day/100g body weight as mean ± standard deviation (n=8). * $p<0.05$ vs. control; # $p<0.05$ vs. T09; $ $p<0.05$ vs. PX.

DISCUSSION

The most important finding in this study is that activation of FXR by the novel agonist PX ameliorates T09 activated LXR-induced hepatic steatosis in a Screbp1c-independent way. Since both nuclear receptor agonists have been shown to exert anti-atherosclerotic actions in rodent models [11,24], their combinatory use may open the way to clinical development of these compounds.

Our results partly confirm the data of Watanabe *et al.* [8]. These authors demonstrated that treatment of mice with the pharmacological LXR agonist T09 and the endogenous FXR activator cholate resulted in attenuation of LXR-agonist-induced lipogenesis *in vivo*, based on determination of mRNA levels of lipogenic genes. After 3 weeks of cholate feeding, hepatic TG levels in these mice were reduced by more than 50% and this was accompanied by decreased expression of *Srebp1c* and other lipogenic genes, indicating reduced lipogenesis. When treating wild-type mice fed a cholate-containing diet for one day with T09 for one additional day, liver weights and plasma TG levels were reduced compared to chow-fed T09-treated mice. Watanabe and coworkers concluded that cholate feeding lowers TG levels *via* attenuation of LXR-induced lipogenesis *in vivo*. Since cholate feeding stimulates the production of proinflammatory mediators by hepatocytes [25] and affects intestinal epithelial barrier function [26], we specifically activated both nuclear receptors using pharmacological compounds in wild-type mice, using a treatment regime of one week. The novel FXR agonist PX [10,11] has been shown to effectively reduce hepatic TG levels and to reduce plaque size area in human CETP transgenic LDLR knockout mice after 12 weeks of treatment. We hypothesized that co-administration of the above mentioned pharmacological LXR and FXR compounds could overcome the hepatic steatosis caused by pharmacological LXR activation alone, *via* inhibition of the classical lipogenic pathway as proposed by Watanabe and coworkers.

Hepatic TG levels were significantly reduced upon dual-treatment compared to T09-treated mice alone, but the levels did not reach hepatic TG contents of control mice (Fig. 1C). In contrast to the results of Watanabe *et al.*, TG lowering upon dual-treatment was accompanied by slightly suppressed hepatic expression *Srebp-1c* but other lipogenic genes were unaffected or even further increased (*Acc1*, *Acc2*,and *Fas*) (Figure 2C). These differential results might be explained by the actions of the plasma membrane-bound G-protein coupled receptor GPBAR1/TGR5, which functions as a BS sensing receptor [27,28] upon cholate feeding. Stimulation of TGR5 with BS activates adenylate cyclase thereby elevating intracellular cyclic AMP

levels that can activate various signaling cascades, involved in proliferation, anti-inflammation, anti-apoptosis, gallbladder relaxation and cholangiocyte secretion (reviewed in [29]). Treatment of obese mice using the specific TGR5 agonist INT-777 for 10 weeks reduced hepatic fatty acid and TG contents [30]. The beneficial effects of co-administration of cholate and T09 seen on hepatic steatosis by Watanabe and coworkers, might be the effect of cholate-induced TGR5 activation, since cholate and its metabolite deoxycholate are potent TGR5 ligands. Another explanation for the discrepancy between our data and those of Watanabe *et al*., concerns the treatment regime. After one day of cholate treatment, lipogenic gene expression was reduced in livers of wild-type mice. However, these effects were almost abolished after one week of cholic acid feeding (*Srebp-1c, Acc, Fas*), which we also observed in livers of PX-treated wild-type mice compared to controls (Fig. 2B). In the short-term experiments of Watanabe *et al*., mice were only treated for one or two days. In our studies we administered the agonists for one week either alone or simultaneously. Since the fatty acids synthesized upon T09-treatment accumulate less in the liver in the dual-treated mice, they have to be redistributed or stored somewhere else in the body. Free fatty acid concentrations were unaltered between the different treatment groups in the plasma compartment (Table 1), indicating that the plasma compartment does not act as a 'reservoir' for surplus fatty acids. Recently, Xu *et al*. [23] identified hepatic caboxylesterase 1 (Ces1g) as a FXR target that regulates lipid homeostasis. Increased hepatic *Ces1*g expression lowered hepatic TG and plasma cholesterol levels while knock-down of hepatic *Ces1g* increased hepatic TG and plasma cholesterol levels. Furthermore, hepatic CES1G has been proposed to exert anti-atherogenic actions by increasing sterol elimination in feces [31]. Activation of FXR using PX significantly induced hepatic *Ces1g* expression (Fig. 2C) and reduced levels of hepatic (Fig. 1C) and plasma TG as well as plasma cholesterol levels (Table 1), possibly caused by the TG hydrolase activity of CES1G. *Ces1g* is also expressed in the intestine and is reported to act as a lipid sensor in enterocytes regulating chylomicron secretion rate by regulating the availability of substrates for intestinal lipoprotein assembly [32]. Intestinal expression of *Ces1g* was reduced upon PX- and dual treatment (Fig. 3), suggestive for less lipoprotein assembly in the intestine which might contribute to the reduced plasma cholesterol levels seen upon PX-treatment (Table 1 and Fig. 1A and B).

Recently, Yuan and coworkers [33] reported that human hepatic CES1 catalyzes the hydrolysis of SRB1-delivered HDL-cholesterol and that SRB1 and CES1 cooperate to enhance movement of HDL-cholesterol into bile and to eliminate cholesterol

from the body. These results are in agreement with our results: FXR activation by PX induced the expression of both hepatic *Srb1* and *Ces1g* and increased FNS loss. However, how exactly hepatic and intestinal CES1G coordinately modulate TG and cholesterol metabolism upon pharmacological FXR activation remains to be elucidated.

An estimation of cholesterol removal from the body *via* the non-biliary route can be made by calculating the amounts of cholesterol that enters the intestine *via* diet and bile and the amount that leaves the body *via* FNS excretion. FNS excretion was massively increased upon PX- and dual treatment compared to controls. The massive FNS excretion seen upon PX- and dual-treatment cannot be explained by increased cholesterol intake (Fig. 4A) or increased biliary cholesterol output (Fig. 4B), since both were similar between controls and PX-treated mice. We did not measure fractional cholesterol absorption in these studies. Taking into account that this fraction is reduced from ~35% to ~20% by PX in chow-fed wild-type mice [11], likely due to a BS pool enriched in hydrophilic muricholates, the massive FNS loss seen upon PX-treatment cannot be explained by reduced absorption. Therefore, part of the cholesterol that is excreted into the feces must originate from the non-hepatobiliary route which includes intestinal cell shedding, transintestinal cholesterol excretion (TICE) [34,35], or locally synthesized cholesterol that is directly excreted. Although intestinal mRNA levels of *HMG-CoA reductase* were slightly induced upon PX-treatment, we consider it unlikely that enhanced *de novo* synthesis provides an important source of FNS. Therefore, we hypothesize that the majority of the FNS has actually been excreted *via* TICE. Again, altered composition of the circulating BS pool may have a role herein.

In conclusion, we have shown that pharmacological FXR activation by PX ameliorates the hepatic TG accumulation seen upon pharmacological LXR activation, possibly *via* upregulation of hepatic *Ces1g* which has TG hydrolyzing capacity. Furthermore, we show that pharmacological FXR activation using PX massively induced FNS excretion in feces, which cannot be explained by differences in dietary cholesterol intake or biliary cholesterol excretion and probably reflects increased TICE. If FXR activity in sum translates into an atheroprotective or proatherogenic effect has not been conclusively answered [36]. Hambruch *et al.* [11] showed that despite an apparent HDL-lowering activity, a FXR agonist with potent effects on transhepatic cholesterol efflux may turn out to be a good candidate for a potential FXR-targeted pharmacotherapy of atherosclerosis or other cholesterol-related diseases. The cholesterol mobilizing ability was already known from LXR activation *via* the actions

of Abca1, Abcg1 and ApoE [37], but this finding is new for FXR. How this stimulation of cholesterol efflux after FXR activation is brought about remains elusive and requires further study.

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