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Pharmacological LXR activation reduces presence of SR-B1 in liver membranes contributing to LXR-mediated induction of HDL-cholesterol☆

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ABSTRACT

Objective: Pharmacological LXR activation has anti-atherosclerotic actions in animal models. Part of these beneficial effects may be explained by accelerated reverse cholesterol transport since both plasma high density lipoprotein (HDL) cholesterol and fecal neutral sterol secretion are higher upon LXR activation. Mechanisms underlying these LXR-mediated effects have not been fully elucidated.
Methods: We investigated the roles of the isoforms LXRα and LXRβ and the HDL cholesterol uptake receptor SR-B1 in modulation of cholesterol metabolism upon treatment of mice with the LXR ligand T0901317.
Results: HDL cholesterol was maximally 60% increased in a time-dependent fashion due to appearance of more and larger HDL particles. Fecal neutral sterol secretion was maximally induced after 1 week treatment. T0901317 treatment induced fecal neutral sterol secretion by ~300% in wild-type but not in *Lxrα* deficient mice. Surprisingly, LXR activation reduced SR-B1 protein amount in hepatic membranes, suggesting that this might contribute to elevated HDL cholesterol. However, T0901317 still elevated plasma HDL cholesterol in *Sr-b1* deficient mice, suggesting that SR-B1 is not the only step involved in LXR-mediated induction of plasma HDL cholesterol. In addition, SR-B1 is not essential for LXR-induced cholesterol removal from the body.
Conclusion: Induction of fecal neutral sterol secretion by T0901317 critically depends on LXRα but not on LXRβ. LXR activation reduces SR-B1 in hepatic membranes, probably partly contributing to elevated HDL cholesterol. SR-B1 is not required to enhance fecal neutral sterol secretion.

1. Introduction

Liver X receptors (LXR) are nuclear receptors that are activated by oxysterols, *i.e.*, oxygenated cholesterol metabolites [1]. Two LXR isoforms have been identified. LXRα (NR1H3) is mainly expressed in the liver and to a lesser extent in intestine and adipose tissue, while LXRβ (NR1H2) is ubiquitously expressed [2,3]. From *in vitro* and *in vivo* studies employing pharmacological LXR ligands like T0901317 [4] it is evident that LXR ligands are potentially promising for treatment of cardiovascular diseases. Pharmacological LXR activation reduces atherosclerotic plaque development in animal models of atherosclerosis (reviewed in [4]). Plasma high density lipoprotein (HDL) cholesterol concentrations were increased upon pharmacological LXR activation [4] and it has therefore been proposed that this treatment induces 'classical' reverse cholesterol transport, *i.e.*, the transport of cholesterol from peripheral tissues (*e.g.*, macrophages) *via* HDL to the liver. Recently, however, van der Veen et al. [5] showed that the increase in fecal neutral sterol secretion induced by LXR activation in mice was mainly due to stimulation of transintestinal cholesterol excretion (TICE), *i.e.*, the direct transfer of cholesterol from the plasma across the intestinal wall into the feces [6].

So far, the mechanism(s) underlying the LXR-mediated increase in plasma HDL cholesterol concentration and the associated changes in HDL composition have not been fully elucidated. We [7] and others [8,9] have shown that LXR activation by pharmacological

Abbreviations: ABC, ATP binding cassette; ApoAI, apolipoprotein AI; FPLC, fast protein liquid chromatography; HDL, high density lipoprotein; LXR, liver X receptor; SR-B1, scavenger receptor-1B; SREBP-1c, sterol-regulatory element-binding protein-1c; TICE, transintestinal cholesterol excretion.

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means results in the appearance of large HDL particles in mice. In the present study, we therefore have evaluated the effects of LXR activation for specific periods of time on plasma HDL-cholesterol metabolism and fecal neutral sterol output in relation to the presence and activity of scavenger receptor B1 (SR-B1) since the plasma HDL cholesterol profiles of LXR agonist-treated wild-type mice closely resemble those observed in non-treated *Sr-b1* deficient mice [10]. In addition, we clarified the respective roles of the two LXR isoforms LXRα and LXRβ in induction of fecal neutral sterol secretion and TICE by employing *Lxrα* deficient mice.

2. Materials and methods

2.1. Animals and in vivo experimental procedures

All mice were housed in a light- and temperature-controlled facility (lights on 6:30 AM–6:30 PM, 21 °C), were fed a standard laboratory chow diet (RMH-B, Abdiets, Woerden, The Netherlands) and had free access to drinking water. Experimental procedures were approved by the Ethics Committees for Animal Experiments of the University of Groningen.

To study the time-dependent effects of LXR activation, male C57Bl/6J mice (Charles River, L'Arbresle Cedex, France) received standard chow containing T0901317 (0.015%, w/w) for 1 day, 1 week or 2 weeks. Untreated controls received non-supplemented laboratory chow. On the last treatment day, 4-h fasted (8–12 AM) animals were sacrificed by cardiac puncture under isoflurane anesthesia. Livers were quickly removed, freeze-clamped and stored at −80 °C. Blood was centrifuged (4000 × *g* for 10 min at 4 °C) and plasma was stored at −20 °C. Feces were collected for 24 h prior to termination.

Cholesterol fluxes were measured in male *Lxrα*$^{-/-}$ mice and their wild-type littermates on a Sv129/OlaHsd C57Bl/6J mixed background [11] as described previously [5,12]. In short, at start of the 2-week T0901317 treatment period, mice received an intravenous dose of 0.3 mg (0.73 μmol) cholesterol-D7 dissolved in Intralipid (20%, Fresenius Kabi, Den Bosch, The Netherlands) and an oral dose of 0.6 mg (1.535 μmol) cholesterol-D5 dissolved in medium-chain triglyceride oil. Blood spots were collected from the tail on filter paper (Schleicher & Schuell No2992, 's Hertogenbosch, The Netherlands) daily for 14 days. At the end of the 2 weeks treatment, mice were anesthesized by intraperitoneal injection with Hypnorm (fentanyl/fluanisone, 1 ml/kg) and Diazepam (10 mg/kg). The gallbladder was cannulated and bile was collected for 30 min. Subsequently, mice were sacrificed by cardiac puncture and the liver and the small intestine were excised. Feces were collected for 48 h prior to termination. Cholesterol was extracted from the blood spots for gas chromatography/mass spectrometry (GC/MS) analysis as described previously [12]. Biliary bile acids were determined by an enzymatic fluorimetric assay [13]. Total cholesterol concentrations in plasma and bile were determined by gas chromatography, as described below.

To study the role of SR-B1 in LXR-mediated effects on cholesterol metabolism, untreated male *Sr-b1*$^{-/-}$ mice on a C57BL/6J background [14] were fasted for 4 h and blood was collected by retro-orbital bleeding. Feces were collected for 24 h prior to retro-orbital bleeding. Next, these mice were fed the diet with 0.015% (w/w) T0901317 for 2 weeks. On the last treatment day, 4-h fasted (8–12 AM) animals were sacrificed by cardiac puncture under isoflurane anesthesia. Livers were quickly removed, freeze-clamped and stored at −80 °C. Blood was centrifuged (4000 × *g* for 10 min at 4 °C) and plasma was stored at −20 °C. Feces were collected for 24 h prior to termination.

2.2. Plasma lipoprotein analysis

Plasma lipoproteins were separated by fast protein liquid chromatography (FPLC) and the total cholesterol content of the collected fractions was determined using a commercially available kit (Roche Diagnostics, Mannheim, Germany).

The FPLC fractions were diluted 1:1 with 2× SDS loading buffer (100 mM Tris–chloride, pH 6.8; 4% SDS; 0.2% bromophenol blue; 20% glycerol; 200 mM dithiothreitol). To determine the apoA-I content, equal volumes of FPLC fractions in SDS loading buffer were subjected to SDS-PAGE on a 26-wells 12.5% gel (Criterion, Bio-Rad). ApoA-I was determined using an antibody against human apoA-I raised in rabbit (Calbiochem, San Diego, CA). Finally, horseradish peroxidase-conjugated anti-rabbit from donkey (Amersham Pharmacia Bioscience, GE Healthcare) and SuperSignal West Pico Chemiluminescent Substrate System (Pierce, Rockford, IL) were used.

2.3. Hepatic gene expression analysis

Total liver RNA was isolated using the TRI Reagent method (Sigma) according to manufacture's protocol. Integrity and concentration of RNA were determined with the Nanodrop spectrophotometer (NanoDrop™ 1000 Spectrophotometer, Thermo Scientific, Waltham, MA). cDNA was obtained using the reverse transcription procedure with Moloney Murine Leukemia Virus-RT (Sigma, St. Louis, MO) with random primers according to the protocol of the manufacturer. cDNA levels were measured in real-time quantitative PCR amplification using an ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA) against a calibration curve of pooled cDNA solutions. Expression levels were normalized for β-actin levels. The sequences of the primers and probes can be found on www.labpediatricsrug.nl and are deposited at RTPrimerDB (www.rtprimerdb.org).

2.4. Fecal neutral sterol and bile acid contents

Collected feces were dried and homogenized. Fifty micrograms of dried feces were boiled in 1 mL alkaline methanol (1 M NaOH/methanol, 1:3 vol/vol) at 80 °C for 2 h. After cooling down, the sample was extracted three times with 3 ml petroleum-ether to collect the neutral sterols. The remaining sample was diluted 1:9 with distilled water and this was used for bile acid isolation by reversed phase (C18) solid phase extraction. The neutral sterols and the bile acids were derivatized to the methyl ester-trimethylsilyl derivatives for gas chromatographic analysis [15]. The prepared fecal neutral sterol and fecal bile acid samples were determined by capillary gas chromatography using an Agilent gas chromatograph (HP 6890) equipped with a 25 m × 0.32 mm CP-Sil-19 fused silica column (Varian B.V., Middelburg, The Netherlands) and a Flame Ionization Detector.

2.5. Immunoblot analysis

Hepatic membrane fractions were prepared as described before [16] and the protein concentrations were determined using the BCA Protein Assay kit (Pierce). Individual samples were mixed with loading buffer, heated for 5 min at 96 °C and subjected to SDS-PAGE. SR-B1 was determined using an antibody against mouse SR-B1 raised in goat (Novus Biologicals, Littleton, CO), PDZK1 was determined using an antibody against mouse PDZK1 raised in rabbit (Abcam, Cambridge, UK). As loading controls, the concentration of the β-subunit of the Na/K-ATPase was determined using an antibody against Na/K-ATPase raised in goat [17]. Finally, horseradish peroxidase-conjugated anti-rabbit from donkey (Amersham Pharmacia Bioscience) or horseradish peroxidase-conjugated anti-goat

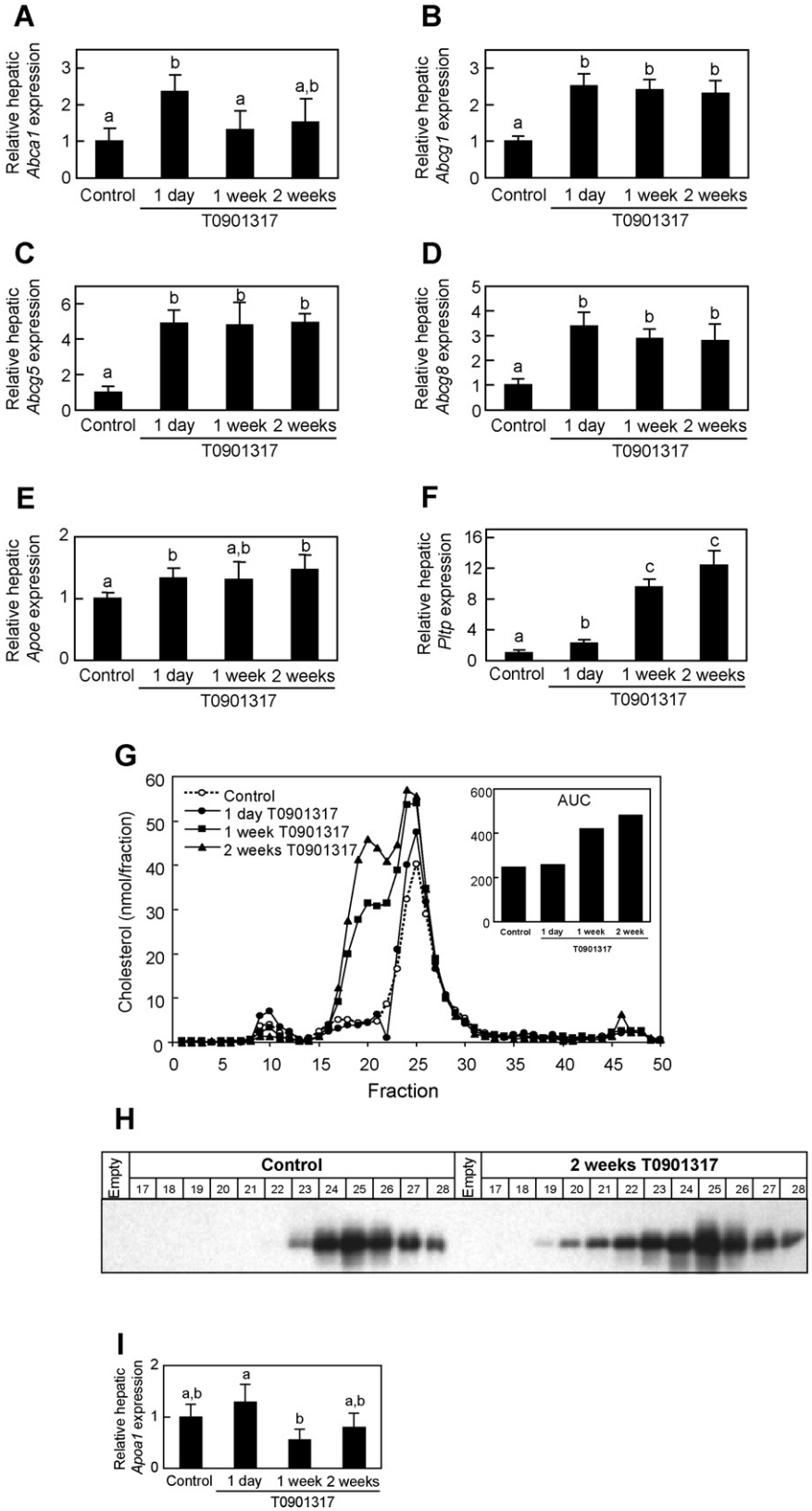

Fig. 1. Pharmacological LXR activation resulted in a time-dependent increase of plasma HDL cholesterol, mainly due to the appearance of large HDL particles. Hepatic gene expression of Abca1 (A), Abcg1 (B), Abcg5 (C), Abcg8 (D), Apoe (E) and Pltp (F) in untreated C57BL/6J mice and C57BL/6J mice treated with the pharmacological LXR ligand T0901317 for 1 day, 1 week or 2 weeks. G. Cholesterol FPLC profiles of untreated C57BL/6J mice and C57BL/6J mice treated with the pharmacological LXR ligand T0901317 for 1 day, 1 week, or 2 weeks. Inset: Area under the curve (AUC) of the cholesterol *vs.* fraction curve. H. Immunoblots of apoA-I in FPLC fractions of untreated C57BL/6J mice and C57BL/6J mice treated with the pharmacological LXR ligand T0901317 for 2 weeks. I. Hepatic gene expression of ApoAI in untreated C57BL/6J mice and C57BL/6J mice treated with the pharmacological LXR ligand T0901317 for 1 day, 1 week or 2 weeks. Values are averages ± SD; $n = 6$; bars without similar symbols are significantly different.

from donkey (Dako, Glostrup, Denmark) and SuperSignal West Pico Chemiluminescent Substrate System (Pierce) were used. The immunoblots were analyzed by densitometry using ImageJ software (http://imagej.nih.gov/ij/).

2.6. Statistics

Differences between groups were tested using a general linear model with corrections for multiple comparisons; $P < 0.05$ was considered significant when two groups were compared; $P < 0.008$ was considered significant when four groups were compared.

3. Results

3.1. The effects of pharmacological LXR activation on hepatic expression of Abca1, Abcg1, Abcg5, and Abcg8 do not depend on duration of treatment

To study the temporal effects of LXR activation on plasma and hepatic cholesterol concentrations, male C57BL/6J mice were treated with the commonly used pharmacological LXR ligand T0901317 for 1 day, 1 week or 2 weeks. To assess efficacy of the treatment protocol, expression of some well-defined LXR target genes encoding for the ATP binding cassettes A1, G1, G5, and G8 (ABCA1, ABCG1, ABCG5, and ABCG8), ApoE and PLTP was determined. As expected, treatment of mice with T0901317 increased hepatic expression of all genes. *Abcg1*, *Abcg5* and *Abcg8* expression all had a maximal induction already reached after one day of LXR activation (Fig. 1B–D) while *Abca1* expression (Fig. 1A) fluctuated throughout the treatment period. T0901317 induced hepatic *Apoe* expression with maximal effect after already 1 day (Fig. 1E) while *Pltp* is induced in a time-dependent manner (Fig. 1F).

3.2. The appearance of large HDL particles but not the induction of fecal neutral sterol secretion depends on the duration of LXR activation

One day of T0901317 treatment did not affect plasma cholesterol concentrations (Fig. 1G). Prolonged treatment, however, resulted in elevated HDL and a time-dependent appearance of cholesterol in FPLC fractions preceding the 'normal' HDL peak (Fig. 1G), indicative for the presence of larger particles. This increase in HDL cholesterol was reflected by the increased plasma total cholesterol concentrations that were 0.96, 1.11, 2.03 and 1.84 mM in control, 1-day, 1-week and 2-week T0901317-treated mice, respectively. We checked whether the 'extra' cholesterol peak represents large HDL by determining its apolipoprotein A-I (apoA-I) protein content by immunoblotting. Fig. 1H shows that, indeed, in FPLC fractions collected from mice treated with T0901317 for 2 weeks, apoA-I was detectable in earlier fractions compared to fractions collected from untreated mice. In addition, T0901317 treatment also increased the amount of apoA-I in the 'normal' HDL fractions. Although T0901317 treatment resulted in increased plasma apoA-I concentrations, *Apoal* gene expression in the liver was not induced by T0901317 (Fig. 1I). One week of T0901317 even slightly reduced hepatic *Apoal* expression.

Fecal bile acid secretion was not affected by T0901317 after one day of treatment, but reached maximal induction after one week (Fig. 2). Similarly, one day T0901317 treatment also did not affect fecal neutral sterol secretion, but both 1- and 2-week treatment regimes resulted in a clear ~4 fold induction (Fig. 2).

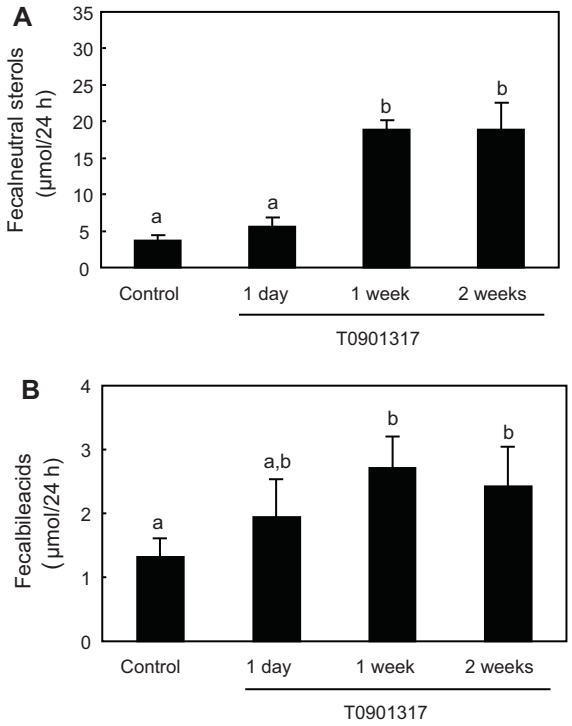

Fig. 2. Pharmacological LXR activation induces fecal neutral sterol and bile acid secretion. The 24-h fecal neutral sterol (A) and bile acid (B) secretion of untreated C57BL/6J mice and C57BL/6J mice treated with the pharmacological LXR ligand T0901317 for 1 day, 1 week, or 2 weeks. Values are averages ± SD; $n = 6$; bars without similar symbols are significantly different.

3.3. The effects of T0901317 on fecal sterol loss and TICE critically depend on the presence of LXRα

To determine the contribution of TICE to the enhanced neutral fecal cholesterol secretion upon T0901317 treatment and to evaluate its dependence on LXRα, we repeated the two-week T0901317 protocol in *Lxrα*$^{-/-}$ mice and their wild-type littermates and combined this with the TICE protocol as described previously [11,12]. The data presented in Fig. 3 show that the induction of plasma cholesterol concentration, biliary cholesterol secretion, fecal neutral sterol secretion, and TICE by T0901317 all critically depend on the presence of LXRα.

3.4. Pharmacological LXR activation reduces SR-B1 protein amount in the hepatic membrane

The appearance of large HDL particles upon pharmacological LXR activation resembles the phenotype of *Sr-b1*$^{-/-}$ mice [10]. Little is known about SR-B1 regulation, yet, PDZK1 plays an important role in stabilizing SR-B1 protein in membranes [18,19]. *Pdzk1*$^{-/-}$ mice have a plasma cholesterol profile comparable to that of *Sr-b1*$^{-/-}$ mice [20]. To study the potential involvement of SR-B1 and/or PDZK1 in the appearance of the large HDL upon LXR activation, we determined their hepatic gene and membranous protein expression.

Hepatic *Sr-b1* mRNA expression was not affected by the LXR agonist treatment (Fig. 4A). One day of T0901317 treatment did not affect SR-B1 protein content in isolated membrane fractions (Fig. 4C and D), but one and two weeks of treatment resulted in a clear reduction of the amounts of mature SR-B1 protein in membranes and the appearance of a lower band in the immunoblot.

T0901317 treatment affected neither hepatic *Pdzk1* gene expression (Fig. 4B) nor appearance of the PDZK1 protein in

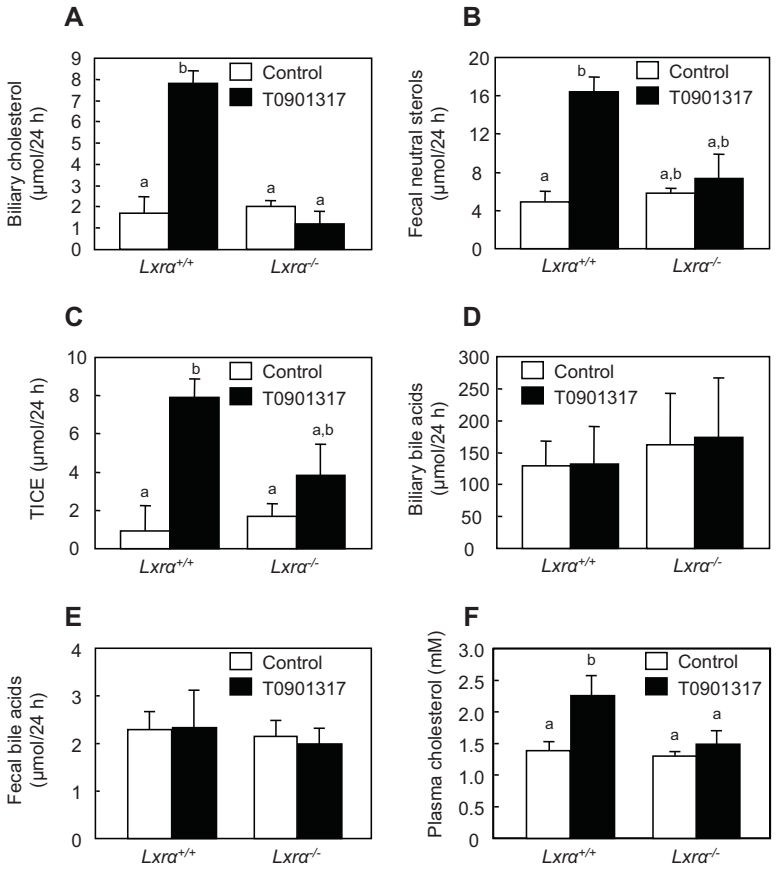

Fig. 3. Effects of pharmacological LXR activation by T0901317 on biliary and fecal neutral sterol secretion depends on the presence of LXRα. Biliary cholesterol secretion (A), fecal neutral sterol secretion (B), transintestinal cholesterol excretion (TICE) (C), biliary bile acid secretion (D), fecal bile acid secretion (E) and plasma cholesterol concentrations (F) of *Lxrα*$^{-/-}$ mice and their wild-type littermates treated with or without the pharmacological LXR ligand T0901317 for 2 weeks. Values are averages ± SD; $n = 4–6$; bars without similar symbols are significantly different.

hepatic membranes after one and two weeks of pharmacological LXR activation (Fig. 4C).

3.5. T0901317 enhances fecal neutral sterol secretion independently of SR-B1

The reduced amount of SR-B1 protein in hepatic membranes upon T0901317 treatment suggest that this might contribute to the elevated plasma HDL cholesterol. To test this, we treated *Sr-b1*$^{-/-}$ mice with T0901317 for 14 days. Treatment of *Sr-b1*$^{-/-}$ mice with the LXR agonist treatment raised the plasma cholesterol concentrations (Fig. 5A; 3.98 *vs.* 5.00 mM plasma total cholesterol, untreated *vs.* 2-week T0901317-treated mice) suggesting that the T0901317 induced reduction of plasma membrane Sr-b1 is not the only reason underlying the HDL increase in wild-type mice. Similarly to wild-types (Fig. 2), T0901317 treatment of *Sr-b1*$^{-/-}$ mice enhanced fecal neutral sterol secretion by 300% but did not affect fecal bile acid secretion (Fig. 5B and C).

4. Discussion

In this study we show for the first time that sustained LXR activation by T0901317 affects homeostasis of the HDL receptor SR-BI in mice. Although *Sr-b1* gene expression levels were unaltered, presence of SR-BI in the hepatic membranous pool reduced. SR-B1 is essential in hepatic uptake of HDL-derived cholesterol (ester) [21]. This novel observation suggests that the time-dependent raise in plasma HDL cholesterol-concentrations with the appearance of more and larger HDL particles is not only a consequence of enhanced ABCA1-mediated production [7,22] but also of impaired SR-B1-mediated uptake. Interestingly, T0910137 treatment of *Sr-b1* deficient mice also raised plasma HDL levels. This can probably be explained by the fact that in the absence of HDL uptake, a small increase in ABCA1 and ABCG1- induced peripheral cholesterol efflux will have a relatively large effect on plasma cholesterol concentrations. In wild-type mice, the decrease in hepatic SR-B1 protein upon T0901317 treatment coincided with enhanced fecal neutral sterol secretion, strongly suggesting that SR-B1 mediated hepatic HDL cholesterol uptake is not required for LXR-induced fecal neutral sterol secretion. Indeed, pharmacological LXR activation also enhanced fecal neutral sterol secretion in *Sr-b1*$^{-/-}$ mice.

Prolonged activation of LXR induced all the phenotypical aspects associated with enhanced RCT: elevated plasma HDL cholesterol (Fig. 1C) and enhanced fecal neutral sterol secretion (Fig. 2), as has repeatedly been shown before (see for review [4]). The enhanced *Apoe* and *Pltp* expression might both have contributed to the appearance of the larger HDL particles. Larger HDL is known to contain ApoE and PLTP can catalyze the conversion of two HDL particles to one larger HDL particle and one small lipid-poor apoA-I particle, pre-β-HDL [23,24]. Until recently, elevated HDL and enhanced fecal neutral sterol secretion upon LXR activation were thought to be mechanistically linked, especially since they appeared together with changes in gene transcription that are indicative for enhanced macrophage-to-liver cholesterol flux and subsequent conversion of cholesterol into bile acids. In line with previous publications [7,22,25], the present study also demonstrates that elevated plasma HDL cholesterol concentrations and enhanced fecal neutral sterol secretion are not linked to each other by definition.

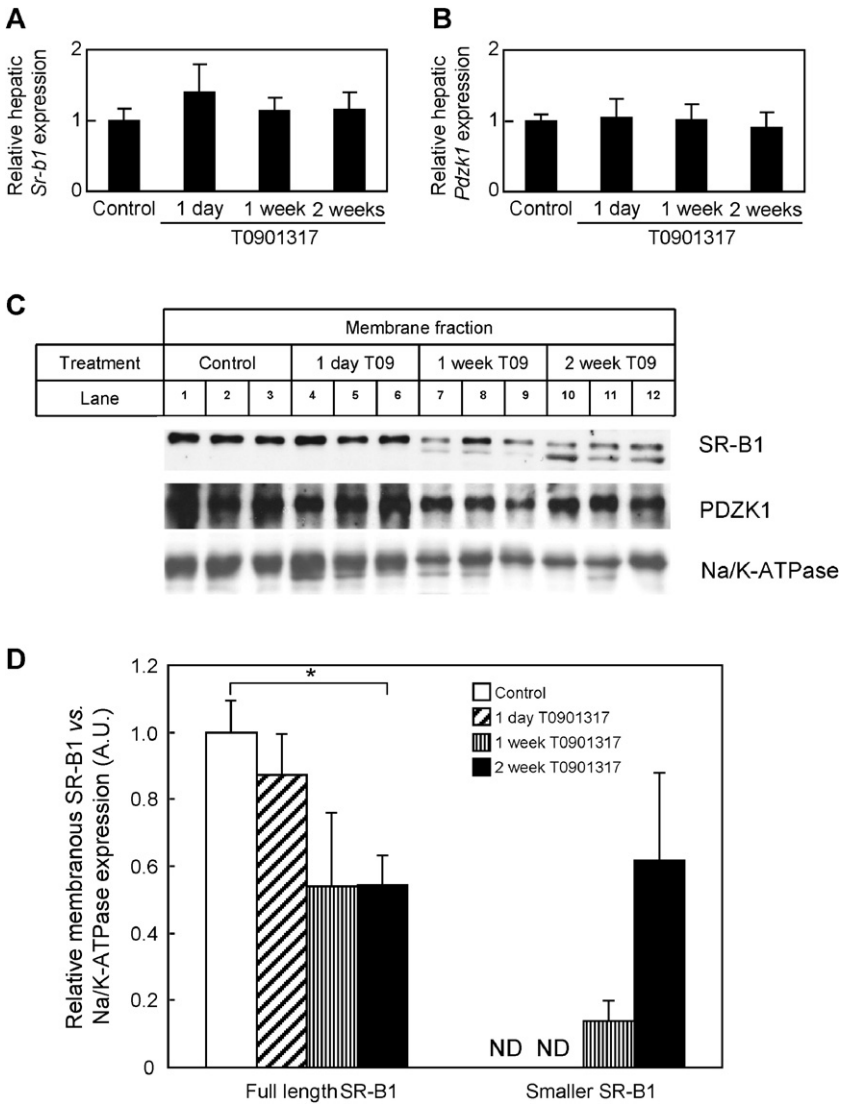

Fig. 4. Disturbed cellular distribution of SR-B1 protein in the liver and reduced PDZK1 protein of mice upon pharmacological LXR activation. A and B. Hepatic gene expression of Sr-b1 (A) and Pdzk1 (B) in untreated C57BL/6J mice and C57BL/6J mice treated with the pharmacological LXR ligand T0901317 for 1 day, 1 week or 2 weeks. Values are averages ± SD; $n = 6$. C. Immunoblot of SR-B1 and PDZK1 in hepatic membranes of untreated C57BL/6J mice and C57BL/6J mice treated with the pharmacological LXR ligand T0901317 for 1 day, 1 week, or 2 weeks. D. Density of the membranous full length and smaller SR-B1 *vs.* Na/K-ATPase calculated using ImageJ software. Values are averages ± SD; $n = 3$; $^*P < 0.008$.

In contrast to biliary bile acid secretion, biliary cholesterol secretion has been shown to be increased upon LXR activation [22] but, when extrapolated to daily secretion, this effect could by no means explain the concomitant induction of fecal neutral sterol secretion. Earlier studies already demonstrated that LXR-induced fecal neutral sterol secretion is only partly dependent on biliary cholesterol secretion [26] and is mainly explained by activation of a process referred to as transintestinal cholesterol excretion or TICE [5]. In the current study, we show for the first time that the effects of T0901317 on cholesterol secretion critically depend on the presence of LXRα. T0901317 did not at all stimulate biliary cholesterol secretion, fecal neutral sterol excretion or TICE in *Lxra*$^{-/-}$ mice (Fig. 3), thus indicating that LXRβ is not involved. Interestingly, intestine-specific activation of LXR has been shown to be anti-atherogenic despite relatively modest induction of fecal neutral sterol secretion [27]. Since whole body LXR activation is associated with hepatic-specific side-effects like hepatic steatosis [7,28], intestine-specific LXR-activation might provide a better option for novel therapeutic strategies.

The T0901317-mediated reduction of SR-B1 protein in hepatic membranes (Fig. 4C and D) is a remarkable finding. Since the immunoblots are from total membranes, including both plasma and internal membranes, we do not know whether pharmacological LXR activation affects redistribution of SR-B1 to external membranes. This can be an area for further study. SR-B1 membrane protein stability is regulated in a complex and not well-understood fashion, although PDZK1 is known to play an important role herein [18,19]. However, membranous PDZK1 proteins were not reduced (Fig. 4C). It has been reported that cholesterol feeding reduced hepatic SR-B1 proteins in a sterol-regulatory element-binding protein-1c (SREBP-1c)-dependent fashion in mice [29]. Since LXR is a main regulator of SREBP-1c transcriptional activity [30], the LXR-SREBP-1c pathway might regulate SR-B1 membrane stability. Strikingly, a lower band appeared in the SR-B1 immunoblot of hepatic membranes upon LXR activation. This lower band might be the unglycosylated form of SR-B1 as previously reported [31], but this requires more investigation.

Our data show that SR-B1-mediated hepatic HDL cholesterylester uptake only had a minor, if any, contribution

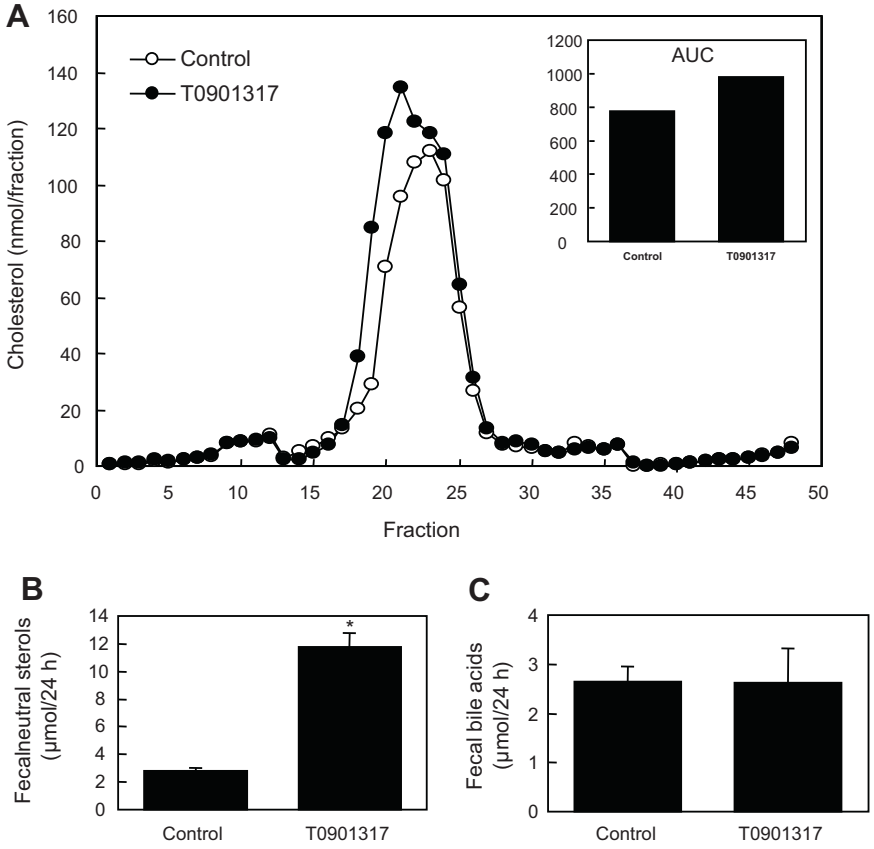

Fig. 5. Elevated plasma cholesterol and fecal neutral sterol secretion upon pharmacological LXR activation do not require SR-B1. A. Cholesterol FPLC profiles of untreated *Sr-b1*$^{-/-}$ mice and *Sr-b1*$^{-/-}$ mice treated with the pharmacological LXR ligand T0901317 for 2 weeks. Inset: Area under the curve (AUC) of the cholesterol *vs.* fraction curve. B-C. The 24-h fecal neutral sterol (B) and bile acid (C) secretion of untreated *Sr-b1*$^{-/-}$ mice and *Sr-b1*$^{-/-}$ mice treated with the pharmacological LXR ligand T0901317 for 2 weeks. Values are averages ± SD; $n = 6$; $^{*}P < 0.05$ *vs.* untreated mice.

to LXR-mediated fecal neutral sterol secretion since treatment of *Sr-b1*$^{-/-}$ mice with T0901317 induced fecal neutral sterol secretion as strong as in wild-type mice (Fig. 5B). This underscores that the HDL-to-feces cholesterol flux is of only minor importance in LXR-induced whole-body cholesterol removal. Indeed, TICE has been shown not to be decreased but to be even increased in *Sr-b1* deficient mice [25].

With respect to the effects of pharmacological LXR activation on plasma HDL-cholesterol concentrations, our studies for the first time report that not only the induction of the classical reverse cholesterol transport (mediated by ABCA1 and ABCG1) but also reduced HDL cholesterol uptake (mediated by SR-B1) might be involved in induced plasma HDL cholesterol upon T0901317 treatment in mice. Plosch et al. [22] reported a 26% reduction of *Sr-b1* gene expression in mice on the DBA1 background upon T0901317 treatment, but no significant reduction in *Sr-b1* in *Abca1* deficient mice on the same background. The difference of the T0901317 treatment on the *Sr-b1* expression between this [22] and our study (Fig. 4A) might be due to the background. There are differences in the backgrounds of the various knockout models we use, but since knockouts were compared with their wild-type littermates, we are able to draw conclusions about the involvement of either LXRα or SR-B1 in pharmacological LXR activation. In line with our observations, Plosch et al. also showed that T0901317 enhanced fecal sterol secretion in both wild-type and the *Abca1* deficient mice [22]. It might be of interest to investigate the effects of pharmacological LXR activation on fecal cholesterol secretion in the *Abca1/Sr-b1* double knockout mice.

Altogether, the data show that LXR activation unexpectedly reduced SR-B1 protein in hepatic membranes, an effect that might explain in part the increased concentration of HDL cholesterol and the larger size of the HDL particles. In addition, SR-B1 is clearly not required for the T0901317-mediated induction of whole body cholesterol removal *via* the feces.

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References

[1] Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 1996;383:728–31.
[2] Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ. LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev 1995;9:1033–45.
[3] Teboul M, Enmark E, Li Q, Wikstrom AC, Pelto-Huikko M, Gustafsson JA. OR-1, a member of the nuclear receptor superfamily that interacts with the 9-cis-retinoic acid receptor. Proc Natl Acad Sci U S A 1995;92:2096–100.
[4] Oosterveer MH, Grefhorst A, Groen AK, Kuipers F. The liver X receptor: control of cellular lipid homeostasis and beyond. Implications for drug design. Prog Lipid Res 2010;49:343–52.
[5] van der Veen JN, van Dijk TH, Vrins CL, et al. Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. J Biol Chem 2009;284:19211–9.
[6] van der Velde AE, Brufau G, Groen AK. Transintestinal cholesterol efflux. Curr Opin Lipidol 2010;21:167–71.
[7] Grefhorst A, Elzinga BM, Voshol PJ, et al. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. J Biol Chem 2002;277:34182–90.
[8] Jiang XC, Beyer TP, Li Z, et al. Enlargement of high density lipoprotein in mice via liver X receptor activation requires apolipoprotein E and is

abolished by cholesteryl ester transfer protein expression. J Biol Chem 2003;278:49072–8.
[9] Cao G, Beyer TP, Yang XP, et al. Phospholipid transfer protein is regulated by liver X receptors in vivo. J Biol Chem 2002;277:39561–5.
[10] Rigotti A, Trigatti BL, Penman M, Rayburn H, Herz J, Krieger M. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. Proc Natl Acad Sci U S A 1997;94:12610–5.
[11] van der Veen JN, Havinga R, Bloks VW, Groen AK, Kuipers F. Cholesterol feeding strongly reduces hepatic VLDL-triglyceride production in mice lacking the liver X receptor alpha. J Lipid Res 2007;48:337–47.
[12] Brufau G, Kuipers F, Lin Y, Trautwein EA, Groen AK. A reappraisal of the mechanism by which plant sterols promote neutral sterol loss in mice. PLoS One 2011;6:e21576.
[13] Murphy GM, Billing BH, Baron DN. A fluorimetric and enzymatic method for the estimation of serum total bile acids. J Clin Pathol 1970;23:594–8.
[14] Wiersma H, Nijstad N, Gautier T, et al. Scavenger receptor BI facilitates hepatic very low density lipoprotein production in mice. J Lipid Res 2010;51:544–53.
[15] Hulzebos CV, Renfurm L, Bandsma RH, et al. Measurement of parameters of cholic acid kinetics in plasma using a microscale stable isotope dilution technique: application to rodents and humans. J Lipid Res 2001;42:1923–9.
[16] Engelking LJ, Kuriyama H, Hammer RE, et al. Overexpression of Insig-1 in the livers of transgenic mice inhibits SREBP processing and reduces insulin-stimulated lipogenesis. J Clin Invest 2004;113:1168–75.
[17] Koopen NR, Wolters H, Voshol P, et al. Decreased Na^+-dependent taurocholate uptake and low expression of the sinusoidal Na^+-taurocholate cotransporting protein (Ntcp) in livers of mdr2 P-glycoprotein-deficient mice. J Hepatol 1999;30:14–21.
[18] Fenske SA, Yesilaltay A, Pal R, et al. Normal hepatic cell surface localization of the high density lipoprotein receptor, scavenger receptor class B, type I, depends on all four PDZ domains of PDZK1. J Biol Chem 2009;284:5797–806.
[19] Yesilaltay A, Kocher O, Pal R, et al. PDZK1 is required for maintaining hepatic scavenger receptor, class B, type I (SR-BI) steady state levels but not its surface localization or function. J Biol Chem 2006;281:28975–80.
[20] Kocher O, Yesilaltay A, Cirovic C, Pal R, Rigotti A, Krieger M. Targeted disruption of the PDZK1 gene in mice causes tissue-specific depletion of the high density lipoprotein receptor scavenger receptor class B type I and altered lipoprotein metabolism. J Biol Chem 2003;278:52820–5.
[21] Trigatti B, Rigotti A, Krieger M. The role of the high-density lipoprotein receptor SR-BI in cholesterol metabolism. Curr Opin Lipidol 2000;11:123–31.
[22] Plosch T, Kok T, Bloks VW, et al. Increased hepatobiliary and fecal cholesterol excretion upon activation of the liver X receptor is independent of ABCA1. J Biol Chem 2002;277:33870–7.
[23] Huuskonen J, Olkkonen VM, Jauhiainen M, Ehnholm C. The impact of phospholipid transfer protein (PLTP) on HDL metabolism. Atherosclerosis 2001;55:269–81.
[24] Rye K-A, Clay M, Barter PJ. Remodelling of high density lipoproteins by plasma factors. Atherosclerosis 1999;145:227–38.
[25] van der Velde AE, Vrins CL, van den Oever K, et al. Regulation of direct transintestinal cholesterol excretion in mice. Am J Physiol Gastrointest Liver Physiol 2008;295:G203–8.
[26] Kruit JK, Plosch T, Havinga R, et al. Increased fecal neutral sterol loss upon liver X receptor activation is independent of biliary sterol secretion in mice. Gastroenterology 2005;128:147–56.
[27] Lo Sasso G, Murzilli S, Salvatore L, et al. Intestinal specific LXR activation stimulates reverse cholesterol transport and protects from atherosclerosis. Cell Metab 2010;12:187–93.
[28] Schultz JR, Tu H, Luk A, et al. Role of LXRs in control of lipogenesis. Genes Dev 2000;14:2831–8.
[29] Niemeier A, Kovacs WJ, Strobl W, Stangl H. Atherogenic diet leads to posttranslational down-regulation of murine hepatocyte SR-BI expression. Atherosclerosis 2009;202:169–75.
[30] Liang G, Yang J, Horton JD, Hammer RE, Goldstein JL, Brown MS. Diminished hepatic response to fasting/refeeding and liver X receptor agonists in mice with selective deficiency of sterol regulatory element-binding protein-1c. J Biol Chem 2002;277:9520–8.
[31] Voshol PJ, Schwarz M, Rigotti A, Krieger M, Groen AK, Kuipers F. Down-regulation of intestinal scavenger receptor class B, type I (SR-BI) expression in rodents under conditions of deficient bile delivery to the intestine. Biochem J 2001;356:317–25.