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Different roles of liver X receptor α and β in lipid metabolism: Effects of an α-selective and a dual agonist in mice deficient in each subtype

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ABSTRACT

Liver X receptor (LXR) α and LXRβ are closely related nuclear receptors that respond to elevated levels of intracellular cholesterol by enhancing transcription of genes that control cholesterol efflux and fatty acid biosynthesis. The consequences of inactivation of either LXR isoform have been thoroughly studied, as have the effects of simultaneous activation of both LXRα and LXRβ by synthetic compounds. We here describe the effects of selective activation of LXRα or LXRβ on lipid metabolism. This was accomplished by treating mice genetically deficient in either LXRα or LXRβ with an agonist with equal potency for both isoforms (Compound B) or a synthetic agonist selective for LXRα (Compound A). We also determined the effect of these agonists on gene expression and cholesterol efflux in peritoneal macrophages derived from wild-type and knockout mice. Both compounds raised HDL-cholesterol and increased liver triglycerides in wild-type mice; in contrast, in mice deficient in LXRα, Compound B increased HDL-cholesterol but did not cause hepatic steatosis. Compound B induced ATP-binding cassette transporter (ABC) A1 expression and stimulated cholesterol efflux in macrophages from both LXRα and LXRβ-deficient mice. Our data lend further experimental support to the hypothesis that LXRβ-selective agonists may raise HDL-cholesterol and stimulate macrophage cholesterol efflux without causing liver triglyceride accumulation.

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1. Introduction

The nuclear receptors LXRα (NR1H3) and LXRβ (NR1H2) are two highly homologous proteins that sense cellular cholesterol excess and regulate transcription of multiple genes of lipid metabolism (for recent reviews, see [1,2]). Both liver X receptor (LXR) subtypes are activated by multiple oxysterols [3], and we have previously demonstrated that 27-hydroxycholesterol is a physiologically relevant LXR agonist in human cells [4]. The LXRs were originally cloned as ''orphan receptors'' in the mid-1990s [3], and over the next few short years a series of discoveries demonstrated the critical importance of LXRs in lipid metabolism. These discoveries were enabled by the use of two types of tools: first, mice genetically engineered to be deficient in LXRα and/or LXRβ [5,6], and second, synthetic LXR agonists. It is important to note that the well-studied synthetic LXR agonists T0901317 [7] and GW3965 [8], as well as the endogenous LXR agonist 27-hydroxycholesterol [4], activate both LXRα and LXRβ with approximately equal potency and efficacy.

In addition to their well-established function as regulators of cholesterol and fatty acid metabolism [9,10], more recent evidence points to additional roles for the LXR nuclear receptors in inflammation [11], glucose metabolism [12–14] and fertility [15]. In the arena of cholesterol metabolism, LXRs regulate multiple sterol transporters, including ABCA1 [3], ABCG1 [1], ABCG5 and ABCG8 [16]. ABCA1 is a lipid pump that rids cells of excess cholesterol; this gene is necessary for maintaining HDL levels, as demonstrated by the very low HDL levels caused by homozygous genetic deficiency in ABCA1 in both mice and humans [17]. Increased transcription of ABCA1 caused by LXR agonists likely contributes to the increase in serum HDL and decrease in atherosclerosis caused by administration of T0901317 [18] or GW3965 [19] to mice. These favorable effects suggest that synthetic LXR agonists might be useful therapeutics in patients with atherosclerotic disease.

LXR regulation of fatty acid metabolism occurs predominantly through transcription of sterol regulatory element-binding protein (SREBP)-1c [20], which is the master regulator of transcription of all the genes of de novo fatty acid biosynthesis [21]. LXR agonists increase mRNA levels for SREBP-1c and its downstream genes, leading to increased levels of serum triglyceride and liver triglyceride content [7]. The increased serum triglyceride has been reported to be transient [22], but it is likely that long-term dosing of LXR agonists will cause unacceptable fatty liver. Therefore, the key challenge in the development of LXR agonists as novel therapeutics is to dissociate the favorable effects on cholesterol metabolism from the unfavorable effects on fatty acid metabolism.

It has been proposed on multiple occasions that subtype-selective LXR activators, in particular LXRβ-selective agonists, may provide an opportunity to dissociate the favorable from the unfavorable effects of LXR agonists (see, e.g. refs. [2,23,24]). The well-studied LXR agonists T0901317 and GW3965 are both dual agonists, i.e., they activate both LXRα and LXRβ. This dual activity is not surprising given the high degree of homology between the ligand-binding domains of LXRα and LXRβ (discussed in ref. [1]). The high degree of homology is reflected in the difficulty in obtaining subtype-specific agonists, and to our knowledge no highly selective compound has been disclosed. Despite this lack of specific compounds, the hypothesis is strongly supported by at least four independent pieces of evidence indicating that LXRα and LXRβ do not perform identical functions in mice. First, DNA microarray studies showed different gene expression profiles in tissues from mice lacking LXRα versus mice lacking LXRβ [25]. Secondly, the induction of lipoprotein lipase in mouse liver by LXR agonists was shown to require the presence of LXRα but not LXRβ, and an LXRβ/RXR heterodimer was shown to bind less efficiently than LXRα/RXR to the lipoprotein lipase promoter [26]. Thirdly, LXRα-deficient mice, but not LXRβ-deficient mice, accumulate hepatic cholesterol upon cholesterol feeding [6]. Although part of the subtype-specific regulation can be rationalized by different relative tissue expression levels of LXRα and LXRβ, bona fide subtype-specific regulation has been demonstrated by promoter studies of the antiapoptotic factor SPα which was found to be selectively upregulated by LXRα [11]. Finally, very recent work indicates that LXRβ is of particular importance for cholesterol homeostasis in mouse testis [15]. In the present work, we further characterize the different roles of the two LXR subtypes in lipid metabolism using the complementary approaches of a novel LXRα-selective agonist and mice deficient in LXRα.

2. Materials and methods

Mice deficient in LXRα and/or LXRβ of mixed genetic background (129/OlaHsd × C57BL/6) were obtained from Deltagen, Inc. (San Carlos, CA). C57BL/6 mice were obtained from Taconic (Germantown, NY). Animals were housed under barrier conditions and all protocols were approved by the Institutional Care and Animal Use Committee at Merck Research Laboratories.

Compound A (4-({[methyl(3-{[7-propyl-3-(trifluoromethyl)-1,2-benzisoxazol-6-yl]oxy}propyl)amino]carbonyl}amino)benzoic acid) and Compound B ({5-[methyl(3-{[7-propyl-3-(trifluoromethyl)-1,2-benzisoxazol-6-yl]oxy}propyl)amino]pyrazin-2-yl}acetic acid) were synthesized at the Merck Research Laboratories in Rahway, NJ (J.W. Szewczyk, A.D. Adams, et al., manuscript in preparation).

2.1. *Determination of mRNA expression in mouse peritoneal macrophages*

Thioglycollate-elicited mouse peritoneal macrophages were prepared as described [27] and plated in 48-well dishes at a density of 1 million cells per well in DMEM supplemented with 10% FBS. Twenty-four hours after culture, cells were treated with Compound A, B or 8-Br-cAMP for 24 h at 37 °C in a humidified atmosphere consisting of 95% air and 5% carbon dioxide. The isolation of mRNA from cells was performed using mRNA Catcher plates (Invitrogen, Carlsbad, CA; cat #7001) according to the manufacturer's instructions. Reverse transcription and PCRs followed the protocols from Applied Biosystems (Foster City, CA) for Taqman Gold RT-PCR and Taqman Universal PCR Master Mix. Sequences of forward primers, reverse primers and probes for ABCA1 and SREBP-1 have been described elsewhere [28]. This primer/probe set for

SREBP-1 recognizes both the 1a and 1c isoforms; it should be noted that the 1c isoform predominates in intact tissues [21]. Probes of the genes of interest were labeled with FAM fluorescent dye. The normalizing gene was the mouse GAPDH tagged with a VIC dye.

2.2. Determination of cholesterol efflux in mouse peritoneal macrophages

Thioglycollate-elicited mouse peritoneal macrophages were plated in 48-well dishes at a density of 1 million cells per well in DMEM supplemented with 10% FBS. Cells were labeled with [^{3}H]cholesterol (10 μCi/ml) 24 h after culture. Cholesterol efflux was measured as previously described [28].

2.3. Determination of HDL-cholesterol

HDL-cholesterol in mouse serum was determined after precipitation of apoB-containing particles with phosphotungstic acid/magnesium chloride (HDL Precipitating Agent, #352-7, Sigma, St. Louis, MO) and subsequent quantitation of the cholesterol in the supernatant using the Wako Cholesterol CII kit (#276-64909, Wako Diagnostics, Richmond, VA). The assay was adapted to a 96-well format using appropriate aliquots of standards, samples, or controls in 100 μl total volume with phosphate-buffered saline, pH 7.4, and mixed with 100 μl kit reagent. The plates were incubated for 30 min at 37 °C and read at 505 nm. Cardiolipin standard controls (#C4571, Sigma) were included to assure assay accuracy.

2.4. Determination of serum triglycerides

Serum triglyceride (TG) concentrations were determined using the Sigma GPO Trinder kit (#337-B, Sigma) adapted to a 96-well format. Briefly, 150 μl of Reagent A was added to 15 μl volumes of standards, samples, or controls, followed by a 10 min incubation at room temperature, and a reading at 540 nm for determination of free glycerol. Next 40 μl of Reagent B was added to each well, followed by a 15 min incubation at 37 °C, and a second reading at 540 nm. This readout yields the sum of triglycerides and free glycerol in the original sample. The true triglyceride level was calculated from the difference between the two measurements. Accutrol Abnormal Controls (#A-3034, Sigma) were included to assure assay accuracy.

2.5. Determination of serum plant sterols

Serum plant sterols (sitosterol and campesterol) were analyzed by gas chromatography–mass spectrometry as described previously [29].

2.6. Determination of hepatic cholesterol

Hepatic cholesterol was analyzed by gas chromatography (GC) as described elsewhere [29] for serum samples with minor modifications. Liver pieces (10–30 mg) were added to glass tubes containing 50 μg epicoprostanol in 10 μl of toluene as an internal standard. The samples were saponified in ethanolic KOH and then derivatized to trimethylsilyl ethers. The derivatized samples thus obtained were redissolved in 50 μl isooctane and analyzed by GC. The amount of cholesterol in the original liver samples was determined using a standard curve.

2.7. Determination of hepatic triglycerides

Triglycerides were extracted using the Bligh and Dyer method [30] from mouse livers that had been snap frozen at harvest. Briefly, approximately 100 mg of liver was homogenized with a Teflon pestle in 1 ml phosphate-buffered saline, pH 7.4, and 0.5 ml of the resulting homogenate was diluted with 2.3 ml saline. Methanol (7 ml) and chloroform (3.5 ml) were added to precipitate the protein. The resulting supernatant was broken to two phases with the addition of an additional 3.5 ml each of chloroform and methanol. After mixing thoroughly, 2.0 ml of the organic lower phase was transferred to a clean tube containing 1 ml of 1% Triton X-100 in chloroform and dried down under argon with low heat. The residue was resolubilized in 0.25 ml distilled water and appropriate aliquots assayed for triglyceride levels using a GPO–PAP reagent (#1488872, Roche Applied Science, Indianapolis, IN). For a 96-well format, 40 μl volumes standards, samples, and controls were incubated with 200 μl of reagent for 10 min at room temperature, then read at 505 nm. Control Serum Level 1 (#410-00101, Wako Diagnostics) aliquots were included to assure assay accuracy.

2.8. Determination of mRNA expression levels in mouse livers

Mouse livers (~100 mg) were homogenized using Lysing Matrix A from Q-biogene (Irvine, CA; cat #6910-100) following the manufacturer's protocol. mRNA was obtained using mRNA Catcher plate as described above. Reverse transcription and PCR conditions followed the protocol from Applied Biosystems. Sequences of forward primers, reverse primers, and probes (respectively) were as follows: *LPL*, CTTTCCAGCCAGGATGCAA, CCACGTCTCCGAGTCCTCTC, and TTGGAGAAGCCATCCGTGTGATTGC; *FASN*, GGCTCAGCATGGTCGCTT, CTCCCGCCAGCTGTCATT, and AACCACCCTCTGGGCATGGCTATCTTCT; *HMGCS1* primers and probes were purchased from Applied Biosystems (Assay on demand ID number Mm00524111m1), and ABCA1 and SREBP-1 primers and probes were as described above.

3. Results

3.1. Differential activation of LXR subtypes by Compounds A and B

We previously described the LXR agonist F3MethylAA, which induced LXR-controlled genes in cultured cells and in vivo [31]. This molecule, however, also activated all three subtypes of PPARs (with EC50s of 327, 7.3, and 133 nM towards PPARα, PPARγ, and PPARδ, respectively, in an assay of coactivator association; data not shown). Derivatives of F3MethylAA were prepared that were LXR-specific, and Fig. 1 shows the chemical structures of two of these analogs, Compounds A and B. Neither Compound A nor Compound B activated any of the three PPAR subtypes, PXR, RXRα, or FXR in coactivator association assays. Compound B but not Compound A was a

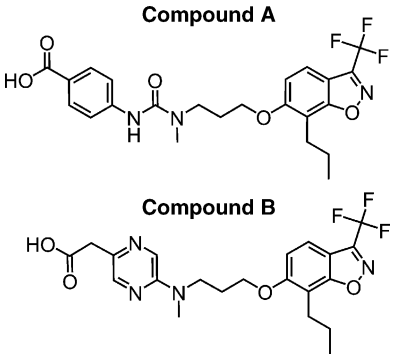

Fig. 1 – Chemical structures of Compounds A and B.

weak FXR antagonist (EC50 ~ 20 μM against 9 μM chenodeoxycholic acid). Fig. 2 shows that Compound B bound to and activated both LXRα and LXRβ, with potency in the mid-nanomolar range. In contrast, Compound A potently bound and activated LXRα, but was relatively inactive on LXRβ in the assays shown in Fig. 2. In the same assays, the commercially available, well-characterized LXR agonist T0901317 had IC50s of 8 and 24 nM, respectively, for LXRα and LXRβ binding, EC50s of 160 and 12 nM for cofactor association, and 126 and 137 nM for transactivation. Maximal responses of T0901317 were similar to Compound B with the exception of cofactor association, where T0901317 was a partial agonist of both LXRα and LXRβ with a maximal response about 35% of that of Compound B and other full agonists (data not shown).

3.2. *Characterization of the effects of LXR agonists in mouse peritoneal macrophages deficient for LXRα, LXRβ, or both*

To assess the effects of Compounds A and B on gene expression driven by wild-type, chromosomally encoded

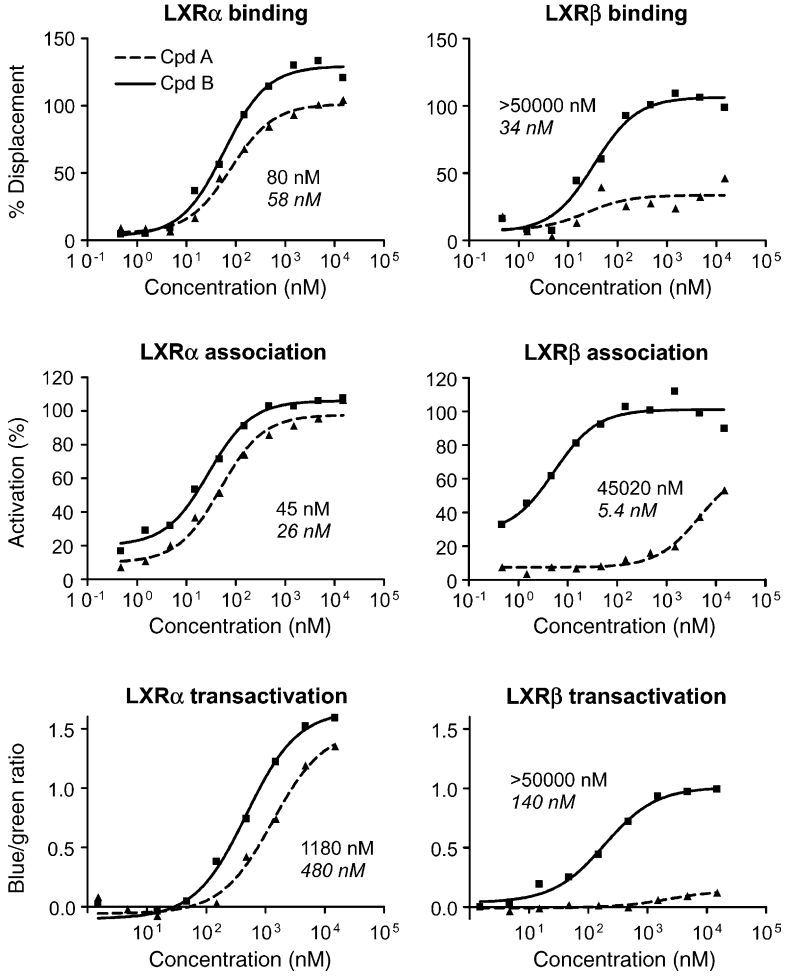

Fig. 2 – Assessment of Compounds A and B in three assays of human LXR binding and activation. Compounds A and B were tested for their ability to bind and activate human LXRα and LXRβ, using three different assays of LXR function. Top panels: competition-binding assays performed using fusion proteins of GST with the ligand-binding domains of the relevant LXR subtype, as described by Menke et al. [31]. Middle panels: assays measuring the ability of Compounds A and B to cause the association of steroid receptor coactivator-1 with the ligand-binding domain of the relevant LXR subtype, in a cell-free assay previously described by Menke et al. [31]. Bottom panels: the results of cell-based transactivation assays using LXR–Gal4 fusion proteins to drive the expression of β-lactamase in an agonist-dependent manner, as described by Chin et al. [45]. IC50 or EC50 values for Compounds A (regular font) and B (italics) are shown in the respective figures.

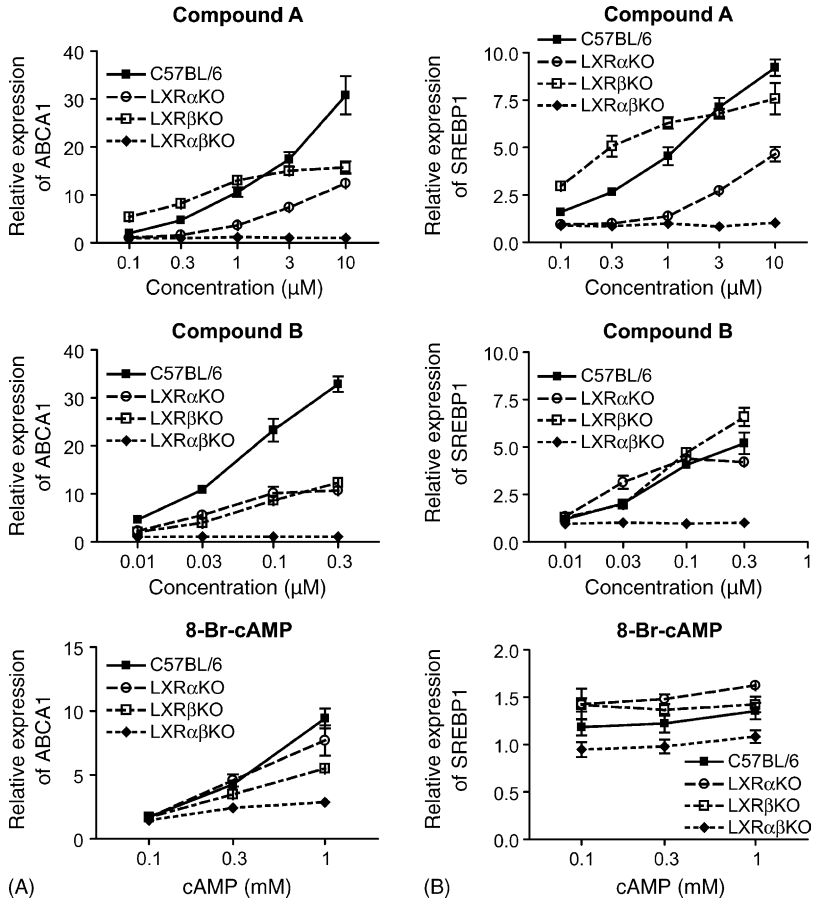

Fig. 3 – Induction of gene expression by LXR agonists and 8-Br-cAMP in macrophages of different genotypes. Thioglycollate-elicited peritoneal macrophages were isolated from mice of the genotypes indicated in the figure (see Section 2 for details). Macrophages were incubated with the indicated concentrations of Compound A, B, or 8-Br-cAMP for 24 h, and cellular mRNA levels were measured by quantitative real-time PCR. Data are presented as expression level relative to the DMSO control, which was arbitrarily set to equal 1. Error bars indicate S.E.M. ($n = 4$). Panel A: results for mRNA levels of ABCA1. Panel B: results for SREBP-1. The relative baseline expression of LXRα was 1.8-fold higher in LXRβ-deficient macrophages than in macrophages from C57BL/6 mice, comparing the vehicle-treated groups. Conversely, baseline LXRβ expression was increased 2.2-fold in macrophages from LXRα-deficient vs. C57BL/6 mice. Mean relative baseline expression of ABCA1 in C57BL/6, LXRα-deficient, LXRβ-deficient, and LXRα/β-deficient macrophages was, respectively, 1, 0.55, 0.71, and 2.1; corresponding values for SREBP-1 were 1, 0.61, 0.53, and 6.2. KO: knockout.

LXR subtypes, we titrated these two molecules on mouse peritoneal macrophages isolated from C57BL/6 mice or mice lacking one or both LXR subtypes. As shown in Fig. 3, both compounds increased mRNA levels for ABCA1 and SREBP-1 in C57BL/6 cells, with potencies consistent with results from the more artificial assays described above. No induction of either gene was seen with either compound in cells lacking both LXR subtypes (Fig. 3). The dual agonist Compound B induced both ABCA1 and SREBP-1 to equal extents in LXRα or LXRβ-deficient macrophages, whereas Compound A was >10-fold less potent in cells deficient in LXRα, as would be expected from the data presented in Fig. 2. There is, however, an important quantitative difference between Figs. 2 and 3 with respect to the degree of specificity of Compound A for LXRα over LXRβ. In the artificial assays of nuclear receptor function, Compound A appeared to be approximately 1000-fold selective for LXRα, whereas the selectivity decreased to approximately 10-fold when Compound A was tested in macrophages expressing only one of the two subtypes. This surprising discordance might be explained by differences in receptor conformation in the natural setting versus the artificial assays that use only the ligand-binding domain of the nuclear receptor. Although human LXRα and LXRβ were used for the compound characterization shown in Fig. 2, species differences are unlikely to explain the reduced selectivity of Compound A for LXRα specificity in mouse macrophages, since similar selectivity for LXRα was observed using the ligand-binding domain from mouse LXRα and LXRβ in the LXR—β-lactamase assay shown in Fig. 2 (data not shown).

3.3. *Characterization of the effects of 8-bromo-cAMP on gene expression in mouse peritoneal macrophages deficient in LXRα, LXRβ, or both*

We also determined the effect of 8-Br-cAMP on ABCA1 and SREBP-1 expression (Fig. 3A and B). 8-Br-cAMP increased

ABCA1 but not SREBP-1 expression in macrophages from C57BL/6 mice. The increase was blunted in macrophages deficient in both LXRα and LXRβ, and intermediate in macrophages deficient in either isoform. This result indicates that 8-Br-cAMP-induced upregulation of ABCA1 is at least in part mediated through LXR, and that either LXR isoform can mediate this effect.

3.4. *Determination of cholesterol efflux in mouse peritoneal macrophages deficient in LXRα, LXRβ, or both treated with Compound A or B*

To determine the consequences of upregulation of ABCA1 mRNA expression by Compounds A and B in macrophages of the different genotypes, we measured efflux of radioactive cholesterol to apoA-I as previously described [28] (Fig. 4). Both Compounds A and B stimulated cholesterol efflux in macrophages from C57BL/6 and LXRβ knockout mice. Compound B increased cholesterol efflux also in LXRα knockout

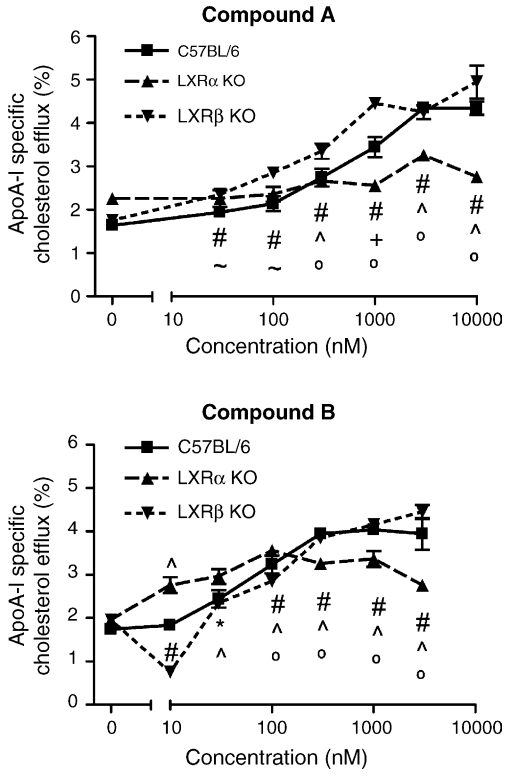

Fig. 4 – Effects of LXR agonists on cholesterol efflux from macrophages carrying different LXR genotypes. Thioglycollate-elicited peritoneal macrophages were harvested from mice of various LXR genotypes, and used for assays of cholesterol efflux as described in Section 2 and ref. [28]. Briefly, cells were incubated with [^{3}H]cholesterol for 24 h, rinsed with medium containing BSA for 24 h, and treated with serum-free medium ± apoA-I (10 μg/ml) and Compound A or B for another 24 h. ApoA-I-dependent cholesterol efflux is shown in the figure. (+, *, ~) $p < 0.05$ and (^, #, °) $p < 0.01$ vs. 0 nM for LXRα KO (knockout), LXRβ KO, and C57BL/6, respectively.

macrophages, indicating that LXRβ has the capacity to drive cholesterol efflux in macrophages. No stimulation of cholesterol efflux by Compound A or B was observed in macrophages lacking both LXRα and LXRβ (data not shown).

3.5. *Characterization of the effects of LXR agonists in mice deficient for LXRα or LXRβ*

To further characterize the different roles of the two LXR subtypes, C57BL/6 mice, or mice deficient for LXRα or LXRβ were administered LXR agonists. A baseline blood sample was obtained from each mouse the day before the study began, and then compounds were administered at a dose of 30 mg/kg (Compound A) or 10 mg/kg (Compound B) of body weight once per day by oral gavage in the morning for 7 days. Blood samples were obtained from each mouse 6 h after the first dose, which we have found to be the time of maximum serum triglyceride response to LXR agonists (data not shown). A terminal blood sample was obtained 6 h after the last dose on day 7. As shown in Fig. 5, in C57BL/6 mice both Compounds A and B significantly increased serum triglyceride at the 6 h time point, and increased HDL-cholesterol and liver triglyceride content after 7 days. These compounds caused different outcomes in LXRα-deficient mice. As would be expected, the LXRα-selective Compound A had no significant effect on either triglyceride or HDL-cholesterol in LXRα-deficient mice compared to vehicle. The dual agonist Compound B significantly increased HDL-cholesterol in LXRα-deficient mice, but this treatment did not change serum triglyceride at the 6 h time point, whereas serum triglyceride was significantly increased after 7 days of dosing. LXRα-deficient mice showed no increase in liver triglyceride content following administration of Compound A, and a relatively minor, 25% increase after Compound B administration. The effects of Compounds A and B, respectively, on serum and liver lipids were very similar in LXRβ-deficient mice and C57BL/6 mice (data not shown). Taken together, these results strongly suggest that activation of LXRβ is sufficient for increasing HDL levels in mice, whereas increases in liver triglyceride content requires activation of LXRα.

We also examined gene expression in the livers of the mice administered LXR agonists. Fig. 6 presents the liver mRNA levels for two critical LXR target genes, ABCA1 and SREBP-1, as well as for other genes involved in lipid metabolism. Both of these genes showed increased mRNA levels in wild-type mice that were administered the LXR dual agonist Compound B, similar to previous findings with T0901317 [22]. The genes shown in Fig. 6 were all induced to a similar extent by Compound B in C57BL/6 mice and mice lacking LXRβ (data not shown; Compound A not tested in LXRβ-deficient mice). Baseline expression levels of ABCA1 and SREBP-1 were very similar in all genotypes (Fig. 6; not shown for LXRβ-deficient mice), whereas baseline expression of lipoprotein lipase, fatty acid synthase, and HMG-CoA synthase was lower in LXRα-deficient than in C57BL/6 mice (Fig. 6); the baseline levels of these genes were very similar in LXRα and LXRβ-deficient mice (not shown). Strikingly, mice lacking LXRα showed a minimal induction of SREBP-1 following administration of not only the LXRα-selective Compound A, which would be expected, but also of the dual agonist Compound B. These data are

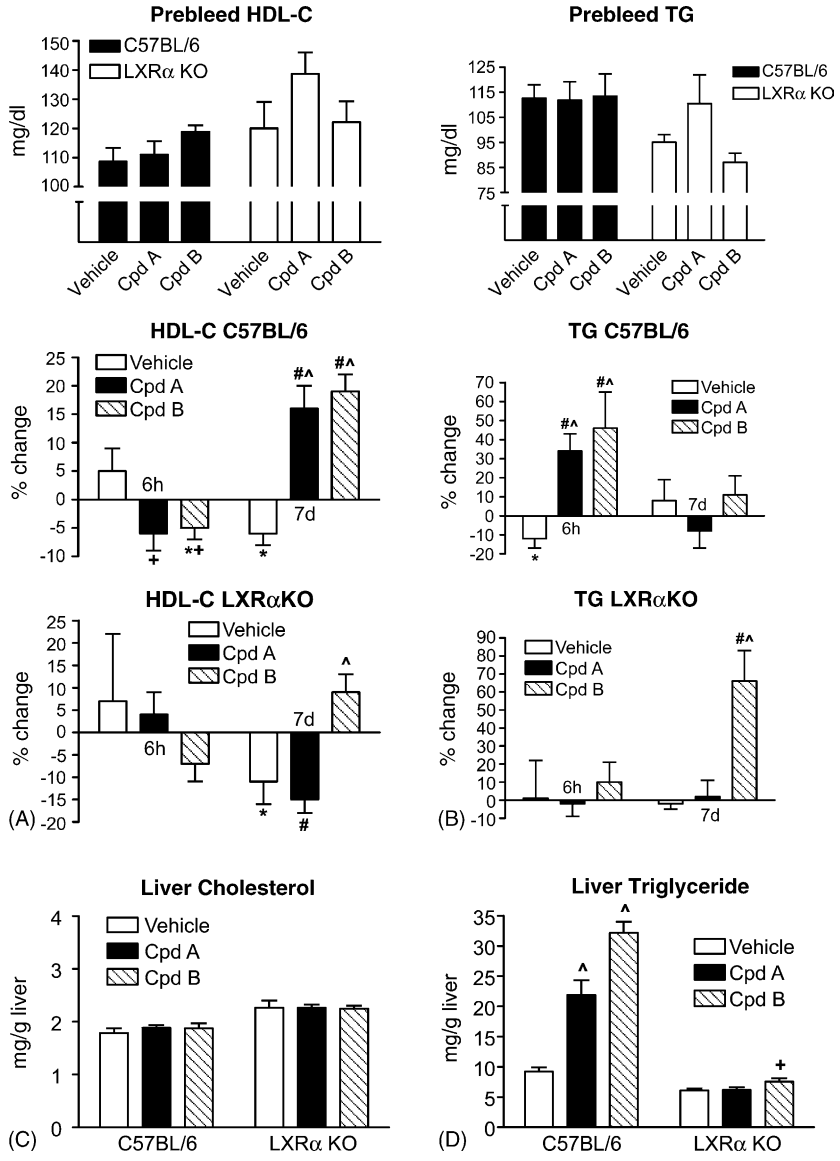

Fig. 5 – Effects of LXR agonists on lipid levels in serum and liver in C57BL/6 mice or mice deficient in LXRα. Wild-type or LXRα-deficient mice (n = 10 per treatment) were administered Compound A, B, or vehicle alone (2% DMSO, 4% Cremophor, and 94% water) by oral gavage once per day in the morning for 7 days. Compounds were administered at a dose of 30 mg/kg (Compound A) or 10 mg/kg (Compound B) of body weight. Blood samples were obtained from each mouse 6 h after the first dose and again 6 h after the last dose on day 7, at which time the animals were killed and samples of liver were obtained. Chow diet was available ad libitum throughout the study. Serum lipid levels for each mouse were normalized to the baseline lipid levels in the same mouse measured in blood samples obtained the day before the study began. Panel A: HDL-cholesterol; panel B: total serum triglyceride (corrected for free glycerol); panel C: liver cholesterol content; panel D: liver triglyceride content. KO: knockout. Means ± S.E.M. are shown. Average baseline and final serum lipid levels, respectively, were as follows: HDL-cholesterol—109 and 102 mg/dl (C57BL/6 mice, vehicle); 111 and 129 mg/dl (C57BL/6 mice, Cpd. A); 119 and 141 mg/dl (C57BL/6 mice, Cpd. B); 120 and 104 mg/dl (LXRα-deficient mice, vehicle); 139 and 117 mg/dl (LXRα-deficient mice, Cpd. A); 122 and 132 mg/dl (LXRα-deficient mice, Cpd. B); TG—113 and 120 mg/dl (C57BL/6 mice, vehicle); 112 and 120 mg/dl (C57BL/6 mice, Cpd. A); 113 and 99 mg/dl (C57BL/6 mice, Cpd. B); 95 and 93 mg/dl (LXRα-deficient mice, vehicle); 110 and 104 mg/dl (LXRα-deficient mice, Cpd. A); 87 and 142 mg/dl (LXRα-deficient mice, Cpd. B). $^{*}p < 0.05$, $^{\#}p < 0.01$ vs. prebleed; $^{+}p < 0.05$, $\wedge p < 0.01$ vs. vehicle same time point.

consistent with the effects on lipids described above. These results suggest that activation of LXRα, but not LXRβ, induces SREBP-1 in mouse liver. Interestingly, this is different from our results in isolated mouse peritoneal macrophages (see Fig. 3), where the presence of either LXR subtype was sufficient for agonist induction of SREBP-1. There have been other reports of LXR activation leading to tissue-specific regulation of genes, including apoE [32] and lipoprotein lipase [26]. In accordance

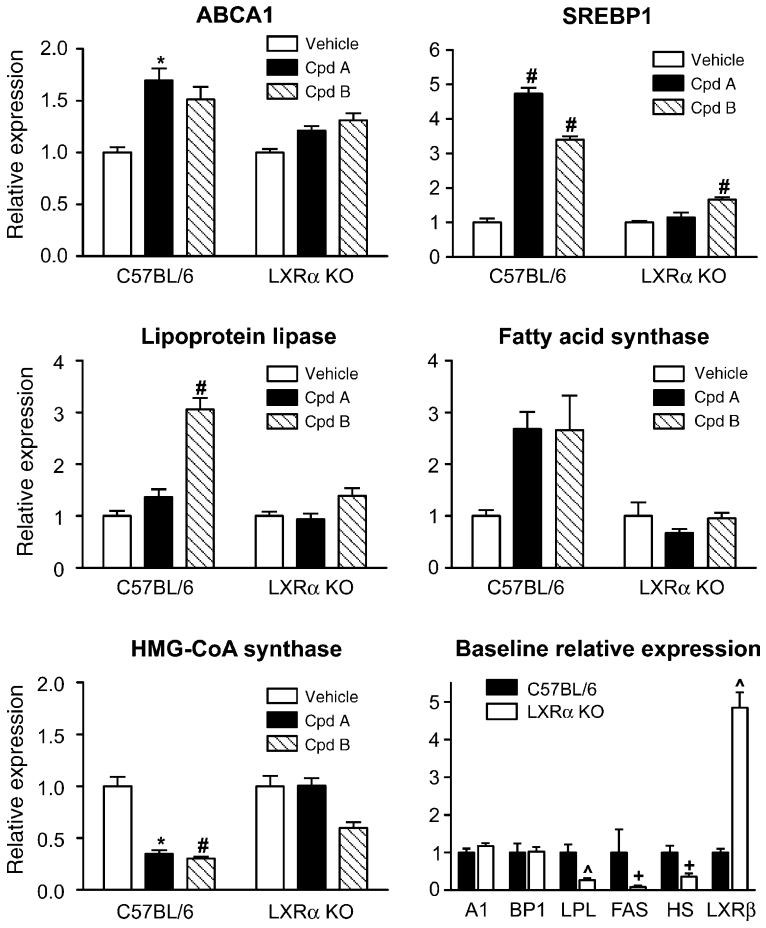

Fig. 6 – Effects of LXR agonists on hepatic mRNA levels for various genes important to lipid metabolism. Hepatic mRNA levels for various genes were measured in liver samples obtained in the study described in Fig. 5. Relative expression levels were normalized to the vehicle group for each genotype; the last panel compares the baseline (vehicle) expression between C57BL/6 mice and LXRα-deficient mice. Mouse genotype, treatment, and mRNA measured are indicated in the figures. A1, ABCA1; BP1, SREBP-1; LPL, lipoprotein lipase; FAS, fatty acid synthase; HS, HMG-CoA synthase; KO, knockout. $^{*}p < 0.05$, $^{\#}p < 0.01$ vs. vehicle; $^{+}p < 0.05$, $^{\wedge}p < 0.01$ vs. C57BL/6 (baseline relative expression graph only).

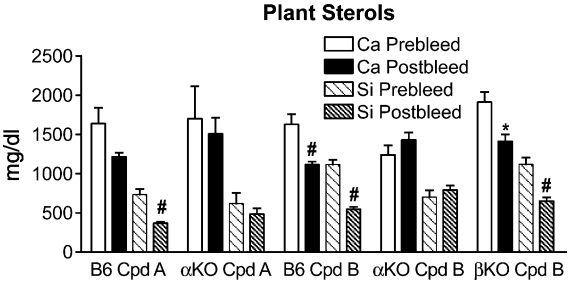

Fig. 7 – Effects of LXR agonists on serum plant sterol levels in mice of various genotypes. Wild-type mice, or mice lacking LXRα or LXRβ (5 per group), were dosed with 30 mg of Compound A or 10 mg of Compound B per kilogram of body weight once per day in the morning by oral gavage for 7 days. Plant sterol levels (campesterol and sitosterol) were measured in blood samples obtained at baseline and 6 h after the last dose. $^{*}p < 0.05$, $^{\#}p < 0.01$ vs. prebleed (same genotype).

with the latter study, Compound B increased lipoprotein lipase mRNA levels in C57BL/6 mice but not LXRα knockout mice [26].

Recent work has shown that LXR agonists can regulate ABCG5 and ABCG8, which work together to rid the body of unwanted plant sterols, and it has previously been shown that the LXR agonist T0901317 reduces plasma plant sterol levels in mice [33]. To determine the possible different roles of the two LXR subtypes in the regulation of serum plant sterol levels, prebleeds and terminal serum samples from the animal study described above were analyzed for the plant sterols campesterol and sitosterol by gas chromatography/mass spectrometry as described in Section 2. As shown in Fig. 7, administration of Compound B to C57BL/6 mice or LXRβ-deficient mice decreased serum levels of both campesterol and sitosterol, whereas there was no significant change in these parameters following administration of Compound B to LXRα-deficient mice. Compound A lowered sitosterol significantly in C57BL/6 but not LXRα-deficient mice. These results indicate that the reduction in circulating plant sterol by LXR agonists in mice is mediated primarily by LXRα.

4. Discussion

Our study confirms and extends previous findings that LXRα and LXRβ have substantially different effects on lipid metabolism in mice. We demonstrate this by two different means: (i) by treating C57BL/6 mice and macrophages with an LXRα-selective compound and (ii) by treating mice or mouse macrophages deficient in LXRα, LXRβ, or both with a potent dual agonist of LXRα and LXRβ. It has been proposed that LXR subtype-specific agonists, in particular LXRβ-specific compounds, may overcome some or all of the harmful effects, mainly hepatic triglyceride accumulation and (transient) hypertriglyceridemia, which have thus far hampered the development of LXR agonists as novel therapeutics [2,23,24]. Our results with the LXRα-selective Compound A further strengthen the widely held notion that most of the lipogenic effects of LXR agonists are mediated through LXRα. Surprisingly, Compound A did not increase hepatic lipoprotein lipase mRNA which has previously been shown to be an LXRα-regulated gene [26]. In macrophages, Compounds A did increase lipoprotein lipase up to 3.2-fold, equal to Compound B (same experiment as in Fig. 3, data not shown). We speculate that a higher degree of LXR activation is required for induction of hepatic lipoprotein lipase than for, e.g. ABCA1, and that with the dosing regimen used for Compound A this threshold was not reached.

Although an LXRβ-specific agonist was not available for this study, our data from LXRα knockout mice treated with Compound B lend further support to the notion that synthetic LXRβ-specific agonists may have an efficacy/side effect profile that warrants development. The effects on serum lipids were moderate both with respect to HDL and triglycerides. The most serious adverse effect of LXR agonists observed in animal models is arguably hepatic steatosis and encouragingly, no major increase in liver triglycerides was found in this experiment; however, the effect of long-term chronic dosing of Compound B in LXRα knockout mice has not been determined. An earlier study of Compound B in LXRα knockout mice using the same dosing regimen yielded similar results (7 days dosing: serum triglycerides, +30% versus vehicle, $p = 0.007$; HDL-cholesterol, +18%, $p = 0.04$; liver triglycerides, +32%, not significant; baseline levels in same mice not determined). The weak FXR antagonist activity of Compound B was likely irrelevant for the lipid changes since the hepatic mRNA expression levels of SHP and BSEP were essentially unchanged (0.96 ± 0.17 and 1.19 ± 0.21 versus vehicle, data not shown). Interestingly, 7 days treatment of LXRα knockout mice with Compound B caused a significant increase of serum triglycerides. In contrast, consistent with published results [22], in C57BL/6 mice Compound B caused only a transient increase in serum triglycerides which was normalized after 7 days. Generally speaking, the normalization cannot be entirely due to reduced fatty acid synthesis: unpublished studies from our group demonstrated an 8-fold increase in short-term in vivo incorporation of ^{3}H from tritiated water in liver triglycerides after 7 days of dosing C57BL/6 mice with a different dual LXR agonist (data not shown); at this time serum triglycerides were normal. A gradual shift of newly synthesized triglycerides from lipoprotein secretion to storage may underlie the transient nature of the serum triglyceride increase, but we speculate that in addition, induction of lipoprotein lipase in liver causing hydrolysis of triglycerides in circulating lipoproteins and subsequent reuptake of free fatty acids may play a role. Although LPL mRNA was not induced by Compound B in LXRα knockout mice, absolute levels were lower than in C57BL/6 mice. It is therefore conceivable that Compound B caused a small increase in fatty acid synthesis and lipoprotein secretion in LXRα knockout mice and that low expression of lipoprotein lipase precluded subsequent normalization of serum triglycerides. It can be noted that an increase of 55 mg/dl serum triglycerides as seen here corresponds to a total of 0.8 mg based on 1.5 ml serum volume; reuptake of that amount by the liver would cause hepatic triglyceride content to rise by only about 10%.

Along the same lines, induction of lipoprotein lipase may also play some part in the etiology to LXR agonist-provoked hepatic triglyceride accumulation [34]. Lipoprotein lipase expression is generally low in liver but can be significantly induced by LXR agonists [26]. Lipoprotein lipase expression per se can cause triglyceride accumulation: genetically engineered overexpression of lipoprotein lipase in skeletal muscle was found to cause accumulation of tissue triglyceride [35]. A similar correlation is reported in the present study: in contrast to Compound A, Compound B induced lipoprotein lipase expression significantly and also caused significantly higher liver triglyceride levels and slightly lower serum triglycerides (not significant) despite similar inductions of fatty acid synthase mRNA. Further studies are required to determine the relative importance of direct deposition of newly synthesized triglycerides and lipoprotein lipase-mediated reuptake from circulating lipoproteins for the etiology of LXR-mediated hepatic steatosis.

Several lines of evidence support the view that the main promise for LXR agonists as antiatherosclerotic therapy lies less with their capacity to increase HDL than with their capacity to increase cholesterol efflux and reduce plaque load at the site of the atherosclerotic lesion. Joseph et al. demonstrated that GW3965, a widely used synthetic LXR agonist, inhibited atherosclerosis in LDL receptor-deficient mice without raising HDL-cholesterol or lowering LDL or VLDL-cholesterol [19], and Terasaka et al. found no correlation between HDL-cholesterol concentrations and atherosclerosis in LDL receptor-deficient mice treated with T0901317, another synthetic LXR agonist [18]. More specifically addressing the question of macrophage LXR involvement in atherosclerosis, Levin et al. recently described experiments in which LDL receptor-deficient mice fed a western diet received bone marrow transplantation from mice of different genotypes [36]. Interestingly, mice that received bone marrow from C57BL/6 mice or LDL receptor-deficient mice responded to 8 weeks treatment with T0901317 by a reduction of aortic lesion area by 57–68%, whereas no significant reduction was seen in mice that received bone marrow from LXRα/β-deficient mice. HDL-cholesterol levels were similar in the different groups and not affected by treatment with LXR agonist [36]. By similar bone marrow transplantation studies, Aiello et al. demonstrated that selective inactivation of ABCA1 in macrophages increased atherosclerosis in apoE-deficient mice [37]. In this context it is tantalizing to note that mouse peritoneal macrophages deficient in either LXRα or LXRβ have been shown to retain

approximately full capacity to induce ABCA1 expression by T0901317 [38,39] and GW3965 [39]. These results were qualitatively replicated in the present study in that Compound B increased ABCA1 expression up to 10-fold in LXRα-deficient macrophages, equal the increase observed in macrophages obtained from LXRβ-deficient mice. In our study, the difference between wild-type and knockout macrophages was larger than in the earlier studies; this may reflect an inherently higher response to LXR agonists in C57BL/6 wild-type mice than in the mixed strain knockout mice. Importantly, the capacity of an LXR agonist to stimulate cholesterol efflux was retained in LXRα-deficient macrophages, albeit to a reduced level. These results indicate that LXRβ has the capacity to substantially increase the expression of ABCA1 and stimulate cholesterol efflux in macrophages. Further studies are required to determine whether selective stimulation of LXRβ would increase whole-body reverse cholesterol transport, e.g. by determining neutral fecal sterol excretion. Nevertheless, in light of our results and what has been described previously by other groups, it appears reasonable to expect that an LXRβ-specific agonist would reduce atherosclerosis in a mouse model by induction of ABCA1 in macrophages. In the absence of an agonist specific for LXRβ, it would therefore be interesting to study whether treatment of mice deficient in LXRα and apoE or the LDL receptor with a dual LXR agonist such as Compound B would reduce atherosclerotic load.

Although our data are encouraging in this respect, LXR dual agonist treatment of LXRα knockout mice cannot be uncritically assumed to fully predict in vivo effects of an LXRβ-selective agonist. In our studies, Compound B raised HDL in LXRα knockout mice, but it has been shown that expression of cholesteryl ester transfer protein, which is present in humans but not in wild-type mice, abolishes HDL-raising by LXR agonists [40]. Hepatic baseline LXRβ expression in LXRα-deficient mice was also higher than in C57BL/6 mice (also observed, to a lesser extent, in macrophages; see Fig. 6 and legend to Fig. 3); this could possibly be due to a compensatory increase in which case the hepatic effects of a true LXRβ-selective agonist may be smaller than predicted from this study. However, since Mangelsdorf and coworkers observed no compensatory increase in LXRβ expression in their LXRα knockout mice [5], we speculate that the different expression levels of LXRβ may rather be due to inherent differences between mixed strain and C57BL/6 mice. As discussed above, however, an LXRβ-selective agonist that does not increase expression of hepatic genes or raise HDL may still have positive clinical effects, provided the agonist is effective in macrophages.

An alternative approach to obtain desired clinical outcomes is suggested by our gene expression studies in macrophages using 8-Br-cAMP. Although it is well known that cyclic AMP and analogs can increase ABCA1 mRNA and protein as well as cholesterol efflux in mouse macrophages [41–43], the mechanism is not characterized in detail. It goes beyond the scope of the current study to investigate this mechanism thoroughly; however, the blunted upregulation of ABCA1 by 8-Br-cAMP in macrophages from LXRα/β double knockout mice together with the intermediate response in single knockout mice suggest that the induction is predominantly LXR-dependent, and that both LXRα and LXRβ can mediate this induction. The effect is likely indirect as the primary response elements for cAMP and LXR in the mouse ABCA1 gene have been shown to be distinct [44]. Interestingly, while in our experiments ABCA1 was induced up to 9-fold by 8-Br-cAMP, SREBP-1 was not induced at all. Although the relevance for regulation of the human ABCA1 gene is still unclear, further exploration of this pathway may uncover hitherto unidentified ways of inducing ABCA1 without concomitant SREBP-1 activation.

In conclusion, our study demonstrates that separate activation of LXRα and LXRβ yields distinctive lipid outcomes in vivo. Most importantly, it lends further support to the hypothesis that a specific agonist of LXRβ would increase ABCA1 expression in macrophages and thereby inhibit atherosclerosis, while keeping deleterious hepatic gene inductions low. For further development of LXRβ agonists into viable therapeutics, it will be important to determine whether this expression pattern is true also in humans. This can be accomplished by treating primary human hepatocytes and macrophages with specific agonists for LXRα or LXRβ, or in the absence of such reagents, with a dual LXRα/β agonist concomitantly with suppression of either LXR isoform by siRNA. Such experiments are currently being conceived in our laboratories.

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