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# Liver X Receptor (LXR)-$\beta$ Regulation in LXR$\alpha$-Deficient Mice: Implications for Therapeutic Targeting

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## ABSTRACT

The nuclear receptors liver X receptor (LXR) LXR$\alpha$ and LXR$\beta$ are differentially expressed ligand-activated transcription factors that induce genes controlling cholesterol homeostasis and lipogenesis. Synthetic ligands for both receptor subtypes activate ATP binding cassette transporter A1 (ABCA1)-mediated cholesterol metabolism, increase reverse cholesterol transport, and provide atheroprotection in mice. However, these ligands may also increase hepatic triglyceride (TG) synthesis via a sterol response element binding protein 1c (SREBP-1c)-dependent mechanism through a process reportedly regulated by LXR$\alpha$. We studied pan-LXR$\alpha/\beta$ agonists in LXR$\alpha$ knockout mice to assess the contribution of LXR$\beta$ to the regulation of selected target genes. In vitro dose-response studies with macrophages from LXR$\alpha-/-$ and $\beta-/-$ mice confirm an equivalent role for LXR$\alpha$ and LXR$\beta$ in the regulation of ABCA1 and SREBP-1c gene expression. Cholesterol-efflux studies verify that LXR$\beta$ can drive apoA1-dependent cholesterol mobilization from macrophages. The in vivo role of LXR$\beta$ in liver was further evaluated by treating LXR$\alpha-/-$ mice with a pan-LXR$\alpha/\beta$ agonist. High-density lipoprotein (HDL) cholesterol increased without significant changes in plasma TG or very low density lipoprotein. Analysis of hepatic gene expression consistently revealed less activation of ABCA1 and SREBP-1c genes in the liver of LXR$\alpha$ null animals than in treated wild-type controls. In addition, hepatic CYP7A1 and several genes involved in fatty acid/TG biosynthesis were not induced. In peripheral tissues from these LXR$\alpha$-null mice, LXR$\beta$ activation increases ABCA1 and SREBP-1c gene expression in a parallel manner. However, putative elevation of SREBP-1c activity in these tissues did not cause hypertriglyceridemia. In summary, selective LXR$\beta$ activation is expected to stimulate ABCA1 gene expression in macrophages, contribute to favorable HDL increases, but circumvent hepatic LXR$\alpha$-dominated lipogenesis.

There is great interest in targeting LXR nuclear receptors and their modulation for the treatment of atherosclerosis. These transcription factors play a critical role in the control of cholesterol homeostasis and have been the topic of several recent reviews (Jaye, 2003; Joseph and Tontonoz, 2003; Tontonoz and Mangelsdorf, 2003; Cao et al., 2004). Their therapeutic potential resides in their ability to dramatically up-regulate ABCA1 transcription and thereby stimulate cholesterol efflux from macrophages. It has been demonstrated that activation of LXR by cognate ligands promotes apoA1-mediated efflux, and this is believed to be a critical first step for the removal of cholesterol from the actual site of atherogenesis in the vasculature (Costet et al., 2000; Repa et al., 2000b; Schwartz et al., 2000).

LXRs behave as cholesterol sensors to stimulate transcription from a number of genes, including ABCA1, ABCG1, apoE, CETP, and LPL, resulting in the coordinate up-regulation of the reverse cholesterol transport (RCT) process. RCT promotes the return of excess cholesterol from peripheral tissues, including arterial lesion sites, to the liver for conversion to bile acids and excretion from the body. As such, the process of RCT plays a central role in maintaining whole-body cholesterol homeostasis. The atheroprotective proper-

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**ABBREVIATIONS:** LXR, liver X receptor; ABCA1, ATP binding cassette transporter A1; VLDL, very low density lipoprotein; CYP7A1, cholesterol 7$\alpha$ hydroxylase; angptl3, angiopoietin-like protein 3; apoCI, apolipoprotein CI; DMEM, Dulbecco's modified Eagle's medium; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CETP, cholesteryl ester transfer protein; TG, triglyceride; RCT, reverse cholesterol transport; SCD1, stearoyl CoA desaturase-1; KO, knockout; FAS, fatty acid synthase; WT, wild-type; PCR, polymerase chain reaction; BSA, bovine serum albumin; FBS, fetal bovine serum; SREBP-1c, sterol-response element binding protein; TO901317, *N*-(2,2,2,-trifluoro-ethyl)-*N*-[4-(2,2,2,-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide; GW3965, 3-(3-(2-chloro-3-trifluoromethylbenzyl-2,2-diphenylethylamino) proproxy) phenylacetic acid.

ties of LXR nuclear receptors also include the regulation of key genes involved in inflammation (Joseph et al., 2003) and several intestinal cholesterol transporters (ABCA1, ABCG5, and ABCG8) limiting cholesterol absorption (Berge et al., 2000; Repa et al., 2000a,b). In mice but not humans, LXRs also induce the expression of cholesterol 7α hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid biosynthesis (Peet et al., 1998; Chiang et al., 2001).

A potential obstacle in the pharmacologic targeting of nuclear receptors as a general class resides in their ability to regulate or integrate numerous gene responses, some of which may be deleterious. In this case, it is known that synthetic LXR agonists can exhibit the adverse property of increasing lipogenesis (Schultz et al., 2000; Grefhorst et al., 2002; Repa et al., 2002a) through transcriptional activation of sterol response element binding protein 1c (SREBP-1c) (Schultz et al., 2000; Yoshikawa et al., 2001; Grefhorst et al., 2002; Repa et al., 2002a), fatty acid synthase (FAS) (Joseph et al., 2002), angiopoietin-like protein 3 (angptl3) (Inaba et al., 2003) and/or inhibition of Apo AV (Jakel et al., 2004). Although purported to be transient, these effects are cause for concern because triglyceride (TG) elevations are an established independent risk factor for atherosclerotic heart disease (Assmann et al., 1998). For this reason, pharmacological modulators are being sought which separate the favorable LXR antiatherogenic properties from the less favorable lipogenic effects. Several possible approaches for achieving this have been put forward in recent reviews, and one strategy commonly cited is by the selective modulation of LXR isoforms (Jaye, 2003; Joseph and Tontonoz, 2003; Lund et al., 2003; Tontonoz and Mangelsdorf, 2003).

The two known receptor subtypes, LXRα and -β, exhibit differential expression patterns and may perform different functional roles. The apparent ubiquitous expression of LXRβ contrasts with preferential expression of LXRα in liver, kidney, macrophages, and intestine. LXRα−/− mice challenged with high-cholesterol diets accumulate hepatic lipid, thus pointing to a dominant role for LXRα in liver (Peet et al., 1998; Alberti et al., 2001). Moreover, genetic ablation of LXRα impairs CYP7A1 induction and hepatic conversion of cholesterol to bile acids. These studies also suggest that it is primarily the LXRα subtype controlling liver lipogenesis though the activation of SREBP-1c transcription (Peet et al., 1998). LXRβ knockout mice handle excess cholesterol as effectively as wild-type mice (Alberti et al., 2001). However, the LXRβ subtype has been implicated in control of basal ABCA1 expression in LXRβ−/− macrophages and regulation of cholesterol efflux (Repa et al., 2000b). A recent report demonstrates that either receptor can play an atheroprotective role in macrophages and that the combined deficiency of both LXRα and LXRβ is required for foam cell-lipid accumulation in aortic lesions (Schuster et al., 2002). These studies imply that LXRβ-selective targeting may avoid detrimental lipogenic effects dominated by LXRα while achieving beneficial effects from ABCA1 gene activation and increased cholesterol efflux in macrophages.

The current studies were undertaken to more definitively characterize the role of the LXRβ isoform in the regulation of selected LXR target genes and control of lipogenesis. For these studies, LXRα−/− mice were treated with pan-LXR α/β agonists that have comparable binding activity for α and β isoforms.

## Materials and Methods

**Ligands.** LXR agonists, Tularik TO901317 (Repa et al., 2000b; Schultz et al., 2000) and Glaxo GW3965 (Laffitte et al., 2001; Collins et al., 2002) were prepared by following standard chemical syntheses from the published literature. Human LDL was obtained from Wake Forest University, School of Medicine (Wake Forest, NC), and [1,2-$^3$H(*N*)]cholesterol was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA).

**In Vivo Studies: Animals and Diet.** Mice of wild-type, LXRα−/−, LXRβ−/−, and LXRα/β−/− genotype have been characterized in detail previously (Alberti et al., 2001; Juvet et al., 2003). Mice used for in vivo experiments and cultured macrophage preparations were Sv129/C57BL/6 hybrids backcrossed on C57BL/6 mice for three generations. Upon receipt, all mice were maintained on a 12-h light/dark cycle and fed a normal chow diet, Rodent Diet 5001 (PMI Nutritionals, St. Louis, MO) ad libitum. Peritoneal macrophages were prepared as described below and represent pools from four to six male mice (25–30 g) from each genotype. Age-matched adult mice (6–8 months) were used for in vivo studies for which ligands were administered once a day in the morning by oral gavage for 3 days. Control animals received vehicle, 1.3% Tween 80/0.25% sodium carboxymethylcellulose. At study termination, mice were fasted for 5 to 6 h, blood was recovered, and plasma was prepared using standard centrifugation techniques. Tissues were collected for RNA preparation and frozen in liquid $N_2$ before storage at −70°C. Animal experiments were approved by the Institutional Animal Care and Use Committee of Wyeth (Collegeville, PA).

**Murine Peritoneal Macrophage Isolation and Culture.** Thioglycollate-elicited peritoneal macrophages were isolated from nonfasted male mice 3 days after peritoneal injection of 4% Brewers thioglycollate media (25 ml/kg) (Joseph et al., 2000). The peritoneal cavity was flushed with 10 ml of ice-cold Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) (10% FBS/DMEM), and cells were pelleted from the medium by centrifugation at 1500 rpm for 15 min (4°C). Cells were resuspended in DMEM containing 10% FBS and plated in 96-well plates ($4 \times 10^5$ cells/well). Nonadherent cells were removed after 5 h. The media were replaced, and peritoneal macrophages were treated with ligands in DMEM containing 5% lipoprotein-deficient serum (Intracel, Frederick, MD). RNA was isolated after 18 to 20 h of ligand treatment.

**RNA Extraction and mRNA Quantitation.** Total cellular RNA was isolated from treated cells cultured in 96-well plates using PrepStation 6100 (Applied Biosystems, Foster City, CA), and RNA was isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). Glycogen (10 μg/ml; Ambion, Inc., Austin, TX) was added to facilitate the recovery of nucleic acid from murine peritoneal macrophages. RNA was resuspended in ribonuclease-free water and stored at −70°C before analysis. RNA concentrations were quantitated with RiboGreen assay (Molecular Probes, Eugene, OR).

Gene-specific mRNA quantitation was performed by real-time PCR on an ABI Prism 7700 Sequence detection system (Applied Biosystems, Foster City, CA) as described previously (Quinet et al., 2004). Samples (50–100 ng) of total RNA were assayed in duplicate or triplicate in 50-μl reactions using one-step reverse transcription PCR. The standard curve method was used to estimate specific mRNA concentrations. PCR results were normalized to 18S ribosomal RNA levels with rodent/human 18S probe and primers purchased commercially (Applied Biosystems). Sequences of gene-specific primer and probe sets designed with Primer Express Software (Applied Biosystems) were published previously for murine ABCA1, SREBP-1c, FAS, and CYP7A1 and human LXRα and SREBP-1c (Quinet et al., 2004). Murine LXRα, LXRβ, stearoyl CoA desaturase-1 (SCD1), angptl3, apoCI, Insig-1, and Insig-2a probe/primer sets are available upon request.

**Measurement of Cholesterol Efflux in Isolated Mouse Peritoneal Macrophages.** Thioglycollate-elicited macrophages were

isolated from wild-type, LXR$\alpha$, and LXR$\beta$ knockout (KO) mice as described above. Macrophages were plated, and cholesterol efflux was measured in adherent cell monolayers as described previously (Quinet et al., 2004), with the following modifications. After 18 h, mouse peritoneal macrophage monolayers were preincubated with medium containing acetylated LDL (50 $\mu$g/ml) and [1,2-$^3$H]cholesterol (5 $\mu$Ci/ml) for 48 h. Cells were washed with phosphate-buffered saline and then incubated with RPMI 1640 medium containing 0.2% BSA in the presence or absence of compounds for 6 h. After an additional wash, human apoA1 (15 $\mu$g/ml) acceptor protein was added to medium with or without ligands (RPMI 1640 minus FBS or BSA). Cellular cholesterol was quantified after 24 h. Quadruplicate aliquots (30 $\mu$l) of incubation medium were removed and filtered through a 0.45-$\mu$M multiscreen plate (Millipore Corporation, Bedford, MA). The radioactivity in the incubation medium was determined by TopCount (PerkinElmer), and the percentage of radiolabeled cholesterol released (% efflux) was calculated as follows: (treatment in medium − control/control) × 100, where the treatment and control values were measured as counts per minute.

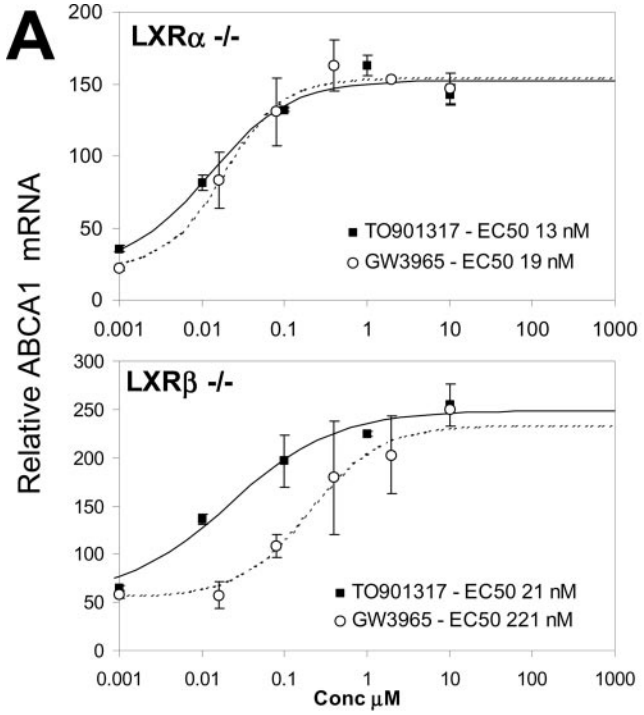
**Hepatocyte Cell Culture.** Mouse hepatoma, Hepa 1–6 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM with high glucose (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin (Invitrogen) and were plated at 60,000 cells/well in 96-well plates 18 to 24 h before ligand addition. LXR ligands were dissolved in dimethyl sulfoxide and added to cells for 18 h. Control cells were treated with vehicle.

**Plasma Lipid and Lipoprotein Analysis.** Plasma lipids, total cholesterol, and triglyceride concentrations were analyzed using a Hitachi 911 Clinical AutoAnalyzer with Boehringer Mannheim cholesterol and triglyceride (glycerol-blanked) reagents (Roche Diagnostics, Indianapolis, IN). Plasma lipoprotein concentrations were determined for individual animals by fast-performance liquid chromatography analysis using Superose 6 columns (Pharmacia, Peapack, NJ). Cholesterol concentrations in column fractions were measured enzymatically with the Boehringer Mannheim reagent. Hepatic lipids, cholesterol, and triglyceride concentrations in liver were determined by Analytics (Gaithersburg, MD).

**Statistical Analysis.** Mean, standard deviation, and statistical significance were determined by one-way analysis of variance using SAS Statistical Analysis Software (SAS Institute, Cary, NC).

## Results

**Dose-Response Evaluation of ABCA1 and SREBP-1c mRNA in Macrophages Isolated from LXR KO Mice.** To assess whether subtle activity differences exist for LXR$\alpha$ versus LXR$\beta$ isoforms, thioglycollate-elicited peritoneal macrophages isolated from LXR$\alpha$−/− and LXR$\beta$−/− mice were treated in vitro with LXR ligands TO901317 or GW3965 for 18 h, and $EC_{50}$ analysis of endogenous gene expression was performed. ABCA1 gene expression results are represented graphically in Fig. 1A. Baseline expression levels seem slightly higher in LXR$\beta$−/− mice, and thus, absolute induction is relatively higher. The LXR pan agonist TO901317 exhibits equal potency and efficacy for ABCA1 stimulation in macrophages expressing single receptor isotypes. GW3965 demonstrates lower functional activity relative to TO901317 in LXR$\beta$−/− macrophages (Fig. 1, A and B). As a result, the $EC_{50}$ value for GW3965 in LXR$\beta$−/− macrophages is 10-fold greater. This difference in regulating endogenous gene expression correlates with its lower potency in LXR$\alpha$ (175 nM) versus LXR$\beta$ (25 nM) peptide recruitment assays as reported by Groot et al. (2005). Collectively, these data extend and largely support previous analyses that used saturating single-point ligand binding conditions to compare efficacy (Repa et al., 2000b; Joseph et al., 2004; Walczak et al., 2004).




B

| Ligand | Gene activity | LXRα -/- % ag | LXRα -/- EC50 μM | LXRβ -/- % ag | LXRβ -/- EC 50 μM |
|---|---|---|---|---|---|
| TO901317 | ABCA1 | 100 | 0.013 | 100 | 0.022 |
| | SREBP-1c | 100 | 0.029 | 100 | 0.039 |
| GW3965 | ABCA1 | 101.4 | 0.019 | 91.8 | 0.221 |
| | SREBP-1c | 123.6 | 0.075 | 93.3 | 0.227 |

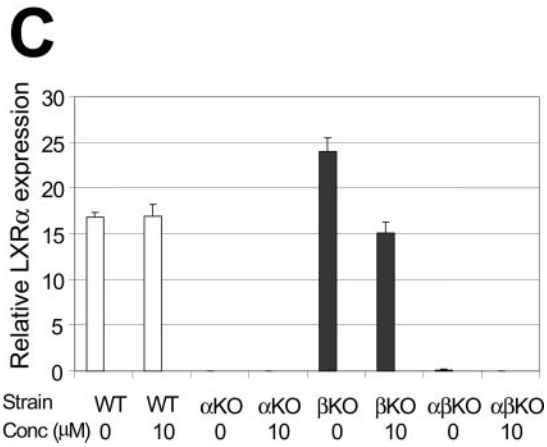



**Fig. 1.** LXR-mediated regulation of ABCA1 and SREBp-1c gene expression in TG-elicited peritoneal macrophages isolated from LXR$\alpha$−/− and LXR$\beta$−/− mice. Real-time PCR analysis of ABCA1 and SREBP-1c mRNA in macrophages treated in vitro with TO901317 or GW3965 for 18 h. A, dose-response analysis of ABCA1 expression in LXR$\alpha$−/− and LXR$\beta$−/− macrophages. B, comparison of LXR-mediated induction of ABCA1 and SREBP-1c mRNA expression in LXR$\alpha$−/− versus LXR$\beta$−/− macrophages; % ag, percentage of agonism or relative efficacy relative to TO901317. C, analysis of LXR$\alpha$ mRNA relative abundance in control or TO901317-treated (10 $\mu$M) macrophages from the four strains of mice ($n$ = 2).

Wild-type macrophages exhibit similar responses to LXRβ−/− cells (data not shown).

These studies also demonstrate that TO901317 and GW3965 stimulate SREBP-1c mRNA gene expression in LXRα-deficient macrophages (Fig. 1B) with efficacy and $EC_{50}$ values very comparable with those for ABCA1. The abundance of SREBP-1c mRNA in macrophages is comparatively lower relative to liver (data not shown). However, upon ligand stimulation, there are comparable SREBP-1c mRNA increases in wild-type (data not shown), LXRα-null, and LXRβ-null macrophages (Fig. 1B). The relative abundance of LXRα mRNA for each cell type was confirmed (Fig. 1C). Expression levels of LXRα mRNA in wild-type and LXRβ-deficient cells were similar and unchanged with ligand treatment. Undetectable levels of the LXRα receptor mRNA were found in double-mutant and LXRα-deficient cells. These studies underscore the importance of both receptor isoforms in the control of ABCA1 gene expression and extend previous observations of SREBP-1c responsiveness to LXRβ regulation in this cell type (Joseph et al., 2004).

**Cholesterol Efflux in LXRα and LXRβ KO Macrophages.** Given that some ABCA1 regulation is through post-translational degradation, it was deemed important to verify that LXR-mediated increases in ABCA1 mRNA translate to increases in apoA1-dependent cellular cholesterol efflux from macrophages. Cholesterol efflux measurements were performed in thioglycollate-stimulated peritoneal macrophages isolated from wild-type, LXRα−/−, and LXRβ−/− genotypes. These studies verify that both LXRα and LXRβ pathways lead to increases in cellular cholesterol efflux to lipid-poor apoA1 acceptors (Fig. 2). The macrophage studies seem to implicate ABCA1 as the primary contributor to cholesterol efflux; however, they do not exclude the participation of other LXR-inducible proteins, such as ABCG1 or apoE as newly lipidated apoA1 particles mature. Wild-type macrophages expressing both isoforms show slightly greater efficacy and potency relative to cells expressing single receptors. Overall, these efflux studies suggest receptor redundancy and a small additive effect upon dual activation.

**LXR-Mediated SREBP-1c Regulation in Murine Hepatocytes.** Hepa 1-6 cells (Peet et al., 1998) were treated with TO901317 and GW3965 to assess whether tissue-specific differences exist with respect to the regulation of SREBP-1c by the LXRβ isoform. Dose-response studies performed in Hepa 1-6 hepatocytes showed robust SREBP-1c induction by LXR pan agonists. SREBP-1c mRNA as measured by real-time PCR yielded potent $EC_{50}$ values of 26 nM for TO901317 and 53 nM for GW3965 (Fig. 3A). Characterization of this murine liver cell line derived from C57BL/6 mice showed predominantly LXRβ expression despite its hepatic origin. The relative abundance of LXRα mRNA in control or TO901317-treated Hepa 1-6 cells was 100-fold lower than levels observed in whole C57BL/6 liver (Fig. 3B) and is more consistent with J774 and RAW267.4 macrophages, cells with little or no LXRα expression (unpublished observations; Joseph et al., 2004). Consistent with their LXRα-deficient phenotype, these hepatocytes fail to up-regulate CYP7A1 in response to synthetic LXR agonists (data not shown). In addition, a 40-fold LXRα-selective ligand with micromolar potency in LXRα-expressing cells was totally inactive in Hepa 1-6 hepatocytes (data not shown). Overall, this characterization suggests a hepatocyte with predominantly LXRβ expression and little LXRα-mediated regulation. It confirms LXRβ-mediated activation of SREBP-1c but fails to explain the divergent regulation observed by others for liver in vivo (Peet et al., 1998; Alberti et al., 2001).

**In Vivo Comparison of LXRα−/− versus Wild-Type Control Mice Treated with Pan Agonists: Lipid and Lipoprotein Profiles.** As part of the systematic effort to characterize SREBP-1c regulation in vivo, 6-month-old, gender-matched LXRα-null and wild-type mice were dosed

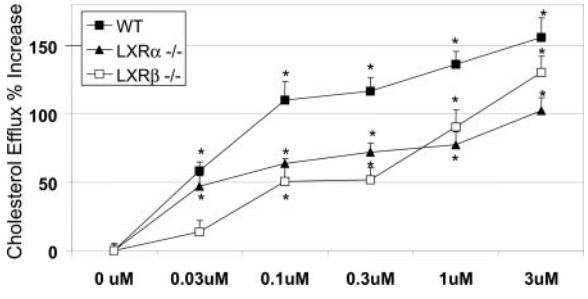



**Fig. 2.** Ligand activation of cholesterol efflux in thioglycollate-stimulated macrophages isolated from mice of WT, LXRα−/−, and LXRβ−/− genotypes. Cholesterol mobilization was measured in cells incubated with acetylated LDL (50 μg/ml) and [$^3$H]cholesterol (5 μCi/ ml) for 48 h followed by 6-h incubation in 0.2% BSA-containing medium with vehicle or ligands. ApoA1 protein (15 μg/ml) was added to the final 24-h incubation. The graph represents dose-response treatment with TO901317. Values represent the means ± S.E.M., $n = 4$ replicates; *, $p < 0.002$.

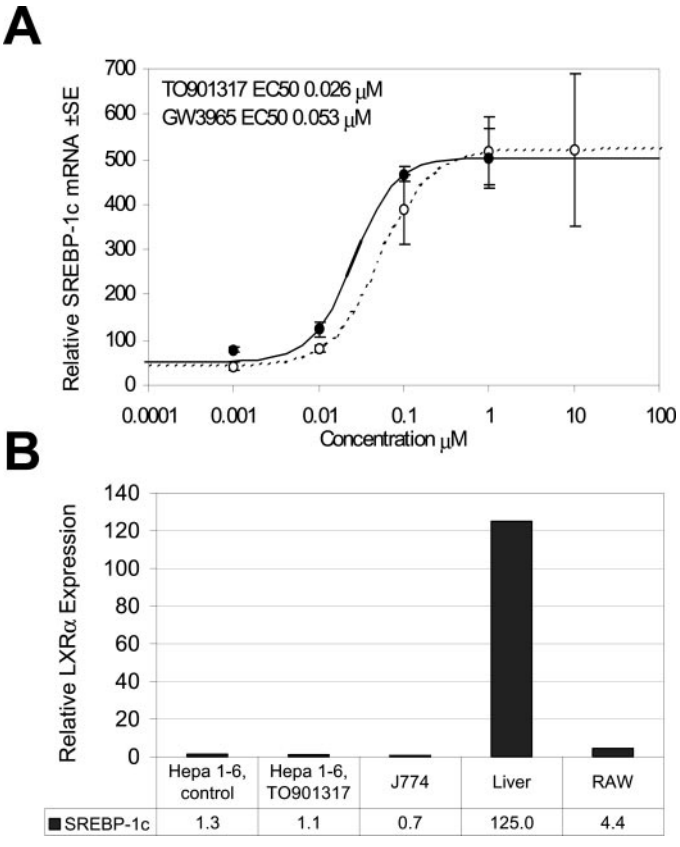



**Fig. 3.** LXR-mediated gene regulation in hepatocyte cell line. A, Hepa 1-6 hepatocytes were dosed with LXR ligands, TO901317, and GW3965 and SREBP-mRNA quantitated by real-time PCR. B, comparison of relative LXRα mRNA expression in several murine cell lines, Hepa 1-6 cells treated with 10 μM TO901317 and untreated controls, murine macrophage cell lines, J774, RAW 264.7, and murine liver.

with LXR ligands for 3 days. Study mice were maintained on standard chow, low in cholesterol (0.02%) to minimize the effects of liver lipid accumulation for LXR$\alpha$−/− mice. In the absence of LXR$\alpha$, these mice are unable to tolerate any dietary cholesterol in excess of what they can synthesize de novo and rapidly accumulate surplus lipid in liver (Peet et al., 1998). Animals were fed ad libitum, and treatment groups received vehicle, TO901317 (5- or 50-mg/kg doses), or GW3965 (10 or 50 mg/kg) as a single oral dose once daily. Comparison of baseline differences between strains of mice show that LDL cholesterol concentrations were 3-fold increased in LXR$\alpha$-null mice relative to WT controls (Table 1) as reported previously (Peet et al., 1998; Schuster et al., 2002). Total cholesterol was 24% higher ($p < 0.01$) and triglycerides 56% lower ($p < 0.001$) in LXR$\alpha$-null mice with no significant difference in HDL cholesterol between the two genotypes (Table 1).

In LXR$\alpha$-null mice treated for only 3 days, significant HDL cholesterol increases were associated with agonist treatment. Increases of 22 and 24% in HDL cholesterol were observed for TO901317 at the 5- and 50-mg/kg doses, respectively. GW3965 showed even greater efficacy, increasing HDL by 32 and 38% in the model at 10- and 50-mg/kg doses, respectively (Table 1). In LXR$\alpha$-null mice, none of the HDL elevations were associated with changes in plasma TG or VLDL cholesterol, and no dose-dependent-effects were observed for total plasma or LDL cholesterol. By comparison, age-matched control mice show a statistically significant HDL cholesterol elevation for the high-dose TO901317 treatment group only, whereas total plasma cholesterol and TGs were increased simultaneously. Both agonists increased VLDL cholesterol almost 2-fold, with higher doses in wild-type control. Thus, the in vivo consequence of dual agonist stimulation of the LXR$\beta$ receptor seems to be a potentially favorable increase in HDL not associated with a marked increase in plasma TGs or VLDL cholesterol.

**Hepatic Lipid Accumulation in LXR$\alpha$−/− versus WT Mice upon Treatment with LXR Agonists.** At the end of the study period, liver weights and liver lipids, both cholesterol and TG, were measured to evaluate the effects after LXR agonist treatment. The comparison of basal liver to body weight ratios (LW/BW) in Table 2 reveals that LXR$\alpha$-null mice, even on chow diets at this mature age, have relatively larger livers (42.5 ± 1.7 versus 52.5 ± 5.8 mg/g, $p < 0.05$). Ligand treatment with TO901317 was associated with liver weight gain in both LXR$\alpha$−/− and WT mice at the higher dose. No significant liver cholesterol increases were observed in either LXR$\alpha$−/− or wild-type mice due to treatment with either LXR agonist (Table 2). Liver TG concentrations were also unchanged in wild-type mice. However, the high-dose GW3965 treatment increased hepatic TGs slightly in LXR$\alpha$-null mice.

**Gene Expression Analysis in LXR$\alpha$−/− Mice Liver Confirms Isoform-Specific Effects.** Various tissue samples recovered from the experiments described above were used to examine isoform-specific effects on gene regulation in vivo. SREBP-1c gene expression and several LXR target genes in liver were analyzed by real-time PCR to allow correlation with observed changes in lipids and lipoproteins. Measurement of CYP7A1 and LXR$\alpha$ gene expression confirms the genotypes of the mice. LXR$\alpha$ was undetectable in hepatic RNA isolated from LXR$\alpha$−/− mice (data not shown). LXR-mediated up-regulation of murine CYP7A1 mRNA was observed only in wild-type mice (Fig. 4) concordant with literature on LXR$\alpha$-null mice (Peet et al., 1998). In liver, the expression of LXR$\beta$ was neither autoregulated by ligand treatment (Fig. 4) nor was its basal mRNA expression levels up-regulated by deletion of the LXR$\alpha$ isoform (data not shown).

Stimulation of ABCA1 gene expression was observed in liver from LXR$\alpha$-null mice treated with pan agonists, albeit the magnitude of activation was small (Fig. 4). In wild-type mice, a maximal 1.9-fold increase of ABCA1 mRNA was associated with a higher dose of TO901317, whereas an equivalent dose generated lipid and lipoprotein changes (see above). The data also show clear up-regulation of SREBP-1c in hepatic tissues from agonist-treated LXR$\alpha$−/− mice. However, SREBP-1c target genes and several other genes involved in hepatic TG biosynthesis or metabolism, including SCD1, angptl3, and apolipoprotein C-I, were not induced in LXR$\alpha$−/− mice but were up-regulated in wild-type liver after ligand treatment (Fig. 4). ApoAV, an SREBP-1c target implicated in TG clearance, was reduced in WT livers after agonist treatment but was largely unresponsive in LXR$\alpha$−/− livers (data not shown).

FAS gene expression, a downstream target of both LXR and SREBP-1c transcription factors, exhibits a lower response in LXR$\alpha$−/− mouse liver relative to wild-type con-

TABLE 1
Plasma lipids and lipoprotein cholesterol concentrations in mice treated with the LXR ligands T0901317 or GW3965 for 3 days.
Values represent mean ± S.E.M.

| Treatment | Dose | *n* | TC | TG | VLDL-c | LDL-c | HDL-c |
|---|---|---|---|---|---|---|---|
| | *mg/kg* | | | | *mg/dl* | | |
| LXR$\alpha$ KO mice | | | | | | | |
| Vehicle | | 6 | 83 ± 1.3 | 21 ± 1.4 | 2.8 ± 0.7 | 28 ± 2.1 | 52 ± 5.6 |
| TO901317 | 5 | 6 | 95 ± 5.0 | 22 ± 1.5 | 3.1 ± 0.3 | 29 ± 2.7 | 63 ± 2.9† |
| TO901317 | 50 | 6 | 89 ± 2.3 | 26 ± 2.6 | 3.4 ± 0.6 | 22 ± 2.3 | 64 ± 2.7† |
| GW3965 | 10 | 6 | 90 ± 1.6 | 28 ± 2.1 | 2.3 ± 0.3 | 20 ± 1.0* | 68 ± 1.5† |
| GW3965 | 50 | 6 | 97 ± 7.1* | 25 ± 3.9 | 2.6 ± 0.4 | 23 ± 4.3 | 71 ± 3.3† |
| Wild-type mice | | | | | | | |
| Vehicle | | 6 | 67 ± 11 | 48 ± 11 | 2.9 ± 0.97 | 9 ± 1.7 | 55 ± 8.9 |
| TO901317 | 5 | 6 | 66 ± 6.6 | 49 ± 14 | 2.9 ± 1.1 | 9 ± 1.8 | 54 ± 4.6 |
| TO901317 | 50 | 6 | 82 ± 5.5** | 78 ± 14† | 5.0 ± 1.0** | 11 ± 1.9 | 66 ± 4.9** |
| GW3965 | 10 | 6 | 73 ± 9.1 | 43 ± 8.7 | 2.2 ± 0.44 | 11 ± 2.8 | 60 ± 6.2 |
| GW3965 | 50 | 6 | 75 ± 9.5 | 57 ± 12 | 4.6 ± 2.4** | 14 ± 3.7** | 56 ± 4.8 |

* $p < 0.5$; ** $p < 0.01$; and † $p < 0.005$ represent significance relative to vehicle control.
TC, total cholesterol; TG, triglycerides; VLDL-C, VLDL cholesterol; LDL-C, LDL cholesterol; HDL-C, HDL cholesterol.

trols. For example, treatment of LXRα-null mice with 50 mg/kg TO901317 increased FAS mRNA 1.9-fold compared with 3.8-fold induction in liver from wild-type mice. None of these changes was associated with increases in either plasma TGs or substantial liver lipid accumulation in LXRα-deficient mice. In livers from wild-type mice, the enhanced gene induction observed for TO901317 relative to GW3965 might be attributed to its greater affinity for LXRα.

TABLE 2
Relative liver weight and lipids in mice treated with LXR ligands for 3 days
Data are expressed as mean ± S.E.M.

| Treatment | Dose | *n* | LW/BW | TC | TG |
|---|---|---|---|---|---|
| | *mg/kg* | | | *mg/g* | |
| LXRα KO mice | | | | | |
| Vehicle | | 6 | 52.0 ± 1.7 | 4.5 ± 0.22 | 8.3 ± 0.49 |
| TO901317 | 5 | 6 | 50.2 ± 1.1 | 4.2 ± 0.17 | 8.3 ± 0.21 |
| TO901317 | 50 | 6 | 55.5 ± 1.1* | 4.2 ± 0.17 | 8.3 ± 0.42 |
| GW3965 | 10 | 6 | 52.4 ± 1.0 | 4.5 ± 0.22 | 9.0 ± 0.37 |
| GW3965 | 50 | 6 | 51.4 ± 0.60 | 4.5 ± 0.34 | 9.8 ± 0.48** |
| Wild-type Mice | | | | | |
| vehicle | | 6 | 42.5 ± 5.8 | 4.0 ± 0.45 | 11.7 ± 0.99 |
| TO901317 | 5 | 6 | 45.1 ± 2.3 | 4.0 ± 0.00 | 12.0 ± 1.05 |
| TO901317 | 50 | 6 | 46.6 ± 3.4* | 4.0 ± 0.26 | 14.3 ± 1.2 |
| GW3965 | 10 | 6 | 42.8 ± 2.9 | 4.0 ± 0.00 | 14.8 ± 1.6 |
| GW3965 | 50 | 6 | 44.2 ± 2.3 | 4.0 ± 0.00 | 14.3 ± 0.56 |

* $p < 0.05$; ** $p < 0.01$. $n = 6$ per group.
LW/BW, liver weight (in milligrams)/body weight (in grams).

**Peripheral Tissues in LXRα-Deficient Mice Show Robust Induction of Both ABCA1 and SREBP-1c mRNA.** Despite apparent low levels of LXRβ-mediated gene activation in LXRα-deficient liver (above), there was significant stimulation of both ABCA1 and SREBP-1c in duodenum and kidney (Fig. 5). Absolute SREBP-1c mRNA basal expression, although quite low in the duodenum (PCR Ct value in the low thirties), increased more than 100-fold upon activation of the LXRβ isoform. A greater apparent potency and efficacy for LXR gene activation were observed in the duodenum for GW3965 relative to TO901317. In the kidney, ABCA1 and SREBP-1c mRNA induction were comparable with maximal increases in the 5- to 10-fold range for LXRα-deficient mice. In peripheral tissues, therefore, similar to isolated peritoneal macrophages from LXRα−/− mice, one fails to observe differential regulation of ABCA1 and SREBP-1c by LXRβ. Base-

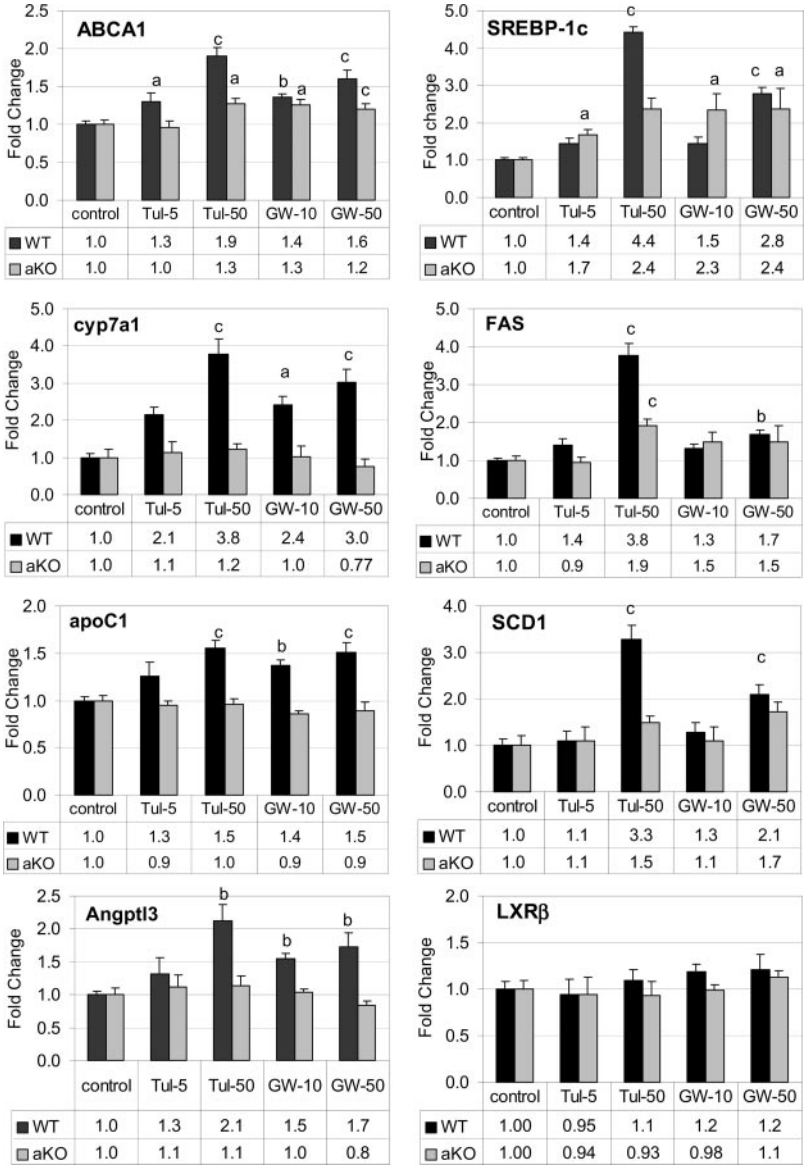



**Fig. 4.** Differential regulation of hepatic LXR target genes in wild-type and LXRα−/− mice. RNA was isolated from livers of mice treated with dual agonists for 3 days: TO901317 (Tul) 5 and 50 mg/kg and GW3965 (GW) 10 and 50 mg/kg. mRNA was quantitated by real-time PCR. RNA amounts were normalized with 18S rRNA, and data are expressed relative to vehicle control. Values represent means ± S.E.M., $n = 6$ mice per group; a, $p < 0.05$; b, $p < 0.01$; c, $p < 0.001$.

line expression levels of ABCA1 and SREBP-1c in kidney were 3- to 4-fold lower and slightly higher in duodenum of LXR$\alpha$-deficient mice relative to WT mice.

**Expression Profiles of Insig mRNAs after Treatment with LXR Agonist.** Transcriptional activation of SREBP-1c expression in LXR$\alpha$−/− livers by pan LXR agonists was associated with little change in SREBP-1c downstream target genes. The results suggest that SREBP-1c is well transcribed, but a transcriptionally active form of the protein may be absent from the nuclei. To investigate the mechanisms involved in regulation of SREBP-1c, Insig-1 and Insig-2a mRNA expression was measured by real-time PCR in LXR$\alpha$−/− and wild-type controls treated with TO901317 and GW3965 (Fig. 6). Insigs promote SREBP retention in the endoplasmic reticulum and, consequently, prevent SREBP precursor cleavage in the Golgi and nuclear translocation of the mature form of the transcription factor (Yang et al., 2002). Both forms of Insig exist in liver and may interfere with SREBP-1c cleavage. In LXR$\alpha$−/− livers, Insig-2a but not Insig-1 mRNA concentrations were increased with TO901317 and GW3965 treatment (Fig. 6A). By contrast, neither Insig-1 nor Insig-2a mRNA was up-regulated in wild-type liver (Fig. 6B). It is noteworthy that Insig-2a was not increased in LXR$\alpha$-null liver with the higher dose of GW3965 which was associated with a small increase in liver TG (Table 2). Thus, elevated Insig-2a levels could explain the absence of SREBP-1c target gene activation in LXR$\alpha$-null animals despite elevated levels of SREBP-1c mRNA.

## Discussion

Quantitative evaluation of nuclear receptor LXR$\beta$ activity performed using isolated peritoneal macrophages from LXR$\alpha$ KO mice demonstrate that the LXR$\beta$ subtype has a role comparable with LXR$\alpha$ for promoting macrophage ABCA1 expression and cholesterol efflux. Dose-response effects in adherent LXR$\alpha$−/− and LXR$\beta$−/− macrophages treated ex vivo with ligands underscore the importance of both receptor isoforms in the control of ABCA1 gene expression as others have suggested based on their efficacy at high doses (Repa et al., 2000b; Joseph et al., 2004; Walczak et al., 2004). The inference from these studies is that LXR$\beta$ possesses efficacy similar to that of LXR$\alpha$ for stimulating cholesterol efflux and potential for mediating the removal of excess cholesterol from lipid-laden macrophages of atherosclerotic lesions. Consistent with such properties, individual LXR subtypes seem to share equivalent atheroprotective roles (Schuster et al., 2002). In aging LXR KO mice (Schuster et al., 2002), aortic neutrophil infiltration was not significantly increased in LXR$\alpha$−/− or LXR$\beta$−/− mice relative to wild-type mice, and combined deficiency of LXR$\alpha$ and -$\beta$ was required for significant macrophage foam cell accumulation in spleen, lung, and the arterial wall. Our studies did not address and clearly do not preclude the contributions of ABCA1-independent, LXR-inducible cholesterol efflux through ABCG1, apoE, or as-yet-unidentified pathways.

Similar to findings reported recently for macrophages treated with LXR and/or retinoid X receptor ligands (Joseph et al., 2004), no obvious selectivity for one LXR isoform was apparent for ABCA1 or SREBP-1c gene induction. These results viewed in combination with reported impaired SREBP-1c expression in LXR$\alpha$−/− liver (Peet et al., 1998; Alberti et al., 2001) suggest that tissue-specific differences may exist for LXR-mediated SREBP-1c regulation. This observation is reminiscent of the estrogen receptor in which unique agonist/antagonist activities are expressed in a cell context-dependent manner (McDonnel, 2004).

In vivo studies provide further insight into the metabolic consequences of LXR$\beta$ activation by the synthetic agonists and extend earlier studies using dietary cholesterol to induce LXR activation (Peet et al., 1998; Alberti et al., 2001). Wild-type mice maintain low hepatic cholesterol in response to cholesterol-rich diets by up-regulating CYP7A1 mRNA and bile acid synthesis. In the present studies, CYP7A1 mRNA is

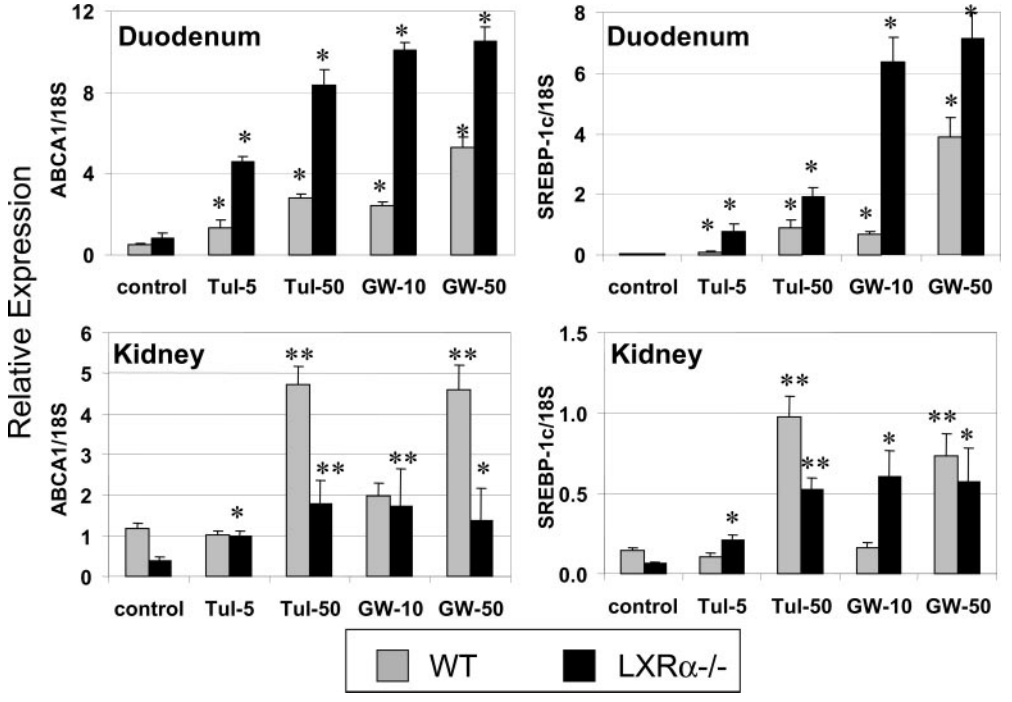



**Fig. 5.** Regulation of SREBP-1c and ABCA1 gene expression in peripheral tissues of LXR$\alpha$−/− mice. RNA was isolated from duodenum and kidney of LXR$\alpha$−/− mice treated with LXR ligands (TO901317, 5 and 50 mg/kg; and GW3965, 10 and 50 mg/kg) for 3 days. ABCA1 and SREBP-1c mRNA was quantitated by real-time PCR. RNA amounts were normalized with 18S rRNA. Values represent relative expression means ± S.E.M., $n = 6$ mice per group, except for kidney LXR$\alpha$−/− ($n = 5$ mice); *, $p < 0.0001$ for duodenum; *, $p < 0.05$ and **, $p < 0.001$ for kidney.

induced in wild-type mice treated with LXR pan agonists TO901317 or GW3965. In LXRα−/− mice treated similarly, there was no corresponding increase in CYP7A1 mRNA (Fig. 4). This hallmark of the LXRα−/− phenotype corroborates the earlier report using dietary cholesterol to induce LXR activation. More importantly, it highlights a potential liability in hepatic metabolism of dietary cholesterol in LXRα-null animals that is likely to affect SREBP gene regulation in this tissue, particularly as cholesterol accumulates (see *Discussion*).

In LXRα null mice, plasma HDL cholesterol was significantly increased after ligand treatment without altering plasma TGs or VLDL (Table 1). HDL cholesterol increases were observed at all doses for LXRα-null animals despite little change in the lipogenic profile in either plasma or liver. By contrast, ligand-induced elevations in plasma HDL cholesterol in wild-type mice occur only in the presence of lipogenic increases in total cholesterol, TGs, and VLDL cholesterol. Although a detailed investigation of the HDL source in LXRα−/− mice is beyond the scope of the current work, recent evidence provided by several studies define the liver as an important source of HDL cholesterol in mice (Basso et al., 2003). Bone marrow transplantation studies in ABCA1-KO mice also establish that macrophage ABCA1-mediated cholesterol efflux has little impact on plasma HDL-C levels (Haghpassand et al., 2001). Based on these combined findings, one might speculate that the observed induction of ABCA1 transporter mRNA by LXRβ may facilitate hepatic cholesterol efflux to the plasma HDL cholesterol pool. However, relative contributions to the HDL fraction from ABCA1 mRNA up-regulation in peripheral tissues or the impact of cholesterol efflux via ABCG1 and apoE are unclear. HDL increases may also reflect reduced particle uptake.

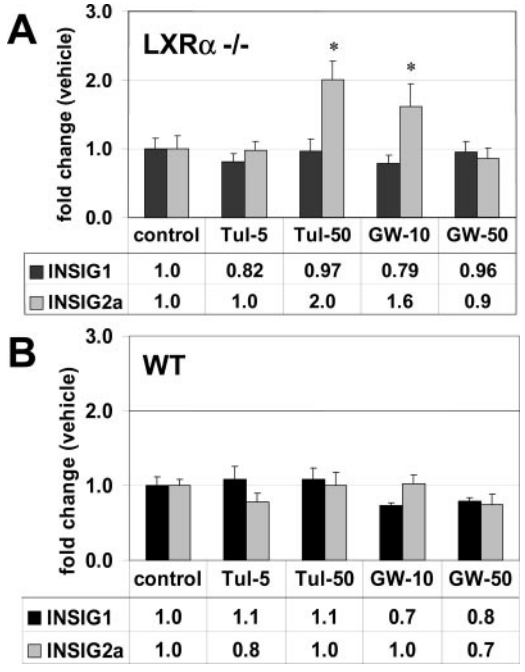



**Fig. 6.** Liver-specific mRNA for Insig-2a up-regulated in LXRα−/− mice but not wild-type mice by LXR agonists. RNA was isolated from livers of LXRα−/− mice (A) or wild-type (B) treated with dual agonists for 3 days: TO901317 (Tul) 5 and 50 mg/kg and GW3965 (GW) 10 and 50 mg/kg. Insig-1 and Insig-2a transcripts were quantitated by real-time PCR. RNA amounts were normalized with 18S rRNA and data expressed relative to vehicle control. Values represent means ± S.E.M., $n = 6$ mice per group; *, $p < 0.05$.

Critical target genes implicated in TG metabolism were not induced in LXRα−/− mice, providing a molecular mechanism for the lack of lipogenic effects. A modest activation of SREBP-1c gene expression was observed in LXRα-null liver relative to controls. However, no mRNA changes were observed for several other genes with lipogenic potential (Fig. 4), such as SCD, angptl3, and apoCI after LXR agonist treatment of null mice. Hepatic FAS, a gene target activated by both LXR, and SREBP-1c transcription factors, also exhibited reduced stimulation relative to control livers. In contrast, large increases in hepatic SREBP-1c, FAS, and SCD1 observed in C57BL/6 wild-type mice treated with TO901317 correlate with hypertriglyceridemia and increases in plasma VLDL lipoproteins.

It seems that the lipogenic potential of LXRα−/− mouse livers is blunted relative to wild-type controls. The fact that GW3965 significantly increases both SREBP-1c and hepatic triglycerides at the 50 mg/kg dose, however, suggests that LXRβ contributes to these hepatic effects and implies that LXRβ-selective agonists may not be completely devoid of hepatic side effects. Insig-2a up-regulation in LXRα-deficient liver provides a post-transcriptional mechanism whereby LXR-mediated induction of SREPB-1c message levels fails to activate the lipogenic cascade of genes leading to TG synthesis. Under conditions of cellular cholesterol excess, SREBPs bind Insig proteins and remain trapped in the endoplasmic reticulum, where proteolytic processing, maturation, and translocation cannot occur (Yang et al., 2002). In LXRα-null mice, SREBP-1c activity may be regulated primarily by cellular factors that inhibit proteolytic processing of the membrane-bound precursor.

The fact that hepatic ATP binding cassette transporters ABCG5 and ABCG8 were not up-regulated in LXRα−/− mice (Repa et al., 2002) provides additional evidence for a more global defect in LXR activation in liver with LXRα deletion. Conceivably, the phenotype reflects LXRα-predominant expression in liver and means that apparent LXR isoform gene selectivity may simply reflect the differential ratios of LXRα versus LXRβ expression in various tissues. In addition, the lack of LXR-mediated responses potentially provides a metabolic basis for hepatic sterol accumulation in LXRα-deficient mice, which is usually attributed largely to CYP7A1. Although there was minimal lipid accumulation in liver in this short-term study, larger liver/body weight ratios were observed at baseline for 6-month-old LXRα−/− mice relative to wild-type mice of the same age.

In contrast to the liver, agonist treatment enhances SREBP-1c gene expression in parallel with ABCA1 in non-hepatic tissues such as duodenum and kidney recovered from LXRα-null mice. SREBP-1c activation may promote fatty acid synthesis necessary for cholesteryl ester formation and intracellular lipid storage (Repa et al., 2000a; Schultz et al., 2000; Yoshikawa et al., 2001) in these peripheral tissues. It is not anticipated that SREPB-1c stimulation in extrahepatic tissue should induce hypertriglyceridemia, and no influence on plasma TG levels was observed in LXRα−/− mice treated with LXR ligands. The differential pattern of hepatic SREBP-1c regulation by LXRβ may reflect the higher concentration of LXRα in liver, as suggested above, or infer a

need for additional liver-specific regulatory factors. Corroborating evidence for equivalent SREBP-1c activation by both receptor isoforms is documented in adipose tissue (Ulven et al., 2004) and in an unpublished study in which small interfering RNA-mediated silencing of LXR$\alpha$ in a fibroblast cell line did not significantly reduce agonist-stimulated SREBP-1c mRNA induction (J. Prestle, personal communication).

Unexpectedly in another publication, LXR activation via synthetic LXR dual $\alpha/\beta$ agonists in monkeys was achieved without evidence of hypertriglyceridemia (Groot et al., 2005). These data suggest that the triglyceride liability may be overstated for higher species possessing CETP and lipoprotein metabolism relevant to humans and further emphasize the lack of predictability of lower species. However, dual $\alpha/\beta$ agonist treatment of CETP-containing species did result in elevations of LDL-C/apoB and an unfavorable shift in lipoprotein profile due to neutral lipid exchange between HDL and apoB-containing lipoproteins by CETP (Groot et al., 2005). Conceivably, LXR $\beta$-selective ligands with their reduced capacity for stimulating VLDL synthesis might display a more favorable lipoprotein profile in higher species, including humans.

The definitive role of LXR$\beta$ in the control of hepatic lipogenesis will ultimately require potent and selective synthetic ligands to probe the function of the receptor. However, the results presented herein suggest that it is possible to achieve tissue-selective gene regulation through LXR$\beta$. The current studies demonstrate that the LXR$\beta$ isoform, through activation of ATP-binding cassette transporter expression, can promote cholesterol transport and its excretion in liver, intestine, and macrophage to regulate cholesterol balance. The results also suggest that selective LXR$\beta$ activation may separate lipogenic effects from antiatherosclerotic potential established for LXR ligands in mice. LXR agonists delay the progression of atherosclerosis (Joseph et al., 2000; Tangirala et al., 2002) and, moreover, can induce regression and stabilization of established lesions in mice (Levin et al., 2005). Recent studies implicating a role for the apoptosis inhibitory factor apoptosis inhibitor expressed in macrophages/Sp$\alpha$/Api6 in atherosclerosis development may provide further justification for selective targeting of LXR$\beta$ (Arai et al., 2005). The path forward may require agonists with better selectivity or pharmacokinetic properties than published ligands (Miao et al., 2004; Quinet et al., 2004; Groot et al., 2005). Whether this is accomplished through isoform-selective agonists or gene- or tissue-specific ligands, such as selective LXR modulators, with a mixed agonist profile remains to be established. Despite these challenges, the potential beneficial outcomes due to LXR receptor modulation continue to fuel great interest in this field.

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