Molecular Pharmacology Fast Forward. Published on July 6, 2006 as doi:10.1124/mol.106.022608

LXRβ Regulation in LXRα Deficient Mice: Implications for Therapeutic Targeting.

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Molecular Pharmacology Fast Forward. Published on July 6, 2006 as DOI: 10.1124/mol.106.022608
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Running title: LXRβ activation induces SREBP-1c gene expression.

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Text pages: 33

Number of Tables: 2

Number of Figures: 6

Number of References: 40

Number of words in Abstract: 249 (Max = 250)

Number of words in Introduction: 730 (Max = 750)

Number of words in Discussion: 1499 (Max = 1500)

[1]The abbreviations used are: ABCA1, ATP binding cassette transporter A1; AIM, apoptosis inhibitor expressed in macrophages (also called Spα or Api6); angiopoietin-like protein 3, angptl3; cyp7a1, cholesterol 7α hydroxylase; CETP, cholesteryl ester transfer protein; EC50, half maximum effective concentration; IC50, half maximum inhibitory concentration; LPDS, lipoprotein deficient serum; RCT, reverse cholesterol transport; SCD1, stearoyl CoA desaturase-1; SREBP-1c, sterol-response element binding protein.

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ABSTRACT

The nuclear receptors LXRα and LXRβ are differentially expressed ligand-activated transcription factors that induce genes controlling cholesterol homeostasis and lipogenesis. Synthetic ligands for both receptor subtypes activate ABCA1-mediated cholesterol metabolism, increase reverse cholesterol transport and provide atheroprotection in mice. However, these ligands may also increase hepatic triglyceride (TG) synthesis via an SREBP-1c-dependent mechanism through a process reportedly regulated by LXRα. We studied pan LXRα/β agonists in LXRα knockout mice to assess the contribution of LXRβ to regulation of selected target genes. *In vitro* dose response studies with macrophages from LXRα -/- and β -/- mice confirm an equivalent role for LXRα and LXRβ in the regulation of ABCA1 and SREBP-1c gene expression. Cholesterol efflux studies verify that LXRβ can drive apoA1-dependent cholesterol mobilization from macrophages. The *in vivo* role of LXRβ in liver was further evaluated by treating LXRα -/- mice with a pan LXRα/β agonist. HDL-cholesterol increased without significant changes in plasma TG or VLDL. Analysis of hepatic gene expression consistently revealed less activation of ABCA1 and SREBP-1c genes in the liver of LXRα null animals than in treated-WT controls. In addition, hepatic Cyp7a1 and several genes involved in fatty acid/ TG biosynthesis were not induced. In peripheral tissues from these LXRα null mice, LXRβ 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 LXRβ activation is expected to stimulate ABCA1 gene expression in macrophages, contribute to favorable HDL increases, but circumvent hepatic LXRα-dominated lipogenesis.

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INTRODUCTION

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 (Cao et al., 2004; Jaye, 2003; Tontonoz and Mangelsdorf, 2003; Joseph and Tontonoz**,** 2003). Their therapeutic potential resides in their ability to dramatically upregulate ABCA1 transcription, and thereby stimulate cholesterol efflux from macrophages. It has been demonstrated that activation of LXR by cognate ligands promotes apoA-1 mediated efflux and this is thought 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 upregulation 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 properties 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 (Repa et al., 2000a,b; Berge et al., 2000). In mice but not man, LXRs also induce 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 (Repa et al., 2002a; Schultz et al., 2000; Grefhorst et al., 2002) through transcriptional

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activation of sterol response element binding protein 1c (SREBP-1c) (Repa et al., 2002a; Schultz et al., 2000; Grefhorst et al., 2002; Yoshikawa et al., 2001), 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 as triglyceride (TG) elevations are an established independent risk factor for atherosclerotic heart disease (Assmann et al., 1998). For this reason, pharmacologic 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; Tontonoz and Mangelsdorf, 2003; Joseph and Tontonoz**,** 2003; Lund et al., 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 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

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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.

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MATERIALS AND METHODS

Ligands. LXR agonists, Tularik TO901317 [*N*-(2,2,2,-trifluoro-ethyl)-*N*-[4-(2,2,2,-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide] (Repa et al., 2000b; Schultz et al., 2000) and Glaxo GW3965 [3-(3-(2-chloro-3-trifluoromethylbenzyl-2,2-diphenylethylamino) proproxy) phenylacetic acid] (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 [1,2-3H(N)]-cholesterol was purchased from PerkinElmer NET-139.

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 3 generations. Upon receipt, all mice were maintained on a 12 h light /12 h 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 a.m. 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 5-6 h, blood was recovered and plasma prepared using standard centrifugation techniques. Tissues were collected for RNA preparation and frozen in liquid N_2 prior to 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. Thioglycolate-elicited peritoneal macrophages were isolated from nonfasted male mice three days after peritoneal injection of 4% Brewers

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thioglycolate media (25 ml/kg) (Joseph et al., 2000). The peritoneal cavity was flushed with 10 ml of ice cold, DMEM containing 10% fetal bovine serum (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 x10^5 cells/well). Non-adherent cells were removed after 5 h. The media was replaced and peritoneal macrophages were treated with ligands in DMEM containing 5% lipoprotein deficient serum (LPDS) (Intracel, Frederick, MD). RNA isolated after 18-20 h 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 isolated from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). Glycogen (10 μg/ml, Ambion, Inc., Austin, TX) was added to facilitate recovery of nucleic acid from murine peritoneal macrophages. RNA was resuspended in ribonuclease-free water and stored at –70^{o}C prior to analysis. RNA concentrations were quantitated with RiboGreen assay, #R-11490 (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 (Quinet et al., 2004). Samples (50-100 ng) of total RNA were assayed in duplicate or triplicate in 50 μl reactions using one-step RT-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, Foster City, CA). Sequences of gene-specific primer and probe sets designed with Primer Express Software (Applied Biosystems, Foster City, CA) were previously published for murine ABCA1, SREBP-1c, FAS, and cyp7a1 and human LXRα and SREBP-1c (Quinet et al., 2004). Murine LXRα, LXRβ, SCD1, angptl3, apoCI, Insig-1 and Insig-2a probe/primer sets are available upon request.

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Measurement of Cholesterol Efflux in isolated MPMs. Thioglycollate-elicited macrophages were isolated from wild-type, LXRα and LXRβ KO mice as described above. Macrophages were plated and cholesterol efflux measured in adherent cell monolayers as previously described (Quinet), with the following modifications. After 18 h, MPM macrophage monolayers were preincubated with medium containing acLDL (50 μg/ml) and [1,2 ^{3}H]-cholesterol (5 μCi/ml) for 48 h. Cells were washed with PBS and then incubated with RPMI 1640 medium containing 0.2% BSA in the presence or absence of compounds for 6 h. Following an additional wash, human ApoA1 (15 μg/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 μl) of incubation medium were removed and filtered through a 0.45 μM multiscreen plate (Millipore, Corp., Bedford, MA). The radioactivity in the incubation medium was determined by TopCount (Perkin Elmer, Shelton, CT) and the percentage of radiolabeled cholesterol released (% efflux) was calculated as: (Treatment cpm in medium minus control cpm / control cpm) X 100.

Hepatocyte cell culture. Mouse hepatoma, Hepa 1-6 cells (ATCC #CRL-1830) were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in Dulbecco's modified Eagle's DMEM with high glucose (Invitrogen, Carlsbad, Ca), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics, 100 U/ ml penicillin and 100 μg ml streptomycin (Invitrogen, Carlsbad, Ca) and were plated at 60,000 cells per well in 96 well plates 18-24 h prior to ligand addition. LXR ligands were dissolved in DMSO 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

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lipoprotein concentrations were determined for individual animals by FPLC 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 (Gathersburg, MD).

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

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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 LXRα vs. β isoforms, thioglycolate-elicited peritoneal macrophages isolated from LXRα -/- and LXRβ -/- mice were treated *in vitro* with LXR ligands TO901317 or GW3965 for 18 h and EC50 analysis of endogenous gene expression performed. ABCA1 gene expression results are represented graphically in Figure 1a. Baseline expression levels appear slightly higher in LXRβ -/- 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 LXRβ -/- macrophages (Fig 1a and b). As a result, the EC50 value for GW3965 in LXRβ -/- macrophages is 10-fold higher. This difference in regulating endogenous gene expression correlates with its lower potency in LXRα (175 nM) vs. LXRβ (25 nM) peptide recruitment assays as reported by Groot et al., 2005. Collectively, these data extend and largely support previous analyses that employed saturating single point ligand binding conditions to compare efficacy (Repa et al., 2000b; Joseph et al., 2004; Walczak et al., 2004). 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 very comparable efficacy and EC50 values to 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

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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 thioglycolate 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 apoA-1 acceptors (Fig. 2). The macrophage studies appear 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 apoA-1 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 Hep1-6 hepatocytes showed robust SREBP-1c induction by LXR pan agonists. SREBP-1c mRNA as measured by real-time PCR yielded potent EC50 values of 26 nM for TO901317 and 53 nM for GW3965 (Fig. 3a). Characterization of this murine liver cell line derived from C57Bl6 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

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in whole c57BL6 liver (Fig. 3b) and is more consistent with J774 and RAW267.4 macrophages, cells with little or no LXRα expression (unpublished observations and Joseph et al., 2004). Consistent with their LXRα-deficient phenotype, these hepatocytes fail to upregulate cyp7a1 in response to synthetic LXR agonists (data not shown). In addition, a 40-fold LXRα selective ligand with μM 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 -/- vs. 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 with LXR ligands for 3 days. Study mice were maintained on standard chow, low in cholesterol (0.02%) to minimize effects of liver lipid accumulation for LXRα -/- mice. In the absence of LXRα, 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 lib 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 LDL-cholesterol concentrations were 3-fold increased in LXRα null mice relative to WT controls (Table 1) as reported (Peet et al., 1998; Schuster et al., 2002). Total cholesterol was 24% higher ($p < 0.01$) and triglycerides 56% lower ($p < 0.001$) in LXRα null mice with no significant difference in HDL cholesterol between the two genotypes (Table 1).

In LXRα null mice treated for only 3 days, significant HDL-c increases were associated with agonist treatment. Increases of 22 and 24% in HDL-c were observed for TO901317 at the 5 and 50 mg/kg doses,

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respectively. GW3965 showed even greater efficacy, raising HDL 32 and 38% in the model at 10 and 50 mg/kg doses, respectively (Table 1). In LXRα 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-c elevation for the high dose TO901317 treatment group only, while total plasma cholesterol and TGs were raised simultaneously. Both agonists raised VLDL-c almost two-fold with higher doses in wild-type control. Thus, the *in vivo* consequence of dual agonist stimulation of the LXRβ receptor appears to be a potentially favorable increase in HDL not associated with a marked increase in plasma TGs or VLDL cholesterol.

Hepatic lipid accumulation in LXRα -/- vs. 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 effects following LXR agonist treatment. The comparison of basal liver to body weight ratios (LW/BW) in Table 2 reveals that LXRα null mice even on chow diets at this mature age have relatively larger livers (42.5 ± 1.7 vs. 52.5 ± 5.8 mg/gm, $p < 0.05$). Ligand treatment with TO901317 was associated with liver weight gain in both LXRα -/- and WT mice at the higher dose. No significant liver cholesterol increases were observed in either LXRα -/- 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 raised hepatic TGs slightly in LXRα null mice.

Gene expression analysis in LXRα -/- 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 LXRα gene expression confirms the genotypes of the mice.

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LXRα was undetectable in hepatic RNA isolated from LXRα -/- mice (data not shown). LXR-mediated upregulation of murine cyp7a1 mRNA was observed only in wild-type mice (Fig 4) concordant with literature on LXRα null mice (Peet et al., 1998). In liver, the expression of LXRβ was neither autoregulated by ligand treatment (Fig. 4) nor was its basal mRNA expression levels upregulated by deletion of the LXRα isoform (data not shown).

Stimulation of ABCA1 gene expression was observed in liver from LXRα 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 upregulation of SREBP-1c in hepatic tissues from agonist-treated LXRα -/- mice. However, SREBP-1c target genes and several other genes involved in hepatic TG biosynthesis or metabolism, including stearoyl CoA desaturase-1 (SCD1), angiopoietin-like protein 3 (angptl3) and apolipoprotein C-I (apoC1), were not induced in LXRα -/- mice but were upregulated in wild-type liver following ligand treatment (Fig. 4). ApoAV, an SREBP-1c target implicated in TG clearance, was reduced in WT livers following agonist treatment but was largely unresponsive in LXRα -/- livers (data not shown).

Fatty acid synthase (FAS) gene expression, a downstream target of both LXR and SREBP-1c transcription factors, exhibits a lower response in LXRα -/- mouse liver relative to wild-type controls. For example, treatment of LXR α null mice with 50 mg/kg of TO901317 increased FAS mRNA 1.9 fold compared with 3.8 fold induction in liver from wild-type mice. None of these changes were 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.

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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 SREBP1c in duodenum and kidney (Fig. 5). Absolute SREBP-1c mRNA basal expression although quite low in the duodenum (Cts in the low 30s) 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 5-10 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. Baseline expression levels of ABCA1 and SREBP-1c in kidney were 3-4 fold lower and slightly higher in duodenum of LXRα-deficient mice relative to WT mice.

Expression profiles of Insig mRNAs following treatment with LXR agonist.

Transcriptional activation of SREBP-1c expression in LXRα -/- 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 Insig2a mRNA expression were measured by real-time PCR in LXRα -/- and wild-type controls treated with TO901317 and GW3965 (Fig. 6). Insigs promote SREBP retention in the ER 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 LXRα -/- 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 were upregulated in wild-type liver (Fig. 6b). Notably, Insig-2a was not increased in LXRα null liver with the higher dose of

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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 LXRα null animals despite elevated levels of SREBP-1c mRNA.

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DISCUSSION

Quantitative evaluation of nuclear receptor LXRβ activity performed using isolated peritoneal macrophages from LXRα knockout (KO) mice demonstrate that the LXRβ subtype has a comparable role to LXRα for promoting macrophage ABCA1 expression and cholesterol efflux. Dose response effects in adherent LXRα -/- and LXRβ -/- 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 LXRβ possesses similar efficacy as LXRα for stimulating cholesterol efflux and potential for mediating removal of excess cholesterol from lipid-laden macrophages of atherosclerotic lesions. Consistent with such properties, individual LXR subtypes appear 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 LXRα –/– or LXRβ -/- mice relative to wild-type mice and combined deficiency of LXRα and β 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 RXR 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 LXRα -/- liver (Peet et al., 1998; Alberti et al., 2001), suggest tissue-specific differences may exist for LXR-mediated SREBP-1c regulation. This observation is reminiscent of the estrogen receptor where unique agonist/antagonist activities are expressed in a cell context-dependent manner (McDonnel, 2004).

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In vivo studies provide further insight into the metabolic consequences of LXRβ 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 upregulating cyp7a1 mRNA and bile acid synthesis. In the present studies, cyp7a1 mRNA is 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, likely to impact SREBP gene regulation in this tissue, particularly as cholesterol accumulates (see discussion below).

Strikingly in LXRα null mice, plasma HDL-c was significantly increased following ligand treatment without altering plasma TGs or VLDL (Table 1). HDL-c 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-c in wild-type mice occur only in the presence of lipogenic increases in TC, TGs and VLDL-c. 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 upregulation in peripheral tissues or the impact of cholesterol efflux via ABCG1 and apoE are unclear. HDL increases may also reflect reduced particle uptake.

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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 following 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 c57Bl6 wild-type mice treated with TO901317, correlate with hypertriglyceridemia and increases in plasma VLDL-lipoproteins.

It appears 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 LXRβ contributes to these hepatic effects and implies that LXRβ-selective agonists may not be completely devoid of hepatic side effects. Insig-2a upregulation 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 ER 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 ABC transporters, ABCG5 and ABCG8, were not upregulated 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

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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 -/- relative to wildtype mice of the same age.

In contrast to the liver, agonist treatment enhances SREBP-1c gene expression in parallel with ABCA1 in nonhepatic 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 where siRNA-mediated silencing of LXRα in a fibroblast cell line did not significantly reduce agonist-stimulated SREBP-1c mRNA induction (personal communication).

Unexpectedly in another publication, LXR activation via synthetic LXR dual α/β 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 man, and further emphasize the lack of predictability of lower species. However, dual α/β 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 β-selective ligands with their

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reduced capacity for stimulating VLDL synthesis might display a more favorable lipoprotein profile in higher species including man.

The definitive role of LXRβ 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 LXRβ. The current studies demonstrate that the LXRβ 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-LXRβ activation may separate lipogenic effects from anti-atherosclerotic potential established for LXR ligands in mice. LXR agonists delay the progression of atherosclerosis (Tangirala et al., 2002; Joseph et al., 2000) 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 AIM/Spα/Api6 in atherosclerosis development may provide further justification for selective targeting of LXRβ (Arai et al., 2005). The path forward may require agonists with better selectivity or pharmacokinetic properties than published ligands (Groot et al., 2005; Quinet et al., 2004). Whether this is accomplished through isoform selective agonists, gene or tissue-specific ligands, such as selective LXR modulators (SLRMs) 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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FOOTNOTES

*The authors would like to thank the Wyeth Bioresource Department for support of *in vivo* studies and Dr. Juergen Prestle (Boehringer-Ingelheim, Germany) for communication of siRNA silencing data prior to publication. The generation of LXR isoform deleted mice was supported by grants from the Swedish Science Council and from KaroBio AB.

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FIGURE LEGENDS

Fig. 1. *LXR-mediated regulation of ABCA1 amd SREBp-1c gene expression in TG-elicited peritoneal macrophages isolated from LXRα -/- and LXRβ -/- 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 LXRα -/- and LXRβ-/- macrophages. B) Comparison of LXR-mediated induction of ABCA1 and SREBP-1c mRNA expression in LXRα -/- vs. LXRβ-/- macrophages (EC50 = concentration of compound that leads to half-maximal activity, % ag = percent agonism or relative efficacy relative to TO901317. C) Analysis of LXRα mRNA relative abundance in control or TO901317-treated (10 μM) macrophages from the four strains of mice (n = 2).

Fig. 2 *Ligand activation of cholesterol efflux in thioglycolate-stimulated macrophages isolated from mice of WT, LXRα -/- and LXRβ -/- genotypes.* Cholesterol mobilization was measured in cells were incubated with acLDL (50 μg/ml) and [^{3}H]-cholesterol (5 μCi/ ml) for 48 h, followed by 6h incubation in 0.2% BSA-containing medium with vehicle or ligands. ApoA1 protein (15 μg/ml) was added to the final 24 hr incubation. The graph represents dose response treatment with TO901317. Values represent the means ± SEM, 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 and RAW 264.7 and murine liver.

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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 expressed relative to vehicle control. Values represent means ± SEM, n = 6 mice per group, ^{a} $p < 0.05$, ^{b} $p < 0.01$, ^{c} $p < 0.001$. Abbreviations: ATP-binding-cassette transporter (ABCA1), angiopoietin-like protein 3 (angptl3), apolipoprotein CI (apoCI), cholesterol 7α hydroxylase (cyp7A1), fatty acid synthase (FAS), liver X receptor β (LXRβ), stearoyl CoA-desaturase (SCD), sterol regulatory element binding protein-1c (SREBP-1c)

Fig. 5. *Regulation of SREBP-1c and ABCA1 gene expression in peripheral tissues of LXRα -/- mice.* RNA was isolated from duodenum and kidney of LXRα -/- mice treated with LXR ligands (TO901317, 5, 50 mg/kg and GW3965, 10, 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 ± SEM, n = 6 mice per group, except for kidney LXRα -/- (n = 5 mice), * $p < 0.0001$ for duodenum and * $p < 0.05$, ** $p < 0.001$ for kidney.

Fig. 6. *Liver specific mRNA for Insig-2a upregulated 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 ± SEM, n = 6 mice per group, * $p < 0.05$.

Table 1 - Plasma lipids and lipoprotein cholesterol concentrations in mice treated with the LXR ligands: T0901317 or GW3965 for 3 days.

Treatment	Dose	*n*	TC mg/dl	TG mg/dl	VLDL-c mg/dl	LDL-c mg/dl	HDL-c mg/dl
LXRα KO Mice							
vehicle	-	6	83 ± 1.3	21 ± 1.4	2.8 ± 0.7	28 ± 2.1	52 ± 5.6
TO901317	5 mg/kg	6	95 ± 5.0	22 ± 1.5	3.1 ± 0.3	29 ± 2.7	63 ± 2.9†
TO901317	50 mg/kg	6	89 ± 2.3	26 ± 2.6	3.4 ± 0.6	22 ± 2.3	64 ± 2.7†
GW3965	10 mg/kg	6	90 ± 1.6	28 ± 2.1	2.3 ± 0.3	20 ± 1.0*	68 ± 1.5†
GW3965	50 mg/kg	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 mg/kg	6	66 ± 6.6	49 ± 14	2.9 ± 1.1	9 ± 1.8	54 ± 4.6
TO901317	50 mg/kg	6	82 ± 5.5**	78 ± 14†	5.0 ± 1.0**	11 ± 1.9	66 ± 4.9**
GW3965	10 mg/kg	6	73 ± 9.1	43 ± 8.7	2.2 ± 0.44	11 ± 2.8	60 ± 6.2
GW3965	50 mg/kg	6	75 ± 9.5	57 ± 12	4.6 ± 2.4**	14 ± 3.7**	56 ± 4.8

Values represent mean ± SEM, * $p < 0.5$, ** $p < .01$, † $p < .005$, represent significance relative to vehicle control.
TC, total cholesterol, TG, triglycerides, VLDL-C, VLDL cholesterol, LDL-C, LDL cholesterol, HDL-C, HDL cholesterol.

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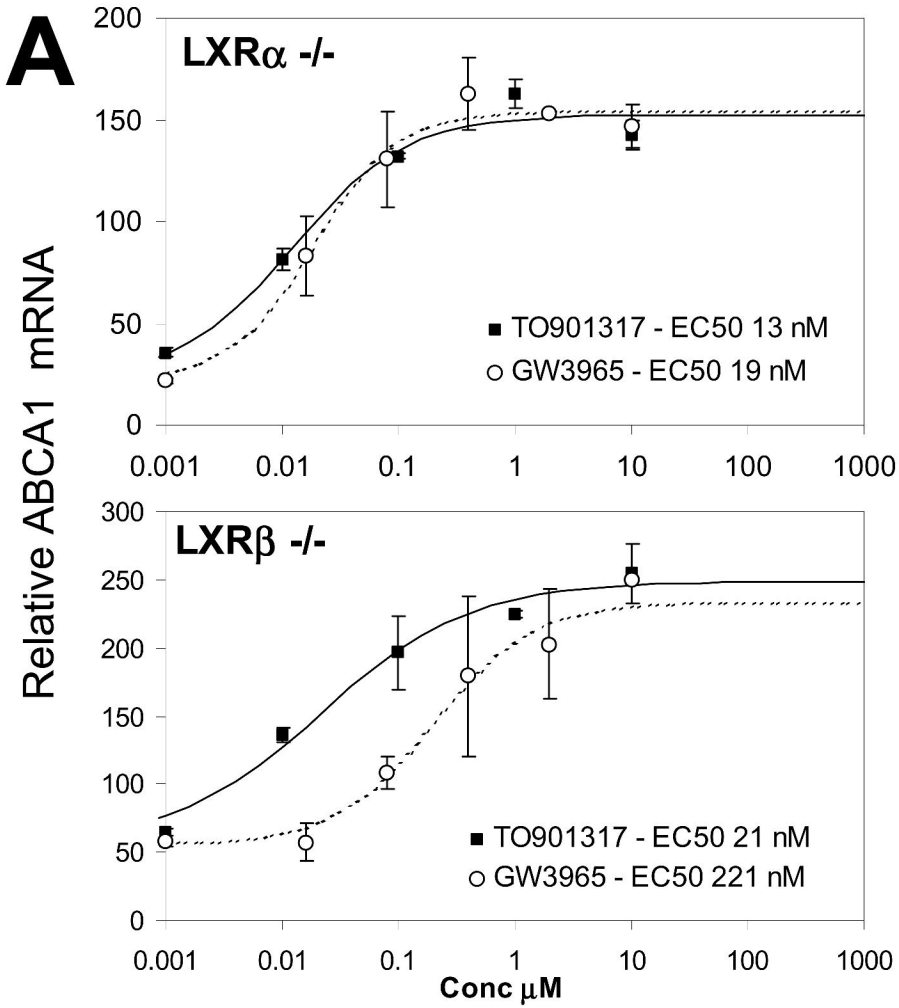
Table 2. Relative liver weight and lipids in mice treated with LXR ligands for 3 days.

Treatment	Dose	*n*	LW/BW mg/gm	TC mg/gm	TG mg/gm
LXRα KO Mice					
vehicle	-	6	52.0 ±1.7	4.5 ±0.22	8.3 ±0.49
TO901317	5 mg/kg	6	50.2 ±1.1	4.2 ±0.17	8.3 ±0.21
TO901317	50 mg/kg	6	55.5 ±1.1*	4.2 ±0.17	8.3 ±0.42
GW3965	10 mg/kg	6	52.4 ±1.0	4.5 ±0.22	9.0 ±0.37
GW3965	50 mg/kg	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 mg/kg	6	45.1 ±2.3	4.0 ±0.00	12.0 ±1.05
TO901317	50 mg/kg	6	46.6 ±3.4*	4.0 ±0.26	14.3 ±1.2
GW3965	10 mg/kg	6	42.8 ±2.9	4.0 ±0.00	14.8 ±1.6
GW3965	50 mg/kg	6	44.2 ±2.3	4.0 ±0.00	14.3 ±0.56

Data expressed as mean ± SEM. * $p < 0.05$, ** $p < .01$. n = 6 per group.
LW/BW = liver weight (mg) / body weight (gm).



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

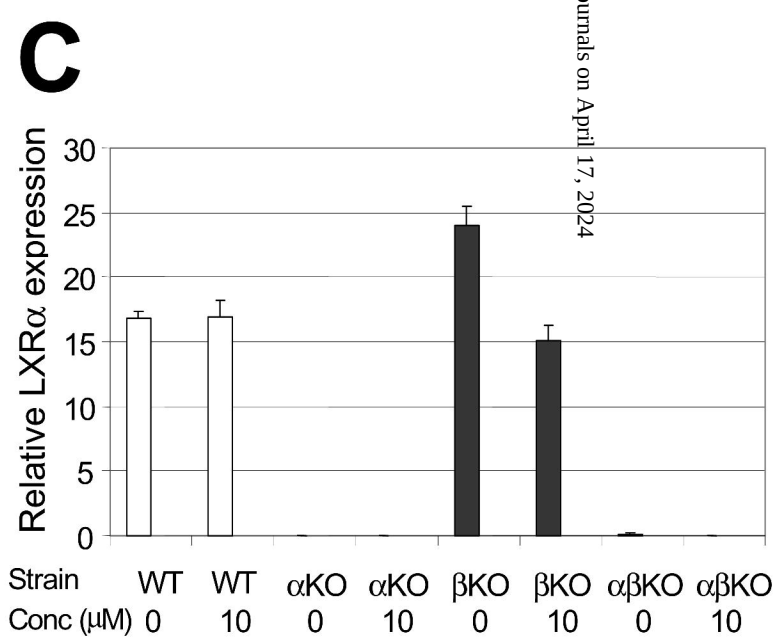


Figure 1

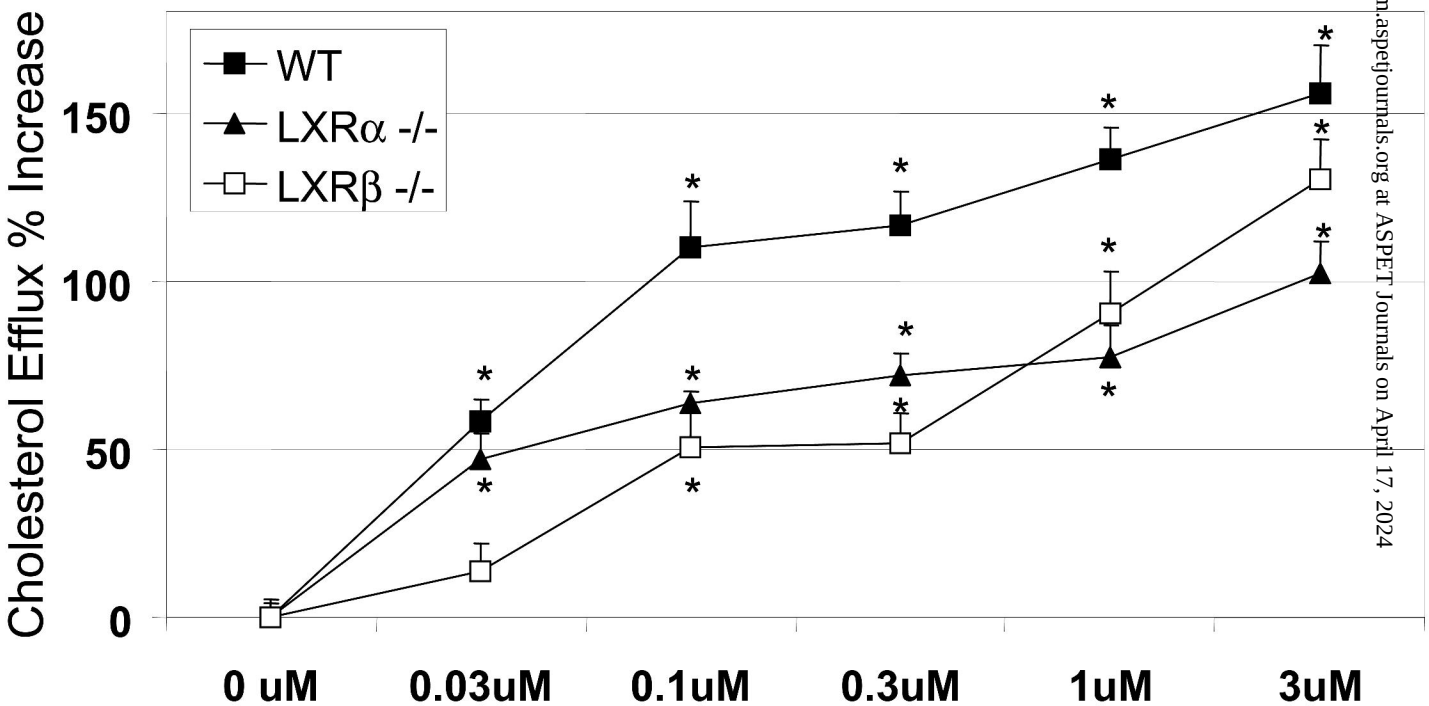


Figure 2

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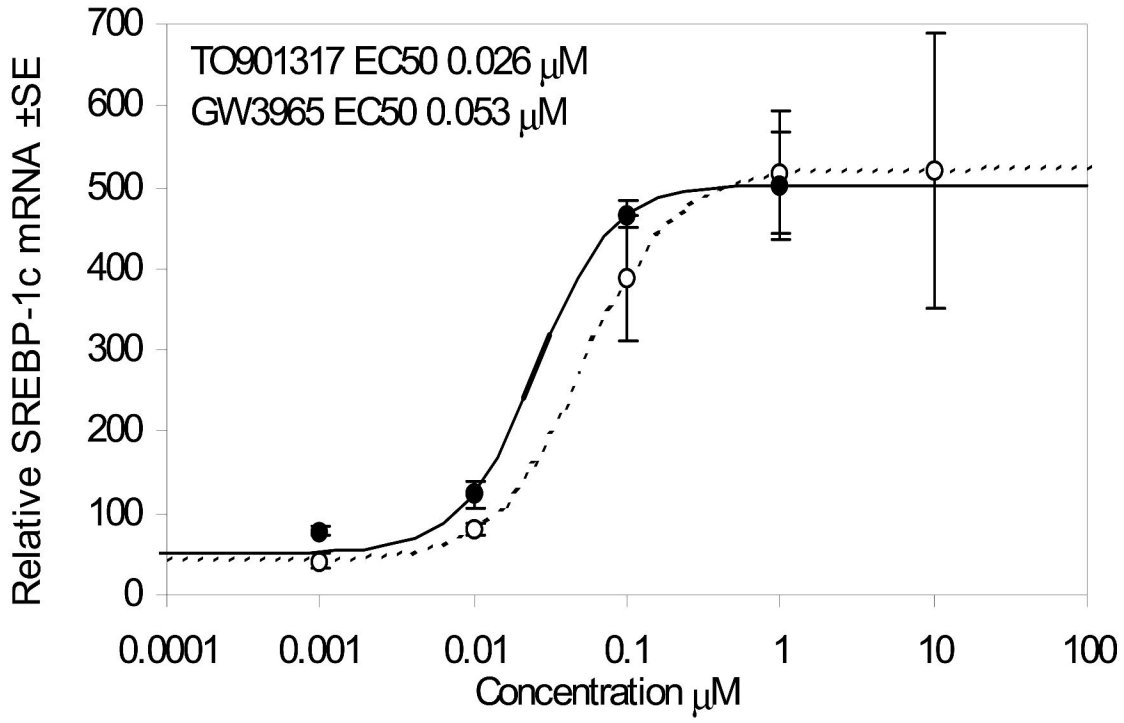
A



B

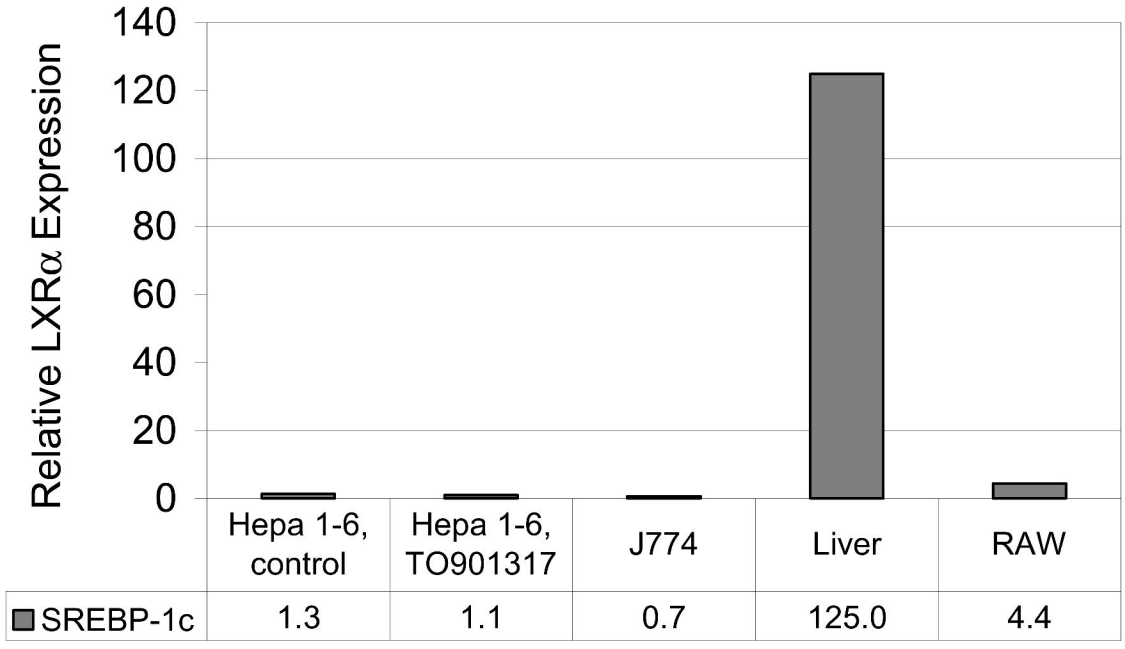


Figure 3

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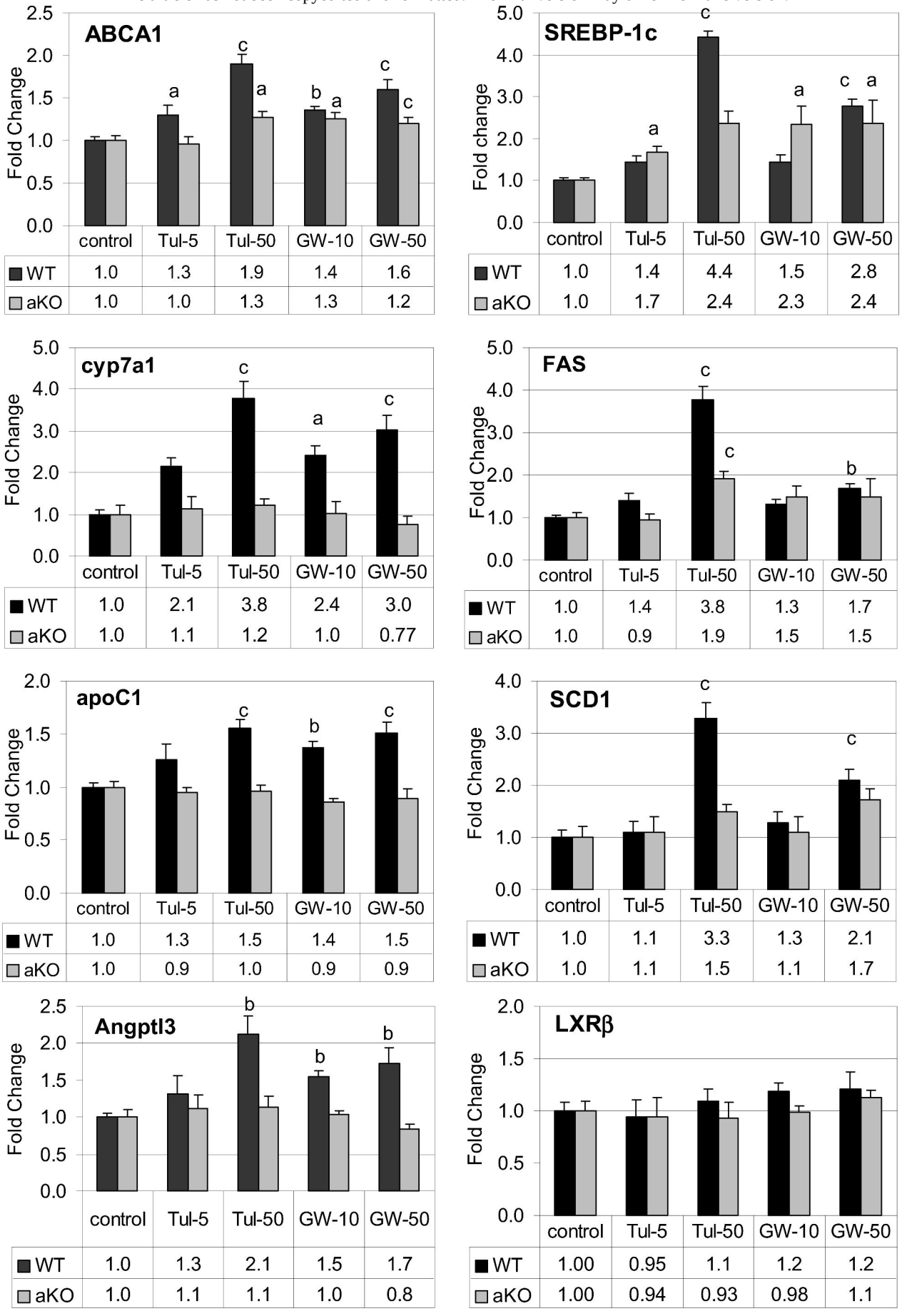

Figure 4

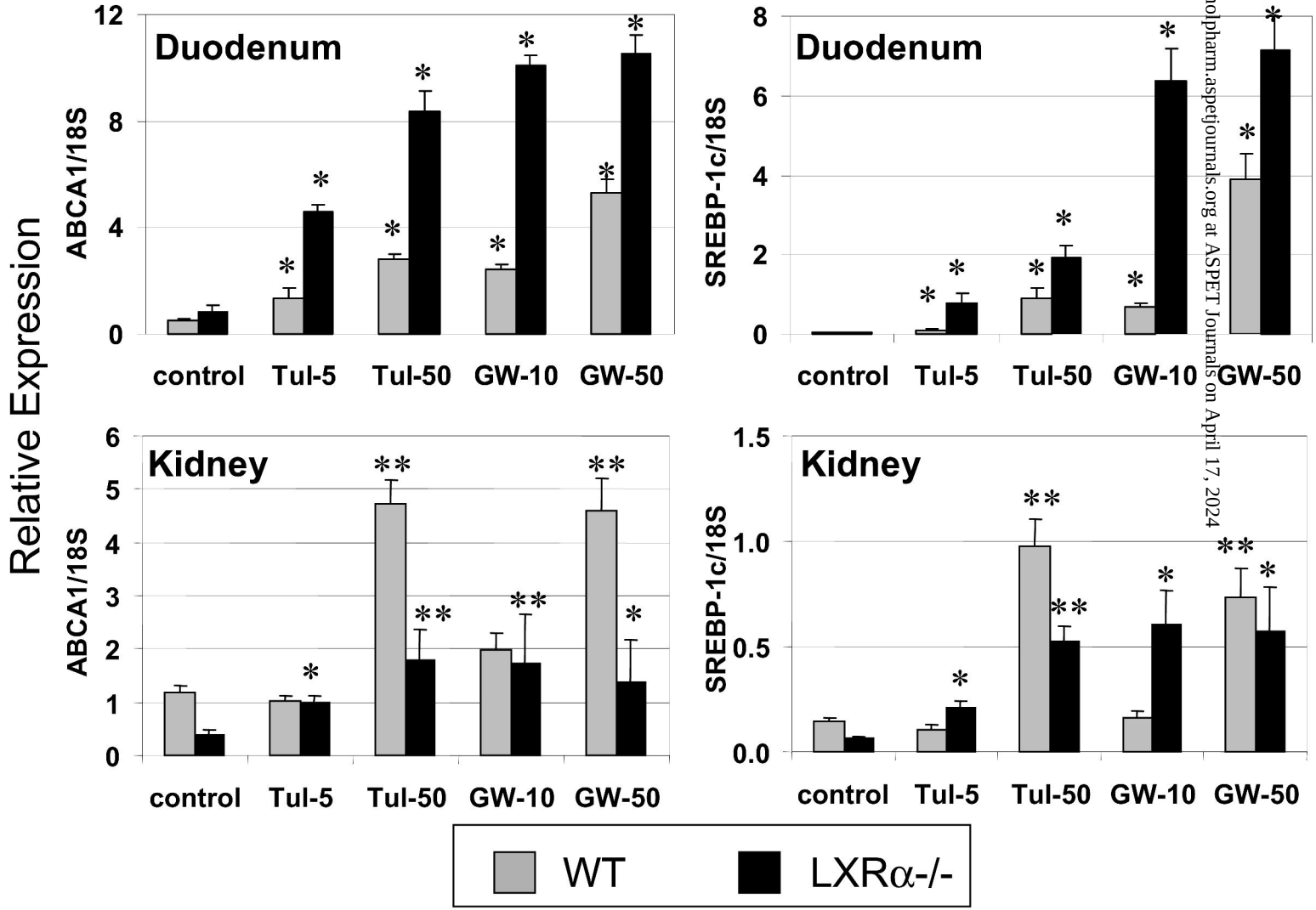

FIGURE 5

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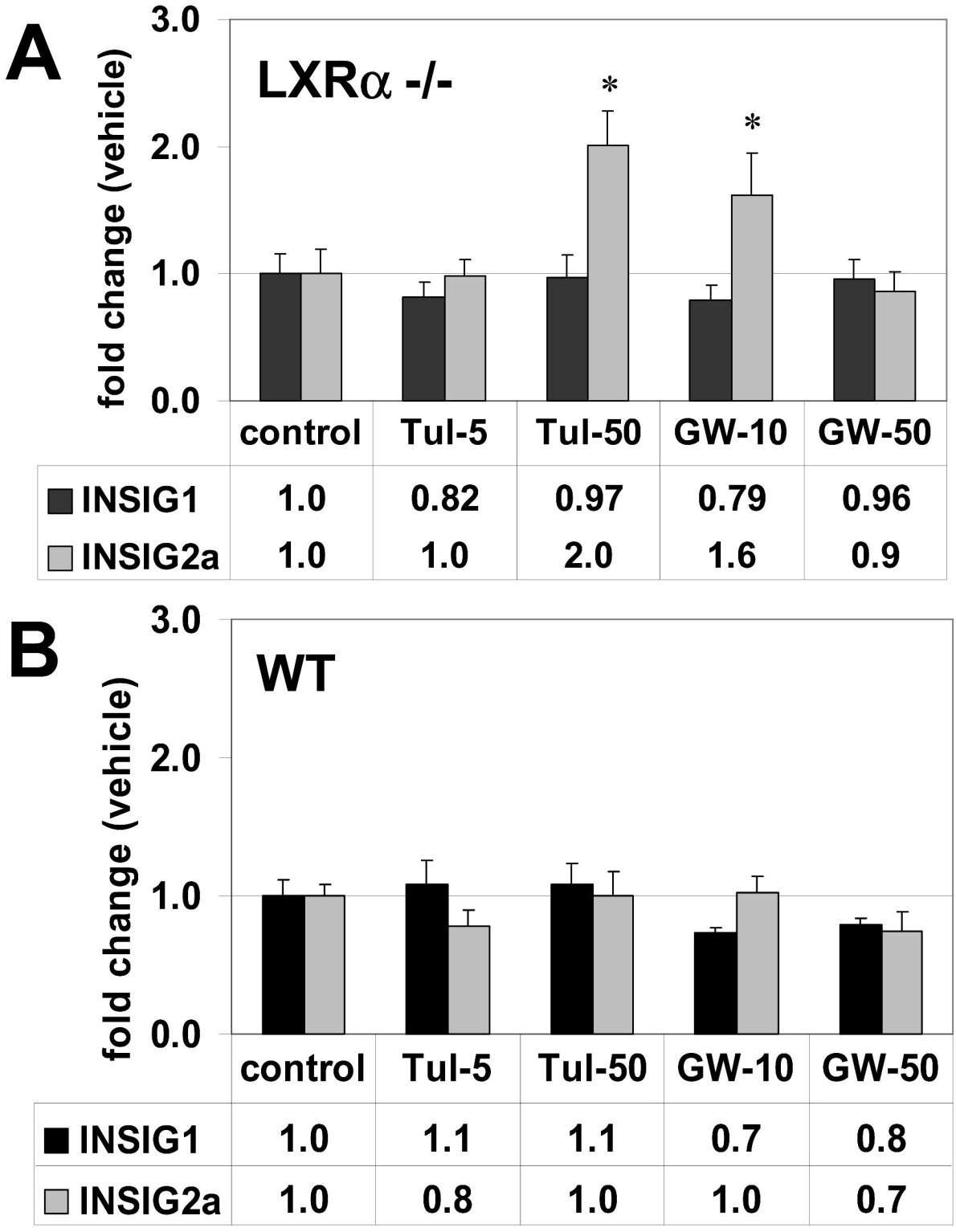

Figure 6