0026-895X/08/7303-891–899
MOLECULAR PHARMACOLOGY
Vol. 73, No. 3
40741/3305119
Mol Pharmacol 73:891–899, 2008
Printed in U.S.A.

Identification of Oxysterol 7α-Hydroxylase (*Cyp7b1*) as a Novel Retinoid-Related Orphan Receptor α (RORα) (NR1F1) Target Gene and a Functional Cross-Talk between RORα and Liver X Receptor (NR1H3)

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Received August 8, 2007; accepted November 30, 2007

ABSTRACT

The retinoid-related orphan receptors (RORs) and liver X receptors (LXRs) were postulated to have distinct functions. RORs play a role in tissue development and circadian rhythm, whereas LXRs are sterol sensors that affect lipid homeostasis. In this study, we revealed a novel function of RORα (NR1F1) in regulating the oxysterol 7α-hydroxylase (Cyp7b1), an enzyme critical for the homeostasis of cholesterol, bile acids, and oxysterols. The expression of *Cyp7b1* gene was suppressed in the RORα null (*ROR*$\alpha^{sg/sg}$) mice, suggesting RORα as a positive regulator of *Cyp7b1*. Promoter analysis established *Cyp7b1* as a transcriptional target of RORα, and transfection of RORα induced the expression of endogenous *Cyp7b1* in the liver. Interestingly, *Cyp7b1* regulation seemed to be RORα-specific, because RORγ had little effect. Reporter gene analysis showed that the activation of *Cyp7b1* gene promoter by RORα was suppressed by LXRα (NR1H3), whereas RORα inhibited both the constitutive and ligand-dependent activities of LXRα. The mutual suppression between RORα and LXR was supported by the in vivo observation that loss of RORα increased the expression of selected LXR target genes, leading to hepatic triglyceride accumulation. Likewise, mice deficient of LXR α and β isoforms showed activation of selected RORα target genes. Our results have revealed a novel role for RORα and a functional interplay between RORα and LXR in regulating *endo*- and xenobiotic genes, which may have broad implications in metabolic homeostasis.

Retinoid-related orphan receptors (RORs, or NR1F1–3), including the α, β, and γ isoforms, were isolated based on their homology to the retinoid receptors (Jetten et al., 2001; Jetten and Joo, 2006). Each of the ROR isoforms has distinct tissue distribution patterns (Carlberg et al., 1994). RORα is widely distributed, with its expression detectable in the cerebellar Purkinje cells, liver, thymus, skeletal muscle, skin, lung, and kidney (Hamilton et al., 1996; Steinmayr et al., 1998). In contrast, RORβ has a more tissue-specific distribution, expressing in the brain, retina and pineal gland (André et al., 1998a; Jetten et al., 2001). RORγ is highly enriched in the thymus, but its expression is also detectable in the kidney, liver, and muscle (Medvedev et al., 1996; Jetten et al., 2001). The functional ligands of RORs remain elusive. It has been suggested that cholesterol and its sulfonated derivatives might function as RORα ligands (Kallen et al., 2002). However, to our knowledge, none of those have been convinc-

This work was supported in part by National Institutes of Health grants CA107011 and ES014626 (to W.X.) and by the Intramural Research Program of the National Institute of Environmental Health Sciences, National Institutes of Health (to A.M.J.). H.G. is supported by postdoctoral fellowship PDF0503458 from the Susan G. Komen Breast Cancer Foundation. Normal human hepatocytes were obtained through the Liver Tissue Procurement and Distribution System, which was funded by National Institutes of Health grant N01-DK70004/HHSN267200700004C.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.107.040741.

ABBREVIATIONS: ROR, retinoid-related orphan receptor; RORE, retinoid-related orphan receptor response element; LXR, liver X receptor; kb, kilobase(s); tk, thymidine kinase; Pcp2, Purkinje cell protein 2; Luc, luciferase; WT, wild type; bp, base pair(s); PCR, polymerase chain reaction; PEI, polyethylenimine; MEM, minimal essential medium; β-Gal, β-galactosidase; DMSO, dimethyl sulfoxide; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; LXRE, liver X receptor response element; SCR1, steroid receptor coactivator 1; VP, viral protein 16; DKO, double knockout; UAS, upstream activation sequence.

ingly demonstrated to be physiological ROR agonists. RORs regulate gene expression by binding as monomers to the ROR response elements (ROREs) found in target gene promoters. A typical RORE is composed of a consensus AGGTCA half-site preceded by an A/T-rich region (Giguère et al., 1994). RORα has also been shown to bind DNA as homodimers (Harding et al., 1997).

Subsequent functional analyses, mainly through the creation and characterization of ROR-deficient mice, have revealed diverse physiological function of RORs. ROR$\alpha^{-/-}$ mice had cerebellar ataxia, a behavioral phenotype also observed in the *Staggerer* (*sg/sg*) mutant mice, which contained a natural deletion in the ligand binding domain of the RORα gene as a result of a frame shift (Hamilton et al., 1996; Steinmayr et al., 1998). The *sg/sg* mice exhibited vascular dysfunction, muscular irregularities, osteoporosis, and immuno abnormalities (Jarvis et al., 2002). The *sg/sg* mice developed severe atherosclerosis and hypo-α-lipoproteinemia when maintained on an atherogenic diet (Mamontova et al., 1998). RORβ is thought to be involved in the processing of sensory information, because ROR$\beta^{-/-}$ mice showed significant phenotypes in circadian behaviors and retinal degeneration (André et al., 1998b). ROR$\gamma^{-/-}$ mice lacked all lymph nodes and Peyer's patches, and they had reduced numbers of thymocytes (Kurebayashi et al., 2000), suggesting that RORγ plays an essential role in lymphoid organogenesis and thymopoiesis. Although both ROR α and γ are expressed in the liver, their hepatic function is largely unknown.

Both liver X receptor (LXR) α and β are nuclear receptors that can be activated by the endogenous oxysterols, such as 22(*R*)-hydroxycholesterol; and by synthetic agonists, such as T0901317 (TO1317) (Schultz et al., 2000) and GW3965 (Collins et al., 2002). LXRs exhibit diverse functions, ranging from cholesterol efflux to lipogenesis and anti-inflammation (Repa and Mangelsdorf al., 2002; Zelcer and Tontonoz, 2006). LXRs have also been explored as therapeutic targets for atherosclerosis (Tontonoz and Mangelsdorf, 2003), diabetes, and Alzheimer's disease (Zelcer et al., 2007) in animal models. We have recently identified several novel LXR target genes. These include the bile acid-detoxifying sulfotransferase *Sult2a9/2a1* (Uppal et al., 2007), estrogen sulfotransferase (*Est*/*Sult1e1*) (Gong et al., 2007), and fatty acid transporter *Cd36* (J. Zhou and W. Xie, unpublished data). We showed that activation of *Sult2a9/2a1* by LXR was associated with increased bile acid detoxification and alleviation of cholestasis (Uppal et al., 2007). In the same study, the expression of *Cyp7b1* was found to be suppressed in LXR-activated mice, but the mechanism for this suppression is unknown (Uppal et al., 2007). Activation of *Est*/*Sult1e1* by LXR led to functional estrogen deprivation and inhibition of estrogen-dependent breast cancer growth (Gong et al., 2007). More recently, we showed that *Cd36* is a LXR target gene and an intact expression of *Cd36* plays an important role in the steatotic effect of LXR agonists (J. Zhou and W. Xie, unpublished data).

In this report, we show that RORα positively and directly regulates the expression of *Cyp7b1*. In addition, we have provided evidence for a functional cross-talk between RORα and LXR in regulating *Cyp7b1* and other target genes controlled by these two receptors.

Materials and Methods

Animals. Heterozygous C57BL/6 *staggerer* (*ROR*$\alpha^{+/sg}$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The *staggerer* (*ROR*$\alpha^{sg/sg}$) mice, a natural mutant mouse strain, contain a 6.5-kb deletion in the *ROR*α gene, resulting in a functional knockout of *ROR*α. *ROR*$\alpha^{sg/sg}$ mice of 8 to 10 weeks of age were used. All animal protocols followed the guidelines outlined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and they were approved by the Institutional Animal Care and Use Committee at the National Institute of Environmental Health Sciences.

Plasmid Constructs and Cell Transfection. The thymidine kinase (*tk*)-Purkinje cell protein 2 (*Pcp2*)/RORE-Luc and *tk-Cyp7b1*/RORE and its mutant variant were generated by insertion of corresponding annealed oligonucleotides into the tk-Luc vector. Three copies of the following response elements were used: *Pcp2*/RORE, 5′-GTTATAGTAACTGGGTCAGGGGACT-3′; *Cyp7b1*/RORE WT, 5′-TATTTTATGCAGGTCAGTGG-3′; and *Cyp7b1*/RORE Mutant, 5′-TATTTTATGCACCTCAGTGG-3′. The 5′ regulatory region (−3500 bp to +125 bp) of mouse *Cyp7b1* was amplified by PCR using mouse liver genomic DNA as the PCR template and the following oligonucleotides: *Cyp7b1* −3500, 5′-TTTGTGAACTTGGCATGACAT-3′ and *Cyp7b1* +125, 5′-TCCCGACGAGCTGGCGGCTC-3′. The PCR-amplified sequence was cloned into the pGL3-basic vector (Promega, Madison, WI). Site-directed mutagenesis was performed by PCR overextension method, and it was confirmed by DNA sequencing (Xie et al., 2000). HepG2 cells were transfected in 48-well plates using the polyethylenimine (PEI) polymer transfection agent (Mu et al., 2005, 2006). For each three-well transfection, the PEI polymer complexes were formed by incubating 0.4 μg of nuclear receptor expression vector or the CMX empty vector, 0.8 μg of reporter gene, 0.3 μg of CMX β-Gal plasmid, and 10 μl of PEI at room temperature for 10 min in a total volume of 300 μl of serum-free minimum essential medium (MEM). The complexes were then diluted with additional 300 μl of serum-free MEM, they were mixed, and then they were applied at 200 μl/well. After 12 h of incubation, the transfection medium was replaced with MEM supplemented with 10% fetal bovine serum and laced with DMSO solvent or drugs. The concentration for all drugs used in transfections is 10 μM. Cells were lysed 24 h later and assayed for luciferase and β-galactosidase activities. The transfection efficiency was normalized against the β-Gal activities. All transfections were performed in triplicate.

Human and Mouse Primary Hepatocyte Preparation and Transfection. Human livers were obtained through the Liver Tissue Procurement and Distribution System (Pittsburgh, PA), and hepatocytes were isolated by three-step collagenase perfusion (Strom et al., 1999). Mouse primary hepatocytes and stellate cells were isolated from 8-week-old female C57BL/6J mice by collagenase perfusion and differential centrifugation (Monga et al., 2005; Mu et al., 2005). Cells were plated on six-well plates and maintained in hepatocyte maintenance medium from Lonza Walkerville, Inc. (Walkersville, MD) supplemented with dexamethasone (10^{-7} M), insulin (10^{-7} M), and gentamicin (50 μg/ml). Mouse primary hepatocytes on each well were transfected with 4 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 24 h of incubation, cells were replaced with fresh hepatocyte maintenance medium for 16 h before RNA harvesting and real-time RT-PCR analysis.

Real-Time RT-PCR Analysis. Total RNA was extracted with TRIzol Reagent (Invitrogen). The cDNA was synthesized from 1.6 μg of total RNA by Superscript3 (Invitrogen), according to the manufacturer's protocol. Aliquots of cDNA were amplified on ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the SYBR Green PCR master mix (Applied Biosystems). The mRNA expression was normalized against the cyclophilin B expression (Zhou et al., 2006).

Electrophoretic Mobility Shift Assay. Receptor proteins were prepared using the transcription/translation in vitro transcription

and translation system (Promega, Madison, WI). The binding reactions were performed as described previously (Saini et al., 2004, 2005). Protein-DNA complexes were resolved by electrophoresis through 5% polyacrylamide gel in 0.5× Tris borate-EDTA at 4°C for 1 to 3 h. For oligonucleotide competition experiments, unlabeled oligonucleotides were added to the reaction at 100-fold molar excess to the radio labeled probes. Electrophoretic mobility shift assay (EMSA) probe sequences are labeled in the figures.

Hydrodynamic Liver Transfection. Six-week-old CD-1 female mice purchased from Charles River Laboratories, Inc. (Wilmington, MA) were each injected with 5 μg of plasmid DNA in 1.6 ml of saline via tail vein (Zhou et al., 2006). Mice were sacrificed 6 h after the injection, and their livers were harvested. Total RNA was extracted and subjected to real-time RT-PCR analysis.

Chromatin Immunoprecipitation Assay. Three-week-old C57BL/6J male mice were sacrificed, and 30 mg of liver tissues from each mouse was subjected to ChIP assay as described previously (Zhou et al., 2006). Tissue lysates were incubated overnight with 1 μg of anti-RORα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C. Parallel samples were incubated with normal IgG as a negative control. The following PCR primers were used: *Cyp7b1* −1026, 5′-AACCTTAGGAAGGAGCCCATGAA-3′; *Cyp7b1* −902, 5′-TGATGAATACTCCATGTGTCAATGAGA-3′; *Cyp7b1* −2900, 5′-GTTTCAAAATAATACATTCAGATCTT-3′; and *Cyp7b1* −2776, 5′-AACAGGTAAAAGACTGATGGACAGGC-3′. ChIP assay using the SRC1 antibody was performed on primary mouse hepatocytes. In this experiment, cells were treated with DMSO or TO1317 (10 μM) for 24 h before formaldehyde cross-linking. Cross-linked DNA was extracted from cells, and ChIP assay was performed using an anti-SRC1 antibody (Santa Cruz Biotechnology, Inc.) The final DNA extracts were amplified by PCR using primer pairs encompassing the *Cyp7b1*/RORE, a distal control *Cyp7b1* promoter region, or the *Est*/LXRE that we have described previously (Gong et al., 2007).

Measurement of Circulating and Tissue Lipid Levels. To measure circulating lipid levels, mice were fasted for 16 h before sacrificing and blood collection. The plasma levels of triglycerides and cholesterol were measured by using assay kits from Stanbio Laboratory (Boerne, TX). To measure liver lipids, tissues were homogenized, and lipids were extracted as described previously (Zhou et al., 2006). The lipid pellets were then dissolved in a mixture of 60 μl of *tert*-butanol and 40 μl of Triton X-100/methanol (2:1). Triglyceride and cholesterol levels were then measured using the Stanbio Laboratory assay kits.

Results

Mice Deficient of RORα Had Decreased Expression of *Cyp7b1* in the Liver. RORα is expressed in the liver, but its hepatic function is largely unknown. To understand the function of RORα in the liver, we compared the hepatic gene expression between the wild-type (WT) and *RORα*$^{sg/sg}$ male mice by microarray analysis using the Agilent mouse 20,000-oligo chips (Agilent Technologies, Palo Alto, CA). The *RORα*$^{sg/sg}$ mice contain a 6.5-kb deletion in the *RORα* gene, resulting in a functional knockout of the *RORα* gene (Hamilton et al., 1996; Steinmayr et al., 1998). We initially observed a 4.2-fold decrease in the expression of *Cyp7b1* in the male *RORα*$^{sg/sg}$ mice (Kang et al., 2007). The microarray results were confirmed and extended by real-time RT-PCR analysis. As shown in Fig. 1A, the hepatic expression of *Cyp7b1* was significantly decreased in both male and female *RORα*$^{sg/sg}$ mice. WT female mice had lower basal expression of *Cyp7b1*, consistent with previous reports (Li-Hawkins et al., 2000; Uppal et al., 2007). The down-regulation of *Cyp7b1* in *RORα*$^{sg/sg}$ male mice was also confirmed by Northern blot analysis (Fig. 1B).

To gain an insight into the hepatic function of RORα and its potential regulation of *Cyp7b1*, we evaluated the expression of *RORα* in isolated liver cell types, including the parenchymal hepatocytes and the mesenchymal/nonparenchymal stellate cells. As shown in Fig. 1C, *RORα* is expressed in the stellate cells at a reduced (approximately 50% of the hepatocytes) but significant level. The expression of *Cyp7b1* and *LXRα* was substantially lower (less than 10% of the hepatocytes) in the stellate cells. The identity of the stellate cells was confirmed by the near absence of *HNF4α* and *Cyp3a11*, two hepatic differentiation markers; and an enriched expression of *Desmin* and *glial fibrillary acidic protein*, two known stellate cell markers (Geerts et al., 2001; Morini et al., 2005).

***Cyp7b1* Is a Transcriptional Target of RORα.** The down-regulation of *Cyp7b1* in *RORα*$^{sg/sg}$ mice suggested that this Cyp isoform might be under the positive control of RORα. To determine whether *Cyp7b1* is a transcriptional target of RORα, we cloned and analyzed the 3.5-kb 5′ flanking region of the mouse *Cyp7b1* gene. As shown in Fig. 2A, this 3.5-kb *Cyp7b1* promoter was responsive to RORα in transient transfection and reporter gene assay. The regulation seemed to be ROR isoform-specific, because cotransfection of RORγ had little effect on the same promoter (Fig. 2A). Deletion analysis localized the RORα-responsive region to −1017 bp to −520 bp (Fig. 2A). Inspection of this region revealed a putative RORE characterized by an AGGTCA

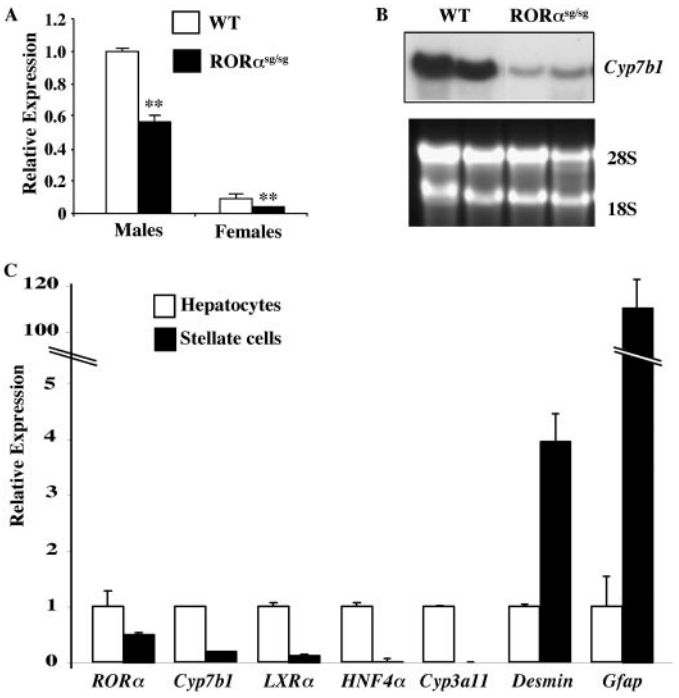

Fig. 1. Mice deficient of RORα had decreased expression of *Cyp7b1* in the liver. A, hepatic expression of *Cyp7b1* in the wild-type and *RORα*$^{sg/sg}$ mice was measured by real-time RT-PCR analysis. Results represent the averages and standard deviation from three mice per group. **, $P < 0.01$, compare to the same sex WT control mice. B, expression of *Cyp7b1* in the WT and *RORα*$^{sg/sg}$ male mice was measured by Northern blot analysis. Ethidium bromide staining of the 28S and 18S rRNA serves as a loading control. Lanes represent RNA samples pooled from three individual mice of each genotype. C, gene expression in isolated primary mouse hepatocytes and stellate cells as measured by real-time RT-PCR. Results represent averages and standard deviation from triplicate assays. The expression level of individual genes in the hepatocytes is arbitrarily set at 1.

half-site flanked by adjacent A/T-rich six nucleotides (Fig. 2B). EMSA was performed to determine the binding of RORs to *Cyp7b1*/RORE using synthesized receptor proteins and ^{32}P-labeled oligonucleotide probe. RORα bound to *Cyp7b1*/RORE efficiently (Fig. 2B), but not to the radiolabeled mutant *Cyp7b1*/RORE (data not shown). This binding was specific, because strong competition of binding was achieved by excess unlabeled wild-type *Cyp7b1*/RORE and *ApoA-V*/RORE, but not by the mutant *Cyp7b1*/RORE (Fig. 2B). *ApoA-V*/RORE is a prototypic RORE derived from the *ApoA-V* gene promoter (Lind et al., 2005). It is noteworthy that RORγ could also bind to *Cyp7b1*/RORE, but the binding was substantially weaker compared with that of RORα (Fig. 2B), consistent with the lack of *Cyp7b1* promoter activation by RORγ (Fig. 2A). The binding of radiolabeled *ApoA-V*/RORE by RORα and RORγ was included as positive controls (Fig.

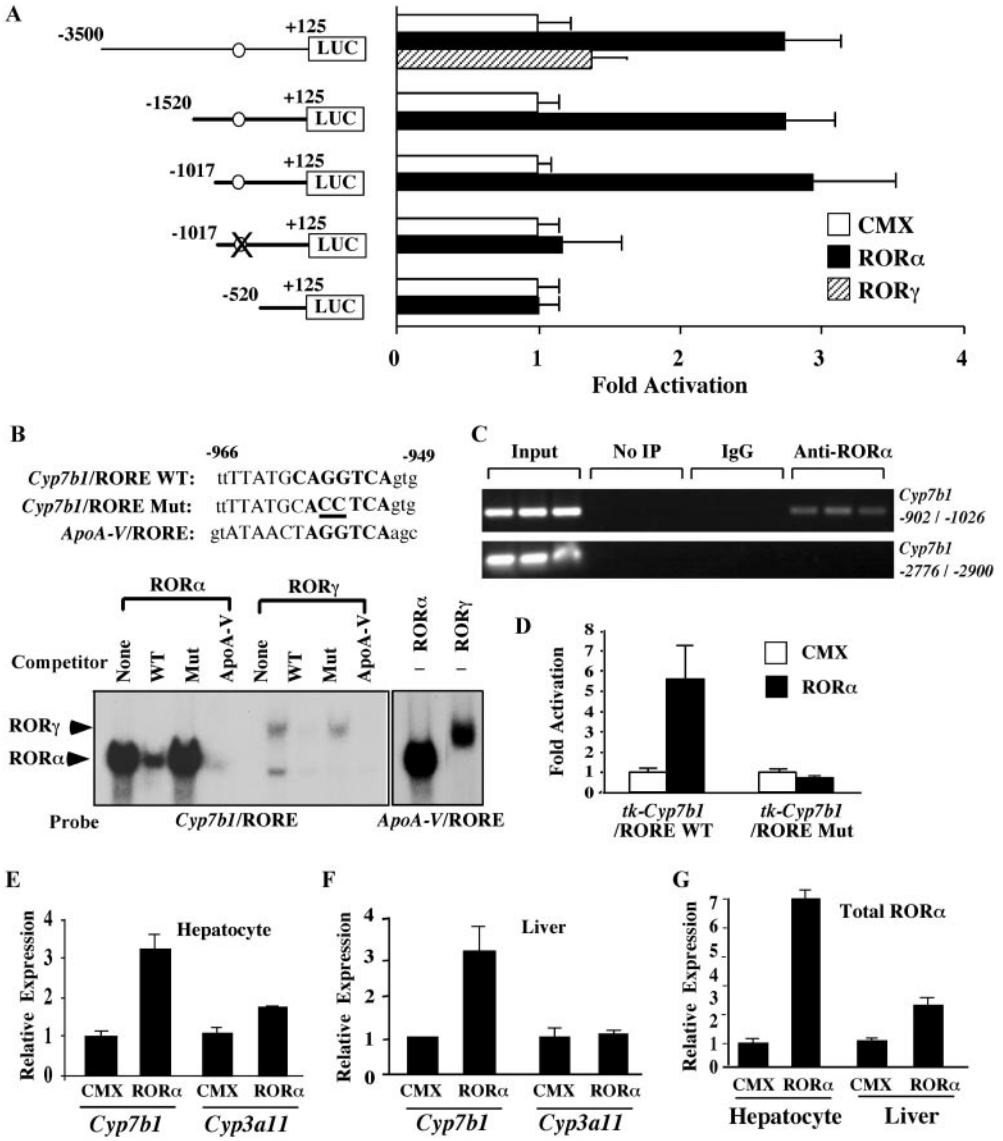

Fig. 2. Regulation of the mouse Cyp7b1 gene promoter by RORα. A, reporter genes containing various lengths of the mouse Cyp7b1 gene promoter were transfected into HepG2 cells in the presence of expression vectors for RORα or RORγ. The position of the putative RORE is labeled. The transfection efficiency was normalized against the β-Gal activity from the cotransfected CMX-β-Gal vector. Normalized luciferase activity in cells transfected with empty expression vector (CMX) was arbitrarily set at 1. Results shown represent the averages and standard deviation from triplicate assays. B, partial DNA sequence of the mouse Cyp7b1 gene promoter. The putative Cyp7b1/RORE sequence is in uppercase letters, and the mutated nucleotides are underlined. The sequence of ApoA-V/RORE is also shown. The binding of RORα and RORγ proteins to ^{32}P-labeled Cyp7b1/RORE was demonstrated by EMSA. In the competition lanes, unlabeled probes were present in 100-fold molar excess relative to the radiolabeled probe. The binding of radiolabeled ApoA-V/RORE by RORα and RORγ was included as positive controls. C, ChIP assay to demonstrate the recruitment of RORα onto the Cyp7b1 gene promoter. Formaldehyde cross-linked DNA was extracted from mouse liver tissues, and ChIP assay was performed using an antibody against RORα or control IgG. The final DNA extracts were amplified by PCR using the primer pairs encompassing either the Cyp7b1/RORE region ($-$902 to $-$1026 bp) or a distal control region ($-$2776 bp to $-$2900 bp) of the Cyp7b1 gene promoter. Lanes represent individual mice. D, the synthetic tk-Cyp7b1/RORE reporter or its mutant variant was transfected into HepG2 cells in the presence of RORα. E, wild-type mouse primary hepatocytes were transiently transfected with empty vector (CMX) or expression vectors for RORα. Total RNA was isolated 40 h after transfection and subjected to real-time RT-PCR analysis to detect the expression of endogenous Cyp7b1 and Cyp3a11 (a nontarget gene control). F, wild-type mouse livers were transfected with empty vector (CMX) or expression vectors for RORα by a hydrodynamic gene deliver method. Mice were sacrificed 6 h after transfection. Liver total RNA was extracted and subjected to real-time RT-PCR analysis to detect the expression of endogenous Cyp7b1 and Cyp3a11. G, expression of transduced RORα in hepatocytes (E) and mouse livers (F) was confirmed by real-time RT-PCR using primers designed to detect total RORα. Results shown in E to G represent averages and standard deviation of three independent experiments.

2B). EMSA analysis showed *Cyp7b1*/RORE was not bound by LXRα (data not shown).

ChIP assay was used to determine whether the endogenous RORα can be recruited onto the *Cyp7b1* promoter in vivo. As shown in Fig. 2C, immunoprecipitation on liver DNA lysate with an anti-RORα antibody revealed the specific recruitment of RORα to a 100-bp sequence encompassing the *Cyp7b1*/RORE. In contrast, no amplification was observed when the same precipitate was amplified using a pair of control primers designed for a distal region approximately 2 kb upstream of *Cyp7b1*/RORE (Fig. 2C).

Transfection-based assays were used to determine whether RORα can transactivate through *Cyp7b1*/RORE. Synthetic luciferase reporter genes, containing three copies of the wild-type or mutant *Cyp7b1*/RORE upstream of a minimal tk promoter (*tk-Cyp7b1*/RORE), were constructed and transfected into HepG2 cells together with the expression vector for RORα. As shown in Fig. 2D, cotransfection with RORα activated the *tk-Cyp7b1*/RORE reporter gene, and this activation was abolished when the *Cyp7b1*/RORE was mutated. This RORE is also required for the activation of the natural 1-kb *Cyp7b1* promoter, because mutation of the RORE in this context also abolished the transactivation by RORα (Fig. 2A).

Finally, we showed that overexpression of RORα in primary hepatocyte cultures by transient transfection (Fig. 2E) or in wild-type mouse livers by a hydrodynamic liver transfection method (Fig. 2F) induced the mRNA expression of the endogenous *Cyp7b1*, but not the control *Cyp3a11* gene. The overexpression of RORα in transfected hepatocytes or livers was confirmed by real-time RT-PCR analysis (Fig. 2G). Taken together, our results strongly suggest that the mouse *Cyp7b1* gene is a transcriptional target of RORα, and this regulation is mediated by *Cyp7b1*/RORE.

The Activation of *Cyp7b1* Promoter by RORα Was Negatively Regulated by LXRα, and RORα and LXRα Were Mutually Suppressive in Reporter Gene Assays. We have previously shown that the expression of *Cyp7b1* was suppressed in LXR-activated mice (Uppal et al., 2007). *CYP7B1* suppression was also seen in primary human hepatocytes treated with LXR agonists (Fig. 3A). However, the mechanism for LXR-mediated *Cyp7b1* suppression is unknown. Having established RORα as a positive *Cyp7b1* regulator, we went on to examine whether LXR suppresses *Cyp7b1* by inhibiting RORα activity. As shown in Fig. 3B, activation of the 1-kb *Cyp7b1* promoter (*pGL-Cyp7b1*) by RORα was suppressed by cotransfection of LXRα, even in the absence of LXR agonists. The inhibitory effect of LXRα was enhanced by the LXR agonist TO1317. Transfection of LXRα alone, even in the absence of LXR agonists, inhibited *Cyp7b1* promoter activity. The inhibitory effect of LXRα, in the presence or absence of TO1317, was largely abolished when the RORE was mutated (Fig. 3B), suggesting that the inhibition was mediated by RORα. The suppression of RORα by LXRα was also seen when a synthetic *tk-Pcp2*/RORE-Luc reporter was used. This reporter contains three copies of RORE derived from the *Pcp2* gene (Matsui, 1997). As shown in Fig. 3C, the activation of *tk-Pcp2*/RORE by RORα was inhibited by ligand-free LXRα, and this inhibition was enhanced by TO1317 or 22(*R*)-hydroxycholesterol, another LXR agonist. LXRα alone had little effect on the basal activity of *tk-Pcp2*/RORE reporter.

It is noteworthy that the LXRα activity was reciprocally suppressed by RORα. As shown in Fig. 3D, cotransfection with LXRα activated the LXR-responsive *tk-MTV* reporter gene as expected (Willy et al., 1995). However, this activation was inhibited by cotransfection of an RORα in a dose-dependent manner, whereas transfection of RORα alone had little effect on *tk-MTV* reporter activity. The ligand-dependent activation of *tk-MTV* by LXRα was also inhibited by the cotransfection of RORα (Fig. 3E). These results suggest that RORα and LXRα are mutually suppressive.

The mutual suppression was also observed when the chimeric Gal4-LXRα and Gal4-RORα receptors were transfected, together with the Gal4-responsive tk-UAS reporter gene. The constitutive activity of Gal4-LXRα was inhibited by the wild type RORα (CMX-RORα) in a dose-dependent manner (Fig. 3F, left), whereas the constitutive activity of Gal4-RORα was inhibited by the wild-type LXRα (CMX-LXRα) in the absence of a ligand (Fig. 3F, right). RORα is known to interact with nuclear receptor coactivators without an exogenously added ligand (Delerive et al., 2002). We showed that LXRα also exhibited ligand-independent interaction with the nuclear receptor coactivator SRC1 as revealed by a mammalian two-hybrid assay, in which the VP fusion receptor of LXRα (VP-LXRα) was cotransfected with Gal4-SRC1 and tk-UAS-Luc (Fig. 3G). Moreover, the VP-LXRα-SRC1 interaction was inhibited by cotransfection of the wild-type RORα (Fig. 3G). ChIP assay on primary mouse hepatocytes showed that SRC1 was constitutively recruited onto *Cyp7b1*/RORE, and this recruitment was decreased in the presence of TO1317 (Fig. 3H). In contrast, the recruitment of SRC1 onto *Est*/LXRE was increased by the TO1317 treatment (Fig. 3H). *Est*/LXRE is a DR-4 type LXR response element found in the mouse *Est* gene promoter (Gong et al., 2007), and *Est* is a gene reciprocally activated in the RORα null mice (Fig. 4). The constitutive recruitment of SRC1 onto *Est*/LXRE was detectable when more PCR template was added or when the PCR cycle number was increased (data not shown). The ligand-independent recruitment of coactivator may account for the constitutive activities of both receptors, and coactivator competition may represent a plausible mechanism for the mutual suppression of transcriptional activity between these two receptors.

Reciprocal and Selective Activation of Target Gene Expression in Mice Deficient of RORα and LXRs. The potential functional cross-talk between RORα and LXR was further investigated in vivo. For this purpose, we measured the expression of LXR target genes and ROR target genes in the *RORα*$^{sg/sg}$ and LXR double knockout (DKO) mice (Peet et al., 1998), respectively. As shown in Fig. 4A, among LXR target genes, the expression of *Est* (Gong et al., 2007), *Sult2a9* (Uppal et al., 2007), *Cd36* (J. Zhou and W. Xie, unpublished data), lipoprotein lipase (Zhang et al., 2001), aldo-keto reductase 1d1 (*Akr1d1*) (Volle et al., 2004), scavenger receptor BI (*SR-BI*) (Malerød et al., 2002), and acetyl CoA carboxylase 1 (*Acc-1*) was significantly induced, whereas the expression of *Srebp-1c* was significantly suppressed in female *RORα*$^{sg/sg}$ mice. When the male *RORα*$^{sg/sg}$ mice were analyzed, the activation of *Est*, *Cd36*, lipoprotein lipase, and *SR-BI*, but not *Sult2a9/2a1*, *Akr1d1*, and *Acc-1*, remained significant (Fig. 4B). The lack of *Sult2a9* activation in male mice may be due to its nearly undetectable basal expression in this sex. Other gender differences include the male-specific activation of fatty acid synthase (*Fas*) and *Cyp7a1* and suppression of stearoyl CoA desaturase-1 (*Scd-1*). The suppres-

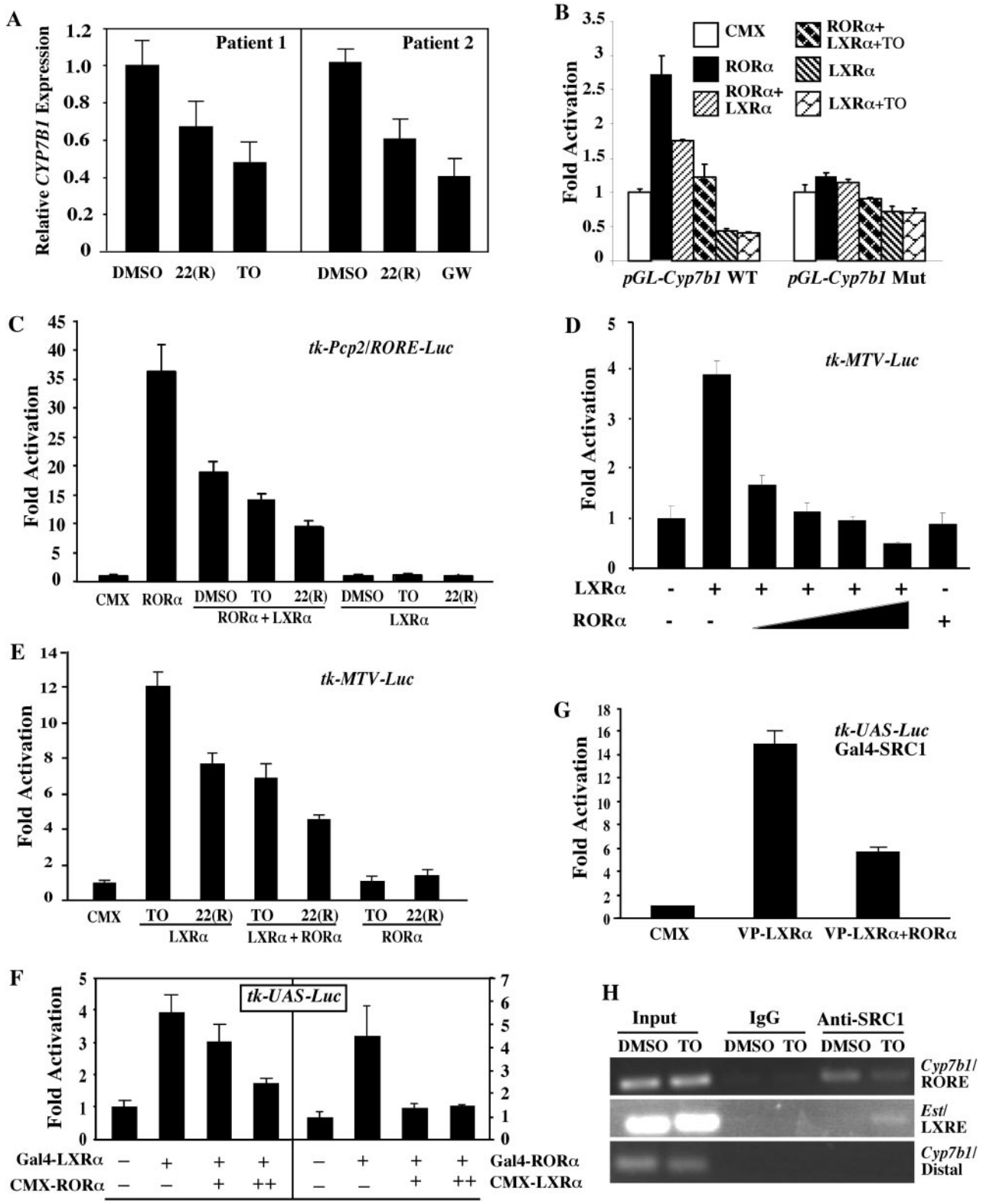

Fig. 3. RORα and LXRα were mutually suppressive in reporter gene assays. A, treatment with LXR agonists (10 μM each) inhibited the expression of *CYP7B1* in primary human hepatocytes as measured by real-time PCR analysis. Patients 1 and 2 are a 41-year-old white female and a 44-year-old white male, respectively. TO, TO1317; 22(*R*), 22(*R*)-hydroxycholesterol; and GW, GW3965. B to E, *pGL-Cyp7b1-Luc* or its RORE mutant variant (B), *tk-Pcp2*/RORE-Luc (C), and *tk-MTV*-Luc (D and E) reporter genes were transiently transfected into HepG2 cells in the presence of expression vectors for indicated receptors or their combinations. Where applicable, transfected cells were treated with DMSO or indicated drugs for 24 h before luciferase assay. Results shown are -fold induction over CMX vector control, and they represent the averages and standard deviation from triplicate assays. Drug concentration is 10 μM. F, HepG2 cells were cotransfected with *tk-UAS* and the indicated receptors or their combinations. Results shown are -fold induction over reporter alone control, and they represent the averages and standard deviation from triplicate assays. G, HepG2 cells were transfected with *tk-UAS*, Gal4-SRC1, and VP-LXRα, in the absence or presence of wild-type RORα (CMX-RORα). H, ChIP assay on primary mouse hepatocytes to demonstrate the recruitment of SRC1 onto the *Cyp7b1* and *Est* gene promoters and the effect of LXR agonist TO1317 (TO) on SRC1 recruitment.

sion of *Srebp-1c* in *RORα*$^{sg/sg}$ male mice was not statistically significant (Fig. 4B). Loss of RORα had little effect on the expression of *ApoE*, *Abcg5*, and *LXRs* in either gender.

When the expression of RORα target genes was measured in the LXR DKO mice, we found that the expression of *BMAL1* (Sato et al., 2004), *ApoA1* (Vu-Dac et al., 1997), and *p21* (Schräder et al., 1996) was induced in LXR DKO mice of both sexes (Fig. 4, C and D). *IKKβ* (Delerive et al., 2001) was induced in female, but not male, LXR DKO mice. The expression of *ApoCIII* (Raspé et al., 2001), *Rev-erbα* (Delerive et al., 2002), and *RORα* was not significantly altered. The mutual activation of target gene expression in RORα null and LXR DKO mice suggests that these two receptors are mutually suppressive in vivo, providing a plausible mechanism for the functional cross-talk between these two receptors.

RORα Null Mice Had Increased Liver Triglyceride Accumulation. LXR is known to activate lipogenic gene expression through Srebp-dependent (Repa et al., 2000) or independent (Chu et al., 2006; Cha and Repa, 2007) mechanisms. The activation of several LXR target genes in *RORα*$^{sg/sg}$ mice prompted us to determine whether loss of RORα affects hepatic lipid accumulation. We found that the average liver concentration of triglyceride was more than doubled in *RORα*$^{sg/sg}$ mice of both genders, compared with their age- and gender-matched wild-type counterparts (Fig. 5A). In contrast, loss of RORα had no significant effect on hepatic cholesterol levels in either sex (Fig. 5A). It is noteworthy that the circulating levels of both triglycerides and cholesterol were significantly decreased in male *RORα*$^{sg/sg}$ mice (Fig. 5B), consistent with previous reports (Raspé et al., 2001; Kang et al., 2007). The decreases in circulating lipids in female *RORα*$^{sg/sg}$ mice were not statistically significant. These results suggest that the gene regulation in *RORα*$^{sg/sg}$ mice is functionally relevant by affecting hepatic triglyceride accumulation.

Discussion

In this study, we have established *Cyp7b1* as a novel RORα target gene. Loss of RORα decreased the basal expression of *Cyp7b1*, whereas transfection of RORα activated *Cyp7b1* gene promoter and induced the expression of endogenous *Cyp7b1*. The activation of *Cyp7b1* gene promoter by RORα was inhibited by LXRα. *Cyp7b1*, a key enzyme in the alter-

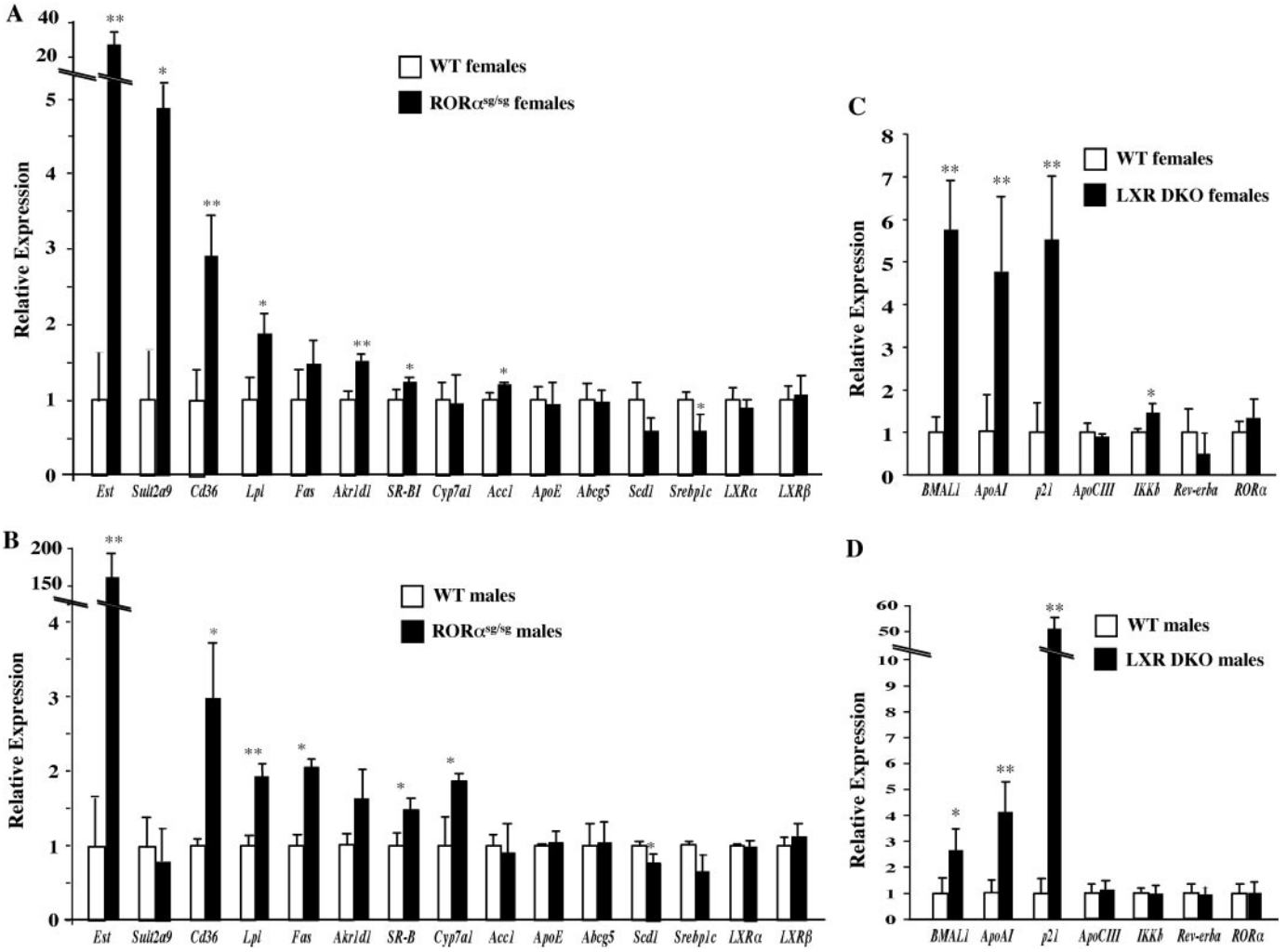

Fig. 4. Reciprocal and selective activation of target gene expression in mice deficient of RORα and LXRs. A and B, real-time RT-PCR analysis on the hepatic expression of LXR target genes in female (A) and male (B) *RORα*$^{sg/sg}$ mice. C and D, real-time RT-PCR analysis on the hepatic expression of ROR target genes in female (C) and male (D) LXR DKO mice. Results represent the averages and standard deviation from three (A and B) or five (C and D) mice per group. *, $P < 0.05$; **, $P < 0.01$, compared with the same-sex WT control mice.

native pathway of cholesterol metabolism to form bile acids, plays an important role in the homeostasis of cholesterol, oxysterols, and bile acids (Schwarz et al., 1998; Chiang, 2004). The oxysterol levels were increased in mice deficient of *Cyp7b1*, presumably as a result of a defect in the conversion of oxysterols to bile acids (Li-Hawkins et al., 2000). It would be interesting to know whether the decreased basal expression of *Cyp7b1* in the *RORα*$^{sg/sg}$ mice is associated with accumulation of oxysterols, the endogenous LXR agonists.

The cross-talk between RORα and LXRα is intriguing. This cross-talk was initially hinted by a remarkable overlap in gene regulation between the *RORα*$^{sg/sg}$ mice and mice whose LXRs were genetically or pharmacologically activated. We then proposed that RORα may normally function as an LXR suppressor, a notion that is supported by the activation of LXR target genes in the *RORα*$^{sg/sg}$ mice. The suppression of LXR by RORα may have broad physiological implications. LXRs are sterol sensors known to promote hepatic lipogenesis. Although lipogenesis is an essential function of the liver, overactivation of the lipogenic pathway is potentially harmful, leading to both local and systemic metabolic disorders. In this regard, the constitutive activity of RORα and its suppression on LXR activity may have offered a mechanism of "checks and balances" to prevent the overactivation of lipogenesis. Indeed, we showed that loss of this suppressor led to the accumulation of hepatic triglycerides (Fig. 5). It is noteworthy that the triglyceride accumulation in the *RORα*$^{sg/sg}$ mice was independent of the activation of *Srebp-1c*, a master lipogenic transcriptional factor and primary target gene of LXR (Repa et al., 2000). Instead, the *RORα*$^{sg/sg}$ mice had increased expression of *Cd36*, a fatty acid transporter known to play a role in pregnane X receptor-mediated and Srebp-independent lipogenesis (Zhou et al., 2006). Cd36 is also essential for LXR agonist-induced hepatic steatosis (J. Zhou and W. Xie, unpublished data). However, we cannot exclude the possibility that the regulation of genes other than *Cd36* may have also contributed to the accumulation of triglycerides in the *RORα*$^{sg/sg}$ mice.

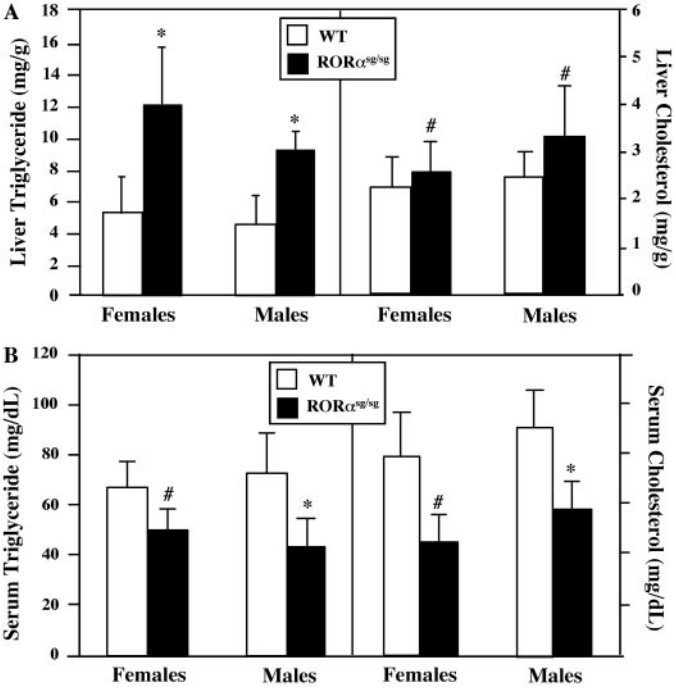

Fig. 5. RORα null mice had increased liver triglyceride accumulation. Triglyceride and cholesterol levels in the liver (A) and serum (B) of the WT and RORa$^{sg/sg}$ mice of indicated sexes. Results represent the averages and standard deviation from three mice per group. *, $P < 0.05$; #, $P > 0.05$, compared with the same-sex WT control mice.

Likewise, loss of LXRs resulted in the activation of several ROR target genes, including the circadian-related *BMAL1* (Sato et al., 2004) and CDK inhibitor *p21* (Schräder et al., 1996). The in vivo consequences of ROR target gene activation in the LXR DKO mice remain to be determined. It is also interesting to note that the mutual activation of target gene expression in the *RORα*$^{sg/sg}$ and LXR DKO mice are gene-specific. The mechanism for this selective gene regulation remains to be determined. Cross-talk between RORα and LXR can involve several mechanisms, including competition for coactivators and DNA binding sites. The inhibition of RORα-mediated *Cyp7b1* activation by LXR seemed to involve coactivator competition, because the *Cyp7b1*/RORE was not bound by LXRα (data not shown), but it was required for the inhibitory effect of LXRα (Fig. 3B). Repression of LXR target genes by RORα may also involve an adjacent or distant RORE.

Another potential implication of the functional cross-talk between RORα and LXR is the role of these two receptors in the survival of Purkinje cells. *Staggerer* mutant mice have a natural deletion in the ligand binding domain of RORα, which is highly expressed in Purkinje cells. The *Staggerer* phenotype is similar to patients with Niemann-Pick disease type C who suffer from a significant loss of Purkinje cells and cerebellar ataxia (for review, see Vanier and Millat, 2003). It has been shown that treatment with LXR agonists relieves Alzheimer's disease (Koldamova et al., 2005; Zelcer et al., 2007), a neurological disorder associated with cerebellar degeneration and loss of Purkinje cells (Sjöbeck and Englund, 2001). For this reason, it was thought that activation of LXR might be beneficial to Purkinje cell survival. If loss of RORα activates LXR, which was strongly suggested by hepatic gene regulation and steatosis in the *RORα*$^{sg/sg}$ mice, one might expect that Purkinje cells may be protected in the *Staggerer* mice. Future studies are warranted to examine the role of RORα and LXR in Purkinje cell survival and its implication in Niemann-Pick disease type C.

In summary, we have revealed a novel function of RORα in regulating metabolic genes, including the cholesterol-metabolizing *Cyp7b1*. The effect of RORα on metabolic gene expression can be achieved by its direct transcriptional regulation (such as *Cyp7b1*), and through its functional cross-talk with the sterol receptor LXR. The metabolic regulatory role of RORα is distinct to the previously known function of this orphan receptor in the structure and function of neuronal and immunological tissues and bones.

Acknowledgments

We thank Dr. David Mangelsdorf for LXR null mouse RNA samples and Drs. Ramalinga Kuruba and Song Li for synthesizing GW3965. We thank Ying Mu for preparation of mouse primary hepatocytes.

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