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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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 (RORαsg/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, RORγ 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 convincingly identified as physiological RORα ligands.

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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; LXRε, 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. RORα/− 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 hypoa-lipoproteinemia when maintained on a atherogenic diet (Mamontova et al., 1998). RORβ is thought to be involved in the processing of sensory information, because RORβ/− mice showed significant phenotypes in circadian behaviors (Andre ´ et al., 1998b). RORγ mice showed significant phenotypes in circadian behaviors and retinal degeneration (Andre ´ et al., 1998b). RORα/− mice lacked all lymph nodes and Peyer's patches, and they exhibited a significant phenotype in circadian behaviors (Andre ´ et al., 1998b). RORγ mice showed significant phenotypes in retinal degeneration (Andre ´ et al., 1998b). RORγ/− 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 RORγ 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 hypoa-lipoproteinemia when maintained on a atherogenic diet (Mamontova et al., 1998). RORβ is thought to be involved in the processing of sensory information, because RORβ/− mice showed significant phenotypes in circadian behaviors (Andre ´ et al., 1998b). RORγ mice showed significant phenotypes in circadian behaviors and retinal degeneration (Andre ´ et al., 1998b). RORα/− 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 oysterolgens, 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 (Repap and Mangelsdorf, 2002; Zeleer and Tontonoz, 2006). LXRs have also been explored as therapeutic targets for atherosclerosis (Tontonoz and Mangelsdorf, 2003), diabetes, and Alzheimer's disease (Zeleer et al., 2007) in animal models. We have recently identified several novel LXR target genes. These include the bile acid-detoxifying subfotransferase Sult2a9/2a1 (Uppal et al., 2007), estrogen subfotransferase (Est/Sult1e1) (Gong et al., 2007), and fatty acid transporter Cd36 (J. Zhou and W. Xie, unpublished data). We found 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 (RORα−/−) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The staggerer (RORα−/−) mice, a natural mutant mouse strain, contain a 6.5-kb deletion in the RORα gene, resulting in a functional knockout of RORα. RORα−/− 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 Institutes 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 cor- responding annealed oligonucleotides into the tk-Luc vector. Three copies of the following response elements were used: Pcp2/RORE, 5′-GGTATAAGTACGTGAGTTGGGACT-3′; Cyp7b1/RORE WT, 5′-TTTTTTATGCAGGTCAGTGG-3′; and Cyp7b1/ROR Mutation, 5′-TTTTTTATGCACCTCAGTGG-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 oligonucloteid: Cyp7b1−3500, 5′-TCTGTTGAACCTTGCAATGCT-3′ and Cyp7b1−125, 5′-CCCGGACGACGCTGCGGCTC-3′. The PCR-ampli- fied sequence was cloned into the pGL3-basic vector (Promega, Madison, WI). Site-directed mutagenesis was performed by PCR overextension method, and the recombinant plasmid was sequenced (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 concentra- tion 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 Tis- sue 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 Lipo- fectamine 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 manu- facturer’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 (Zhao 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 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′-ACCTTAGAAGGAGCCATGGA-3′; Cyp7b1 −902, 5′-TGTAGAATCTCCATGGCTCAATGGA-3′; Cyp7b1 −2900, 5′-GTTCAAAAATATACATTGACATCTT-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 extract 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 kit.

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<sup>−/−</sup> male mice by microarray analysis using the Agilent mouse 20,000-oligo chips (Agilent Technologies, Palo Alto, CA). The RORα<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> 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 assay. Deletion analysis localized the RORα-responsive region to −1017 bp to −520 bp (Fig. 2A). Inspection of this region revealed a putative RORE motif regulated by an AGGTCA
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 32P-labeled oligonucleotide probe. RORs 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. 2B).
TRANSECTION-ASESSED ASYR TO DETERMINE WHETHER THE ENDOGENOUS RORα CAN TRASACTIVATE THROUGH CYP7BI//ORE. SYNTHETIC LUCERENE REPORTER GENES, CONTAINING THREE COPIES OF THE WID LETYPE OR MUTANT CYP7BI//ORE UPSTREAM OF A MINIMAL T1 PROMOTER (tk-CYP7BI//ORE), WERE CONSTRUCTED AND TRANSFECTED INTO HEPG2 CELLS TOGETHER WITH THE EXPRESSION VECTOR FOR RORα. AS SHOWN IN FIG. 2A, COTRANSFECION WITH RORα ACTIVATED THE tk-CYP7BI//ORE REPORTER GENE, AND THIS ACTIVATION WAS ABOLISHED WHEN THE CYP7BI//ORE WAS MUTATED. THIS RORE IS ALSO REQUIRED FOR THE ACTIVATION OF THE NATURAL 1-kb CYP7BI promoter, BECAUSE MUTATION OF THE RORE IN THIS CONTEXT ALSO ABOLISHED THE TRANSACTIVATION BY RORα (FIG. 2A).

FINALLY, WE SHOWN THAT OXPRESSION OF RORα IN PRIMARY HEPATOCYTE CULTURES BY TRANSIENT TRANSFECION (FIG. 2E) OR IN WILD-TYPE MOUSE LIVERS BY A HYDRODYNAMIC LIVER TRANSFECION METHOD (FIG. 2F) INDUCED THE mRNA EXPRESSION OF THE ENDOGENOUS CYP7BI, BUT NOT THE CONTROL CYP3A11 GENE. THE OXPRESSION OF RORα IN TRANSFECTED HEPATOCYTES OR LIVERS WAS CONFIRMED BY REAL-TIME RT-PCR ANALYSIS (FIG. 2G). TOGETHER, OUR RESULTS STRONGLY SUGGEST THAT THE MOUSE CYP7BI GENE IS A TRANSCRIPTIONAL TARGET OF RORα, AND THIS REGULATION IS MEDITATED BY CYP7BI//ORE.

THE ACTIVATION OF CYP7BI 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 CYP7BI WAS SUPPRESSED IN LXR-ACTIVATED MICE (UPPAL ET AL., 2007). CYP7BI SUPPRESSION WAS ALSO SEEN IN PRIMARY HUMAN HEPATOCITIES TREATED WITH LXR AGONISTS (FIG. 3A). HOWEVER, THE MECHANISM FOR LXR-MEDIATED CYP7BI SUPPRESSION IS UNKNOWN. HAVING ESTABLISHED RORα AS A POSITIVE CYP7BI REGULATOR, WE WENT ON TO EXAMINE WHETHER LXR SUPPRESSES CYP7BI BY INHIBITING RORα ACTIVITY. AS SHOWN IN FIG. 3B, ACTIVATION OF THE 1-kb CYP7BI PROMOTER (pGL-CYP7BI) BY RORα WAS SUPPRESSED BY COINSTALLATION OF LXRα, EVEN IN THE ABSENCE OF LXR AGONISTS. THE HINCTION EFFECT OF LXRα WAS ENHANCED BY THE LXR AGONIST TO1317. TRANSFECION OF LXRα ALONE, EVEN IN THE ABSENCE OF LXR AGONISTS, INHIBITED CYP7BI PROMOTER ACTIVITY. THE HINCTION 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-Ptcp2/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-Ptcp2/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-Ptcp2/RORE REPORTER.

IT IS NOTWEARY THAT THE LXRα ACTIVITY WAS RECIPROCALLY SUPPRESSED BY RORα. AS SHOWN IN FIG. 3D, COINSTALLATION WITH LXRα ACTIVATED THE LXR-RESPONSIVE tk-MTV REPORTER GENE AS EXPECTED (WILLY ET AL., 1995). HOWEVER, THIS ACTIVATION WAS INHIBITED BY COINSTALLATION OF AN RORα IN A DOSE-DEPENDENT MANNER, WHEREAS TRANSFECION OF RORα ALONE HAD LITTLE EFFECT ON tk-MTV REPORTER ACTIVITY. THE LIND-DEPENDENT ACTIVATION OF tk-MTV BY LXRα WAS ALSO INHIBITED BY COINSTALLATION OF RORα (FIG. 3E). THESE RESULTS SUGGEST THAT RORα AND LXRα ARE MUTUALLY SUPPRESSIVE.

THE MUTUAL SUPPRESSION WAS ALSO SEEN 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 SHOWN 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 COINSTALLATED WITH GAL4-SRC1 AND tk-UAS-Luc (FIG. 3G). MOREOVER, THE VP-LXRα-SRC1 INTERACTION WAS INHIBITED BY COINSTALLATION OF THE WILD-TYPE RORα (FIG. 3G). CHIP ASSAY ON PRIMARY MOUSE HEPATOCYTES SHOWED THAT SRC1 WAS CONSTITUTIVELY RECRUITED ONTO CYP7BI//ORE, 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).

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 LXRα. 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 RORα TARGET GENES IN THE RORα<sup>p<sup>sg</sup>/sg</sup> AND LXR DOUBLE KNOOUT (DKO) MICE (PEET ET AL., 1998), RESPECTIVELY. AS SHOWN IN FIG. 4A, AMONG LXR TARGET GENES, THE EXPRESSION OF Est (GONG ET AL., 2007), Sul2a9 (UPPAL ET AL., 2007), Cd36 (J. ZHOU AND W. XIE, UNPUBLISHED DATA), LIPOPROTEIN LIPOSE (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<sup>p<sup>sg</sup>/sg</sup> MICE. WHEN THE MALE RORα<sup>p<sup>sg</sup>/sg</sup> MICE WERE ANALYZED, THE ACTIVATION OF Est, Cd36, LIPOPROTEIN LIPOSE, AND SR-BI, BUT NOT Sul2a9/sul2a9, Akrd1, AND Acc-1, REMAINED SIGNIFICANT (FIG. 4B). THE LACK OF Sul2a9 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 SUPPRESS-
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-null 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), ApoAl (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-erba (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-null 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-null 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-null mice (Fig. 5B), consistent with previous reports (Raspé et al., 2001; Kang et al., 2007). The decreases in circulating lipids in female RORα-null mice were not statistically significant.

These results suggest that the gene regulation in RORα-null 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-

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**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-null 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<sup>sg/sg</sup> 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<sup>sg/sg</sup> mice and mice whose LXRαs 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<sup>sg/sg</sup> mice. The suppression of LXR by RORα may have broad physiological implications. LXRαs 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<sup>sg/sg</sup> mice was independent of the activation of LXR by RORα by RORα or RORα<sup>sg/sg</sup> mice. The suppression of LXR by RORα may have broad physiological implications. LXRαs 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<sup>sg/sg</sup> mice was independent of the activation of LXR by RORα by RORα or RORα<sup>sg/sg</sup> mice. The suppression of LXR by RORα may have broad physiological implications. LXRαs 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 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.

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**References**


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