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Liver X receptor-α mediates cholesterol efflux in glomerular mesangial cells

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Wu, Jing, Yahua Zhang, Nanping Wang, Linda Davis, Guangrui Yang, Xian Wang, Yi Zhu, Matthew D. Breyer, and Youfei Guan. Liver X receptor-α mediates cholesterol efflux in glomerular mesangial cells. *Am J Physiol Renal Physiol* 287: F886–F895, 2004. First published July 27, 2004; doi:10.1152/ajprenal.00123.2004.—Lipid-mediated injury plays an important role in the pathogenesis of many renal diseases including diabetic nephropathy. Liver X receptor-α (LXRα) is an intracellular sterol sensor that regulates expression of genes controlling cholesterol absorption, excretion, catabolism, and cellular efflux. The present study was aimed at examining the role of LXRα in cholesterol metabolism in glomerular mesangial cells. A 1,561-bp fragment of full-length rabbit LXR cDNA was cloned. The deduced protein sequence exhibited 92.4 and 89.2% identity to human and mouse LXRα, respectively. Tissue distribution studies showed that rabbit LXRα was expressed in the liver, spleen, and kidney. In situ hybridization and RT-PCR assays further indicated that LXRα mRNA was widely expressed in the kidney and present in every nephron segment including the glomeruli. To determine intrarenal regulation of LXRα, rabbits were treated with thiazolidinedione (TZD) peroxisome proliferator-activated receptor-γ (PPARγ) agonists, which have been previously shown to enhance LXRα expression via PPARγ and increase cholesterol efflux in macrophages. The results showed that glomerular LXRα expression was markedly induced by TZDs. In cultured rabbit mesangial cells, LXRα mRNA and protein were detected by RT-PCR and immunoblotting. Treatment of mesangial cells with a specific LXRα agonist, TO-901317, significantly increased basal and apolipoprotein AI-mediated cholesterol efflux and markedly enhanced the promoter activity of an LXRα target gene, ATP-binding cassette transporter A1 (ABCA1). In conclusion, LXRα is expressed in renal glomeruli and functionally present in mesangial cells where its activation mediates cholesterol efflux via ABCA1. These data suggest that LXRα may be a potential therapeutic target for treating lipid-related renal glomerular disease.

glomeruli; ATP-binding cassette transporter A1

LIVER X RECEPTORS (LXRs) BELONG to the nuclear receptor and transcription factor superfamily and exist in two isoforms including LXRα and LXRβ (40). The two isoforms are differentially expressed in several tissues. LXRα is highly expressed in the liver, intestine, and the kidney, whereas LXRβ is ubiquitously present in most tissues examined (40). LXRs form heterodimers with the retinoic acid receptor RXRα, which bind to characteristic DNA sequences termed LXR-response elements (LXRE) located in the 5′-flanking region of target genes. Target genes of LXRs include ATP-binding cassette transporter (ABC) A1, ABCG5, ABCG8, apolipoprotein E (ApoE), cholesterol ester transport protein, lipoprotein lipase, fatty acid synthase, and sterol-response element-binding protein 1c, suggesting that LXRs are key players in lipid and cholesterol metabolism (33, 40). Recent reports demonstrated that an LXR-specific activator increased HDL cholesterol in mice (37), and gene disruption of LXRα and LXRβ resulted in increased LDL cholesterol and a decrease in HDL cholesterol in plasma, with a massive lipid accumulation in the liver, spleen, and lung (31, 38). It has also been reported that LXRs are highly expressed in monocytes/macrophages, where they may enhance cholesterol efflux via increasing expression of ABCA1 (33). In addition, LXR activation results in increased ApoE expression (15, 23), thereby stimulating the ApoE-dependent cholesterol efflux in macrophages. These findings suggest LXRs may serve as a sensor for metabolites of lipid and cholesterol metabolism and play an important role in cholesterol homeostasis in macrophages.

Hyperlipidemia accelerates the progression of renal disease (29, 30), and lipid-lowering therapies reverse this effect (1, 16, 19, 21). There is increasing evidence suggesting that glomerulosclerosis shares similar pathogenic mechanisms with atherosclerosis, where accumulation of intracellular cholesterol and triglycerides in vascular smooth muscle cells (VSMCs) and infiltrating monocytes leads to formation of foam cells (1). In chronic kidney disease, glomerular cells mimic these characteristics, and foam cell-like changes are frequently present in glomerular sclerotic lesions (9, 29). Thus attenuating intracellular cholesterol accumulation in glomerular cells provides an attractive target for slowing the progression of glomerular sclerosis. As seen in VSMCs and macrophages, lipid homeostasis in glomerular cells is also under tight metabolic regulation (1). Increased uptake of oxidized LDL and decreased lipid efflux coordinately lead to foam cell formation in cultured mesangial cells (34–36). Because LXRα activation induces the expression of ABCA1, a key gene in cholesterol efflux from macrophages, it may also play a critical role in cholesterol homeostasis in the kidney, especially in the glomeruli. The present study was designed to examine the expression of LXRα in the kidney and determine its role in cholesterol metabolism in cultured glomerular mesangial cells.

MATERIALS AND METHODS

Chemical reagents and animals. TO-901317 was purchased from Cayman Chemicals (Ann Arbor, MI) and dissolved in absolute ethanol. Troglitazone (provided by Warner Lambert/Parke-Davis Pharmaceutical Research, Ann Arbor, MI), rosiglitazone (a gift from SmithKline Beecham, West Sussex, UK), and an experimental thiazolidinedione, i.e., 5-{4-[2-(5-methyl-2-phenyl-4-oxazoly)-2-hydroxyethoxy] benzyl-2,4-thiazolidinone}, henceforth referred to as TZD3 (Merck Pharmaceutics), were dissolved in DMSO at a concentration of 30 mM. Male

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New Zealand White rabbits were treated for 2 wk with troglitazone or rosiglitazone mixed with rabbit chow (Purina Diet, Indianapolis, IN) at the doses of 200 and 10 mg·kg^{-1}·day^{-1}, respectively (13). TZD3 compound was given by gavage at the dose of 3 mg·kg^{-1}·day^{-1} (13). The use of all animals in this study was approved by the Vanderbilt Animal Care and Use Committee (protocol M/03/105).

cDNA cloning of a full-length rabbit LXRα. Total RNA was purified from rabbit liver using TRIzol reagent (GIBCO BRL), treated with RNAse-free DNAse I (Promega), and reverse-transcribed to single-strand cDNA using Molony murine leukemia virus reverse transcriptase and 2.5 μM of random hexamers according to the manufacturer's protocol (GeneAmp RNA PCR kit, PerkinElmer Cetus, Norwalk, CT). The synthesized cDNA was then used to amplify a portion of rabbit LXRα cDNA using a pair of selective primers designed from conserved sequences of mouse and human LXRα. The upstream sense primer was 5′-CGT CCA CAA AAG CGG AAA AAG-3′, and the downstream antisense primer was 5′-GTC TGC AGA GAA GAT GCT GAT-3′ (41, 44). PCR reactions were carried out in 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 μM primers at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for 30 cycles in a PerkinElmer Cetus 2400 thermal cycler. The predicted 869-bp fragment was ligated into pCR II 2.1 vector (Invitrogen) and sequenced. BLAST and CLUSTAL analysis revealed that this cDNA sequence was homologous to the coding region of human and mouse LXRα (41, 44).

5′-Rapid amplification of cDNA ends (RACE) was used to obtain the 5′-ends of rabbit LXRα cDNA using the above cloned fragment of rabbit LXRα. Three gene-specific antisense primers were designed: *primer 1* (5′-CTT CAG CAG GGC GAT TTG GTC-3′), nested *primer 2* (5′-GCT CCT CCT CCT GTC GCT TCA-3′), and nested *primer 3* (5′-GCC CCT TTT TCG GCT TTT GTG-3′). One microgram of total RNA from rabbit liver (see above) was used as a template for the first-strand cDNA synthesis, with *primer 1* and 200 U SuperScript II reverse-transcriptase according to the manufacturer's description (GIBCO BRL). The first-strand cDNA was purified and tailed with dCTP using terminal deoxynucleotidyl transferase (TdT). Amplification of the 5′-terminal cDNA sequence was performed with *primer 2* and the abridged anchor primer, and the product was used in a second nested PCR with the internal *primer 3* and the abridged universal amplification primer (94°C for 30 s, 58°C for 1 min, 72°C for 2 min for 35 cycles). PCR products were subcloned into pCR II vector (Invitrogen) and then sequenced.

Two gene-specific sense primers for 3′-RACE were synthesized for obtaining the sequence downstream of the known LXRα cDNA: a 3′-primer (*primer 4*; 21 mer: 5′-CCA AAT CGC CCT GCT GAA GAC-3′) and a nested primer (*primer 5*; 21 mer: 5′-GAG GTG ATG CTG CTG GAG ACG-3′). One microgram of liver total RNA was reverse-transcribed with the adapter primer and 200 U SuperScript II reverse-transcriptase at 42°C (3′-RACE system, GIBCO BRL) according to the manufacturer's protocol. Two microliters of cDNA were used for the first round of PCR amplification with the abridged universal amplification primer and *primer 4*. A nested PCR was performed with internal *primer 5* and universal amplification primer (94°C for 30 s, 58°C for 40 s, and 72°C for 3 min for 35 cycles). A single 3′-RACE product was purified (Qiaquick gel extraction kit, Qiagen) and subcloned for sequencing.

Construction of an expression vector containing a full-length LXRα and determination of its activity. A full-length rabbit LXRα was obtained by RT-PCR using a pair of primers located in the regions of the 5′-untranslated region (UTR) and 5′-coding region (5′-AGA GAT GTC CCT GTG GCT GG-3′) and 3′-UTR (5′-AGC CTG GGG GCC TCA GCT TT-3′). A 1,403-bp fragment of full-length rabbit LXRα cDNA was subcloned into pRc/CMV2 vector (LXRα/CMV) and sequenced (Invitrogen). To determine whether the cloned full-length LXRα encodes a functional protein, an LXRE3x TK-luciferase reporter (a gift provided by Dr. Barry Forman at the City of Hope) was transfected with or without LXRα expression vector into a hepatocarcinoma cell line, HepG2 cells. Luciferase activity was measured using the method described below.

Solution hybridization/RNase protection assays. To determine the tissue distribution of LXRα mRNA expression in the rabbit, a 289-bp rabbit LXRα cDNA fragment were amplified using a pair of primers (sense primer: 5′-CCG AAG ATG CTG GGG AAC GAG-3′; antisense primer: 5′-GCT CCT CCT CCT GTC GCT TCA-3′). The RT-PCR product was then subcloned into pCRII vector, and a specific riboprobe was synthesized. RNase protection assays were performed as previously described (13). Briefly, the plasmids containing rabbit LXRα (289 bp) and GAPDH (174 bp) (13) were linearized with appropriate restriction enzymes. Radiolabeled riboprobes were synthesized from 1 μg of linearized plasmids in vitro using a MAXIscript kit (Ambion) for 1 h at 37°C in a total volume of 20 μl. The reaction buffer contained 10 mM DTT, 0.5 mM ATP, CTP, and GTP, 2.5 mM of UTP, and 5 μl of 800 Ci/mmol [α-^{32}P]UTP at 10 mCi/ml (DuPont-New England Nuclear, Boston, MA). Hybridization buffer included 80% deionized formamide, 100 mM sodium citrate, pH 6.4, and 1 mM EDTA (RPA II, Ambion). Twenty micrograms of total RNA from various rabbit tissues were incubated at 45°C for 12 h in hybridization buffer with 5×10^4 cpm labeled riboprobes. After hybridization, RNase digestion was carried out at 37°C for 30 min, and protected fragments were precipitated and separated on a 6% polyacrylamide gel at 200 V for 4 h. The gel was exposed to Kodak XAR-5 film overnight at −80°C with intensifying screens.

In situ hybridization of LXRα. Male New Zealand White rabbits were treated with or without troglitazone (200 mg·kg^{-1}·day^{-1}), rosiglitazone (10 mg·kg^{-1}·day^{-1}), or TZD3 (3 mg·kg^{-1}·day^{-1}) orally for 2 wk. An ^{35}S-labeled antisense riboprobe was generated from a 289-bp PCR fragment of LXRα (see above) hybridized to the section and then washed as previously described (13). Slides were dehydrated with graded ethanol containing 300 mM ammonium acetate, dipped in emulsion (Ilford K5, Knutsford, Cheshire, UK), and exposed for 4–5 days at 4°C. After developing in Kodak D-19, slides were counterstained with hematoxylin. Photomicrographs were taken using a Zeiss Axioskop microscope with either darkfield or brightfield optics.

Microdissection of rabbit nephron fragments. The method of renal tubule dissection was reported previously (11). Male New Zealand White rabbits weighing ~2 kg were anesthetized intramuscularly with a mixture of ketamine (44 mg/kg) and xylazine (11 mg/kg). The animals were then euthanized. The renal arteries were cannulated and perfused with 60–80 ml of chilled PBS [containing (in mM) 90 NaCl, 5 KCl, 1 $MgSO_4$, and 2 $CaCl_2$] plus BSA (0.5% wt/vol) and type I collagenase (0.1% wt/vol), as well as the protease inhibitors aprotinin (0.005% wt/vol) and bacitracin (0.08% wt/vol). The kidneys were removed and sliced into 1-mm-thick sections and incubated in the collagenase-containing buffer for 45–60 min in a 37°C water bath while O_2 was bubbled through the solution. The slices were then transfered to chilled PBS plus BSA without $CaCl_2$ or collagenase, and the nephron segments were obtained by microdissection.

RT-PCR analysis of LXRα expression in microdissected tubules. Total RNA was purified from microdissected tubules using TRIzol reagent (GIBCO BRL). RNA was then quantified spectrophotometrically. Equal amounts of RNA (1 μg) were reverse-transcribed as described above. The cDNA was amplified using the primers used for amplifying a 289-bp LXRα fragment (see above). For RNA loading control, rabbit GAPDH primers (sense: 5′-CTG ACC TGC CGC CTG GAG AAA-3′; antisense: 5′-GGG TCT GGG ATG GAA ACT GTG-3′) were also utilized to amplify a 411-bp fragment. PCR reactions were carried out in 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 1 μM primers at 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s for 30 cycles.

Isolation of rabbit glomeruli and culture of glomerular mesangial cells. Glomeruli were isolated by fractional sieving of minced cortex using 125- and 75-μm mesh wire. Rabbit glomerular mesangial cells were cultured according to a previous report (13). Cells were cultured in DMEM media supplemented with 10% FBS (GIBCO BRL). To test whether LXRα is constitutively expressed in mesangial cells, cells were cultured until complete confluence, and total RNA and protein were extracted for RT-PCR analysis and immunoblot assay, respectively. To examine endogenous LXRα activity in the mesangial cells, 70% confluent cells cultured in 24-well plates were transfected with

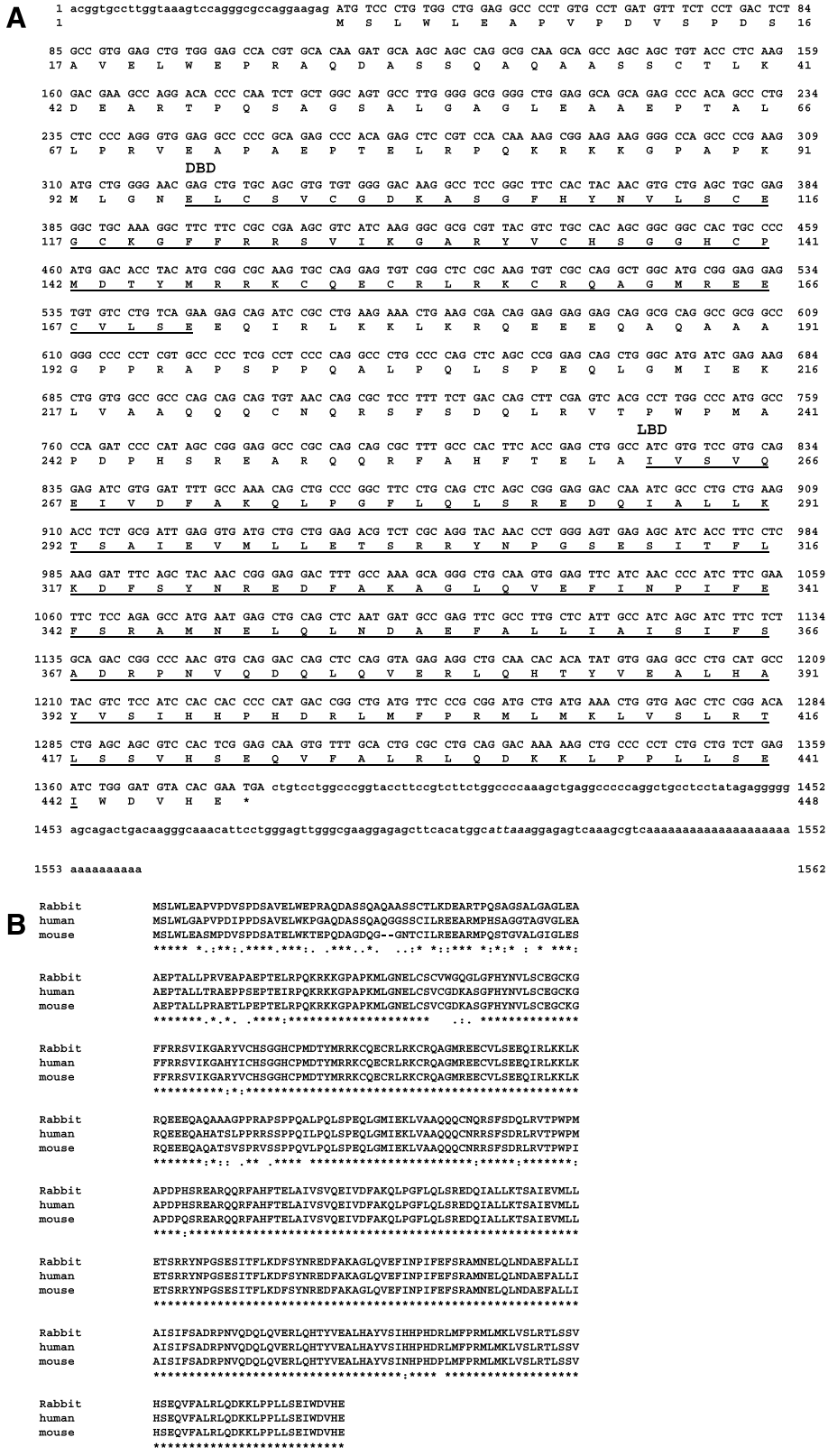


Fig. 1. *A*: nucleotide and deduced amino acid sequence of rabbit liver X receptor-α (LXRα). The DNA-binding domain (DBD) and ligand-binding domain (LBD) are underlined, and the polyadenylation sequence is shown in italics. *B*: alignment of the deduced amino acid sequence of rabbit LXRα (rabLXRα) with human (hLXRα) and mouse LXRα (mLXRα). Sequences were deduced from cDNA sequences and aligned using the CLUSTALV program. *, Identical sequence; ·, conserved sequence; -, gap in sequence alignment. GenBank accession no. (pending).

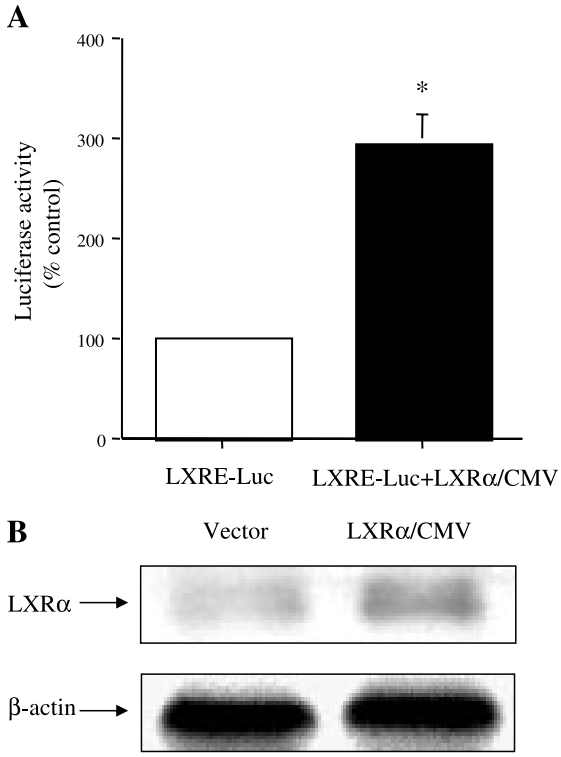


Fig. 2. *A*: luciferase reporter assay demonstrating the cloned full-length LXRα encodes a functional protein capable of binding to an LXR-response element (LXRE) consensus sequence and transactivating LXRE promoter-driven luciferase activity in cultured hepatocarcinoma cell line HepG2. HepG2 cells were cotransfected with LXRE3x-luciferase reporter plasmid with control vector (pRC/CMV2) or rabbit LXRα expression vector (LXRα/CMV). Twenty-four hours after transfection, cells were lysed for measurement of luciferase reporter activity. Values are means ± SD of 4 wells in a single experiment representative of 3 independent experiments ($n = 4$, $^*P < 0.001$). *B*: overexpression of the cloned rabbit full-length LXRα increases LXRα protein expression as assessed by immunoblotting. Note that ~51- and 42-kDa bands, corresponding to predicted full-length LXRα protein and β-actin, respectively, were observed. β-Actin was utilized as a protein loading control.

ABCA1-luciferase reporter (see below) in presence or absence of 10 μM TO-901317, an LXR-specific agonist, for 24 h.

Transient transfections and luciferase reporter assays. Cultured HepG2 cells and mesangial cells were transfected with LXREx3 TK-Luc, a reporter construct containing three copies of LXRE from the mouse mammary tumor virus long tandem repeat, or a human ABCA1-luciferase construct (pABCA1–116-luc) (26) using Effectene transfection reagent as recommended by the supplier (Qiagen, Valencia, CA). After incubation for 24 h, the transfection mixture was replaced with complete media containing either ethanol or TO-901317 (10 μM). After 24 h, cells were harvested in 1× luciferase lysis buffer (Dual Luciferase Kit, Promega), and relative light units were determined using a luminometer (Mono light 2010, Analytical Luminescence Laboratory, San Diego, CA).

Immunoblot analysis of LXRα expression. Mesangial cells were cultured in 10% FCS DMEM medium until complete confluence. As a positive control, cells were infected with an adenovirus expressing human LXRα adenovirus constructed as previously reported (43) for 24 h. Cells were lysed in SDS-PAGE sample buffer (120 mM Tris·HCl, pH 6.5, 4% SDS, 5 mM DTT, and 20% glycerol) followed by repetitive aspiration. The lysate was boiled for 3 min, and protein concentration was measured by BCA protein assay (Pierce, Rockford, IL). Ten micrograms of each protein sample were loaded onto 10% SDS-PAGE minigel and run at 100 V. Proteins were transferred to a nitrocellulose membrane at 22 V overnight at 4°C. The nitrocellulose membranes were washed three times with PBS and incubated in blocking buffer (Tris-buffered saline which contained 150 mM NaCl, 50 mM Tris, 0.05% Tween 20 detergent, and 5% nonfat dry milk, pH 7.5) for 1 h at room temperature. The membranes were then incubated in goat anti-human (mouse) LXRα polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:200 in blocking buffer for 2 h at room temperature. Antibody labeling was visualized by addition of chemiluminescence reagent (DuPont-New England Nuclear) and exposure of the membrane to Kodak XAR-5 film.

Assessment of cholesterol efflux in cultured mesangial cells. The cholesterol efflux was assessed as described with minor modifications (26). Briefly, rabbit renal glomerular mesangial cells in 12-well plates were treated with the LXR agonist TO-901317 (10 μM) in 10% FBS supplemented with DMEM for 24 h and then labeled with [^{3}H]cholesterol (0.2 μCi/ml) for 6 h. Cells were gently washed three times with PBS containing 0.1% BSA. Then, fresh serum-free medium with or without ApoA-I (10 μg/ml) was added, and the cells were incubated for 2 h. After incubation, the medium was centrifuged to remove any detached cells. The cells were washed and lysed in 1 N NaOH. Aliquots of medium and cell lysates were assayed by liquid scintillation. The results represent radioactivity in medium as a percentage of the total (medium + cell lysate). Data are expressed as means ± SD of three experiments in triplicate.

Statistical analysis. In all experiments, data were evaluated for significance by one-way ANOVA using Minitab software or Student's *t*-test. $P < 0.05$ is considered statistically significant.

RESULTS

cDNA cloning of full-length rabbit LXRα isoform. A 869-bp fragment of LXRα was amplified by RT-PCR from rabbit liver using a pair of primers designed from conserved sequences in the DNA-binding domain (DBD) and ligand-binding domain (LBD) of mouse and human LXRα, yielding a region of rabbit LXRα with high homology to human and mouse LXRα as previously reported (41, 44). 5′-RACE yielded a single 298-bp fragment and shares high similarity to the human and mouse LXRα sequence. 3′-RACE yielded a single 641-bp fragment. This product overlaps the original 869-bp PCR product by 219 bp and extends the rabbit LXRα 3′-sequence by 422 bp. The sequence includes a termination codon TGA in the open reading frame, as well as a 182-bp 3′-UTR with a poly(A) tail of 30 adenosine nucleotides and a putative polyadenylation signal, suggesting the complete LXRα sequence was obtained.

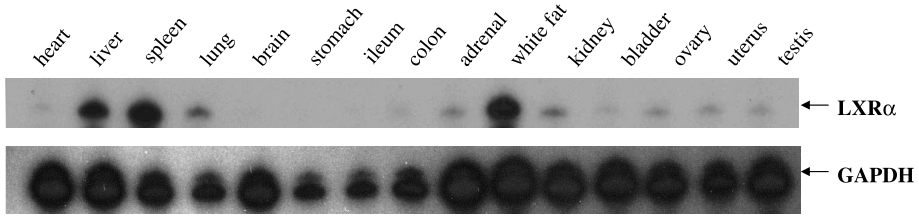


Fig. 3. RNase protection assay showing the distribution of LXRα and GAPDH mRNA in normal rabbit tissues. Twenty micrograms of total RNA from various rabbit tissues were utilized for the nuclease protection assay. The protected fragments were electrophoretically separated on a 6% agarose/7 M urea gel. The film was exposed for 24 h. A 100-bp ladder was used as a size marker. RNA loading was corrected by normalizing to GAPDH mRNA expression (*bottom*).

Assembly of the RACE product sequences provided a full-length rabbit LXRα consisting of 1,561 bp (Fig. 1*A*). This sequence encodes a predicted polypeptide of 447 amino acids with an estimated molecular mass of 51 kDa (Fig. 1*A*). Comparison of the amino acid sequence of rabbit LXRα with human and mouse homologs revealed 92.4 and 89.2% identity, respectively (Fig. 1*B*). A putative DBD (amino acids 96–171) and LBD (amino acids 262–442) are among the conserved regions in the rabbit LXRα sequence using the National Center for Biotechnology Information Conserved Domain Search (Fig. 1*A*). Transfection studies using a rabbit LXRα expression vector (LXRα/CMV) demonstrated that LXRα/CMV markedly enhanced LXRE3x-luciferase reporter activity, consistent with enhanced LXRα protein expression (Fig. 2). These results suggest the cloned rabbit LXRα sequence encodes a protein capable of binding to the LXRE consensus sequence and capable of activating downstream gene transcription.

Tissue distribution of LXRα in the rabbit. An RNase protection assay (Fig. 3) revealed that LXRα was selectively expressed in many rabbit tissues. LXRα mRNA was highly expressed in the liver, spleen, and adipose tissue, followed by lung, kidney, and adrenal gland. Lower levels of LXRα mRNA expression were observed in reproductive organs (ovary, uterus, and testis), the urinary bladder, large intestine, and heart with very little expression in the brain, stomach, and small intestine.

Intrarenal localization of LXRα in rabbit kidney. In agreement with the RNase protection assay shown in Fig. 3, in situ hybridization also revealed expression of LXRα in rabbit kidney (Fig. 4*A*). As shown in Fig. 4*A*, specific LXRα mRNA signals were detected throughout the kidney using an antisense riboprobe, whereas no signal was obtained using a sense control riboprobe. This widespread expression was further confirmed by the fact that the expression of LXRα was found in every segment along the nephron as assessed by RT-PCR (Fig. 4*B*). In addition, high-magnification images clearly showed that LXRα mRNA was expressed in the renal glomeruli and appeared to be present in all three major glomerular cell types, including mesangial cells, endothelial cells, and podocytes (Fig. 4*C*).

Effect of TZDs on LXRα expression in rabbit glomeruli. To determine whether endogenous glomerular LXRα can be regulated in vivo, we examined the effect of oral treatment with TZD PPARγ agonists troglitazone (200 mg·kg^{-1}·day^{-1}), rosiglitazone (10 mg·kg^{-1}·day^{-1}), or TZD3 (3 mg·kg^{-1}·day^{-1}) for 2 wk on renal expression of LXRα, a well-documented PPARγ direct target gene (6). As assessed by in situ hybridization, treatment with the TZD PPARγ ligands resulted in a marked increase in LXRα expression in the glomeruli (Fig. 5). Consistent with their pharmacological properties, TZD3 was more potent than rosiglitazone and troglitazone in inducing LXRα gene expression (troglitazone < rosiglitazone < TZD3) (4). Interestingly, induction of LXRα by PPARγ agonists was predominantly observed in the glomeruli as opposed to collecting ducts, where PPARγ is also highly expressed (12, 13).

LXRα expression in cultured glomerular mesangial cells. To further characterize the intraglomerular expression of LXRα, rabbit glomerular mesangial cells were cultured and examined for LXRα at the mRNA level and for protein expression. RT-PCR analysis (Fig. 6*A*) and immunoblot assays (Fig. 6*B*) confirmed the expression of LXRα in cultured mesangial cells.

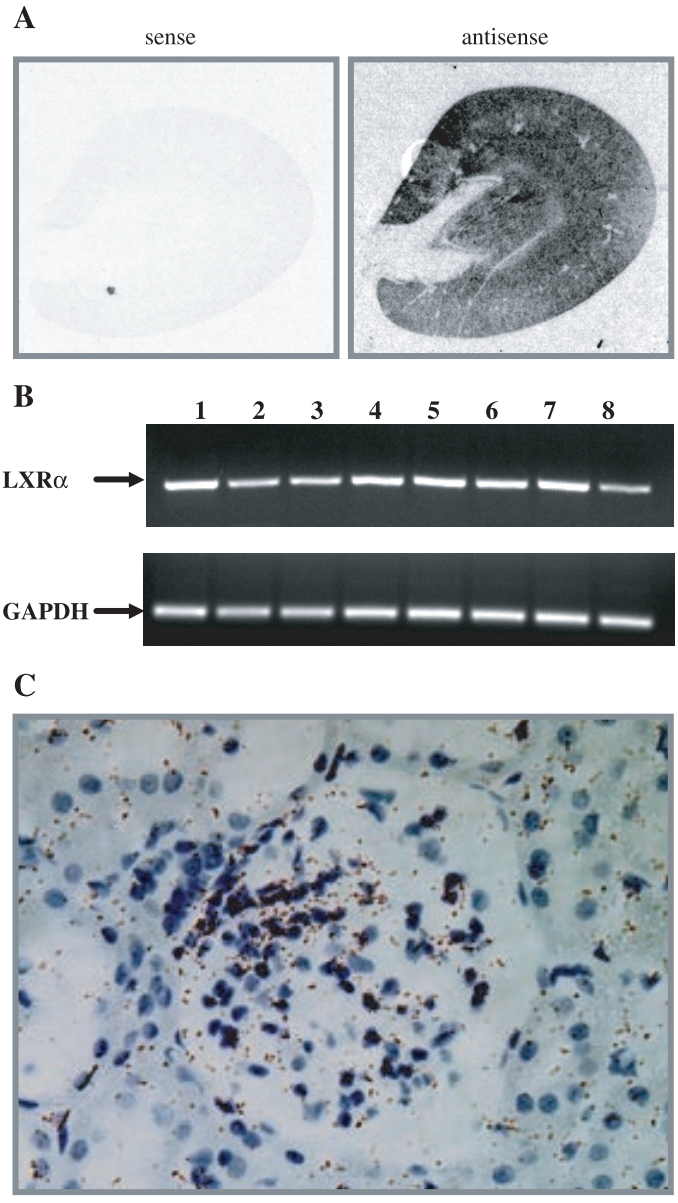
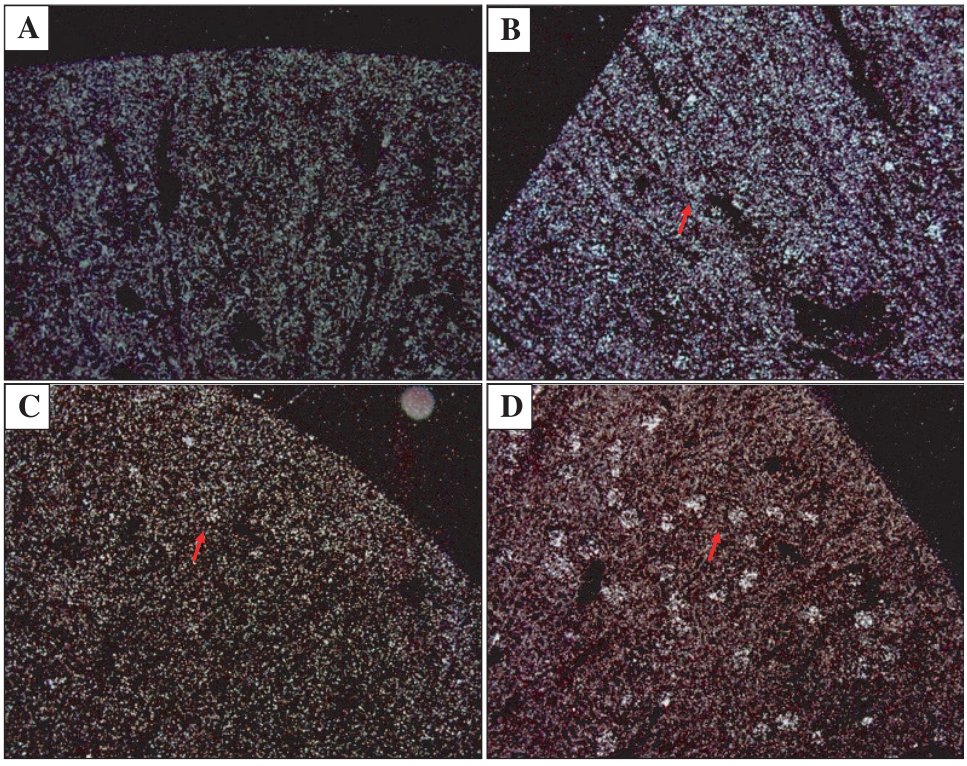


Fig. 4. Determination of LXRα mRNA expression in rabbit kidney. *A*: autoradiograph demonstrating ubiquitous expression of LXRα mRNA expression in rabbit kidney. Dark black areas indicate regions of hybridization with the antisense riboprobe (*right*). In contrast, no hybridization was seen using a sense probe (*left*). *B*: RT-PCR analysis demonstrating distribution of LXRα (*top*) and GAPDH mRNA (*bottom*) along rabbit nephron. PCR products were electrophoretically separated on a 1% agarose gel. RNA loading was corrected by normalizing to GAPDH mRNA expression. *Lanes 1–8*: glomeruli, proximal convoluted tubule, descending limb of the Henley's loop, medullary thick ascending limb (TAL), cortical TAL, distal convoluted tubule, cortical collecting duct, and medullary collecting duct, respectively. *C*: photomicrograph (brightfield, ×400) showing in situ hybridization for LXRα mRNA expression in normal rabbit glomeruli. Brown grains indicate hybridization signals of LXRα mRNA over glomeruli. Note that LXRα mRNA appears to be expressed in all 3 major cell types of glomeruli, i.e., mesangial cells, endothelial cells, and podocytes.

Mesangial cells displayed the expected PCR fragment of 289 bp for LXRα mRNA and a ~51-kDa protein band of LXRα protein, confirming that LXRα is expressed in glomerular mesangial cells.



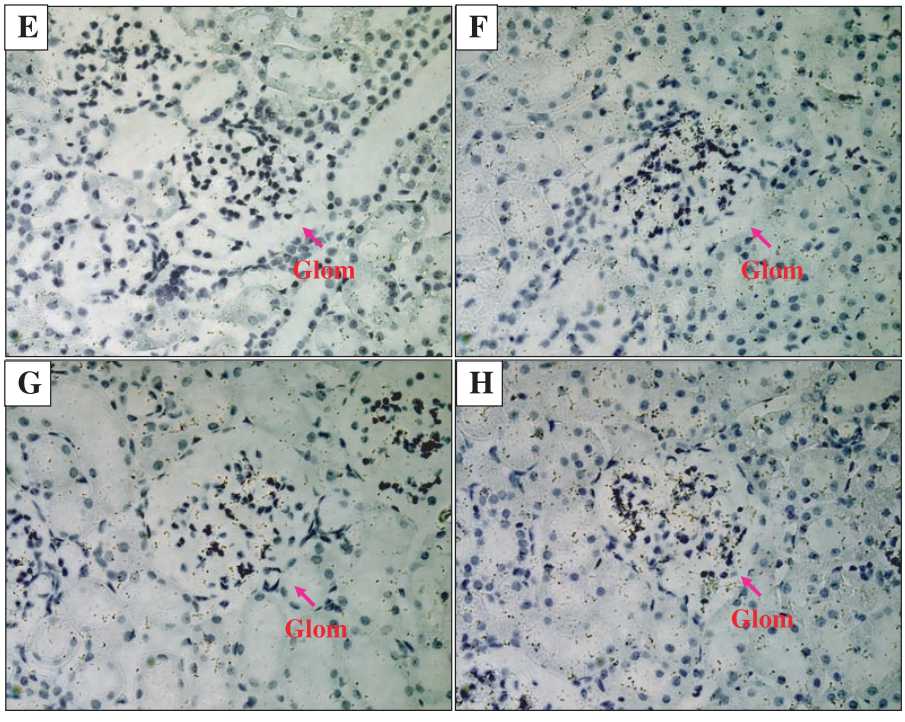


Fig. 5. In situ hybridization showing an induction of LXRα mRNA level in rabbit glomeruli after treatment with troglitazone (200 mg·kg^{-1}·day^{-1}), rosiglitazone (10 mg·kg^{-1}·day^{-1}), or the thiazolidinedione TZD3 (3 mg·kg^{-1}·day^{-1}) for 2 wk. *A–D*: autoradiograms showing glomerular localization of LXRα mRNA in the kidneys of rabbits receiving no treatment (*A*), troglitazone (*B*), rosiglitazone (*C*), and TZD3 (*D*). Note selective induction of LXRα in glomeruli (white grains, arrows; *B*). *E–H*: ×400 brightfield illumination of the kidneys from normal rabbits (*E*) or rabbits treated with troglitazone (*F*), rosiglitazone (*G*), and TZD3 (*H*). Black grains indicate LXRα mRNA radiolabeling in the glomeruli (Glom; arrows).

LXRα ligand-induced cholesterol efflux in cultured glomerular mesangial cells. The effect of a LXRα-specific agonist, TO-901317, on ApoA-I-mediated cholesterol efflux was assessed in cultured rabbit mesangial cells. As shown in Fig. 7*A*, the basal spontaneous efflux of cholesterol in primary cultured mesangial cells was ~8%. ApoA-I significantly increased cholesterol efflux in untreated cells ($P < 0.05$), and LXR activation by TO-901317 markedly increased ApoA-I-mediated cholesterol efflux ($P < 0.05$), suggesting there is basal ABCA1 activity and that LXR activation can upregulate ABCA1 function in these cells. However, when we compared the net change between the TO-901317+ApoA1 group and the

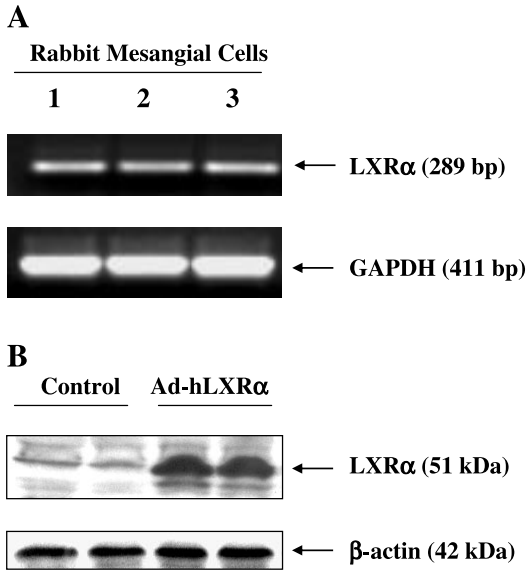


Fig. 6. LXRα is expressed in cultured rabbit glomerular mesangial cells. *A*: LXRα mRNA expression was detected using RT-PCR. A 289-bp rabbit LXRα cDNA fragment and a 411-bp GAPDH cDNA fragment were amplified. *Lanes 1–3*: 3 independent preparations of renal glomeruli from 3 individual rabbits. *B*: LXRα protein expression was detected in cultured mesangial cells. Cell lysates were prepared and analyzed for LXRα protein by immunoblotting. *Bottom*: blot was stripped and reprobed with a specific anibody against β-actin. Rabbit mesangial cells were treated with (2 *right* lanes) or without (2 *left* lanes; control) an adenovirus containing a full-length human LXRα cDNA (Ad-hLXRα). Note that ~51-kDa bands corresponding to predicted full-length LXRα protein were observed in untreated mesangial cells as well as in Ad-hLXRα-treated cells. β-Actin was utilized as a control for protein loading.

TO-901317 group vs. the net change between the ApoA1 group and control group, the net change did not reach statistical significance. Because cellular cholesterol homeostasis is tightly controlled by the balance of uptake, synthesis, esterification, and efflux (34), one possible explanation is that TO-901317 pretreatment altered lipid metabolism and increased the basal cholesterol efflux, which suggests another LXR-targeting gene, e.g., sterol-response element-binding protein 1c, may also play a role.

Enhanced transcriptional activity of ABCA1 by LXRα activation in mesangial cells. To explore the molecular mechanism underlying the LXRα-induced cholesterol efflux in mesangial cells, we examined the effect of LXRα activation on the gene expression driven by ABCA1 with a promoter reporter assay. Cultured rabbit mesangial cells were transfected with a luciferase reporter gene driven by the human ABCA1 gene promoter (pABCA1–116-luc) and then treated with the LXRα agonist TO-901317. As shown in Fig. 7*B*, exposure to TO-901317 for 24 h significantly increased the promoter activities of ABCA1 gene by 240% compared with controls ($P < 0.01$).

DISCUSSION

The present studies provide novel evidence that LXRα is expressed in vivo in renal glomeruli and in cultured mesangial cells, where this receptor may promote cholesterol efflux. To facilitate further investigation into the physiological and pathophysiological roles of this receptor in the kidney, we cloned a rabbit LXRα. The cloned LXR isoform corresponds to human and mouse LXRα (41, 44). The predicted amino acid sequence for the rabbit LXRα is highly conserved compared with these other species with ~90% identity between all species (41, 44). As with other nuclear receptors, the rabbit LXRα contains a putative DBD and an LBD. Transfection with an expression vector containing the cloned full-length rabbit LXRα activated the LXRE 3x-driven luciferase reporter in a heptocarcinoma cell line, HepG2, thereby documenting functional activity of this clone. Successful cloning of the rabbit LXRα cDNA sequence in the present study enabled us to further examine LXRα expression in rabbit tissues using a nuclease protection assay. The RNase protection assay demonstrated high levels of LXRα expression in white fat tissue, spleen, and liver with relatively low but significant expression levels in the lung, adrenal gland, ovary, testis, and kidney. This expression pattern is consistent with known roles for LXRα in promoting cholesterol metabolism in these tissues (2, 10, 39). Furthermore, by examining the expression of LXRα in normal kidneys and kidneys from TZD-treated rabbits using in situ hybridization and RT-PCR, we found that LXRα mRNA was widely

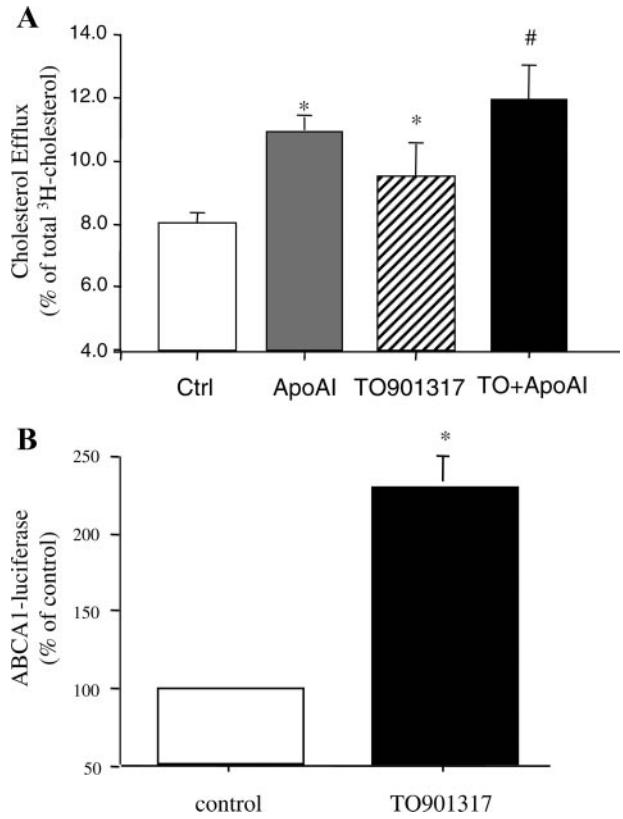


Fig. 7. *A*: effect of LXRα agonist TO-901317 (TO) on cholesterol efflux. Rabbit renal glomerular mesangial cells in 12-well plates were treated with TO-901317 (10 μM) in 10% FBS-supplemented DMEM for 24 h and then labeled with [^{3}H]cholesterol (0.2 μCi/ml) for 6 h. After a 2-h incubation with apolipoprotein AI (ApoAI), radioactivity levels in the medium and cell lysates were assayed by liquid scintillation counting. The results are given as media radioactivity as a percentage of total medium + cell lysate radioactivities. Values are means ± SD of 3 experiments in triplicate. *$P < 0.05$ vs. control. #$P < 0.05$ vs. ApoAI alone and TO-901317 alone. *B*: luciferase reporter activity demonstrating activation of human ATP-binding cassette transporter A1 (hABCA1) promoter by the LXRα agonist TO-901317 in cultured rabbit mesangial cells. Mesangial cells were transfected with LXRE3x-luciferase reporter plasmid. Twenty-four hours after transfection, cells were treated with TO-901317 (10 μM) for 24 h. Values are means ± SD of 4 wells in a single experiment representative of 3 independent experiments ($n = 4$, *$P < 0.01$).

expressed along the nephron. Furthermore, LXRα expression in the glomeruli was greatly enhanced after a 2-wk treatment with three different TZDs including troglitazone, rosiglitazone, and TZD3. These findings suggest that LXRα was expressed in the kidney, and its expression can be selectively induced in the glomeruli. The expression of LXRα in the glomeruli is consistent with the possibility that this nuclear receptor may play an important role in lipid homeostasis in renal glomeruli.

As in other vasculature, renal glomeruli are continuously exposed to high levels of endogenous plasma lipids including physiological cholesterol-containing lipoproteins and pathophysiological oxidized LDL (oxLDL). Normally, the pathways for cholesterol synthesis, uptake, and efflux are in equilibrium, maintaining stable intracellular lipid levels (1). Under pathophysiological circumstances including hypercholesterolemia, hypertriglyceridemia, and inflammation, the cholesterol influx pathway is enhanced through increased expression of scavenger receptor-A (SR-A) and CD36/SR-BII, while the cholesterol efflux pathway is inhibited, likely via decreased expression of ABCA1. This leads to intracellular lipid accumulation and, ultimately, foam cell formation (1, 35). Growing evidence has indicated that lipid-mediated renal injury or "glomerular atherosclerosis" shares similar pathogenic mechanisms with atherosclerosis (20, 35) and accelerates the progression of renal disease (29). It has been previously shown that glomerular cells, especially mesangial cells, possess characteristics similar to macrophages and VSMCs and are capable of taking up native and oxLDL through the LDL receptor (LDLR) and the scavenger receptors (SR-A, SR-BI, and SR-BII), contributing to the formation of glomerular foam cells characterized by massive intracellular lipid accumulation under inflammatory conditions (35, 36). To date, the underlying mechanism contributing to glomerular mesangial cell lipid homeostasis remains poorly characterized. The present studies clearly demonstrated that glomerular mesangial cells have functional LXRα expression. Activation of LXRα by its specific agonist TO-901317 markedly increased ApoAI-mediated cholesterol efflux in cultured mesangial cells, suggesting that LXRα may participate, at least in part, in cholesterol transport in renal mesangial cells.

LXRα is an essential transcription factor controlling cholesterol catabolism in the liver and modulating cholesterol efflux in hepatocytes, intestinal epithelial cells, VSMCs, and macrophages (18). To date, more than a dozen LXR target genes have been identified, and the majority of the known target genes are involved in lipid metabolism. Among them is the ABC protein ABCA1, a membrane transporter mediating removal of cholesterol from the cells. ABCA1 has been shown to be directly regulated by LXRα and to mediate LXRα activation-induced cholesterol efflux in macrophages (18, 22). In the present studies, we found LXRα is also expressed in renal glomeruli and cultured glomerular mesangial cells. Moreover, using a human ABCA1 promoter-driven luciferase reporter system, we were able to demonstrate that activation of LXRα increased ABCA1 transcriptional activity. These observations suggest that mesangial LXRα is functional and may promote cholesterol efflux by increasing mesangial ABCA1 expression. This conclusion is supported by the fact that LXRα activation by its agonist TO-901317 stimulated cholesterol efflux in cultured mesangial cells.

Although it seems clear that LXRα is expressed in glomeruli, little is known regarding its regulation in the kidney. It has been previously reported that LXRα transcription is under direct control by another metabolic nuclear receptor, PPARγ, in macrophages, possibly through binding to a consensus PPRE sequence in the LXRα promoter region (6, 10). As a result, PPARγ agonists markedly increased LXRα expression in macrophages (6). The present studies showed that three distinct TZD PPARγ agonists upregulate LXRα in renal glomeruli in vivo, further supporting control of renal LXRα expression by PPARγ. Although we and other groups have previously demonstrated that renal glomerular cells, including mesangial cells, podocytes, and endothelial cells, have functional PPARγ expression (13, 27), at present it remains largely uncertain why only glomerular LXRα is selectively induced by PPARγ activation. One possible explanation is that renal

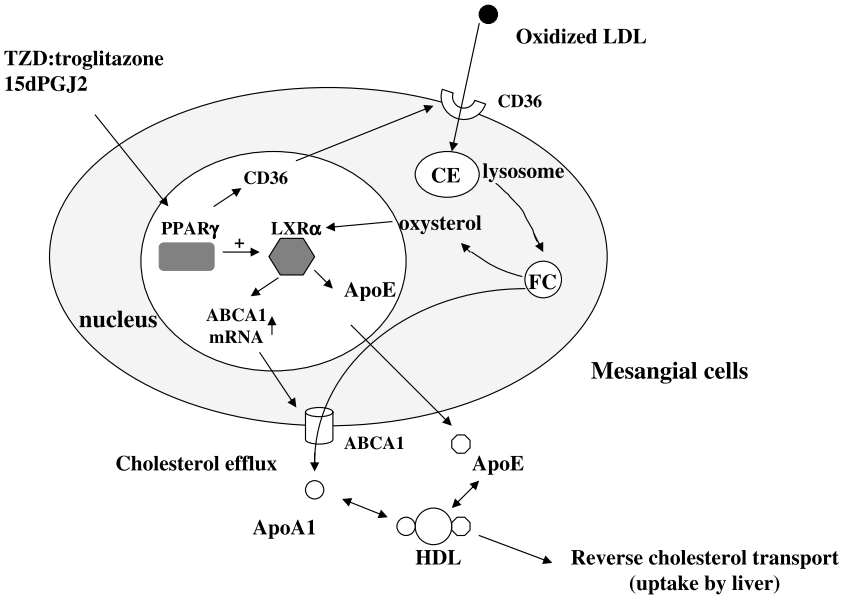


Fig. 8. Schematic illustration of the mechanisms by which LXRα enhances cholesterol efflux (CE) in mesangial cells. Mesangial cells take up native LDL via the LDL receptor (LDLR) or modified LDL through scavenger receptors. Free cholesterol (FC) is then released from the endocytosed LDL particles in lysosomes and then activates the LXRα receptor. Activation of LXRα by free cholesterol induces the synthesis of the cholesterol transporter ABCA1 and the important lipoprotein ApoE, thereby stimulating cholesterol removal from mesangial cells. Because LXRα is a direct target gene of peroxisome proliferator-activated receptor-γ (PPARγ), PPARγ ligands, including TZD, rosiglitazone, and pioglitazone, may also increase cholesterol efflux through PPARγ-LXRα-ABCA1 pathway in mesangial cells, contributing to their beneficial effect on glomerular disease.

glomeruli possess a unique nuclear receptor cofactor complex that allows PPARγ to modulate LXRα transcription. Accumulating evidence suggests that coactivators and corepressors are critical for controlling the expression and activity of the nuclear receptors in a given cell or tissue (24, 32). It remains to be determined whether particular cofactors are differentially expressed in glomeruli and other parts of the nephron.

Accumulating evidence also suggests that PPARγ activity may play a protective role in atherogenesis and glomerulosclerosis (27, 42). The fact that PPARγ promotes adipogenesis raised the worrisome possibility that PPARγ might promote foam cell formation in both vascular and nonvascular tissues, including the kidney, in patients receiving TZD treatment. In contrast, recent data from animal and clinical studies indicate that TZD PPARγ ligands consistently decrease foam cell formation in atherosclerotic lesions independently of changes in circulating lipid levels, blood pressure, and glycemic control (8, 25). The underlying beneficial mechanisms may be due to increased cholesterol efflux from macrophages by enhanced expression of the ABAC1 gene, as a consequence of activating the PPARγ-LXR-ABAC1 pathway (6, 7). In the present study, we demonstrated that a functional PPARγ-LXRα-ABAC1 pathway may also exist in renal glomeruli, especially glomerular mesangial cells. Activation of PPARγ upregulates LXRα expression in glomeruli, and LXRα-driven ABCA1 expression is enhanced, resulting in increased cholesterol removal from mesangial cells. Thus, although PPARγ is involved in both the influx (oxLDL-CD36 pathway) and efflux (LXR-ABAC1 pathway) of cholesterol in macrophages, the net effect appears to be removal of cholesterol from mesangial cells, thereby reducing intracellular lipid accumulation and foam cell formation. This observation further supports the idea that the beneficial effects of PPARγ activators on glomerular disease could be mediated by activation of the glomerular PPARγ-LXR-ABCA1 pathway (3, 5, 14, 17, 27, 28) (Fig. 8).

In summary, we reported the cloning of rabbit LXRα and provided evidence that endogenous LXRα activity is associated with renal glomeruli in vivo. Functional activity of glomerular LXRα in vivo was supported by demonstrating the capacity of TZD PPARγ ligands to induce its expression in renal glomeruli. We also demonstrated endogenous LXRα activity in cultured glomerular mesangial cells, where activation of this receptor enhances ABCA1 expression and cholesterol efflux. These results suggest that renal glomerular LXRα or, more specifically, LXRα in glomerular mesangial cells, could provide a direct target for ameliorating intracellular lipid accumulation in glomerulopathies, including lipid-induced glomerular injury and diabetic nephropathy.

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