Activation of the Nuclear Receptor LXR by Oxysterols Defines a New Hormone Response Pathway*

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Accumulation of cholesterol causes both repression of genes controlling cholesterol biosynthesis and cellular uptake and induction of cholesterol 7α-hydroxylase, which leads to the removal of cholesterol by increased metabolism to bile acids. Here, we report that LXRα and LXRβ, two orphan members of the nuclear receptor superfamily, are activated by 24(S),25-epoxycholesterol and 24(S)-hydroxycholesterol at physiologic concentrations. In addition, we have identified an LXR response element in the promoter region of the rat cholesterol 7α-hydroxylase gene. Our data provide evidence for a new hormonal signaling pathway that activates transcription in response to oxysterols and suggest that LXRs play a critical role in the regulation of cholesterol homeostasis.

Cholesterol (CH) is a major structural constituent of cellular membranes and serves as the biosynthetic precursor for bile acids and steroid hormones. Animal cells can obtain CH endogenously through de novo synthesis from acetyl-CoA or exogenously through receptor-mediated endocytosis of low density lipoproteins. Cells must balance the internal and external sources of CH so as to maintain mevalonate biosynthesis while at the same time avoiding the accumulation of excess CH, which can result in diseases such as atherosclerosis, gallstones, and several lipid storage disorders (1).

CH homeostasis is maintained in part through feedback regulation of the low density lipoprotein receptor gene and at least two genes encoding enzymes in the CH biosynthetic pathway, 3-hydroxy-3-methylglutaryl coenzyme A synthase and 3-hydroxy-3-methylglutaryl coenzyme A reductase (2). Although increases in dietary CH lead to the inhibition of expression of these genes in vivo, it remains unclear whether CH or CH metabolites are responsible for this inhibition (2). Experiments performed in vitro using several different cell lines have indicated that derivatives of CH that are oxygenated on the CH side chain are significantly more potent in the suppression of sterol biosynthesis than CH (3). These oxysterols are produced through the actions of P450 enzymes in various metabolic pathways including bile acid synthesis in the liver and sex hormone synthesis in the adrenal glands. The in vitro activities of oxysterols together with their presence in vivo suggests that oxysterols may serve in metabolic feedback loops to regulate CH homeostasis.

Although CH and its oxysterol metabolites can repress gene transcription, in at least one instance dietary CH has been shown to stimulate gene expression. Expression of the cholesterol 7α-hydroxylase (CYP7A) gene, which encodes the enzyme responsible for the initial and rate-limiting step in the conversion of CH to bile acids (4), is up-regulated in rats fed a CH-rich diet (5–7). This stimulatory effect provides a regulatory mechanism whereby excess dietary CH can be converted to more polar bile acids for subsequent removal from the body. Although the molecular mechanism is unknown, induction of CYP7A expression in the presence of CH occurs at the level of gene transcription (8, 9).

A variety of CH derivatives, including steroid hormones and vitamin D, exert effects on gene expression through interactions with members of the nuclear receptor superfamily (10). Members of this family function as ligand-activated transcription factors by binding to short stretches of DNA, termed hormone response elements, present in the regulatory regions of target genes. In addition to the nuclear receptors with known ligands, this superfamily includes a large number of structurally related members that contain DNA binding domains and putative ligand binding domains but lack identified ligands, the so-called “orphan receptors.”

In this report, we have identified a binding site for the orphan nuclear receptor LXRα in the proximal promoter region of the rat CYP7A gene. LXRα and the closely related orphan receptor LXRβ are broadly expressed and bind to DNA as heterodimers with the retinoid X receptors (RXRs) (11–14). As part of an effort to identify natural LXR ligands, we have found that the oxysterols 24(S),25-epoxycholesterol and 24(S)-hydroxycholesterol activate both LXRα and LXRβ at concentrations consistent with those found in tissue extracts. Our results provide evidence for a novel oxysterol signaling pathway regulating hepatic CH homeostasis that may also be important in mediating other cellular and developmental effects of CH.

MATERIALS AND METHODS

Chemical Reagents—24(S),25-epoxycholesterol, 24(R),25-epoxycholesterol, 24(S)-hydroxycholesterol, and 24(R)-hydroxycholesterol were prepared as described previously (20, 21). 24-Ketocholesterol was synthesized from cholic acid by conversion to the Weinreb amide and reaction with isopropyl magnesium chloride. All other compounds were obtained through commercial sources (Sigma; Steraloids).

Plasmids—To generate the plasmids pSG5-mLXRα, pSG5-hLXRβ, and pSG5-hRXRα, the cDNAs encoding the murine LXRs, human LXRG, or human RXRα were inserted into the expression vector pSG5 (Stratagene). The GAL4-LXR constructs contain in the pSG5 expression vector the translation initiation sequence and amino acids 1–76 of the glucocorticoid receptor fused to the DNA-binding domain of the yeast
transcription factor GAL4 (amino acids 1–147) and the SV40 large T antigen nuclear localization signal (APKKKKRKKKV). The cDNAs encoding amino acids 164–447 and 157–440 of the human LXRs and LXRβ were amplified by polymerase chain reaction and inserted C-terminal to the nuclear localization sequence to generate the plasmids pSG5GAL4-LXRa and pSG5GAL4-LXRβ, respectively. The reporter plasmids (CYP7-LXRE), tkt-CAT and (DR-4)-tkt-CAT were generated by inserting four copies of the CYP7-LXRE (5’-gatcCCTTGGTCTCAGTCAAGTGC) or the DR-4-LXRE (5’-gatcCCTTTGCTCACAGTCAAGTGC) into the BamHI restriction site of pBlCAT2 (29).

**cotransfection assay**—CV-1 cells were plated in 24-well plates in Dulbecco’s modified Eagle’s medium supplemented with 10% charcoal-stripped fetal calf serum at a density of 1.2 × 10^5 cells/well. In general, transfection mixtures contained 33 ng of receptor expression vector, 100 ng of reporter plasmid, 200 ng of β-galactosidase expression vector (pCH110, Pharmacia Biotech Inc.), and 166 ng of carrier plasmid. Cells were transfected overnight by lipofection using Lipofectamine (Life Technologies Inc.) according to the manufacturer’s instructions. The medium was changed to Dulbecco’s modified Eagle’s medium supplemented with 10% delipidated calf serum (Sigma), and compound was added for 24 h. Cell extracts were prepared and assayed for chloramphenicol acetyltransferase (CAT) and β-galactosidase activities as described previously (30).

**gel mobility shift assay**—LXRa, LXRβ, and RXRα were transcribed and translated in vitro using pSG5-mLXRα, pSG5-mLXRβ, and pSG5-hRXRa as templates and the TNT coupled transcription/translation system (Promega). Gel mobility shift assays (20 μl) contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.1% Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 0.2 μg of poly(dI-dC), 2.5 μl each of in vitro synthesized LXRa or LXRB, and RXR proteins. The total amount of reticulocyte lysate was maintained constant in each reaction (5 μl) through the addition of unprogrammed lysate. After a 10-min incubation on ice, 1 ng of 32P-labeled oligonucleotide was added, and the incubation continued for an additional 10 min. DNA-protein complexes were resolved on 4% polyacrylamide gel in 0.5 × TBE (1 × TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at −70 °C. Liver nuclear extracts were prepared as described previously (31). In experiments performed with liver nuclear extracts, 5 μg of extract was used, the amount of poly(dI-dC) in each reaction was increased to 8 μg, and DNA-protein complexes were resolved on 8% polyacrylamide gels. The following double-stranded oligonucleotides were synthesized and used in the gel mobility assay (sense strand, mutated nucleotides underlined): CYP7-LXRE, GATCCCTTTGCTCACAGTCAAGTGC (mCYP7-LXRE, in which the second half-site has been mutated to the GR recognition sequence, are shown). For comparison, the sequences of the idealized DR-4-LXRE and the mCYP7-LXRE, in which the second half-site has been mutared to the GR recognition sequence, are shown. Arrows indicate potential response element half-site motifs. B, LXR binds with high affinity to the CYP7-LXRE as a heterodimer with RXR. Gel mobility shift assays are shown in which mLXRα (upper panel), hRXRa (lower panel), hRXRa, or a combination of LXR and RXR were incubated as indicated with radiolabeled probes corresponding to CYP7-LXRE or DR-4-LXRE. Specificity of the binding complex was tested by adding a 5- or 25-fold molar excess of CYP7-LXRE, DR-4-LXRE, or mCYP7-LXRE as indicated.

**results and discussion**

The CYP7A Promoter Contains an LXR-RXR Heterodimer Binding Site—Previous work had identified a region of the CYP7A promoter that interacts with nuclear proteins and contains a motif that closely resembles known binding sites for members of the nuclear receptor family (15, 16). This putative response element is composed of a nearly perfect tandem repeat of the AGTTCA motif (Fig. 1, upper panel). To investigate whether the DR-4 motif in the CYP7A promoter could function as an LXR response element, we performed a series of gel retardation assays. First, to verify that the in vitro synthesized receptor proteins were functional, we used an oligonucleotide in which two nucleotides in the 5’ half-site of the CYP7A direct repeat sequence were mutated to obtain an idealized, tandem repeat of the AGTTCA motif (Fig. 1A, DR4-LXRE). Consistent with previous findings (11–14), a strong complex was formed when either LXRa or LXRβ was added together with RXR, indicating that high affinity DNA binding required the formation of LXR-RXR heterodimers (Fig. 1B, lane 3). Interestingly, a weaker complex migrating slightly faster than the LXR-RXR heterodimer was observed when LXRa was used in the absence of RXR (Fig. 1B, lane 1), suggesting the weak binding of an LXR homodimeric complex. In experiments performed with an oligonucleotide containing the CYP7A DR-4 element (Fig. 1A), strong binding was observed with both the LXRa-RXR and LXRβ-RXR heterodimers (Fig. 1B, lane 6). This binding was sequence-specific because an excess of either the unlabelled CYP7 oligonucleotide or the idealized DR-4-LXRE oligonucleotide (Fig. 1B, lanes 7–10) competed efficiently for binding to the probe, but no competition for binding was seen with an oligonucleotide in which the 3’ half-site had been mutated to a consensus glucocorticoid receptor half-site (mCYP7-LXRE) (Fig. 1, A and B, lanes 11 and 12). As observed with the DR-4-LXRE, a weak and slightly faster migrating complex of a potential LXRa homodimer was seen when LXRa was added alone to the CYP7A direct repeat element (Fig. 1B, lane 4). Based on these data, we refer to the DR-4 of the CYP7A promoter as the CYP7-LXRE.

LXRa is abundantly expressed in the liver (11, 12), the site of CYP7A expression. To determine whether endogenously expressed LXRa bound to the CYP7-LXRE, we prepared nuclear extracts from rat liver for use in gel retardation assays. A strong, shifted complex was observed in gel assays performed with the CYP7-LXRE and liver extracts (Fig. 2B, lane 3) that migrated at the same position as the one obtained with in vitro synthesized LXRa and RXR protein (Fig. 2B, lane 1). A portion of this complex was supershifted (Fig. 2B, lane 4) upon the addition of a pool of monoclonal antibodies that specifically recognizes the LXRa but not the LXRβ ligand binding domain (Fig. 2A). An analogous supershifted complex was seen in experiments performed with these antibodies and in vitro syn-

**fig. 1. LXR response element in the proximal CYP7A promoter. A, schematic representation of the CYP7A gene and the sequences of the CYP7-LXRE located between nucleotides −72 and −57. For comparison, the sequences of the idealized DR-4-LXRE and the mCYP7-LXRE, in which the second half-site has been mutated to the GR recognition sequence, are shown. Arrows indicate potential response element half-site motifs. B, LXR binds with high affinity to the CYP7-LXRE as a heterodimer with RXR. Gel mobility shift assays are shown in which mLXRα (upper panel), hRXRa (lower panel), hRXRa, or a combination of LXR and RXR were incubated as indicated with radiolabeled probes corresponding to CYP7-LXRE or DR-4-LXRE. Specificity of the binding complex was tested by adding a 5- or 25-fold molar excess of CYP7-LXRE, DR-4-LXRE, or mCYP7-LXRE as indicated.**
the position of the antibody supershifted LXR
pathway or in the presence of testosterone or progesterone (Fig. 2A). In vitro synthesized LXRα or LXRβ with radiolabeled CYP7-LXRE probe in the presence or the absence of a pool of monoclonal antibodies (Ab-LXRα) generated against the LXRα ligand binding domain (amino acids 164–447). The arrow indicates the position of the antibody supershifted LXRα-RXR heterodimer. No supershifted complex was seen when the pool of antibodies was incubated with LXRβ-RXRα or PPAR-RXRα (data not shown). 2.4-LXRα binding domain (amino acids 164–447). The arrow indicates the position of the antibody supershifted LXRα-RXR heterodimer.

Oxysterols Activate LXRα and LXRβ—The presence of a binding site for LXR in the promoter of the CYP7α gene, which encodes the rate-limiting enzyme responsible for the conversion of CH to bile acids, prompted us to test a comprehensive set of CH precursors and metabolites including bile acids, oxysterols, and steroids for their ability to activate LXRα and LXRβ (Fig. 3A). The compounds were initially tested using chimeric receptor proteins containing the LXRE DNA binding domain of the yeast transcription factor GAL4 fused to the single receptor at a concentration of 10 μM. As shown in Fig. 3A, neither of the LXR chimeras was activated in response to CH or its precursors lanosterol and desmosterol. Interestingly, however, significant activation of both the LXRα and LXRβ was observed in transfected cells treated with the oxysterols 20(S), 22(R)-, and 24(S)-hydroxycholesterol (Fig. 3A). 25- and 27-hydroxycholesterol had relatively little or no effect on either LXR chimera (Fig. 3A). Notably, activation of the LXRα was more efficient with the LXRα chimera (data not shown). In contrast, no activation of the LXRβ chimera was seen in the presence of additional, downstream intermediates in the steroid hormone biosynthetic pathway or in the presence of testosterone or progesterone (Fig. 3A). 24-Hydroxycholesterol, its bile acid derivatives chenodeoxycholic acid and cholic acid, or bile salts also failed to activate the LXRα chimera (data not shown). In addition, steroids with the 3α-hydroxyl configuration were inactive (data not shown).

In the various hydroxycholesterol derivatives, we also examined the activity of 24(S),25-epoxycholesterol. We performed dose response analysis using expression plasmids for full-length LXRα or LXRβ. Reporter constructs were generated that contained four copies of either the CYP7-LXRE or the idealized DR-4-LXRE driving CAT gene expression. In preliminary experiments performed with 10 μM 24(S),25-epoxycholesterol, we found that LXRα induced expression of both...
In summary, we have demonstrated that the LXRs are activated by the oxysterols 24(S),25-epoxycholesterol and 24(S)-hydroxycholesterol at concentrations consistent with those found in tissues. It is well known that oxysterols suppress de novo CH biosynthesis as well as cellular uptake of CH (1). However, oxysterols have a number of other biological effects including marked effects on DNA synthesis and cell growth and proliferation (25). Our data suggest that some of these effects may be mediated through the LXRs. It is interesting that the nuclear receptor family member most closely related to the LXRs is the insect ecysone receptor (12, 13, 15). Ecysone is a sterol hydroxylated at the 22 and 25 positions that functions as a temporal signal to coordinate tissue-specific morphogenetic changes during insect development (27). The use of nuclear receptor pathways as a means to transduce the effects of oxygenated sterols thus appears to have been conserved throughout a large part of evolution, from insects to man.

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FIG. 4. Dose response analysis of LXRα and LXRβ with both enantiomers of 24-hydroxycholesterol and 24,25-epoxysterol. Expression plasmids for mLXRs (open symbols) or hLXRs (closed symbols) were cotransfected into CV-1 cells with the CYP7α-LXRE or DR-4-LXRE reporter constructs, respectively. Transfected cells were treated for 24 h with the indicated concentrations of 24(S),25-epoxycholesterol (circles) or 24( R),25-epoxycholesterol (squares) (A) or of 24(S)-hydroxycholesterol (circles) or 24(R)-hydroxycholesterol (squares) (B). Normalized CAT activity was determined and plotted as the percentage of the maximal response obtained.

the CYP7α-LXRE-CAT and DR-4-LXRE-CAT reporter constructs but that LXRβ only efficiently induced expression of the DR-4-LXRE-CAT reporter construct (data not shown). We speculate that LXRβ may not bind to the CYP7α-LXRE with sufficient affinity to activate reporter expression. Subsequent dose response analyses with LXRs and LXRβ were performed with the CYP7α-LXRE-CAT and DR-4-LXRE-CAT reporter constructs, respectively. LXRs and LXRβ responded to 24(S),25-epoxycholesterol with EC50 values of 7.5 and 1.5 μM, respectively (Fig. 4A). 24(R),25-Epoxycholesterol, which has not been found to occur naturally, was significantly less active with an EC50 greater than 10 μM on both LXRs. 24(S)-Hydroxycholesterol had an activation profile similar to that of 24(S),25-epoxycholesterol on the LXRs, activating LXRs and LXRβ with EC50 values of 7 and 1.5 μM, respectively (Fig. 4B). The synthetic isomer 24(R)-hydroxycholesterol was at least 1 order of magnitude less active. 20(R)-Hydroxycholesterol, 22(R)-hydroxycholesterol, and 24-ketonecholesterol displayed EC50 values for LXR activation that were greater than 10 μM (data not shown).

Based upon these data, we suggest that 24(S),25-epoxycholesterol, which is abundant in liver, and 24(S)-hydroxycholesterol, which is abundant in brain, may function as endogenous activators of LXRs and LXRβ. We note that both LXRs are expressed in the liver. Interestingly, Northern analyses have demonstrated that LXRβ is expressed in the brain (14). More detailed in situ studies have shown that LXRβ is broadly expressed in fetal brain and that its expression becomes more restricted in postnatal and adult brains (26). Taken together, these findings support the suggestion that 24(S)-hydroxycholesterol may serve in the development and function of the brain via interactions with LXRβ (22).