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Regulation of Human 3α-Hydroxysteroid Dehydrogenase (AKR1C4) Expression by the Liver X Receptor α

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ABSTRACT

Type I human hepatic 3α-hydroxysteroid dehydrogenase (AKR1C4) plays a significant role in bile acid biosynthesis, steroid hormone metabolism, and xenobiotic metabolism. Utilization of a hidden Markov model for predictive modeling of nuclear hormone receptor response elements coupled with chromatin immunoprecipitation/microarray technology revealed a putative binding site in the *AKR1C4* promoter for the nuclear hormone receptor known as liver X receptor α, (LXRα [NR1H3]), which is the physiological receptor for oxidized cholesterol metabolites. The putative LXRα response element (LXRE), identified by chromatin immunoprecipitation, was ~1.5 kilobase pairs upstream of the transcription start site. LXRα was shown to bind specifically to this LXRE and mediate transcriptional activation of the *AKR1C4* gene, leading to increased AKR1C4 protein expression. These data suggest that LXRα may modulate the bile acid biosynthetic pathway at a unique site downstream of CYP7A1 and may also modulate the metabolism of steroid hormones and certain xenobiotics.

Human 3α-hydroxysteroid dehydrogenases (3α-HSD), members of the aldo-keto reductase (AKR) enzyme superfamily, are essential enzymes in metabolic pathways, including the biosynthesis of bile acids and the metabolism of steroid hormones and xenobiotics. AKRs are soluble NAD(P)(H) oxidoreductases that reduce aldehydes and ketones to primary and secondary alcohols. Type I 3α-HSD (AKR1C4) displays a pattern of liver-specific expression and functions both to produce intermediates in bile acid biosynthesis and inactivate circulating steroid hormones (Penning et al., 2000). AKR1C4 protects against circulating steroid hormone excess by catalyzing the reduction of 3-ketosteroids to 3α-hydroxysteroids (Jin and Penning, 2007). In addition, AKR1C4 catalyzes the production of 3α,7α,12α-trihydroxy-5β-cholestane, a committed precursor within the bile acid biosynthetic pathway, from 7α,12α-diihydroxy-5β-cholestan-3-one.

The liver X receptors (LXRα [NR1H3] and LXRβ [NR1H2]) are members of the nuclear hormone receptor (NHR) superfamily and function as ligand-activated transcription factors. Both LXRα and LXRβ are expressed in the liver (with LXRα levels significantly higher than LXRβ levels) and function as physiological receptors for oxidized cholesterol metabolites, oxysterols. LXRs play an important role in the regulation of bile acid biosynthesis by either directly (most rodents) or indirectly (primates) regulating the expression of CYP7A1, the rate-limiting enzyme in bile acid biosynthesis (Chiang, 2004). Because production of bile acids from cholesterol is the metabolic pathway for degradation of cholesterol, LXRs serve as "cholesterol sensors" to provide levels of CYP7A1 expression that maintain appropriate concentrations of cholesterol. In addition to their roles in bile acid biosynthesis, LXRs regulate an array of genes involved in cholesterol and lipid synthesis and transport (Michael et al., 2005).

In this study, we use a combination of LXRE predictive mod-

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ABBREVIATIONS: HSD, hydroxysteroid dehydrogenase; AKR, aldo-keto reductase; LXR, liver X receptor; LXRE, liver X receptor response element; T0901317, *N*-[4-(1,1,1,3,3,3-hexafluoro-2-hydroxypropan-2-yl)phenyl]-*N*-(2,2,2-trifluoroethyl)benzenesulfonamide; DMSO, dimethyl sulfoxide; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; RXR, retinoid X receptor; ChIP, chromatin immunoprecipitation; BAP, bacterial alkaline phosphatase; bp, base pair(s); T1317, *N*-[4-(1,1,1,3,3,3,-hexafluoro-2-hydroxypropan-2-yl)phenyl]-*N*-(2,2,2-trifluoromethyl)benzene sulfonamide; siRNA, small interfering RNA; HMM, hidden Markov model.

eling and ChIP/microarray technology to identify *AKR1C4* as a potential direct target gene of the nuclear hormone receptor LXRα. LXRα directly regulates the expression of the human *AKR1C4* gene. Because AKR1C4 is involved in bile acid biosynthesis, our finding suggests a novel mechanism by which oxysterols might modulate the rate of bile acid production and, hence, cholesterol degradation. In addition, AKR1C4 plays a key role in hepatic metabolism of steroid hormones; therefore, it is possible that oxysterols influence this pathway via activation of LXR.

Materials and Methods

Cell Culture and mRNA/Protein Measurement. HuH7 and HepG2 cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 (1:3) supplemented with 10% fetal bovine serum at 37°C under 5% CO_2. Twenty-four hours before treatment, HuH7 and HepG2 cells were plated in six-well plates in serum-free media (Dulbecco's modified Eagle's medium/Ham's F12, 1:3) at a density of 1×10^6 cells/well. Cells were stimulated with the LXRα agonist T0901317 (1 μM in 0.1% DMSO). Total cellular RNA was isolated 24 h after stimulation. cDNA synthesized from mRNA was quantified by TaqMan analysis as described previously (Savkur et al., 2005; Stayrook et al., 2005). AKR1C4 protein levels were assessed by Western blot using an antibody from Abnova (Taipei, Taiwan). LXRα and control siRNA was obtained from Ambion (Austin, TX).

Plasmid Construction. Full-length untagged LXRα and LXR$\alpha^{\Delta AF2}$ were constructed by cloning PCR-amplified DNA fragments into the BamHI and EcoRV sites of pcDNA3.1D/V5-His-TOPO (Invitrogen, Carlsbad, CA). The plasmid expressing FLAG-tagged LXRα was constructed by inserting the PCR product for LXRα into 3xFLAG pCMV7.1 (Sigma, St. Louis, MO). The 3α-HSD-tk-Luc reporter plasmid was generated by PCR amplification of human genomic DNA (−1899 to −1226 of the human *AKR1C4* promoter region) (Clontech, Mountain View, CA) as the template. The resulting PCR product was inserted between the MluI and BglII sites upstream of the minimal thymidine kinase promoter linked to a luciferase gene (pTA-Luc; Clontech). The mutation of the LXR binding site within the *AKR1C4* promoter was obtained by site-directed mutagenesis (Stratagene, La Jolla, CA). All constructs were verified by DNA sequence analysis.

Transient Cotransfection Experiments. Twenty-four hours before transfection, HuH7 cells were plated in serum-free medium in 96-well plates at a density of 15×10^3 cells/well. Each transfection contained 25 ng of the 3α-HSD-tk-Luc reporter and receptor as described in the figure legend using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN). Sixteen hours after transfection, cells were stimulated with T0901317 (1 μM in 0.1% DMSO). Twenty-four hours after treatment, the luciferase activity was measured using standard luciferase substrate reagents.

Electrophoretic Mobility Shift Assays. LXRα and RXRα were expressed using coupled in vitro transcription and translation. EMSAs were performed using the [α-^{32}P]dCTP labeled 3α-HSD LXRE oligonucleotide. Competition assays were performed using various amounts of the unlabeled wild type or the mutant oligonucleotide as described previously (Burris et al., 1995).

Chromatin Immunoprecipitation Assays. HuH7cells transfected with either 3×FLAG-tagged LXRα or 3×FLAG-tagged bacterial alkaline phosphatase (BAP) were subjected to ChIP using standard procedures with M2 mouse monoclonal anti-FLAG antibody (Sigma). The *3α-HSD* promoter region was PCR-amplified using the oligonucleotides 5′-TAATCTCTTGCCAGTGGCATCT-3′ (forward) and 5′-CACACAGAGTGTCATCAGCAGG-3′ (reverse) to yield a 168-bp product that was analyzed by electrophoresis on a 2% agarose gel. Endogenous LXRα and acetylated histone H3 chIP analysis was performed in HepG2 cells treated with DMSO or T1317 (1 μM) for 48 h using an anti-LXRα antibody (Abcam, Cambridge, MA) or anti-acetylated histone H3 antibody (Millipore, Billerica, MA). Precipitated DNA was quantitated using quantitative PCR with the primers indicated above for the promoter or the following *AKR1C4* primers for the acetylated histone H3 ChIP: forward, CTGCCTCCTGAGTTTAAGCAAT; reverse, CAAGGCGGTAGGTTTACTTGAG.

ChIP-Chip Analysis. ChIP/microarrays were performed from HuH7 cells transfected and incubated for 48 h with FLAG-tagged LXRα or FLAG-tagged control vector. Chromatin immunoprecipitations and chromosomal tiling array hybridization was performed by GENpathway, Inc. (San Diego, CA). Immunoprecipitated and amplified chromatin was hybridized to GeneChip Human Tiling 2.0R Array Set (Affymetrix, Inc., Santa Clara, CA). Microarray slides were scanned with the Affymetrix scanner (model 3000) and processed with Affymetrix GeneChip Operating Software (GCOS v1.4). The acquired .CEL files were analyzed with the MAT software v2.0 (Johnson et al., 2006). The NCBI36 assembly of the human genome (http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid = 9606) was used to map all Affymetrix probe sets from the GeneChip Human Tiling 2.0R Array Set. The signal intensities of the input DNA and the FLAG-control immunoprecipitated DNA were used as a normalization factor. The bandwidth parameter was set to 1000 bp, and the maximum gap between positive probes was 300 bp. The threshold p value was set to 10^{-5}. The genomic intervals with p values below this threshold were then retrieved from the genomic database and analyzed by the LXRE.HMM predictive model that was developed for the identification of LXR response elements (Varga and Su, 2007) to pinpoint candidate LXREs. The LXRE.HMM score cutoff was set to 2.5. Subsequently, the candidate LXREs were compared with known LXREs for further classification. The results from the HMM model and the MAT analysis were integrated and visualized using the Integrated Genome Browser (ver. 4.56; Affymetrix).

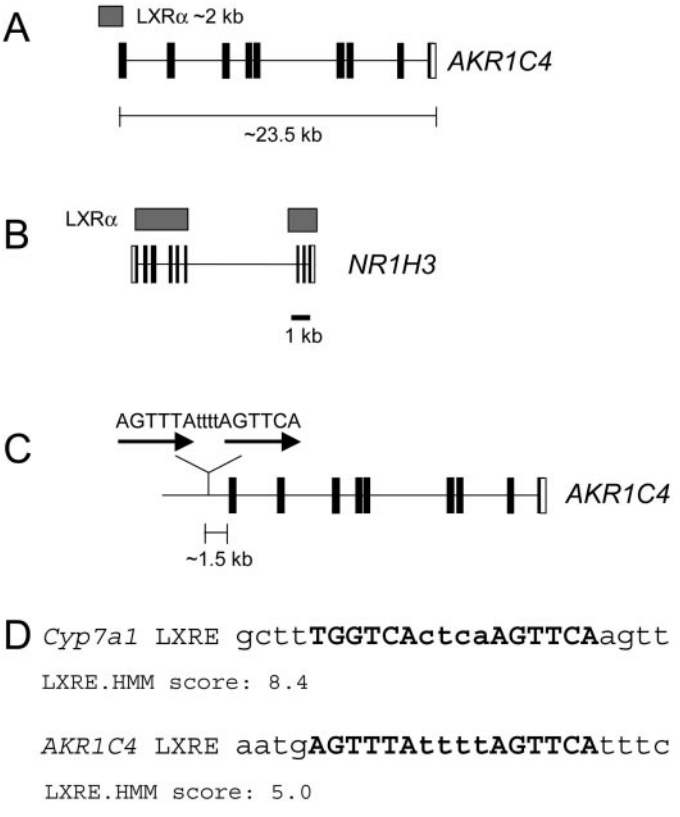

Fig. 1. Identification of a putative LXRE immediately upstream of the *AKR1C4* gene. A, diagram of the *ABCA1* gene indicating regions of significant LXRα occupancy identified in the ChIP-chip experiment. B, diagram of the *AKR1C4* gene indicating regions of significant LXRα occupancy identified in the ChIP-chip experiment. C, sequence of the DR4 element identified by HMM in the A*KR1C4* gene. D, sequence comparison of the putative *AKR1C4* LXRE to the *Cyp7a1* LXRE. HMM scores are indicated.

Results

By using predictive response element modeling and whole-genome analysis of LXRα occupancy sites using ChIP/microarray technology, we identified a relatively short region (~2 kb) immediately 5′ of the *AKR1C4* gene in chromosome 10p15-p14 that displayed significant signal (Fig. 1A). We also identified LXRα occupancy of several well characterized direct LXRα target genes, including *NR1H3* (Laffitte et al., 2001; Whitney et al., 2001; Li et al., 2002) (Fig. 1B), indicating that the Chip/microarray technology was successful. Analysis of this region in the LXRα occupancy site within the *AKR1C4* gene with the LXRE.HMM model revealed a single putative binding site with two direct repeating half-sites and a 4-bp spacer (DR4) located between positions 1548 and 1533 upstream of the transcription start site. The putative binding site exhibited similarities to the LXRE previously identified in the *Cyp7A1* gene (Fig. 1B) (Lehmann et al., 1997); the second half-site of the direct repeat was identical to the second half-site of the Cyp7A1 LXRE, whereas the first half-site contained three conserved nucleotides. Two of these three conserved nucleotides were also shown to be completely conserved across all known LXREs. The high degree of nucleotide conservation corroborated the hypothesis that the predicted DR4 site is a novel LXR binding site. To determine whether LXR was capable of binding to the putative *AKR1C4* LXRE, EMSAs were performed using in vitro translated LXRα and RXRα (Fig. 2). A radiolabeled oligonucleotide spanning the putative LXRE (−1552; Fig. 2B) was not able to bind to either LXRα or RXRα alone; however, the LXRα/RXRα heterodimer bound as illustrated by a shifted protein/DNA complex (Fig. 2). Furthermore, this shifted band was efficiently competed by the unlabeled oligonucleotide, indicating that LXRα/RXRα heterodimer binds to this response element in a specific manner in vitro. Previous studies studying the orientation of LXRα/RXRα heterodimer on DNA have demonstrated that LXRα occupies the 3′ half-site of the response element (Willy and Mangelsdorf, 1997). To determine the specificity of the LXRE in binding to LXRα, the LXR binding region within the LXRE was mutated (Fig. 2). Competition binding analysis of this mutant unlabeled oligonucleotide revealed that the shifted band using the radiolabeled wild-type oligonucleotide was not inhibited by even a 100-fold molar excess of the unlabeled mutant oligonucleotide, further demonstrating the specificity of binding of the LXRα/RXRα heterodimer to the LXRE in the *AKR1C4* promoter.

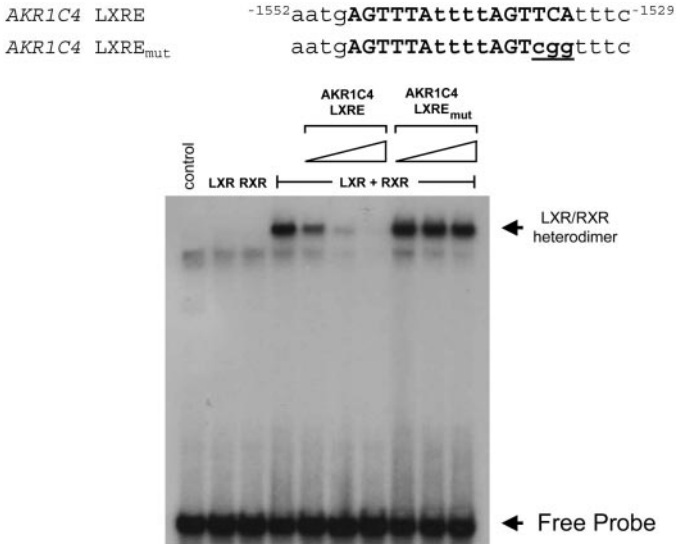

Fig. 2. LXRα binds to the *AKR1C4* LXRE. The sequence of the oligonucleotide probe corresponding to the putative LXR response element from *AKR1C4* and a mutated derivative of the LXR binding site (*AKR1C4* LXRE$_{mut}$; mutated bases are underlined in lower case). EMSA was performed using in vitro transcribed/translated LXRα and RXRα, as indicated, and the radiolabeled *AKR1C4* LXRE (−1552). Competition EMSA was performed with increasing concentrations (5×, 25×, and 100×) of unlabeled *AKR1C4* LXRE (−1552) or *AKR1C4* LXRE$_{mut}$ (−1552$_{mut}$). The positions of the shifted LXRα/RXRα complex and free probe are indicated.

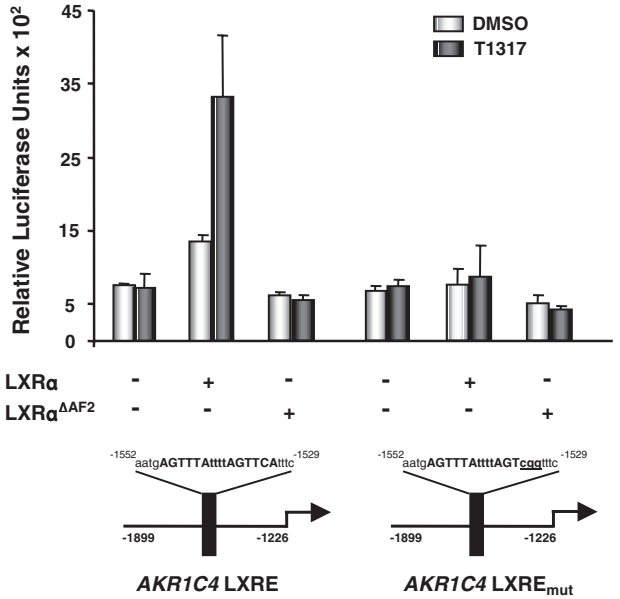

Fig. 3. Identification of a functional LXR response element within the *AKR1C4* promoter. HuH7 cells were transfected with the human wild-type (*AKR1C4* LXRE) or the mutated derivative of the LXR binding site (*AKR1C4* LXRE$_{mut}$; mutated bases are underlined in lower case) luciferase reporter plasmid (25 ng) along with 25 ng of a plasmid expressing either full-length LXRα (LXRα) or a plasmid expressing a helix 12 deleted LXRα (LXRα$^{\Delta AF2}$). Sixteen hours after transfection, cells were treated with DMSO or the LXR agonist (T0901317; 1 μM). Twenty-four hours after treatment, cells were harvested and the luciferase activity was measured.

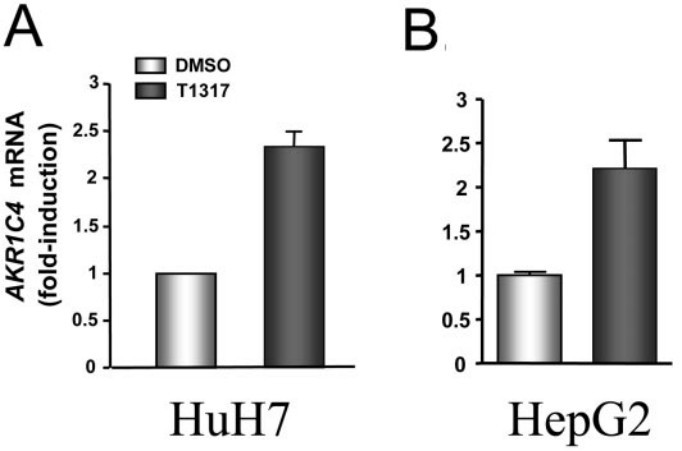

Fig. 4. Induction of *AKR1C4* by LXR agonists in human hepatoma cells. HuH7 and HepG2 cells were treated with T0901317 (1 μM) or DMSO for 24 h of HuH7 (A) or 6 h of HepG2 (B) followed by assessment of AKR1C4 mRNA expression. Values were normalized to 18S rRNA (HuH7) or *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase; HepG2) expression.

To characterize its LXR responsiveness, a fragment of the *AKR1C4* promoter (−1899 to −1226) containing the LXRE was cloned upstream of a minimal promoter directing the expression of a luciferase reporter. As shown in Fig. 3, when cotransfected into HuH7 hepatoma cells along with LXRα, there is a LXR ligand-dependent induction of luciferase activity. If a mutant of LXRα lacking the ability to activate transcription (helix 12 deletion-LXR$\alpha^{\Delta H12}$) is used instead of WT LXRα, the LXR dependence is lost. The identical mutation within the *AKR1C4* LXRE that eliminated LXR/RXR binding was introduced into the *AKR1C4* promoter reporter and cotransfected into HuH7 cells. All responsiveness to LXRα was lost, indicating that this LXRE confers LXR responsiveness to the promoter (Fig. 3).

We next examined the ability of the LXR ligand T1317 to induce *AKR1C4* expression in two human hepatoma cell lines, HuH7 and HepG2. Cells were treated for 24 h with 1 μM T1317 followed by quantitation of AKR1C4 mRNA by quantitative PCR. Figure 4A shows that *AKR1C4* mRNA increases approximately 2.3-fold in HuH7 cells in response to LXR ligand treatment. Consistent with this observation, *AKR1C4* mRNA is induced by the same degree in HepG2 cells by the LXR ligand (Fig. 4B). This level of induction is consistent with the degree of induction of other direct LXR target genes, such as PLTP (Laffitte et al., 2003). To confirm that LXRα occupies the LXRE within the *AKR1C4* promoter in vivo, we performed a ChIP assay in HuH7 cells that were transiently transfected with an expression vector expressing FLAG-tagged LXRα or FLAG-tagged BAP as the control. As illustrated in Fig. 5, LXRα occupancy was observed within the *AKR1C4* promoter and no signal was detected in the BAP control. LXRα occupancy of the site was ligand-independent. LXR agonist treatment (T1317) also induced AKR1C4 protein expression (~2.3-fold) in HepG2 cells after 48 h of treatment (Fig. 6A). Induction of *AKR1C4* mRNA was also detected after 48 h of treatment (~1.6-fold) with T1317, and the induction was LXRα-dependent because knock-down of LXRα with specific siRNA (~50% decrease in LXRα expression; Fig. 6B) significantly reduced the T1317 effect (Fig. 6, C and D). We examined the occupancy of the LXRE within the *AKR1C4* promoter by endogenously expressed LXRα in HepG2 cells by ChIP analysis as shown in Fig. 6E. LXRα was detected in the absence of the agonist, and addition of T1317 led to a significant increase in LXRα occupancy (Fig. 6E). In addition, we observed an increase in histone H3 acetylation of the *AKR1C4* gene when we treated HepG2 cells with T1317 (Fig. 6F). These data, taken together, indicate that the *AKR1C4* gene is directly regulated by LXRα and that regulation is ligand-dependent.

Discussion

Type I or hepatic 3α-HSD (AKR1C4) is an essential enzyme required for both biosynthesis of bile acids and metabolism of steroid hormones within the liver. In addition, AKR1C4 plays an important role in the metabolism of certain xenobiotics (Jin and Penning, 2007). In this study, we identified a putative LXRE in the proximal promoter of the *AKR1C4* gene using the LXRE.HMM predictive model coupled with LXR ChIP/microarray analysis. The LXRE.HMM prediction and subsequent analysis of nucleotide conservation of the binding site signature showed that the putative LXRE exhibited similarities to other DR4 classes of functional LXREs identified in other LXR responsive genes, such as the *Cyp7A1* gene (Lehmann et al., 1997). The *AKR1C4* LXRE bound directly to an RXR/LXR heterodimer, and when a fragment of the *AKR1C4* promoter was cloned upstream of a reporter, it conferred LXR- and LXR ligand-dependent reporter expression. In addition, *AKR1C4* mRNA expression was induced in two human hepatoma cell lines upon treatment with a specific LXR agonist, T1317. Thus, the human *AKR1C4* gene is directly regulated by LXRα. Given the role of LXR as a receptor for oxysterols, the above results provide unique insight into novel mechanisms of regulation of bile acid synthesis and steroid hormone metabolism via cholesterol metabolites.

Conversion of cholesterol to bile acids provides an essential method for elimination of cholesterol from animals. Cyp7A1 catalyzes the first and rate-limiting step in the classic pathway for bile acid production (Chiang, 2004). High-cholesterol

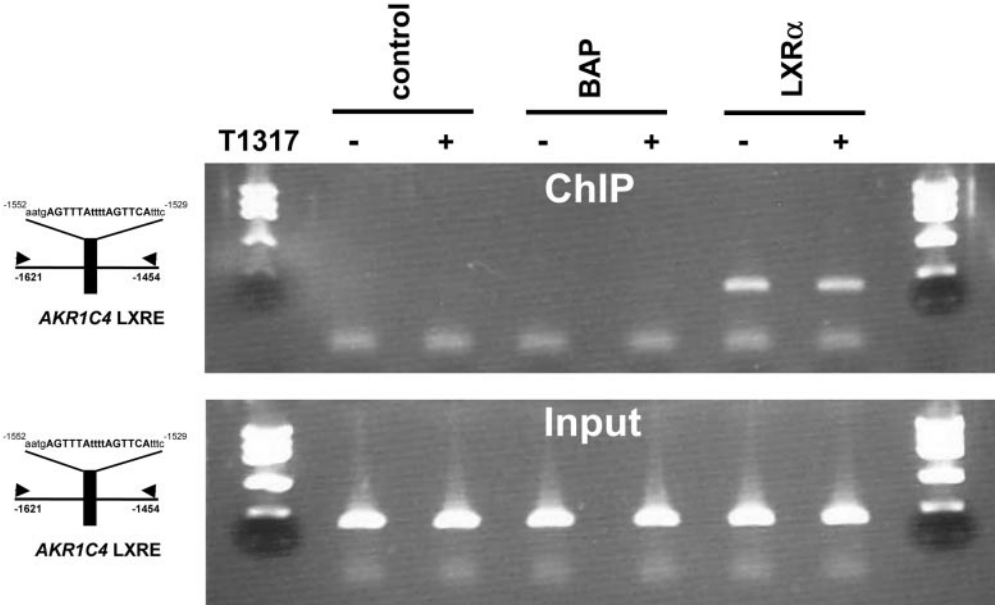

Fig. 5. LXRα binds to the AKR1C4 LXRE in vivo. Untransfected HuH7 cells (control) or cells transfected with 3×FLAG-tagged LXRα (LXRα-or 3×FLAG-tagged BAP) in the absence or presence of the LXR agonist T0901317 (T1317; 1 μM) were subjected to ChIP (upper panel) using an anti-FLAG antibody as described under *Materials and Methods*. In vivo AKR1C4 promoter occupancy was assessed by PCR amplification of the *AKR1C4* promoter regions using the forward and reverse primer sets. The lower panel (Input) represents DNA before immunoprecipitation that has been amplified using the same set of primers.

diets induce *Cyp7A1* expression in rats and mice, and the induction of this gene is mediated via oxysterol metabolites of cholesterol-activating LXR, which directly regulates transcription of *Cyp7A1* via a LXRE located in its promoter (Janowski et al., 1996; Lehmann et al., 1997). Thus, these rodents efficiently induce *Cyp7A1* expression in response to a high-cholesterol diet, increase cholesterol conversion to bile acids, and adapt to the diet with little effect on plasma cholesterol levels. In contrast, humans develop hypercholesterolemia in response to a high-cholesterol diet, which is consistent with the observation that *Cyp7A1* is not induced in this state as a result of the lack of conservation of the LXRE within the gene's promoter (Chiang et al., 2001; Agellon et al., 2002). Human *CYP7A1* expression is actually repressed by LXR agonists, an effect that was recently shown to be an indirect effect of LXR mediated via induction of *hSHP* expression (Goodwin et al., 2003). Our observation that human *AKR1C4* gene expression is induced by LXR ligands provides an additional point of potential regulation of bile acid synthesis downstream of Cyp7A1 by oxysterol metabolites of cholesterol. It is unclear whether this regulatory pathway functions in rodents. Rats seem to have a single 3α-HSD gene (AKR1C19) whereas humans have four: *AKR1C4*, *AKR1C3*, *AKR1C2*, and *AKR1C1* (Penning et al., 2003). Thus, direct comparison of the function and regulation of these pathways between the species is difficult.

Beyond its role in regulation of bile acid synthesis, AKR1C4 is an important enzyme involved in steroid hormone metabolism. Together with 5α- and 5β-HSDs, 3α-HSD is primarily responsible for the metabolism of most steroids (Penning, 1997). In the liver, AKR1C4 plays this important 3α-HSD role. Therefore, LXR and its ligands, oxidized cholesterol metabolites, may play a role in increasing the rate of metabolism of steroid hormones. Because cholesterol is the common precursor for biosynthesis of the steroid hormones, the fact that oxysterols that are thought to be general indicators of cholesterol levels or potentially regulating the degradation of the steroid hormones via LXR suggests that a feedback loop may be operating.

In addition, AKR1C4 is an important enzyme in the metabolism of several drugs, including tibolone and naltrexone.

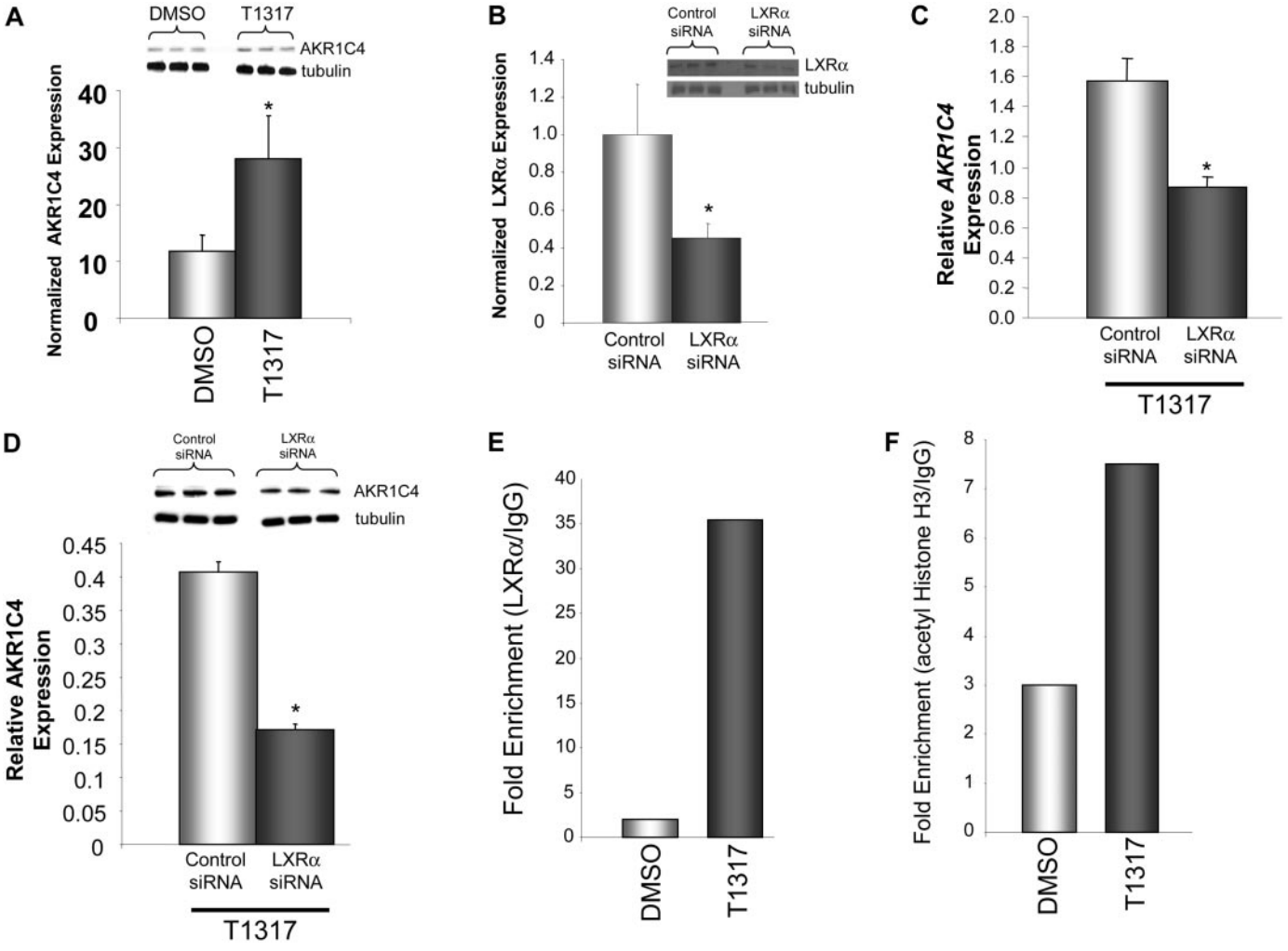

Fig. 6. LXRα regulates AKR1C4 protein levels. A, Western blot illustrating a 2.3-fold increase in AKR1C4 protein expression in response to T1317 treatment for 48h. B, Western blot illustrating the degree of loss of LXRα protein in response to siRNA treatment in HepG2 cells. C, relative to control (DMSO alone; expression = 1.0), T1317 induced *AKR1C4* mRNA expression approximately 1.6-fold (48-h treatment). Transfection of cells with a selective siRNA directed against LXRα substantially reduced the induction. Treatments received either control siRNA or selective LXRα siRNA as described under *Materials and Methods*. D, analysis of AKR1C4 protein expression under conditions identical to C. E, ChIP analysis of the LXRE in the *AKR1C4* promoter illustrating LXRα occupancy in HepG2 cells. F, ChIP analysis of the *AKR1C4* gene indicating increased histone H3 acetylation in response to LXR agonist treatment of HepG2 cells.

Along with AKR1C1 and AKR1C2, AKR1C4 is responsible for conversion of the selective estrogen receptor modulator tibolone to the estrogenic form of the drug 3α/β-hydroxytibolone (Steckelbroeck et al., 2004). AKR1C4 is also responsible for conversion of the opiate antagonist naltrexone to the longer half-life metabolite 6β-naltrexol (Porter et al., 2000). AKR1C4 is also involved in the activation of carcinogenic polycyclic hydrocarbons as well as in the detoxification of nicotine-derived carcinogens (Jin and Penning, 2007). Thus, activation of LXRα may lead to modulation of the metabolism of these xenobiotics.

In summary, we have shown that the human *AKR1C4* gene is regulated by LXRα. The regulation is mediated by specific binding of the receptor to a LXRE located in the promoter of the *AKR1C4* gene. LXRα/RXR heterodimers occupy this site in vivo and directly increase the expression of this gene in response to LXR ligands. These data suggest that LXRα may modulate the bile acid biosynthetic pathway at a unique site downstream of CYP7A1 and may also modulate the metabolism of steroid hormones and certain xenobiotics. Because LXR has been demonstrated to be a potential drug target for a variety of disorders, including dyslipidemia, diabetes, and inflammation (Cao et al., 2003; Michael et al., 2005; Chintalacharuvu et al., 2007), these data indicate that LXR agonists may have side effect profiles that include alterations in steroid hormone degradation and xenobiotic metabolism.

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