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Liver X Receptor (LXR) Regulation of the *LXRα* Gene in Human Macrophages*

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The nuclear oxysterol receptors LXRα (NR1H3) and LXRβ (NR1H2) coordinately regulate the expression of genes involved in the transport and catabolism of cholesterol. In macrophages, LXR stimulates the transcription of genes encoding transporters involved in cholesterol efflux, which may limit the transformation of these cells into foam cells in response to lipid loading. Here, we report that natural and synthetic LXR ligands induce the expression of the *LXRα* gene in primary human macrophages and differentiated THP-1 macrophages. This regulation was not observed in primary human adipocytes or hepatocytes, a human intestinal cell line, or in any mouse tissue or cell line examined. The human *LXRα* gene was isolated, and the transcription initiation site delineated. Analysis of the *LXRα* promoter revealed a functional LXR/RXR binding site ~2.9 kb upstream of the transcription initiation site. We conclude that LXRα regulates its own expression in human macrophages and that this response is likely to amplify the effects of oxysterols on reverse cholesterol transport. These findings underscore the importance of LXR as a potential therapeutic target for the treatment of atherosclerosis.

The characterization of two closely related ligand-activated transcription factors, named liver X receptor (LXR)[1] α (NR1H3) and LXRβ (NR1H2) (1–4), has provided important insights into the mechanisms underlying cellular and whole-body cholesterol homeostasis (5). The LXRs are activated by cholesterol derivatives and metabolites, including oxysterols such as 24(*S*),25-epoxycholesterol and 22(*R*)-hydroxycholesterol (6–10), and bind as heterodimers with the 9-*cis* retinoic acid receptor (RXR; NR2B1) to direct repeat four (DR-4) type sequence motifs, termed LXR response elements (LXREs), in the regulatory regions of target genes (1–4). Whereas LXRβ is expressed in most tissues, LXRα is abundantly expressed in a more restricted set of tissues including the liver, kidney, spleen, intestine, and in macrophages (1–4). Over the past several years, the LXRs have been shown to regulate a number of genes involved in cholesterol absorption, transport, and excretion. For example, in the rodent liver, LXRα stimulates the transcription of cholesterol 7α hydroxylase (CYP7A1) (11), the rate-limiting enzyme in the classical pathway for the conversion of cholesterol to bile acids (12, 13). When fed a cholesterol-rich diet, mice lacking functional LXRα failed to up-regulate *Cyp7a1* and accumulated copious amounts of cholesterol in their livers (11). Thus, LXRα is critical for the elimination of excess cholesterol from the mouse liver.

More recently, the LXRs have been shown to have a central role in the regulation of reverse cholesterol transport, a process whereby excess cholesterol is transferred in high-density lipoprotein (HDL) particles from peripheral tissues to the liver for elimination from the body (14). The biology of the LXRs and their cholesterol transporter target genes is of particular disease relevance in macrophages since the accumulation of lipids in these cells is a critical step in their transformation into foam cells in fatty streak lesions in atherosclerotic vasculature subendothelium (15, 16). One of the principal vehicles for lipid accumulation is oxidized low density lipoprotein (oxLDL), which contains a number of oxysterol LXR ligands (17). In macrophages, the LXRs regulate the expression of the adenosine triphosphate-binding cassette (ABC) proteins A1 and G1 (18–22), which serve as free-cholesterol and phospholipid translocators enabling cholesterol efflux from the macrophage onto various acceptors, including nascent, cholesterol-poor HDL (18, 21, 23–26). Defective ABCA1 expression in humans is the basis for Tangier disease and is associated with cholesterol accumulation in peripheral tissues along with a near-total lack of cellular cholesterol efflux and plasma HDL (27–31). LXR-dependent regulation of cholesterol efflux may prove to be an important antiatherogenic process amenable to pharmacological intervention with LXR agonists.

In studies performed in macrophages and macrophage cell lines, we have discovered that one of the genes regulated by the LXRs is *LXRα*. Notably, this positive feedback loop is specific to human macrophages. We hypothesize that this regulatory pathway increases the magnitude of the biological response to rising cellular levels of cholesterol and other components of oxLDL and, thus, represents an important component of macrophage lipid physiology.

EXPERIMENTAL PROCEDURES

Cell Culture—THP-1 cells were maintained in suspension for passage and growth in RPMI 1640 (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (FBS, Irvine Scientific, Santa Ana, CA), 100 units/ml penicillin/100 μg/ml streptomycin (Irvine Scientific), 1 mM sodium pyruvate (Invitrogen), and 55 μM β-mercaptoethanol (Sigma). Passaging was performed every 3–4 days at a 1:4 dilution. For experiments, 1×10^6 cells/well were plated in 6-well plates in media supplemented with 100 ng/ml phorbol 12-myristate-13-acetate (Sigma) to induce differentiation. Cells were maintained in this media for 5 days prior to treatment with LXR agonists. RAW 264.7 cells were main-

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[1] The abbreviations used are: LXR, liver X receptor; LXRE, LXR response elements; RXR, 9-*cis* retinoic acid receptor; HDL, high-density lipoprotein; oxLDL, oxidized low-density lipoprotein; ABC, adenosine triphosphate-binding cassette; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; PBMC, peripheral blood mononuclear cells; RACE, rapid amplification of cDNA ends; RTQ-PCR, real-time quantitative polymerase chain reaction; kb, kilobase(s); bp, base pair(s).

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tained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Irvine Scientific), 2 mM L-glutamine (Invitrogen) and 100 units/ml penicillin/100 μg/ml streptomycin (Invitrogen). Passaging was performed by scraping every 3–4 days at 1:3 dilutions. Cells were plated at 5×10^5 cells/well into 6-well plates or 5×10^6 cells into T75 flasks and dosing was begun when the cells had grown to ~50% confluency. FHs74 cells were grown in Hybri-Care medium (ATCC, Manassas, VA) supplemented with 10% FBS and 30 ng/ml epidermal growth factor PBS (Sigma). Cells were passaged by trypsinization every 3–4 days at a 1:3 dilution. Cells were plated at 3×10^5 cells/well into 6-well plates or 3×10^6 cells into T75 flasks. Treatments began when cells were ~50% confluent. Primary human adipocytes (Zen-Bio, Research Triangle Park, NC) were supplied in 6-well plates in a proprietary culture media. After cells arrived, the media was changed and the cells allowed to recover overnight, whereupon drug treatments commenced. Cells were ~60–80% confluent at this time. Primary human hepatocytes preplated in 6-well plates were obtained from either Stephen Strom (University of Pittsburgh) or Clonetics (Walkersville, MD). Hepatocyte media consists of Williams' E (Invitrogen) supplemented with 2 mM L-glutamine and ITS-G (insulin-transferrin-selenium-G, Invitrogen). Primary human macrophages were isolated and cultured as follows. Human blood was freshly drawn into heparin-treated tubes and diluted 1:3 with phosphate-buffered saline containing 2 mM EDTA. Peripheral blood mononuclear cells (PBMC) were collected by ficoll density separation by overlaying 25 ml of diluted blood on 25 ml of LymphoPrep reagent (Nycomed Pharma AS, Asker, Norway). After centrifugation according to the LymphoPrep protocol, the leukocyte layer was collected. Monocytes were isolated from PBMC by an indirect magnetic cell isolation technique. In this system, non-monocyte PBMC cell subsets are labeled with subset-specific antibody-coated magnetic beads (Monocyte Isolation Kit, Miltenyi Biotec, Auburn, CA) and then separated from unlabeled monocytes with a magnet (VarioMACS separator, Miltenyi Biotec). Unretained cells were found to be >95% monocytes by flow cytometry. Postseparation, monocytes were washed and maintained overnight at 1×10^6 cells/well in 6-well plates in DMEM containing 10% FBS, 2 mM glutamine, 100 units/ml penicillin/100 μg/ml streptomycin, and 0.1 ng/ml GM-CSF (BD PharMingen, Franklin Lakes, NJ).

Drug Treatments—Cultured cells and cell lines were generally dosed with compounds dissolved in the same culture media in which they were grown. Treatments included the LXRα/β agonists GW3965 (32), T0901317 (19), 22(*R*)-hydroxycholesterol (6, 7), or 24(*S*),25-epoxycholesterol (7). Each cell line was treated according to an optimized schedule. Generally, the culture media was replaced with media containing vehicle (Me_2SO or ethanol) or 1–10 μM drugs at 0 h. FHs74 cells were harvested for RNA isolation 48 h after the initial dosing; human macrophages and hepatocytes were treated a second time at 24 h and harvested 4 h later; THP-1 and RAW 264.7 cells were dosed a second time at 24 h and then harvested 24 h later; adipocytes were treated a second time at 48 h and harvested 4 h later.

Rapid Amplification of 5′-cDNA Ends (RACE)—The transcriptional initiation site of the human LXRα gene was determined by rapid amplification of cDNA ends (5′-RACE) using a 5′/3′ RACE kit (Roche Molecular Biochemicals) and THP-1 total RNA prepared as described below. The primers used were based on the 5′-end of the published human LXRα cDNA (2). The sequences are as follows: SP1, 5′-GGCCCCCAGCCACAAGGACAT-3′; SP2, 5′-CTCTTCCTGGAGCCCT-3′; SP3, 5′-CATTACCAAGGCACTG-3′. SP1–3 are nested primers with SP1 positioned the furthest downstream.

Animal Treatments and Tissue Collection—Male C57-Bl/6 mice maintained on a regular chow diet were dosed twice daily by oral gavage with 0.5% methyl cellulose or 10 mg/kg GW3965 suspended in 0.5% methyl cellulose. After 2 days dosing, peritoneal macrophages, liver, and intestine were collected. Peritoneal macrophages were harvested by flushing the abdominal cavity with 10 ml ice-cold DMEM containing 10% FBS (Irvine Scientific). The media was withdrawn and spun at $3000 \times g$ for 10 min in a tabletop centrifuge. Cell pellets were lysed in TRIzol reagent (Invitrogen) and RNA extracted according to the manufacturer's instructions. Intestine samples were removed and flushed with ice-cold saline. Liver and intestine samples were snap-frozen in liquid nitrogen and held at −80 °C prior to RNA isolation.

Real-time Quantitative Polymerase Chain Reaction (RTQ-PCR)—Total RNA samples were diluted to 100 μg/ml and treated with 40 units/ml RNA-free DNase-I (Ambion, Austin, TX) for 30 min at 37 °C followed by inactivation at 75 °C for 5 min. Samples were quantitated by spectrophotometry or with the RiboGreen assay (Molecular Probes, Eugene, OR) and diluted to a concentration of 10 ng/μl. Samples were assayed in duplicate 25-μl reactions using 25 ng of RNA/reaction with PerkinElmer chemistry on an ABI Prism 7700 (Applied Biosystems, Foster City, CA). Gene-specific primers were used at 7.5 or 22.5 pmol/reaction and optimized for each gene examined, and the gene-specific fluorescently tagged probe was used at 5 pmol/reaction. In this system, the probe is degraded by *Taq* polymerase during the amplification phase, releasing the fluorescent tag from its quenched state; amplification data is expressed as the number of PCR cycles required to elevate the fluorescence signal beyond a threshold intensity level. Fold induction values were calculated by subtracting the mean threshold cycle number for each treatment group from the mean threshold cycle number for the vehicle group and raising 2 to the power of this difference.

Northern Blot Protocol—Northern blot analysis was performed exactly as described elsewhere (33). Briefly, 10 μg of total RNA was electrophoresed in a denaturing agarose gel and transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech) according to the manufacturer's instructions. Human LXRα and LXRβ cDNA corresponding to the ligand-binding domain was labeled with [α-^{32}P]dCTP by random priming using a commercially available system (Megaprime, Amersham Pharmacia Biotech). Blots were sequentially probed with radiolabeled LXRα, LXRβ, and β-actin (CLONTECH Laboratories, Palo Alto, CA) using standard techniques.

Electrophoretic Mobility Shift Assays—DNA-receptor protein interactions were examined by electrophoretic mobility shift assays as described elsewhere (33). Competitor oligonucleotides were added at 5-, 15-, or 75-fold molar excess (mutant oligonucleotide added only at 75-fold molar excess). The binding reactions were resolved on a pre-electrophoresed 0.4 × TBE, 4% polyacrylamide gel at room temperature. Human LXRα, LXRβ, and RXRα proteins were synthesized from pSG5-hLXRα, LXRβ, and RXRα using the TNT T7-coupled reticulocyte system (Promega, Madison, WI). The oligonucleotides used in the experiments described here were as follows (sense strand only, with overhang and mutated nucleotides in lowercase and underlined, respectively: Rat CYP7A1; 5′-gatcCTTTGGTCACTCAAGTTCAAGT-3′, LXRE1; 5′-agctTGAATGACCAGCAGTAACCTCAGC-3′, mutLXRE1; 5′-agctTGAATG*TT*CAGCAGTA*TT*CTCAGC-3′.

Generation of Reporter Constructs—A 3.5-kb *Apa*I fragment of the human *LXRα* 5′-flanking domain was cloned directly upstream of a luciferase reporter gene in a modified pGL3-Basic vector (Promega) containing an *Apa*I site in the polylinker. This construct, named pGL3-hLXRα-3027/463, contains bases −3027 to +463 of the human *LXRα* gene and was obtained by *Apa*I digest of bacterial artificial chromosome clone RP1117G12 (GenBank™ AC018410). This construct was modified to generate deletion and point mutants as follows. pGL3-hLXRα-2677/463 was generated by digestion of the parent construct with *Sac*I and lacks a fragment extending from the *Sac*I site in the polylinker upstream of the insert to position −2677 within the insert; this construct lacks LXRE1. In pGL3-hLXRα-3027/463mut, four bases within LXRE1 from the parent construct were mutated using the Transformer site-directed mutagenesis system (CLONTECH Laboratories) and the following mutation primer (mutated nucleotides underlined): 5′-CAGGGGGTGAATGTTCAGCAGTATTCTCAGCAGCTTGC-3′. The pGL3-hLXRα-3027/463-Pst deletion mutant lacks LXRE2 and LXRE3 and was generated by digestion of the parent construct with *Pst*I to exclude a fragment from bases −2895 to −217; pGL3-hLXRα-3027/463-Pst-mut was generated by *Pst*I digestion of the pGL3-hLXRα-3027/463mut mutant.

Reporter Construct Transactivation Assays—HepG2 cells were cultured and transfected exactly as described elsewhere (33). All luciferase values were normalized to secreted placental alkaline phosphatase and are expressed as fold activation over the activity of the no receptor/vehicle condition for each construct.

RESULTS

As part of a comprehensive search for LXR target genes in macrophages, we discovered that *LXRα* itself was up-regulated in differentiated THP-1 cells treated with the potent, synthetic LXR agonists GW3965 (32) and T0901317 (19) as assessed by Northern blot analysis (Fig. 1). In multiple experiments, *LXRα* up-regulation in THP-1 cells averaged 4.2- and 6.2-fold with GW3965 and T0901317, respectively, as measured by RTQ-PCR (Table I). The natural LXR agonists 22(*R*)-hydroxycholesterol and 24(*S*),25-epoxycholesterol also robustly up-regulated *LXRα* (Table I), whereas the inactive enantiomer 22(*S*)-hydroxycholesterol (6, 7) had no effect on gene expression in THP-1 cells or any other cells examined (data not shown). In sharp contrast, *LXRβ* expression was not regulated in THP-1

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cells by treatment with the synthetic LXR agonists (Fig. 1).

Additional experiments were performed to explore the tissue and species specificity of this effect. LXRα, ABCA1, and ABCG1 mRNA levels were assessed by RTQ-PCR in primary human hepatocytes, adipocytes, and the human intestinal cell line FHs74 treated with various synthetic and natural LXR agonists. Gene expression was also analyzed in drug-treated murine macrophage-like RAW 264.7 cells and primary peritoneal macrophages obtained from mice treated with GW3965. Whereas expression of the known LXR target genes *ABCA1* and *ABCG1* was substantially up-regulated by LXR agonists, little or no change was observed in *LXRα* expression levels (Table I). Similar results were obtained in additional tissues from drug-treated mice including liver and intestine (data not shown). In contrast, all three *LXR* target genes were up-regulated in primary human monocyte-derived macrophages (Table I), confirming the original results obtained with THP-1 cells. Overall, these results suggest the effect is specific to macrophages of human origin. *LXRβ* was not regulated by LXR agonists in any of the cells, cell lines, or tissues examined (data not shown).

The genomic structure and promoter regions of the mouse *LXRα* and *LXRβ* genes have recently been examined in detail (34). To begin dissecting the human *LXRα* 5′-flanking region, DDBJ/EMBL/GenBank high throughput genomic sequence data bases were queried with nucleotides corresponding to mouse *Lxrα* exon 1 (34). This search identified a BAC encompassing the 5′-end of the human *LXRα* gene (GenBank™ AC018410). The region surrounding the murine *Lxrα* transcription initiation site (bases −248 to +521) was found to be highly homologous (~75%) to that in the human *LXRα* gene. To determine the transcriptional initiation site for the human LXRα gene, 5′-RACE was performed using total RNA prepared from THP-1 cells and primers located at the 5′-end of the published LXRα cDNA (Fig. 2*B*). Despite the high degree of sequence homology between the mouse and human *LXRα* genes, the positions of the major transcription initiation sites were not conserved. The vast majority (20 of 22 clones) of cDNA ends that were obtained by 5′-RACE started ~320 bp downstream from the mouse transcription initiation site (Fig. 2*A*). These clones delineated a novel exon (designated exon 1B) of the human *LXRα* gene that is 134 bp in length and is not present in the previously described human LXRα cDNA (2). A search of the DBEST data base of GenBank/EMBL/DDBJ sequences from EST Divisions demonstrated that nearly all entries containing sequence corresponding to the human *LXRα* translational start site extend upstream to this same region and that none of them contains a sequence homologous to exon 1 of the mouse *Lxrα* gene (data not shown). The 3′-donor site of exon 1B lies 1050 bp upstream of the 5′-acceptor site of exon 2, which contains the translational start site (Fig. 2, *A* and *B*). Two 5′-RACE clones mapped an additional exon (exon 1A, bases −290 to −261) in the region of the human *LXRα* gene corresponding to exon 1 of the murine gene (Fig. 2*A*). Exon 1 of the murine *Lxrα* gene contains two alternative splice donor sites (1a and 1b, positions +84 and +152, respectively). The splice donor site of the human exon 1A was identical to splice donor site 1a of the mouse *Lxrα* gene (34). It is notable that the transcription initiation site in the murine *Lxrα* gene was delineated using RNA prepared from liver (34), while the RNA used in this study was isolated from a macrophage-derived cell line. Hence, the observed differences may be the result of

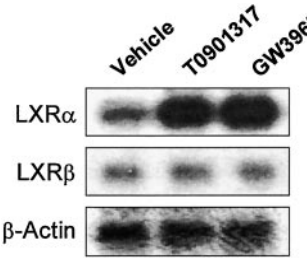

FIG. 1. **LXRα is up-regulated in THP-1 cells in response to synthetic LXR agonists.** Total RNA (10 μg) was prepared from differentiated THP-1 cells treated for a total of 48 h with vehicle or 1 μM of the indicated drugs. Northern blot analysis was performed with radiolabeled probes for human LXRα (*upper panel*), LXRβ (*middle panel*), and glyceraldehyde-3-phosphate dehydrogenase.

TABLE I

RTQ-PCR analysis of LXRα, ABCA1, and ABCG1 expression in various cells and cell lines treated with various synthetic and natural LXR agonists

Cultured cells were treated with 1–10 μM of the indicated compounds, while peritoneal macrophages were obtained from mice dosed orally with 10 mg/kg GW3965. Gene expression levels were calculated from raw RTQ-PCR data as described under "Experimental Procedures" with the vehicle group expression set to 1.0 arbitrary unit. Data presented are averaged from multiple determinations on samples from one to six individual experiments. First entry, THP-1 cell data; middle entries, additional human and mouse cells and cell line data; last entry, primary human macrophage data. ND, not determined.

Cell or cell line	Drug	Fold regulation		
		LXRα	ABCA1	ABCG1
THP-1 human macrophage-like cell line	GW3965	4.2	9.2	60.1
	T0901317	6.2	12.2	10.3
	24(*S*),25-epoxycholesterol	3.9	4.0	65.4
	22(*R*)-hydroxycholesterol	4.9	4.4	4.6
Human hepatocytes	GW3965	1.1	5.1	39.7
	T0901317	0.7	3.1	32.4
Human adipocytes	GW3965	1.5	7.8	5.7
	T0901317	1.5	6.0	5.0
	24(*S*),25-epoxycholesterol	1.1	3.7	4.1
FHs74 intestinal cell line	GW3965	1.1	7.3	83.1
	T0901317	0.9	8.5	91.8
	24(*S*),25-epoxycholesterol	0.9	9.2	154.9
	22(*R*)-hydroxycholesterol	1.1	10.0	114.6
RAW 264.7 murine macrophage-like cell line	GW3965	1.1	8.9	4.2
	24(*S*),25-epoxycholesterol	0.8	4.0	3.6
	22(*R*)-hydroxycholesterol	1.3	4.2	2.8
Mouse peritoneal macrophages	GW3965	1.6	6.2	10.7
Human macrophages	GW3965	2.5	7.6	113.8
	T0901317	3.6	16.2	118.0
	22(*R*)-hydroxycholesterol	3.3	3.1	ND

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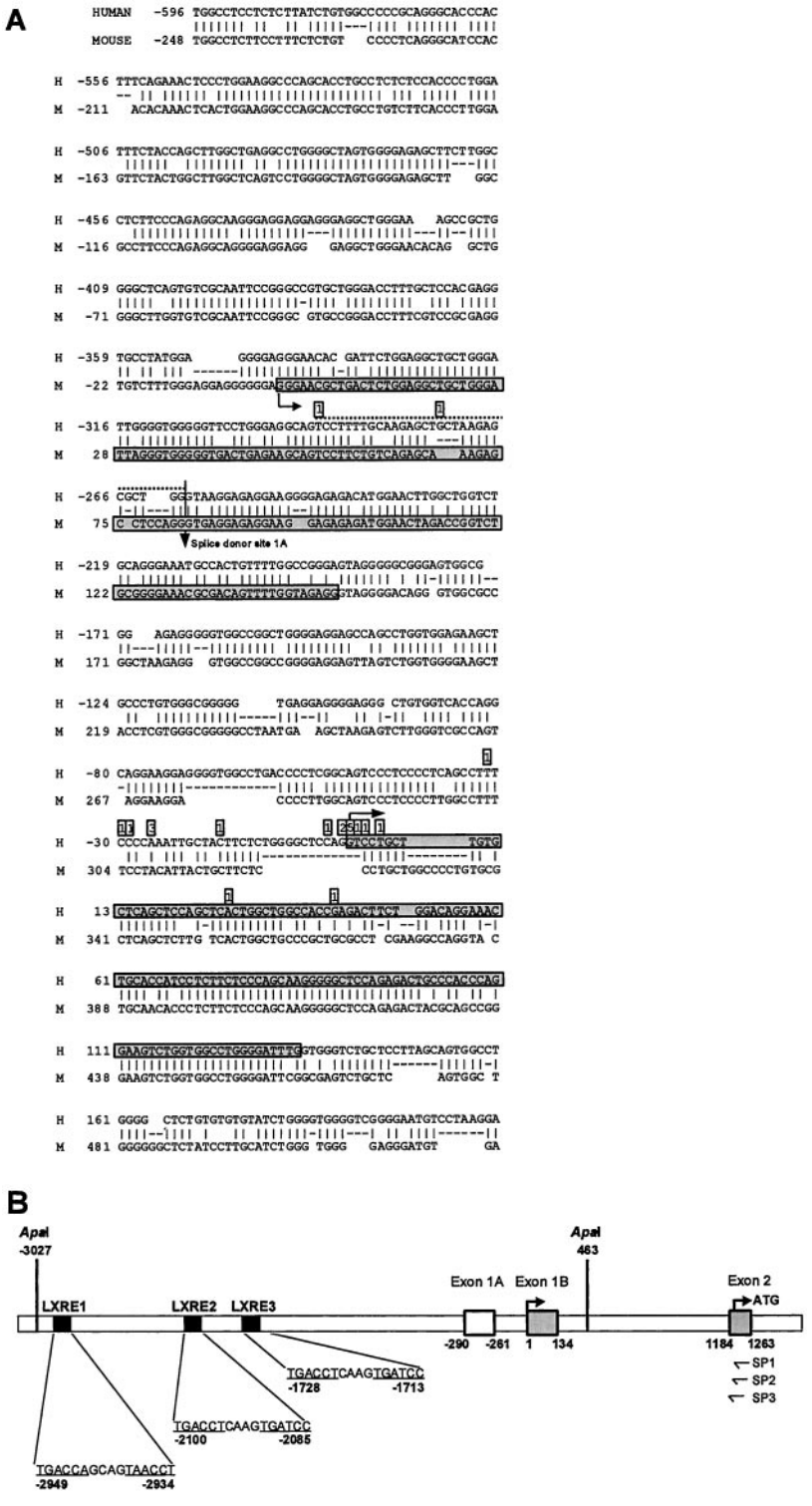

FIG. 2. **The human *LXRα* 5′-flanking region.** *A,* alignment of the human *LXRα* 5′-flank with the mouse gene indicating mouse exon 1 (34) (*shaded box on lower strand*), human exon 1A (*dashed line on upper strand*), and human exon 1B (*shaded box on upper strand*) as deduced from 5′-RACE and EST data base analysis. The number and position of cDNA ends as delineated by 5′-RACE are shown in *boxes* above the sequence. Sequence numbering is expressed relative to the transcriptional initiation site for each gene (indicated with an *arrow*). Exon 2 of the human LXRα gene, which contains the translation start site, lies ~1 kb downstream of the sequence depicted here. *B,* schematic of the human *LXRα* 5′-flanking region depicting the three potential LXREs (*black boxes*), exon 1A and exons 1B and 2 (*open* and *shaded boxes*, respectively). The indicated *Apa*I restriction sites were used to generate a reporter construct containing this portion of the 5′-flanking region. The positions of the primers used for 5′-RACE (SP1, SP2, and SP3) are shown.

tissue-specific transcriptional events. Both the human and mouse *LXRα* genes contain in-frame stop codons in exon 2 (34, 35) that prevent any contribution from exons 1A or 1B to the sequence of the LXRα protein.

Using a computer search algorithm, we identified three potential LXREs that conformed to the DR-4 consensus sequence in the 3 kb of sequence upstream from the human transcription initiation site. These sites were termed LXRE1, 2, and 3 (Fig. 2*B*) Interestingly, the LXRE2 and 3 sites are perfect duplicates, and moreover, the region extending ~200 bp upstream and ~100 bp downstream of the two LXREs is highly conserved (~75% homology). The functional significance of this observation is not clear. To explore the transcriptional activity of these LXREs in their genomic context, an *Apa*I fragment of the *LXRα* gene containing exon 1 and upstream sequence covering all three putative response elements was inserted into a luciferase reporter gene vector (pGL3-Basic) directly upstream of the luciferase gene (named pGL3-hLXRα-3027/463; Figs. 2*B* and 3). Deletion and point mutants of this construct were generated and are depicted in Fig. 3. Reporter construct activity was examined in transfection assays in a hepatocellular carcinoma cell line, HepG2. The transfections were performed in a liver cell line because of the difficulty of transfecting human macrophage cells. HepG2 cells were transfected with each reporter

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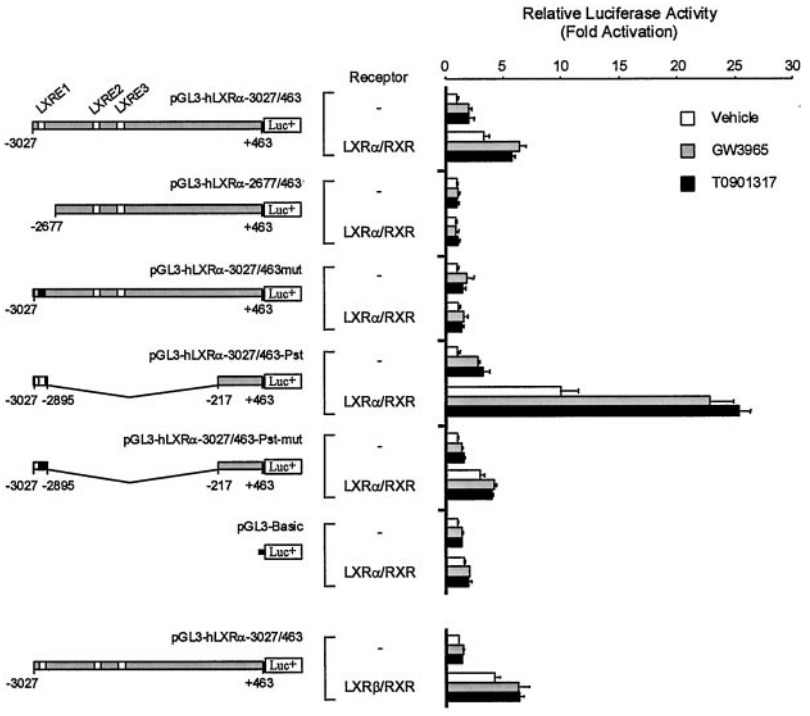

FIG. 3. **Activation of human LXRα reporter constructs by LXR agonists.** Luciferase reporter constructs containing a 3.5-kb *Apa*I fragment of the intact *LXRα* upstream flanking region (bases −3027 to +463) or various mutations of this construct were transfected into HepG2 cells. Control cultures were transfected with the backbone pGL3-Basic vector. Cells were cotransfected either with an empty expression vector (pSG5) or expression vectors containing human RXRα and either human LXRα or LXRβ as indicated and then treated with vehicle (0.1% Me_2SO) or 1 μM either GW3965 or T0901317 for 48 h. Data are expressed as fold induction over the normalized luciferase activity measured in the vehicle-treated/no receptor group. *Error bars* represent the mean ± S.D. from four separate transfection experiments.

construct either without exogenous expression of receptors or in the presence of hRXRα and either hLXRα or hLXRβ expression vectors. Ligand treatments included vehicle or the synthetic LXR agonists GW3965 and T0901317. As shown in Fig. 3, pGL3-hLXRα-3027/463 was activated in a receptor- and ligand-dependent fashion. Only weak induction of reporter activity in response to the LXR agonists was detected in HepG2 cells transfected with this reporter plasmid alone. Cotransfection of LXRα and RXRα expression plasmids resulted in an ~2-fold increase in reporter activity (Fig. 3). Addition of either LXR agonist to these cells increased reporter activity an additional 2-fold, for a total of 5- to 7-fold activation compared with the no receptor/vehicle condition. Virtually identical results were obtained when an LXRβ expression plasmid was substituted for LXRα (Fig. 3).

To determine which of the three putative LXREs underlies LXR-mediated reporter activation, mutants were constructed that deleted or mutated LXRE1 or deleted both LXRE2 and LXRE3. The parent construct was compared side-by-side to each mutant construct both in the absence and presence of LXRα/RXR and either vehicle, GW3965, or T0901317 (Fig. 3). Compared with the parent construct (pGL3-hLXRα-3027/463), no basal or ligand-induced activity was observed with the mutant in which LXRE1 is deleted (pGL3-hLXRα-2677/463) (Fig. 3). Very similar data were obtained with the pGL3-hLXRα-3027/463mut, in which four nucleotides of LXRE1 are mutated Fig. 3). Deletion of LXRE1 also resulted in a loss of induction in response to LXRβ (data not shown). In contrast, deletion of nucleotides −2895 to −217 in the *LXRα* promoter, which includes LXRE2 and LXRE3 (pGL3-hLXRα-3027/463-Pst), did not reduce the activity of the *LXRα* promoter. Instead, the basal and ligand-induced activities of this reporter construct were substantially increased over the parent construct (Fig. 3). We speculate that the deleted region contains a repressor element that may suppress LXR-dependent induction in cells other than macrophages. Site-directed mutagenesis of LXRE1 in this construct (pGL3-hLXRα-3027/463-Pst-mut) eliminated the increases seen in basal and ligand-induced activities (Fig. 3). Overall, these results demonstrate that LXRE1 is the principal LXRE mediating LXR control of *LXRα* expression.

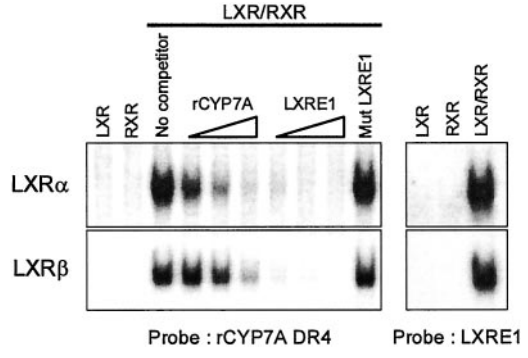

FIG. 4. **Electrophoretic mobility-shift analysis of LXRE1.** Competition experiments were performed using a radiolabeled oligonucleotide probe corresponding to the DR4 LXRE from the rat *CYP7A1* promoter (*left panels*) or LXRE1 from the human LXRα gene (*right panels*). In addition to probe, binding reactions contained *in vitro* translated human RXRα and/or LXRα (*top panels*) or LXRβ protein (*bottom panels*). Some reactions also contained competitor oligonucleotides corresponding to either the rat *CYP7A1* DR4 or LXRE1 from the human LXRα promoter as indicated. Competitor rat CYP7A1 and LXRE1 oligonucleotides were added at 5-, 15-, or 75-fold molar excess, while the mutant LXRE oligonucleotide was added at 75-fold molar excess over the radiolabeled probe.

We next sought to determine whether LXR/RXR heterodimers bind directly to LXRE1. Electrophoretic mobility-shift assays were performed with *in vitro* synthesized LXRα, LXRβ, and RXRα. As expected, both the LXRα/RXRα and LXRβ/RXRα heterodimers bound efficiently to a radiolabeled LXRE derived from the rat *CYP7A1* promoter (Fig. 4) (7). An oligonucleotide corresponding to LXRE1 competed efficiently with the rat CYP7A1 probe for binding to both the LXRα/RXRα and LXRβ/RXRα heterodimers. Under the conditions employed, the LXRE1 oligonucleotide competed better than the unlabeled rat CYP7A1 oligonucleotide (Fig. 4). An oligonucleotide harboring a mutated LXRE1 failed to compete for binding (Fig. 4). Using radiolabeled LXRE1, we confirmed that both the LXRα/RXRα and LXRβ/RXRα heterodimers bind directly to LXRE1 (Fig. 4). Together, these data provide evidence that the

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human *LXRα* gene is regulated directly by the LXR/RXR heterodimers.

DISCUSSION

LXR is known to regulate a number of genes involved in cholesterol homeostasis (5, 36). The experiments described in this report reveal *LXRα* as a novel LXR target gene in human macrophages. We hypothesize that this autoregulation increases the expression of LXRα target genes such as *ABCA1*, *ABCG1*, and apolipoprotein (Apo) E in response to oxysterols derived from oxLDL (18–21, 37). The coordinate increase in the expression of these genes could then increase the efflux of excess cholesterol from macrophages onto acceptor proteins such as ApoA1 and ApoE for transport to the liver and elimination from the body (14, 36). Thus, the LXRα autoregulatory loop provides a mechanism for efficiently amplifying the effects of oxysterols in macrophages and promoting reverse cholesterol transport. Interestingly, we did not observe stimulation of *LXRβ* gene expression by LXR agonists, indicating that autoregulation is restricted to the *LXRα* gene. The physiological importance of this difference remains to be determined. The *LXRα* gene was also recently shown to be directly regulated by peroxisome proliferator-activated receptor γ, which is activated by fatty acids and their metabolites (38). Thus, *LXRα* gene expression is under the control of multiple receptors that sense dietary status. In cell-based reporter assays, both the LXRα and LXRβ subtypes were capable of stimulating transcription of the *LXRα* promoter. Since LXRα and LXRβ are both expressed in macrophages, these data suggest that activation of either subtype can initiate this regulatory cascade.

Among the human cells examined, *LXRα* was only up-regulated in primary macrophages. This macrophage-specific effect may be due to the restricted expression of positively acting transcription factors or coactivators in this cell type. Alternatively, tissue-specific repressors, perhaps interacting with a region of the *LXRα* promoter bracketed by the *Pst*I sites exploited in the deletion mutation experiments described above, may prevent induction in tissues where excessive activation of LXR target genes could be detrimental. Notably, we failed to detect a similar autoregulation of *LXRα* gene expression by LXR agonists in murine macrophages or other mouse tissues or cell lines. The basis for these species differences is currently unknown. We speculate that the dynamic response to oxysterols as measured by cholesterol efflux may be greater in human macrophages than in rodent cells, perhaps reflecting an evolutionary response to the cholesterol-rich diet of humans as compared with rodents.

One possible implication of these findings is that polymorphisms in LXRE1 or other regulatory elements of the *LXRα* promoter may impair the gene response to lipid loading. Thus, individuals harboring these mutations may have an increased risk of developing atherosclerosis. Interestingly, familial combined hyperlipidemia, a polygenic lipid disorder associated with the early onset of coronary artery disease, was recently found to have genetic linkage with a region of chromosome 11 that contains the *LXRα* gene (39). It remains to be determined whether mutations in either the *LXRα* gene or its regulatory regions contribute to this disease.

In conclusion, we have demonstrated that *LXRα* is a direct target gene of LXRα and LXRβ in human macrophages. Regulation is mediated by a single LXRE located 2.9 kb upstream of a previously unrecognized exon in the human *LXRα* gene. LXRα regulation is not seen in other human cell types or any murine tissue or cell examined. This macrophage-specific phenomenon may form an important component of the body's response to elevated cholesterol levels in general and may help prevent the transformation of macrophages into foam cells by amplifying the process of reverse cholesterol transport.

Addendum—Laffitte *et al*. (40) have also recently described autoregulation of the LXRα gene promoter.

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