27-Hydroxycholesterol Is an Endogenous Ligand for Liver X Receptor in Cholesterol-loaded Cells*

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The nuclear receptors liver X receptor α (LXRα) (NR1H3) and LXRβ (NR1H2) are important regulators of genes involved in lipid metabolism, including ABCA1, ABCG1, and sterol regulatory element-binding protein-1c (SREBP-1c). Although it has been demonstrated that oxysterols are LXR ligands, little is known about the identity of the physiological activators of these receptors. Here we confirm earlier studies demonstrating a dose-dependent induction of ABCA1 and ABCG1 in human monocyte-derived macrophages by cholesterol loading. In addition, we show that formation of 27-hydroxycholesterol and cholestenoic acid, products of CYP27 action on cholesterol, is dependent on the dose of cholesterol used to load the cells. Other proposed LXR ligands, including 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 24(S),25-epoxycholesterol, could not be detected under these conditions. A role for CYP27 in regulation of cholesterol-induced genes was demonstrated by the following findings. 1) Introduction of CYP27 into HEK-293 cells conferred an induction of ABCG1 and SREBP-1c; 2) upon cholesterol loading, CYP27-expressing cells induce these genes to a greater extent than in control cells; 3) in CYP27-deficient human skin fibroblasts, the induction of ABCA1 in response to cholesterol loading was ablated; and 4) in a coactivator association assay, 27-hydroxycholesterol functionally activated LXR. We conclude that 27-hydroxylation of cholesterol is an important pathway for LXR activation in response to cholesterol overload.

The nuclear receptors LXRα (NR1H3) and LXRβ (NR1H2) have recently been shown to be important regulators of cholesterol and fatty acid metabolism (reviewed in Ref. 1). Originally identified as orphan receptors (1), LXR isoforms were subsequently shown to bind and be activated by certain oxysterols, oxygenated derivatives of cholesterol (2–4). Among candidates proposed, 24(S),25-epoxycholesterol is one of the most potent activators (5). At least in certain cell culture systems, this compound is formed via a shunt in the cholesterol biosynthetic pathway (6). Other proposed activators include 20(S)-hydroxycholesterol and 22(R)-hydroxycholesterol (2). These compounds are formed as transient intermediates in steroid hormone synthesis; thus, their distribution is limited.

The first demonstration of the importance of LXRα for maintenance of cholesterol homeostasis came with results from studies using mice deficient in that receptor (7). Upon feeding a high cholesterol diet, the livers of the LXRα−/− mice become engorged with cholesterol, which was shown to be caused by a failure to up-regulate CYP7A1 and bile acid synthesis as a response to cholesterol overload. Subsequently, several genes of importance for cholesterol and fatty acid metabolism have been shown to be responsive to LXR. Included in that list is a group of genes involved in reverse cholesterol transport (ABCA1,2 ABCG1,2 ApoE, and CETP) (8–14), and LXR has indeed been proposed to be a coordinator of HDL cholesterol catabolism and bile acid synthesis (8). Also regulated by LXR is SREBP-1c (15, 16), a master regulator of fatty acid synthesis.

Among LXR-responsive genes, ABCA1 is of particular interest since the recent discovery that mutations in this gene are the underlying cause of Tangier disease (17–20). This condition is characterized by defective formation of nascent HDL and extremely low levels of circulating apoA-I-containing lipoproteins. Before the realization that ABCA1 is an LXR-responsive gene, it had been shown that loading of human monocyte-derived macrophages with cholesterol would induce the expression of that gene and, conversely, that cholesterol unloading would decrease the expression (21). These cells do not synthesize steroid hormones and therefore not 20(S)- or 22(R)-hydroxycholesterol. We also found it counterintuitive that overload of cholesterol, well known to down-regulate early steps in cholesterol biosynthesis, would induce formation of intermediates in this pathway, including 24(S),25-epoxycholesterol, and hypothesized that some other derivative of cholesterol would mediate the activation of LXR in cholesterol-loaded cells.

Stellar 27-hydroxylase, encoded by the CYP27 gene, is an enzyme with multiple functions (recently reviewed in Ref. 22). In the liver, its primary function is to hydroxylate bile acid intermediates such as cholestane-3α,7α,12α-triol, thereby initiating side chain degradation. In extrahepatic tissues and in liver, the enzyme hydroxylates cholesterol to yield 27-hydroxycholesterol2 and cholestenoic acid. 27-Hydroxycholesterol is the most abundant hydroxycholesterol in human circulation, with average concentrations of about 0.4 μM in normal subjects (23).
The absence of a functional sterol 27-hydroxylase is the cause of the rare inherited metabolic disorder cerebrotendinous xanthomatosis (CTX) (24, 25). CTX patients have a complex phenotype, partly reflecting the importance of CYP27 for bile acid synthesis (26). Thus, high levels of toxic bile acid intermediates circulate in these patients. There are, however, other features of CTX that are not readily explained by the bile acid biosynthetic defect but are compatible with the notion of a defective formation of endogenous LXR ligands. For example, intracellular cholesterol is elevated in CTX patients, and premature atherosclerosis is relatively common despite low to normal LDL cholesterol levels. A single study (27), which has not been confirmed (28), reported very low HDL levels in CTX patients. This would be expected if the absence of CYP27 caused a low intracellular LXR tone leading to low ABCA1-mediated high density lipoprotein formation.

To determine the identity of the LXR ligands formed as a response to cholesterol overload, we treated human primary monocyte-derived macrophages with acetylated LDL and determined oxysterol formation. A dose dependence between cholesterol loading, formation of 27-hydroxycholesterol and cholestenoic acid, and activation of LXR-responsive genes was observed. In a coactivator association experiment, 27-hydroxycholesterol was shown to activate LXR. We were then able to show that transient or stable transfection of CYP27 into HEK293 cells confers activation of LXR-responsive genes. Finally, in primary fibroblasts derived from a patient deficient in CYP27 activity, LXR activation in response to cholesterol loading was impaired. We conclude that intracellular 27-hydroxylation of cholesterol is an important pathway for the generation of LXR agonists upon cholesterol loading.

### EXPERIMENTAL PROCEDURES

#### Materials

All of the DNA-modifying enzymes were purchased from Life Technologies, Inc. Vectors and cells (293-Tet-Off) for the generation of tetracycline-regulated stably transfected cells were obtained from CLONTECH (Palo Alto, CA). The expression vector pcDNA3.1 was obtained from Invitrogen (Carlsbad, CA). Human low density lipoprotein was obtained by sequential ultracentrifugation as described (29). Acetylation of LDL was performed according to the method of Goldstein et al. (30). Steroids were obtained from Sigma, Steraloids, (Newport, RI), or Research Plus (Bayonne, NJ). Cholestenoic acid was kindly provided by Dr. Norman B. Javitt (New York University, New York, NY).

#### Cell Culture

Human skin fibroblasts from a CTX patient were kindly provided by Dr. Ingemar Björkhem (Huddinge University Hospital, Huddinge, Sweden). This patient has been extensively characterized, and the mutation has been identified (R441W). This mutation has also been confirmed (28), reported very low HDL levels in CTX patients. This would be expected if the absence of CYP27 caused a low intracellular LXR tone leading to low ABCA1-mediated high density lipoprotein formation.

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#### RNA Isolation

Total RNA was prepared using Trizol reagent (Life Technologies) according to the instructions given by the manufacturer, with the exception that 1-bromo-3-chloropropane (Sigma) was substituted for chloroform. The RNA was treated with DNase (Ambion) before analysis by real-time quantitative RT-PCR.

#### Real Time Quantitative RT-PCR (TaqMan)

Real time quantitative PCR analysis (35) was used to determine the relative levels of ABCA1, ABCG1, SREBP-1c, CYP27, SCD, LXRα, and LXRβ mRNA. Reverse transcription and PCRs were performed according to the manufacturer’s instructions (Applied Biosystems; TaqMan Gold RT-PCR protocol and TaqMan Universal PCR Master Mix). Sequence-specific amplification was detected with an increasing fluorescent signal of FAM (reporter dye) during the amplification cycle. Amplification of the mRNA for the human 23-kDa highly basic protein (HBP), also called ribosomal protein L13α, was performed in the same reaction on all samples tested as an internal control for variations in RNA amounts. Levels of the different mRNAs were subsequently normalized to HBP mRNA levels.

#### Primers and Fluorogenic Probes

Oligonucleotide primers and TaqMan probes were designed using Primer Express software (Applied Biosystems) and were synthesized by Applied Biosystems. See Table I for sequences of probes and primers.

#### cDNA Cloning

A full-length expressible cDNA encoding human CYP27 was obtained by single-tube RT-PCR using RNA from human primary monocyte-derived macrophages. A Titan RT-PCR kit (Roche Molecular Biochemicals) was used according to the instructions supplied by the manufacturer. Primers used were AAAGGGGCGCGGCGCGGAGCACAACCAT (forward primer, includes a NotI restriction site) and AAGAATTCTGAGTCCTTAGAGACCCCGCAAA (reverse primer, includes an EcoRI restriction site). The following program was used for the Perkin-Elmer 9800 thermocycler: reverse transcription at 50 °C for 30 min, denaturing at 94 °C for 2 min, 10 cycles of denaturing at 94 °C for 30 s, annealing at 62 °C for 30 s, elongation at 68 °C for 30 s, and 30 more cycles with the elongation time increased by 5 s, followed by 30 cycles of 94 °C for 30 s, 62 °C for 30 s, and 68 °C for 30 s. The product was purified from agarose gel, the amplified fragment was cloned into the expression vector pcDNA3.1-His5 using the Topo TA cloning kit from Invitrogen, yielding plasmid pcDNA3.1-CYP27. A full-length cDNA encoding the human STAR protein was obtained by RT-PCR and cloned into pcDNA3.1-His5 in the same manner, using human adrenal gland mRNA (CLONTECH). The primers used were CCACCAATCGCCA- CATTTCGCCAGAA (forward primer) and CAGTTGGGAAACAGCAG- GCTGTGCTTC (reverse primer), and the temperature program was identical to that used for HAVEPCR. The resulting plasmid was designated as pcDNA3.1-hSTAR. The endogenous adrenocorticotropin was included in the reverse primer for both cDNA and STAR to prevent incorporation of a His tag in the expressed protein, which may have interfered with activity.

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3 I. Björkhem, personal communication.
| Gene  | Forward primer | Reverse primer | Probe Description | Accession no. |
|-------|----------------|----------------|-------------------|--------------|
| ABCA1 | TGTCCAGTCCAGTAATGGTTCTGT | AAGCGAGATATGGTCCGGATT | 6FAM-ACACCTGGAGAGAAGCTTTCAACGAGACTAACC-TAMRA | NM_005502 |
| ABCG1 | TGCAATCTTGTGCCATATTTGA | CCAGCCGACTGTTCTGATCA | 6FAM-TACCACAACCCAGCAGATTTTGTCATGGA-TAMRA | XM_016998 |

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/H9251 LXR expression by the LXR agonist 24-

cle (ethanol) control. After 72 h of incubation, cells were harvested to
determine loading efficiency by GC-MS and gene expression by real
time PCR as described above. For determination of induction of gene
determination for real time quantitative RT-PCR as described above.

A Tetracycline-suppressible HEK293 Cell Line Expressing Human CYP27—The insert from pcDNA3.1-hCYP27 was subcloned into the plasmid pTRE2 (CLONTECH) using standard molecular biology techniques (36), yielding plasmid pTRE2-hCYP27. This plasmid encodes human sterol 27-hydroxylase under the control of a tetracycline response element and a minimal CMV promoter. This plasmid and plasmid pTRE-hy (CLONTECH), which expresses a hygromycin resistance gene, were cotransfected into 293-Tet-Off cells (CLONTECH), a stable cell line enabling the suppression by tetracycline or doxycycline of genes under control of a tetracycline response element. LipofectAmine (Life Technologies) was used as a transfection reagent according to the instructions given by the manufacturer. After 3 weeks of selection in medium C supplemented with 2 μg/ml doxycycline, several clones were isolated. The clones were screened for CYP27 expression by plating on 60-mm dishes at a density of 1 million cells/dish and cultured in medium C for 48 h in the presence or absence of 2 μg/ml doxycycline. RNA was prepared and analyzed by quantitative real time RT-PCR for induction of CYP27 in the absence of doxycycline, and medium was collected and analyzed by GC-MS for accumulation of 27-hydroxycholesterol and cholestenoic acid. One clone, designated XF-4, showed a 3-fold induction of CYP27 mRNA expression in the absence of doxycycline. Medium concentrations of 27-hydroxycholesterol and cholestenoic acid were 72 and 10 nmol/liter, respectively, in the suppressed state and 90 and 140 nmol/liter, respectively, in the induced state after a 48-h incubation in the presence of 10% fetal bovine serum.

**An HEK293 Cell Line Expressing Human CYP27 and Human StAR Constitutively and a Control Cell Line—**The inserts from pcDNA3.1-hCYP27 and pcDNA3.1-hStAR were subcloned into the plasmid pIRES (CLONTECH) using standard molecular biology techniques. The resulting plasmid, p27ST, encodes the human CYP27 and the human STAR, separated by an IRES, under the control of a CMV promoter. The IRES functions as a ribosomal start site; therefore, translation can be initiated at the beginning of the mRNA transcript as well as following the IRES. This feature allows for the simultaneous functional expression of both of the two proteins when transfected into a suitable cell line. The plasmid p27ST was used to transfect 293-Tet-Off cells using LipofectAmine as described above. After 3 weeks of selection in medium C supplemented with 2 μg/ml doxycycline, several clones were isolated and propagated. The different isolated cell lines were plated on 60-mm dishes at a density of 1 million cells/dish and cultured for 24 h in medium C, and the medium was collected and analyzed by GC-MS for the presence of cholestenoic acid and 27-hydroxycholesterol. One cell line, designated XF-13, that did not have detectable 27-hydroxylase activity was saved and propagated as a control line. Another cell line, designated XF-12, demonstrated a constitutive sterol 27-hydroxylase activity, and after culturing for 24 h in medium C, the final concentration of cholestenoic acid was 220 nmol/liter (27-hydroxycholesterol not determined). This cell line was saved, propagated, and used in further experiments.

**Cholesterol Loading in CTX and Normal Skin Fibroblasts**

CTX and normal skin fibroblasts were cultured and cholesteryl-loaded as described by Francis et al. (37). Briefly, cells were set up in medium A and were allowed to reach confluence before loading with cholesterol or treatment with LXR agonist. For determination of induction of gene expression by cholesterol loading, cells were washed twice with phosphate-buffered saline, and the medium was changed to medium A (like medium C but without serum) supplemented with 30 μg/ml cholesterol. After 2 h of incubation, cells were harvested to determine loading efficiency by GC-MS and gene expression by real time PCR as described above. For determination of induction of gene expression by the LXR agonist 24(S),25-epoxycholesterol, cells were incubated with that compound (10 μM) or vehicle (ethanol) control for 24 h. After incubation, cells were harvested to determine gene expression by real time PCR as described above.

**Cholesterol Loading in HEK293 Cells Constitutively Expressing CYP27 and StAR**

The stable cell line XF-12 and a control cell line XF-13 were cultured in medium C to reach 80% confluence. Cholesterol loading was carried out in medium F (like medium C but without serum) supplemented with 30 μg/ml cholesterol or vehicle (ethanol) control. After 24 h of incubation, loading efficiency and gene expression were determined as described above.
Gas Chromatography-Mass Spectrometry

GC-MS was performed using a ThermoQuest GCQ instrument equipped with an RTX-5MS column (30 m x 0.25 mm inner diameter, 0.25-µm phase thickness; Restek Corp., Bellefonte, PA). The gas chromatography program was 180 °C for 1 min, followed by a temperature gradient of 20 °C/min to 290 °C and a final elution at 290 °C for 20 min. The injector was operated in the split mode (1:10 split), and the temperature was kept at 275 °C. Helium was used as the carrier gas at a constant flow rate of 1 ml/min. The instrument was operated in the electron ionization mode with the electron energy set to 70 eV. The ion trap was scanned between m/z 50 and 550 at the rate of 1 scan/1.5 s except when used for quantification of cholesterol, when the ion trap was used for the selected ion monitoring of m/z 458 and 464.

Determination of 27-Hydroxycholesterol and Cholestenoic Acid

To aliquots of cell media, defined amounts (500–1000 ng of each) of 22(S)-hydroxycholesterol and cholestenic acid (internal standards for 27-hydroxycholesterol and cholestenoic acid, respectively) were added. The medium (2–3 ml) was extracted with 3 volumes of chloroform/methanol (2:1, v/v), and the organic phase was collected and separated into a neutral fraction containing neutral oxysterols and an acidic fraction containing cholestenic acid and cholestenoic acid (38). The neutral fraction was further purified using Isolute Silica columns as described (23), the exception that 6 instead of 8 ml of 0.5% isopropyl alcohol in hexane was used to elute cholesterol. 27-Hydroxycholesterol and other neutral oxysterols were then eluted with 2 ml of 30% isopropyl alcohol in hexane. Prior to analysis by GC-MS, purified neutral fractions were converted to TMS ethers using Sigma Sil-A (60 °C, 1 h), and acidic fractions were methylated using trimethylsilyldiazomethane (30 min at room temperature) followed by conversion to TMS ethers. Ions used for quantitation were 22(35S)-hydroxycholesterol, m/z 355; 27-hydroxycholesterol, m/z 456; cholestenic acid, m/z 370; and cholestenoic acid, m/z 412. 27-Hydroxycholesterol was quantitated using a standard curve. No cholestenic acid standard was available, and this compound was quantitated using the assumption that the fragment of m/z 412 was generated to the same proportion as the analogous fragment of m/z 370 in the homologous internal standard cholestenic acid.

Determination of Cholesterol and Oxysterols in Cell Extracts

Cells were extracted twice (10 min with shaking each time) with 1 ml of hexane/isopropyl alcohol/water (3:2:0.1, v/v/v) 5 cm2 of cell surface area. Extracts were pooled; internal standard (126,27,3H2-holesterol (Medical Isotopes, Inc., Concord, NH) or 22(S)-hydroxycholesterol and cholestenoic acid as described above) was added for quantitative determination of cholesterol or 27-hydroxycholesterol and cholestenoic acid, respectively, and the samples were taken to dryness under a stream of argon. For determination of oxysterols, the extract was then partitioned into neutral and acidic fractions, purified on Isolute Silica columns, and derivatized and analyzed by GC-MS as described above. For the experiment shown in Fig. 1, no internal standard was added, and the separation into an acidic and a neutral fraction prior to purification on Isolute Silica columns was omitted. For determination of total cholesterol, the extract was sonicated for 1 h at 50 °C in a solution of 3 weight % KOH in 90% ethanol. One volume of water, 5 ml of isopropanol, and the mixture was extracted with two volumes of hexane. The extract was taken to dryness under argon, derivatized with Sigma Sil-A as above, and analyzed by GC-MS. The amount of cholesterol in the original sample was determined using a standard curve.

RESULTS

Identification of Oxysterols in Cholesterol-loaded Human Primary Monocyte-derived Macrophages—The observations that the LXR-responsive genes ABCA1 and ABCG1 (9–13) are induced by cholesterol loading in human monocyte-derived macrophages (21, 39) imply that these cells synthesize unknown endogenous LXR ligands. To determine the identity of the relevant oxysterol ligands, we examined oxysterol profiles in human primary macrophages. Cells were cholesterol-loaded with acetylated LDL, with a control group receiving regular medium. After 72-h incubation, media were saved and cellular lipids were extracted and analyzed for content of cholesterol and oxysterols. As expected, treatment of cells with acetylated LDL led to an accumulation of intracellular cholesterol, mainly in esterified form (Table II). GC-MS analysis of cell lipid extracts from nonloaded cells revealed the presence of barely detectable amounts of several autoxidation products of cholesterol as well as 27-hydroxycholesterol (Fig. 1A). The amount of these oxysterols was greatly enhanced in cholesterol-loaded cells (Fig. 1B). The composition of oxysterols in medium was similar to that in cell lipid extracts, although the relative amount of 27-hydroxycholesterol was higher (data not shown). Notable was the absence of detectable amounts of 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, and 24(S),25-epoxycholesterol, three compounds that have been suggested as endogenous ligands for LXR. To ensure that methodological problems were not preventing the detection of those compounds, we carried out the extraction, column separation, and GC-MS analysis of 1 µg of each of these compounds, and we were able to detect all of them satisfactorily (data not shown). While autoxidation products of cholesterol were formed in the greatest abundance under these experimental conditions, we chose to turn our interest primarily to 27-hydroxycholesterol. The reasons for this were several: autoxidation products of cholesterol are absent or occur at very low levels in human plasma (23, 40); 27-hydroxycholesterol is a major oxysterol in human atherosclerotic tissue (41, 42); and the most abundant cholesterol autoxidation products are known to be poor binders/activators of LXR (2–4).

27-Hydroxycholesterol Is an Agonist of LXRα and LXRβ—In order to determine whether 27-hydroxycholesterol is a functional activator of LXR, we utilized a fluorescence resonance energy transfer-based coactivator association assay (34). This assay measures the ligand-dependent interaction of the LXR isoforms with the coactivator SRC-1 in an in vitro system. A dose-dependent response was obtained using both LXRα and LXRβ with an EC50 for 27-hydroxycholesterol of 85 and 71 nM, respectively. When using 24(S),25-epoxycholesterol as a standard, 27-hydroxycholesterol was found to be a partial agonist of both LXRα and LXRβ, with the activity reaching a plateau at 25 and 21% of maximum, respectively (Fig. 2). Cholestenic acid is a recognized downstream metabolite of 27-hydroxycholesterol formed by multiple hydroxylations of cholesterol by the CYP27 enzyme; therefore, we decided to determine the affinity of the LXR receptors to this compound as well. However, neither of the two LXR isoforms was activated significantly by cholestenic acid in our assay (data not shown).

Intracellular Concentration of 27-Hydroxycholesterol—We next determined the intracellular concentration of 27-hydroxycholesterol and cholesterol in human primary monocyte-derived macrophages by GC-MS, using 22(S)-hydroxycholesterol and cholestenic acid, respectively, as internal standards. The amount of 27-hydroxycholesterol was 37 ± 2.4 and 115 ± 17 ng/mg protein (mean ± S.E., n = 3) in normal and cholesterol-loaded human macrophages, respectively. The intracellular space of HEK293 cells, another mammalian cell type, has been determined to be 6.3 µl/mg of protein (43). Using that value and assuming even distribution of 27-hydroxycholesterol throughout...

| Table II Cholesterol content in macrophages |
|--------------------------------------------|
| Cholesterol content | Free cholesterol |
| Normal | 63 ± 3.5 | 35 ± 1.2 |
| Loaded | 802 ± 45 | 51 ± 2.7 |

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out the cytosol, the intracellular concentration of 27-hydroxycholesterol could be estimated to be 15 ± 1.0 and 45 ± 6.8 μM (mean ± S.E., n = 3) in unloaded and loaded cells, respectively. While these values can only be regarded as approximations, they are high enough to support a role for 27-hydroxycholesterol as a physiological activator of LXR as judged from both the previous experiment in which the bulk of 27-hydroxycholesterol and cholestenoic acid was found in the medium and published data showing that both of these compounds are efficiently excreted from cells (44), we decided to only analyze the medium for 27-hydroxylated cholesterol. In order to obtain a dosed cholesterol-loading of human monocyte-derived macrophages, we treated the cultured cells with different concentrations of acetylated LDL for 72 h. At the end of the incubation period, groups of cells were harvested and cholesterol and protein content was determined. (Fig. 3A). The medium was harvested, and the levels of 27-hydroxycholesterol and cholestenoic acid were determined. (Fig. 3B). Cells in duplicate wells were harvested for RNA preparation, and expression levels of the known LXR-responsive genes ABCA1 and ABCG1 were determined using real time PCR (TaqMan®; Fig. 3C). As can be seen in Fig. 3, this experiment established a clear dose dependence for cholesterol loading, formation of 27-hydroxycholesterol and cholestenoic acid, and expression levels of ABCA1 and ABCG1. There was no significant change (p > 0.05) in the expression levels of LRα, LRβ, and CYP27 with cholesterol loading (data not shown).

**Induction of ABCA1, ABCG1, and SREBP-1c by Exogenous 27-Hydroxycholesterol and by Transient Transfection of CYP27**—We next wanted to see whether transfection of CYP27 into cultured cells would lead to induction of LXR-responsive genes. To that end, we cloned the coding region of the cDNA for human CYP27 into a mammalian expression vector by RT-PCR, using Ac-LDL-loaded human monocyte-derived macrophages as the source for RNA. The resulting construct, containing human CYP27 under the control of a CMV promoter, was then used to transfect HEK293 cells. In order to increase the formation of 27-hydroxylated cholesterol, we co-transfected a plasmid encoding the StAR protein, which is known to enhance cholesterol delivery to the mitochondria where the CYP27 enzyme is located. As shown in Fig. 4, transfection of recombinant human CYP27 and StAR resulted in a modest increase in the mRNA levels of ABCA1, ABCG1, and SREBP-1c as compared with control cells, with concomitant accumulation of 27-hydroxycholesterol and cholestenoic acid in the medium. The addition of 5 μM 27-hydroxycholesterol to the cell medium likewise led to a small increase in the mRNA levels for the same genes, whereas the documented LXR agonist 22(R)-OH-cholesterol induced the same genes to a higher degree (Fig. 4). The effects with transfected cells were, however, modest. Since

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**FIG. 1. Effects of cholesterol loading on oxysterol synthesis in human monocyte-derived macrophages.** A and B, GC-MS chromatograms obtained from cellular lipid extracts from control (A) and cholesterol-loaded (B) human monocyte-derived macrophages. The cells were cultured for 72 h in a medium containing 12% human serum with or without the addition of 200 μg/ml acetylated LDL, after which cellular lipids were extracted and oxysterol fractions were purified on a cartridge column, TMS-derivatized, and analyzed by GC-MS. To exclude the possibility that potential LXR ligands with polarity close to that of cholesterol would go undetected, cholesterol was deliberately not completely eluted before the collection of an oxysterol fraction from the cartridge column. Peaks annotated in B represent structures identified by retention time and mass spectrum. The arrows in A denote the retention times of 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 24(S),25-epoxycholesterol, respectively. (Due to partial decomposition in the GC injector, 24(S),25-epoxycholesterol gives rise to several peaks in the chromatogram (64).) None of these compounds could be detected in cell or medium extracts from control or cholesterol-loaded cells.

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**FIG. 2. 27-Hydroxycholesterol is a functional agonist of LXR.** Dose-response curve for 27-hydroxycholesterol activity in the LXRα (diamonds) and LXRβ (squares) coactivator (SRC-1) association assay described under “Experimental Procedures.”
of CYP27 in a Tet-responsive 293 Cell Line—To overcome the problems associated with transient transfections, an HEK293 cell line that stably expresses CYP27 under control of the TET promoter was developed as described under “Experimental Procedures.” This cell line, designated XF-4, confers constitutive expression of human CYP27 that can be largely suppressed by adding tetracycline or its analog doxycycline to the medium. As a control, we used the cell line XF-13, which had undergone the same transfection and selection procedure as the XF-4 cells but did not have detectable CYP27 activity. We incubated these cells in the presence or absence of doxycycline (2 μg/ml) for 24 h using a serum-free medium, since the absence of an extracellular acceptor has been shown to reduce the efflux of 27-hydroxycholesterol and cholestenoic acid from cells significantly (44). The expression levels of CYP27 mRNA in XF-4 cells with and without doxycycline were 8.6- and 34-fold higher, respectively, than that of XF-13 cells (Fig. 5A). The relative mRNA levels for ABCG1 in XF-13, XF-4-suppressed, and XF-4-induced cells were 1.0, 1.1, and 2, respectively (Fig. 5B), and the corresponding numbers for SREBP-1c were 1.0, 0.78, and 1.4, respectively. An induction of ABCG1 in the absence of doxycycline was also seen in three other cell lines with regulated expression of CYP27 that were generated in parallel with XF-4 (data not shown; SREBP-1c not determined). The message levels of ABCA1 were too low to be reliably detected. To summarize, in both stable and transient transfections, introduction of CYP27 led to the induction of LXR-responsive genes (Figs. 4 and 5).

Impaired Up-regulation of ABCA1 in CYP27-deficient Human Primary Fibroblasts in Response to Cholesterol Loading—Having thus demonstrated that overexpression of CYP27 can lead to induction of LXR-responsive genes, we next asked the question of whether deletion of CYP27 from a cell line in which it is normally expressed would lead to an impaired LXR-mediated response upon cholesterol loading. We took advantage of

the transfection efficiency was only about 30% (data not shown), we hypothesized that the true induction of LXRE-responsive genes may be higher but masked by the absence of response from the majority of cells that did not become transfected.

Induction of ABCG1 and SREBP-1c by Regulated Induction

FIG. 4. Effect of transient transfection of CYP27 and the addition of exogenous 22(R)- and 27-hydroxycholesterol on expression levels for ABCA1, ABCG1, and SREBP-1c mRNAs. Human embryonic kidney 293 cells were set up on day 0 as described under “Experimental Procedures.” On day 1, they were transfected with an empty plasmid (Mock, 220HC, 270HC) or with an equimolar mix of plasmids encoding human CYP27 and StAR cDNAs. After recovery for 24 h, the medium was changed to a serum-free medium supplemented with 22(R)-hydroxycholesterol or 27-OH-cholesterol as indicated to a final concentration of 5 μM, with the remaining dishes receiving the same amount of vehicle. After 24 h of incubation, cells were harvested for RNA preparation and real time quantitative RT-PCR analysis. The figure shows the relative amount of mRNA for ABCA1, ABCG1, and SREBP-1c from the different transfected cells (n = 3; mean ± S.E.). The amounts were normalized to those of HBP in the same cells, and the value was arbitrarily set to 1 in the mock-transfected cells receiving vehicle. #, p < 0.01; *, p < 0.05 versus mock. The results are representative of several independent experiments.

FIG. 3. Effects of cholesterol loading of human macrophages on synthesis of 27-hydroxycholesterol and cholestenoic acid and on gene expression. Effects of treatment with acetylated LDL of human monocyte-derived macrophages on intracellular cholesterol levels (A), accumulation of 27-hydroxycholesterol and cholestenoic acid in media (B), and expression levels of ABCA1 and ABCG1 (C). On day 0, cells were set up as described under “Experimental Procedures.” After 8 h, the medium was supplemented with Ac-LDL to the final concentration (μM) indicated in the figure. On day 3, conditioned media were harvested for determination of 27-hydroxycholesterol and cholestenoic acid, and cells were harvested for determination of cholesterol and protein or for RNA preparation and real time PCR analysis as described under “Experimental Procedures.” The RNA expression levels were normalized to those of HBP in the same RNA sample (n = 3; mean ± S.E.).
the fact that human fibroblasts have a small but detectable CYP27 activity (45) and that fibroblasts from CTX patients, who lack CYP27, may be cultured. We cultured primary human fibroblasts from one control subject and one CTX patient and incubated them in the presence or absence of 30 μg/ml of doxycycline to suppress expression of CYP27. After 24 h of incubation, serum was withdrawn from all of the cells. Twelve h later, the cells were harvested, and RNA were prepared for quantitative real time PCR analysis. The figure shows expression levels of CYP27 (A) and ABCG1 and SREBP-1c (B) in XF-13 cells (Mock), XF-4 cells with suppressed CYP27 expression, and induced XF-4 cells, respectively. The amounts were normalized to those of HBP in the same cells, and the value was arbitrarily set to 1 in the control cells. #, p = 0.01 versus XF-4 suppressed (n = 3; mean ± S.E.).

of ABCA1 in nonloaded normal and CTX cells were comparable (Fig. 6A), whereas the level of the SREBP-1c message in nonloaded CTX cells was 0.4-fold of that in nonloaded normal cells. The levels of the ABCG1 message were too low to be reliably detected in these cells. In order to ascertain that the LXR signaling pathway was not impaired in CTX fibroblasts, we incubated control and CTX cells for 24 h in the presence or absence of 10 μM of the LXR ligand 24(S),25-epoxycholesterol (+24EC) or vehicle (−24EC) for 24 h as described under “Experimental Procedures.” RNA was isolated from the cells, and gene expression levels were quantitated by real time quantitative PCR. A and B show the effect of cholesterol loading and 24(S),25-epoxycholesterol, respectively, on gene expression. The amounts were normalized to those of HBP in the same cells, and the value was arbitrarily set to 1 for untreated normal cells. #, p < 0.01 versus cholesterol (A) or without 24(S),25-epoxycholesterol (B). * , p < 0.05 (n = 3; mean ± S.E.); NS, not significant. The results are representative of three independent experiments.
plasmid encoding human CYP27 and human StAR, separated by an IRES, under the control of a CMV promoter, followed by hygromycin selection as described under “Experimental Procedures.” XF-12 cells have a high constitutive 27-hydroxylase activity. As a control, we used the cell line XF-13, which had undergone the same transfection and selection procedure as the XF-12 cells but did not have detectable CYP27 activity. We incubated XF-12 and XF-13 cells in the presence or absence of 30 μg/ml cholesterol or vehicle for 24 h in a serum-free medium as described under “Experimental Procedures.” RNA was isolated from the cells, and gene expression levels were quantitated by real-time quantitative PCR. The cell medium was collected, and the concentrations of oxysterols were determined by GC-MS.

**DISCUSSION**

Sterol 27-hydroxylase, encoded by the CYP27 gene, is a ubiquitously expressed enzyme, although the highest expression is found in the liver (46). Its importance for bile acid synthesis has long been documented (for a review, see Ref. 47). Its function in extrahepatic tissues, however, has been less clear. Over the last several years, it has become apparent that nonhepatic cells can eliminate cholesterol by CYP27-mediated formation of 27-hydroxycholesterol and cholestenoic acid, which are then excreted (44). This represents an alternative pathway for elimination of intracellular cholesterol that may be of quantitative importance in certain cells. We now demonstrate an additional function for sterol 27-hydroxylase in generating important endogenous activators for LXR. Our evidence comes from multiple experimental approaches: endogenous production of 27-hydroxycholesterol in normal human primary macrophages, transient and stable transfection of CYP27, and studies on human cells lacking CYP27. In all cases, we showed that 27-hydroxylation of cholesterol creates LXR ligands. In particular, we have demonstrated the role of CYP27 in mediating the cellular response to cholesterol overload. Furthermore, 27-hydroxycholesterol was shown to be a potent partial agonist of both LXRα and LXRβ in a fluorescence resonance energy transfer-based coactivator association assay.

To demonstrate that CYP27 generates LXR ligands in cells, we used the messages for the ABC transporters ABCA1 and ABCG1 as well as the lipid biosynthesis regulator SREBP-1c as endogenous reporter genes for LXR. Whereas under the experimental conditions used in this study, ABCA1 and ABCG1 levels appear to faithfully mirror the intracellular LXR tone, the situation for SREBP-1c is more complex. Thus, in our cell systems, ABCA1 and ABCG1 messages were invariably induced by both exogenous LXR ligands and cholesterol loading. We also consistently saw an induction of SREBP-1c message by exogenous LXR agonists; however, cholesterol loading of fibroblasts resulted in a small but statistically significant reduction in that message. While it goes beyond the scope of this study to investigate the regulation of SREBP-1c, the data are in agreement with the notion that this gene may be negatively regulated by a sensing mechanism for intracellular cholesterol in addition to and distinct from the LXR pathway. Evidence for such a model was recently presented in a study in which it was demonstrated that the mouse SREBP-1c gene contains a sterol regulatory element that is essential to the basal promoter activity and that confers responsiveness to activation by nuclear SREBPs (48).

This mechanism is predicted to confer increased expression of SREBP-1c under conditions when cholesterol synthesis is elevated, and vice versa. Consequently, the absence of induction of SREBP-1c message by cholesterol loading in human primary fibroblasts with or without CYP27 is still compatible with our primary conclusion that 27-hydroxylation is an important mechanism for the generation of endogenous LXR ligands.

This complexity of the regulation of SREBP-1c may also help explain the apparent absence of decreased LXR tone observed in mice with a targeted disruption in the CYP27 gene (49, 50). These mice have increased hepatic expression of SREBP-1c and downstream target genes (50) as well as of CYP7A1, which in the mouse is an LXR target gene (7). CYP27−/− mice have very low bile acid synthesis (49) and, possibly as a consequence thereof, negligible intestinal cholesterol absorption (50), which they compensate for with a severalfold increase in cholesterol synthesis in the liver and elsewhere (50). As discussed above,
such a condition would be expected to be accompanied by an increase in the SREBP-1c message. The up-regulation of CYP7A1 seen in CYP27−/− mice was attributed to reduced pressure on the farnesoid X receptor by its endogenous ligands, bile acids, relieving the repression on the CYP7A1 gene indirectly exerted by that receptor (50). It is possible that these mechanisms may have overridden any reduction in LXR tone that would have been expected to result in decreased SREBP-1c and CYP7A1 expression.

As shown here, CYP27 can mediate LXR activation as a response to cholesterol loading, and this may be the dominant mechanism for induction of LXR-responsive genes in human macrophages. Sterol 27-hydroxylase may accordingly protect these cells from cholesterol overload by two concurrent but separate mechanisms. One of these, described previously (44, 51–53) utilizes the increased solubility of 27-hydroxycholesterol and cholestenoic acid to excrete these compounds from the cell. The second mechanism, described here, operates via activation of LXR, stimulating ABCA1-mediated reverse cholesterol transport. Both of these pathways would be expected to be impaired in CTX patients, lacking sterol 27-hydroxylase activity. This is compatible with the observation of increased incidence of atherosclerosis in CTX patients (26). Standard therapy for CTX patients consists of oral supplementation of chenodeoxycholic acid, which suppresses endogenous bile acid synthesis and the accumulation of toxic intermediates but does not address the possible impairment in cholesterol efflux. Our present results suggest that CTX patients might benefit from supplementation of a synthetic LXR agonist in addition to the current therapy to compensate for the impaired formation of endogenous LXR ligands.

There exists limited information on humans with elevated plasma 27-hydroxycholesterol. There is a single case report of one patient homozygous for a nonsense mutation in the CYP7B1 gene, which encodes the major catabolic enzyme for 27-hydroxycholesterol. The patient had plasma concentrations of 27-hydroxycholesterol that were more than 1000-fold greater than normal (54). This patient suffered liver failure and died at 5 months of age. More moderate increases (2–3-fold) in plasma 27-hydroxycholesterol have recently been reported in patients with Smith-Lemli-Opitz syndrome (SLOS) (55). SLOS is caused by mutations in the 7-dehydrocholesterol 7-reductase gene, which catalyzes the last step in the biosynthesis of cholesterol, and patients have very low levels of total cholesterol and of all lipoproteins (56). Unfortunately, SLOS is a severe and pleiotropic disease. For both SLOS and CYP7B1 deficiency, therefore, the severe nature of the inborn errors prevent a clear understanding of the metabolic effects of high levels of 27-hydroxycholesterol. In contrast, mice with targeted disruption of CYP7B1 have a grossly normal appearance and exhibit 3–4-fold elevated levels of 27-hydroxycholesterol (57). No significant changes in lipoprotein profile were found in these animals; however, there was a small increase in protein levels of CYP7A1, which in the mouse is an LXR-responsive gene. It would be interesting to know whether there is an increased expression of ABCA1 or ABCG1 in these animals. Thus, for both CYP7B1 and CYP27, a genetic deficiency causes less dramatic phenotypes in mice than in humans. This may imply the existence of other, compensating hydroxylase activities in mice.

From the present study, we cannot say with certainty whether 27-hydroxycholesterol or cholestanolic acid is the more significant endogenous ligand for LXR. While cholestanolic acid failed to induce LXR-SRC-1 association in our hands, recent studies have demonstrated activation of LXR by that compound using other assays (58). Nevertheless, circumstantial evidence suggests that 27-hydroxycholesterol is the more important of the two candidates discussed here. Cholestanolic acid is very efficiently excreted from cells (44), and whereas we only found trace amounts of intracellular cholestanolic acid in cholesterol-loaded human macrophages, 27-hydroxycholesterol was present in significant amounts. This is in agreement with previously published results (59). Likewise, in culture medium from human skin fibroblasts, 27-hydroxycholesterol but not cholestanolic acid could be detected. Finally, recent data show that the cholestanolic acid present in the circulation originates mainly from the lung (60), which would not be expected from an endogenous ligand of a ubiquitously expressed receptor. It should be pointed out that 27-hydroxycholesterol has previously been shown to bind or activate LXR in various assays (2, 4, 5).

It is likely that endogenous ligands for LXR are formed by other pathways in addition to 27-hydroxylation of cholesterol. In particular, recent findings suggest that intermediates in the cholesterol biosynthetic pathway serve as LXR ligands. In a recently published study, inhibition of cholesterol synthesis with compactin in the rat hepatoma cell line McA-RH7777 led to a down-regulation of the mRNA for the LXR-responsive gene SREBP-1c (61). This block could be circumvented by the addition of mevalonate or exogenous LXR ligands. It was concluded that endogenous ligands for LXR were formed as intermediates of cholesterol biosynthesis. 27-Hydroxycholesterol is not an intermediate in the synthesis of cholesterol, and, at least in that particular cell line, it is likely that there are other endogenous ligands for LXR, whose concentrations reflect the rate of cholesterol synthesis in the cell. One suggested (62) candidate compound reflecting cholesterol synthesis is 24(S),25-epoxycholesterol, which is postulated to be formed via a shunt in the cholesterol biosynthetic pathway (6) and is a known potent LXR agonist (2). This compound has been found in human liver (63) and recently also in rat liver (64), albeit at very low concentrations. We could not, however, detect that compound in extracts from human monocyte-derived macrophages, possibly due to a very low need for de novo sterol synthesis in these nonproliferating cells. In conclusion, it appears likely that intracellular LXR tone may be set by two parameters: the degree of cholesterol loading of the cell, mediated by 27-hydroxylated cholesterol as shown here, and the rate of cholesterol synthesis, mediated by 24(S),25-epoxycholesterol or some other cholesterol biosynthesis intermediate. Work toward the positive identification of the latter type of LXR ligand is currently under way in our laboratory.

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