Sterol Intermediates from Cholesterol Biosynthetic Pathway as Liver X Receptor Ligands*

The liver X receptors (LXRs) are ligand-activated transcription factors that regulate the expression of genes controlling lipid metabolism. Oxysterols bind LXRs with high affinity in vitro and are implicated as ligands for the receptor. We showed previously that accumulation of selected dietary sterols, in particular stigmasterol, is associated with activation of LXR in vivo. In the course of the defining of structural features of stigmasterol that confer LXR agonist activity, we determined that the presence of an unsaturated bond in the side chain of the sterol was necessary and sufficient for activity, with the C-24 unsaturated cholesterol precursor sterols desmosterol and zymosterol exerting the largest effects. Desmosterol failed to increase expression of the LXR target gene, ABCA1, in LXRα/β-deficient mouse fibroblasts, but was fully active in cells lacking cholesterol 24-, 25-, and 27-hydroxylase; thus, the effect of desmosterol was LXR-dependent and did not require conversion to a side chain oxysterol. Desmosterol bound to purified LXRα and LXRβ in vitro and supported the recruitment of steroid receptor coactivator 1. Desmosterol also inhibited processing of the sterol response element-binding protein-2 and reduced expression of hydroxymethylglutaryl-CoA reductase. These observations are consistent with specific intermediates in the cholesterol biosynthetic pathway regulating lipid homeostasis through both the LXR and sterol response element-binding protein pathways.

The liver X receptors (LXRα and LXRβ) are ligand-dependent transcription factors belonging to the nuclear hormone receptor superfamily (1). Although the two transcription factors are encoded by separate genes, LXRα and LXRβ share 78% similarity within the ligand-binding domains (2). LXRβ (NR1H2) is ubiquitously expressed, whereas LXRα (NR1H3) has a more restricted distribution with the highest expression observed in the liver, adipose tissue, intestine, kidney, and macrophages (3). LXRs regulate multiple genes involved in lipid metabolism, including those involved in sterol transport (4, 5) and fatty acid biosynthesis (6–8). More recently, LXRs have been found to play a role in the regulation of glucose metabolism (9, 10), immunity, and cellular responses to various environmental stresses (11–14).

To function as a transcription factor, LXR must heterodimerize with retinoid X receptor (RXRs) and then bind to LXR-response elements in target genes. The LXR-response elements consist of two direct hexanucleotide repeats separated by four nucleotides (DR4 element) (3). The binding of LXR or RXR ligands results in a conformational change in the heterodimer and recruitment of nuclear receptor coactivators such as steroid receptor coactivator-1 (SRC-1), resulting in the activation of gene transcription (16). Several compounds have been identified that are potent LXR agonists, including various oxysterols (17, 18). Position-specific monooxidation of the sterol side chain leads to high affinity binding and activation of LXR (19). Analysis of the crystal structure of the LXRα:RXR heterodimer is consistent with an important role for the hydroxyl group at the 22, 24, or 27 positions in the cholesterol side chain in ligand binding (20). Accordingly it has been speculated that naturally occurring oxysterols, such as 20(S), 22(R), 24(S), 25-, and 27-hydroxycholesterol and 24(S),25-epoxycholesterol are endogenous LXR ligands (17, 18, 21–23).

Previously, we showed that the accumulation of noncholesterol sterols increased the expression of the LXR target gene Abca1 in the adrenal glands of mice (24). Studies in a cultured adrenal cell line (Y1–BS1) revealed that expression of ABCA1 and of an LXR-reporter construct was stimulated by the dietary plant sterol, stigmasterol, but not by sitosterol (24). Stigmasterol has a ring structure identical to cholesterol, but contains a double bond between C-22 and C-23 and an ethyl group attached to C-24 in the side chain. Unlike the oxysterols that activate LXR, stigmasterol does not have a hydroxyl group in the side chain. Stigmasterol also interferes with the processing of sterol response element binding protein-2 (SREBP-2) (24), a central regulatory factor in cholesterol metabolism (25). SREBP-2 is synthesized in the endoplasmic reticulum, and is...
transported to the Golgi complex in association with the SREBP cleavage activating protein (SCAP). When cells are replete with cholesterol, the SCAP-SREBP complex associates with INSIG, an ER resident protein, resulting in retention of the complex. Reduction of cellular cholesterol is associated with a dissociation of SCAP and SREBP from INSIG, and the SCAP-SREBP complex is transported to the Golgi complex where SREBP is proteolytically cleaved to release the transcriptionally active form of the protein.

To further characterize the mechanism by which stigmasterol activates LXR and suppresses the SREBP pathway, we investigated the relative ability of other structurally related sterols with modified side chains to activate LXR and suppress SREBP cleavage. Unsaturation within the side chain was associated with LXR activation and desmosterol, a late intermediate in the cholesterol biosynthetic pathway, was identified as a potent activator of LXR in cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The ent-desmosterol was prepared as described previously (26) and other sterols were from Sigma or Steroloids (Newport, RI); ketoconazole, triparanol, 58-035, and sodium arachidonate were from Sigma, and [26,27,3H]-24(S),25-epoxycholesterol (72 Ci/mmol) was obtained from PerkinElmer Life Sciences. T0901317 was purchased from Cayman Chemical (Ann Arbor, MI). Polyclonal antibodies against the bovine low density lipoprotein receptor (LDLR) (27), SREBP-2 (28), and a monoclonal antibody (IgG-A9) against HMG-CoA reductase (29) were generated as described. Polyclonal anti-calnexin antibody was obtained from Stressgen (Victoria, BC, Canada) and anti-ABCA1 antibody was obtained from Novus Biologicals (Littleton, CO).

**Cell Culture**—Chinese hamster ovary (CHO)-7 cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate (medium A) supplemented with 5% normal calf lipoprotein-poor serum (NCLPPS). SRD-12B cells, which lack site-1 protease (30), and SRD-13A cells, which lack SCAP (31), were maintained in medium A supplemented with 1 mM mevalonate, 20 μM oleate, 5 μg/ml cholesterol, 500 μg/ml G418. SRD-14 cells, which lack INSIG-1, were maintained in medium A supplemented with 5% NCLPPS and 10 μg/ml G418. SRD-14 cells, which lack site-1 protease (30), and SRD-13A cells, which lack SCAP (31), were maintained in medium A supplemented with 1 mM mevalonate, 20 μM oleate, 5 μg/ml cholesterol, 500 μg/ml G418. SRD-14 cells, which lack INSIG-1, were maintained in medium A supplemented with 5% NCLPPS and 10 μg/ml G418. Mouse embryonic fibroblast (MEF) cells were generated from Lxra/β double knock-out mice (6), and from 24-, 25-, and 27-hydroxylase triple knockout mice. The triple knock-out mice were developed in the laboratory of David W. Russell by breeding the 24-hydroxylase knock-out mice (33) with the 27-hydroxylase (34) and 25-hydroxylase knock-out mice.5 The cells were grown in Dulbecco’s modified Eagle’s medium, containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate (medium B) supplemented with 10% fetal calf serum. All cells were grown at 37 °C in 8–9% CO₂.

**SCAP-INSIG-1 Binding Assay**—The interaction between SCAP and INSIG-1 was examined using Blue Native-PAGE as described (35) except that 250 mM sucrose and 1% Nonidet P-40 were used in a buffer system for the membrane protein preparation instead of 250 mM sorbitol and 2% digitonin.

**Lipid Chemistries**—The levels of neutral sterols in cells were measured as follows: after 24 h of sterol treatment, cells were washed twice with 10 ml of phosphate-buffered saline containing 0.1% bovine serum albumin. Cells were then rinsed twice with 10 ml of phosphate-buffered saline and collected by lysis in 1 ml of 0.1 N NaOH. Ten-microliter aliquots of cell lysates were used to assay protein concentration by BCA (Pierce) and neutral sterols by gas chromatography-mass spectroscopy. A total of 200 μl of cell lysate was added to 1 ml of 33% KOH (in ethanol) containing 10 μg of epicoprostanol as internal standard. After saponification at 70 °C for 1 h, 1 ml of water and 2 ml of petroleum ether were added to each sample. Samples were vigorously vortexed, centrifuged, and the organic phases were collected and dried under nitrogen. Sterols were derivatized using trimethylsilane (Pierce) and assayed by gas chromatography-mass spectroscopy as described (36). Oxysterols were analyzed by liquid chromatography-mass spectrometry. A total of 200 μl of cell lysate was added to a fresh tube containing 1.6 ml of water, 2 ml of chloroform, and 2 ml of methanol. Samples were vigorously vortexed, centrifuged, and the organic phases (lower) were collected and dried under nitrogen. Samples were then resuspended in methanol and loaded onto a reverse-phase high performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) using a binary solvent system consisting of solvent A (methanol) and solvent B (water), both with 5 mM ammonium acetate (37). Gradient elution was performed on a Luna C18 reverse-phase HPLC column (1 × 150-mm, 3 μm particle size; Phenomenex, Torrance, CA) at a flow rate of 0.25 ml/min and a temperature of 25 °C. The gradient began at 85% B for 1 min, then increased from 85% B to 100% B over 5 min, maintained at 100% B for 10.5 min, and then re-equilibrated to the starting conditions for 5 min. The HPLC was coupled to a 4000 QTrap triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with an electrospray ionization source. The mass spectrometer was operated in selected reaction monitoring mode with transitions optimized for each sterol of interest. The ionization voltage was 5500 V, curtain, nebulizing, and turbo gas was 15, 80, and 20 p.s.i., respectively; collision energy and declustering potential were optimized for each selected reaction monitoring transition and ranged from 10–40 to 50–120 V, respectively. The curtain, collision, and turbo gases were nitrogen when air was used for the nebulizing gas. Selected reaction monitoring transitions were optimized for each analyte and consisted of either the [M + NH₄]⁺ or [M + H]⁺ adduct ion and loss of one or two waters, [M + H₂O]⁻ or [M + H₂H₂O]⁺, respectively. Ionization characteristics of sterols are source and platform dependent; parameters must be optimized for each instrument. Example product ion mass spectra are shown at lipidmaps.org. Quantitation was performed with stable-isotope dilution using d3-25-hydroxycholesterol (27,27,27-d3, cholest-5-en-3β,25-diol; 99%) as the surrogate and d7-7-oxocholesterol (25,26,26,26,27,27,27-d7, 7-oxo-cholest-5-en-3β-ol, 99%) as the internal standard (Avanti Polar Lipids, Alabaster, AL). All solvents were HPLC grade or better.

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5 G. Liang, unpublished data.
Endogenous Ligands for LXR

**FIGURE 1. Effect of stigmasterol on ABCA1 expression and SCAP/INSIG-1 interaction in CHO cells. A, CHO-7 cells were plated at a density of 7 × 10^5 cells in 10-cm dishes and on day 2 the medium was replaced with medium containing 10 μM compactin and 50 μM mevalonate. After 16 h, cells were treated with 0.1 μM T0901317, 125 μM sitosterol, 125 μM stigmasterol + 200 μM arachidonate. After 16 h, immunoblot analysis was performed on the membrane proteins using a polyclonal antibody to ABCA1 as described under “Experimental Procedures.” B, SRD-13A cells, which do not express SCAP, were plated at a density of 3 × 10^5 on day 1, and from wild type and SRD-12B, SRD-13A, SRD-14 cells, and from wild type and Lxrβ/−−/− MEF cells as described (40).**

### Promoter Reporter Assay for LXR Activity—Activation of LXR was measured using a reporter assay as described (24).

Briefly, CHO-7 cells were grown to 50–60% confluence in 6-well plates, transfected with 1.5 μg of plasmid DNA including 0.2 μg of pCMX-Gal4-LXRα, 0.8 μg of pMH100X4-TK-luc, and 0.5 μg of pCMX-Bgalactosidase using FuGENE 6 (Roche Applied Science), then incubated in medium A containing 10 μM compactin and 50 μM mevalonate supplemented with 5% NCLPPS. Sterols (cholesterol, sitosterol, desmosterol, or lanosterol) were added in ethanol to a final concentration of 1 μM. After 20 h, cells were washed and switched to medium containing 1% hydroxypropyl cyclodextrin. After incubation for 5 h, cells were pooled for measurement of SCAP-INSIG-1 complex formation by Blue Native-PAGE and immunoblot analysis as described under “Experimental Procedures.”

### GST Pulldown Assays—CDNA encoding the nuclear boxes of human SRC-1 (amino acids 595–771) was cloned into the GST fusion vector pGEX5X-2 (Amersham Biosciences). GST-SRC-1 fusion protein was expressed in BL21(DE3) cells (Promega) and affinity purified onto glutathione-Sepharose 4B beads according to the manufacturer’s protocol (Amersham Biosciences). [35S]-Labeled human LXRα and human LXRβ proteins were generated by the TnT Quick Coupled Transcription/Translation System (Promega). GST pulldown assays were performed as previously described (41) with slight modifications. A total of 2 μg of GST-SRC-1 fusion protein was bound to glutathione-Sepharose 4B beads and equilibrated in binding buffer (20 mM Tris-HCl, pH 7.9, 150 mM KCl, 4 mM MgCl2, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40, 2 mg/ml bovine serum albumin). Bound GST protein was then incubated with labeled LXRα or LXRβ for 4 h at 4 °C in the presence or absence of 1 or 10 μM desmosterol or 1 μM T0901317. After washing with binding buffer five times, beads were resuspended in SDS-PAGE sample buffer, and loaded onto a 10% SDS-PAGE gel for electrophoresis. Bands for bound proteins were visualized by autoradiography.

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### Statistical Analysis—Numerical data are reported as mean ± S.E.

#### RESULTS

**Stigmasterol Increases ABCA1 Expression and Enhances the Formation of SCAP-INSIG-1 Complexes in CHO Cells**—Stigmasterol disrupts cholesterol homeostasis by activating LXR and suppressing the SREBP pathway (24). To further define the action of stigmasterol on these pathways, we examined the effect of stigmasterol on expression of the LXR target gene ABCA1, and on the interaction between SCAP and INSIG-1 in

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**TABLE 1.**

| Condition | ABCA1 | Calnexin |
|-----------|-------|----------|
| Control   | 100   | 100      |
| Stigmasterol | 120   | 90       |
| Cholesterol | 110   | 95       |

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**Figure 2.**

**A** is a graph showing the expression levels of ABCA1 and Calnexin under different conditions. **B** is a Western blot showing SCAP-INSIG-1 complex formation in CHO cells treated with stigmasterol. The figure shows that stigmasterol increases ABCA1 expression and enhances the formation of SCAP-INSIG-1 complexes.
CHO-7 cells. Stigmasterol treatment increased ABCA1 protein levels 4–5-fold, whereas sitosterol did not increase ABCA1 expression (Fig. 1A). Addition of arachidonate, an LXR antagonist (42), abolished the effect of stigmasterol on ABCA1 levels, which is consistent with stigmasterol increasing ABCA1 expression by activating LXR.

To probe the mechanism by which stigmasterol suppresses SREBP cleavage (24), we used Blue Native gel electrophoresis to examine the effect of stigmasterol on the interaction between SCAP and INSIG-1 (Fig. 1B). SRD-13A cells, which fail to express SCAP, were co-transfected with SCAP and INSIG-1. In the absence of added sterols, no SCAP-INSIG-1 complex was observed and all INSIG-1 appeared in an unbound form. In cells treated with 25-hydroxycholesterol, a potent suppressor of the SREBP pathway, the amount of INSIG-1 in the unbound form was reduced, and a SCAP-INSIG-1 complex was detected. Stigmasterol also promoted the formation of a SCAP-INSIG-1 complex, whereas sitosterol did not. These findings suggest that stigmasterol suppresses the SREBP pathway by promoting the formation of SCAP-INSIG-1 complexes in the ER.

Sterols with Unsaturated Side Chains Up-regulate ABCA1 in CHO-7 Cells—Sitosterol and stigmasterol differ only by the presence of a double bond at C-22 in the side chain of stigmasterol. To determine whether the effect of stigmasterol on ABCA1 expression is conferred by the Δ22 moiety, we compared the effects of two other Δ22 sterols (22-dehydrocholesterol and brassicasterol) and their structural homologs with saturated side chains (cholesterol and campesterol) on ABCA1 levels in cultured CHO-7 cells (Fig. 2A). The Δ22 sterols (22-dehydrocholesterol and stigmasterol) increased ABCA1 expression more than the homologs with saturated side chains. Brassicasterol did not increase ABCA1 levels despite having a Δ22 double bond in its side chain. This may reflect the orientation of the C-24 methyl group in brassicasterol, which is in the 24(S), rather than the 24(R) configuration.

Next we examined whether the position of the double bond in the sterol side chain influenced the effect on ABCA1 expression. CHO-7 cells were treated with 25 μM cholesterol, 20-dehydrocholesterol, 22-dehydrocholesterol, 24-dehydrocholesterol, or 24,28-methylene cholesterol (fucosterol) for 16 h (Fig. 2B). ABCA1 protein levels were increased in cells treated with 20-dehydrocholesterol, 22-dehydrocholesterol, and 24-dehydrocholesterol, but not in cells treated with fucosterol. The largest increase in ABCA1 protein level was seen in the cells treated with 24-dehydrocholesterol. The greater potency of 24-dehydrocholesterol is not due to preferential accumulation of this sterol, because each of the sterols administered accumulated to comparable levels in the cells (data not shown). 24-Dehydrocholesterol is an intermediate in the cholesterol biosynthetic pathway and is commonly known as desmosterol.

Desmosterol Induces a Dose-dependent Increase in ABCA1 Levels in CHO-7 Cells—ABCA1 protein levels were consistently increased within 4 h after addition of 25 μM desmosterol to CHO-7 cells and continued to increase for at least 16 h (Fig. 2C). Desmosterol also reduced expression of the SREBP target genes LDLR and HMGCR, as indicated by the decreased levels of LDLR and HMGCR protein at the 16-h time point. Accordingly, all subsequent sterol treatments were performed for...
A dose-response experiment indicated that increased expression of ABCA1 was readily apparent at 1.2 μM desmosterol, the lowest concentration tested, and increased progressively with increasing concentrations (Fig. 2D). Conversely, expression of the LDLR decreased progressively with increasing concentrations of desmosterol. Expression of HMGCR was almost completely abolished at a desmosterol concentration of 2.5 μM.

To determine whether other sterol intermediates in the cholesterol biosynthetic pathway (Fig. 3A) stimulate expression of ABCA1, we incubated CHO-7 cells with 7-dehydrocholesterol, zymosterol, lathosterol, or lanosterol, as well as desmosterol and cholesterol. Desmosterol and zymosterol increased ABCA1 expression, but addition of the other cholesterol precursor sterols (7-dehydrocholesterol, lathosterol, and lanosterol) did not (Fig. 3B). Zymosterol treatment increased cellular concentrations of both zymosterol and desmosterol, consistent with zymosterol being metabolized to desmosterol (Fig. 3A and Table 1). Treatment with 2.5 μM desmosterol resulted in a 15-fold increase in cellular desmosterol, but did not increase cellular levels of zymosterol. These results indicate that sterols that increase cellular levels of desmosterol increase ABCA1 expression. Therefore, subsequent experiments focused on the role of desmosterol in LXR regulation.

**Increased Expression of ABCA1 by Desmosterol Is Mediated by LXR and Not by the SREBP Pathway**—Desmosterol treatment increased ABCA1 expression and reduced HMGCR levels and LDLR levels in CHO-7 cells (Fig. 2C), suggesting that desmosterol, like stigmasterol, acts on both the LXR and the SREBP pathways. To assess the roles of these pathways in the response of ABCA1 to desmosterol, we assayed ABCA1 expression in cells in which these pathways were disrupted.

Immunoblotting of nuclear SREBP-2 in cultured CHO-7 cells revealed that desmosterol treatment reduced SREBP-2 processing (Fig. 4A), confirming that desmosterol suppresses the SREBP pathway. Interestingly, zymosterol did not suppress SREBP-2 cleavage in these cells. To determine whether the desmosterol-induced increase in ABCA1 expression requires the SREBP pathway, cells in which SREBP processing is either disrupted by inactivating site 1

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**FIGURE 3. Overview of the cholesterol and 24(S),25-epoxycholesterol synthetic pathways.** A, the enzyme inhibitors used in the experiments described in the text are highlighted. Sterols that up-regulated expression of ABCA1 are shown in bold. B, ABCA1 protein levels in CHO-7 cells treated with sterol intermediates in the cholesterol biosynthetic pathway. CHO-7 cells were prepared as described in the legend to Fig. 1, and treated with 2.5 μM of the indicated sterols, 25-hydroxycholesterol (2.5 μM), or T0901317 (0.1 μM). After 16 h, the cells were harvested and processed for immunoblot analysis of ABCA1 protein levels. Cellular sterol levels are provided in Table 1. The experiment was repeated twice and similar results were obtained.
protease (SRD-12B cells) (43) or SCAP (SRD-13A) (31) or augmented by inactivating INSIG-1 (SRD-14 cells) (32) were treated with desmosterol; increased ABCA1 levels were seen in association with desmosterol treatment in all three cell lines (Fig. 4B). Therefore neither suppression nor activation of the SREBP pathway altered either basal or desmosterol-induced expression of ABCA1.

The role of LXR was examined using embryonic fibroblasts (MEF) from mice lacking both LXRα and LXRβ (Lxrαβ/−/−). Desmosterol (2.5 μM) markedly increased both the protein and the mRNA levels of ABCA1 in wild type MEF cells, but this response was abolished in Lxrαβ/−/− MEF cells (Fig. 5, A and B). Thus LXR is required for the desmosterol-induced increase in ABCA1 expression. Messenger RNA levels of ABCG1, another LXR target gene (9, 44), were also increased in wild type MEF cells but not in Lxrαβ/−/− MEF cells treated with desmosterol (Fig. 5B). Messenger RNA levels of genes in the fatty acid biosynthetic pathway (fatty acid synthase, stearoyl-CoA desaturase-1, and SREBP-1c, which are increased in liver in response to LXR agonists (6, 45, 46)), were not increased in response to desmosterol in wild type or Lxrαβ/−/− MEF cells (Fig. 5B).

Desmosterol-Induced Activation of LXR Does Not Require Accumulation of 24-, 25-, or 27-Hydroxycholesterol or 24(S),25-Epoxycholesterol—Oxysterols are proposed to function as endogenous ligands for LXR (17). To determine whether desmosterol-induced LXR activation is mediated by the formation of 24-, 25-, or 27-hydroxycholesterol, the three most abundant side chain oxysterols (47), we treated MEF cells that were deficient in cholesterol 24-, 25-, and 27-hydroxygenase (provided by David W. Russell, University of Texas, Southwestern) with cholesterol, desmosterol, and zymosterol (2.5 μM) for 16 h. ABCA1 levels increased in wild type MEF cells and in the 24-, 25-, and 27-hydroxylase-deficient MEF cells with desmosterol and to a lesser extent with zymosterol treatment (Fig. 6A). Thus desmosterol stimulates LXR without being converted to 24-, 25-, or 27-hydroxycholesterol.

To assess the effect of endogenously synthesized desmosterol on LXR target genes we inhibited desmosterol reductase (DHCR24) in wild type and 24-, 25-, and 27-hydroxylase-deficient MEF cells using triparanol (Fig. 6B), and small interfering RNA (Fig. 6C). Both treatments were associated with a 10–40-fold increase in cellular levels of zymosterol and desmosterol (documented by gas chromatography-mass spectroscopy, data not shown) and were accompanied by an increase in the expression of ABCA1. Similar results were obtained using triparanol in peritoneal macrophages from the two mouse strains (Fig. 6B). Thus, increasing endogenous desmosterol levels increases the expression of ABCA1.

Next we tested whether desmosterol stimulated LXR by promoting the accumulation of 24(S),25-epoxycholesterol, another potent LXR ligand (18). Cellular levels of 24(S),25-epoxycholesterol increased ~1.5-fold in CHO-7 cells treated with 2.5 μM desmosterol for 16 h (Table 2). Treatment with the sterol 14α-demethylation inhibitor ketoconazole resulted in greater increases in cellular 24(S),25-epoxycholesterol levels (Table 2) but did not increase ABCA1 expression (Fig. 6D). Thus, increased levels of 24(S),25-epoxycholesterol are not invariably associated increases in ABCA1 expression. This finding suggests that the effect of desmosterol on ABCA1 expression is not mediated by 24(S),25-epoxycholesterol.
Enantioselective interactions between sterols and proteins are enantioselective, whereas interactions between sterols and lipids show little or no enantioselectivity (48). To determine whether the effect of desmosterol on LXR activation was enantioselective, we incubated CHO-7 cells with desmosterol and with the enantiomer ent-desmosterol. No increase in ABCA1 expression was seen in cells treated with the enantiomer of desmosterol at a concentration of 2.5 μM, and expression was only modestly increased at a concentration of 12.5 μM (Fig. 7),
Sterols in CHO-7 cells treated with inhibitors of cholesterol biosynthesis

Values are micrograms/mg of cellular protein.

| Treatment | Cholesterol | Lathosterol | Lanosterol | Desmosterol | Zymosterol | 7-DC<sup>a</sup> | 24(S),25-EC<sup>b</sup> |
|-----------|-------------|-------------|------------|-------------|-------------|----------------|-----------------|
| Control   | 19.42       | 0.10        | 0.05       | 1.51        | 0.25        | 1.64           | 0.22            |
| Desmosterol (2.5 μM) | 12.25       | 0.00        | 0.03       | 4.30        | 0.24        | 0.70           | 0.34            |
| Compactin (5 μM) | 14.45       | 0.01        | 0.01       | 0.38        | 0.00        | 1.10           | 0.16            |
| Ketoconazole (5 μM) | 13.38       | 0.02        | 4.73       | 0.92        | 0.00        | 1.00           | 0.62            |
| Triparanol (5 μM) | 14.21       | 0.21        | 0.25       | 1.41        | 12.31       | 0.94           | 2.02            |
| S8-035 (5 μM) | 22.78       | 0.29        | 0.10       | 1.57        | 0.49        | 1.73           | 0.29            |
| T0901317 (0.1 μM) | 18.11       | 0.12        | 0.05       | 1.47        | 0.30        | 1.48           | 0.20            |

<sup>a</sup>7-DC, 7-dehydrocholesterol.

<sup>b</sup>24(S),25-EC, 24(S),25-epoxycholesterol.

**FIGURE 7.** Desmosterol-stimulated increase in ABCA1 expression is enantiospecific. CHO-7 cells were prepared as described in the legend to Fig. 1, and then treated with 2.5 and 12.5 μM cholesterol, desmosterol, or ent-desmosterol for 16 h. Cell membranes were isolated and subjected to immunoblotting as described under “Experimental Procedures.”

suggesting that desmosterol activates LXR through interaction with protein(s), rather than with lipids.

**Desmosterol Binds Directly to LXRα and LXRβ and Induces Recruitment of the Cofactor SRC-1**—A ligand competition assay was used to test for direct interaction between desmosterol and the ligand binding domains of LXRα and LXRβ. Sigmoidal competition curves were obtained using 24(S),25-epoxycholesterol and desmosterol (Fig. 8A): the binding affinity of desmosterol was ~6-fold lower than that of 24(S),25-epoxycholesterol for both LXRα and LXRβ.

To determine whether the binding of desmosterol to LXR induced recruitment of the cofactor SRC-1, a GST-SRC-1 fusion protein was immobilized on glutathione-Sepharose 4B beads and mixed with 35S-labeled LXRα or LXRβ in the absence or presence of desmosterol or T0901317. After 4 h, beads were washed several times and bound proteins were eluted and separated on 10% polyacrylamide gels. The amount of 35S-LXR protein in the eluates was determined by autoradiography. Desmosterol enhanced association of both LXRα and LXRβ with the GST-SRC-1 fusion protein in a dose-dependent manner (Fig. 8B).

The effect of desmosterol on the interaction between LXR and SRC-1 was then examined in cultured cells using a luciferase reporter assay, as described previously (24). At a concentration of 25 μM (10 μg/ml), desmosterol increased LXR-mediated transcription by ~6-fold and this activation was inhibited by arachidonate in a dose-dependent manner. At equivalent concentrations, cholesterol, sitosterol, and lanosterol had no effect on LXR activation (Fig. 8C).

**DISCUSSION**

The major finding of this study is that desmosterol, the Δ<sup>24</sup> precursor of cholesterol, activates the nuclear receptor LXR. Desmosterol increased the expression of ABCA1 in wild type cells and in cells in which the SREBP pathway was disrupted, but not in LXR<sup>−/−</sup> cells. The action of desmosterol on ABCA1 expression was enantioselective and was observed in cells lacking enzymes that generate three side chain oxysterols. Desmosterol treatment increased the cellular content of 24(S),25-epoxycholesterol, a well characterized activator of LXR in vitro (18), but treatment of cells with ketoconazole failed to increase expression of the LXR target gene ABCA1 despite causing an even greater increase in cellular levels of 24(S),25-epoxycholesterol. Desmosterol displaced 24(S),25-epoxycholesterol from the ligand binding domains of LXRα and LXRβ and promoted the interaction between LXR and SRC-1. These findings indicate that LXR activation by desmosterol is not secondary to displacement of cholesterol from membranes and does not require conversion of desmosterol to a known oxysterol ligand. Rather, desmosterol binds directly to LXR and stimulates recruitment of cofactors that facilitate LXR-mediated gene transcription. Taken together, the results suggest that desmosterol is an endogenous ligand for LXR.

The present study was motivated by our previous finding that accumulation of plant sterols disrupts adrenal cholesterol homeostasis in Abcg5/Abcg8<sup>−/−</sup> mice, resulting in a marked reduction in the cholesterol content of this organ (24). The disruption of adrenal cholesterol homeostasis observed in vivo was recapitulated in cultured mouse adrenal cells (Y1-BS1 cells) treated with the plant sterol stigmasterol. Stigmasterol increased expression of ABCA1 and reduced protein levels of HMG-CoA reductase and LDLR in the cultured mouse adrenal cells (24). Arachidonate suppressed the effect of stigmasterol on ABCA1 expression in CHO-7 cells (Fig. 1) as well as mouse adrenal cells (24), suggesting that stigmasterol up-regulates ABCA1 by activating the LXR:RXR heterodimer (42), as previously proposed (24). Here we further probed the molecular mechanism responsible for the inhibitory effect on SREBP processing of stigmasterol treatment.

SREBP-2 is escorted by SCAP from the ER to the Golgi complex in cells depleted of cholesterol, resulting in cleavage and release of the active transcription factor. In cells that are replete with cholesterol, the SCAP-SREBP complex is retained in the ER in association with INSIG-1 (25). The current studies show that stigmasterol inhibits activation of SREBP by promoting the association of SCAP-SREBP with INSIG-1 in the ER, resulting...
Endogenous Ligands for LXR

In reduced levels of expression of SREBP target genes, including HMGCR and the LDLR. The effect of stigmasterol may be direct or indirect. Stigmasterol may bind directly to SCAP and promote its association with INSIG-1, as has been shown for cholesterol (35). Alternatively, stigmasterol may act indirectly, perhaps by displacing cholesterol from the plasma (or ER) membrane (49), thereby increasing the amount of cholesterol bound to SCAP and promoting the SCAP-INSIG interaction.

In contrast to stigmasterol, sitosterol had no effect on ABCA1 levels or on the formation of the SCAP-INSIG-1 complex. Stigmasterol has the same nuclear ring structure as cholesterol but has a Δ24 bond and a C-24-ethyl group on the side chain. Sitosterol, like stigmasterol, has an ethyl group at C-24, but does not have a Δ24 bond. To determine the structural requirements for sterol-mediated activation of LXR, we examined the effects of other sterols with modified side chains on ABCA1 levels in cultured cells. We found that unsaturation of the cholesterol side chain was necessary and sufficient for the LXR-activating effects of stigmasterol: the presence of a double bond at C-20, C-22, and C-24 of cholesterol was associated with activation of LXR, with the Δ24 bond in desmosterol having the most potent effect (Fig. 2B).

Desmosterol also decreased the expression of two SREBP-2 target genes, LDLR and HMG-CoA reductase (Fig. 2, C and D). Interestingly, brassicasterol, a plant sterol with a Δ22 side chain on ABCA1 levels suggested that LXR may be regulated by endogenous desmosterol, or by another sterol intermediate. Among the precursor sterols tested, only the Δ24 unsaturated sterols desmosterol and zymosterol activated expression of ABCA1. Neither the early Δ22 precursor lanosterol, nor later precursors with saturated side chains (lathosterol and 7-dehydrocholesterol) elicited an increase in ABCA1 expression (Fig. 3B).

Alternatively, desmosterol treatment may stimulate ABCA1 expression by increasing cellular levels of 24(S),25-epoxycholesterol or other oxysterols, which are potent LXR agonists (18). Cellular levels of 24(S),25-epoxycholesterol were increased by treatment with triparanol, but ketonazole treatment resulted in a comparable increase in cellular levels of 24(S),25-epoxycholesterol and yet failed to stimulate expression of ABCA1 (Fig. 6D). Moreover, the effects of desmosterol were preserved in cells lacking three enzymes known to be required for side chain hydroxylation of cholesterol; 24-, 25-, and 27-hydroxylase (Fig. 6A). Nonetheless, it remains possible that desmosterol activates LXR by promoting the synthesis of another oxysterol.

The effects of desmosterol on ABCA1 expression were enantiospecific, consistent with desmosterol mediating its effect by interacting with an enantioselective molecule, presumably a protein. We show here that desmosterol binds directly to LXRα and LXRβ with a Kᵦ ~ 5-fold higher than that of 24(S),25-epoxycholesterol, whereas cholesterol competed very weakly for binding to the nuclear receptors, with an apparent Kᵦ 100-fold higher.

**Figure 8.** Desmosterol binds LXR and promotes recruitment of SRC-1 and activation of LXR. A, Hix-hLXRα(LBD) or His₅-hLXRβ(LBD) immobilized on SPA beads was incubated with 25 nM [³H]24(S),25-epoxycholesterol. Increasing concentrations of unlabeled cholesterol (▲), desmosterol (●), or 24(S),25-epoxycholesterol (○) were added and the radioactivity remaining on the beads was detected by scintillation counting. Competition curves were plotted as the percentage of radioactivity remaining on the beads. Values from beads void of competitor represented 100% binding. Results represent mean ± S.E. of three replicate dishes. B, GST-SRC-1 fusion protein was conjugated to glutathione-Sepharose 4B beads and incubated with labeled LXRα or LXRβ for 4 h. The beads were washed with PBS, and the radioactivity remaining was measured. C, CHO-7 cells were plated on day 0 at a density of 2 × 10⁶ cells in 6-well plates. On day 1, cells were cotransfected with pCMX/Gal4-LXRα (0.2 μg/well), pHM100X4-TK-luc (0.8 μg/well), and pCMX-β-galactosidase (0.5 μg/well). Cells were incubated for 16 h and the luciferase activities were measured as described under “Experimental Procedures.” The experiment was repeated 3 times with similar results.
REFERENCES

1. Repa, J. J., and Mangelsdorf, D. J. (2002) *Nat. Med.* 8, 1243–1248
2. Alberti, S., Steffensen, K. R., and Gustafsson, J. A. (2000) *Gene (Amst.)* 243, 93–103
3. Willy, P. J., Umesono, K., Ong, E. S., Evans, R. M., Heyman, R. A., and Mangelsdorf, D. J. (1995) *Genes Dev.* 9, 1033–1045
4. Yu, L., York, J., von Bergmann, K., Lutjohann, D., Cohen, J. C., and Hobbs, H. H. (2003) *J. Biol. Chem.* 278, 15565–15570
5. Repa, J. J., Berge, K. E., Pomajdl, C., Richardson, J. A., Hobbs, H., and Mangelsdorf, D. J. (2002) *J. Biol. Chem.* 277, 18793–18800
6. Repa, J. J., Liang, G., Ou, J., Bashmakov, Y., Lobaccaro, J. M., Shimomura, I., Shan, B., Brown, M. S., Goldstein, J. L., and Mangelsdorf, D. J. (2000) *Genes Dev.* 14, 2819–2830
7. Schultz, J. R., Tu, H., Luk, A., Repa, J. I., Medina, J. C., Li, L., Schwendner, S., Wang, S., Thoollen, M., Mangelsdorf, D. J., Lustig, K. D., and Shan, B. (2000) *Genes Dev.* 14, 2831–2838
8. Yoshikawa, T., Shimano, H., Amemiya-Kudo, M., Yahagi, N., Hasty, A. H., Matsuzaka, T., Okazaki, H., Tamura, Y., Iizuka, Y., Ohashi, K., Osuga, J., Harada, K., Gotoda, T., Kimura, S., Ishibashi, S., and Yamada, N. (2001) *Mol. Cell. Biol.* 21, 2991–3000
9. Laffitte, B. A., Chao, L. C., Li, J., Walczak, R., Hummasti, S., Joseph, S. B., Castrillo, A., Wilpitz, D. C., Mangelsdorf, D. J., Collins, J. L., Saiz, E., and Tontonoz, P. (2003) *Proc. Natl. Acad. Sci. U.S.A.* 100, 5419–5424
10. Chen, G., Liang, G., Ou, J., Goldstein, J. L., and Brown, M. S. (2004) *Proc. Natl. Acad. Sci. U.S.A.* 101, 11245–11250
11. Joseph, S. B., Castrillo, A., Laffitte, B. A., Mangelsdorf, D. J., and Tontonoz, P. (2003) *Nat. Med.* 9, 213–219

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Endogenous Ligands for LXR

than that of 24,25-epoxysterolesterol. Thus, the introduction of a double bond in the side chain of cholesterol is sufficient to confer high affinity LXRs binding. The finding that desmosterol increased ABCA1 expression to a greater extent than did other side chain-ununsaturated steroids may reflect higher affinity binding to LXRs. The binding of desmosterol to LXR also promoted recruitment of the co-factor SRC-1. These data are consistent with the notion that desmosterol binds LXR and promotes the recruitment of transcriptional coactivators required for LXR activity.

The results of this study suggest that some steroids, such as stigmasterol, disrupt cholesterol homeostasis because they are structural analogs of endogenous regulators of LXR and SREBP. The ATP-binding cassette transporters ABCG5 and ABCG8 have been found in the genomes of all vertebrates characterized to date, but do not appear to be present in invertebrates such as *Drosophila* that do not synthesize cholesterol. The rigorous exclusion of sterols other than cholesterol in vertebrates may be essential for the maintenance of normal cholesterol homeostasis.
Endogenous Ligands for LXR

42. Ou, J., Tu, H., Shan, B., Luk, A., DeBose-Boyd, R. A., Bashmakov, Y., Goldstein, J. L., and Brown, M. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 6027–6032
43. Sakai, J., Rawson, R. B., Espenshade, P. J., Cheng, D., Seegmiller, A. C., Goldstein, J. L., and Brown, M. S. (1998) Mol. Cell 2, 505–514
44. Kennedy, M. A., Venkateswaran, A., Tarr, P. T., Xenarios, I., Kudoh, J., Shimizu, N., and Edwards, P. A. (2001) J. Biol. Chem. 276, 39438–39447
45. Joseph, S. B., Laffitte, B. A., Patel, P. H., Watson, M. A., Matsukuma, K. E., Walczak, R., Collins, J. L., Osborne, T. F., and Tontonoz, P. (2002) J. Biol. Chem. 277, 11019–11025
46. Sun, Y., Hao, M., Luo, Y., Liang, C. P., Silver, D. L., Cheng, C., Maxfield, F. R., and Tall, A. R. (2003) J. Biol. Chem. 278, 5813–5820
47. Russell, D. W. (2000) Biochim. Biophys. Acta 1529, 126–135
48. Westover, E. J., and Covey, D. F. (2004) J. Membr. Biol. 202, 61–72
49. Lange, Y., Ye, J., and Steck, T. L. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 11664–11667
50. Berge, K. E., Tian, H., Graf, G. A., Yu, L., Grishin, N. V., Schultz, J., Kwitterovich, P., Shan, B., Barnes, R., and Hobbs, H. H. (2000) Science 290, 1771–1775