Selective Up-regulation of LXR-regulated Genes ABCA1, ABCG1, and APOE in Macrophages through Increased Endogenous Synthesis of 24(S),25-Epoxycholesterol*

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Liver X receptor (LXR) activation represents a mechanism to prevent macropage foam cell formation. Previously, we demonstrated that partial inhibition of oxidosqualene:lanosterol cyclase (OSC) stimulated synthesis of the LXR agonist 24(S),25-epoxycholesterol (24(S),25-epoxy) and enhanced ABCA1-mediated cholesterol efflux. In contrast to a synthetic, nonsteroidal LXR activator, TO-901317, triglyceride accumulation was not observed. In the present study, we determined whether endogenous 24(S),25-epoxy synthesis selectively enhanced expression of macrophage LXR-regulated cholesterol efflux genes but not genes that regulate fatty acid metabolism. TPH-1 human macrophages incubated with the OSC inhibitor (OSCi) RO0714565 (15 nM) significantly reduced cholesterol synthesis and maximized synthesis of 24(S),25-epoxy. Endogenous 24(S),25-epoxy increased ABCA1, ABCG1, and APOE mRNA abundance and consequently increased cholesterol efflux to apoA1. In contrast, OSCi had no effect on LXR-regulated genes LPL (lipoprotein lipase) and FAS (fatty acid synthase). TO-901317 (≥10 nM) significantly enhanced expression of all genes examined. OSCi and TO-901317 increased the mRNA and precursor form of SREBP-1c, a major regulator of fatty acid and triglyceride synthesis. However, conversion of the precursor to the active form (nSREBP-1c) was blocked by OSCi-induced 24(S),25-epoxy but not by TO-901317 (≥10 nM), which instead markedly increased nSREBP-1c. Disruption of nSREBP-1c formation by 24(S),25-epoxy accounted for diminished FAS and LPL expression. In summary, endogenous synthesis of 24(S),25-epoxy selectively up-regulates expression of macrophage LXR-regulated cholesterol efflux genes without stimulating genes linked to fatty acid and triglyceride synthesis.

Macrophage-derived cholesteryl ester-rich foam cells develop within the arterial wall as a result of excessive internalization of lipoproteins, which subsequently promote early atherosclerotic plaque formation. In addition, foam cells enhance susceptibility to plaque rupture within advanced stage lesions, leading to further atherosclerosis complications (1, 2). The ligand-activated nuclear receptors known as liver X receptors (LXRs), whose natural ligands are oxysterols, regulate the expression of genes involved in lipid homeostasis through binding to LXR response elements (LXREs) within the promoter of several responsive genes. These include ABCA1 (ATP-binding cassette A1), ABCG1, and APOE (apolipoprotein E), which mediate cellular cholesterol efflux from human and mouse macrophages to extracellular acceptors (3). Additionally, activated LXR increases expression of SREBP-1c (sterol regulatory element-binding protein 1c), FAS (fatty acid synthase), and LPL (lipoprotein lipase), which together act to stimulate cellular free fatty acid synthesis and free fatty acid uptake, respectively, leading to enhanced triglyceride synthesis (4–6). SREBP-1c is itself a master regulator of genes involved in lipogenesis, such as LPL (7) and FAS (8), and is also self-regulating, since it positively influences SREBP-1c transcription (9).

In vivo experiments using a synthetic, nonsteroidal LXR agonist, TO-901317, established that LXR activation is integral in attenuating atherosclerosis (10, 11). Although the precise underlying mechanisms are not fully understood, reduction in macrophage foam cell formation by enhancing cholesterol efflux probably plays a significant role. LXR activation in mice directly stimulates reverse cholesterol transport as determined by tracing radiolabeled sterols from macrophages to plasma high density lipoprotein and subsequently into the liver and bile (12). Macrophage-specific deletion of LXRs in mice results in enhanced atherogenesis (13), whereas liver-specific LXRα overexpression reduces atherosclerosis (14), further reinforcing the importance of LXR activation in slowing disease progression.

*This work was supported by Canadian Institutes of Health Research Grant MT-8014 and Heart and Stroke Foundation of Ontario (HSFO) Grant PG-4484. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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5 The abbreviations used are: LXR, liver X receptor; LXRE, LXR response element; SRE, sterol regulatory element; SREBP, sterol regulatory element-binding protein; 24(S),25-epoxycholesterol; OSC, oxidosqualene:lanosterol cyclase; nSREBP-1, nuclear SREBP-1; SCAP, sterol cleavage-activating protein; INSIG, insulin-signaling protein; DMHCA, N,N-dimethyl-3β-hydroxycholenamide.

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The major deleterious effect of treatment of mice with synthetic LXR agonists is a massive increase in plasma and liver triglyceride (10, 11, 15, 16). Lipogenesis and triglyceride accumulation is enhanced by highly potent synthetic LXR agonists that up-regulate expression of SREBP-1c and consequently such downstream genes as FAS and LPL, which, in addition to LXREs, contain sterol response elements (SREs) in their promoter regions (7, 8). Selective activation of LXR-regulated genes involved in macrophage cholesterol efflux and ultimately separation of the two LXR-regulated metabolic pathways of cholesterol efflux and triglyceride accumulation represents a potential mechanism for the attenuation of macrophage foam cell formation.

The discovery of endogenously produced oxysterols, such as 24(S),25-epoxycholesterol (24(S),25-epoxy), as potent, naturally occurring LXR-agonists (17), permitted the identification of oxysterol-activated LXRs as central regulators of cholesterol homeostasis (3). Partial inhibition of 2,3-oxidosqualene:lanosterol cyclase (OSC), a cholesterol biosynthesis enzyme downstream of the statin target hydroxymethylglutaryl-CoA reductase, has been shown to substantially reduce cholesterol synthesis while simultaneously maximizing 24(S),25-epoxy synthesis (reviewed in Ref. 18). Previously, we demonstrated that inhibition of OSC by RO0714565 at a dose that maximizes 24(S),25-epoxy synthesis enhanced cholesterol efflux from cultured mouse macrophages, an effect associated with an LXR-induced increase in the expression of ABCA1 and ABCG1. In contrast to the synthetic, nonsteroidal LXR agonist TO-901317, OSC inhibition did not lead to an accumulation of cellular triglycerides, possibly due to the lack of increase in nuclear SREBP-1 (nSREBP-1) (19). However, the underlying mechanism for this effect was not determined.

In the present study, we hypothesized that enhanced synthesis of 24(S),25-epoxy within a physiologically relevant range would lead to functionally significant increases in the expression of LXR-regulated genes ABCA1, ABCG1, and APOE and thus cholesterol efflux, whereas FAS, LPL, and SREBP-1c would be relatively unaffected. Human THP-1 macrophages were exposed to an OSC inhibitor (OSCi) at a dose that maximizes endogenous 24(S),25-epoxy synthesis. ABCA1, ABCG1, and APOE expression were increased, as was cholesterol efflux. However, expression of genes involved in fatty acid metabolism, FAS and LPL, was not increased; nor was there an increase in fatty acid synthesis, triglyceride synthesis, or LPL activity. Furthermore, OSCi-induced 24(S),25-epoxy or exogenously added 24(S),25-epoxy increased SREBP-1c mRNA and precursor SREBP-1 (pSREBP-1). However, unlike higher concentrations of TO-901317, the OSCi-induced 24(S),25-epoxy or exogenous 24(S),25-epoxy blocked conversion of the inactive pSREBP-1 to its active nuclear form, which probably accounts for the absence of enhanced FAS and LPL gene expression.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—The human THP-1 macrophage-like cell line was obtained from American Type Culture Collection (Manassas, VA). For experiments, cells were cultured at 4.0 × 10^6 cells/35-mm plate (Falcon Scientific, BD Biosciences) or 3.0 × 10^7 cells/100-mm plate in RPMI 1640 supplemented with 10% fetal bovine serum (Sigma), β-mercaptoethanol (5 × 10^-5 m), 100 units/ml penicillin, and 100 μg/ml streptomycin and differentiated with 300 nM phorbol 12,13-dibutyrate (PDB) for 1 week prior to use in experiments as described (20). The OSCi designated as RO0714565 with an I_(50) of 5 nM for human liver microsomal OSC activity was provided by J. Aebi (F. Hoffmann-La Roche AG (Pharmaceuticals Division, Basel, Switzerland)) and dissolved in Me_2SO. In some experiments, cells were incubated with exogenous 25-hydroxycholesterol (25-OH) (Sigma), 24(S),25-epoxy, or 22(R)-hydroxycholesterol (22(R)-OH) (Steraloids Inc., Newport, RI) dissolved in 100% ethanol or the synthetic, nonsteroidal LXR ligand TO-901317 (Sigma) dissolved in Me_2SO. LDL was isolated from normal human subjects by differential ultracentrifugation and acetylated (acLDL) using acetic anhydride as described (21), and the extent of modification was confirmed by alterations in electrophoretic mobility (22).

**Cellular Analyses**—To examine cellular 24(S),25-epoxy and cholesterol synthesis, THP-1 cells were incubated for 24 h in RPMI 1640 with 5% human lipoprotein-deficient serum (LPDS), 300 nM PDB, and 1 μCi/ml [1-14C]acetic acid in the presence of Me_2SO alone (=0.5% of total cell medium) or increasing concentrations of OSCi, RO0714565 (0.5–1000 nM). Lipids were extracted, saponified, and separated by TLC using Whatman Partisil® 60 TLC plates in hexane/diethyl ether/ acetic acid (60:40:1, v/v/v). A PhosphorImager and ImageQuant software (Amersham Biosciences) were used for quantification of the cholesterol and 24(S),25-epoxy bands (19, 23). 24(S),25-epoxy was identified by comparing the R_f value with a pure standard (19).

The synthesis of cholesterol, fatty acid, or triglyceride was measured in THP-1 cells following a preincubation for 24 h in RPMI 1640 with 5% human LPDS and 300 nM PDB along with selected doses of LXR agonists or the OSCi (15 μM). For a subsequent 5-h incubation, 1 μCi/ml [1-14C]acetic acid or 0.04 nCi/ml [1-14C]oleic acid (Amersham Biosciences) complexed with fatty acid-free bovine serum albumin in a molar ratio of 5:36:1 (Sigma) were added in the presence or absence of LXR agonists or OSCi as described (24). The synthesis of cholesterol and fatty acid during the 5 h after the preincubation was measured by incorporation of [1-14C]acetic acid into these lipids. Saponified cellular lipid extracts were separated, and cholesterol was identified by TLC using Whatman Partisil® 60 TLC plates in petroleum ether/diethyl ether/ acetic acid (84:15:1, v/v/v) and quantitated as described previously (19). Fatty acids were measured from a second extraction following acidification of the infranatant of the saponified lipid extract as described previously (25). Triglyceride synthesis was measured over a 5-h period by measuring incorporation of [1-14C]oleic acid into cellular triglyceride present in nonsaponified cellular lipid extracts following separation by thin layer chromatography (24). LPL activity in the medium of THP-1 cells was determined following a 24-h incubation with OSCi or the indicated LXR agonists (as described above) as the release of free fatty acids from intralipid, an exogenous lipid source (26). Cholesterol efflux was measured using 5 µg of cholesterol/ml of acLDL in the presence of OSCi or selected doses of LXR agonists as described previously (19).
mRNA Abundance—THP-1 cells were incubated for 24 h in RPMI 1640 with 5% LPDS, 300 nM PDB and in the presence of OSCi (at selected concentrations) or LXR agonists, and subsequently, total RNA was isolated using Trizol reagent (Invitrogen). Samples of total RNA were analyzed with an S1 nuclease protection assay to measure ABCA1, ABCG1, APOE, LPL, and FAS mRNA abundance relative to glyceraldehyde phosphate dehydrogenase (GAPDH) as described previously (19, 27). The target sequences for the S1 nuclease assay were as follows: ABCA1 (GenBank™ accession number NM_0005502, bases 6896–6935); ABCG1 (GenBank™ accession number NM_207629, bases 2042–2088); APOE (GenBank™ accession number NM_000041, bases 79–127); LPL (GenBank™ accession number NM_000237, bases 502–552); FAS (GenBank™ accession number NM_004104, bases 443–505); and GAPDH (GenBank™ accession number BC029618, bases 730–745).

SREBP-1c mRNA quantitation was performed by quantitative real-time RT-PCR (qRT-PCR) on an ABI Prism (model 7900HT) Sequence Detection System (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Samples of total RNA (75 ng) were assayed in 20–μl reactions in triplicate per treatment group on a 384-well plate using a one-step qRT-PCR protocol for SREBP-1c quantitation. ABCA1 and FAS mRNA abundance was confirmed using qRT-PCR. ABCA1 and FAS mRNA was measured from total RNA (10 μg) reverse transcribed using the Applied Biosystems High Capacity cDNA archive kit according to the manufacturer’s protocol. cDNA (20–30 ng) was assayed in 20-μl reactions using the qRT-PCR protocol from Applied Biosystems. The standard curve method was used to determine mRNA abundance. Expressions of ABCA1, FAS, and SREBP-1c were each normalized to GAPDH expression. The SREBP-1c primer and probe set was obtained from Applied Biosystems, and the sequences were as reported previously (28): forward primer (5′–3′), AGGGCGGCGCAGAT; reverse primer (5′–3′), GGTGTGTGATGAAGCTGAGATGTG; probe (5′–3′), TCGAAAATGTCGACATGTCGGC (GenBank™ accession number NM_001005291). The human ABCA1, FAS, and GAPDH primer and probe sets were obtained from Taqman® Assays-on-Demand (Applied Biosystems).

Immunoblot Analysis and Densitometry—Immunoblot analyses of ABCA1 and SREBP-1c were conducted according to previously described methods (19). Briefly, THP-1 cells were incubated for 24 h in RPMI 1640 with 300 nM PDB and 5% LPDS in the presence of the OSCi, 24(S),25-epoxy, 22(R)-OH, 25-OH, TO901317, or acetylated LDL. Total cell lysates were fractionated into postnuclear and nuclear fractions as described (29) and separated by SDS-PAGE (6% acrylamide for ABCA1 and 10% for SREBP-1). Proteins were transferred to polyvinylidene difluoride membranes and immunoblotted as described previously (19). A polyclonal anti-ABCA1 antibody (Novus Biologicals, Littleton, CO) or a monoclonal anti-SREBP-1 antibody (Neomarkers, Fremont, CA) were used to probe cellular postnuclear fractions. Blots were reprobed with a polyclonal anti-β-actin antibody (Cell Signaling, Danvers, MA). Nuclear fractions were probed with the monoclonal anti-SREBP-1 antibody, and blots were reprobed using a polyclonal antilamin A/C antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Quantification analysis of the developed films was performed using an imaging densitometer (Bio-Rad Quantity One Software). ABCA1 or pSREBP-1 from postnuclear fractions was normalized to β-actin, whereas nSREBP-1 from nuclear fractions was normalized to lamin A/C.

Statistical Analysis—Data are expressed as means ± S.E. and were analyzed using one-way analysis of variance with significance set at p < 0.05 followed by Bonferroni post hoc analysis to determine significantly different treatment groups from control.

RESULTS

Inhibition of OSC Decreases Cholesterol Biosynthesis While Simultaneously Increasing Endogenous Production of 24(S), 25-EpOxy—Inhibition of OSC with RO0714565 has previously been shown to decrease cholesterol biosynthesis and increase 24(S),25-epoxy production in J774.1 mouse macrophages (19). Consistent with this result, OSCi in human THP-1 macrophages decreased cholesterol biosynthesis over a 24-h period in a dose-dependent manner with a maximum decrease of 80% observed at 1 μM (p < 0.05 versus control) (Fig. 1A). The IC50 value for inhibition of cholesterol synthesis was ~7.5 nM. A biphasic response in 24(S),25-epoxy synthesis was observed; as the concentration of the OSCi was increased, maximal synthesis was achieved at 15 nM RO0714565 (p < 0.05 compared with base line) (Fig. 1A). At concentrations greater than 15 nM, OSCi became increasingly inhibited, such that 24(S),25-epoxy levels decreased to base line. A concentration of 15 nM RO0714565 was therefore selected for subsequent experiments.

Exogenous Oxysterols Decrease Cholesterol Biosynthesis, whereas TO-901317 Has No Effect—The exogenous addition of oxysterols to mammalian cells inhibits the transcription, increases degradation, and decreases activity of hydroxymethylglutaryl-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis, resulting in a reduction in cholesterol biosynthesis (30). THP-1 cells were incubated for 24 h with 24(S),25-epoxy, 22(R)-OH, TO901317, and the OSCi. Cholesterol synthesis was measured following an additional 5 h with the indicated compound and radiolabeled acetate. Exogenous 24(S),25-epoxy and 22(R)-OH significantly reduced cellular cholesterol synthesis to 37 and 52% of control, respectively, whereas the OSCi reduced cholesterol biosynthesis to 15% of control (p < 0.05) (Fig. 1B). In contrast, all three doses of TO-901317 showed no significant reduction in cholesterol biosynthesis (Fig. 1B).

OSCi Enhances Transcription of ABCA1, ABCG1, and APOE and Increases Cholesterol Efflux to ApoAI—THP-1 macrophages incubated for 24 h with the OSCi (RO0714565) at 15 nM, a dose that maximized 24(S),25-epoxy synthesis, enhanced the expression of ABCA1 to a maximal level of 1.9-fold compared with control (p < 0.05) (Fig. 2A). At 0.01 nM, the OSCi had no effect on ABCA1 expression, whereas at 100 nM OSCi, ABCA1 mRNA abundance was decreased significantly (p < 0.05 compared with control) (Fig. 2A). We reasoned that the increased expression of ABCA1 at 15 nM OSCi would represent the anticipated maximum within the physiological context of enhanced endogenous synthesis of 24(S),25-epoxy. Doses of exogenous 24(S),25-epoxy (1 μM), 22(R)-OH (2 μM), and TO-901317 (2 μM) were incubated with OSCi (15 nM) for 24 h, and mRNA abundance was normalized to β-actin. Consistent with the exogenous addition of oxysterols to mammalian cells inhibits the transcription, increases degradation, and decreases activity of hydroxymethylglutaryl-CoA reductase, a rate-limiting enzyme in cholesterol biosynthesis, resulting in a reduction in cholesterol biosynthesis (30). THP-1 cells were incubated for 24 h with 24(S),25-epoxy, 22(R)-OH, TO-901317, and the OSCi. Cholesterol synthesis was measured following an additional 5 h with the indicated compound and radiolabeled acetate. Exogenous 24(S),25-epoxy and 22(R)-OH significantly reduced cellular cholesterol synthesis to 37 and 52% of control, respectively, whereas the OSCi reduced cholesterol biosynthesis to 15% of control (p < 0.05) (Fig. 1B). In contrast, all three doses of TO-901317 showed no significant reduction in cholesterol biosynthesis (Fig. 1B).

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were selected in order to achieve the same magnitude of up-regulation of ABCA1 that was observed with 15 nM OSCi (p < 0.05 compared with control) (Fig. 2A). Higher doses of 24(S),25-epoxy (5 and 10 μM) increased ABCA1 expression 4.4-and 7.9-fold (p < 0.05). Higher doses of TO-901317, namely 10 nM and 1 μM, were chosen to examine pharmacological activation of LXR and were found to increase expression of ABCA1 3.1- and 8.0-fold, respectively (Fig. 2A). It should be noted that in previously published studies, the most common doses of TO-901317 used in cultured cells for pharmacological activation of LXR ranged between 1 and 10 μM (5, 6, 11, 28).

The OSCi resulted in a 3.4-fold increase in ABCA1 protein relative to control following normalization to β-actin. Exogenous 24(S),25-epoxy (1, 5, and 10 μM) increased ABCA1 protein abundance 3.5-, 8.8-, and 9.8-fold, respectively, and 22(R)-OH (2 μM) increased ABCA1 protein 1.6-fold (Fig. 2B). TO-901317 at doses of 2 nM, 10 nM, and 1 μM increased ABCA1 protein 3.0-, 6.2-, and 8.2-fold, respectively (Fig. 2B). No significant differences were detected among treatments in total cell protein or the protein content of the postnuclear fractions (data not shown), indicating that the interventions used did not affect cell viability.

THP-1 cells were incubated with increasing concentrations of acLDL to confirm the increase of ABCA1 protein levels in cholesterol-loaded cells. Compared with control cells (LPDS only), acLDL at 5, 50, and 100 μg of cholesterol/ml increased ABCA1 protein levels in the postnuclear fraction 1.8-, 2.7-, and 2.9-fold, respectively (Fig. 2B).

OSC inhibition increased mRNA abundance of ABCG1 and APOE 2.3- and 1.6-fold, respectively (p < 0.05), whereas exogenous 24(S),25-epoxy, 22(R)-OH, and TO-901317 (2 nm) increased ABCG1 and APOE to a similar degree (p < 0.05 compared with control) (Fig. 3A). However, the increase in APOE in the presence of 22(R)-OH (2 μM) and TO-901317 (2 nm) was not significant (Fig. 3A). APOE mRNA abundance...
was significantly increased 3-fold (p < 0.05) in the presence of 10 nM TO-901317. The OSCi-enhanced expression of ABCA1, ABCG1, and APOE was associated with a 1.4-fold increase in efflux of cholesterol to exogenously added apoAI (p < 0.05), whereas no effect was observed in the absence of apoAI (Fig. 3B). Exogenous 24(S),25-epoxy, 22(R)-OH, and 20 nM TO-901317 increased cholesterol efflux to apoAI 1.6-fold (p < 0.05 compared with control), 2.0-fold, and 1.4-fold, respectively (Fig. 3B). TO-901317 at higher doses (10 nM) increased cholesterol efflux to apoAI 2.6-fold (p < 0.05) (Fig. 2D).

OSCi or Exogenous 24(S),25-Epox Does Not Increase FAS mRNA Abundance, Fatty Acid Synthesis, or Triglyceride Synthesis—OSCi-induced 24(S),25-epoxy production, unlike the synthetic LXR agonist TO-901317, does not stimulate triglyceride accumulation following activation of LXR-regulated genes within J774.1 mouse macrophages (19). We therefore examined FAS expression and fatty acid and triglyceride synthesis in human THP-1 macrophages incubated with either the OSCi or the control LXR agonists. The OSCi had no significant impact on FAS mRNA abundance; nor did either exogenous 24(S),25-epoxy (1 or 5 μM), 22(R)-OH, or low dose TO-901317 (Fig. 4A). The higher 10 nM and 1 μM doses of TO-901317 increased expression of FAS 1.7- and 3.5-fold, respectively (p < 0.05 compared with control).

OSCi, 24(S),25-epoxy, 22(R)-OH, or lower dose TO-901317 did not alter fatty acid synthesis (Fig. 4B) or triglyceride synthesis (Fig. 4C), which coincided with the observed absence of change in FAS mRNA abundance for each treatment. In contrast, both 10 nM and 1 μM doses of TO-901317 significantly
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A.

B.

FIGURE 5. LPL mRNA abundance and LPL activity in the presence of OSCi, exogenous oxysterols, and TO-901317 in THP-1 macrophages. A, cells were incubated for 24 h in RPMI supplemented with 5% LPDS and with vehicle (n = 7) or in the presence of OSCi (15 nM, n = 6), 24(S),25-epoxy (1 μM, n = 5), 22(R)-OH (2 μM, n = 7), or TO-901317 (2 nM (n = 7), 10 nM (n = 4), or 1 μM (n = 1)). LPL mRNA abundance is expressed as a ratio of GAPDH mRNA and plotted as a percentage of control (mean ± S.E.). *, p < 0.05 compared with control. B, LPL activity was measured in cell medium after a 24-h incubation with vehicle (n = 5), OSCi (15 nM, n = 4), 24(S),25-epoxy (1 μM, n = 4), 22(R)-OH (2 μM, n = 4), or TO-901317 (2 nM (n = 5), 10 nM (n = 5), or 1 μM (n = 4)) in the presence or absence of 1.67 units of heparin/ml. Values are mean ± S.E. and plotted as a percentage of total LPL in control cells. Cell surface-bound LPL activity is calculated from the difference between total LPL (in the presence of heparin) and secreted LPL (without heparin) activities. *, p < 0.05 compared with control.

increased both fatty acid synthesis 1.5- and 2.1-fold relative to control (Fig. 4B) and triglyceride synthesis 1.2- and 1.3-fold relative to control (all p < 0.05) (Fig. 4C).

OSC Inhibition Has No Effect on LPL Gene Expression or LPL Activity—LPL expression in macrophages enhances accumulation of fatty acids upon hydrolysis of triglyceride-rich lipoproteins and increases the subsequent uptake of cholesterol ester rich remnant particles, thus promoting foam cell formation (26). When we examined LPL mRNA abundance in the presence of OSCi, we found no significant change in mRNA levels compared with control (Fig. 5A). Furthermore, there was no difference in total or cell-bound (heparin-releasable) LPL activity with the OSCi (Fig. 5B). 24(S),25-epoxy, 22(R)-OH, or 2 nM TO-901317 had no effect on LPL mRNA abundance (Fig. 5A) or on total or cell-bound LPL activities (Fig. 5B). TO-901317 at 10 nm and 1 μM doses increased LPL mRNA 1.6- (p < 0.05) and 3.7-fold, respectively (Fig. 5A). Total LPL activity was significantly increased 1.3-fold with 10 nM TO-901317 and 1.4-fold with 1 μM TO-901317, whereas cell-bound LPL activity increased 1.3- and 1.5-fold with 10 nm and 1 μM TO-901317 (all p < 0.05), respectively (Fig. 5B). Secreted LPL activity was unaffected by all treatments (Fig. 5B).

SREBP-1c mRNA and pSREBP-1 Are Increased by OSC Inhibition, yet Processing of pSREBP-1 into nSREBP-1 Is Inhibited—Transcriptional regulation of lipogenic genes can occur through LXR-mediated binding to LXRE or more indirectly through LXR-activated expression of SREBP-1c and subsequent binding of nSREBP-1c to SREs within the promoter (31). However, maximal up-regulation of lipogenic gene expression requires cooperative binding of both an agonist-bound LXR and an nSREBP-1c to LXRE and SRE, respectively (5). Since OSCi or exogenous oxysterols did not increase the mRNA abundance of either LPL or FAS, SREBP-1c mRNA and SREBP-1 protein abundance were measured within macrophages in order to elucidate the mechanism preventing expression of genes implicated in triglyceride accumulation. SREBP-1c mRNA was increased significantly 1.4-fold in the presence of OSCi (p < 0.05 compared with control) (Fig. 6A). Exogenous 24(S),25-epoxy at doses of 1 and 5 μM increased SREBP-1c mRNA 1.7- and 2.3-fold, respectively, whereas 22(R)-OH and 2 nM TO-901317 enhanced SREBP-1c transcript abundance 1.3- and 1.6-fold (both p < 0.05 compared with control). Furthermore, TO-901317 up-regulated SREBP-1c expression in a dose-dependent manner 4.2- and 6.9-fold at concentrations of 10 nm and 1 μM (p < 0.05) (Fig. 6A). SREBP-1a mRNA abundance was unaffected by the OSCi or exogenous 24(S),25-epoxy (data not shown).

Immunoblot analysis of the postnuclear fraction of cell homogenates revealed a band at ~125 kDa corresponding to pSREBP-1 and a ~67-kDa protein in the nuclear fraction corresponding to active nSREBP-1 (Fig. 6B). pSREBP-1 was elevated 1.7-fold by the OSCi, whereas 1 and 5 μM doses of 24(S),25-epoxy increased pSREBP-1 1.9- and 2.4-fold, respectively (p < 0.05 versus control) (Fig. 6C). The increases in pSREBP-1 with 22(R)-OH and 2 nM TO-901317 were not significant. TO-901317 at 10 nm and 1 μM resulted in a marked 3.0- and 5.2-fold increase, respectively (both p < 0.05 versus control) (Fig. 6C). In contrast, the OSCi, exogenous 24(S),25-epoxy (1 μM), or TO-901317 (2 nm) did not change nSREBP-1 levels compared with control (vehicle alone), whereas 22(R)-OH significantly decreased nSREBP-1 abundance (p < 0.05 versus control). The higher dose of 24(S),25-epoxy (5 μM) also had no effect on nSREBP-1 and contrasted with the higher doses of TO-901317 (10 nm and 1 μM), which elevated nSREBP-1 2.1- and 2.8-fold, respectively (p < 0.05 versus control) (Fig. 6C).

Previously, 25-OH has been shown to decrease mature nSREBP-1 protein levels in Chinese hamster ovary cells (32) and CaCo-2 intestinal epithelial cells (33). Therefore, we sought to confirm this finding in THP-1 macrophages. Compared with 24(S),25-epoxy (Fig. 6C), increasing concentrations of 25-OH had little or no effect on pSREBP-1 (Fig. 7). This reflects the observation that 25-OH is a relatively weak LXR agonist, having only 25% of the LXR activation potential of 24(S),25-epoxy (17). Levels of nSREBP-1 decreased dose-dependently following exposure of cells to 25-OH, demonstrating inhibition of pSREBPI processing (Fig. 7).
DISCUSSION

In the present study, we clearly demonstrate that partial inhibition of OSC in human THP-1 macrophages not only reduces cholesterol biosynthesis but selectively activates LXR-regulated genes through endogenous synthesis of the LXR agonist 24(S),25-epoxy. Specifically, ABCA1, ABCG1, and APOE expression were significantly increased, which was associated with enhanced cellular cholesterol efflux. In contrast, the expression of FAS and LPL and the synthesis of fatty acid and triglyceride and LPL activity were unaffected. This lack of increased expression of genes involved in fatty acid metabolism is in part mediated by blocking the conversion of pSREBP-1c into its active nuclear form. Without nSREBP-1c, cooperative initiation of transcription with activated LXR does not occur, thereby providing a mechanism to account for the absence of FAS and LPL expression.

The selective activation of LXR-regulated genes we observed in human THP-1 cells confirms and extends our previous experiments in J774.1 mouse macrophages (19). In J774.1 cells, ABCA1 and ABCG1 transcription were both enhanced, leading to increased cholesterol efflux, whereas cellular triglyceride synthesis and abundance and LPL activity were unchanged. The mechanism for this differential activation of LXR-regulated genes was not fully elucidated. In the current study, despite the significant OSC-induced increase in SREBP-1c mRNA and pSREBP-1, cellular levels of the active nuclear form did not increase, indicating that conversion was inhibited. Our results are consistent with a mechanism whereby the selective increase in LXR-regulated gene expression was due to the OSC-induced synthesis of 24(S),25-epoxy. Similar results were obtained in cells incubated with exogenous 24(S),25-epoxy, at a concentration (1 μM) that increased ABCA1 expression to the same extent as cells incubated with the OSC. Importantly, at higher doses of 24(S),25-epoxy, ABCA1 expression increased even further; however, nSREBP-1 protein and FAS expression remained unchanged, supporting the concept that 24(S),25-epoxy inhibits the formation of nSREBP-1.

In contrast, the nonsteroidal synthetic LXR activator, TO-901317, at concentrations of 10 nM or greater showed no selectivity and increased the expression of all LXR-regulated genes in J774.1 cells.

FIGURE 6. Effect of OSC, exogenous oxysterols, or TO-901317 on SREBP-1c mRNA abundance and precursor and active nuclear forms of SREBP-1 protein levels. THP-1 cells were incubated for 24 h in RPMI supplemented with 5% LPDS and vehicle alone or OSC (Me2SO), 24(S),25-epoxy (ethanol), 22(R)-OH (ethanol), or TO-901317 (Me2SO). A, SREBP-1c mRNA values are calculated as the ratio of SREBP-1c to GAPDH mRNA and expressed as a percentage of control (mean ± S.E.). *, p < 0.05 compared with control (n = 5). B, representative immunoblot of SREBP-1 precursor (pSREBP-1, 125 kDa), in the postnuclear fraction, using β-actin (45 kDa) as a loading control. Levels of active nSREBP-1 (~ 67 kDa), were determined in the nuclear fraction of cells, using lamin A/C (67 kDa) as a loading control. C, immunoblot band intensities of pSREBP-1 (n = 9, except 5 μM 24(S),25-epoxy, n = 6) and nSREBP-1 (n = 8, except 2 μM 22(R)-OH and 5 μM 24(S),25-epoxy, n = 5) were quantitated by densitometry. Values are expressed as mean ± S.E. and plotted as a ratio of control. *, p < 0.05 compared with vehicle alone for pSREBP-1. †, p < 0.05 compared with vehicle alone for nSREBP-1.
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![Effect of 25-OH on the precursor and active, nuclear form of SREBP-1 protein levels. THP-1 cells were incubated for 24 h in RPMI supplemented with 5% LPDS and ethanol alone or increasing concentrations of 25-OH (6.25, 12.5, and 25 μM). Immunoblots of postnuclear and nuclear cell fractions were probed for pSREBP-1 and nSREBP-1, respectively. pSREBP-1 bands were normalized to β-actin, and nSREBP-1 bands were normalized to lamin A/C. Representative immunoblots are shown, and the values above each lane represent the quantity of normalized SREBP-1 expressed as a ratio of control values.](image)

Genes examined. Importantly, the increased SREBP-1c mRNA and pSREBP-1 protein led to a significant increase in nSREBP-1 and consequently FAS and LPL mRNA abundance. At low concentrations of TO-901317 (2 nM), the expression of ABCA1, ABCG1, and SREBP-1c were increased in THP-1 cells to the same extent as with the OSCi. Furthermore, LPL and FAS mRNA, fatty acid, and triglyceride synthesis and pSREBP-1 were not significantly affected, suggesting that 2 nM was below the threshold for activation of genes involved in fatty acid metabolism. Alternatively, this may reflect gene-specific recruitment of co-activators and dissociation of co-repressors by LXR and depend on the type of agonist bound (34).

Inhibition of SREBP processing by cholesterol and oxysterols is the major homeostatic mechanism that balances cholesterol and fatty acid metabolism within cells (35). In order to produce the active nSREBP, the precursor protein must be chaperoned by sterol cleavage-activating protein (SCAP) from the endoplasmic reticulum to the Golgi in order to access specific proteins. When cholesterol is abundant within the cell, it binds directly to SCAP at the sterol-sensing domain, which recruits insulin signaling protein (INSIG) to bind and retain the SCAP-SREBP complex in the endoplasmic reticulum, thus preventing formation of the nSREBP and decreasing expression of SREBP-regulated genes (36). Our results demonstrate that in human macrophages, the oxysterol, 25-OH, was also capable of inhibiting pSREBP processing to nSREBP in a dose-dependent manner. Previous studies have demonstrated that the mechanism for blocking SREBP processing by 25-OH is indirect. 25-OH binds to a putative protein that enhances SCAP-INSIG binding and thus promotes retention of pSREBP within the endoplasmic reticulum (32). It is possible that inhibition of pSREBP-1 processing by OSCi-induced 24(S),25-epoxy acts through a similar mechanism.

The structure of TO-901317 (37) differs substantially from oxysterols and cholesterol (32), which potentially does not allow TO-901317 to bind SCAP or the putative SCAP-INSIG binding protein. The essential part of the sterol that mediates binding to SCAP or a SCAP-INSIG-binding protein was shown to be the 3β-hydroxy group (32), which is absent from TO-901317 (37). The difference in structure between TO-901317 and the endogenous, naturally occurring 24(S),25-epoxy appears to mediate the selectivity in gene expression patterns.

Analysis of the promoter regions of ABCA1, ABCG1, and APOE has led to the identification, in each, of an LXRE capable of binding LXR, leading to transcriptional activation in the presence of oxysterols or synthetic LXR agonist (38–40). The activation of these three genes by SREBP-1 has not been directly assessed; presumably, they are not responsive to SREBP-1, since SREs have not been identified for ABCA1 or APOE (41, 42). SRE-like elements with only slight similarity to functionally active SREs have been identified for ABCG1 (40). Therefore, genes that contain LXREs (4–6) as well as functionally active SREs (5, 7–9), such as SREBP-1c, LPL, and FAS appear to require both LXR and SREBP-1c acting in a synergistic manner to maximize transcription (5) and consequently to increase lipogenesis and lipid uptake. In contrast to oxysterols, the inability of TO-901317 to prevent SREBP processing, as demonstrated in the present study, results in enhanced formation of nSREBP-1c from an abundance of precursor, leading to increased expression of lipogenic genes and cellular triglyceride accumulation. Endogenous production of 24(S),25-epoxy activates LXR but blocks SREBP-1 processing, thus preventing up-regulation of FAS or LPL expression. Fig. 8 summarizes the proposed mechanism of selective activation of LXR-regulated genes by 24(S),25-epoxy versus the nonselective LXR agonist TO-901317.

Recently, a synthetic oxysterol LXR agonist N,N-dimethyl-3β-hydroxycholenamide (DMHCA) was shown to selectively activate LXR-regulated genes in THP-1 macrophages (28). Compared with TO-901317 (10 μM), DMHCA increased ABCA1 and LXRs expression to a similar extent (~10-fold); however, the increases in SREBP-1c (5-fold) and FAS (1.3-fold) were only half of that observed for TO-901317. Although DMHCA increased cholesterol efflux 1.6-fold in THP-1 cells, the effects of DMHCA on levels of SREBP-1 protein, fatty acid synthesis, and triglyceride accumulation were not reported (28). Our results are consistent with these observations and suggest that LXR-activation by molecules based on an oxysterol structure is more selective for the activation of ABCA1 expression compared with SREBP-1c and FAS.

Studies in mice confirm the physiological role of LXR as a mediator of cholesterol metabolism in which synthetic LXR agonists, including TO-901317, provide at least some protection from atherosclerosis and hypercholesterolemia (11) by enhancing reverse cholesterol transport (12). The propensity of nonsteroidal LXR agonists to induce hepatic steatosis and
plasma hypertriglyceridemia, most likely through up-regulation of SREBP-1c (37), detracts significantly from the development of LXR agonists as therapeutic agents (11, 15, 16). Based on our in vitro studies, the inhibition of OSC in vivo of cholesterol biosynthesis and the concurrent enhanced synthesis of 24(S),25-epoxy could prove advantageous for the reduction of atherogenesis while maintaining normal lipid concentrations in liver and plasma. Recently, we reported that administration of an OSC to pigs decreased hepatic and plasma cholesterol without increasing triglycerides due, in part, to selective activation of LXR-regulated genes, including ABCG5 and ABCG8 in liver and jejunum (43). Whether this benefit extends to atheroprotection remains to be determined.

In summary, our results demonstrate that partial inhibition of OSC provides a dual mechanism of action, maintenance of cholesterol homeostasis via reducing cholesterol biosynthesis while supplying substrate for the alternate oxysterol synthesis pathway. Partial OSC inhibition enhances 24(S),25-epoxy production, which selectively activates LXR-regulated genes involved in cholesterol efflux without a concomitant increase in fatty acid or triglyceride synthesis. The OSCi, RO0714565, was designed to inhibit the prokaryotic squalene-hopene cyclase, based on the assumption that the human counterpart (OSC) was homologous in structure. The recent crystallization of human OSC, which has revealed that current OSCis are suboptimal inhibitors (44), should enable the design and synthesis of more effective inhibitors intended to optimally increase the synthesis of 24(S),25-epoxy. OSC inhibition therefore provides a mechanism to avert foam cell formation through selective activation of genes that promote cholesterol efflux as a result of enhanced endogenous synthesis of the LXR agonist 24(S),25-epoxy.

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