Enhanced Synthesis of the Oxysterol 24(S),25-Epoxycholesterol in Macrophages by Inhibitors of 2,3-Oxidosqualene:Lanosterol Cyclase

A Novel Mechanism for the Attenuation of Foam Cell Formation

Andrea H. Rowe,* Carmen A. Argmann,* Jane Y. Edwards, Cynthia G. Sawyez, Olivier H. Morand, Robert A. Hegele, Murray W. Huff

Abstract—Oxysterols are key regulators of lipid metabolism and regulate gene expression by activating the liver X receptor (LXR). LXR plays a vital role in macrophage foam cell formation, a central event in atherosclerosis. It is known that addition of exogenous oxysterols to cultured macrophages activates LXR, leading to increased expression of ABCA1 and cholesterol efflux. In this study, we tested the novel hypothesis that stimulation of endogenous oxysterol synthesis would block foam cell formation induced by atherogenic lipoproteins. Macrophage synthesis of 24(S),25-epoxycholesterol, a potent LXR ligand, increased 60-fold by partial inhibition of 2,3-oxidosqualene:lanosterol cyclase (OSC), a microsomal enzyme in both the cholesterol biosynthetic pathway and the alternative oxysterol synthetic pathway. When macrophages were challenged with human hypertriglyceridemic VLDL (HTG-VLDL), cellular cholesteryl ester accumulation increased 12-fold. This was reduced dramatically, by 65%, after preincubation with an OSC inhibitor (OSCi). The HTG-VLDL–induced accumulation of macrophage TG (70-fold) was unaffected by the OSCi or exogenous 24(S),25-epoxycholesterol, an effect associated with suppression of SREBP-1 processing. By contrast, TO901317, a synthetic LXR agonist, increased cellular TG significantly and markedly increased SREBP-1 processing. OSC inhibition decreased HTG-VLDL uptake through downregulation of LDL-receptor expression, despite substantial inhibition of cholesterol synthesis. Furthermore, OSC inhibition significantly upregulated ABCA1 and ABCG1 expression, which led to enhanced macrophage cholesterol efflux, an effect mediated through LXR activation. Therefore, increased macrophage synthesis of endogenous oxysterols represents a new mechanism for the dual regulation of LXR- and SREBP-responsive genes, an approach that inhibits foam cell formation without detrimental effect on TG synthesis.

Key Words: oxidosqualene:lanosterol cyclase ■ oxysterols ■ liver X receptors ■ ATP binding cassette A1 ■ SREBP-1

The uptake of apolipoprotein (apo) B–containing lipoproteins by macrophages within the arterial wall results in cholesteryl ester (CE) deposition and foam cell formation, a hallmark of early and late atherosclerotic lesions.1 Foam cell formation is the result of an imbalance in processes mediating lipoprotein uptake and cholesterol efflux.2 The importance of cholesterol efflux in the maintenance of macrophage cellular cholesterol homeostasis arose from elucidation of mutations in the ATP binding cassette protein (ABC) A1 in Tangier disease, which is characterized by macrophage cholesteryl accumulation (reviewed by Attie et al3). ABCA1 is a transmembrane protein that controls the transfer of cholesterol and phospholipids to apoA1, the initial step in HDL formation and reverse cholesterol transport (reviewed by Tall et al;4 Francis et al,5 and Oram6). The discovery of ABCA1 and its key role in regulating macrophage cholesterol efflux has stimulated development of therapeutic interventions that enhance the removal of cholesterol from arterial cells as a strategy to reduce atherosclerosis.

Expression of ABCA1 is transcriptionally controlled and increases in response to cellular cholesterol, which is dependent on the ligand-activated nuclear hormone receptors, liver

© 2003 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000097606.43659.F4
ABCG1, apoE, lipoprotein lipase (LPL), fatty acid synthase, metabolism through induction of genes encoding ABCA1, coordinate the regulation of both cholesterol and fatty acid metabolism. Indeed, LXRs are expressed in activated macrophages, and the addition of exogenous oxysterols or protelytic processing of SREBP-1c results in macrophage ABCA1 expression leading to increased cholesterol efflux independent of cholesterol loading and regulate LXR-sensitive genes, including ABCA1.

Macrophage foam cell formation can be induced by VLDL and their remnants (REM), isolated from patients with moderate hypertriglyceridemia (HTG) in both their native and oxidized forms. Native HTG-VLDL and VLDL-REM induce macrophage lipid accumulation via a 2-step process. TG accumulation occurs via interaction of HTG-VLDL with cell-surface LPL, resulting in core TG hydrolysis, cellular free fatty acid uptake, and reesterification. CE accumulation results from CE-rich remnant uptake by LDL receptors (LDL-R), and lipoprotein-derived free cholesterol (FC) is utilized, reesterified, and stored as CE droplets or effluxed from the cell via ABCA1.

In the present study, we tested the hypothesis that partial inhibition of OSC in macrophages would attenuate CE accumulation and foam cell formation induced by HTG-VLDL. Exposure of macrophages to OSC inhibitors dramatically decreased cholesterol synthesis while concomitantly increasing endogenous synthesis of 24(S),25-epoxy. This resulted in significant attenuation of macrophage CE accumulation by two mechanisms: (1) reduced HTG-VLDL uptake through decreased LDL-R expression and (2) enhanced ABCA1 and ABCG1 expression leading to increased cholesterol efflux, mediated by LXR activation. Macrophage TG metabolism was unaltered, an effect associated with suppression of SREBP-1 processing. Thus, this study provides a new mechanism by which cellular oxysterol synthesis can be induced without cholesterol loading to modulate LXR-responsive genes such as ABCA1.

Materials and Methods

Subjects and Lipoprotein Isolation

Subjects were recruited from the Lipid Clinics at the London Health Sciences Center (London, Ontario, Canada). Studies were approved by the University of Western Ontario Health Science Standing Committee on Human Research. VLDL (Sf 20 to 400) and LDL (Sf 0 to 12) were isolated from plasma of type IV hypertriglyceridemic subjects and normal subjects, respectively, and VLDL-REM and acetylated LDL (AcLDL) were prepared.

Cell Culture

The following cells were used in this study: J774A.1 (murine macrophages), THP-1 (human macrophages), HepG2 (human hepatocytes), and mouse peritoneal macrophages isolated from LDL-R–null mice and their control C57Bl/6 mice. Cells were cultured as previously outlined. Several OSC inhibitors (OSCi), designated Ro-48-8071, Ro-61-3479, Ro-71-3852, and Ro-71-4565 (all in DMSO), were tested, which inhibit OSC in rat hepatic microsomes.
with IC\textsubscript{50} of 8.1, 17.2, 4.4, and 28.4 nmol/L, respectively (F. Hoffmann-La Roche AG, Pharmaceuticals Division). Cell viability and protein contents were not affected at any dose tested. In some experiments, cells were incubated with 24(S),25-epoxy, 22(S)-OH, and protein contents were not affected at any dose tested. In some experiments, cells were incubated with 24(S),25-epoxy, 22(R)-OH, and protein contents were not affected at any dose tested. In some experiments, cells were incubated with 24(S),25-epoxy, 22(S)-OH, and protein contents were not affected at any dose tested. In some experiments, cells were incubated with 24(S),25-epoxy, 22(S)-OH, and protein contents were not affected at any dose tested.

Data are expressed as mean±SEM. Statistical analyses were performed using 2-sample \( t \) tests and 1-way ANOVA, with significance at \( P<0.05 \).

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

### Results

**OSC Inhibitors Decrease Cholesterol Biosynthesis and Enhance Endogenous Synthesis of 24(S),25-Epoxo**

Each OSCi tested dose-dependently decreased cholesterol synthesis, with a maximal inhibition of \(-90\% (P<0.05) \) (Figure 2). IC\textsubscript{50} for cholesterol synthesis inhibition ranged from 5 to 30 nmol/L. By comparison, 22(R)-OH maximally decreased cholesterol biosynthesis by 40% \( (P<0.05) \) (data not shown).

The effects of OSCi on synthesis of MOS, DOS, and 24(S),25-epoxy were determined after TLC separation (Figure 3A). The 24(S),25-epoxy band was identified by comparison with purified standards. Previous studies demonstrated that 24(S),25-epoxy was the only oxysterol synthesized in J774A.1 macrophages. Radiolabeled cellular lipids were separated by TLC and quantified by use of a phosphorimager \((n=3)\). A, Imaged TLC plate depicting separation of MOS, DOS, cholesterol, and 24(S),25-epoxy. B, Cellular levels of 24(S),25-epoxy, DOS, and MOS \((n=3)\), calculated as dpm/mg cell protein, are plotted as percent \( \text{mean±SEM} \) of control (no OSCi). \( *P<0.05 \) vs control. The vertical dashed line indicates the OSCi dose that produced the maximal level of endogenous 24(S),25-epoxy and is the dose used in subsequent experiments.

The effects of OSCi on synthesis of MOS, DOS, and 24(S),25-epoxy were determined after TLC separation (Figure 3A). The 24(S),25-epoxy band was identified by comparison with purified standards. Previous studies demonstrated that 24(S),25-epoxy was the only oxysterol synthesized in OSCi-treated hepatocytes.\(^{13}\) Ro-71-4565 dose-dependently increased 24(S),25-epoxy synthesis, with a maximal increase of 64-fold at 1 \( \mu \text{mol/L} \) \((P<0.05, \text{Figure 3B})\). Higher OSCi concentrations \((>2 \mu \text{mol/L})\) decreased 24(S),25-epoxy such that at 10 \( \mu \text{mol/L} \), levels were 20% of those observed with 1 \( \mu \text{mol/L} \). MOS and DOS increased dose-dependently at OSCi concentrations \(>1 \mu \text{mol/L} \) to maximum levels at 10 \( \mu \text{mol/L} \) (Figure 3B). These results are consistent with the higher affinity of OSC for DOS compared with MOS,\(^{15}\) such that partial inhibition of OSC \(<1 \mu \text{mol/L} \text{ Ro-71-4565}) results in increased conversion of MOS to DOS and enhanced formation, via OSC, of 24(S),25-epoxy (Figure 1). Therefore, 1 \( \mu \text{mol/L} \) of Ro-71-4565 was used in subsequent experiments. All OSCis tested were quantitatively similar (data not shown).

### OSCi Decreases Macrophage Cellular Lipid Accumulation Induced by HTG-VLDL

J774A.1 macrophages incubated with HTG-VLDL demonstrated substantial accumulation of cellular Oil Red O–
stained lipid droplets, indicative of cytoplasmic neutral lipids (Figure 4A). Cells treated with Ro-71-4565 and HTG-VLDL demonstrated a marked reduction in neutral lipid inclusions (Figure 4B). J774A.1 macrophages incubated with HTG-VLDL increased CE accumulation 12-fold, compared with control (P<0.05, Figure 4C). Preincubation with Ro-71-4565, significantly decreased HTG-VLDL–induced CE accumulation in a dose-responsive manner, with maximal inhibition (63%) at 1 μmol/L. Higher doses resulted in marked attenuation of this decrease such that cellular CE accumulation was reduced by only 24% at 10 μmol/L of Ro-71-4565. Similarly, VLDL–REM– and LDL–induced CE accumulation in macrophages was reduced significantly by preincubation with Ro-71-4565 (data not shown). OSC inhibitors had no significant effect on the 70-fold increase in TG accumulation induced by HTG-VLDL except at the 1 μmol/L dose, at which an 11% increase was observed (P<0.05, Figure 4D). By comparison, macrophages incubated with HTG-VLDL and exogenous 24(S),25-epoxy resulted in a dose-dependent decrease in CE (−40% at 4 μmol/L; Figure 4E). TG mass was unaffected (Figure 4F). Macrophages preincubated with either 22(R)-OH or the synthetic LXR agonist TO901317 reduced HTG-VLDL–induced CE mass by 61% and 63%, respectively (P<0.05, Figure 4E). However, TG accumulation was increased 35% with 22(R)-OH and 47% to 52% with TO901317 (all P<0.05, Figure 4F).

Macrophage FC concentrations were unaffected by HTG-VLDL alone (33±3 versus 31±4 μg/mg cell protein); however, FC mass decreased by 14% (P<0.001) in cells preincubated with Ro-71-4565. Preincubation of macrophages with exogenous 24(S),25-epoxy or TO901317 had no effect on cellular FC (data not shown). HTG-VLDL enhanced CE accumulation in mouse peritoneal macrophages isolated from C57Bl/6 mice by 2.2-fold (P<0.05). This was inhibited 60% (P<0.05) by preincubation of cells with Ro-71-4565 (1 μmol/L). In mouse peritoneal macrophages from LDL–R–null mice, HTG-VLDL increased cellular CE by only 10% above baseline. Ro-71-4565 prevented this small increase in CE accumulation; however, the TG increase was unchanged in both cell types (data not shown).

**Effect of OSCi on Cholesterol Esterification**

No significant effect on oleate incorporation into CE was observed during the first 5-hour incubation of macrophages with Ro-71-4565. However, after 19 hours of preincubation with 1 μmol/L Ro-71-4565, CE synthesis was reduced by 44% (P<0.05, data not shown). This indicates that OSC inhibitors did not directly affect acylCoA:cholesterol acyltransferase activity but reduced cholesterol esterification secondary to cholesterol synthesis inhibition.

**OSC Inhibition Does Not Affect TG Synthesis or LPL Activity**

Incubation of macrophages with Ro-71-4565 had no effect on oleate incorporation into TG, LPL activity (Figures 5A and 5B), or fatty acid synthesis (data not shown). Exogenous 24(S),25-epoxy also had no significant effect on TG synthesis (Figure 5A). In contrast, 22(R)-OH and TO901317 significantly increased TG synthesis (Figure 5A). Furthermore, 22(R)-OH increased LPL activity, whereas Ro-71-4565 had no significant effect (Figure 5B). LPL activities correlated with the effect of these compounds on TG accumulation (Figure 4F).

SREBP-1, primarily SREBP-1c, is a potent activator of genes involved in fatty acid and TG metabolism. Therefore, we examined expression and activation of SREBP-1. SREBP-1 mRNA was significantly increased in macrophages incubated with Ro-71-4565, 24(S),25-epoxy, or TO901317 (Figure 6A). Although our probe did not distinguish between SREBP-1a and SREBP-1c, it is likely that the increased mRNA represents SREBP-1c, because LXR agonists do not affect mouse SREBP-1a expression. Immunoblot analyses indicated that the precursor form of SREBP-1 also increased with each treatment (Figure 6B). However, TO901317 markedly increased the active, nuclear form of SREBP-1 (4-fold),
whereas Ro-71-4565 and exogenous 24(S),25-epoxy produced only minor increases (H110211.4-fold, Figure 6C).

Regulation of ABCA1 and ABCG1 mRNA and ABCA1 Protein by OSC Inhibition
Cholesterol efflux to apoAI is mediated by ABCA1 and ABCG1, both of which can be induced by oxysterol-dependent LXR activation. Ro-71-4565 significantly increased ABCA1 and ABCG1 mRNA (2- and 2.8-fold, respectively, both P<0.05) (Figures 7A and 7B). Similarly, exogenous 24(S),25-epoxy and TO901317 significantly induced both ABCA1 and ABCG1 mRNA. Immunoblot analyses demonstrated similar increases in ABCA1 protein (Figure 7C).

OSCi Induces Cholesterol Efflux via LXR
The OSCi-induced expression of ABCA1 and ABCG1 and reduction in cellular cholesterol in cells challenged with HTG-VLDL were determined under conditions in which cells were not preloaded with cholesterol. Therefore, we examined the effect of OSCi on [3H]cholesterol efflux in J774A.1 macrophages preincubated with [3H]cholesterol and minimal AChLDL (5 µg TC/mL). Compared with control, apoAI stimulated cholesterol efflux 3.0-fold (Figure 7D). In cells preincubated with Ro-71-4565, cholesterol efflux was enhanced a further 38% (P<0.05). Similarly, exogenous 24(S),25-epoxy increased efflux to apoAI by 53% (P<0.05) compared with apoAI alone. TO901317 and 22(R)-OH also enhanced cholesterol efflux. In addition, incubation of human THP-1 macrophages with Ro-71-4565 (10 nmol/L) significantly increased 24(S),25-epoxy synthesis and enhanced cholesterol efflux to apoAI. Similar values for cholesterol efflux were observed in response to exogenous 24(S),25-epoxy (4 µmol/L; data not shown).

A role for LXR in the induction of cholesterol efflux by 24(S),25-epoxy was demonstrated in J774A.1 macrophages coinubated with either ECHS or 22(S)-OH, two specific LXR antagonists. Both ECHS and 22(S)-OH completely inhibited the increase in apoAI-mediated cholesterol efflux
induced by Ro-71-4565, 24(S),25-epoxy, and TO901317 (Figure 7D and data not shown). To confirm that partial OSC inhibition results in LXR-mediated gene expression, HepG2 cells were transfected with a reporter construct driven by an LXR response element. LXR activity was markedly increased in cells treated with either Ro-71-4565, 24(S),25-epoxy, or TO90317 (Figure 7E). Collectively, these results establish that increased cellular 24(S),25-epoxy, subsequent to OSC inhibition, enhances ABCA1 expression and macrophage cholesterol efflux via LXR-activation.

OSC inhibition had no effect on cholesterol efflux to apoAI in macrophages loaded with high amounts of cholesterol (see online Figure IA in the data supplement, available at http://www.circresaha.org). By contrast, TO901317 significantly increased cholesterol efflux to apoAI (online Figure IA). This inconsistency is explained by the observation that in cholesterol-loaded cells, both cholesterol and 24(S),25-epoxy synthesis were inhibited >90% (online Figures IB and IC). The OSCi further reduced cholesterol synthesis to undetectable levels and importantly, was unable to increase 24(S),25-epoxy synthesis. These results correlated with an absence of ABCA1 mRNA induction by the OSCi in these cells above that observed with cholesterol loading alone (online Figure ID).

Effect of OSC Inhibition on LDL-R mRNA and Activity

Some oxysterols can decrease LDL-R expression through suppression of SREBP activation, a process independent of LXR.8 We therefore examined whether OSCi-induced 24(S),25-epoxy synthesis would decrease LDL-R expression and activity. Ro-71-4565 reduced LDL-R mRNA 42% (P<0.05). By comparison, exogenous 24(S),25-epoxy decreased LDL-R mRNA 50% (P<0.05) (Figure 8A). By contrast, the LXR agonist TO901317 increased LDL-R mRNA 25%. Ro-71-4565 also significantly reduced 125I-LDL specific binding and uptake (∼25 to ∼54%, P<0.05, Figure 8B). Similar reductions were observed with 22(R)-OH treatment.

Discussion

Oxysterols play an important role in regulating genes involved in lipid metabolism and modulate the development of atherosclerosis.6,7 Oxysterols activate LXR, which increases the expression of genes involved in foam cell formation, including ABCA1, ABCG1, apoE, and SREBP-1c.6 Some oxysterols also suppress SREBP activation.8 SREBP-1c is a potent activator of genes involved in biosynthesis and esterification of unsaturated fatty acids, whereas SREBP-2 primarily activates genes involved in cholesterol metabolism, including the LDL-R and HMG-CoA reductase.8,24,25 In this study, we demonstrate that partial inhibition of OSC enhanced endogenous oxysterol synthesis, resulting in attenuated macrophage foam cell formation induced by the atherogenic lipoprotein HTG-VLDL. Our results indicate a mechanism involving (1) decreased lipoprotein uptake through inhibition of LDL-R expression and activity and (2) increased ABCA1 and ABCG1 expression. These effects were directly correlated with the extent of OSC inhibition and
Rowe et al. OSCi Attenuates Macrophage Foam Cell Formation

24(S),25-epoxy synthesis and inhibition of HTG-VDL-induced foam cell formation is biphasic. Peak cellular concentrations of 24(S),25-epoxy achieved with Ro-71-4565 correlate exactly with maximal inhibition of macrophage CE accumulation. However, the effect is significantly attenuated as the extent of OSC inhibition increases, whereas cholesterol synthesis remains inhibited. Thus, reduced HTG-VDL-induced CE accumulation results from increased 24(S),25-epoxy synthesis and not inhibition of cholesterol synthesis. Furthermore, this observation reveals an elegant feedback mechanism for regulation of endogenous synthesis of a potent regulatory oxysterol. Further OSC inhibition blocks the conversion of DOS to 24(S),25-epoxylanosterol, preventing the formation of 24(S),25-epoxy. This is in contrast to the effect of exogenous oxysterols or TO901317, in which no biphasic response was observed.

LDL-R expression and activity were reduced by OSC inhibition and exogenous 24(S),25-epoxy, contributing to the decrease in lipoprotein uptake and cellular CE accumulation. This provides evidence that 24(S),25-epoxy mediates transcriptional regulation of the LDL-R in OSCi-treated cells. LDL-R downregulation occurred despite inhibition of cholesterol synthesis. Depletion of cellular cholesterol would normally increase LDL-R expression; however, this response occurs when oxysterol concentrations are low, thereby stimulating SREBP-2 processing. 8 Exogenous 24(S),25-epoxy is known to suppress SREBP-2 processing, 24 supporting the concept that OSCi-induced 24(S),25-epoxy synthesis also suppressed SREBP-2 processing, resulting in the downregulation of LDL-R expression. 8 The importance of the LDL-R in macrophage foam cell formation is demonstrated in atherosclerosis-susceptible mice, whereby transplantation of irradiated C57Bl/6 mice with LDL-R–null bone marrow significantly decreased diet-induced atherosclerosis. 27

Oxysterols regulate transcription of key genes in cellular lipid homeostasis in part through activation of LXR. 6,9 24(S),25-Epoxy is a potent LXR ligand, 8 known to activate the LXR-responsive genes ABCA1 10 and ABCG1. 6 Cholesterol loading of macrophages induces ABCA1 expression via LXR, and recently it was determined that the oxysterol responsible was 27-hydroxycholesterol, whereas 22(R)-OH and 24(S),25-epoxy, two oxysterols believed to be endogenous ligands for LXR, were undetected. 10 We demonstrate clearly that in the absence of cholesterol loading, inhibition of OSC increased 24(S),25-epoxy synthesis, leading to increased ABCA1 and ABCG1 expression, ABCA1 protein, and cholesterol efflux. LXR is implicated in this process, because LXR antagonists completely attenuated the OSCi-induced increase in apolipoprotein-mediated cholesterol efflux, and partial inhibition of OSC resulted in an increase in LXR-mediated gene expression.

In macrophages preincubated with small amounts of cholesterol, OSC inhibition markedly increased cholesterol efflux, similar to that observed with exogenous 24(S),25-epoxy, 22(R)-OH, or TO901317. Therefore, OSCi-induced cholesterol efflux contributed to attenuated CE accumulation in macrophages exposed to HTG-VDL. However, in macrophages loaded with high concentrations of cholesterol, OSC inhibition did not induce cholesterol efflux, whereas the nonsterol LXR agonist TO901317 was effective. This was

Increases in endogenous synthesis of the regulatory oxysterol 24(S),25-epoxy.

Macrophages take up HTG-VDL, resulting in significant CE and TG deposition, demonstrating the atherogenic potential of these lipoproteins. 16 The novel aspect of the present study is that partial OSC inhibition in macrophages increases 24(S),25-epoxy synthesis, leading to significant reductions in HTG-VDL–induced CE accumulation, an observation supported by marked decreases in Oil Red O–stained cytoplasmic lipid droplets. Similar decreases in CE mass were measured in macrophages treated with exogenous 24(S),25-epoxy, suggesting that the effects of OSC inhibition on cellular CE accumulation are primarily a result of the regulatory activity of endogenous 24(S),25-epoxy. Reductions in HTG-VDL–induced CE accumulation were observed in macrophages incubated with the synthetic LXR ligand TO901317, indicating that endogenously synthesized 24(S),25-epoxy was acting, in part, through LXR activation.

An important concept emerging from this study is that 24(S),25-epoxy synthesis and inhibition of HTG-VDL–

ª BioMed Central Ltd. 2017
because of decreased cholesterol synthesis via feedback inhibition of HMG-CoA reductase by AcLDL-derived cholesterol, thus preventing Ro-71-4565 from enhancing 24(S)25-epoxy synthesis and subsequent ABCA1 upregulation. Collectively, our experiments suggest that OSCIs may be beneficial for enhancing cholesterol efflux and preventing foam cell formation during the lipoprotein uptake phase but may be less effective once macrophages become cholesterol loaded.

A concern with LXR agonists as therapeutic agents is the potential to increase liver and plasma TG concentrations through LXR activation of fatty acid synthase directly or via the activation of SREBP-1c, a transcriptional regulator of TG and fatty acid synthesis. OSCi-induced 24(S),25-epoxy synthesis had no appreciable effect on macrophage TG or free fatty acid synthesis, accumulation of cellular TG by HTG-VLDL, or LPL activity. Furthermore, exogenous 24(S),25-epoxy did not influence TG accumulation induced by HTG-VLDL. In contrast, 22(R)-OH or the synthetic LXR agonist TO901317 induced both LPL activity and TG synthesis. The OSCi, exogenous 24(S),25-epoxy and TO901317 significantly increased both SREBP-1 mRNA and precursor protein levels. However, only TO901317 resulted in a marked increase in active, nuclear SREBP-1; the OSCi and exogenous 24(S),25-epoxy appeared to suppress SREBP-1 processing. Although other oxysterols, including 25-hydroxycholesterol, are known to suppress SREBP-1 processing, this has not been reported for 24(S),25-epoxy. These observations provide a potential mechanism for the lack of effect of OSC inhibition on macrophage TG metabolism. Recently, Joseph et al demonstrated in mice that a synthetic LXR agonist decreased atherosclerosis but only transiently increased TGs, suggesting that alteration of TG metabolism may not affect the atheroprotective effect of LXR activation. Whether this applies to humans is unknown. Nevertheless, the lack of effect of OSC inhibition on TG metabolism is a potential therapeutic advantage.

In the present study, we clearly show that partial OSC inhibition decreases macrophage uptake of native lipoproteins and CE accumulation through decreased LDL-R expression and enhanced expression of ABCA1 and ABCG1, leading to increased cholesterol efflux. Therefore, increased macrophage synthesis of endogenous oxysterols represents a new mechanism for dual regulation of LXR- and SREBP-responsive genes, an approach that inhibits foam cell formation without detrimental effect on TG synthesis. Thus, OSC inhibition within vascular wall macrophages represents a potential new strategy for the prevention of atherosclerosis.

Acknowledgments

This work was supported by a grant from the Canadian Institutes of Health Research (CIHR; MT 8014 to M.W.H.). A.H.R. is the recipient of an Ontario Graduate Scholarship in Science and Technology, and C.A.A. is the recipient of a CIHR Studentship, M.W.H. and R.A.H. are Career Investigators of the Heart and Stroke Foundation of Ontario, and R.A.H. holds a Canada Research Chair (Tier 1) in Human Genetics. We thank Allison Kulchycki, Cara Ooi, and Janine van Gils for their expert technical assistance. OSC inhibitors were prepared by J. Aebi and H. Dehmflow, F. Hoffmann-La Roche Ltd.

References


Enhanced Synthesis of the Oxysterol 24(S),25-Epoxycholesterol in Macrophages by Inhibitors of 2,3-Oxidosqualene: Lanosterol Cyclase: A Novel Mechanism for the Attenuation of Foam Cell Formation
Andrea H. Rowe, Carmen A. Argmann, Jane Y. Edwards, Cynthia G. Sawyez, Olivier H. Morand, Robert A. Hegele and Murray W. Huff

Circ Res. 2003;93:717-725; originally published online September 25, 2003;
doi: 10.1161/01.RES.0000097606.43659.F4

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/93/8/717

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2003/10/15/93.8.717.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Expanded Materials and Methods: 

Cell Culture

J774A.1 (murine macrophages), THP-1 (human macrophages) and HepG2 (human hepatocytes) cells were obtained from American Type Culture Collection. Mouse peritoneal macrophages (MPMs) were isolated from LDL-R-null mice (Jackson Laboratories) and their control C57Bl/6 mice (Charles River). Cells were cultured as previously outlined.1-4 Several OSC inhibitors (OSCi) designated: Ro-48-8071, Ro-61-3479, Ro-71-3852 and Ro-71-4565 (all in DMSO) were tested, which inhibit OSC in rat hepatic microsomes with IC_{50}s of 8.1, 17.2, 4.4 and 28.4 nmol/L, respectively (F. Hoffmann-La Roche AG, Pharmaceuticals Division, Basel, Switzerland). Cell viability and protein contents were not affected at any dose tested. In some experiments, cells were incubated with 24(S),25-epoxy (in ethanol, Steraloids), 22(R)-OH (ethanol, Sigma), 22(S)-OH (LXR antagonist, ethanol, Sigma) the LXR antagonist 5α,6α-epoxycholesterol-3-sulfate5 (ECHS, ethanol, from the Ben May Institute for Cancer Research, University of Chicago, Chicago, IL) or the synthetic LXR ligand, TO901317 (DMSO, Sigma).

Cellular Lipid Synthesis

The incorporation of [1-14C]-oleic acid (Amersham Biosciences, Piscataway, NJ) into cellular CE, in J774A.1 macrophages, was measured as previously described.6 Incorporation of [1-14C]-acetic acid (Amersham Biosciences) into nonsaponifiable lipids (cholesterol, MOS, DOS,
24(S),25-epoxy, free fatty acids) was determined in J774A.1 cells as described by Morand et al.\(^7\) Briefly, cells were incubated for 24 h in the presence of 1 µCi \([1-^{14}C]\)-acetic acid. Lipids were extracted, saponified and separated by TLC in nhexane/diethyl ether/acetic acid (60:40:1, v/v/v). Lipids were visualized using a phosphorimager and quantified using Image Quant Software (Molecular Dynamics, Sunnyvale, CA).

TG synthesis was determined using a previously described method with minor modifications.\(^8\) Following a 19 h preincubation of cells with drug or vehicle (10% FBS), each dish received 0.04 µCi of \([1-^{14}C]\)-oleic acid (Amersham Biosciences) and 0.05 mmol/L of cold oleic acid complexed with fatty acid free bovine serum albumin (FAF:BSA, Sigma, Oakville, ON), with drug or vehicle, for an additional 5 h (5% LPDS \(^2\)). Lipids were then extracted and the radioactivity incorporated into TG was determined following TLC separation.

**Cellular Lipid Mass**

J774A.1 macrophages were preincubated with the OSCi, oxysterol or LXR agonist for 24 h in media supplemented with 5% LPDS. Cells were incubated for a further 16 h with fresh media and compounds in the absence or presence of HTG-VLDL or VLDL-REM (50 µg of lipoprotein total cholesterol (TC)/mL medium). Cellular CE, TC, FC, TG and protein mass were determined as previously described.\(^3\) In some experiments cells growing on sterile coverslips in 35 mm wells (Falcon Scientific, VWR, Mississauga, ON) were preincubated with an OSC inhibitor (24 h), incubated with HTG-VLDL (16 h) and then fixed (10% neutral buffered formalin), stained with Oil Red O and counterstained with Harris’ modified hematoxylin, to visualize neutral lipids.\(^9\)

**LPL Activity and \(^{125}\text{I}\)-LDL Binding, Uptake and Degradation**

LPL activity and \(^{125}\text{I}\)-LDL binding, uptake and degradation studies were performed as described elsewhere.\(^3,4\)

**mRNA Abundance**

J774A.1 macrophages were incubated for 24 h in media supplemented with 5% LPDS and an OSCi, oxysterol or TO901317. Total RNA was isolated using Trizol reagent (Invitrogen,
Burlington, ON), and the mRNA abundances of ABCA1, ABCG1, LDL-R and SREBP-1 were determined by an S1 nuclease protection assay as previously described. Briefly, custom primers (Invitrogen) were generated corresponding to murine ABCA1 (GenBank® accession #XM_124268.1, bases 198-237); ABCG1 (GenBank® accession #NM_009593, bases 1499-1545); LDL-R (GenBank® accession #X64414, bases 419-477) and SREBP-1 (GenBank® accession #NM_004176.2 bases 3967-4010) and 5'-end labeled with \( {\gamma}^{32}P \)-ATP (Amersham Biosciences). Oligonucleotides of interest were simultaneously hybridized with an oligo for GAPDH to total RNA from either control or treated cells and incubated over night at 55°C. Following hybridization samples were incubated with S1 nuclease (200 U, 20 min, 37°C, Roche Diagnostics, Laval, QC), ethanol precipitated and probes were separated by denaturing PAGE (19%). Bands were visualized using a phosphorimager, quantified using Image Quant software and the mRNA abundances for genes of interest were determined relative to the amount of GAPDH mRNA.

**SREBP-1 and ABCA1 Immunoblot Analyses**

J774A.1 macrophages (100 mm dishes, Falcon Scientific, VWR, Mississauga, ON) were incubated in the absence or presence of an OSCi, 24(S),25-epoxy or TO901317, for 24 h (5% lipoprotein deficient serum). Cells were then scraped into 500 µL of lysis buffer (10 mmol/L Tris/HCL, 10 mmol/L NaCl, 3 mmol/L MgCl2, 0.5% IGEPAL) containing aprotinin (100 units/mL), ALLN (2 µg/mL), leupeptin (0.1 mmol/L), PMSF (2 mmol/L), pepstatin (5 µg/mL) and benzamidine (250 µg/mL) and homogenized by 15 passes through a 25 gauge needle. The postnuclear supernatant was obtained by centrifugation (500 g, 4°C, 10 min). The remaining pellet was washed once with lysis buffer and then resuspended in hypertonic buffer (10 mmol/L Hepes, 0.42 mol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT) containing protease inhibitors (as above) and incubated at 4°C for 30 min. The nuclear fraction was obtained by centrifugation in a TLA120.2 rotor (Beckman Instruments, Mississauga, ON) at 100 000 g for 30 min at 4°C. Protein from the postnuclear or nuclear fractions were mixed with
SDS loading buffer and subjected to SDS/PAGE on either a 4.5% (ABCA1, 60 µg postnuclear fraction) or 8% (SREBP-1, 40 µg nuclear and 80 µg postnuclear fraction) gel.

Proteins were then transferred electrophoretically to PVDF membranes. For ABCA1 detection, membranes were blocked overnight at 4°C with commercial blocking buffer (1% w/v in tris buffered saline (TBS), Roche Diagnostics, Laval, PQ), and then incubated with a polyclonal antibody for ABCA1 (1:500, Novus Biologicals Inc, Littleton, CO) for 2 h, then washed, and incubated with a peroxidase-conjugated anti-(rabbit IgG) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). For SREBP-1 detection, membranes were blocked overnight at 4°C with 5% (w/v) non-fat dried milk in TBS:0.1% Tween, and then incubated with a monoclonal antibody for SREBP-1 (1:200, Medicorp Inc, Montreal, PQ) for 2 h, then washed and incubated with a peroxidase-conjugated anti-(mouse IgG) antibody (Santa Cruz Biotechnology). ABCA1 or SREBP-1 expression was detected using BM Chemiluminescence Blotting Substrate (Roche Diagnostics). Quantification of developed films was performed using an Imaging Densitometer (GS-700; Bio-Rad Laboratories, Mississauga, ON).

**Cholesterol Efflux Assay**

J774A.1 or THP-1 (0.16 µmol/L phorbol dibutyrate, 5 days, Sigma) macrophages were incubated with AcLDL (5 µg TC/mL) in medium containing 0.2% FAF:BSA and [1α,2α (n)-3H]cholesterol (1 µCi/mL, Amersham Biosciences) for 24 h and designated as non-cholesterol loaded cells. In some experiments, J774A.1 cells were incubated with [3H]-cholesterol and 100 µg TC/mL of AcLDL and were designated as cholesterol-loaded cells. Cells were then washed with phosphate buffered saline (PBS) and incubated with Ro-71-4565, oxysterols, or TO901317 in the presence or absence of LXR antagonists (ECHS (10 µM) or 22(S)-OH (100 µM)). After 24 h, macrophages were incubated with fresh compound and medium with 0.2% FAF:BSA alone, or with the addition of apoA1 (10 µg/mL, Sigma) for 16 h. Cholesterol efflux was determined as described previously.4,10

**Dual Luciferase Reporter Assay**
The TK-LXRE3-luc construct containing three copies of the LXR response element upstream of a luciferase reporter (prepared by Dr. D Mangelsdorf, University of Texas Southwestern, Dallas, Texas) and the TK promoter-Renilla luciferase construct, pRL-TK, were provided by Dr. D. Ory, Washington School of Medicine, St. Louis, Missouri.\textsuperscript{11,12} HepG2 cells were seeded in 35 mm wells (Falcon Scientific) overnight (50% confluent) and transiently cotransfected with 1 $\mu$g/mL TK-LXRE3-luc and 0.1 $\mu$g/mL pRL-TK using Lipofectamine reagents (Invitrogen) and the manufacturer’s protocol. Following transfection (24h) cells were re-fed LPDS and incubated with vehicle alone or Ro-71-4565, 24(S),25-epoxy or TO901317 (24 h). Cells were then harvested and Firefly and Renilla luciferase activities measured using the Dual-Luciferase Reporter Assay System (Promega). The relative luciferase activity (rlu) was then calculated by normalizing LXRE promoter-driven Firefly luciferase activity to control Renilla activity. Data from all experiments are presented as the rlu (mean ± SEM) from two independent sets of experiments, each with duplicate measurements.
References


Online Figure 1: The effect of cholesterol-loading on cellular responses mediated by OSC inhibition. A, J774A.1 macrophages were cholesterol-loaded by incubation of cells with 100 µg total cholesterol (TC)/mL of acetylated LDL (AcLDL) and [³H]-cholesterol. Following a 24 h incubation of cells with DMSO, Ro-71-4565 (1 µmol/L) or TO901317 (1 µmol/L) and an additional 16 h of cells with drug or vehicle with FAF:BSA alone or plus apoAI (10 µg/mL), the percent [³H]cholesterol effluxed to the media versus the total [³H]cholesterol (cell plus media) was determined. The incorporation of [¹⁴C]-acetic acid into (B) cholesterol (n=4) and (C) 24(S),25-epoxycholesterol (24(S),25-epoxy, n=3) was determined in J774A.1 macrophages incubated with DMSO, Ro-71-4565 (1 µmol/L), AcLDL (100 µg TC/mL), or AcLDL plus Ro-71-4565 for 24 h. Cellular lipids were extracted, saponified, separated by thin layer chromatography and the amount of radioactivity determined. Data were calculated as dpm/mg cell protein and plotted as the percent (mean ± SEM) of control values (vehicle alone). ABCA1 mRNA abundance (D) was determined by nuclease protection in cells incubated with vehicle alone or Ro-71-4565 (1 µmol/L), AcLDL (100 µg TC/mL), AcLDL plus 24(S),25-epoxy (all n=3) or AcLDL plus 22(R)-OH (10 µmol/L, n=1) for 24 h. Data is presented as the ratio of ABCA1/GAPDH and plotted as percent of control (mean ± SEM). ND: not detected. *P<0.05 as compared to control.
Online Figure I

A. Cholesterol Efflux (cholesterol-loaded cells)

B. Cholesterol Synthesis

C. [24(S),25-epoxy]

D. ABCA1 mRNA

Control + AcLDL + Ro-71-4565 AcLDL+ Ro-71-4565 + TO901317 AcLDL+22(R)-OH