Statins Inhibit Synthesis of an Oxysterol Ligand of the Liver X Receptor in Human Macrophages With Consequences for Cholesterol Flux

Jenny Wong, Carmel M. Quinn, Andrew J. Brown

Objective—Cholesterol efflux from macrophages in the artery wall, a key cardioprotective mechanism, is largely coordinated by the nuclear oxysterol-activated liver X receptor, LXRα. We investigated the effect of statins on LXR target gene expression and cholesterol efflux from human macrophages.

Methods and Results—In human macrophages (THP-1 cell line and primary cells), the archetypal statin, compactin, greatly reduced mRNA levels of 2 LXR target genes, ABCA1 and ABCG1 mRNA, as well as decreased cholesterol efflux. Commonly prescribed statins also downregulated LXR target gene expression in THP-1 cells. We provide several lines of evidence indicating that statins decrease expression of LXR target genes by inhibiting the synthesis of an oxysterol ligand for LXR, 24(S),25-epoxycholesterol. When THP-1 cells were cholesterol-loaded via incubation with acetylated low-density lipoprotein, synthesis of 24(S),25-epoxycholesterol was greatly reduced and the downregulatory effect of compactin on ABCA1 mRNA levels and cholesterol efflux was lost.

Conclusions—Our results suggest that statins may downregulate cholesterol efflux from nonloaded human macrophages by inhibiting synthesis of an oxysterol ligand for LXR. Further work is needed to determine how relevant our observations are to arterial foam cells in vivo.

Key Words: statins • human macrophage • liver X receptor • 24(S),25-epoxycholesterol • THP-1 cells • human monocyte-derived macrophages • pleiotropic

Statins are highly effective in lowering serum cholesterol concentrations and in reducing the risk of coronary heart disease and stroke.1 There is a growing body of clinical and experimental evidence that statins exert additional benefits beyond cholesterol reduction.2,3 Intensive efforts are underway to study the pleiotropic, cholesterol-independent effects of statins on the vasculature.2,3

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The macrophage is a key vascular cell involved in the development of atherosclerosis. Cholesterol-laden macrophages (foam cells) have essential functions in all phases of atherosclerosis, from development of the fatty streak to processes that ultimately contribute to plaque rupture and myocardial infarction. Because mammalian cells cannot degrade the sterol fused-ring structure, excess sterols undergo elimination from the body principally by biliary excretion. Therefore, macrophages must export cholesterol to extracellular acceptors for transport to the liver.4

The liver X receptor (LXR), NR1H3 or LXRα, plays an important role in coordinating cholesterol efflux from the macrophage. As a member of the nuclear receptor family of ligand-dependent transcription factors, LXRα activates transcription of target genes in response to the binding of certain oxidized forms of cholesterol (oxysterols).5,6 LXRα forms a heterodimer with the retinoid X receptors to bind to specific response elements in the promoters or enhancers of target genes. The genes encoding several important proteins that facilitate cholesterol removal from macrophages are targets of LXR, including ABCA1, ABCG1, apolipoprotein E (apoE), and LXRα itself. ABCA1 and ABCG1 are members of the ATP-binding cassette superfamily of transporter proteins. Mutations in ABCA1 were recently identified as the basis for Tangier disease, a rare autosomal disorder characterized by very low levels of high-density lipoprotein and premature atherosclerosis. Genetic and biochemical evidence indicate that ABCA1 is important for mediating efflux of cholesterol and phospholipids from macrophage-foam cells in atherosclerotic lesions.5,7 Less is known about the half-transporter, ABCG1, but it is also implicated in facilitating cholesterol efflux from macrophages.7

The crystal structure of the LXRα ligand-binding domain has been solved. The topography of the pocket suggests a common anchoring of certain oxysterols with oxygenated...
side chains. Different oxysterol ligands for LXR have been proposed to be produced in a tissue-specific manner; e.g., 24(S),25-epoxycholesterol (24,25EC), until recently, was detected only in the liver, 24(S)-hydroxycholesterol in the brain, and 20(S)-hydroxycholesterol and 22(R)-hydroxycholesterol in the adrenals. 27-Hydroxycholesterol was proposed as one candidate ligand for LXR activation in macrophages. However, others have reported that 27-hydroxycholesterol is not an efficient activator of human LXR. Therefore, the identity of an oxysterol ligand(s) for LXR, endogenously produced by the human macrophage, remains unclear.

It is commonly assumed that oxysterol LXR ligands will be produced from preformed cholesterol, as believed to be the case for 27-hydroxycholesterol. However, synthesis of an oxysterol ligand could also occur by de novo synthesis in the mevalonate pathway, as was shown for hepatic synthesis of 24,25EC. Forman et al have shown that statins can reduce mevalonate pathway, as was shown for hepatic synthesis of an oxysterol ligand(s) for LXR, endogenously produced by the human macrophage, remains unclear.

Methods

Reagents and Cell Culture
Chemicals and reagents used are detailed elsewhere (please see http://atvb.ahajournals.org). Cells were grown at 37°C in a 5% CO₂ atmosphere in RPMI 1640 supplemented with pénicillin/streptomycin 100 U/100 µg/mL, L-glutamine (2 mmol/L), and various sera as described. As a model for human macrophages, we used the widely used monocytic cell line THP-1, differentiated with phorbol 12-myristate 13-acetate. Human monocyte-derived macrophages (HMDM) were prepared from white cell buffy coat concentrates from healthy donors as described. For detailed culturing conditions, please see online supplement.

Cholesterol Efflux
After prelabeling THP-1 cells or HMDMs with [³H]-cholesterol (1 µCi/mL) while maintaining treatment(s). Cholesterol efflux was expressed as the radioactivity appearing in the media as a percentage of total radioactivity (cells plus media).

Thin-Layer Chromatography
Thin-layer chromatography (TLC) was conducted for assessment of cholesterol and 24,25EC synthesis using a method detailed by Morand et al with minor modifications. Cells were incubated in the presence or absence of apoAI (25 µg/mL) while maintaining treatment(s). Cholesterol efflux was expressed as the radioactivity appearing in the media as a percentage of total radioactivity (cells plus media).

RNA Extraction and mRNA Quantitation
Cells were harvested for total RNA using Tri Reagent according to the manufacturer’s instructions (Sigma) and quantitative (“real-time”) reverse transcriptase-polymerase chain reaction (QRT-PCR) was performed using an ABI 7700 Sequence Detector and analyzed using ABI Prism Sequence Detector Soft-ware v1.6.3 (PE Biosystems) as detailed online.

Western Blotting
To measure ABCA1 protein expression, membrane fractions were prepared as described and samples (50 µg per lane) were analyzed by SDS-PAGE (ABCA1, 7.5%; transferrin receptor, 10%). Protein

Results

Compactin Decreases Expression of LXR Target Genes, ABCA1 and ABCG1, in THP-1 Human Macrophages
We hypothesized that if oxysterol ligands for LXR are derived from de novo synthesis, inhibition of the committed step in this pathway, catalyzed by 3-hydroxy-3-methylglutaryl (HMG) coenzyme A (CoA) reductase, should reduce LXR ligand production and, hence, expression of LXR target genes. Cells were treated with varying concentrations of compactin (0 to 50 µmol/L) were incubated with phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 cells for 24 hours. mRNA levels for (A) ABCA1 and ABCG1 and (B) HMG-CoA reductase and the LDL receptor measured using QRT-PCR, normalized to PBGD mRNA levels. Data are presented relative to vehicle-treated controls and are means ± SEM (n=3 replicate cultures). Statistically significant effects (P<0.05 by unpaired t test) started to be observed at 0.05 µmol/L compactin for ABCG1, HMG-CoA reductase, and the LDL receptor, and at 0.5 µmol/L compactin for ABCA1.
tein (LDL) receptor and HMG-CoA reductase increased in a concentration-dependent manner (Figure 1B). Maximal effects for all genes examined were observed at 5 μmol/L compactin, a concentration that was used in subsequent experiments.

**Compactin Decreases ABCA1 Protein Levels and Cholesterol Efflux From THP-1 Human Macrophages**

To determine whether the statin-induced reduction of ABCA1 translates to decreased protein levels, we incubated cells in the presence and absence of compactin (5 μmol/L) and measured ABCA1 protein levels in membrane fractions by Western blotting. Two bands were observed migrating in the vicinity of the deduced molecular weight of ABCA1 (≈220 kDa) in agreement with others. Compactin treatment greatly reduced ABCA1 protein levels (Figure 2A) in concordance with the downregulatory effect of the statin at the mRNA level (Figure 1A). Another plasma membrane-bound protein, the transferrin receptor, was probed as a test for specificity and found to be unaffected by compactin treatment (Figure 2A). We then tested whether a diminished protein level of ABCA1 resulted in reduced function. Cells were incubated with [3H]-cholesterol and membrane efflux was induced by incubating with the cholesterol acceptor, apoAI, for 24 hours. Compactin treatment did not affect total radio-labeling or cell protein levels (data not shown). Figure 2B shows that cholesterol efflux induced by apoAI was ablated in the presence of compactin. These results suggest that compactin inhibited apoAI induced cholesterol efflux from THP-1 human macrophages by decreasing ABCA1 mRNA and protein expression levels.

**24(S),25-Epoxycholesterol Is a Potent Activator of LXR Target Genes in THP-1 Human Macrophages**

In agreement with previous studies in cotransfected CV-1 cells and a ligand-binding assay, we showed that 24,25EC is a potent activator of LXR target genes in THP-1 macrophages. Of a panel of natural oxysterol ligands tested, 24,25EC upregulated the LXR target genes, ABCA1 and ABCG1, to the greatest extent. Another LXR target gene, LXRα, responded to LXR ligands and decreased with compactin treatment.

24(S),25-Epoxycholesterol is an important LXR Ligand Produced in THP-1 Human Macrophages

24,25EC is derived from a shunt in the mevalonate pathway in which 2,3(S)-monooxidosqualene (MOS), rather than undergoing cyclization to lanosterol, is converted to 2,3(S):22(S),23-dioxidosqualene (DOS) (Figure 3A). DOS is then cyclized into 24(S),25-epoxylanosterol, which ultimately leads to the formation of 24,25EC. Because the cyclase preferentially cyclizes DOS over MOS, incomplete inhibition of oxidosqualene cyclase explains how the squalene epoxides can be channeled into 24,25EC, a finding observed in HepG2 liver cells and recently extended to the murine macrophage cell line, J774A.1.17 We repeated this observation in [14C]-acetate–labeled J774A.1 murine macrophages using a different cyclase inhibitor, GW534511X (Figure 3B), and further extended this finding to the human macrophage cell line, THP-1 (Figure 3C). Identification of 24,25EC was confirmed chemically and by mass spectrometry. In THP-1 cells, levels of synthesized 24,25EC increased in the presence of low concentrations of cyclase inhibitor (0.1 to 1.0 nM) but decreased at higher concentrations (100 to 1000 nM) (Figure 3C). This profile was similar to the mRNA levels of ABCA1 and ABCG1 observed in response to increasing concentrations of the cyclase inhibitor (Figure 3D). THP-1 human macrophages also produced 24,25EC under control or basal conditions (Figure 3C and 3E). Densitometry of the phosphorimages indicated that under basal conditions, THP-1 macrophages synthesized 24,25EC in a ratio of ≈0.9:100 relative to cholesterol. Cholesterol and 24,25EC were the only 2 sterols
detected under basal conditions (data not shown). Treatment with compactin decreased synthesis of both 24,25EC and cholesterol (Figure 3E), again reflecting the LXR target gene expression data (Figure 1A). 24,25EC production, in the presence of either compactin or the cyclase inhibitor, was strongly correlated with the expression of LXR target genes, ABCA1 and ABCG1 (Figure 3F). Together, these data indicate that compactin may decrease expression of LXR target genes by inhibiting production of the potent LXR ligand, 24,25EC.

Exogenous 24(S),25-Epoxycholesterol Overcomes Compactin-Induced Reduction of LXR Target Gene Expression in THP-1 Human Macrophages

To further explore how compactin decreases expression of LXR target genes, we tested whether the effect could be reversed by the addition of mevalonate, the product of HMG-CoA reductase. Exogenous mevalonate restored transcription of the LXR target genes, ABCA1 and ABCG1 (Figure 4A and 4B). Also, we tested if mevalonate addition could overcome blockade of an enzyme distal to HMG-CoA reductase (squalene epoxidase) using the inhibitor GR144000X (Figure 3A). At the concentration used (5 μmol/L), GR144000X completely blocked synthesis of both 24,25EC and cholesterol (data not shown). Exogenous mevalonate failed to restore transcription of ABCA1 (Figure 4A) and ABCG1 (Figure 4B) in the presence of the squalene epoxidase inhibitor. Together, these data indicate that the LXR ligand is produced downstream of this enzyme. We then...
tested if addition of 24,25EC reversed the effect of inhibiting either HMG-CoA reductase or squalene epoxidase. When either enzyme was inhibited, the addition of 24,25EC normalized expression of ABCA1 and ABCG1 (Figure 4C and 4D) and cholesterol efflux (data not shown), lending further support to the contention that 24,25EC is a significant LXR ligand in human macrophages whose generation is inhibited by compactin.

**Effect of Other Statins on Expression of LXR Target Genes in THP-1 Human Macrophages**

We used compactin in our investigations because it is the archetypal statin. To ensure that the suppression of ABCA1 and ABCG1 expression is not restricted to compactin, we tested a panel of statins, most of which are in clinical use. 24,25EC synthesis and expression of ABCA1 and ABCG1 mRNA displayed a marked dose-related decrease with all statins tested. Therefore, the downregulation of LXR target genes that contribute to cholesterol efflux represents a general effect of statins.

**Effect of Compactin on Expression of LXR Target Genes in and Cholesterol Efflux From Human Monocyte-Derived Macrophages**

We determined if compactin had comparable effects in primary human macrophages to those we observed in THP-1 cells. Although cholesterol and 24,25EC were synthesized under our culturing conditions (10% whole serum) (Figure 5A), the 24,25EC band was very faint, and the ratio of 24,25EC relative to cholesterol was considerably less than observed for nonloaded THP-1 cells (t=2.01). Partial inhibition of oxidosqualene cyclase with 1 nM GW534511X greatly enhanced 24,25EC synthesis (Figure 5A), clearly demonstrating that primary human macrophages can produce this oxysterol ligand for LXR. Compactin treatment decreased both cholesterol and 24,25EC synthesis, and these effects were maximal at 5 μmol/L. Compactin treatment (5 μmol/L) also significantly decreased ABCA1 mRNA expression (Figure 5B) and cholesterol efflux (Figure 5C). Addition of 24,25EC greatly increased ABCA1 mRNA levels and cholesterol efflux and abolished the compactin effect. Therefore, our key findings in THP-1 cells were also observed in primary human macrophages.

**Effect of Compactin on Expression of LXR Target Genes in and Cholesterol Efflux From THP-1 Human Macrophages Loaded With Cholesterol**

Up to this point, our studies have been performed in non-loaded THP-1 macrophages and HMDMs. Cholesterol-laden macrophages are arguably more relevant to atherogenesis. However, cholesterol loading might be expected to shut down synthesis of cholesterol and 24,25EC. Therefore, we tested whether statins also decrease ABCA1 mRNA levels in THP-1 macrophages that have been loaded with cholesterol via incubation with acetylated low-density lipoprotein (AcLDL). Consistent with the lack of 24,25EC synthesis observed with AcLDL-loading, compactin had little effect on mRNA levels of ABCA1. Furthermore, compactin treatment had no effect on apoAI-stimulated cholesterol efflux from AcLDL-loaded THP-1 cells. Therefore, the inhibitory effect of statins on cholesterol efflux is lost with extensive cholesterol loading of macrophages.

**Discussion**

Statins are a structurally related group of HMG-CoA reductase inhibitors that are widely used to treat hypercholester-
emia. However, there is growing interest in the action of statins beyond cholesterol reduction. For example, statins have been reported to exert a number of cholesterol-independent effects on macrophages such as inhibiting secretion of matrix metalloproteinases and downregulating scavenger receptor class A expression. In support of recent studies, we showed that statins decrease expression of several key LXR target genes that are involved in cholesterol removal from the macrophage. ABCA1 is particularly important in facilitating cholesterol efflux from the macrophage as evidenced from the virtually absent high-density lipoprotein cholesterol levels and the increased atherosclerosis seen in ABCA1 deficiency (Tangier disease). We demonstrated that the statin-mediated reduction in ABCA1 mRNA levels produced a drastic change in function, namely, a marked reduction in ABCA1 protein levels and ablation of cholesterol efflux to apoAI. The effect was observed both in a human macrophage cell line (THP-1) as well as in primary human macrophages (HMDMs). Furthermore, we provided compelling evidence that statins inhibit these processes by interfering with the generation of an oxysterol ligand for LXR.

We presented several lines of evidence implicating 24,25EC as a significant LXR ligand in the human macrophage. 24,25EC was a potent activator of LXR target genes in human macrophages, superior to other natural oxysterol ligands tested, and comparable to a potent synthetic ligand. In an experiment using exogenous mevalonate, we showed that endogenous LXR ligand was produced de novo, downstream from HMG-CoA reductase and squalene epoxidase. Squalene epoxidase is a secondary rate-limiting enzyme in sterol biosynthesis and converts squalene to MOS and catalyzes the conversion of MOS to DOS in the initial step of the shunt pathway, which ultimately forms 24,25EC (Figure 3A). Consequently, we showed that exogenous 24,25EC restored transcriptional activity of LXR target genes both in the presence of compactin or an inhibitor of squalene epoxidase. Moreover, in experiments using compactin and an oxidosqualene cyclase inhibitor, we showed that there was a strong linear relationship between 24,25EC synthesis and LXR target gene expression. Taken together, our data indicate that 24,25EC is an endogenous LXR ligand produced by human macrophages and that statins interfere with this production.

Our data have potential implications for the clinical use of statins considering that oxysterol activation of LXR has been ascribed a significant anti-atherogenic role. Whereas most administered statins are cleared rapidly by the liver, their pleiotropic vascular effects suggest that extrahepatic cells such as the macrophage are also exposed to statins. Considering that peak plasma concentrations achieved for most statins range from 0.1 to 1 μmol/L, effects in the present study were observed at therapeutically relevant concentrations (0.05 to 0.5 μmol/L).

Statins decrease cholesterol synthesis and downregulate scavenger receptor class A expression, effects that would be predicted to be anti-atherogenic. We observed that statins upregulated the LDL receptor and HMG-CoA reductase. We also observed that statins downregulated key efflux pathways (ABCA1, ABCG1) in nonloaded human macrophages. Reduced cholesterol efflux would be predicted to be pro-atherogenic. However, the effect of statins was lost when macrophages were extensively cholesterol-loaded. These conflicting observations beg the question: do statins accentuate or ameliorate macrophage cholesterol accumulation within the arterial wall?

In this initial report, we have not attempted to measure the effect of statins on cellular cholesterol status. From the literature, statins appear to have varying effects on macrophage cholesterol levels, depending on culturing conditions. In the present study, AcLDL-loading of THP-1 cells tended to increase ABCA1 expression in agreement with previous studies, but drastically inhibited the mevalonate pathway, including synthesis of 24,25EC. These results suggest that human macrophages may produce different ligands for LXR, depending on the supply of exogenous cholesterol versus the activity of the shunt pathway. 27-hydroxycholesterol is unlikely to be a significant ligand in THP-1 cells because these cells, unlike primary human macrophages, have very low expression of CYP27A1 and produce negligible 27-hydroxycholesterol (Quinn CM, Brown AJ, Kritiharies L, Jessup W, unpublished observations).

Further work is required to evaluate the role of cholesterol status on the effects of statins on LXR target genes, and to determine how relevant our observations are to arterial foam cells in vivo. In terms of future studies to assess the clinical implications of the effect of statins on LXR target genes, studies in mice may not be particularly helpful, considering the substantial differences in lipid metabolism between mice and humans, particularly regarding the regulation of LXRα.

Although there is no doubt that statins exert a net benefit, their efficacy potentially may be negated to some extent by reducing LXR ligand formation and hence impairing reverse cholesterol transport, at least in nonloaded human macrophages. With the evolving interest in the cholesterol-independent vascular effects of statins, it is imperative that we explore and understand all interactions between statins and the macrophage, even those that may be potentially negative.

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References


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SUPPLEMENTARY INFORMATION:

Establishing the identity of 24(S),25-Epoxycholesterol as produced by THP-1 human macrophages

Methods

Cell culture and TLC: THP-1 cells (one to two 6-well plates) were PMA-differentiated in 10% FCS as described in the main text. They were incubated for 24 h (10% LPDS) in the presence of an oxidosqualene cyclase inhibitor (1 nM GW534511X) in media containing [14C]-acetate (2 µCi/well). [14C]-acetate was excluded from samples to be analyzed by mass spectrometry. Neutral lipid extracts were separated using TLC with a mobile phase of n-hexane: diethyl ether: acetic acid (60:40:1 v/v/v), and visualized using a phosphoimager (48 h exposure). The band corresponding to putative 24,25EC was excised from the TLC plate using methanol (1 ml/band) followed by diethyl ether (1 ml/band). The eluted bands were separated from residual silica by addition of water (0.4 ml/band) and n-hexane (1 ml/band). The organic phase was evaporated under nitrogen and used as described.

Lithium Aluminium Hydride Reduction: A portion of the dried sample was re-dissolved in tetrahydrofuran (3 ml). Lithium aluminium hydride (20 mg) was added and the mixture was refluxed (~40°C; 24 h) whilst stirring. The solvent was evaporated under nitrogen and extracted (1 ml water, 2.5 ml each of methanol, n-hexane and diethyl ether). The organic phase was evaporated under nitrogen, re-dissolved in n-hexane: diethyl ether (1:1 v/v) applied to TLC. After separation with a mobile-phase of n-hexane: ethyl acetate (1:1 v/v), radioactive bands were visualized using a phosphoimager (48 h exposure). Authentic standards (30-100 µg) were visualized by spraying the plate with 10% (w/v) CuSO₄ in 8.5% (v/v) phosphoric acid, and briefly heating.

Hydrolysis by perchloric acid: Two equal portions of the dried sample were re-dissolved in tetrahydrofuran (500 µl), followed by two drops of water. Perchloric acid (100 µl) was added to one sample while water (100 µl) was added to the other. Standard 24,25EC aliquots (2 x 200 µg) were treated in an identical way. After stirring (4°C, 24 h), a saturated solution of sodium bicarbonate (1 ml) was added, followed by phosphate-
buffered saline (1 ml) and diethyl ether (7 ml). The organic phase was evaporated, run on TLC, and bands visualized as above.

**Mass spectrometry:** For electron impact (EI) mass spectrometry (MS), solutions of an authentic 24,25EC standard and the sample (eluted from the putative 24,25EC band), were deposited on a rhenium wire and heated inside the mass spectrometer (Finnigan Polaris Q Ion Trap). The vapor was ionized with an electron beam at 70eV. For atmospheric pressure chemical ionization (APCI) mass spectrometry, the standard and sample were dissolved in dichloromethane and applied directly to the APCI probe in a Finnigan LCQ Ion Trap mass spectrometer.

**Results and Discussion**
The compound eluted from this putative 24,25EC band co-chromatographed with authentic standard under three different solvent systems (including reverse-phase), but separately from known oxysterol LXR ligands (data not shown). Moreover, we found that this putative 24,25EC band undergoes chemical reactions that are known and specific for 24,25EC. We found that the compound is converted to 25-hydroxycholesterol by treatment with lithium aluminium hydride (Figure I). Furthermore, we found that under mild acid treatment, the epoxide undergoes hydrolysis to produce a more polar TRIOL (Figure II). Mass spectrometry provided further proof that this band is 24,25EC. EI-MS showed the same molecular ion at m/z 400 as authentic standard (data not shown). APCI-MS results in proton transfer and hence an increase in 1 m/z unit. Figure III shows the Selective Ion Monitoring measurements carried out on 401 (MH+) and 383 (MH+-H2O). Both standard and sample gave similar results. Therefore, we have confirmed the identity of 24,25EC, and showed that THP-1 human macrophages produced 24,25EC under these culturing conditions. It should be noted that our methods for identifying 24,25EC do not distinguish between the 24(S),25EC and 24(R),25EC epimers. But on the basis of previous work in human liver tissue and their relative potencies as LXR ligands, we assume that this is the S-epimer.

Supplemental Figure I.
Reduction of 24(S),25-epoxycholesterol (24,25EC) to 25-hydroxycholesterol (25HC) using lithium aluminium hydride (LiAlH₄)
Supplemental Figure II.
Hydrolysis of 24(S),25-epoxycholesterol (24,25EC) with perchloric acid (PCA). The main product is a TRIOL. Dehydration products probably account for the less polar bands.
Supplemental Figure III: APCI-Mass spectrometry of 24(S),25-epoxycholesterol

A. Standard (SIM: 380.5-385.5)  \( \text{MH}^+ - \text{H}_2\text{O} \)

B. Standard (SIM: 400-403)  \( \text{MH}^+ \)

C. Band (SIM: 380.5-385.5)

D. Band (SIM: 400-403)
Statins inhibit synthesis of an oxysterol ligand for the Liver X receptor in human macrophages with consequences for cholesterol flux.

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Methods

Reagents

Chemicals and reagents used are listed below with the supplier. From Amersham Biosciences: [2-¹⁴C]-acetic acid, sodium salt (specific activity: 56 mCi/mmol); [1α,2α(n)-³H]-cholesterol (specific activity: 49 Ci/mmol); ECL reagent. From Invitrogen: L-glutamine, oligo dT; penicillin/streptomycin, SuperScript II. From Peprotech (UK): Macrophage-Colony Stimulating Factor (M-CSF). From Sequoia Research Products (Oxford, UK): atorvastatin, fluvastatin, lovastatin, (p)itavastatin, pravastatin, rosuvastatin, simvastatin. From Sigma: fatty acid-free bovine-serum albumin fraction V (BSA); compactin; deoxynucleotide triphosphate; 20(S)-hydroxycholesterol; 22(R)-hydroxycholesterol; phorbol 12-myristate 13-acetate (PMA); Tri Reagent; oligonucleotides were synthesized by Sigma-Genosys. From Steraloids: 24(S),25-epoxycholesterol; 27-hydroxycholesterol. From Trace Biosciences: RPMI 1640; DMEM. From miscellaneous companies: fetal calf serum (FCS; Gibco BRL); iQ SyBr Green Supermix (BioRad); RNasin (Promega); UltimaGold scintillation fluid (Packard Bioscience). All solvents were of HPLC grade (EM Science). GR144000X (squalene epoxidase inhibitor), GW534511X (oxidosqualene cyclase inhibitor) and GW3965 (LXR ligand) were kind gifts of
Glaxo-SmithKline. Human lipoprotein-deficient serum (LPDS) and acetylated low-density lipoprotein (AcLDL) were prepared from normolipidemic human peripheral blood \(^1\). Apolipoprotein AI (apoAI) was prepared as described \(^2\). The anti-ABCA1 antibody was an affinity purified rabbit polyclonal antipeptide antibody raised against a partial peptide sequence of the human ABCA1 protein (Novus Biologicals), and the anti-transferrin receptor antibody was an affinity purified mouse monoclonal antipeptide antibody raised against residues 3-28 of the human transferrin receptor protein (Zymed). Peroxidase-conjugated affinity purified secondary antibodies were obtained from Jackson ImmunoResearch Laboratories.

**Cell Culture**

Cells were grown at 37°C in a 5% CO\(_2\) atmosphere in RPMI 1640 supplemented with penicillin/streptomycin 100U/100 µg/ml, L-glutamine (2 mM), and various sera as described below. As a model for human macrophages, we employed the widely-used, monocytic cell-line THP-1, differentiated with PMA. Human monocyte-derived macrophages (HMDM) were prepared from white cell buffy coat concentrates from healthy donors as described \(^3\). THP-1 cells and the murine macrophage cell-line, J774A.1, were obtained from The American Type Culture Collection (Manassas, VA, USA), and were plated at 1.2x10\(^6\) cells/ml in media containing 10% (v/v) Fetal Calf Serum (FCS). THP-1 cells were plated (and maintained) in the presence of PMA (50 ng/ml; 72 h) to promote differentiation into macrophages. Purified monocytes were differentiated by plating at 1.5 x 10\(^6\) cells/ml in media containing 10% (v/v) heat-inactivated whole human serum and M-CSF (25 ng/ml) for 9 days. Following differentiation, the HMDMs were washed and incubated for 3 days in 10% FCS and treated with compactin and oxysterol ligand in media containing 10% FCS for 24
hours. Treatments of differentiated THP-1 cells and J774A.1 cells were conducted for 24 h in media containing 10% or 5% (v/v) lipoprotein-deficient serum (LPDS) for THP-1 cells or J774A.1 cells, respectively. All treatments were added to cells in absolute ethanol or dimethyl sulfoxide and compared with vehicle-only controls. Cells were washed twice in phosphate-buffered saline (PBS) at room temperature before harvesting. For efflux assays, media was retained.

**Cholesterol Efflux**

THP-1 cells were differentiated in the presence of \([^{3}H]\)-cholesterol (1 µCi/ml; 12 well plates; 72 h), then incubated in the presence or absence of compactin (5 µM) together with \([^{3}H]\)-cholesterol (1 µCi/ml; 24 h). For AcLDL-loading experiments, THP-1 cells were differentiated (72 h), incubated in 10% LPDS in the presence of \([^{3}H]\)-cholesterol (1 µCi/ml) with or without AcLDL (150 µg/ml; 48 h). After cholesterol-loading, cells were washed twice with PBS then incubated in 10% LPDS containing \([^{3}H]\)-cholesterol (1 µCi/ml; 24 h) in the presence or absence of compactin (5 µM; 24 h). HMDMs differentiated for 9 days were labelled with \([^{3}H]\)-cholesterol (1 µCi/ml; 72 h) in 10% FCS. Treatments (compactin and/or 24,25EC) were continued in 10% FCS containing \([^{3}H]\)-cholesterol (1 µCi/ml). Efflux was determined for both THP-1 cells and HMDMs by changing the media to RPMI 1640 containing BSA (0.1% v/v) in the presence or absence of apoAI (25 µg/ml), whilst maintaining treatment(s). After incubation (24 h), media was collected, centrifuged (16 000 g; 5 min) to remove non-adherent cells, and effluxed \([^{3}H]\)-cholesterol determined by scintillation counting (Packard Tri-Carb 2100 TR Liquid Scintillation Analyzer) using UltimaGold scintillation fluid (5 ml). Cells were washed twice with PBS, lysed in NaOH
(1.0 ml; 0.2 M) and an aliquot assayed for \(^{3}\text{H}\)-cholesterol by scintillation counting. Cholesterol efflux was expressed as the radioactivity appearing in the media as a percentage of total radioactivity (cells+media). To determine if all of the radioactivity in the efflux media could be attributed to cholesterol alone, we performed TLC on the lipids extracted from apoAI-stimulated efflux media taken from non-loaded and AcLDL-loaded THP-1 cells. Radioactive bands were visualized using a phosphoimager (10 day exposure). Sensitivity was such that we would have been able to detect minor bands comprising as little as 1% of the radioactivity present in the cholesterol band. However, we observed only one band co-chromatographing with authentic cholesterol, indicating that sterol-metabolites of cholesterol do not significantly contribute to the radioactivity effluxed from these cells.

**Thin Layer Chromatography**

TLC was conducted for assessment of cholesterol and 24,25EC synthesis using a method detailed by Morand and colleagues \(^4\) with minor modifications. Cells were incubated in the presence or absence of inhibitors (various statins or GW534511X) together with \(^{14}\text{C}\)-acetate (2 µCi/well; 6 well plates; 24 h). The medium was aspirated and cells were washed twice with PBS. Cells were lysed in KOH in methanol (1.2 ml; 10% w/v), incubated (4°C, 20 min), followed by addition of water (1.4 ml). Butylated-hydroxy toluene/ethylenediamine tetraacetic acid (EDTA; 20 mM of each final concentration) was added to each sample, followed by saponification (70°C, 1 h). After cooling, neutral lipids were extracted twice with n-hexane: diethyl ether (2 ml; 1:1 v/v). Extracts were dried, re-dissolved in n-hexane: diethyl ether (100 µl; 1:1 v/v) and separated by TLC with a mobile phase of n-hexane: diethyl ether: acetic acid (60:40:1 v/v/v). For visualization, TLC plates were exposed to X-ray film
(Hyperfilm Amersham Biosciences: -80°C, 48-96 h) or analyzed by phosphoimaging (FLA 500, Fujifilm).

**RNA Extraction and mRNA Quantitation**

Cells were harvested for total RNA using Tri Reagent according to the manufacturer’s instructions. Concentrations of total RNA were measured by spectrophotometry (260 nm; Pharmacia ‘DNA Calculator’). For each reverse-transcription reaction, 1 µg total RNA was reverse transcribed to cDNA using oligo dT primers and SuperScript II reverse transcriptase according to the manufacturer’s protocol for first strand cDNA synthesis. Quantitative (‘real-time’) Reverse Transcriptase-PCR (QRT-PCR) was performed using an ABI 7700 Sequence Detector and analyzed using ABI Prism Sequence Detector Soft-ware v1.6.3 (PE Biosystems). iQ SyBr Green Supermix was used as the amplification system. Primer pairs used to amplify ABCA1, ABCG1, apoE, LXRα and porphobilinogen deaminase (PBGD) cDNAs are listed in Table I.

Each of the primer sets span an intron/exon boundary to avoid amplification of possible genomic DNA. PCR products were verified by sequencing. Each amplification mixture contained 12.5 µl of 2x iQ SyBr Green Supermix, 10 pmol each of forward and reverse primers (Table 1) and 1 µl of cDNA (from 20 µl reverse-transcription reaction), made up to 25 µl total volume with nuclease-free water. QRT-PCR thermocycling parameters were 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 56°C for 1 min (with the exception of apoE at 60°C). The change in gene expression levels was determined by normalizing the mRNA levels of ABCA1, ABCG1, apoE and LXRα to the mRNA level of PBGD. Standard curves were conducted for all primer sets to ensure
measurements were within the ‘linear’ range. Melting curve analysis was performed to confirm production of a single product in each reaction.

**Western Blotting**

To measure ABCA1 protein expression, membrane fractions were prepared as described. Briefly, THP-1 cells in 75 cm² flasks were washed in PBS and the cell pellet resuspended in buffer A (1.0 ml: 250 mM sucrose, 10 mM HEPES-KOH at pH 7.4, 1.5 mM MgCl₂, 10 mM KCl, 5 mM EDTA, and 5 mM EGTA), containing protease inhibitors. Cell pellets were lysed by passing through a syringe needle (23 gauge, 30 times) and then centrifuged (1000 g, 7 min, 8°C). The post-nuclear supernatant containing the cell membranes was ultracentrifuged (100 000 g, 30 min, 10°C) and the pellet washed with buffer A without sucrose and resuspended in RIPA buffer (100 µl:50 mM Tris-HCl at pH 7.5, 125 mM NaCl; 1.0% w/v deoxycholate; 0.5% v/v triton X100; 0.1% w/v SDS). Aliquots were retained for determination of protein concentration by the bicinchoninic acid method (BCA assay, Pierce) or for electrophoresis. Samples (50 µg per lane) were analyzed by SDS-PAGE (ABCA1, 7.5%; transferrin receptor, 10%). Protein was transferred onto nitrocellulose membrane (Hybond C, Amersham Biosciences) and incubated with blocking solution (5% (w/v) non-fat milk, 0.1% (v/v) Tween-20 in PBS; 1 h). The anti-ABCA1 (1:1000) and the anti-transferrin receptor (1:2000) antibodies were incubated with the membranes (4°C; overnight). After washing, the membranes were incubated with peroxidase-conjugated affinity purified secondary antibodies (1:5000; 1 h). After further washing, bound antibodies were visualised by chemiluminescense (ECL reagent) and exposed to film (Hyperfilm, Amersham Biosciences).
Results

24(S),25-Epoxycholesterol is a potent activator of LXR-target genes in THP-1 human macrophages

In agreement with previous studies in co-transfected CV-1 cells and a ligand-binding assay, we showed that 24,25EC is a potent activator of LXR-target genes in THP-1 macrophages. Of a panel of natural oxysterol ligands tested, 24,25EC up-regulated the LXR-target genes, ABCA1 and ABCG1, to the greatest extent (Figure IA). 24,25EC up-regulated ABCA1 and ABCG1 to a similar degree as the synthetic ligand, GW3965 (Figure IB). Another LXR-target gene, LXRα, responded to both LXR ligands and decreased with compactin-treatment (Figure IB). Expression of apoE showed a similar trend but the effects of LXR ligands or compactin were not statistically significant due to greater variability in the QRT-PCR data (not shown). Despite some variation in the magnitude of the compactin or 24,25EC effect on ABCA1 and ABCG1 expression between experiments, overall the changes in relative mRNA expression were clear and robust (Table I).

Effect of other statins on expression of LXR-target genes in THP-1 human macrophages

We used compactin in our investigations because it is the archetypal statin. To ensure that the suppression of ABCA1 and ABCG1 expression is not restricted to compactin, we tested a panel of statins, most of which are in clinical use. Pitavastatin (also called itavastatin) is a newly developed and fully synthetic statin that shows high selectivity for the liver and a long half-life in vivo. All statins tested are potent inhibitors of HMG-CoA reductase and apart from pravastatin, all showed complete inhibition of cholesterol and 24,25EC synthesis at 5
µM (Figure II). Being much more hydrophilic than other statins\textsuperscript{12}, pravastatin may not partition as readily into the cells. Expression of ABCA1 and ABCG1 mRNA (Figure II) displayed a marked dose-related decrease with all statins tested. Similarly, we found that these statins generally decreased the mRNA expression of two other LXR-target genes involved in cholesterol efflux, apoE and LXRα (data not shown). Therefore, the down-regulation of LXR-target genes that contribute to cholesterol efflux represents a general effect of statins.

**Effect of compactin on expression of LXR-target genes in and cholesterol efflux from AcLDL-loaded THP-1 human macrophages**

Up to this point, our studies have been performed in non-loaded THP-1 macrophages and HMDMs. Cholesterol-laden macrophages are arguably more relevant to atherogenesis. However, cholesterol-loading might be expected to shut down synthesis of cholesterol and 24,25EC. Therefore, we tested whether or not statins also decrease ABCA1 mRNA levels in THP-1 macrophages that have been loaded with cholesterol via incubation with AcLDL. AcLDL-loading greatly reduced synthesis of cholesterol which was completely abolished with compactin treatment (Figure IIIA). Similarly, synthesis of 24,25EC was greatly reduced with AcLDL-loading and was absent in combination with compactin. Interestingly, partial inhibition of oxidosqualene cyclase with 1 nM GW534511X resulted in detectable 24,25EC synthesis under conditions of AcLDL-loading (Figure IIIA), indicating that even under conditions of extensive cholesterol-loading, THP-1 macrophages have the capacity to synthesize this oxysterol. Consistent with the lack of 24,25EC synthesis observed with AcLDL-loading, compactin had little effect on mRNA levels of ABCA1 (Figure IIIB) and
ABCG1 (data not shown). Furthermore, compactin-treatment had no effect on apoAI-stimulated cholesterol efflux from AcLDL-loaded THP-1 cells (Figure IIIC). Therefore, the inhibitory effect of statins on cholesterol efflux is lost with extensive cholesterol-loading of macrophages.

Further acknowledgements

Special thanks to Ingrid Gelissen, Wendy Jessup and Malcolm Lyons for critically reading an earlier version of our manuscript.
References


Figure Legends

Figure I. Effect of compactin and LXR ligands on mRNA expression of selective LXR-target genes in THP-1 human macrophages.

LXR ligands (10 \( \mu \text{M} \)) or compactin (5 \( \mu \text{M} \)) were incubated with PMA-differentiated THP-1 cells for 24 h. mRNA levels were measured using QRT-PCR, normalised to PBGD mRNA levels. Data are presented relative to vehicle-treated controls and are means ± SEM (n = 3 and n = 6 replicate cultures for Panels A and B, respectively). LXR ligands: 20(S)HC, 20(S)-hydroxycholesterol; 22(R)HC, 22(R)-hydroxycholesterol; 24(S)HC, 24(S)-hydroxycholesterol; 24,25EC, 24(S),25-epoxycholesterol; 27HC, 27-hydroxycholesterol; GW3965, synthetic LXR ligand. (A) For all oxysterol ligands, expression of ABCA1 and ABCG1 were significantly different from control (p<0.05 by unpaired t-test). (B) For all treatments, expression of all three LXR-target genes were highly statistically-different from control (p<0.001 by unpaired t-test).

Figure II. Clinically-relevant statins decrease mRNA expression of LXR-target genes, ABCA1 and ABCG1, in THP-1 human macrophages.

PMA-differentiated THP-1 cells were incubated for 24 h in the presence or absence of a panel of statins (0.5 and 5 \( \mu \text{M} \)). Upper panel: mRNA levels of ABCA1 and ABCG1 were measured using QRT-PCR, normalised to PBGD mRNA levels. Data are presented relative to vehicle-treated controls and are means ± SEM (n = 3 replicate cultures). Lower panel: PMA-differentiated THP-1 cells were incubated with various statins in media containing \[^{14}\text{C} \]-acetate. Neutral lipid extracts were separated using TLC and visualized using autoradiography (48 h exposure).
Figure III. Cholesterol-loading of THP-1 human macrophages decreases 24(S),25-epoxycholesterol synthesis and counteracts the effect of compactin in decreasing ABCA1 mRNA and cholesterol efflux.

PMA-differentiated THP-1 cells were incubated for 48 h in the presence or absence of AcLDL (150 µg/ml). Cells were then incubated for 24 h with or without compactin (CPN; 5 µM) or the oxidosqualene cyclase inhibitor, GW534511X (1 nM). (A) For cholesterol and 24,25EC synthesis, this 24 h incubation with or without treatments was also in media containing [14C]-acetate. Neutral lipid extracts were separated using TLC and visualized using autoradiography (48 h exposure). (B) ABCA1 mRNA levels were measured using QRT-PCR, normalised to PBGD mRNA levels. Values are means ± SEM from 4 separate experiments, apart from compactin-treatment without AcLDL-loading where values are means ± ½ range from two separate experiments. * Compactin significantly decreased ABCA1 mRNA levels in non-loaded cells for both experiments (p<0.05 by unpaired t-test). (C) For efflux experiments, [3H]-cholesterol was included in the loading and treatment incubations. Efflux of [3H]-cholesterol was determined for 24 h in serum-free media containing BSA (0.1% w/v) in the presence or absence of apoAI (25 µg/ml). Efflux is expressed as the percentage of total cell cholesterol released into the medium. Values are means ± ½ range from two separate experiments. *Compactin significantly decreased cholesterol efflux in non-loaded cells for both experiments (p<0.005 by unpaired t-test).
<table>
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<tr>
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<th>Primer Sequence</th>
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TABLE II. Summary of the Effects of Compactin and 24(S),25-Epoxycholesterol on LXR-target genes, ABCA1 and ABCG1, in THP-1 Human Macrophages

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<td>Compactin (5 µM)</td>
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<td>0.093 ± 0.029</td>
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<td>ABCG1</td>
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<td>0.100 ± 0.025</td>
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<td>ABCG1</td>
<td>4</td>
<td>5.1 ± 1.1</td>
<td>3.0 – 7.1</td>
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Compactin or 24(S),25-epoxycholesterol were incubated with PMA-differentiated THP-1 cells for 24 h. mRNA levels were measured using QRT-PCR, normalised to PBGD mRNA levels. Data are values from n separate experiments presented relative to vehicle-treated controls. Treatment with compactin or 24(S),25-epoxycholesterol significantly altered ABCA1 and ABCG1 mRNA levels relative to control (p<0.05 by paired t-test).
Figure II

Relative mRNA Expression

Cholesterol 24,25EC

Control  Compactin  Simvastatin  Lovastatin  Pravastatin  Fluvastatin  (P)Itavastatin  Rosuvastatin  Atorvastatin
**Figure III**

**A.**

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<td>-</td>
<td>-</td>
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<tr>
<td>24,25EC →</td>
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**B.**

Relative ABCA1 mRNA Expression

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<td>AcLDL -</td>
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**C.**

[^3]H]-Cholesterol Efflux (%)

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<td>apoAI +</td>
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