Atorvastatin decreases lipoprotein lipase and endothelial lipase expression in human THP-1 macrophages

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Abstract **Macrophage-derived lipases are associated with atherosclerosis in human and animal studies. Despite numerous non-lipid-lowering effects of statins, their effect on macrophage LPL and endothelial lipase (EL) expression has not been investigated. In the present study, atorvastatin and simvastatin dose-dependently decreased LPL and EL expression as well as Rho, liver X receptor α (LXRα), and nuclear factor κB (NF-κB) activation in THP-1 macrophages. Atorvastatin-reduced LPL and EL expression was only partially recovered by mevalonate cotreatment, indicating that mechanisms independent of reductase inhibition may be present. By contrast, Rho activation by lysophosphatidyl acid further decreased LPL and EL expression in the presence or absence of atorvastatin. Another Rho activator, farnysyl pyrophosphate, decreased EL expression only in the absence of atorvastatin. LXRα activation by T0901317 and 22(*R*)-hydroxycholesterol not only rescued but also significantly increased LPL expression in the presence and absence of atorvastatin, respectively, whereas LXRα inhibition by 22(*S*)-hydroxycholesterol decreased LPL expression. By contrast, EL expression was suppressed by LXRα activation in the presence or absence of atorvastatin. NF-κB inhibition by SN50 was associated with an ~30% reduction of EL expression. Furthermore, atorvastatin treatment significantly attenuated the lipid accumulation in macrophages treated with oxidized LDL. We conclude that atorvastatin reduces LPL and EL expression by reducing the activation of LXRα and NF-κB, respectively.**—Qiu, G. and J. S. Hill. **Atorvastatin decreases lipoprotein lipase and endothelial lipase expression in human THP-1 macrophages. *J. Lipid Res.* 2007.** 48: **2112–2122.**

Supplementary key words 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor • simvastatin • Rho protein • liver X receptor • nuclear factor κB

Macrophages play a pivotal role in the development and progression of atherosclerosis. In response to cellular signals, monocytes adhere to endothelial surfaces and are recruited into the intima of the vascular wall, where they differentiate into macrophages and ultimately transform into foam cells in the presence of excess lipid. Lipid-laden macrophages then become an important source for a variety of cytokines and bioactive products, such as tumor necrosis factor-α, interleukins, chemoattractants, adhesion molecules, and matrix metalloproteases, which collectively can accelerate the progression of the atherosclerotic lesion (1, 2).

By hydrolyzing triglycerides and phospholipids, LPL and endothelial lipase (EL) assume a critical role in lipoprotein metabolism. Through the hydrolysis of triglycerides, LPL simultaneously promotes the clearance of triglyceride-rich lipoproteins and directs lipid into the HDL pool (3). EL has been regarded as an important regulator of HDL metabolism, primarily through its ability to hydrolyze phospholipids (4, 5). The presence of LPL and EL protein has been observed in human atherosclerotic plaques, especially in macrophages (6, 7). An accumulating body of evidence also supports a proatherogenic role of macrophage-derived LPL. The LPL level in macrophages derived from subjects with an increased risk of atherosclerosis, such as heterozygous familial hypercholesterolemia or type 2 diabetes, has been reported to be higher compared with that in healthy controls (8, 9). Also, LPL overexpression in macrophages has been associated with an increase in aortic atherosclerosis in cholesterol-fed transgenic rabbits as well as apolipoprotein E (apoE) or LDL receptor knockout mice (10–12). Although conflicting results were reported with regard to the influence of EL in mouse models of atherosclerosis (13, 14), an increase of EL protein was found in human subjects with the metabolic syndrome, and in a separate cohort, plasma EL levels were associated with the concentration of inflammatory markers (15, 16).

HMG-CoA reductase inhibitors (satins) effectively reduce cholesterol and apoB-containing lipoprotein levels by blocking the endogenous cholesterol synthesis through the competitive inhibition of HMG-CoA reductase, and they are used extensively in the treatment of hypercholes-

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terolemia. Beyond their lipid-lowering effects, statins are also credited with pleiotropic bioactivities, including the improvement of endothelial dysfunction, the alleviation of inflammation and oxidative stress, and the stabilization of the atherosclerotic plaque (17). The non-lipid-lowering effects of statins are often attributed to their ability to influence signaling pathways, among which Rho proteins, liver X receptor (LXR), and nuclear factor κB (NF-κB) are most studied (18–20).

There is a relative paucity of knowledge regarding statin influence on lipase expression in macrophages. In clinical studies, the systemic administration of high-dose simvastatin (80 mg daily) was accompanied by a 49% increase in preheparin plasma lipase activity and a 21% increase in postheparin LPL activity (21). Moreover, atorvastatin and pravastatin increased preheparin LPL mass in type II diabetics with hypercholesterolemia, and the magnitude of LPL increase by simvastatin in rabbit was even greater, a 72% increase in LPL activity (22, 23). Because of its ubiquitous distribution and enormous reserve, adipocytes may be the major source explaining the increase in plasma LPL after statin treatment. Consistent with this hypothesis, increased LPL expression and activity were reported in 3T3-L1 preadipocytes when treated with pitavastatin, simvastatin, and atorvastatin (24, 25). However, the expression of LPL or EL has not been explored in human macrophages after statin treatment.

In the present study, the expression of LPL and EL in THP-1 macrophages was investigated after statin treatment. We then further explored the mechanism by which atorvastatin mediated its effect on lipase expression by assessing the commonly affected intracellular signaling molecules, including Rho protein, LXRα, and NF-κB. The experimental results revealed that atorvastatin decreases macrophage LPL and EL expression by modifying LXRα and NFκB, respectively.

METHODS

Reagents

Purified atorvastatin was provided by Pfizer, and mevalonate (MEV; M4667), farnesylpyrophosphate (FPP; F6892), and lysophosphatidic acid (LPA; L7260) were purchased from Sigma-Aldrich to activate Rho protein. *Clostridium botulinum* exoenzyme C3 (G-130) from BioMol was used to inhibit Rho protein. T01901317 (575310; Calbiochem) and 22(*R*)-hydroxycholesterol (HO-Chol; 89355; Cayman Chemical) were used as synthetic and natural activators of LXRα, respectively. Cell membrane-permeable SN50 was obtained from Calbiochem for NF-κB inhibition.

Cell culture and treatment

THP-1 monocytes (American Type Culture Collection) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% antibiotics-antimycotics (Invitrogen), 1 mM sodium pyruvate, and 1.5% sodium bicarbonate at 37°C, 95% air and 5% CO_2, and used within 20 passages for experiments. Phorbol 12-myristate 13-acetate (PMA; P8139; Sigma-Aldrich) was added at a final concentration of 100 nM for 48 h to differentiate THP-1 monocytes into macrophages. Atorvastatin was then added to cultured macrophages at various concentrations for 24 h. Macrophages were treated with Rho activators (200 μM MEV, 20 μg/ml FPP, 2.5 μM LPA), Rho inhibitor (5 U/ml exoenzyme C3), LXRα activators (1 μM T0901317 and 2.5 μM HO-Chol), or NF-κB inhibitor SN50 (18 μM) in the absence and presence of atorvastatin.

Measurement of HMG-CoA reductase activity

HMG-CoA reductase activity was evaluated by measuring the incorporation of [1-^{14}C]acetic acid into cholesterol. Briefly, macrophages after 2 days of PMA stimulation were treated with various concentrations of atorvastatin in 5% FBS-RPMI 1640 for 19 h, then 1 μCi of [1-^{14}C]acetic acid was loaded for an additional 5 h. After three washes in buffer A (0.15 M NaCl, 50 mM Tris, pH 7.4, and 0.2% BSA) and three washes in buffer A without BSA, in situ lipid extraction was performed by two 30 min incubations with 1.0 ml of hexane-isopropyl alcohol (3:2, v/v), and the cell remnants were disrupted in 0.1 N NaOH for protein quantitation. The pooled extraction solution solvents from each extraction were dried under nitrogen, resuspended in 200 μl of chloroform-methanol (2:1, v/v), and spotted on Fisherbrand Silica Gel plastic plates, on which TLC was carried out in petroleum ether-diethyl ether-acetic acid (84:15:1, v/v/v). The radioactivity of total cholesterol (cholesteryl ester and free cholesterol) was adjusted for protein concentration and plotted, with the radioactivity of control (atorvastatin-untreated) cells set as 100%.

Rho pull-down assay

A Rho activation assay kit (17-294; Upstate) was used to perform a Rho pull-down assay. Briefly, 2×10^7 macrophage cells under different treatment conditions were lysed in Mg^{2+} lysis/wash (MLB) buffer on ice for 15 min after two washes of ice-cold Tris-buffered saline. Cell lysates were then collected after removing cell debris by centrifugation. A GTPase protein binding domain/agarose slurry (30 μl) was added to cell lysates to bind Rho protein and incubated for 45 min at 4°C with gentle agitation. Rho-bound agarose beads were pelleted by brief centrifugation (10 s, 14,000 *g*, 4°C) and washed with 1 ml of MLB three times, resuspended in 40 μl of 2× Laemmli reducing sample buffer plus 2 μl of 1 M dithiothreitol, and then boiled for 5 min before gel electrophoresis and Western blotting.

Nuclear extraction and NF-κB ELISA

THP-1 monocytes (1×10^7 cells) were cultured and treated as mentioned above. After two washes with PBS, 0.5 ml of hypotonic buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1% proteinase inhibitor cocktail (Sigma), 1 mM DTT, and 0.4% (Octylphenoxy)polyethoxyethanol Octylphenyl-polyethylene glycol] was added into the culture dish and incubated for 10 min at room temperature, and then the cells were disrupted by pipetting and scraped into microtubes. Nuclei were pelleted at 4°C at 14,000 rpm for 3 min and disrupted in 50 μl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol, 1% proteinase inhibitor cocktail, and 1 mM DTT) at 4°C for 2 h with vigorous shaking, and supernatants were collected after centrifugation at 4°C and 14,000 rpm for 5 min. The NF-κB p50/p65 transcription factor colorimetric assay (SGT510; Chemicon) was used for the measurement of active NF-κB in nuclear extracts according to the manufacturer's instructions. Tumor necrosis factor-α-treated HeLa whole cell extract and negative oligonucleotide capture probe were used for positive and negative controls, respectively.

Trioleinase assay

Trioleinase activity was used to assess LPL activity, using a triolein emulsion containing radiolabeled triolein as described

previously (26). Heparin-challenged conditioned media were used for the assay, and LPL activity was represented as an apoC-II-dependent, salt-sensitive lipase activity.

RNA extraction and real-time quantitative RT-PCR

The RNeasy Plus mini kit (74134; Qiagen) was applied to extract total RNA according to the manufacturer's instructions. QuantiTect™ Probe RT-PCR master mix and RT mix (204443; Qiagen) with primer sets of 18S RNA (Hs99999901_s1; Applied Biosystem), LPL (Hs00173425; Applied Biosystem), or EL (Hs00195812_m1; Applied Biosystem) were mixed in a 9 μl reaction system according to the manufacturer's instructions, and 1 μl of RNA sample was added for RT-PCR amplification. Real-time quantitative RT-PCR was performed on an ABI Prism® 7900 platform with reaction parameters set as follows: 50°C for 30 min for cDNA synthesis, 95°C for 15 min to inactivate reverse polymerase, and then 40 cycles of 95°C for 15 s and 60°C for 60 s. A standard curve was created from serially diluted samples from concentrated RNA. LPL and EL were normalized by the use of an internal control (18S RNA).

Western blotting

Based on our observations, most of the secreted lipase was bound on the cell surface; thus, cell lysates without heparin challenge were used for lipase detection. Cell lysates (20 μg) were mixed with sample buffer and loaded on a 7.5% SDS-PAGE gel after 5 min of boiling. After 45–60 min of electrophoresis at 200 V, gels were blotted onto polyvinylidene difluoride membranes for 1.5 h at 100 V. After 1 h of blocking with Superblock buffer (Pierce), polyvinylidene difluoride membranes were incubated with primary antibody (1:1,000 dilution) of mouse anti-LPL monoclonal antibody (5D2; a kind gift from Dr. John Brunzell), rabbit EL polyclonal antibody (100030-1; Cayman Chemical), or LXR polyclonal antibody (NB 400-157; Novus Biologicals) overnight at 4°C and then incubated with HRP-conjugated mouse or rabbit secondary antibody (1:1,000 dilution; Pierce) for 1 h after three vigorous washes. Blots were developed by chemiluminescence using Supersignal West Femto maximum sensitivity substrate (Pierce). Chemiluminescent signals were captured and quantitated with the ChemiGenius^2 system.

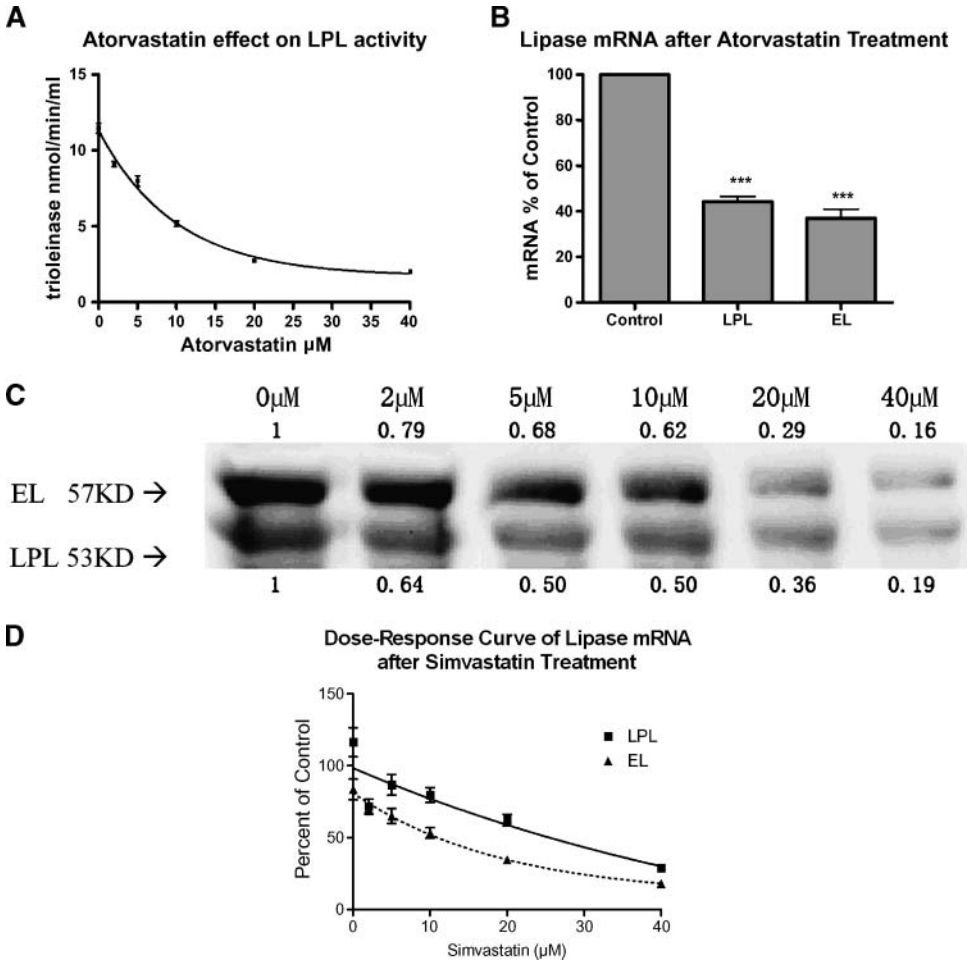

Fig. 1. THP-1 monocytes (5×10^5 cells) were seeded on 12-well plates, differentiated into macrophages by 48 h of stimulation with phorbol 12-myristate 13-acetate (PMA), and then treated with atorvastatin or simvastatin at various concentrations for 24 h. Heparin-challenged culture medium was analyzed for the salt-sensitive, apolipoprotein C-II-dependent fraction of trioleinase activity. Total RNA was isolated for RT-PCR. Twenty micrograms of cell lysates was used for Western blot. A: Atorvastatin dose-dependently decreased trioleinase activity (n = 4). B: LPL and endothelial lipase (EL) mRNA after atorvastatin (20 μM) treatment. C: LPL and EL protein on Western blot (representative of three independent blots). D: LPL and EL mRNA after simvastatin treatment at various concentrations. Data are given as mean ± SEM.

Foam cell formation evaluated by Oil Red O staining

EL-suppressed or -overexpressing macrophages were incubated in RPMI 1640 medium containing 20 μg/ml LDL for 24 h. Cultured macrophages were then washed once with PBS and fixed in 4% paraformaldehyde-PBS for 10 min. After rinsing with 60% isopropanol, macrophages were stained with 0.3% Oil Red O in 60% isopropanol for 10 min and then washed with 60% isopropanol again; thereafter, macrophages were counterstained with hematoxylin for 3 min. After copious washing with water, macrophages were photographed with a microscope at 200× magnification.

RESULTS

Atorvastatin decreases LPL and EL mRNA and protein levels in THP-1 macrophages

THP-1-derived macrophages were treated under variable concentrations of atorvastatin in a range from 2 to 40 μM, which reduced HMG-CoA reductase activity by 38% at 2 μM, 47% at 10 μM, and 66% at 20 μM. LPL activity, as represented by the proportion of salt-sensitive and apoC-II-dependent activity, displayed a dose-dependent decrease with an increase in atorvastatin concentration, with a reduction of 55% at 10 μM, 76% at 20 μM, and 82% at 40 μM (**Fig. 1A**). In-well phospholipase activity was determined to measure total phospholipase activity after atorvastatin treatment, which was decreased by 56% at 20 μM atorvastatin (data not shown). LPL mRNA was decreased by 56% and EL mRNA was decreased by 63% at 20 μM atorvastatin (Fig. 1B). The LPL Western blot was consistent with the trioleinase activity results, exhibiting a decreased LPL mass in a dose-dependent manner, with decreases of 50% at 10 μM, 64% at 20 μM, and 82% at 40 μM (Fig. 1C). Similarly, Western blot of EL protein showed a dose-dependent decrease of EL mass, with a reduction of 38% at 10 μM, 71% at 20 μM, and 84% at 40 μM (Fig. 1C). Because the activity and mass of LPL and EL were not reduced substantially beyond 20 μM and no cytoxicity was observed at this dose (data not shown), an atorvastatin concentration of 20 μM was used in subsequent experiments. Furthermore, we applied simvastatin to THP-1 macrophages and observed similar dose-dependent reductions of LPL and EL mRNA levels (Fig. 1D).

Atorvastatin decreases Rho, LXRα, and NF-κB activity

In an attempt to determine the underlying mechanism by which atorvastatin suppresses LPL and EL expression in macrophages, we explored the intracellular signaling pathway of the small G protein Rho, LXR, and NF-κB. Activated Rho protein and LXRα levels were dose-dependently decreased by atorvastatin, with a 20 μM concentration associated with 76% and 56% reductions, respectively (**Fig. 2A**, B). Measurement of NF-κB indicated that 20 μM atorvastatin decreased NF-κB activity by >80% (Fig. 2C).

Rho inactivation by atorvastatin does not mediate a decrease in EL or LPL expression in THP-1 macrophages

To assess whether decreased Rho activation was responsible for the observed reduction in LPL and EL expression

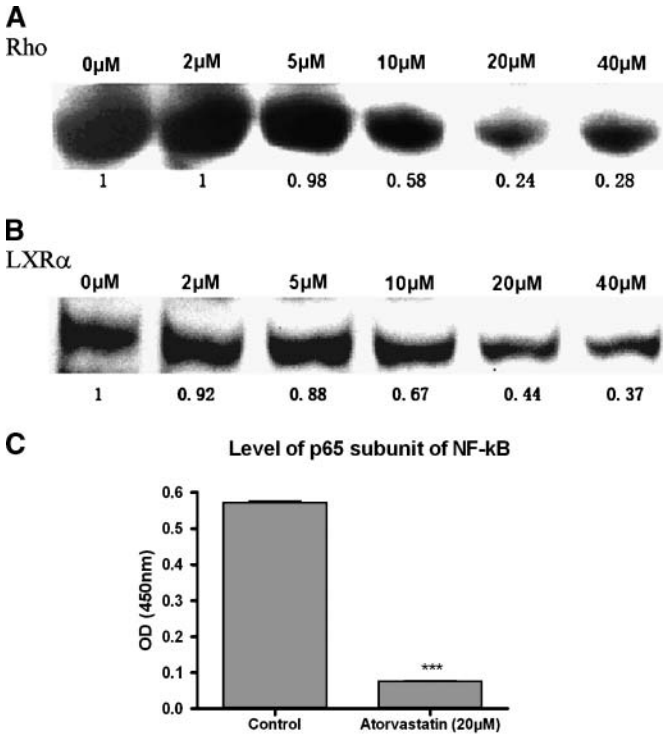

Fig. 2. A: Rho was analyzed by pull-down assay followed by Western blot. B: Liver X receptor (LXR) was analyzed by Western blot. C: Nuclear extracts from 1×10^7 macrophages were measured for the nuclear factor κB (NF-κB) p65 subunit by ELISA (n = 3). Data are given as mean ± SEM. * $P < 0.001$. OD, optical density.

after atorvastatin treatment, we used Rho activators (MEV, FPP, and LPA) in an attempt to recover LPL and EL levels. In the presence of atorvastatin, Rho activation by MEV partially salvaged LPL mRNA and activity, although LPL protein was not affected significantly as assessed by Western blotting, whereas FPP did not increase LPL mRNA, activity, or protein mass, and LPA even further decreased LPL mRNA, activity, and mass (**Fig. 3A–**C). To further assess the relationship of Rho with LPL expression, we treated macrophages with Rho activators and a Rho inhibitor (exoenzyme C3) in the absence of atorvastatin. Interestingly, Rho activation by MEV and FPP stimulation did not noticeably change LPL mRNA; however, LPA stimulation was associated with a 35% decrease of LPL mRNA (Fig. 3D). In parallel with mRNA expression, LPL mass on the Western blot was not altered appreciably, although LPA was inclined to decrease LPL mass (Fig. 3F). In addition, exoenzyme C3 did not change LPL mRNA or protein levels (Fig. 3D, F). Although statistically significant, the reduction of trioleinase activity by either Rho stimulation or inhibition after MEV, FPP, LPA, and exoenzyme C3 was modest (12–19%), with the maximal reduction associated with LPA treatment (Fig. 3E). The discrepancy between trioleinase activity and LPL mRNA and protein levels may be attributed to the lack of complete specificity of the trioleinase activity assay for LPL. Based on these observations, Rho activation, particularly

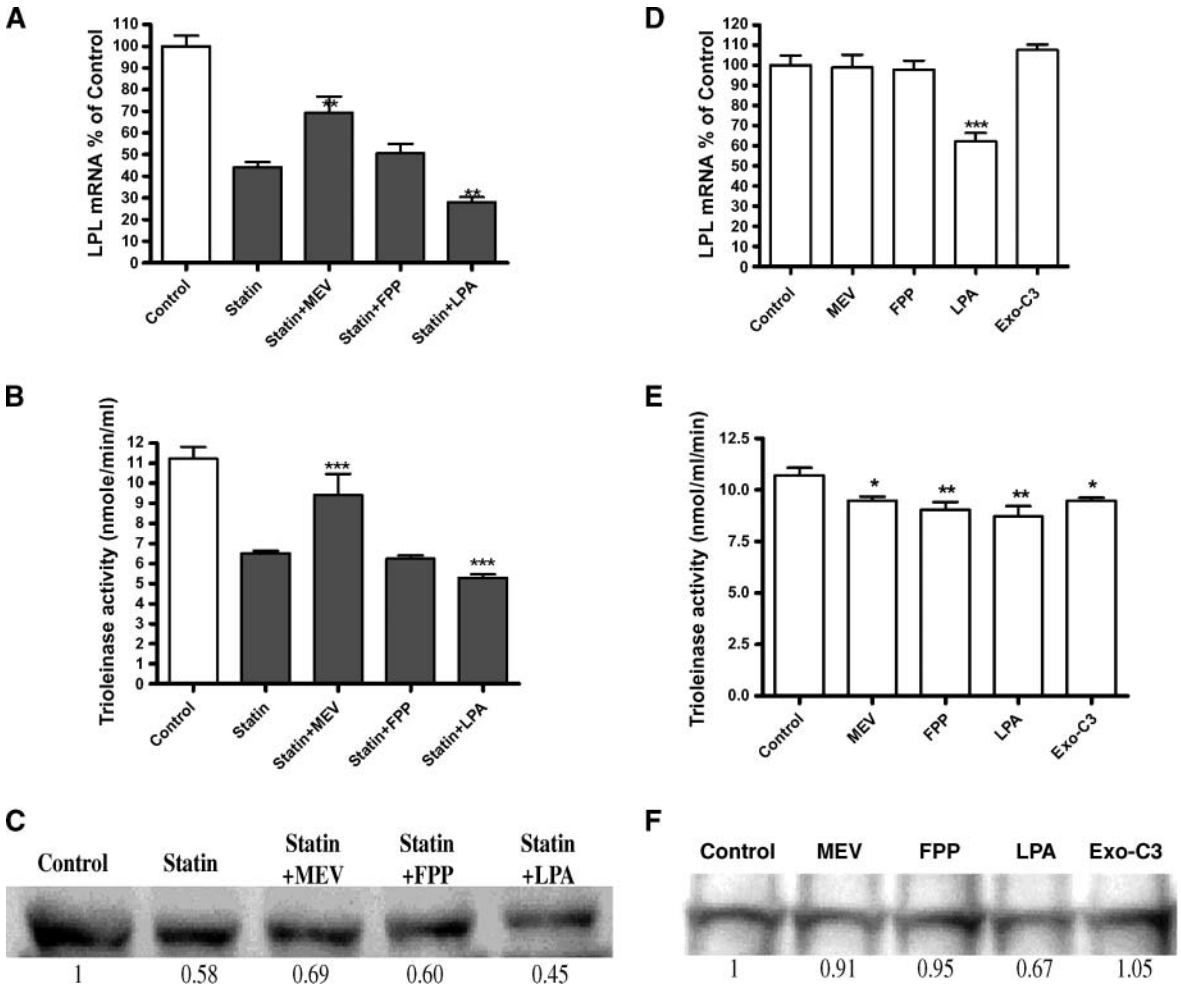

Fig. 3. LPL expression in macrophages after Rho activation (MEV, mevalonate; FPP, farnesylpyrophosphate; LPA, lysophosphatidic acid) or inhibition (Exo-C3, C3 exoenzyme) in the presence (closed bars) or absence (open bars) of 20 μM atorvastatin. A–C: LPL mRNA (n = 8) (A), trioleinase activity (n ⩾ 4) (B), and Western blot (representative of three independent experiments) (C) after Rho activator treatment in the presence of 20 μM atorvastatin. D–F: LPL mRNA (n = 8 for Rho activators and n = 4 for exoenzyme C3) (D), trioleinase activity (n ⩾ 4) (E), and Western blot (representative of three independent experiments) (F) after the treatment of Rho activators and inhibitor in the absence of atorvastatin. Statistical comparisons between statin and statin plus additional treatments are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for A and B; statistical comparisons between the control (no statin treatment) and treatments are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for C and D. Data are given as mean ± SEM.

stimulation by LPA, tended to decrease LPL expression, and a causative relationship between Rho inactivation and LPL suppression after atorvastatin treatment could not be established.

As far as EL expression was concerned, MEV treatment partially rescued EL mRNA expression in the presence of atorvastatin (58% of control compared with 37% of control in atorvastatin; **Fig. 4A**), but no significant differences were observed by Western blot analysis (Fig. 4B). FPP stimulation did not increase EL mRNA and protein levels, and LPA even tended to further depress EL mRNA and protein levels (Fig. 4A, B). In the absence of atorvastatin, Rho activation by MEV, FPP, and LPA consistently decreased EL mRNA and protein levels, whereas Rho inhibition by exoenzyme C3 did not change either EL mRNA or protein level (Fig. 4C, D). These results suggest that atorvastatin-induced Rho inhibition was not responsible for the observed reduction in EL expression.

LXRα inhibition by atorvastatin mediates LPL, but not EL, suppression

To investigate the potential role of LXRα in LPL and EL expression, we applied the LXRα agonists T0901317 and 22(*R*)-hydroxycholesterol to THP-1 macrophages in the presence and absence of atorvastatin. Strikingly, the inhibition of LPL mRNA, activity, and protein by atorvastatin was completely rescued by the synthetic LXRα agonist T0901317 (130, 119, and 128% of control) and was partially recovered by the natural agonist 22(*R*)-hydroxycholesterol (84, 99, and 118% of control) (**Fig. 5A**–C). Moreover, LXRα activation by both natural and synthetic substrates [T0901317 and 22(*R*)-hydroxycholesterol] significantly increased LPL mRNA (47% and 19%), activity (44% and 34%), and mass (29% and 35%) in the absence of atorvastatin (Fig. 5A, B, D). Furthermore, LXRα inhibition by 22(*S*) hydroxycholesterol decreased LPL mRNA in both the presence and absence of atorvastatin

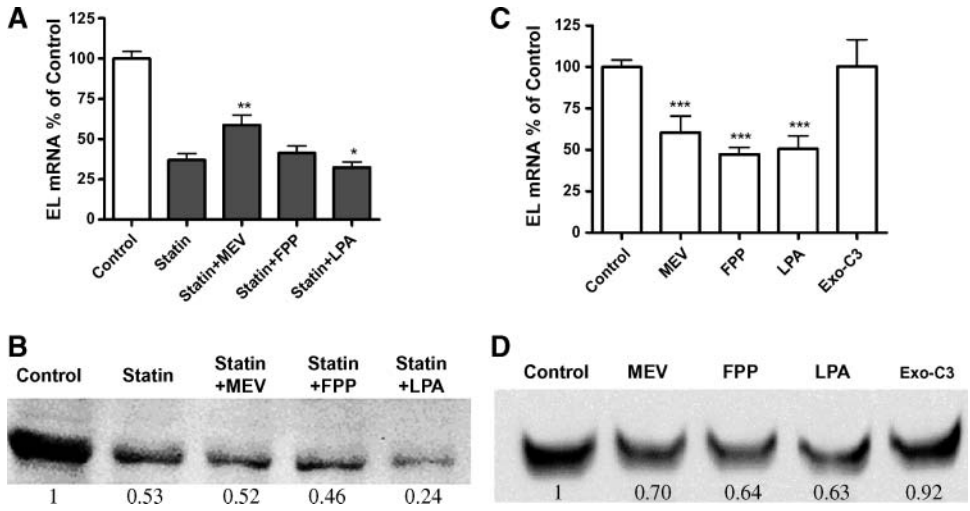

Fig. 4. EL expression in macrophages after Rho activation (MEV, FPP, LPA) or inhibition (Exo-C3) in the presence (closed bars) or absence (open bars) of 20 μM atorvastatin. A, C: EL mRNA by real-time quantitative RT-PCR after Rho activation or inhibition in the presence or absence of 20 μM atorvastatin, respectively (n = 6). B, D: Western blot of EL protein after Rho activation or inhibition in the presence or absence of 20 μM atorvastatin, respectively (representative of three independent Western blots). Statistical comparisons between statin and statin plus additional treatments are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for A; statistical comparisons between the control (no statin treatment) and treatments are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ for C. Data are given as mean ± SEM.

(Fig. 5E). These observations strongly support the idea that atorvastatin-induced LPL suppression is mediated by LXRα suppression.

By contrast, LXRα activation was not able to rescue the suppressive effects of atorvastatin on EL expression. Treatment with the natural substrate 22(*R*)-hydroxycholesterol amplified the suppressive effect of atorvastatin, further depressing EL mRNA and protein, with only 2% and 15% of control levels remaining, respectively (Fig. 5F, G), whereas the synthetic substrate T0901317 did not have any appreciable effects in the presence of atorvastatin. In the absence of atorvastatin, LXRα activation by T0901317 and 22(*R*)-hydroxycholesterol suppressed EL mRNA expression by 55% and 92%, respectively, and EL protein by 60% and 49%, respectively (Fig. 5F, H).

EL suppression by atorvastatin is mediated by NF-κB inhibition

To further address the mechanism by which atorvastatin reduces EL expression, we then explored the role of NF-κB. When macrophages were treated with the NF-κB inhibitor SN50 (SN50 treatment at 18 μM resulted in a 54% reduction of NF-κB activity), EL mRNA expression was reduced by 29% with a corresponding 28% decrease in protein levels, as observed with Western blots (**Fig. 6A**, B). In view of the inhibitory effect of atorvastatin on NF-κB, it is plausible to postulate that atorvastatin may mediate EL suppression through decreased NF-κB activity. In addition, the effects of Rho and LXRα activation on NF-κB were also assessed. NF-κB activity was inhibited to varying degrees from 37% to 55% upon Rho activation by MEV, FPP, and LPA, whereas Rho inhibition by exoenzyme C3 did not change the NF-κB level (Fig. 6C). Furthermore, LXRα activation by T0901317 and 22(*R*)-hydroxycholesterol exerted the same effect as Rho activation on NF-κB activity, with 42% and 77% decreases, respectively (Fig. 6C). These results may explain in part the previous observations of Rho and LXRα activators decreasing EL expression.

Atorvastatin attenuates the transformation of macrophages into foam cells

To explore the effects of atorvastatin on foam cell formation, macrophages were treated with 20 μg/ml oxidized LDL for 24 h in the presence and absence of 20 μM atorvastatin. After Oil Red O staining, it was apparent that untreated macrophages internalized a greater amount of lipid (**Fig. 7A**) compared with atorvastatin-treated macrophages, in which only fine lipid particles/droplets were dispersed in the cells (Fig. 7B). These observations demonstrated that atorvastatin treatment can inhibit the development of macrophage-derived foam cells.

DISCUSSION

The non-lipid-lowering effects of statins have been studied in numerous cell models for a wide range of biologic functions. However, the effects of statins on lipase expression in macrophages have not been addressed. We demonstrate in the present study that LPL and EL expression in human macrophages were markedly reduced by atorvastatin; this effect may apply to other statins, as the dose-dependent reductions of both LPL and EL were also observed after simvastatin treatment.

As statins are competitive inhibitors of HMG-CoA reductase, the metabolism of a variety of downstream cholesterol intermediates is affected. In particular, diphosphates

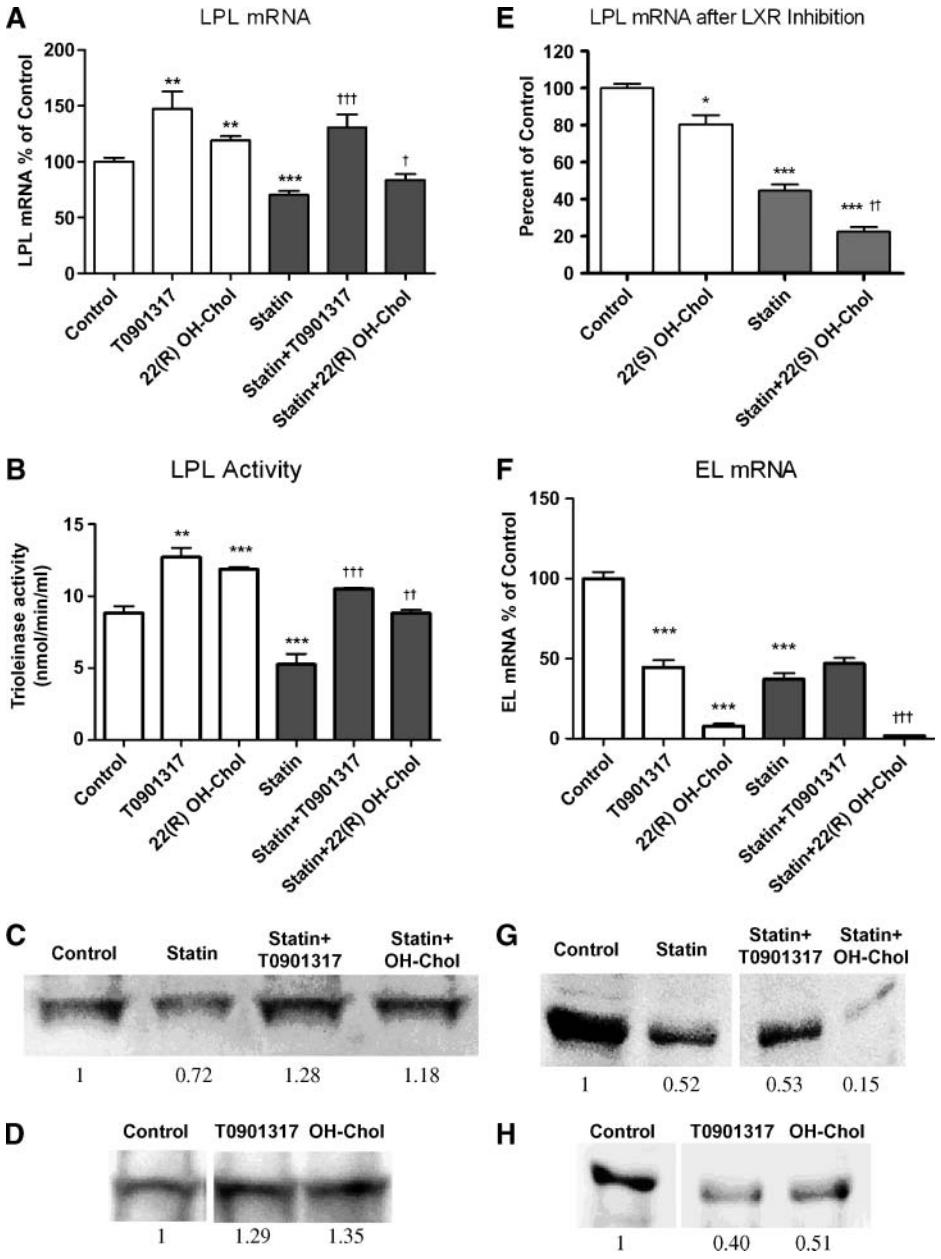

Fig. 5. LPL and EL expression in macrophages after LXRα activation [T0901317 and 22(*R*)-hydroxycholesterol {22(R) OH-Chol}] or inhibition [22(*S*)-hydroxycholesterol {22(S) OH-Chol}] in the presence (closed bars) or absence (open bars) of 20 μM atorvastatin. A, B: LPL mRNA and trioleinase activity after LXRα activation in the absence and presence of 20 μM atorvastatin (n = 4). C, D: LPL Western blot after LXRα activation in the presence and absence of 20 μM atorvastatin, respectively (representative of three independent blots). E: LPL mRNA after LXRα inhibition in the absence and presence of atorvastatin (n = 3). F: EL mRNA after LXRα activation in the absence and presence of 20 μM atorvastatin (n = 6). G, H: EL Western blot after LXRα activation in the presence and absence of 20 μM atorvastatin, respectively (representative of three independent blots). Statistical comparisons between the control and treatments are indicated as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; statistical comparisons between the statin and statin plus treatments are indicated as follows: † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$). Data are given as mean ± SEM.

such as FPP and geranyl pyrophosphate have been shown to mediate the prenylation and subsequent activation of Rho proteins (27). Similarly, because cholesterol is a precursor to oxysterol, statins also decrease this known endogenous activator of LXRα (20). Because LXRα can be autoregulated (28), the inactivation of LXRα consequent to oxysterol reduction after statin treatment leads to decreased protein expression, as demonstrated in this study. To determine whether either of these transcription factors was responsible for the observed suppression of LPL and EL in THP-1 macrophages, we applied a series of known activators and inhibitors of these described pathways.

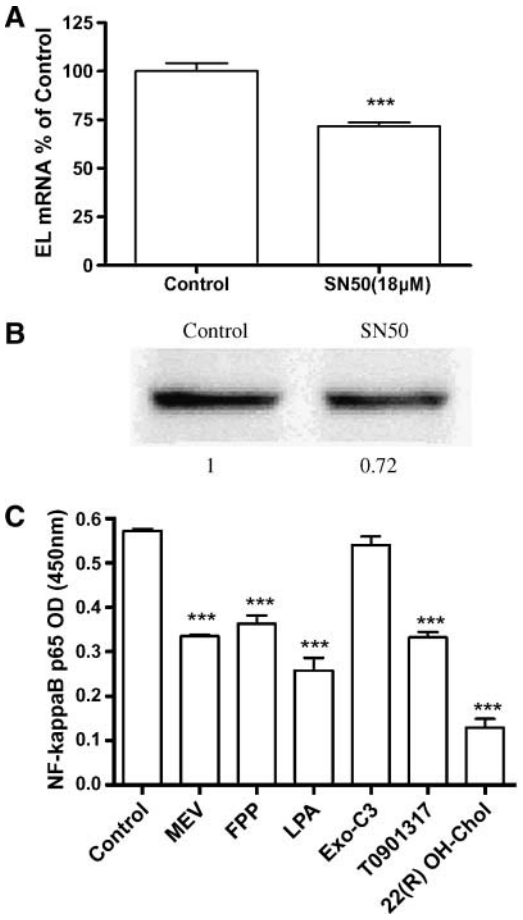

Fig. 6. EL expression after NF-κB inhibition by SN50 and NF-κB levels after Rho and LXRα activation. EL was evaluated by real-time quantitative RT-PCR for mRNA (A; n = 4) and Western blot for protein (B; n = 2). NF-κB levels were measured after Rho and LXRα activation (C; n = 3). *** $P < 0.001$. Data are given as mean ± SEM. OD, optical density.

We observed that activation of Rho proteins did not rescue the suppressive effects on lipase suppression induced by atorvastatin treatment and, in some cases, caused a further depression in lipase expression. However, MEV treatment was able to only partially salvage LPL and EL mRNA levels in the presence of atorvastatin, suggesting that HMG-CoA reductase-independent effects may exist. Previous reports have indicated that statins can mediate effects that appear to be MEV-insensitive (29, 30). In particular, it has been reported that these reductase-independent effects may be related by the ability of statins to bind to a β_2-integrin to mediate an inflammatory response in vivo (30). Furthermore, Rho inhibition by exoenzyme C3 did not disturb LPL or EL expression, suggesting that Rho is not the mediator for atorvastatin action on lipase expression in macrophages. It is possible that the suppressive effects of Rho activators on lipase expression may be mediated through the transcription factor Oct-1, which interacts with the common *cis*-acting octamer element 5′-ATTTGCAT-3′ on target genes (31). The binding site for Oct-1 has been identified and characterized at position −46 bp in the proximal human LPL promoter (32). Once phosphorylated on the homeodomain, its DNA binding activity could be inhibited in vivo and in vitro (33, 34). Patients with a T→C substitution mutation in this region have low plasma LPL activity caused by an inability of Oct-1 binding (35). Furthermore, adipocytes, when treated with tumor necrosis factor-α, have a reduced expression of LPL related to the dissociation of an Oct-1-like molecule from the Oct-1 consensus sequence on the LPL gene (36). Rho activation may lead to the phosphorylation of Oct-1 and retard its translocation from the cytosol to the nucleus, subsequently decreasing LPL transcription. The octamer sequence for Oct-1 binding is also found within the EL gene (37), but its functional relationship has not been investigated.

LXRα activation using synthetic and natural agonists increased LPL mRNA and activity in the absence of atorvastatin and rescued LPL suppression in the presence of atorvastatin, whereas LXRα inhibition decreased LPL mRNA in both the presence and absence of atorvastatin. Consistent with this observation, Zhang et al. (38) reported that a functional DR4 LXR response element is present in the intronic region between exons 1 and 2 in the LPL gene and that gene expression could be modified by oxysterol. Furthermore, mice fed a diet containing high cholesterol or an LXRα-selective agonist exhibited a significant increase in LPL expression in liver and macrophages, whereas a defective response of LPL expression was observed in LXR-deficient mice (38). Thus, it appears that atorvastatin may reduce the expression of LPL by decreasing LXRα activation.

By contrast, LXRα activation was associated with a decrease in EL expression in the presence or absence of atorvastatin. It has been shown previously that the synthetic LXRα agonist T0901317 suppressed EL expression in human endothelial cells, whereas 22(*R*)-hydroxycholesterol had no effect (39). Moreover, another natural activator of LXRα, 24(*S*)-hydroxycholesterol, resulted in EL downregulation at the mRNA and protein levels in brain capillary endothelial cells (40). An LXRα response element has not been identified within the EL gene, so the mechanism of the influence of LXRα on gene expression remains unclear. In addition, the peroxisome proliferator-activated receptor γ (PPARγ) agonist pioglitazone also reduces EL expression. Because of cross-talk between LXRα and PPARs (41), the effect of EL inhibition by LXRα agonists could be mediated by subsequent PPARγ activation.

Because LXRα did not appear to be responsible for EL suppression after atorvastatin treatment, we investigated NF-κB, another transcription factor associated with EL expression. It has been reported that binding sites for NF-κB were present in the 5′ region of −1,250 bp of the EL promoter, and cotransfection experiments using a luciferase reporter gene with the EL promoter in NIH-3T3 cells demonstrated the direct regulation by NF-κB (42). In the present study, we demonstrated in THP-1 macrophages that atorvastatin dramatically inactivates NF-κB. The inhibitory effect of statins on NF-κB has been observed with lovastatin and simvastatin in human vascular endo-

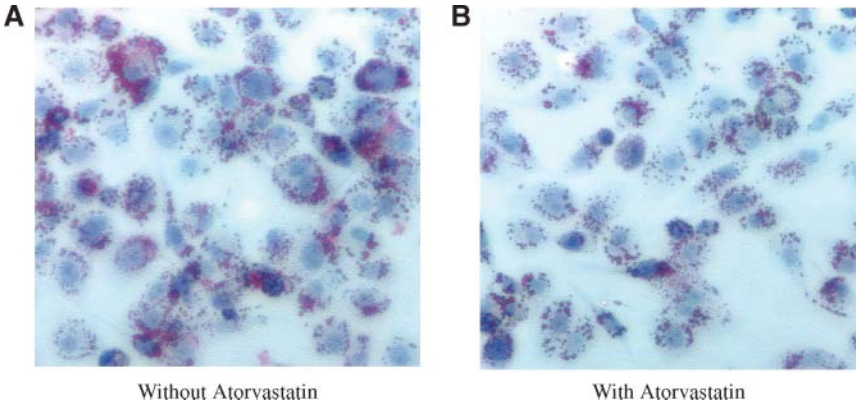

Fig. 7. Oil Red O staining of oxidized LDL-treated macrophages. Macrophages treated with PMA for 48 h were incubated with 20 μg/ml oxidized LDL for an additional 24 h and then stained with Oil Red O. Oil Red O staining shows clustered and aggregated lipids in atorvastatin-untreated macrophages (A), in comparison with the diffuse distribution of fine lipid particles in atorvastatin-treated macrophages (B).

thelial and smooth muscle cells (43–45). Furthermore, it has been proposed that the ability of statins to scavenge free oxygen radicals as well as to stimulate the production of endothelial nitric oxide production could inhibit NF-κB by the induction and stabilization of I-κB-α (45). In the present study, inhibition of NF-κB by SN50 resulted in decreased EL mRNA and protein levels. Consistent with this observation, NF-κB activation by interleukin-1β and tumor necrosis factor-α was shown to induce the expression of EL mRNA and protein in human umbilical vein endothelial cells, whereas the NF-κB inhibitor SN50 attenuated this response (46). Further investigation re-

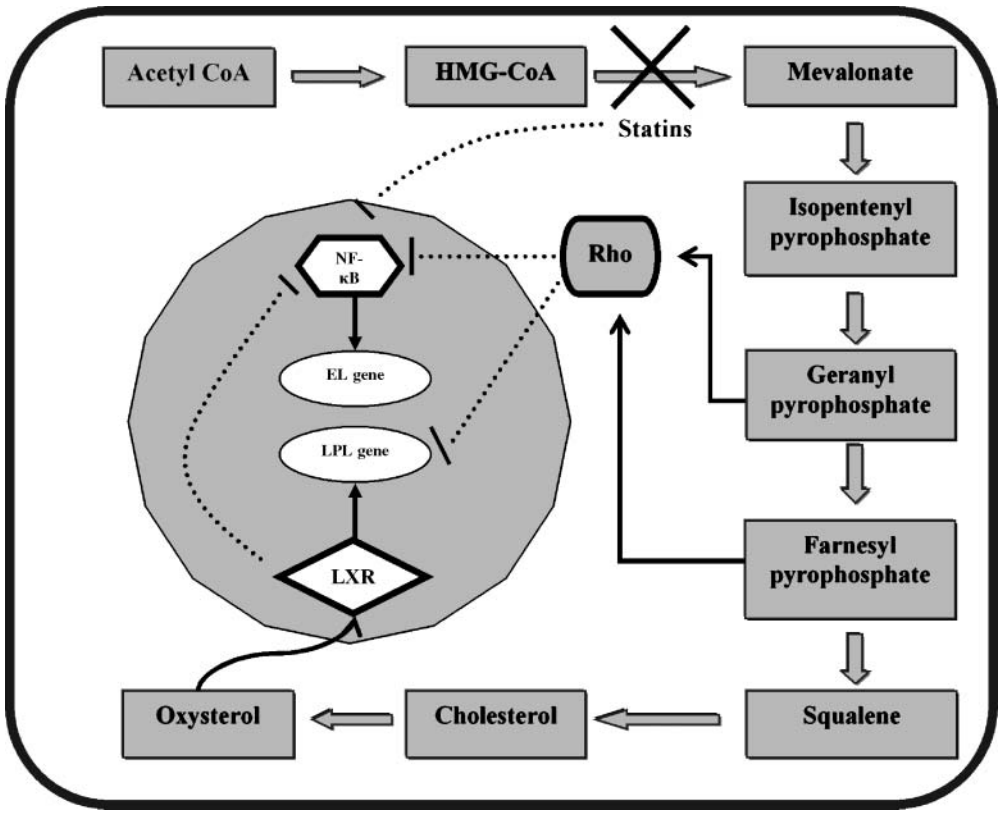

Fig. 8. Schematic illustration of the signaling pathways involved in LPL and EL expression in THP-1 macrophages. The LPL gene contains an LXR response element within the promoter region such that it is positively regulated by LXRα. Statins inhibit cholesterol synthesis and decrease oxysterol production and thus decrease LXRα activation and subsequent LPL expression. A consensus sequence for NF-κB binding is located upstream of the EL gene and positively regulates EL expression; thus, NF-κB suppression by statins leads to EL downregulation. Rho and LXRα activators may also inhibit EL expression by inhibiting NF-κB.

vealed that the treatment of both Rho and LXRα activators led to the marked reduction of NF-κB levels, perhaps explaining in part why Rho and LXRα activation caused EL suppression. By contrast, NF-κB appears to be less critical in the regulation of LPL, as NF-κB inhibition by SN50 did not cause any change of LPL expression (data not shown) and a NF-κB binding site has not been identified in the promoter area of the LPL gene.

As demonstrated in Fig. 7, atorvastatin treatment attenuated macrophage-derived foam cell formation. Previously, LPL was demonstrated to be able to promote macrophage-derived foam cell formation (12, 47). In CHO cells, EL also plays a facilitating role in LDL internalization independent of its catalytic activity (48). Moreover, findings from our laboratory have demonstrated a relationship between EL expression and LDL binding and uptake in THP-1 macrophages (our unpublished data). Consequently, the reduced cholesterol accumulation observed in THP-1 macrophages may be attributable in part to the reduced expression of EL and LPL. Of course, additional effects of statins that have been reported, such as decreased expression of lipoprotein receptors (49, 50), could also influence foam cell formation.

The data in the present study support different intracellular signaling pathways responsible for the regulation of lipase expression in THP-1 macrophages (**Fig. 8**). It appears that the expression of LPL and EL is influenced by LXRα and NF-κB, respectively, whereas NF-κB is negatively regulated by Rho and LXRα. Atorvastatin treatment decreases LPL and EL expression in THP-1 macrophages by reducing the formation of oxysterol (a LXRα ligand) and inhibiting NF-κB, respectively, which likely contributes to the ability of atorvastatin treatment to mitigate lipid accumulation in macrophages. As such, the ability of atorvastatin to decrease LPL and EL expression in THP-1 macrophages may be an additional mechanism that offers protection from the development of atherosclerosis.

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