Suppression of endothelial or lipoprotein lipase in THP-1 macrophages attenuates proinflammatory cytokine secretion[S]

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Abstract LPL and endothelial lipase (EL) are associated with macrophages in human atherosclerotic lesions, and overexpression of LPL in mouse macrophages is associated with a greater extent of atherosclerosis. To investigate potential mechanisms by which macrophage-derived lipase expression may mediate proatherogenic effects, we used lentivirus-mediated RNA interference to suppress the expression of either LPL or EL within THP-1 macrophages. After suppression of either LPL or EL, significant decreases in the concentration of interleukin-1β, interleukin-6, monocyte chemoattractant protein-1, and tumor necrosis factor-α were observed. Incubation of THP-1 macrophages with either mildly or extensively oxidized LDL consistently decreased cytokine expression, which was additive to that contributed by lipase suppression. Decreased lipase expression was also associated with an altered lipid composition, with reduced percentages of cholesterol (unesterified and esterified), triglycerides, and lysophosphatidylcholine. Microarray data indicated a decreased expression of proinflammatory genes, growth factors, and antiapoptotic genes. By contrast, there was an increased expression of lipoprotein receptors (scavenger receptor 1, low density lipoprotein receptor, scavenger receptor class B type I, and CD36). Thus, we conclude that the suppression of either LPL or EL decreases proinflammatory cytokine expression and influences the lipid composition of THP-1 macrophages. These results provide further insight into the specific metabolic and potential pathological roles of LPL and EL in human macrophages.—Qiu, G., A. C. Ho, W. Yu, and J. S. Hill. **Suppression of endothelial or lipoprotein lipase in THP-1 macrophages attenuates proinflammatory cytokine secretion. *J. Lipid Res.* 2007.** 48: **385–394.**

Supplementary key words monocyte • inflammation • microarray • RNA interference • lentivirus

From early fatty streak lesions to advanced plaques, macrophages are integral to the development and progression of atherosclerosis. It is well recognized that a variety of proinflammatory cytokines are expressed in activated macrophages, including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), IL-8, IL-1β, and monocyte chemoattractant protein-1 (MCP-1). These cytokines have been shown to promote lesion progression by aggravating endothelial dysfunction, recruiting additional inflammatory cells, or stimulating smooth muscle proliferation (1).

LPL primarily hydrolyzes triglycerides contained within chylomicrons and very low density lipoproteins, whereas endothelial lipase (EL) preferentially hydrolyzes phospholipids within HDL (2). The expression of LPL and EL has been associated with macrophages within human atherosclerotic lesions (3–5). Although the specific roles of these lipases in atherosclerosis likely depend on their tissue localization, accumulating evidence supports a proatherogenic role of macrophage-derived lipases. LPL expression was observed to be increased in human macrophages isolated from patients with either heterozygous familial hypercholesterolemia or type II diabetes (6, 7). These observations are substantiated by animal studies in which macrophage-specific expression of human LPL promotes the extent of aortic atherosclerosis in transgenic apolipoprotein E knockout mice and rabbits (8–10). Also, despite the presence of a proatherogenic lipid profile, EL deficiency was associated with an ~70% decrease in athero-

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Abbreviations: EL, endothelial lipase; IL, interleukin; lyso-PC, lysophosphatidylcholine; MCP-1, monocyte chemoattractant protein-1; MOI, multiplicity of infection; NF-κB, nuclear factor-κB; OxLDL, oxidized low density lipoprotein; PMA, phorbol 12-myristate 13-acetate; PPARγ, peroxisome proliferator-activated receptor γ; TBARS, thiobarbituric acid-reacting substances; TNF-α, tumor necrosis factor-α.

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sclerotic disease area in apolipoprotein E knockout mice compared with controls (11). More recently, in a cohort of healthy subjects with a family history of premature coronary heart disease, increased human plasma EL concentrations were significantly associated with the metabolic syndrome and subclinical coronary heart disease (12).

The proatherogenic nature of LPL and EL in macrophages has been largely ascribed to their noncatalytic or bridging function, which mediates the interaction between lipoproteins and cell surfaces. An exogenous source of LPL has been shown to facilitate the binding and uptake of LDL by THP-1 monocytes and macrophages, possibly by involving various proteoglycans and lipid rafts (13–15). Similarly, endogenously produced EL in transfected Chinese hamster ovary cells enhances the binding and cellular processing of plasma lipoproteins via a heparan sulfate proteoglycan-mediated route (16). Moreover, EL and LPL can play a role as adhesion molecules through the interaction with heparan sulfate proteoglycans to facilitate the binding of monocytes onto the surface of endothelial cells (17–19).

There is little information on the effect of endogenous lipase expression on the secretion of proinflammatory cytokines in human macrophages. LPL expression in mouse macrophages and adipocytes is decreased by interferon-γ, TNF-α, lipopolysaccharide, IL-11, IL-1, and IL-6 (20–22). Regarding EL, IL-1β and TNF-α have been shown to stimulate its expression in endothelial cells (23). However, very few studies have addressed how lipase expression may affect cytokine expression. Exogenous LPL has been shown to induce TNF-α by increasing TNF-α mRNA transcription and stability (24, 25). To our knowledge, there are no reports on the influence of EL expression on cytokine expression.

In this study, we have applied lentivirus-mediated RNA interference to suppress the expression of either LPL or EL in THP-1-derived macrophages. We observed that the expression of LPL and EL in THP-1 macrophages is positively correlated with proinflammatory cytokine expression in THP-1 macrophages.

METHODS

Cell culture

THP-1 monocytes were purchased from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% FBS, 1 mM sodium pyruvate, 1.5% sodium bicarbonate, and 1% antibiotic-antimycotic. HEK 293 cells were cultured in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic. All cells were incubated in a humidified incubator at 95% air and 5% CO_2. Experiments performed with THP-1 monocytes were used within 20 passages.

Lentiviral production

Candidate shRNA sequences corresponding to either EL or LPL sequences were selected using web-based siRNA design tools (**Table 1**). A scrambled shRNA sequence with no known homology to human genes served as a control. Two shRNA oligonucleotides were annealed in buffer (1 mM $MgCl_2$, 20 mM Tris-HCl, pH 8.0) and then ligated into *Bam*HI-*Bse*RI sites of the pSHAG vector (a kind gift from Dr. Gregory Hannon, Cold Spring Harbor Laboratory). Selected positive constructs (designated pSHAG-shRNA) were sequenced after initial screening by *Hind*III digestion. The lentiviral vector pHR-CMV-eGFP engineered with the Gateway® system (a kind gift from Dr. Alice Mui, University of British Columbia) was the final destination vector, accepting the U6 promoter-shRNA fragment from the pSHAG-shRNA vector via the Gateway® reaction. Selected positive constructs (designated pHR-shRNA) were confirmed by DNA sequencing. Lentivirus was produced by cotransfecting pHR-shRNA with packaging vectors (pMD.G and pCMV.ΔR8.2; kind gifts from Dr. Alice Mui) into HEK 293 cells at a ratio of 4:3:1 pHR-shRNA/pCMV/pMD.G. Viral supernatants were collected for 4 consecutive days and concentrated by ~50-fold using a Centricon Plus-20 centrifugal filter device (cutoff molecular mass of 100,000 kDa; Millipore). Viral titration was conducted on HEK 293 cells with serial dilution of the viral stock. The lentiviral titer was calculated as transduction units/ml = (average cell number at the time of transduction × percent of green fluorescent protein-positive cells)/100 × dilution factor.

LDL oxidation and cell treatment

Native LDL (Biomedical Technologies, Inc.) was incubated with 5 mM $CuSO_4$ for 2 h or overnight at 37°C for mildly and extensively oxidized low density lipoprotein (OxLDL), respec-

TABLE 1. Sequences of shRNA oligonucleotides for LPL, EL, and control constructs

Name	Target Site	Sequence
LPL-shRNA forward	470	ACATTGGAGTCTGGTTCTCTCTTGTACAGAAGCTTGTGTATAAGAGGGAGCCAGATTCCAATGTCATTTTTTT
LPL-shRNA reverse	470	GATCAAAAAAATGACATTGGAATCTGGCTCCCTCTTATACACAAGCTTCTGTACAAGAGAGAACCAGACTCCAATGTCG
EL-shRNA forward	598	GGAGCCAGTCAACCACAACTACATTGGCGAAGCTTGGTCAGTGTAGTTGTGGTTGGCTGGCTCCTCCTTTTTT
EL-shRNA reverse	598	GATCAAAAAAGGAGGAGCCAGCCAACCACAACTACACTGACCAAGCTTCGCCAATGTAGTTGTGGTTGACTGGCTCCCG
Scramble-shRNA forward	None	TGTTCATATGTCTGCTCTTGTAGCAGTAGAAGCTTGTACTGCTGCAGGAGCAGACGTATGAGCACGTTTTTTT
Scramble-shRNA reverse	None	GAGCAAAAAAACGTGCTCATACGTCTGCTCCTGCAGCAGTACAAGCTTCTACTGCTACAAGAGCAGACATATGAACACG

EL, endothelial lipase.

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tively. The reaction was terminated by adding EDTA to a final concentration of 100 nM. Subsequently, LDL was reisolated using a PBS-preequilibrated PD-10 desalting column (GE Healthcare). The protein concentration of the LDL preparation was measured using a bicinchoninic acid protein assay kit (Pierce). The extent of oxidation of LDL was evaluated by thiobarbituric acid-reacting substances (TBARS) assay (ZeptoMetrix Co.). Briefly, 100 μg of OxLDL was added to a test tube containing 100 μl of SDS solution followed by the addition of 2.5 ml of thiobarbituric acid/buffer reagent. After mixing and heating at 95°C for 1 h, the specimens were centrifuged at 3,000 rpm for 15 min, and the absorbance of the supernatant was determined at 532 nm using a spectrophotometer. The amount of TBARS was determined by comparison with a standard of malondialdehyde equivalents. An LDL preparation with a TBARS value of ~20–30 nmol/ml malondialdehyde equivalents was classified as mildly OxLDL, whereas TBARS values of >50 nmol/ml malondialdehyde equivalents were classified as extensively OxLDL. THP-1 monocytes (5×10^5 cells) were seeded onto a 12-well plate and then transduced by lentivirus at multiplicity of infection (MOI) of 20 on the same day. Phorbol 12-myristate 13-acetate (PMA) was added to the cells to a final concentration of 100 nM at 2 days after lentiviral transduction to stimulate the differentiation of the monocytes into macrophages. Subsequently, medium was collected for cytokine analysis every 24 h for 3 consecutive days. OxLDL (50 μg/ml) was added to the macrophage culture 24 h before PMA stimulation.

Lipase activity assay

Trioleinase activity was measured using a triolein emulsion containing radiolabeled triolein as described previously (26). An in-well phospholipase assay was used to evaluate the phospholipase activity. Briefly, the phospholipase substrate bis-BODIPY FL C_{11}-PC (B7701; Invitrogen) was sonicated in PBS and then added to cell culture to a final concentration of 2 μg/ml. After 4 h, culture medium aliquots were collected for the measurement of fluorescence intensity at excitation and emission wavelenghts of 488 nm and 530 nm, which was normalized for total cell protein.

Real-time quantitative RT-PCR

Three days after PMA stimulation, total RNA was isolated using the RNAqueous-4PCR kit (Ambion). The reaction system was assembled using the SuperScript™ III One-Step RT-PCR kit with Platinum® Taq DNA polymerase (Invitrogen) and Assay-On-Demand™ Gene Expression primer sets of 18S rRNA, LPL, and EL (Applied Biosystems). Real-time quantitative PCR was performed on the ABI Prism® 7900 platform. PCR parameters were 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. 18S rRNA served as an internal reference.

Western blot

Cell lysates were collected in RIPA buffer supplemented with protease inhibitor cocktail (Sigma) for Western blot. Equal amounts of total protein (20 μg) were loaded onto a 10% Bis-Tris gel and electrophoresed for 1 h at 200 V. Gels were blotted onto polyvinylidene difluoride membranes for 1 h at 100 V. After 1 h of blocking with Superblock buffer (Pierce), polyvinylidene difluoride membranes were incubated with a primary antibody (1:1,000 dilution; Novus) overnight at 4°C and then incubated with HRP-conjugated 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).

Cytokine ELISA

The quantitation of secreted proinflammatory cytokines was performed by ELISA (BioSource). IL-1β, IL-6, IL-8, MCP-1, and TNF-α were analyzed from aliquots of conditioned medium from THP-1 macrophages according to the manufacturer's instructions. Briefly, aliquots of 100 μl were added to a precoated microplate containing 100 μl of dilution buffer and incubated for 3 h at room temperature. Wells were washed six times before 100 μl of biotinylated anti-target solution was added for 45 min. Microplates were washed again before incubation with 100 μl of streptavidin-HRP working solution at room temperature for 45 min. A stabilized chromogen solution (100 μl) was added and incubated in the dark for 30 min after six wash steps. Absorbance was read at 450 nm after adding 100 μl of stop solution.

Lipid analysis by HPLC

Lentivirus-transduced macrophages were detached using 1% EDTA in PBS (T4174; Sigma) and collected in a glass tube. After a PBS wash, cells were resuspended in 3 ml of chloroform-methanol (1:2) and an additional 0.8 ml of distilled water and vortexed vigorously. After 30 min of incubation at room temperature, the mixture was centrifuged at 3,000 rpm for 5 min, and the organic phase was transferred into a filter paper-lined funnel. The filtrates were dried under a nitrogen gas stream, redissolved in 80 μl of chloroform-methanol (1:2), transferred onto an HPLC column with 50 μl of betulin reference standard, and dried under nitrogen. The dried sample was redissolved in HPLC solvent (4:6:1:1 chloroform-methanol-hexane-acetone in volume ratio) for analysis. Lipid composition was quantified using the method described by Innis and Dyer (27).

Microarray

To evaluate gene expression after lipase suppression, an atherosclerosis-specific microarray was carried out according to the manufacturer's instructions using a copy RNA synthesis kit (GA-029; SuperArray) and an atherosclerosis-specific hybridization chamber (OHS-038; SuperArray). Total RNA was extracted from three individual wells with the same treatment and pooled for cDNA synthesis. In brief, total RNA (3 μg) was synthesized into cDNA, and the latter was then transcribed into copy RNA with the incorporation of biotin-16-UTP. Hybridization was performed in an atherosclerosis-specific hybridization chamber using purified labeled copy RNA, and the membrane was developed using CDP-Star substrate and photographed with the ChemiGenius 2 chemiluminescence system. The expression of genes was analyzed by GEArray® Expression Analysis software, and each gene is represented by four spots on the microarray membrane.

RESULTS

Lipase suppression by lentivirus-mediated RNA interference

Transduction efficiency of lentivirus with THP-1 monocytes was evaluated by flow cytometry. At a MOI of 10, a transduction efficiency of 83% was observed, whereas 100% efficiency was achieved at a MOI of 20 (data not shown). All subsequent experiments were performed with a MOI of 20. Compared with transduction with a lentivector containing a shRNA scrambled sequence, constructs targeting LPL and EL resulted in 83% and 76% decreases in mRNA levels, respectively (**Fig. 1A, B**). LPL

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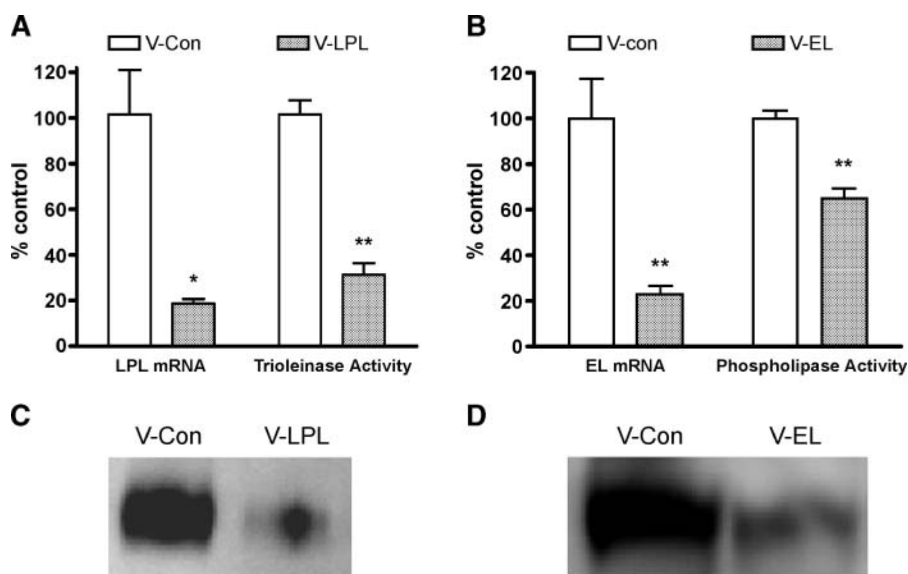

Fig. 1. Lipase suppression in human THP-1 macrophages. Monocytes were transduced by lentivirus for the suppression of each target lipase. Heparin-containing medium, mRNA, and cell lysates were collected after 3 days of phorbol 12-myristate 13-acetate (PMA) treatment for lipase activity assay, quantitative RT-PCR, and Western blot analysis, respectively. Data are presented as means ± SEM (n ⩾ 4). Western blot results are representative of three individual experiments. A: Compared with those in macrophages transduced by lentiviral scramble shRNA (V-Con), LPL mRNA and activity were suppressed by 82% and 70% in cells transduced by lentiviral LPL shRNA (V-LPL); LPL suppression was also evident by Western blot. B: Transduction of lentiviral endothelial lipase (EL) shRNA (V-EL) suppressed EL mRNA by 76% compared with V-Con treatment. Total phospholipase activity was decreased by only 35% after V-EL treatment, and >70% decrease at the protein level was detected by Western blot. * $P < 0.01$, ** $P < 0.001$.

suppression resulted in a 70% decrease in salt-sensitive trioleinase activity (Fig. 1A), whereas EL suppression was associated with a 35% reduction in phospholipase activity, a nonspecific assay of all sources of phospholipase activity (Fig. 1B). There was no suppression or compensatory upregulation detected for the nontargeted lipase in each case, as assessed by mRNA measurements and trioleinase and phospholipase activity (data not shown). Also, Western blotting of immunoprecipitated EL and LPL from heparin-treated medium indicated marked decreases for each lipase protein after viral transduction (Fig. 1C, D).

Cytokine expression secondary to lipase suppression

The concentrations of five proinflammatory cytokines were monitored in THP-1 macrophages over 3 days after suppression of either LPL or EL (see supplementary Tables I–III). Under control conditions, there were marked increases in IL-6, MCP-1, and TNF-α during the observed time period (see supplementary Table I). However, only modest differences were observed for IL-1β and IL-8. Significant decreases were observed for IL-1β for all time points after LPL suppression and at the 24 and 48 h time points for EL suppression ($P < 0.05$ in all cases). Similar decreases in cytokine levels after lipase suppression were observed for IL-6, MCP-1, and TNF-α (see supplementary Table I). Consistent decreases in IL-8 were observed only under EL-suppressed conditions. When experiments were performed in the presence of various oxidized forms of LDL, similar decreases in cytokine levels were observed under lipase-suppressed conditions (see supplementary Tables II, III). There were decreases for all five cytokines measured for at least one time point for LPL- and EL-suppressed macrophages incubated with mildly OxLDL (see supplementary Table II). This effect was attenuated somewhat when macrophages were incubated with extensively OxLDL (see supplementary Table III).

Cytokine expression secondary to treatment with OxLDL

Compared with control conditions, the treatment of THP-1 macrophages with mildly and extensively OxLDL (72 h of incubation) was associated with lower cytokine levels of IL-1β, IL-6, MCP-1, and TNF-α, with observed decreases of 28% and 23%, 38% and 62%, 21% and 25%, and 34% and 66%, respectively (**Fig. 2**). A similar trend was observed for the measured additional time points (see supplementary Tables I–III). However, no significant differences were observed for IL-8 after OxLDL treatment. The same pattern of decreased cytokine levels after OxLDL treatment was also observed for LPL-suppressed cells but was less consistent under conditions of EL suppression (Fig. 2). To determine whether OxLDL treatment influenced lipase expression, LPL and EL mRNAs were quantified throughout the 3 day PMA stimulation period and after the treatment of OxLDL. Both mildly and extensively OxLDL consistently and significantly decreased EL mRNA expression by ∼45–50% throughout monocyte differentiation, whereas LPL mRNA level did not appear to be affected (**Fig. 3A, B**).

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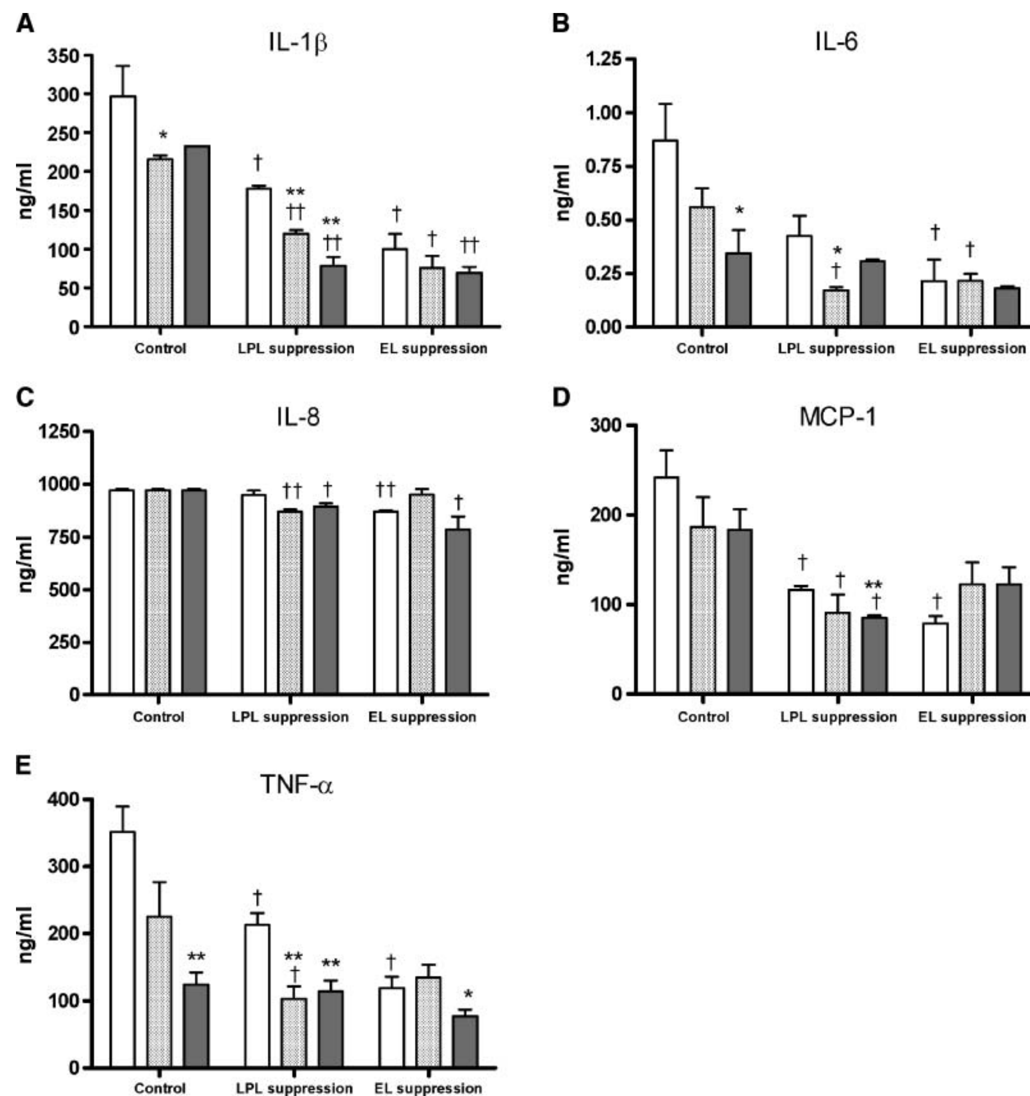

Fig. 2. Cytokine expression in THP-1 macrophages after LPL or EL gene suppression. THP-1 monocytes were transduced with lentivirus containing either a scrambled shRNA sequence (control) or targeted sequences for LPL (LPL suppression) or EL (EL suppression). Macrophages were stimulated by PMA for 48 h in the presence of extensively oxidized low density lipoprotein (OxLDL; black bars), mildly OxLDL (gray bars), or no treatment (white bars). A: Interleukin (IL)-β. B: IL-6. C: IL-8. D: Monocyte chemoattractant protein-1 (MCP-1). E: Tumor necrosis factor-α (TNF-α). Lipase-suppressed cells were compared with control conditions for each treatment. Data are presented as means ± SEM (n = 3). Statistical comparisons between the presence and absence of LDL treatments are indicated as follows: * $P < 0.05$, ** $P < 0.01$; statistical comparisons between the presence and absence of lipase suppression are indicated as follows: † $P < 0.05$, †† $P < 0.01$.

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Lipid composition after lipase suppression

The lipid composition of lipase-suppressed THP-1 macrophages was altered significantly (**Fig. 4**). After 3 days of differentiation by PMA treatment, relative decreases in total cholesterol by 39% and 38%, in cholesteryl esters by 38% and 40%, in unesterified free cholesterol by 40% and 36%, and in triglycerides by 15% and 28% were observed in LPL- and EL-suppressed macrophages, respectively. After LPL and EL suppression, the relative lysophosphatidylcholine (lyso-PC) concentrations were decreased by 15% and 20%, respectively. Correspondingly, the relative levels of phosphatidylcholine were increased by 29% and 39% for LPL and EL suppression, respectively. Other phospholipids, including phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine, also tended to increase after LPL and EL suppression.

Microarray analysis of atherosclerosis pathway-specific genes

Microarray analysis of 110 atherosclerosis-specific genes was carried out on EL- and LPL-suppressed macrophages. The suppression of either LPL or EL in macrophages changed the gene expression (>1.0- or <−1-fold change, or absent) of ~50 of the 110 probed genes contained

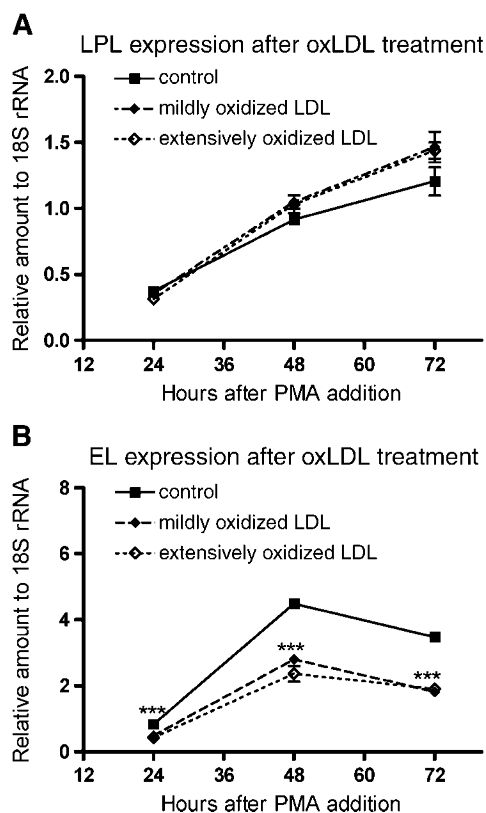

Fig. 3. Effects of OxLDL on lipase expression in THP-1 macrophages. OxLDL was added into cell culture 24 h before PMA stimulation, and mRNA was extracted every day after PMA stimulation for 3 consecutive days. A: Macrophage LPL expression after OxLDL treatment. B: Macrophage EL expression after OxLDL treatment. Data are presented as means ± SEM (n = 4). *** $P < 0.001$.

within the microarray (see supplementary Table IV). The top 10 upregulated and downregulated genes are listed in **Table 2**. Analysis of the microarray data indicated that several proinflammatory cytokines, including IL1-β, IL-6, IL-8, and TNF-α, were downregulated by 1.84-, 2.47-, 4.35-, and 3.72-fold, respectively, for LPL suppression and by 0.96-, 1.89-, 1.15-, and 0.91-fold, respectively, for EL suppression. Interestingly, transforming growth factor β1, one of the modulatory proteins to mitigate the inflammatory response, was upregulated by 3.11- and 1.43-fold in LPL- and EL-suppressed macrophages, respectively. Expression of many of the genes involved in cell adhesion and the extracellular matrix, including chemokine (C-C motif) ligand 2, 20, and 5, intercellular adhesion molecule-1, fibronectin, and integrin α2, β5, and β7, was diminished by varying degrees (from 0.84- to 5.06-fold), whereas the expression of integrin α5, αX, and β2 increased to varying extents (from 0.67- to 3.26-fold). Analysis of the microarray data also indicated marked decreases in the expression of growth factors and antiapoptotic genes (M-CSF, VEGF, and BCL2). The expression of nuclear receptors and transcription factors was also altered in LPL- and EL-suppressed cells, as seen with 2.35- and 1.56-fold decreases for peroxisome proliferator-activated receptor γ (PPARγ) and 7.08- and 7.01-fold increases for nuclear factor-κB (NF-κB). Also, low density lipoprotein receptor, scavenger receptor 1, and scavenger receptor class B type I were increased in LPL-suppressed macrophages (3.17-, 0.44-, and 1.30-fold, respectively) and EL-suppressed cells (2.72-, 1.92-, and 1.44-fold, respectively). In addition, CD36 was also upregulated in the presence of EL suppression (2.11-fold).

DISCUSSION

LPL and EL have been shown to have significant effects on lipid metabolism and to influence the development of lesions in mouse models of atherosclerosis. In this study, we used a lentivirus-mediated RNA interference approach to investigate the effect of LPL and EL suppression in cytokine secretion and cellular lipid composition. To dif-

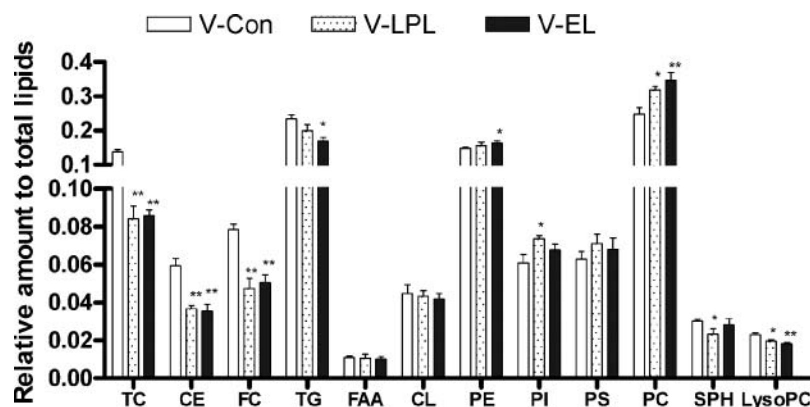

Fig. 4. Effects of LPL or EL suppression on lipid composition in THP-1 macrophages. Cell lipids were extracted from lipase-suppressed and control cells after 3 days of PMA stimulation and analyzed by HPLC. Results are presented as ratios of each lipid mass to total lipid mass (means ± SEM; n ≥ 3). TC, total cholesterol; CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride; FFA, free fatty acid; CL, cardiolipin; PE, [txt]phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine; SPH, sphingomyelin; lyso-PC, lysophosphatidylcholine. * $P < 0.05$, ** $P < 0.01$.

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TABLE 2. Top 10 upregulated and downregulated genes in human atherosclerosis microarray for lipase-suppressed macrophages

	LPL Suppression			EL Suppression		
Category	Gene	Full Name of Gene	Fold Change	Gene	Full Name of Gene	Fold Change
Downregulated genes	IL-8	Interleukin 8	4.35	ITGA2	Integrin α2	2.94
	NPY	Neuropeptide Y	3.94	PTGS2	Prostaglandin-endoperoxide synthase 2	2.48
	TNF-α	Tumor necrosis factor-α	3.72	NPY	Neuropeptide Y	2.40
	ITGA2	Integrin α2	3.38	FGF2	Fibroblast growth factor 2	2.18
	FN1	Fibronectin 1	3.02	IL-1α	Interleukin 1α	1.84
	SOD1	Superoxide dismutase 1	3.02	IL-6	Interleukin 6	1.84
	CCL5	Chemokine (C-C motif) ligand 5	2.97	ITGB5	Integrin β5	1.71
	TNFAIP3	Tumor necrosis factor-α-induced protein 3	2.47	FGB	Fibrinogen β chain	1.63
	EGR1	Early growth response 1	2.43	PPARγ	Peroxisome proliferator-activated receptor γ	1.56
	PPARγ	Peroxisome proliferator-activated receptor γ	2.35	CCL20	Chemokine (C-C motif) ligand 20	1.56
Upregulated genes	NF-κB	Nuclear factor κB	7.09	NF-κB	Nuclear factor κB	7.01
	LDLR	Low density lipoprotein receptor	3.17	ITGA5	Integrin α5	3.26
	TGF-β1	Transforming growth factor β1	3.11	LDLR	Low density lipoprotein receptor	2.72
	ITGB2	Integrin β2	2.39	BCL2L1	BCL2-like 1	2.71
	ITGA5	Integrin α5	1.62	BID	BH3 interaction domain	2.47
	SR-BI	Scavenger receptor class B type I	1.30	F7	Coagulation factor VII	2.42
	ITGAX	Integrin αX	0.86	CD36	CD36	2.11
	F7	Coagulation factor VII	0.57	ICAM-1	Intercellular adhesion molecule 1	2.04
	SRA	Scavenger receptor 1	0.44	SRA	Scavenger receptor 1	1.92
	—	—	—	ITGAX	Integrin αX	1.48

Genes that are expressed in control but absent in lipase-suppressed cells are not listed.

fering extents, IL-1β, IL-6, IL-8, MCP-1, and TNF-α were found to be decreased in the presence of endogenous lipase suppression. Lipase suppression was also accompanied by decreases in the concentrations of cellular cholesterol, cholesteryl ester, triglycerides, and lyso-PC.

This study indicates that lipase suppression causes the decreased expression of proinflammatory cytokines such as IL-1β, IL-6, MCP-1, and TNF-α as well as the decreased production of lyso-PC. Decreased expression of proinflammatory cytokines in macrophages could be ascribed to diminished lyso-PC production secondary to lipase suppression. LPL and EL are capable of hydrolyzing phospholipids, although EL has been shown to more readily hydrolyze phospholipids contained within synthetic substrates (28). The incubation of artificial phospholipid emulsion with LPL generated hydrolytic products with preference for lipolysis at the 1-acyl ester bond of phosphatidylcholine (29). Furthermore, phosphatidylcholine content on cell membranes was increased in LPL-deficient patients because of decreased hydrolysis of phospholipids by LPL (30). The hydrolysis of phosphatidylcholine liberates lyso-PC, which possesses numerous biological activities. For example, lyso-PC has been shown to induce the expression of MCP-1, IL-8, and RANTES by human vascular endothelial cells and smooth muscle cells and to promote the chemotaxis of monocytes, neutrophils, and lymphocytes (31, 32). Lyso-PC promotes proinflammatory changes in monocytes through stimulating arachidonic acid release (33). IL-1β expression was also augmented in macrophages by lyso-PC, which acts on G2A, a receptor for lyso-PC (34, 35). Thus, it is possible that lipase suppression results in decreased hydrolysis of phospholipids and subsequent lyso-PC production, which results in decreased proinflammatory cytokine expression.

It is also possible that a reduction in the noncatalytic function of these lipases influences cytokine regulation. Previously, it was demonstrated that lipases are capable of facilitating lipid uptake and accumulation by lipase-expressing target cells, including macrophages (15, 36). As observed in this study, the relative amounts of cholesterol and triglycerides were decreased in lipase-suppressed macrophages. Moreover, studies from our laboratory have indicated decreased binding of native LDL and OxLDL after lipase suppression (unpublished data). We speculate that intracellular lipids may be depleted after lipase suppression as a result of a reduced uptake of extracellular lipid. Intracellular cholesterol is used for a multiplicity of cellular functions, including the maintenance of cell membrane integrity, hormonal production, cytokine production, and intracellular signaling molecules. Furthermore, intracellular lipids can influence cytokines at the gene expression level. Lipid loading increases oxidative stress and, as a result, IL-1β is rapidly induced in foam cells (37). Meanwhile, free cholesterol-loaded macrophages are considered to be an abundant source of TNF-α, IL-6, and IL-8 (38, 39). Thus, a decreased concentration of intracellular lipids after lipase suppression may also mediate decreases in cytokine expression.

Microarray analysis also indicated decreased expression levels of proinflammatory cytokines consistent with the measured cytokine concentrations. Although IL-8 protein levels did not appear to be appreciably altered by targeted LPL suppression, marked decreases in IL-8 mRNA levels were observed. It is possible that further regulation at

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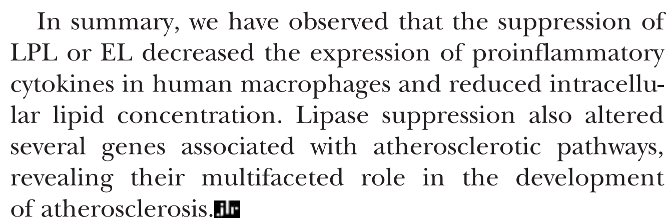
posttranslational or catabolic stages may have affected this relationship. In addition, we found that some key nuclear factors, such as PPARγ and NF-κB, were markedly changed. It is already known that hydrolytic products of lipids and intracellular lipid contents are the key regulators of PPARs and NF-κB. Coincubation of LPL with LDL or VLDL dramatically increases PPARα activity in correspondence to free fatty acid release, and transgenic mice overexpressing LPL had increased peroxisome proliferation (40). In addition, marked PPARα activation is observed after LPL is administered or systemically released by heparin in vivo (40). EL provides an alternative pathway for FFA production, which is further incorporated into cells and then activates PPARα (41, 42). Released arachidonic or linoleic acid can be the agonist for the PPAR/retinoid X receptor heterodimer in macrophages (43). Moreover, the cholesterol accumulation in cells elicits its conversion to oxysterol, which is a potent endogenous activator for LXR and downstream PPAR and retinoid X receptor (44). Because lipase suppression would be consistent with decreased free fatty acid liberation and intracellular lipid accumulation, decreased expression of PPARs would be expected. NF-κB activation could be associated with the decreased concentration of hydrolytic lipid products and PPAR inactivity. Consistent with this hypothesis are results indicating that linoleic acid released by lipases is capable of inhibiting NF-κB in macrophages (45). As a result of a negative transcriptional regulation of NF-κB, PPAR activation abrogates NF-κB activation in macrophages (46).

Strikingly, microarray analysis of gene expression also revealed the upregulated expression of lipoprotein receptors (scavenger receptor 1, low density lipoprotein receptor) and the decreased expression of mitogenic and antiapoptotic genes, such as a variety of growth factors and BCL2. It is well documented that a source of exogenous lipid is required to maintain cell growth and proliferation. Generation of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate from phospholipids as well as ceramide derived from sphingomyelin triggers the progression of cell cycles. In response to a decrease in intracellular lipids from lipase suppression, lipoprotein receptor expression may be upregulated, whereas NF-κB may be stimulated to increase the expression of lipoprotein receptors (47, 48). Also, a lack of lipids may alter cell proliferation and trigger apoptosis.

Oxidized lipoproteins have been well recognized as proatherogenic because of their enhanced receptor-mediated endocytosis by target cells, mostly macrophages in atherosclerotic lesion. In our study, both mildly and extensively OxLDL consistently decreased IL-1β, IL-6, MCP-1, and TNF-α from 24 to 72 h after PMA stimulation. However, the role of oxidized lipoproteins on cytokine expression is not consistent among several studies. Increased expression of cytokines, including IL-1β, IL-6, IL-10, and IL-12, TNF-α and -β, and interferon-γ, was observed after treatment with OxLDL challenge (49, 50). However, treatment with either mildly or extensively oxidized lipoproteins for 48 h consistently decreased proinflammatory cytokine expression in macrophages in this study. Kim and colleagues (51) have demonstrated that the production of proinflammatory cytokines, including inducible NOS and NO, MCP-1 and TNF-α, was suppressed in an oxidation-dependent manner when microglia, one of the differentiated microphages in brain, were treated with either 2,2-Œ-azobis(2-amidimopropane) dihydrochloride or copper sulfate-prepared OxLDL. In addition, the lipopolysaccharide-stimulated expression of interferon-γ, IL-12, IL-1β, and IL-6 was repressed when mouse macrophages were preincubated or coincubated with extensively OxLDL (52–54). In this study, OxLDL treatment suppressed EL expression in macrophages; thus, the reduced cytokine concentrations may in part be mediated by decreased EL expression. The discrepancy of the reported effects of OxLDL on cytokine expression may be related to the extent of oxidation of lipoproteins as well as the amount and treatment duration.

In summary, we have observed that the suppression of LPL or EL decreased the expression of proinflammatory cytokines in human macrophages and reduced intracellular lipid concentration. Lipase suppression also altered several genes associated with atherosclerotic pathways, revealing their multifaceted role in the development of atherosclerosis.



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