Inhibition of LPL Expression in Human Monocyte-Derived Macrophages Is Dependent on LDL Oxidation State

A Key Role for Lysophosphatidylcholine

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Abstract—The regulation of macrophage lipoprotein lipase (LPL) secretion and mRNA expression by atherogenic lipoproteins is of critical relevance to foam cell formation. LPL is present in arterial lesions and constitutes a bridging ligand between lipoproteins, proteoglycans, and cell receptors, thus favoring macrophage lipoprotein uptake and lipid accumulation. We investigated the effects of native and of oxidized lipoproteins on the expression of LPL in an in vitro human monocyte-macrophage system. Exposure of mature macrophages (day 12) to highly copper-oxidized human low density lipoprotein (LDL) (100 μg protein per milliliter) led to marked reduction in the expression of LPL activity (−62%, P<0.01) and mRNA level (−47%, P<0.05); native LDL, acetylated LDL, and LDL oxidized for <6 hours were without effect. The reduction in LPL activity became significant at a threshold of 6 hours of LDL oxidation (−31%, P<0.05). Among the biologically active sterols formed during LDL oxidation, only 7β-hydroxycholesterol (5 μg/mL) induced a minor reduction in macrophage LPL activity, whereas 25-hydroxycholesterol was without effect. By contrast, lysophosphatidylcholine, whose LDL content increased in parallel with the degree of oxidation, induced significant reductions in LPL activity and mRNA levels at concentrations of 2 to 20 μmol/L (−34% to −53%, P<0.01). Our results demonstrate that highly oxidized LDL (>6-hour oxidation) exerts negative feedback on LPL secretion in human monocytes-macrophages via a reduction in mRNA levels. By contrast, native LDL and mildly oxidized LDL (<6-hour oxidation) did not exert a feedback effect on LPL expression. We speculate that the content of lysophosphatidylcholine and, to a lesser degree, of 7β-hydroxycholesterol in oxidized LDLs is responsible for the downregulation of LPL activity and mRNA abundance in human monocyte-derived macrophages and may therefore modulate LPL-mediated pathways of lipoprotein uptake during conversion of macrophages to foam cells. (Arterioscler Thromb Vasc Biol. 1998;18:1172-1180.)

Key Words: macrophage foam cells ■ lipid peroxidation ■ reverse transcription–polymerase chain reaction ■ mRNA ■ lipoprotein lipase

Lipid-laden foam cells, derived principally from monocyte-derived macrophages, are a characteristic feature of atherosclerotic plaques at all stages of their evolution.1 Transformation of macrophages to foam cells results from the cellular uptake of native and modified TG-rich lipoproteins (chylomicron remnants and VLDL) and of modified LDL after their retention in the arterial intima upon binding to extracellular matrix components.2-5 The mechanism of the conversion of macrophages to foam cells containing large amounts of cholesterol, choleseryl esters, and TGs is presently the subject of extensive investigation.6 The uptake of lipoproteins by such cells involves several receptors of distinct specificity, including scavenger receptors,7 CD36,8 and Fc receptors9 for oxLDL; the LDL receptor; the LDL receptor-related protein receptor;10,11 the VLDL receptor for TG-rich lipoproteins;12 and, last, membrane-binding proteins for uptake of certain TG-rich lipoproteins.13 These cellular uptake mechanisms are rendered complex by the contribution of additional factors such as heparan sulfate proteoglycans of the matrix and cell plasma membranes and equally by LPL,14 which may serve to “anchor” lipoproteins to the cell surface.

LPL is a key enzyme of lipoprotein metabolism that hydrolyzes the TG component of chylomicrons and VLDL.15 Moreover, LPL can act as a ligand to create a “bridge” between TG-rich lipoproteins and several receptors of the LDL family.16 LPL may also serve as a molecular bridge between LDL and proteoglycans.17-19 Growing evidence for the implication of LPL in lipoprotein uptake within arterial tissue is emerging. Indeed, LPL mRNA was recently shown to be expressed in the vessel wall in areas rich in macrophages and foam cells.20-22 Considered together, these find-

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Selected Abbreviations and Acronyms

• apo = apolipoprotein
• LPL = lipoprotein lipase
• (lyso)PC = (lyso)phosphatidylcholine
• 25-OH = 25-hydroxycholesterol
• 7β-OH = 7β-hydroxycholesterol
• ox = oxidized
• PCR = polymerase chain reaction
• RT = reverse transcription
• TG = triglyceride

Oxidized lipoproteins (OxLDLs) are characteristic constituents of both early and advanced atherosclerotic lesions. A major fraction of aortic LDL has been detected in mildly oxidized form and as such, is potentially atherogenic, given its capacity to induce secretion of monocyte chemotactic protein-1 (MCP-1), monocyte-endothelial adherence, and expression of macrophage-colony stimulating factor. LPL preanchored to endothelial cells binds mildly oxLDL with high affinity. Moreover, addition (50- to 100-fold) and uptake (20-fold) of mildly oxLDL but did not enhance the uptake of highly oxLDL. In contrast with extensive data on the interactions between LPL and lipoproteins, cell receptors, and proteoglycans, there is a paucity of information on the regulation of LPL secretion and mRNA expression in human macrophages by native and modified LDL.

In view of the potential pathophysiologic role of LPL in the mechanisms governing the uptake of LDL and of oxLDL by macrophages, our aim was to determine the effect of such lipoproteins on LPL secretion and mRNA levels in human monocyte–derived macrophages. In particular, we examined the effect of the degree of oxidative modification of LDL on LPL secretion and mRNA expression in human macrophages by native and modified LDL.

Methods

Materials

Leukocyte-depleted sera and buffy coats for monocyte isolation were obtained from the Hospital Transfusion Center, RPMI-1640 culture medium and PBS were supplied by BioWhittaker. Pools of human sera (100 to 150 donors) for use in cell culture media were supplied by ATGC. Nutridoma HU medium was supplied by Boehringer Mannheim. Restriction enzymes, plasmids, and molecular-weight markers for DNA size (Promega) were used according to the manufacturer’s specifications. The Megaprime random-primer kit was purchased from Boehringer Mannheim. Restriction enzymes, plasmids, and molecular-weight markers for DNA size (Promega) were used according to the manufacturer’s specifications. The Megaprime random-primer kit was supplied from Clontech Laboratories. The labeled nucleotides [α-32P]dATP (3000 Ci/mmol) and glycerol tri(9,10-n)-H)oleate (680.8 GBq/nmol) were purchased from Amersham and Dupont de Nèmes Division–NEN. The following antibodies were from Dako: anti-CD68 (clone KP1), anti-CD14 (clone TÜK4), anti-CD3 (clone T3–4B5), and goat anti-mouse FITC-conjugated IgG. The chromogenic Limulus amebocyte lysate assay for lipopolysaccharide was purchased from Biogenec. The assay kits for LDH and protein bicinchoninic acid assay reagents were purchased from Boehringer Mannheim and Pierce Interchim, respectively. All other reagents were from Sigma Chemical Co.

Isolation and Culture of Human Monocyte–Derived Macrophages

Human mononuclear cells were isolated from freshly drawn blood samples of individual healthy donors (2 to 35 mL) as described earlier. In brief, blood diluted with PBS (1:1, vol/vol) was carefully loaded onto a Ficoll gradient under sterile conditions. After an initial centrifugation step (20 minutes at 2700g), monocytes were collected at the interface and then washed 3 times at room temperature in PBS containing 0.1% EDTA (successively at 1000g, 340g, and 160g for 10 minutes) and then once in PBS alone for 10 minutes. Finally, the cell pellet was resuspended in RPMI-1640 medium containing gentamicin (40 μg/mL) and glucose (0.05%). A typical monocyte preparation yielded 100 to 300×10⁶ cells per donor. The cells were distributed at a density of 1×10⁶ cells well in Primaria 24-well plastic culture dishes (for LPL activities and lipid determinations) and 3×10⁵ cells well in Primaria 6-well dishes (for RNA assays). After 45 minutes of adherence, the cells were washed twice with PBS; finally, fresh medium containing 10% pooled human sera was added. At day 12 of culture, monocyte-derived macrophages (denoted macrophages) were washed 3 times with PBS and then incubated for defined time intervals with LDL, oxLDL, or acLDL in the aforementioned culture medium to which 1% Nutridoma had been added instead of human serum. For determination of LPL activity, heparin (Choay) (10 U/mL) was added at the same time as the lipoproteins and incubated for 24 hours. LPL activity released into the medium was at least 10-fold higher in the presence of heparin; cell-associated activity was then barely detectable. When LPL secretion was measured during macrophage maturation, the medium containing heparin was replaced 24 hours before the day indicated in the time-course study. All cell cultures incubated with lipoproteins, oxysterols, or lysoPC were carried out in a humidified 37°C incubator (5% CO2). Cell viability was measured by trypan blue exclusion and by the release of LDH activity (5% to 10%) into the medium. At the end of the incubation period (6 or 24 hours), medium from the 24-well plates was immediately taken and stored at −80°C for determination of LPL activity. Cells were washed 3 times with cold PBS and further incubated overnight with 0.1N NaOH for cell protein determination by the bicinchoninic acid method. After 6 hours of incubation, total RNA from 6-well plates was immediately extracted with RNA plus.

Characterization of Human Monocyte–Derived Macrophages

Monocytes and macrophages were characterized with specific antibodies that were detected by indirect immunostaining. CD14 and CD68 were used as specific markers for monocytes and CD3 for lymphocytes. At day 12 of culture, all adherent cells were CD68-positive and CD3-negative, thus indicating the absence of T cells.

Lipoprotein Purification and Chemical Modifications

Normolipidemic plasma of healthy blood donors was used to isolate LDL by sequential ultracentrifugation. Native LDLs (d=1.024 to 1.050 g/mL) were centrifuged at their upper limiting density and thereafter exhaustively dialyzed at 4°C against degassed 0.01 mol/L PBS at pH 7.4. Aliquots of LDL were taken to check the purity of each preparation as described elsewhere. Protein content was determined by the assay of Lowry et al. Copper-oxidized LDLs were prepared under sterile conditions by incubating 500 μg LDL protein per milliliter in PBS containing 2.5 μmol/L CuCl₂ for 48 hours.
hours or for the indicated times at 37°C. At the end of the incubation period, oxLDLs were extensively dialyzed at 4°C against PBS (pH 7.4). AcLDLs were prepared with acetic anhydride as described by Basu et al.13 All lipoprotein fractions were dialyzed against PBS buffer at pH 7.4, then against RPMI-1640, and subsequently filtered through a 0.22-µm filter (Millipore). The time course of the copper-induced oxidation of LDL was deduced from the spectrophotometric measurement of conjugated-diene formation at 234 nm. The net electric charge of both native LDL and oxLDL at pH 8.6 was determined by electrophoresis in agarose gel.14 The electrophoretic mobility was measured as the electrophoretic mobility (REM) of oxLDL relative to that of native LDL (REM). Determination of the content of thiobarbituric acid–reacting substances, expressed as malondialdehyde (MDA) equivalents,37 and of lipoperoxides (LPOs)38 in oxLDL provided an estimation of the degree of lipid oxidation. Endotoxin content of oxLDL was measured with the chromogenic Limulus amebocyte lysate assay and was found to be <50 pg/100 μg oxLDL protein.

LPL Assay

LPL activity in the cell incubation medium was assayed with a trioleate-lysoPC emulsion.19 Aliquots (50 to 100 μL) of cell incubation medium were incubated with 100 μL of a substrate emulsion mixture containing 2.64 mmol/L glycerol trioleate (NEN; specific activity, 680.8 GBq/nmol), 0.21 mmol/L L-α-lysoPC, 0.4% BSA, 0.2 mol/L Tris HCl at pH 8.1, and 0.3 mL heat-inactivated human serum for 3 mL of emulsion. The enzymatic reaction was carried out in a shaking bath for 60 minutes at 30°C. The reaction was stopped by successively adding 3.25 mL of solvent mixture (methanol/chloroform/heptane, vol/vol/vol, 1.41/1.25/1) and 1.02 mL of borate buffer at pH 10.40 Liberated radiolabeled fatty acids were counted in the upper phase. One unit of enzyme activity corresponded to 1 nmol of free fatty acid liberated per minute per milligram of cellular protein.

RNA Isolation, First-Strand cDNA Synthesis, and RT-PCR

Total RNA was isolated from adherent macrophages with RNA Plus, and its concentration was determined by spectrophotometry at 260 nm. First-strand cDNA synthesis was performed with 5 or 10 μg of total RNA. Immediately before use, RNA was heated for 5 minutes at 70°C in the presence of RNasin (2 U), oligo(dT) (2 μg), and antisense oligonucleotide LPL-3 (1 μg) (CATTCTTCACAGAATTTCATGC) in aqueous solution in a total volume of 30 μL after denaturation. RT was performed at 37°C for 2 hours in a total volume of 50 μL containing 1× reverse transcriptase buffer, 0.5 mmol/L dNTP, 10 mmol/L DTT, and 500 U of SuperScript M-MLV reverse transcriptase. Detection and quantification of LPL mRNA were performed by RT-PCR in the presence of two specific oligonucleotides, LPL-1 (5′-AGATGTTCTGTGATGGACAC-3′) and LPL-2 (5′-CTGCACATAAGACACCTTCTC-3′). The incubation volume was adjusted to 50 μL by adding master mix components to the first-strand cDNA dilutions; the final concentrations were 1× Taq DNA polymerase buffer, 0.2 mmol/L dNTP, 200 ng oligonucleotides of each upstream and downstream primer, and 0.5 U of Taq DNA polymerase. Incubations were performed in a Schleicher & Schuell Omnigene thermal cycler, starting with a cycle at 94°C for 5 minutes followed by 35 successive cycles of 1 minute at 94°C, 45 seconds at 55°C, and 1 minute at 72°C successively; these cycles were followed by 1 cycle for 7 minutes at 72°C before storage. The PCR products were analyzed by fractionation of 10-μL aliquots on a 3% agarose/TAE gel. Control samples analyzed in the absence of reverse transcriptase were free from genomic DNA. Competitive PCR experiments were performed in the presence of DNA-mimic actin or GAPDH from Clontech Laboratories (0.01 to 1 amol per assay) for 30 cycles at 60°C as described in Reference 41 and according to Clontech recommendations.

Analysis of LDL Phospholipids by Thin-Layer Chromatography

LDL lipids were extracted with chloroform/methanol (2:1, vol/vol) as described.42 The lipid extracts were dried under N2, redissolved in 200 μL of CHCl3-CH3OH (2:1, vol/vol), and separated by thin-layer chromatography on silica gel 60 plates (Merck) with a solvent mixture of chloroform/methanol/water (65/35/6, vol/vol/vol) as the mobile phase. Lipid spots were visualized with I2 vapor. Phospholipid identification was performed by cochromatography with known standards of L-α-PC from egg yolk (Sigma) and L-α-lysoPC from egg yolk (Sigma). After complete disappearance of the I2 color, bands were scraped from the plates and eluted from the silica gel with CHCl3-CH3OH (2:1, vol/vol). Aliquots were dried and resuspended in isopropanol, and phospholipids were quantified with a commercial kit (Boehringer Mannheim).

Statistical Analysis

Data were expressed as mean±SEM. Differences were examined by the paired Student’s t test. A value of P<0.05 denoted a significant difference. All experiments were repeated at least 3 times with different cell and lipoprotein preparations. Representative experiments are indicated in the text.

Results

Induction of LPL Expression During Human Monocyte-Macrophage Maturation

LPL activity could not be accurately determined in human monocytes either in suspension or after 1 hour of cell adhesion, nor was it detected in THP-1 and J774 premonocytic cell lines because values were at the limit of detection. LPL activity, as measured by the activity secreted during 24 hours in the presence of 10 U/mL heparin, was measurable in monocyte incubation medium only after 24 hours (day 1) of adhesion to the plastic wells (Figure 1A). Thereafter, secreted activity increased progressively up to day 8 of culture, was maintained at day 14, but decreased slightly thereafter. For this reason, all further experiments were conducted between 12 and 14 days of cell culture. LPL mRNA was detected in trace amounts after 1 hour of monocyte adhesion (day 0) but increased rapidly at day 1 and, like enzyme activity, increased to day 8, declining at day 14 (Figure 1B). Surprisingly, the level of LPL mRNA was lower on day 2 than on day 1. A similar phenomenon was also observed for scavenger receptor I and II mRNAs (D.S. et al, unpublished results, 1996) and may be linked to the initial differentiation of monocytes to macrophages, at which stage a small proportion of macrophages detached from the culture dishes.

Effect of Modified LDL on Human Macrophage LPL Activity and mRNA Expression

Macrophages obtained after 12 days of culture were incubated with native LDL (100 μg protein per milliliter) or with LDL from the same preparation that had either undergone copper-mediated oxidation for 48 hours (oxLDL) or been modified by acetylation (acLDL) as described in the Methods section. Native LDL and acLDL had no significant effect on either LPL activity (Figure 2A) or mRNA level (Figure 2B). In contrast, oxLDL exerted marked inhibition of LPL activity (−62%, P<0.01) and induced reduction in the mRNA level (−47%, P<0.05); such inhibition was shown to be dose dependent (Figure 3A and 3B). The observation that acLDL had no impact on LPL activity and mRNA abundance indicated that modified
with actin, synthesis of cDNA specific to LPL was expressed as counts per minute of amplified products. Values are mean±SEM of 3 separate experiments, each performed in triplicate.

LDL in which the lysine residues of apoB100 had undergone derivatization exerted no detectable effect on LPL expression and led us to postulate that a lipid component formed during LDL oxidation might be involved in such regulation. We verified that such inhibition was not due to the potential cytotoxicity of oxLDL, because under our conditions, LDH release was identical for all LDL preparations (<10% release into the medium versus cell-associated LDH activity). In addition, cell protein content was not affected, and the expression of actin and scavenger receptor I and II mRNAs remained unaffected or slightly increased after oxLDL treatment, as reported earlier. Moreover, in the same experiments, inhibition of platelet-activating factor receptor gene expression by oxLDL was reversible, as shown by an additional 24-hour incubation in medium devoid of oxLDL. If cytotoxicity has been demonstrated in vitro for mouse macrophages incubated with LDL oxidized for 24 hours with 5 μmol/L CuSO₄, oxLDL prepared under our conditions (48 hours with 2.5 μmol/L CuCl₂, followed by extensive dialysis) had no detectable effect on macrophage morphology, as evaluated by scanning electron microscopy (G. Le Naour et al, unpublished findings, 1997).

**Figure 1.** Time-related changes in LPL activity and LPL mRNA expression in adherent human macrophages. LPL activity secreted into the cell incubation medium over 24 hours in the presence of 10 mU/mL heparin (A) and LPL mRNA (B) was measured on day 0 after 1 hour of cell adhesion (day 0) and subsequently on days 1, 2, 6, 8, and 14 of culture. LPL activity was measured in the same 6-well plates as those used for RNA extraction. Total RNA was extracted, transcribed, and subjected to PCR with specific oligonucleotides for actin and LPL. Competitive PCR experiments were performed in the presence of mimic DNA from Clontech Laboratories (0.01 to 4 amol per assay) in the presence of 5 μL of first-strand cDNA diluted 100-fold with oligonucleotides specific to actin. Different amount of cDNA were assayed for PCR linearity. After normalization of LPL mRNA levels with actin, synthesis of cDNA specific to LPL was expressed as counts per minute of amplified products. Values are mean±SEM of 3 separate experiments, each performed in triplicate.

**Figure 2.** Effect of modified LDL on LPL activity and mRNA levels in human macrophages. Adherent macrophages at 12 days of culture were treated with LDL (100 μg protein per milliliter) for 24 hours in the presence of 10 U/mL heparin for determination of cell medium LPL activity (A) and for 6 hours for mRNA level (B). Native LDL (natLDL), LDL oxidized for 48 hours (ox₄₈hLDL), and acetylated LDL (acLDL) were prepared as described in Methods and extensively dialyzed before addition to the cells. After normalization with actin, LPL cDNA synthesis was expressed as percentage of initial amount. Values are mean±SEM of 3 separate experiments, each performed in triplicate. Significant differences over control values are indicated by *P<0.05 and **P<0.01.

**Effect of Ox₄₈h LDL on the Stability of LPL mRNA in Monocyte-Derived Macrophages**

The stability of LPL mRNA in monocyte-derived macrophages was evaluated with actinomycin D (5 μg/mL), a specific inhibitor of mRNA synthesis. Macrophages were first incubated in the presence or absence of oxLDL (ox₄₈hLDL, 100 μg protein per milliliter) for 1 hour before addition of actinomycin D. After 1, 2, or 4 hours of incubation, control and treated macrophages were washed and total mRNA extracted. LPL mRNA was measured by RT-PCR (Figure 4). No significant difference in the stability of the LPL mRNA was observed under both conditions.

**Effect of the Degree of Oxidation of LDL on Macrophage LPL Activity and mRNA Expression**

Ox₄₈hLDLs were compared with LDLs from the same plasma sample but oxidized for shorter periods (1, 2, 3, 4, or 6 hours) in the presence of 10 μmol/L CuCl₂, followed by extensive dialysis.
were treated with increasing doses of ox 48hLDL and incubated for 24 hours in the presence of 10 U/mL heparin before determination of mRNA levels (B). After normalization with actin, LPL cDNA synthesis was calculated by *P<0.05 and **P<0.01.

6 hours). Although the oxidative resistance of LDL varied among the different LDL preparations, similar values were obtained for the formation of LPOs, MDA, and modification of REM as a function of the period of oxidation (Table). REM increased progressively with the period of oxidation, whereas MDA levels increased after 2 hours and remained stable thereafter until 48 hours. LPO levels increased until 6 hours. LPL activity was markedly decreased (60%) at lysoPC concentrations of 2 hour–oxidized LDL (ox2 hours LDL) and were similar to those detected under control conditions, whereas incubation with ox6 hours LDL led to mRNA levels that were comparable with those obtained with ox2 hours LDL (~70% and ~45% of control levels) (Figure 5B). Thus, a threshold degree of oxidation (6 hours or more) appears necessary to inhibit LPL expression in human monocyte–derived macrophages.

**Effect of 7β-OH and 25-OH on LPL Activity in Human Macrophages**

Among the complex products of lipid oxidation, two oxysterols, namely 7β-OH and 25-OH, at 5 μg/mL concentration have been recently shown to inhibit both the release of LPL activity (~50%) and the expression of LPL mRNA (~60% and ~70% for each oxysterol, respectively) when added to human monocyte–derived macrophages cultured for 7 days. Under our incubation conditions, 7β-OH but not 25-OH added at a concentration of 5 μg/mL induced a significant diminution of LPL activity (~27%, P<0.05) (Figure 6). On incubation of macrophages for 12 days under the same conditions as those of Mattsson Hultén et al,45 ie, medium containing 10% human and 10% fetal calf serum during cell growth followed by 10% fetal calf serum for incubation with sterols, we detected a significant decrease in LPL activity (~25%, P<0.05) with 7β-OH but a greater reduction (~52%, P<0.01) with 25-OH. The difference in the response of macrophage LPL to 25-OH appears to result from the addition of fetal serum to the incubation medium, thereby introducing growth factors that may potentiate 25-OH toxicity.

**Formation of LysoPC During LDL Oxidation and Effect on LPL Activity in Human Macrophages**

LysoPC concentrations increased 2-fold after 6 hours and 4-fold after 48 hours of LDL oxidation, whereas PC contents decreased by 43% and 69% in the respective LDL populations (Table). Such variations have also been reported by other authors.46,47 LysoPC was tested at final concentrations of 2, 10, and 20 μmol/L, corresponding to levels that were in the range of concentrations added as ox6 hours LDL. We observed a clearcut inhibition of LPL activity (~34% with 2 μmol/L, P<0.01 and ~53% with 20 μmol/L, P<0.01) to suppress in the absence of any detectable toxic effect (Figure 7A). LPL mRNA abundance was markedly decreased (~60%) at lysoPC concentrations as low as 2 μmol/L (Figure 7B).

**Discussion**

For the first time, the regulation of LPL at the level of transcription and of the activity of the enzyme protein has been shown to be significantly downregulated by highly
copper-oxidized LDL in human monocyte-derived macrophages. These effects are dose dependent and appear to be independent of the chemical modification of apoB100, because acLDL showed no effect in our human cell system. Consistent with the enrichment of oxLDL in both lysolipids and oxysterols, we were able to demonstrate that lysoPC exerted significant and dose-dependent inhibitory effects on both LPL activity and LPL mRNA abundance. By contrast, and although 7β-OH exerted an inhibitory effect on LPL activity and mRNA expression, the degree of inhibition induced by oxysterols was substantially less. Furthermore, the inhibitory effect exerted by 25-OH depended specifically on culture conditions.

The observation that acLDL did not modify LPL expression in human monocyte-derived macrophages indicates that chemical derivatization of apoB100, which occurs during LDL oxidation, is not directly implicated in the inhibition exerted by oxLDL. The active moiety in oxLDL therefore appears to be a lipid. In this context, it is relevant that recent experiments on CD36 cDNA-transfected cell lines have shown that delipidated oxLDLs do not bind to the CD36 receptor, which normally recognizes oxLDL. Also, chloroform extracts of oxLDL lipids were shown to activate

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**Time Course of Evolution of Oxidation Parameters and Phospholipid Content in LDL During In Vitro Oxidation by Copper Ions**

<table>
<thead>
<tr>
<th></th>
<th>Native LDL</th>
<th>Ox2hLDL</th>
<th>Ox6hLDL</th>
<th>Ox48hLDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPOs, nmol/mg LDL protein</td>
<td>4.88±1.75</td>
<td>66.0±1.43</td>
<td>278±6.17</td>
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<tr>
<td>MDA, nmol equivalent MDA/mg LDL protein</td>
<td>3.83±1.22</td>
<td>24.2±8.91</td>
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<tr>
<td>PC, nmol/mg LDL protein</td>
<td>596±49.9</td>
<td>325±28.2</td>
<td>339±12.5</td>
<td>186.1±23.8</td>
</tr>
<tr>
<td>LysoPC, nmol/mg LDL protein</td>
<td>36.9±3.37</td>
<td>56.1±3.80</td>
<td>73.1±8.37</td>
<td>148.1±1.65</td>
</tr>
<tr>
<td>REM</td>
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<td>1.7±0.2</td>
<td>3.3±0.25</td>
<td>4.46±0.150</td>
</tr>
</tbody>
</table>

Native LDLs were oxidized by incubation at 37°C at a concentration of 0.5 mg LDL protein per milliliter with 2.5 μmol/L CuCl₂ for 2, 6, or 48 hours (h). REM, malondialdehyde MDA, lipoperoxides LPOs, PC, and LysoPC contents were determined in different LDL preparations. Results are mean±SEM with the number of LDL preparations in parentheses.

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**Figure 5.** Impact of degree of oxidation of LDL on LPL activity and mRNA in human macrophages. Adherent macrophages were treated with 100 μg LDL protein per milliliter of either native (nat) LDL or LDL oxidized for 1, 2, 3, 4, 6, or 48 hours (h) with copper as described in Methods. Cells were incubated with different LDL preparations for 24 hours in the presence of 10 U/mL heparin for determination of LPL activity in the cell medium (A). LPL mRNA levels were measured after 6 hours of incubation in the presence of LDL oxidized for 0, 2, 6, or 48 hours (B). After normalization with actin, LPL cDNA synthesis was expressed as percentage of initial amount. Values are mean±SEM of 3 separate experiments, each performed in triplicate. Significant differences over control values are indicated by *P<0.05 and **P<0.01.

**Figure 6.** Effect of oxysterols on LPL activity in human macrophages. Adherent macrophages were incubated for 24 hours in the presence of 10 U/mL heparin and 5 μg/mL of either 25-OH or 7β-OH. Control medium contained 0.1% ethanol (used as sterol solvent). Cells were grown either in medium supplemented with 10% human serum and incubated in medium containing 1% Nutridoma (left part of figure) or in medium supplemented with 10% human serum and 10% fetal calf serum and then incubated with medium containing 10% fetal calf serum alone. Values for cell medium LPL activity are mean±SEM of a representative experiment, each condition repeated 6 times. Significant differences over control values (100%) are indicated by *P<0.05 and **P<0.01.
Oxidized LDL, LysoPC, and Macrophage LPL Expression

Figure 7. Dose-dependent response of LPL activity and mRNA levels to increasing concentrations of lysoPC in human macrophages. Adherent macrophages were incubated with increasing concentrations of lysoPC for 24 hours in the presence of 10 U/mL heparin for determination of LPL activity in the cell medium (A). LPL mRNA levels were measured after 6 hours of incubation (B). Control medium contained 0.1% ethanol (used as solvent for lysoPC). After normalization with actin, LPL cDNA synthesis was expressed as percentage of initial amount. Values for LPL activity are mean±SEM of 1 representative experiment; each point represents the mean of 6 determinations in separate wells. For LPL mRNA, values are mean±SEM of 3 separate experiments, each performed in triplicate or quadruplicate. Significant differences over control values are indicated by **P<0.01.

Mitogen-activated protein kinase activity, as did intact ox-LDL.\textsuperscript{49} Considered together, these observations suggest a direct role of oxidized lipids in the cellular binding and metabolic effects of oxLDL but do not exclude the possibility that the oxidative modification of apoB100 may also play a role. Indeed, an enhanced binding of moderately oxLDL (after long-term storage in the absence of EDTA) to LPL anchored to the endothelial cell matrix has been shown to result from modification of apoB100.\textsuperscript{45}

Moderately copper-oxidized LDL (<6 hours) was without effect on both LPL activity and mRNA levels. By contrast, LDL obtained after prolonged copper oxidation (6 or 48 hours) significantly decreased the secretion of active LPL activity by cultured macrophages. In this respect, it is relevant that the lysoPC content of oxLDL increased 2-fold after 6 hours of copper-mediated oxidation, attaining a 4-fold elevation after 48 hours in our experiments; in parallel, the proportion of PC, its precursor, declined over the same period of time. Indeed, as much as 50% of LDL PC can be converted to lysoPC during oxidation.\textsuperscript{50} As reported herein, addition of lysoPC to our monocyte-macrophage system induced significant reductions in LPL activity and mRNA abundance. The concentrations used (2 to 20 μmol/L) were in the range of lysoPC levels detected in LDL during their progressive oxidation (from 5.6 to 15 nmol/100 μg LDL protein), thereby suggesting that lysoPC is a potent mediator of the inhibitory effect of oxLDL on macrophage LPL expression.

Insight into the biological activity of lysoPC in oxLDL has been gained from experiments in which the endogenous content of lysoPC has been increased by the action of phospholipase A2. Thus, phospholipase A2–treated LDL becomes more negatively charged and enriched in lysoPC and thereafter is more efficiently endocytosed\textsuperscript{41} via the macrophage scavenger pathway.\textsuperscript{52} If acLDL, which is devoid of lysoPC, is also treated with phospholipase A2, then it acquires lysoPC levels and mitogenic properties similar to those of oxLDL.\textsuperscript{52} We also treated native LDL with phospholipase A2 and measured both an increase in its lysoPC content and a decrease in macrophage LPL activity (~47%, P<0.01) when cells were incubated with phospholipase-treated LDL (S.G. et al, unpublished findings, 1997). Thus, lysoPC either added alone or formed endogenously in LDL mimics certain biological properties of oxLDL, such as the inhibition of LPL activity secreted by macrophages. By analogy with the former observations, the difference we observed between oxLDL, which decreased LPL expression in monocyte-macrophages, and acLDL, which was without effect, could be explained on the basis of the distinct lysoPC contents of these LDL preparations. Indeed, the lysoPC content of acLDL was 26.5±2.18 nmol/mg LDL protein\textsuperscript{6} and did not differ from that of native LDL. The exact mechanism by which lysoPC decreases LPL activity and mRNA levels remains to be defined. Nonetheless, lysoPC has been demonstrated to activate the adenyl cyclase system in human platelets, in a megakaryoblastic cell line, and in monocyte-like THP-1 cells\textsuperscript{53} and to potentiate the diacylglycerol-induced activation of protein kinase C,\textsuperscript{54} thereby suggesting that this molecule is an important lipid messenger in macrophages.

Recently, it has been shown that cholesterol hydroperoxides are among the initial products of copper oxidation in LDL; subsequently, oxysterols attain elevated levels at 5 hours.\textsuperscript{55} The content of 7β-OH and 25-OH in LDL preparations, prepared in our laboratory under exactly the same oxidative conditions as in the current study, increased from initial levels of 7.8 and 5.7 ng/mg LDL protein, respectively, to 354 and 9.3 ng/mg LDL protein after 6 hours and from 41 284 and 878 ng/mg protein LDL after 48 hours of copper oxidation.\textsuperscript{44} Such levels correspond to 4.1 μg of 7β-OH and 0.09 μg of 25-OH per 100 μg of oxLDL protein and were in the range of concentrations tested herein. Mattson Hultén et al\textsuperscript{40} showed that both of these oxysterols were potent inhibitors of the expression of human macrophage LPL activity and mRNA mass, while under our conditions, only 7β-OH had a biological effect. Such discrepancies may have arisen from addition of fetal calf serum to the incubation medium by the aforementioned authors\textsuperscript{45}; the underlying mechanism remains indeterminate, however. The cytotoxicity of oxysterols to various cell types has been documented and evaluated in human monocyte–derived macrophages,\textsuperscript{38} in which oxysterols may activate apoptosis. At the low dose of oxysterols (5 μg/mL) that we used, toxicity was not detectable; however, they became toxic at the higher concentrations that we tested (>7.5 μg/mL). In vivo, several oxysterols have been identi-
fied in atherosclerotic lesions, whereas lysoPC is detectable in the lesions of animals fed an atherogenic diet and in human atheromatous plaque. Moreover, lipid peroxidation/protein adducts have also been detected in macrophages in human atherosclerotic plaques. Although the respective roles of these oxidized components of oxLDL remain to be established in vivo, their presence in plaques suggests that they may influence the overall macrophage phenotype and LPL expression in particular.

With respect to the potential atherogenic action of LPL, mildly oxLDL (<6 hours) did not modify enzyme expression in human macrophages; by contrast, uptake of such LDL was markedly enhanced (20-fold) in J774 macrophages on addition of exogenous, purified bovine LPL. From this observation and from our own findings, we speculate that LPL is particularly efficient in promoting macrophage uptake of mildly oxLDL because LPL expression is not downregulated by the latter. In vivo, LDLs isolated from plaques and fatty streaks are moderately oxidized. Thus, higher uptake and sustained LPL secretion could reinforce the atherogenic process associated with mildly oxLDL. By contrast, highly oxLDLs, which induce reduction in macrophage-associated LPL activity and mRNA levels, are internalized by macrophages via pathways such as the scavenger receptor and the CD36-oxLDL binding protein and which are distinct from that mediated by LPL-proteoglycan complexes. Highly oxLDL appears therefore to transform macrophages into foam cells through several mechanisms, which include inhibition of cholesterol efflux and inactivation of lysosomal proteases.

Our data thus suggest that progressive oxidation of LDL is associated with at least two distinct mechanisms of induction of foam cell formation.

In summary, the current study demonstrates that LPL expression (enzyme activity and mRNA levels) is differentially modulated in human monocyte-derived macrophages by potentially atherogenic oxLDL that accumulates in the arterial wall. Highly oxLDL downregulates LPL expression in a dose-dependent manner, thereby reflecting the inhibitory action of LDL-associated lysoPC. In contrast, mildly oxLDL did not downregulate LPL expression; therefore, their binding and uptake by macrophages are markedly increased given the high affinity of LPL for such LDL. These investigations demonstrate that the degree of oxidation of LDL is intimately related to the mechanism of their binding and uptake by macrophages and in turn, to the role of LPL in this process. Clearly, LPL play a key role in the cellular and molecular mechanisms that regulate foam cell formation.

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