Suppression of Endothelin-1 Secretion by Lysophosphatidylcholine in Oxidized Low Density Lipoprotein in Cultured Vascular Endothelial Cells

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Oxidatively modified low density lipoprotein (oxidized LDL), an atherogenic lipoprotein that exists in the atherosclerotic arteries, has been shown to alter endothelial cell functions. In the present study, we examined the effects of oxidized LDL on the production of endothelin-1-like immunoreactivity (ET-1-LI) by the cultured vascular endothelial cells from porcine aorta and human umbilical vein. Incubation with oxidized LDL resulted in a dose-dependent suppression of ET-1-LI release by both endothelial cells. Oxidized LDL also inhibited thrombin-mediated stimulation of ET-1-LI secretion. However, native LDL had no effects on ET-1-LI secretion. A lipid extract from oxidized LDL, but not from native LDL, inhibited ET-1-LI secretion, indicating that the lipid component of oxidized LDL was required for the inhibition of ET-1-LI secretion. Oxidative modification of LDL was associated with degradation of a substantial amount of phosphatidylcholine to lysophosphatidylcholine (LPC). Pretreatment with defatted albumin, which is an acceptor for hydrophilic lipids including LPC, reduced LPC concentration in oxidized LDL to that in native LDL and simultaneously prevented the inhibitory effects of oxidized LDL on ET-1-LI secretion. Incubation with synthetic LPC (palmitoyl), but not with synthetic phosphatidylcholine (dipalmitoyl), suppressed ET-1-LI secretion by the endothelial cells. No cell death was observed during the incubations as judged by the trypan blue exclusion test, and protein synthesis of the endothelial cells was not affected by lipids or lipoproteins at a concentration at which suppression of ET-1-LI was observed. We concluded that LPC in oxidized LDL causes suppression of ET-1-LI release, which may counteract the vasoconstrictive properties of atherosclerotic arteries. (Circulation Research 1992;71:614–619)

**Key Words** • endothelin • lipoprotein • atherosclerosis • radioimmunoassay

Endothelin-1 (ET-1) is a 21-amino acid vasoconstrictor peptide with mitogenic properties that has been isolated from the supernatant of cultured vascular endothelial cells. This peptide is considered to play an important role in the regulation of systemic and local vascular tone. However, it remains unknown whether atherosclerosis and hypercholesterolemia have influences on the endothelial production of ET-1.

On the other hand, low density lipoprotein (LDL), which is the major carrier of cholesterol, is well known as an important atherogenic factor. Considerable attention has been focused on oxidatively modified LDL (oxidized LDL) as a more atherogenic factor, because oxidized LDL can cause accumulation of cholesterol esters in the cells of the macrophage system more rapidly than does native LDL (N-LDL) and, in addition, induces cytotoxicity in the cultured endothelial cells. Furthermore, there has been increasing evidence that oxidized LDL is present in the subendothelial space of atherosclerotic lesions. Recently, it has been demonstrated that transferred lysophosphatidylcholine (LPC) from oxidized LDL to endothelial surface membranes can produce endothelium-dependent vasoregulatory impairments closely resembling those of atherosclerotic arteries.

The present study was designed to examine the effects of oxidized LDL on the secretion of ET-1-like immunoreactivity (ET-1-LI) by the cultured vascular endothelial cells from porcine aorta and human umbilical vein, considering the possible relevance of ET-1 to atherosclerosis and hypercholesterolemia.

**Materials and Methods**

**Preparation of Lipids**

LDL (density, 1.019–1.063) was isolated by ultracentrifugation from freshly harvested normal human plasma with EDTA (1 mg/ml). Oxidized LDL was prepared by 24-hour incubation of N-LDL (150 µg/ml)
with confluent cultured porcine vascular endothelial cells in serum-free F-10 medium\textsuperscript{8,12,13} (EC-LDL). Thiobarbituric acid–reactive substance in the culture medium after the incubation with endothelial cells averaged 2.2±0.2 nmol malondialdehyde equivalents/ml medium, whereas thiobarbituric-reactive substance averaged 0.7±0.1 nmol malondialdehyde equivalents/ml in the culture medium after the incubation in cell-free control dishes treated otherwise identically. Modified LDL was reisolated from the harvested culture medium using ultracentrifugation at a density of 1.21 for 24 hours. Electrophoretic mobility of EC-LDL relative to that of N-LDL was 1.8±0.1. Before use, N-LDL from storage and treated LDL were extensively dialyzed against phosphate-buffered saline containing 20 μM butylated hydroxytoluene and 50 μM EDTA over 24 hours at 4°C. Some aliquots of the harvested culture medium containing EC-LDL were sterilely incubated with 100-fold excess of defatted albumin (0.15 mg LDL protein per 15 mg albumin) at 37°C for 3 hours, and then LDL was reisolated from the supernatant after ultracentrifugation at a density of 1.21 for 24 hours and dialyzed (albumin-treated EC-LDL). The incubated albumin with EC-LDL was also recovered from the infranatant after ultracentrifugation and then dialyzed (“treated albumin”). During the recoveries of albumin-treated EC-LDL and treated albumin, care was taken to avoid contamination with each other, and gel electrophoresis confirmed that there was no contamination. Lipids were extracted from the LDL preparations, treated albumin, and nontreated albumin (albumin without any treatment) with chloroform-methanol (2:1 by volume) and dried under N\textsubscript{2}.\textsuperscript{14} Extracted lipids from lipoproteins and albumin were analyzed by thin-layer chromatography using silica-gel G plates developed with a solvent mixture containing chloroform-methanol-water (25:10:1 by volume).\textsuperscript{11} Lipid bands representing phosphatidylcholine and LPC were eluted, and phospholipid phosphorus in eluates was measured according to the method of Bartlett (Kugiyama et al,\textsuperscript{11} Steinbrecher et al,\textsuperscript{12} and Bartlett\textsuperscript{15}). Extracted lipids and phospholipids were used for the bioassay experiments after sonication in phosphate-buffered saline. Copper-oxidized LDL was prepared by incubation of N-LDL with 5 μM Cu\textsuperscript{2+} in phosphate-buffered saline at 37°C for 24 hours and reisolated in the same manner as described above.\textsuperscript{12} Thiobarbituric-reactive substance in the culture medium after the incubation with Cu\textsuperscript{2+} averaged 4.1±0.2 nmol malondialdehyde equivalents/ml medium, and electrophoretic mobility of copper-oxidized LDL relative to that of N-LDL was 3.2±0.2. Synthetic phosphatidylcholine (dipalmityl) and LPC (palmitoyl) were obtained from Sigma Chemical Co., St. Louis, Mo. Oxyesters, such as 7-ketocholesterol and 7β-hydroxysterol (Steraloids Inc., Wilton, Conn.), were dissolved in ethanol and added in various concentrations to culture medium with 0.1% defatted albumin. Cholesterol content was measured by a colorimetric procedure (Wako Chemical Ltd., Tokyo), and protein content was determined by the Lowry method\textsuperscript{16} using bovine serum albumin as a standard.

Cell Culture and Study Protocol

Endothelial cells harvested from porcine aorta were cloned and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C under a humidified atmosphere of 95% air–5% CO\textsubscript{2}. Endothelial cells from human umbilical vein were grown in Medium 199 with 15% fetal calf serum and endothelial growth factor (Collaborative Research, Inc., Bedford, Mass.). At confluence, the endothelial cells appeared as typical "cobblestone"-patterned monolayers. Porcine aortic endothelial cells in the fifth passage and human umbilical vein endothelial cells in the second passage were used for the present experiments. After washing three times with Hanks' balanced salt solution, confluent monolayer endothelial cells (≈5×10\textsuperscript{5} cells) were preincubated with protein-free medium for 1 hour, and lipoproteins and lipids were added with or without 20 units/ml thrombin (Sigma) and incubated for the indicated time. We confirmed the negativity of endotoxin in lipoproteins in each of the experiments by chromogenic limulus lysate test. After incubation, the culture media were removed and directly applied to the radioimmunoassay. To determine the percentage of viable cells, endothelial cells after the incubations with lipids and lipoproteins for 8 hours were stained with a 0.25% solution of trypan blue.

Radioimmunoassay for ET-1

The preparation of monoclonal antibody and the procedure of radioimmunoassay for ET-1 were described previously.\textsuperscript{17,18} In brief, 50 μl sample or standard ET-1 (Peptide Institute Inc., Minoh, Japan), 50 μl monoclonal antibody (final dilution, 3.0×10\textsuperscript{2}/1), and 100 μl assay buffer were incubated for 24 hours at 4°C, followed by the addition of 50 μl [\textsuperscript{3}H]-ET-1 (3,000 cpm, Amersham, England) and further incubation for 24 hours. The separation of the bound from free ligands was performed by the dextran-coated charcoal method. This radioimmunoassay showed 100% cross-reactivities with endothelin-2 and big ET-1 and 60% cross-reactivity with endothelin-3. This radioimmunoassay system worked in the presence of lipoproteins and lipids, since they did not affect the radioimmunoassay system for ET-1.

Protein Synthesis

Protein synthetic rate in the porcine endothelial cells was measured by incorporation of [\textsuperscript{3}H]leucine into secreted and cellular trichloroacetic acid–precipitable radioactivity.\textsuperscript{19} Confluent endothelial cells were incubated in the serum-free medium containing 10 μCi/ml [\textsuperscript{3}H]leucine with or without EC-LDL or LPC for 8 hours. Synthesized proteins in the medium and in the cells were measured separately. Secreted protein into the medium was precipitated by addition of 500 μl cold 15% trichloroacetic acid. After removing the medium, the cell layers were dissolved by the addition of 0.05% Triton X-100 and subsequent sonication. The protein content of the cells was then precipitated by the addition of trichloroacetic acid. The precipitated proteins in the medium and in the cell layers were washed three times in trichloroacetic acid and were then dissolved in 200 μl of 1N NaOH. The radioactivity was determined in a liquid scintillation counter.

Endotoxin Levels in Lipoproteins

The chromogenic limulus lysate test (Toxicolor test, Seikagaku Kogyo, Tokyo) was used to measure the
levels of endothelin in lipoproteins. The lipoprotein (100 μg LDL protein/100 μl) was exposed to 200 μl of 0.32 M perchloric acid at 37°C for 20 minutes to remove limulus lysate-test—interfering factors. After centrifugation, the supernatant was neutralized with an equal volume of 0.18 M NaOH and used for the test. A sample (100 μl) was added to a 100-μl aliquot of Toxicolor test (a lyophilized mixture of amebocyte lysate from Tachyplesus tridentatus and the chromogenic substrate, Boc-Leu-Gly-Arg-p-nitroanilide) and incubated at 37°C for 30 minutes. Absorbance was measured at 545 nm after diazotization by adding 500 μl of the following reagents: 0.4 g/l sodium nitrite in 1N HCl, 3.0 g/l ammonium sulfamate, and 0.7 g/l N-(1-naphthyl)ethylenediamine dihydrochloride.

Statistical Analysis

Statistical significance was determined using Student’s t test for unpaired data. A value of p<0.01 was considered to indicate a significant difference. Values represent mean±SD of six independent experiments.

Results

Spontaneous and Stimulated ET-1-LI Secretion

Spontaneous secretions of ET-1-LI by endothelial cells from porcine aorta and human umbilical vein for 8 hours of incubation were 490±56 and 262±34 pg/5×10⁶ cells, respectively. Thrombin (20 units/ml) significantly increased the production of ET-1-LI to 805±60 pg/5×10⁶ cells per 8 hours in porcine aortic endothelial cells and to 392±53 pg/5×10⁶ cells per 8 hours in human umbilical vein endothelial cells, respectively.

Effects of LDLs and Associated Lipids

EC-LDL, but not N-LDL, suppressed ET-1-LI secretion by the endothelial cells. A time course of the effect of EC-LDL on the production of ET-1-LI by the porcine aortic endothelial cells is shown in Figure 1. EC-LDL inhibited ET-1-LI secretion in a dose-dependent manner (Figure 2). EC-LDL, but not N-LDL, suppressed thrombin-mediated stimulation of ET-1-LI secretion.

(Abbreviations)

**ET**: endothelin-1-like

**EC-LDL**: endothelial cell-modified low density lipoprotein

**N-LDL**: native low density lipoprotein

**LPC**: lysophosphatidylcholine

**ET-1-LI**: endothelin-1 like immunoreactivity

**LDL**: low density lipoprotein

*Significant difference at p<0.01 vs. control.
from EC-LDL: ET-1-LI secretion by the porcine endothelial cells with a lipid extract from treated albumin (a lipid extract from 10 mg treated albumin, an equivalent dose of albumin incubated with 100 µg EC-LDL protein/ml culture medium) was 391 ± 23 pg/5 × 10⁶ cells per 8 hours (p < 0.01 versus the control value), whereas that with a lipid extract from albumin without any treatment at the same dose was 465 ± 36 pg/5 × 10⁶ cells per 8 hours (NS versus the control value). Furthermore, LPC (palmitoyl, 3–15 nmol/ml culture medium [3–15 µM]; for comparison, 100 µg protein of oxidized LDL contains 48 nmol LPC), which is increased in EC-LDL, suppressed basal (Figure 5) and thrombin-mediated stimulation of ET-1-LI secretion in a dose-dependent manner, whereas phosphatidylcholine (dipalmitoyl, 3–15 nmol/ml culture medium [3–15 µM]) had no effects on the secretion of ET-1-LI. Oxysterols,20,21 7-ketocholesterol (1 nM–1 µM), and 7β-hydroxycholesterol (1 nM–1 µM), which are postulated to be present in EC-LDL, were ineffective on ET-1-LI secretion (Table 2). The effects of LDLs and associated lipids on the secretion of ET-1-LI were almost the same between porcine aortic endothelial cells and human umbilical vein endothelial cells (Table 2). Copper-oxidized LDL (50 µg protein/ml) also inhibited ET-1-LI secretion from porcine aortic endothelial cells and human umbilical vein endothelial cells (252 ± 20 and 171 ± 28 pg/5 × 10⁶ cells per 8 hours, respectively).

Protein Synthesis
EC-LDL and copper-oxidized LDL did not affect protein synthesis (trichloroacetic acid-precipitable radioactivity) in the medium or in the cells at a concentration up to 50 µg LDL protein/ml. EC-LDL at a concentration of 100 µg protein/ml decreased protein synthesis in the cells to 77% of the control values but did not decrease protein synthesis in the medium. LPC up to 15 µM affected protein synthesis neither in the medium nor in the cells (Table 3).

Effect of the Same Batch of Oxidized LDL
Because of the variability in oxidation of LDL, we examined the effects of the same batch of oxidized LDL on ET-1-LI secretion and protein synthesis. The same batch of EC-LDL and copper-oxidized LDL at a concentration of 50 µg protein/ml decreased ET-1-LI secretion to 64±5% and 54±4% of the control value (p<0.01, n=6), respectively, whereas they did not inhibit protein synthesis in the cells (96±9% and 102±7% of the control value, respectively; NS, n=6).

Morphological Analysis
The endothelial cell number was not changed, and no morphological changes were observed in the porcine endothelial cells during the incubations for 8 hours with oxidized LDL up to 100 µg protein/ml and LPC up to 15 µM. Trypan blue staining (0.25% solution) revealed that no cell death occurred after the incubations for 8 hours.

**Table 1. Phosphatidylcholine and Lyosphosphatidylcholine in Low Density Lipoprotein Preparations and Treated Albumin**

<table>
<thead>
<tr>
<th></th>
<th>PC (nmol/mg protein)</th>
<th>LPC (nmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-LDL</td>
<td>1,030±110</td>
<td>30±10</td>
</tr>
<tr>
<td>EC-LDL</td>
<td>510±60</td>
<td>480±50</td>
</tr>
<tr>
<td>Alb-EC-LDL</td>
<td>470±40</td>
<td>20±10</td>
</tr>
<tr>
<td>Treated albumin</td>
<td>0.6±0.1</td>
<td>3.1±0.1</td>
</tr>
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</table>

PC, phosphatidylcholine; LPC, lysophosphatidylcholine; N-LDL, native low density lipoprotein; EC-LDL, endothelial cell-modified low density lipoprotein; Alb-EC-LDL, albumin-treated EC-LDL, treated albumin, isolated from the incubation mixture of defatted albumin and EC-LDL. Values are mean±SD (n=21). Albumin without any treatment had no detectable phospholipids.

**Figure 4.** Bar graphs indicating effects of lipid extracts of low density lipoproteins on the production of endothelin-1–like immunoreactivity (ET-1-LI) by porcine aortic endothelial cells after an 8-hour incubation. A lipid extract from endothelial cell–modified low density lipoprotein (EC-LDL lipid, 50 µg cholesterol/ml) suppressed both ET-1-LI secretion (left panel) and thrombin-mediated stimulation of ET-1-LI secretion (right panel). A lipid extract from native low density lipoprotein (N-LDL lipid, 50 µg cholesterol/ml) was ineffective. *Significant difference at p<0.01 vs. control.

**Figure 5.** Graph showing effects of synthetic lysophosphatidylcholine (LPC [palmitoyl]) and phosphatidylcholine (PC [dipalmitoyl]) on the basal production of endothelin–1-like immunoreactivity (ET-1-LI) by porcine aortic endothelial cells. LPC, but not PC, suppressed ET-1-LI secretion in a dose-dependent manner. Each point represents the mean ± SD of six independent experiments. *Significant difference at p<0.01.
hours with oxidized LDL up to 100 μg protein/ml and LPC up to 15 μM.

**Limulus Lysate Test**

To ensure oxidized LDL–induced inhibition of ET-1 secretion, endotoxin levels were assayed in lipoproteins using a chromogenic limulus lysate test. Endotoxin levels in N-LDL and oxidized LDL were less than 10 pg/100 μg LDL protein.

**Discussion**

In the present study, we have demonstrated that oxidized LDL suppressed production of ET-1-LI by the vascular endothelial cells in a dose-dependent manner, whereas N-LDL had no effects on ET-1-LI secretion. Sonicated lipid extracts exerted effects on ET-1-LI secretion similar to those obtained from the corresponding lipoproteins. These results indicate that effects of oxidized LDL observed in our experiments are not mediated by an apoprotein-dependent mechanism, but its active components are present in the lipid fractions. It has been reported that oxidative modification of LDL is associated with degradation of a substantial amount of phosphatidylcholine to LPC and that after treatment with albumin, a potent acceptor for hydrophobic lipids, most of LPC in oxidized LDL is transferred to albumin, as shown in Table 1. The present study demonstrated that albumin-treated EC-LDL attenuated its suppressive effects on ET-1-LI secretion and that a lipid extract from treated albumin, which was reisolated from the infranatant of the incubation mixture of defatted albumin and EC-LDL, and acquired LPC from EC-LDL (Table 1) produced very similar inhibitory effects on ET-1-LI secretion as a lipid extract from EC-LDL. These observations further confirm that the inhibitory component(s) in oxidized LDL is present in the lipid fraction and is capable of transferring from oxidized LDL to albumin. Furthermore, synthetic LPC (palmitoyl) elicited the inhibitory effect on the secretion of ET-1-LI. It should be emphasized that oxidation of lipids in LDL yields a variety of amphiphiles, such as lysolipids, lipid peroxides, and oxygcnated forms of cholesterol. However, the present study showed that oxysterols such as 7-ketocholesterol and 7β-hydroxycholesterol had no effects on the secretion of ET-1-LI. These results indicate that transferable hydrophilic lipids, especially LPC, account for the oxidized LDL-induced suppression of ET-1-LI secretion.

Incubation with high concentrations of oxidized LDL for more than 24 hours has been reported to induce nonspecific cell injury or death. In the present study, however, incubation of endothelial cells with lipoproteins or lipids for 8 hours did not affect cell viability as judged by the morphological changes and trypan blue exclusion test. Furthermore, oxidized LDL inhibited protein synthesis neither in the medium nor in the cells at a concentration up to 50 μg protein/ml, a level at which inhibition of ET-1-LI production was observed. LPC up to 15 μM did not affect protein synthesis in the medium or in the cells. Since oxidized LDL at a concentration of 100 μg protein/ml suppressed protein synthesis, cell injury by oxidized LDL may partly play a role in the inhibition of ET-1-LI secretion at high concentrations of more than 100 μg protein/ml. Thus, it is unlikely that cell injury or death after the incubation with oxidized LDL is responsible for the mechanism by which oxidized LDL suppresses ET-1-LI secretion by the endothelial cells.

Reports demonstrated that oxidized LDL increased the production of ET-1 by endothelial cells and macrophages. However, they did not examine the effects of lipid components in oxidized LDL. Oxidized LDL...
was reported to facilitate the uptake of endotoxin in endothelial cells, and endotoxin was known to increase ET-1-LI secretion from the endothelial cells. Endotoxin contamination in oxidized LDL can easily occur by the usual manipulation (authors’ observation). However, we confirmed the negativity of endotoxin contamination in lipoproteins used in the present study by chromogenic limulus lysate test.

A previous study (Berliner et al27) showed that some endothelial functions could be altered by minimally modified LDL, which has low levels of thiobarbituric-reactive substance (2–5 nmol malondialdehyde/mg LDL cholesterol) and is taken up by the LDL receptor. Although the present study did not demonstrate oxidative degradation of apoprotein B, the level of thiobarbituric-reactive substance indicates that EC-LDL used in the present study seems to be distinct from minimally modified LDL, because the thiobarbituric-reactive substance level in EC-LDL in our study is almost 10 times higher than that in minimally modified LDL. Berliner et al showed that this level of thiobarbituric-reactive substance is no longer recognized by the LDL receptor but is recognized by the scavenger receptor, and the oxidation of thiobarbituric-reactive substance is extensive.

Oxidized LDL has been shown to exist in the subendothelial space of atherosclerotic arteries. Recent reports have shown that oxidized LDL impairs endothelium-dependent relaxation and enhances the responses of arterial smooth muscle to vasoconstrictors, indicating that oxidized LDL increases arterial vascular tone. The present study, however, has shown that LPC in oxidized LDL causes suppression of ET-1-LI secretion, which may neutralize the vasoconstrictive properties of atherosclerotic arteries. Further studies are needed to investigate the relation between oxidized LDL and ET-1.

References
Suppression of endothelin-1 secretion by lysophosphatidylcholine in oxidized low density lipoprotein in cultured vascular endothelial cells.

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