Transferable Lipids in Oxidized Low-Density Lipoprotein Stimulate Plasminogen Activator Inhibitor-1 and Inhibit Tissue-Type Plasminogen Activator Release From Endothelial Cells

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Decreased fibrinolytic activity has been reported in atherosclerotic cardiovascular diseases. To determine whether oxidized low-density lipoprotein (Ox-LDL), which accumulates in atherosclerotic arteries, modulates the endothelial fibrinolytic system, cultures of human umbilical vein endothelial cells were incubated with low-density lipoproteins or lipids, and levels of plasminogen activator inhibitor-1 (PAI-1) and tissue-type plasminogen activator (t-PA) antibodies in the conditioned medium were measured by enzyme-linked immunosorbent assay. Ox-LDL (30 μg protein/mL) and its extracted lipid (50 μg cholesterol/mL) stimulated PAI-1 release by 42±3% and 29±3% of control cultures, respectively, whereas Ox-LDL and its lipid inhibited t-PA release by 42±4% and 53±3% of control cultures, respectively. Native LDL and its lipid were inactive on their release. Ox-LDL depleted of hydrophilic lipids, which was prepared by the incubation with defatted albumin (an acceptor for hydrophilic lipids), lost both the stimulatory action on PAI-1 and the inhibitory action on t-PA. The extracted lipid from the incubated albumin, which has been found to accept the hydrophilic lipids from Ox-LDL, gained the stimulatory action on PAI-1 and the inhibitory action on t-PA. Ox-LDL depleted of lyso-phosphatidylcholine (LPC), which was prepared by the incubation with phospholipase B, lost the stimulatory effect on PAI-1, whereas the inhibitory effect on t-PA remained present in the Ox-LDL depleted of LPC. The incubation with synthetic palmitoyl LPC (10 μM) stimulated PAI-1 release by 85±7% of control. 25-Hydroxycholesterol (50 μM) and 7-ketocholesterol (50 μM), both of which were generated in Ox-LDL and were found to be transferable from Ox-LDL to defatted albumin by the analysis using gas chromatography-mass spectrometry, inhibited t-PA release by 26±3% and 31±3% of control cultures, respectively. The level of PAI activity in the conditioned medium also increased after the incubation with Ox-LDL or LPC but not native LDL. The results indicate that Ox-LDL stimulates PAI-1 release by the transferable hydrophilic lipid(s), especially LPC, whereas Ox-LDL inhibits t-PA release by oxysterols or other transferable lipid(s) from Ox-LDL to albumin rather than LPC. Lipid products in Ox-LDL may impair endothelial fibrinolysis. (Circulation Research 1993;73:335-343)

KEY WORDS • oxidized low-density lipoprotein • lyso-phosphatidylcholine • oxysterol • plasminogen activator inhibitor-1 • tissue-type plasminogen activator

Atherosclerosis is associated with the alteration of various endothelial functions. We and others have recently demonstrated that acetylcholine-induced endothelium-dependent arterial relaxation (EDR) is impaired in human atherosclerotic coronary arteries. Oxidized low-density lipoprotein (Ox-LDL), an atherogenic lipoprotein that exists in atherosclerotic arterial walls, has been shown to cause the alteration of various cellular functions (reviewed in Reference 7). It has been recently shown that Ox-LDL induces the impairment of EDR, which mimics that observed in atherosclerotic arteries, suggesting that Ox-LDL may contribute to the atherosclerotic impairment of EDR.

It is well known that atherosclerotic coronary artery diseases are frequently associated with thrombotic episodes at the luminal surface of the coronary arteries. We have shown that decreased fibrinolytic activity in the circulating plasma may play an important role in the mechanisms of acute thrombus formation in patients with coronary artery diseases. Net fibrinolytic activity is the result of the balance between the levels of plasminogen activator inhibitor-1 (PAI-1) and tissue-type plasminogen activator (t-PA), both of which are released from vascular endothelial cells. One may
expect that Ox-LDL could modulate the production and secretion of PAI-1 and t-PA from endothelial cells, considering the possible relevance of Ox-LDL to the decreased fibrinolytic activity observed in atherosclerotic cardiovascular diseases. The oxidative modification of low-density lipoprotein (LDL) is associated with many changes in the chemical compositions of LDL, including degradation of apolipoprotein B-100, generation of lipid peroxides and oxysterols, and conversion of phosphatidylcholine to lysophosphatidylcholine (LPC). The present study was, therefore, designed to determine whether Ox-LDL modulates the secretions of PAI-1 and t-PA antigens from cultures of human umbilical vein endothelial cells (HUVECs) and to define which component(s) in Ox-LDL may affect their secretions.

**Materials and Methods**

**Cell Culture**

Primary cultures of HUVECs were obtained by collagenase digestion and were incubated into 12-well plates (Corning) coated with bovine skin gelatin (0.5 mg/mL). Cells were grown to confluence in medium 199 with 15% fetal calf serum, endothelial growth supplement (Collaborative Research, Inc, Bedford, Mass), 80 μg/mL heparin, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere of 95% air–5% CO₂. After reaching confluence, the medium was replaced with medium 199 containing 5% fetal calf serum and antibiotics without growth factor for 48 hours before the experiments. Only primary cultures of HUVECs were used for the experiments. Confluent cultures of HUVECs exhibited the typical cobblestone morphology, and most of those cells contained factor VIII–related antigen as determined using indirect immunofluorescence.

**LDL Preparations**

LDL (density, 1.019 to 1.063 g/mL) was isolated by ultracentrifugation from pooled fresh EDTA (1 mg/mL) plasma harvested from normocholesterolemic persons not ingesting pharmacological amounts of antioxidants. Ox-LDL was prepared by the incubation of native LDL (N-LDL, 100 μg LDL protein/mL) with 5 μM Cu²⁺ in phosphate-buffered saline (PBS) at 37°C for 24 hours as reported previously. Thiobarbituric acid–reactive substances (TBARS) in the incubation mixture averaged 5.1±0.4 nmol malondialdehyde (MDA) equivalents/mL incubation mixture. Electrophoretic mobility of Ox-LDL relative to that of N-LDL was 3.1±0.3. Ox-LDL was resolubilized from the incubation mixture using ultracentrifugation for 24 hours at 4°C (density, 1.21 g/mL). Some aliquots of the collected incubation mixture containing Ox-LDL before subjecting to the ultracentrifugation were used for preparing Ox-LDL depleted of hydrophilic lipids or LPC. The aliquots of the incubation mixture containing Ox-LDL were sterilely incubated at 37°C for 2 hours with 100-fold excess of defatted albumin (0.1 mg LDL protein/10 mg albumin), an acceptor for hydrophilic lipids; then Ox-LDL was recovered from the supernatant after ultracentrifugation for 24 hours at 4°C (density, 1.21 g/mL), yielding albumin-treated Ox-LDL (Alb-Ox-LDL). The incubated albumin with Ox-LDL was also recovered from the infranatant albumin fraction after the ultracentrifugation (treated albumin). During the recoveries of Alb-Ox-LDL and treated albumin, care was taken to avoid contamination with each other, and no contamination was confirmed by the gel electrophoresis. To determine whether LPC is responsible for the biological effects of Ox-LDL, Ox-LDL depleted of LPC was also prepared by treatment of the aliquots of the incubation mixture containing Ox-LDL with phospholipase B (4 U/mL) for 2 hours at 37°C. After the treatment, Ox-LDL was resolubilized with the ultracentrifugation as described above, yielding phospholipase B–treated Ox-LDL (PBlb-Ox-LDL). Control LDL was prepared by the incubation of N-LDL in copper-free incubation mixture, otherwise treated identical to Ox-LDL. Ox-LDL with limited extent of oxidation was prepared by the incubation of N-LDL with 5 μM Cu²⁺ in the combination with 20 μM butylated hydroxytoluene (BHT-Ox-LDL), otherwise treated identical to Ox-LDL. Minimally oxidized LDL was prepared by the stock of N-LDL in the refrigerator at 4°C for 4 months. TBARS in control LDL, BHT-Ox-LDL, and minimally oxidized LDL were 6±2, 9±2, and 7±1 nmol MDA equivalents/mg LDL protein, respectively. Acetylation of LDL (acetyl-LDL) was prepared as described by Basu et al. N-LDL from storage and the treated LDLs were extensively dialyzed against PBS containing 20 μM butylated hydroxytoluene and 50 μM EDTA for 24 hours at 4°C just before use for bioassay. A part of lipid peroxides and hydrophilic products generated in Ox-LDL may be lost during the dialysis, which is aimed to remove Cu²⁺ and potassium bromide, since Ox-LDL after the dialysis contained only traces of TBARS, namely approximately 1 nmol MDA equivalents/mg LDL protein. Therefore, in some experiments, Ox-LDL was prepared by incubation of N-LDL with cultured porcine endothelial cells as in our previous reports. Endothelial cell–modified LDL (EC-LDL) without the ultracentrifugation and dialysis, which contained 24±3 nmol MDA equivalents/mg LDL protein, was directly applied to the bioassay experiments. The extent of LDL oxidation is reported to be variable, although it was very reproducible in our experiments. Therefore, the effects of Alb-Ox-LDL and PBlb-Ox-LDL were examined by comparison with the effect of the same batch of Ox-LDL used for the preparations of Alb-Ox-LDL and PBlb-Ox-LDL. Lipoprotein-free incubation mixtures subjected to the same preparative manipulations as the lipoprotein-containing mixtures were used as controls. The negativity of endotoxin contamination in the lipoprotein preparations was confirmed by the chromogenic Limulus test. Endotoxin levels in all of the lipoprotein preparations used in the present study were less than 10 pg/100 μg LDL protein, at which level there could be no effect on PAI-1 and t-PA release from endothelial cells.

Lipids were extracted from the LDL preparations, treated albumin, and “control albumin” (the albumin recovered from the lipoprotein-free incubation mixture containing defatted albumin and 5 μM Cu²⁺ with the same preparative manipulation as treated albumin, serving as a control) with chloroform-methanol (2:1 by volume) and dried under N₂. Extracted lipids were dispersed in PBS by sonication just before use for the bioassay experiments. Protein concentration was determined by the Lowry method using bovine serum albumin as a standard.
measured by a calorimetric procedure (Wako Chemical Ltd, Tokyo, Japan).8,17,18

Protocol of the Bioassay Experiments and Assay of PAI-1 and t-PA Levels

Confluent monolayers of HUVECs in 12-well plates (3×10^5 cells per well) were washed three times with Hanks balanced salt solution and then preincubated with 1 mL serum-free medium 199 for 1 hour. Thereafter, one of the LDL and lipid preparations was added with or without other additives and incubated for the indicated time. After the incubation, the conditioned medium was collected and then centrifuged at 15 000g for 10 minutes to remove cell debris, made 0.01% with Tween 80, and frozen at −80°C until use. To determine the viable cells after the incubations, cultures of the endothelial cells were stained with a 0.25% solution of trypan blue after the incubations. Cell counts were made after detachment by 0.05% trypsin-EDTA in triplicate with a hemocytometer.

Antigen levels of PAI-1 and t-PA in the conditioned medium were determined by the double antibody sandwich methods with enzyme-linked immunosorbent assay using commercially available kits (TintElize PAI-1, Biopool Inc, Umeå, Sweden; Asserachrom tPA, Diagnostica Stago Inc, Francoville, France) as described previously by us12,13 and others.20 The assays detect both the free and bound proteins. Standard curves obtained from the assay kits showed that the low limit of the measurable range was 1 ng/mL in PAI-1 and 0.1 ng/mL in t-PA. PAI activity (an active form) in the conditioned medium was determined by the method of Verheijen et al13 and was expressed in international units (IU) of t-PA neutralized as described in our previous reports.12,13 The presence of lipoproteins or lipids did not interfere with the assay systems.

Analysis of LPC and Oxysterol

The lipids extracted from the preparations were separated by thin-layer chromatography using silica-gel G plates developed with a solvent mixture containing chloroform-methanol-water (25:10:1 by volume) for the phospholipid analysis.8,17,18 For oxysterol analysis, the lipid extracts were also separated by thin-layer chromatography with benzene–ethyl acetate (3:1 by volume) after addition of 5α-cholestanol as an internal standard.22 Lipid bands were visualized by iodine vapor for the phospholipid analysis or by 2,7-dichlorofluorescein spray for the oxysterol analysis. The lipid band representing LPC was scraped and eluted, and LPC phosphorus in the eluents was quantitated by Bartlett's procedure.8,17,18,23 Lipid bands representing oxysterols and cholesterol were also scraped and eluted. The oxysterols and cholesterol in the eluents were converted to trimethylsilyl derivatives and then analyzed by selected ion monitoring using a gas chromatograph–mass spectrometer (model 5989, Hewlett-Packard Co, Palo Alto, Calif) equipped with a capillary column (OV-17, 0.32 mm id, 25 m).24,25 Column temperature was programmed from 130° to 270°C at a rate of 20°C/min. Oxysterols and cholesterol were identified by the ions at mass to charge ratio (m/z) 327 for the trimethylsilyl ether of 25-hydroxycholesterol, m/z 357 for that of 5α-cholestanol, m/z 367 for that of 7-ketocholesterol, m/z 456 for those of 7α-hydroxycholesterol and 7β-hydroxycholesterol, and m/z 459 for that of cholesterol. The identity of the peaks was confirmed by comparison of retention times relative to 5α-cholestanol with those of the authentic standard of oxysterols and cholesterol.

Protein Synthesis Rate

Endothelial cell protein synthesis was determined by measuring the incorporation of [3H]leucine into trichloroacetic acid (TCA)–precipitable radioactivity in the cell layer and conditioned medium.26 Briefly, confluent endothelial cells were incubated in the serum-free medium containing 10 μCi/mL [3H]leucine with or without Ox-LDL, LPC, or oxysterols for 8 hours. After the incubation, the protein in the conditioned medium was precipitated by 15% TCA. The cell layers were dissolved by the addition of 0.05% Triton X-100 and subsequent sonication. The protein in the cell layer was then precipitated by 15% TCA. The precipitated protein in the medium and cell layers was washed four times in 15% TCA and was then dissolved in 200 μL of 1N NaOH. The radioactivity was then counted. Total protein synthesis rate was calculated as the net radioactivity in the precipitations of the medium and cell layer.

Materials

All reagents for cell culture were obtained from GIBCO, Grand Island, NY. Endothelial growth supplement (bovine, pituitary) was from Collaborative Research. Radioactive materials were from Amersham International, Tokyo, Japan. Oxysterols were from Steraloids Inc, Wilton, NH. Phospholipase B (P-8914) and other chemicals were from Sigma Chemical Co, St Louis, Mo. The assay kit for the chromogenic Limulus test (Toxicolor test) was from Seikagaku Kogyo, Tokyo, Japan. Synthetic phospholipids were dispersed in PBS by sonication just before use. Oxysterols were dissolved in ethanol, and various concentrations of oxysterols were then made in serum-free medium 199 with 0.1% defatted albumin. The culture medium containing oxysterols was added into the incubation medium (serum-free medium 199) with 0.1% defatted albumin. The final concentration of ethanol in the incubation medium was less than 0.1%.

Statistical Analysis

All values were expressed as mean±SEM. Statistical evaluation of the data was performed by Student’s t test for paired or unpaired observations. When more than two groups were compared, analysis of variance was used. A value of P < .05 was considered significant.

Results

Effects of LDLs and Their Associated Lipids on Release of PAI-1 and t-PA

The incubation of endothelial cells with Ox-LDL increased PAI-1 antigen level in the conditioned medium, whereas Ox-LDL decreased t-PA antigen level in the medium as shown in Figs 1 and 2. A time course of the stimulatory effect of Ox-LDL on the release of PAI-1 from endothelial cells is shown in Fig 3. The lipid extracted from Ox-LDL also increased PAI-1 antigen level in the medium, while it decreased t-PA in the medium (Figs 4 and 5). N-LDL and its extracted lipid
were inactive on the release both of PAI-1 and t-PA (Figs 1 through 5). Control LDL, BHT-Ox-LDL, and minimally oxidized LDL had no effect on their release (for PAI-1: control [n=16], 92±6 ng/3×10^5 cells/8 h; control LDL [30 μg protein/mL, n=5], 99±5 ng/3×10^5 cells/8 h; BHT-Ox-LDL [30 μg protein/mL, n=5], 101±7 ng/3×10^5 cells/8 h; minimally oxidized LDL [30 μg protein/mL, n=5], 94±5 ng/3×10^5 cells/8 h; for t-PA: control [n=14], 1.9±0.1 ng/3×10^5 cells/8 h; control LDL, 1.7±0.2 ng/3×10^5 cells/8 h; BHT-Ox-LDL, 1.8±0.1 ng/3×10^5 cells/8 h; minimally oxidized LDL, 1.9±0.2 ng/3×10^5 cells/8 h). As shown in Figs 1 and 2, the maximal effects of Ox-LDL on the release of PAI-1 and t-PA were obtained with the concentration of 30 to 50 μg LDL protein/mL. EC-LDL (30 μg protein/mL) also significantly stimulated PAI-1 release by 34±4% of control cultures and inhibited t-PA release by 48±4% of

**Fig 1.** Graph showing the effects of various concentrations of oxidized (Ox) low-density lipoprotein (LDL) and native LDL on tissue plasminogen activator (t-PA) antigen levels in the conditioned medium. The cultures of human umbilical vein endothelial cells were incubated with Ox-LDL (●) or native LDL (○) for 8 hours, and then the conditioned media were collected. Ox-LDL, but not native LDL, decreases t-PA levels. Each point represents the mean±SEM of five to seven independent experiments. *Significant difference at P<.01 versus control (incubation without LDL).

**Fig 2.** Graph showing the effects of various concentrations of oxidized (Ox) low-density lipoprotein (LDL) and native LDL on tissue-type plasminogen activator (t-PA) antigen levels in the conditioned medium. The cultures of human umbilical vein endothelial cells were incubated with Ox-LDL (●) or native LDL (○) for 8 hours, and then the conditioned media were collected. Ox-LDL, but not native LDL, decreases t-PA levels. Each point represents the mean±SEM of five to seven independent experiments. *Significant difference at P<.01 versus control (incubation without LDL).

**Fig 3.** Time course of plasminogen activator inhibitor-1 (PAI-1) release from the endothelial cells. LDL, low-density lipoprotein; Ox-LDL, oxidized LDL. Cultures of human umbilical vein endothelial cells were incubated for up to 12 hours with or without LDL (30 μg protein/mL). Each point represents the mean±SEM of triplicate experiments.

**Fig 4.** Bar graph showing the effects of incubation with the low-density lipoprotein (LDL) and lipid preparations on plasminogen activator inhibitor-1 (PAI-1) antigen levels in the conditioned medium. N-lipid and Ox-lipid (50 μg cholesterol/mL), the lipids extracted from native LDL (N-LDL) and oxidized LDL (Ox-LDL), respectively; Alb Ox-LDL (30 μg protein/mL), Ox-LDL after treatment with 100-fold excess of defatted albumin and resolation by ultracentrifugation; treated Alb-lipid, the lipid extract of 7.5 mg protein of the infranatant albumin fraction from the incubation mixture of Ox-LDL and defatted albumin after ultracentrifugation in 1 mL incubation medium (an equivalent dose of albumin incubated with 75 μg protein Ox-LDL/mL incubation medium); control Alb-lipid, the lipid extract of 7.5 mg protein of the defatted albumin from the lipoprotein-free incubation mixture with the same preparative manipulation as treated Alb-lipid. The cultures of human umbilical vein endothelial cells were incubated with one of the LDL and lipid preparations for 8 hours, and then the conditioned media were collected. N-LDL and Ox-LDL were incubated with the concentration of 30 μg protein/mL. * Significant difference at P<.01 versus control (n=6 to 16).
TABLE 1. Plasminogen Activator Inhibitor Activity in the Conditioned Medium After Incubation With Low-Density Lipoproteins or Lipids

<table>
<thead>
<tr>
<th>Lipoprotein or Lipid</th>
<th>PAI activity (IU/3x10^5 cells/8 h)</th>
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<tbody>
<tr>
<td>Control</td>
<td>1.6±0.2</td>
</tr>
<tr>
<td>Native LDL</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>3.3±0.3*</td>
</tr>
<tr>
<td>LPC</td>
<td>3.7±0.4*</td>
</tr>
<tr>
<td>PC</td>
<td>1.9±0.2</td>
</tr>
</tbody>
</table>

PAI, plasminogen activator inhibitor; control, incubation of the endothelial cells with lipoprotem-free incubation mixtures subjected to the same preparative manipulations as the lipoprotein-containing mixtures; LDL, low-density lipoprotein; Ox-LDL, oxidized LDL; LPC, lysophosphatidylcholine (10 μM); PC, phosphatidylcholine (10 μM). LDLs were incubated with the concentration of 30 μg LDL protein/mL. Values are mean±SEM (n=6 to 12). *P<.01 versus control.

FIG 5. Bar graph showing the effects of the low-density lipoprotein (LDL) and lipid preparations on tissue-type plasminogen activator (t-PA) antigen levels in the conditioned medium. N-lipid and Ox-lipid (50 μg cholesterol/mL), the lipids extracted from native LDL (N-LDL) and oxidized LDL (Ox-LDL), respectively; Alb Ox-LDL (30 μg protein/mL), Ox-LDL after treatment with 100-fold excess of defatted albumin and resolation by ultracentrifugation; treated Alb-lipid, the lipid extract of 7.5 mg protein of the infranatant albumin fraction from the incubation mixture of Ox-LDL and defatted albumin after ultracentrifugation in 1 mL incubation medium (an equivalent dose of albumin incubated with 75 μg protein Ox-LDL/mL incubation medium); control Alb-lipid, the lipid extract of 7.5 mg protein of the defatted albumin from the lipoprotein-free incubation mixture with the same preparative manipulation as treated Alb-lipid. The cultures of human umbilical vein endothelial cells were incubated with one of the LDL and lipid preparations for 8 hours, and then the conditioned media were collected. N-LDL and Ox-LDL were incubated with the concentration of 30 μg protein/mL. *Significant difference at P<.01 versus control (n=6 to 16).

control cultures. Furthermore, the level of PAI activity in the conditioned medium also increased after the incubation with Ox-LDL but not N-LDL (Table 1). The stimulatory effect of Ox-LDL on PAI-1 release was abolished by incubation with cycloheximide or actinomycin D (incubation with Ox-LDL alone; control cultures). Furthermore, the level of PAT activity was increased after the incubation mixture with Ox-LDL (albumin to LDL protein ratio was 100:1); control albumin, defatted albumin isolated from the lipoprotein-free incubation mixture with the same preparative manipulation as treated albumin; ND, not detectable. Values are mean±SEM (n=8 to 12).

TABLE 2. Lysophosphatidylcholine in the Preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Lysophosphatidylcholine (nmol/mg protein)</th>
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<tbody>
<tr>
<td>Native LDL</td>
<td>20±10</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>480±30</td>
</tr>
<tr>
<td>Alb-Ox-LDL</td>
<td>20±10</td>
</tr>
<tr>
<td>Plb-Ox-LDL</td>
<td>30±10</td>
</tr>
<tr>
<td>Treated albumin</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>Control albumin</td>
<td>ND</td>
</tr>
</tbody>
</table>

LDL, low-density lipoprotein; Ox-LDL, oxidized LDL; Alb-Ox-LDL, albumin-treated Ox-LDL; Plb-Ox-LDL, phospholipase B-treated Ox-LDL; treated albumin, defatted albumin isolated from the incubation mixture with Ox-LDL (albumin to LDL protein ratio was 100:1); control albumin, defatted albumin isolated from the lipoprotein-free incubation mixture with the same preparative manipulation as treated albumin; ND, not detectable. Values are mean±SEM (n=8 to 12).

The oxidation of lipids in LDL yields a variety of amphiphiles, such as LPC, lipid peroxides, and oxysterols, some of which are capable of transferring from Ox-LDL to albumin, a potent acceptor of hydrophilic lipids, as shown in Table 2, Fig 6, and in our previous reports.8,17,18,27 To determine whether the transferable hydrophilic lipids are responsible for the effects of Ox-LDL on PAI-1 and t-PA release, the effect of Alb-Ox-LDL, which was depleted of the hydrophilic lipids, was examined. As shown in Figs 4 and 5, Alb-Ox-LDL lost both of the stimulatory effect on PAI-1 and the inhibitory effect on t-PA release. The lipid extract of treated albumin exerted the stimulatory and inhibitory effects on PAI-1 and t-PA release, respectively, mimicking the effects observed with the lipid extract of Ox-LDL. These results indicate that the transferable lipids from Ox-LDL to the defatted albumin account for the effects of Ox-LDL on the release of PAI-1 and t-PA.

The incubation of the endothelial cells with acetylated LDL, a ligand for the scavenger receptor, tended to increase PAI-1 release, but the increase was not statistically significant, whereas t-PA release was significantly increased after the incubation with acetylated LDL (for PAI-1; control [n=16], 92±6 ng/3x10^5 cells/8 h; acetylated LDL [30 μg protein/mL, n=6], 104±7 ng/3x10^5 cells/8 h [P<NS]; for t-PA; control [n=14], 1.9±0.1 ng/3x10^5 cells/8 h; acetylated LDL [30 μg protein/mL, n=6], 4.1±0.3 ng/3x10^5 cells/8 h [P<.01]). Coincubation with fucoidan, a scavenger receptor blockade, did not influence the stimulatory effect of Ox-LDL on the release of PAI-1 (incubation with Ox-LDL [30 μg protein/mL, n=16] alone, 131±5 ng/3x10^5 cells/8 h; incubation with Ox-LDL+fucoidan [30 μg/mL, n=6], 123±8 ng/3x10^5 cells/8 h [P=NS]).

**Effects of LPC and Oxysterols**

LPC is generated by the action of an intrinsic LDL-associated phospholipase A₂ during the oxidative modification of LDL.28 (Table 2). To determine whether LPC is responsible for the effects of Ox-LDL on PAI-1 and t-PA, the effects of Plb-Ox-LDL, which was depleted of LPC (Table 2), were examined. As shown in
Table 3, Plb-Ox-LDL lost the stimulatory activity on PAI-1, while the inhibitory activity on t-PA remained present in Plb-Ox-LDL. The lipid extract of Plb-Ox-LDL was also inactive on PAI-1 release (control

Table 3. Effects of Low-Density Lipoprotein Preparations and Lipids on Plasminogen Activator Inhibitor-1 and Tissue-Type Plasminogen Activator Secretion

<table>
<thead>
<tr>
<th>PAI-1 antigen</th>
<th>t-PA antigen (ng/3×10⁶ cells/8 h)</th>
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<tbody>
<tr>
<td>Control</td>
<td>94±5</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>131±5*</td>
</tr>
<tr>
<td>Plb-Ox-LDL</td>
<td>93±6</td>
</tr>
<tr>
<td>LPC</td>
<td>170±12*</td>
</tr>
<tr>
<td>PC</td>
<td>84±5</td>
</tr>
</tbody>
</table>

PAI-1, plasminogen activator inhibitor-1; t-PA, tissue-type plasminogen activator; control, incubation of the endothelial cells with lipoprotein-free incubation mixtures subjected to the same preparative manipulations as the lipoprotein-containing mixtures; Ox-LDL, oxidized low-density lipoprotein (LDL); Plb-Ox-LDL, phospholipase B–treated Ox-LDL; LPC, lysophosphatidylcholine (10 μM); PC, phosphatidylcholine (10 μM). Values are mean±SEM. LDLs were incubated with the concentration of 30 μg protein/mL.

*P<.01 versus control (n=6 to 20).

Figure 6. Selected ion monitoring of the ions at mass to charge ratio (m/z) 327 for the trimethylsilyl ether of 25-hydroxycholesterol, m/z 357 for that of 5α-cholestanone, m/z 367 for that of 7-ketocholesterol, m/z 456 for those of 7α-hydroxycholesterol and 7β-hydroxycholesterol, and m/z 459 for that of cholesterol from the extracts of oxidized (Ox) low-density lipoprotein (LDL), albumin-treated Ox-LDL (Alb-Ox-LDL), treated albumin, and control albumin. 1, 7α-Hydroxycholesterol; 2, 7β-hydroxycholesterol; 3, 25-hydroxycholesterol; 4, 7-ketocholesterol. Alb-Ox-LDL is Ox-LDL after treatment with defatted albumin and reisolation by ultracentrifugation. Control albumin is the infranatant albumin fraction recovered from the incubation mixture of Ox-LDL and defatted albumin after ultracentrifugation. Control albumin is the defatted albumin recovered from the lipoprotein-free incubation mixture with the same preparative manipulation as treated albumin. The ratios of peak intensities of 25-hydroxycholesterol and 7-ketocholesterol relative to that of cholesterol decrease in Alb-Ox-LDL as compared with those in Ox-LDL, and the signals corresponding to those of 25-hydroxycholesterol and 7-ketocholesterol are observed in the extract of treated albumin but not in that of control albumin.

The analysis with gas chromatography–mass spectrometry demonstrated that 25-hydroxycholesterol, 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-
hydroxycholesterol were found in Ox-LDL but not N-LDL (Fig 6). Furthermore, 25-hydroxycholesterol and 7-ketocholesterol were found to be transferable from Ox-LDL to albumin. The generation of these oxysterols in Ox-LDL and the transferability of 25-hydroxycholesterol and 7-ketocholesterol were confirmed to be consistent by four independent experiments using different batches of Ox-LDL and N-LDL. The incubation of the endothelial cells with 25-hydroxycholesterol or 7-ketocholesterol decreased t-PA release in a dose-dependent manner (control, 2.0±0.1 ng/3x10^5 cells/8 h; 25-hydroxycholesterol [50 μM], 1.5±0.1 ng/3x10^5 cells/8 h [P<.01 versus control]; 7-ketocholesterol [50 μM], 1.4±0.2 ng/3x10^5 cells/8 h [P<.01 versus control]; n=6 to 8). The results indicated that 25-hydroxycholesterol and 7-ketocholesterol may contribute to the inhibitory effects of the transferable lipids in Ox-LDL on t-PA release from endothelial cells.

**Protein Synthesis and Cell Viability**

To investigate the effect of Ox-LDL, LPC, and oxysterols on the total protein synthesis rate by endothelial cells, the incorporation of [3H]leucine into the TCA-precipitable radioactivity in the cells and medium was analyzed after the incubation of endothelial cells with LPC, oxysterols, or the same batch of Ox-LDL used in the experiment examining the effects on PAI-1 and t-PA release. Both Ox-LDL and LPC were found to increase the total protein synthesis rate as shown in Fig 7. 25-Hydroxycholesterol and 7-ketocholesterol at the concentration of 50 μM suppressed the total protein synthesis rate by 4±1% and 7±2% of control cultures, respectively. However, 25-hydroxycholesterol and 7-ketocholesterol at the concentration of 75 μM significantly suppressed the total protein synthesis rate by 14±3% and 53±6% of control cultures, respectively. Trypan blue staining revealed that no cell death was found after the incubations for 8 hours with Ox-LDL up to the concentration of 100 μg/mL, LPC up to 15 μM, and the oxysterols up to 50 μM.

**Discussion**

The present study demonstrated that Ox-LDL stimulated PAI-1 release while it inhibited t-PA release from the cultured HUVECs. Furthermore, these effects of Ox-LDL were caused by the transferable lipids from Ox-LDL to albumin and were not mediated by the scavenger receptor--dependent mechanism. Oxidative modification of LDL is associated with production of various hydrophilic lipids, which are transferable from Ox-LDL to albumin. Furthermore, these effects of Ox-LDL were caused by the transferable lipid(s) from Ox-LDL to albumin. Certain oxysterols are known to be cytotoxic, as shown in the present study, and modulate endothelial functions.

The present study using gas chromatography–mass spectrometry showed that Ox-LDL contained 25-hydroxycholesterol and 7-ketocholesterol, both of which were transferable from Ox-LDL to albumin and inhibited t-PA release. These results indicate that 25-hydroxycholesterol and 7-ketocholesterol participate in the inhibitory effect of Ox-LDL on t-PA release from endothelial cells. Oxidation of lipids in LDL also yields a variety of fatty acid peroxides, some of which are also capable of transferring to albumin. It remains to be determined whether the lipid peroxides affect PAI-1 and t-PA release from endothelial cells. The present study, however, showed that EC-LDL, which contained lipid peroxides, exerted very similar and equivalent effects on PAI-1 and t-PA release as those obtained with the reisolated and dialyzed Ox-LDL, which contained a smaller amount of lipid peroxides. Thus, it is less likely that lipid peroxides play a major role in the observed effects of Ox-LDL on PAI-1 and t-PA release, although we cannot completely exclude the potential contribution of lipid peroxides to the actions of Ox-LDL. LPC, one of the transferable lipids in Ox-LDL, did not mimic the inhibitory effect exerted by Ox-LDL on t-PA release, but it stimulated t-PA release. The stimulatory effects of LPC and acetyl-LDL on t-PA release make it difficult to interpret the mechanism of the inhibitory action of Ox-LDL on t-PA. The inhibitory action exerted by oxysterols or other hydrophilic lipid(s) in Ox-LDL probably overwhelms the stimulatory actions by LPC and the scavenger receptor–mediated process.

The present study demonstrated that both Ox-LDL and LPC stimulated cell protein synthesis in primary cultures of HUVECs. Furthermore, the present experiments using cycloheximide and actinomycin D showed that protein synthesis and mRNA were required for the stimulatory actions of Ox-LDL and LPC on PAI-1 release, suggesting that the increased levels of PAI-1 may reflect de novo synthesis of PAI-1 through induc-
tion of mRNA. LPC and other hydrophilic lipids are capable of transferring to accessible membrane or macromolecular acceptors through the aqueous phase.29 Recently, we have shown that LPC in Ox-LDL is transferred and incorporated into endothelial cells in an apoprotein-independent manner,8,27 and the transferred LPC directly activates protein kinase C in HUVECs and modulates endothelial functions through a pathway involving protein kinase C activation.24 Several studies have shown that protein kinase C activation results in PAI-1 synthesis and release from endothelial cells.35,36 Currently, we are in the process of determining whether activation of protein kinase C by LPC may be one of the possible mechanisms of the intracellular regulations responsible for the stimulatory effect of Ox-LDL on PAI-1 release. The present study demonstrated that the maximal effects of Ox-LDL on the release of PAI-1 and t-PA were obtained with the concentration of 30 to 50 μg LDL protein/mL, and there was the absence of a dose effect between 30 and 100 μg LDL protein/mL. The transfer process of amphiphiles is reported to be almost independent on the concentrations of the donor (Ox-LDL) and the acceptor (endothelial cell surface membrane),29 which may be one of the possible reasons explaining the lack of a dose effect between 30 and 100 μg LDL protein/mL.

A recent study has reported that ultraviolet-treated LDL also increased PAI-1 secretion from cultured endothelial cells.37 Chemical modifications in ultraviolet-treated LDL are, however, quite different from those in cell-induced Ox-LDL or ion-treated Ox-LDL as used in the present study.38 The study did not clarify the active component(s) in modified LDL, which caused the increase of PAI-1 secretion.

Several clinical studies have revealed that the decreased fibrinolytic activity in the circulating plasma is associated with angina pectoris and previous myocardial infarction.12,13,39,40 The fibrinolytic activity in the circulating plasma is regulated by the balance between the levels of PAI-1 and t-PA, both of which are produced in various tissues, including endothelial cells, and secreted into the circulating plasma.14,15,41 It remains undetermined whether the fibrinolytic state on the endothelial cell surface may be altered in atherosclerotic arteries. A recent report has shown that PAI-1 gene expression increases in the luminal endothelial cells of the atherosclerotic arteries,62 suggesting that endothelial fibrinolytic activity may be impaired in the atherosclerotic arterial walls. The study, however, did not clarify the mechanism(s) explaining the induction of PAI-1 expression in the endothelial cells of atherosclerotic arteries. Our study suggests that LPC in Ox-LDL, which exists in the subendothelial space of the atherosclerotic arterial walls, may contribute to the increased PAI-1 gene expression in the endothelial cells of the atherosclerotic arteries. Furthermore, the present study demonstrated that Ox-LDL and LPC increased PAI-1 activity, which plays a predominant role in the fibrinolytic activity. Impairment of endothelial fibrinolysis by Ox-LDL could promote fibrin deposition or the thrombotic formation on the surface of atheroma plaques, resulting in the vascular occlusion or further progress of atherosclerosis.

In conclusion, Ox-LDL stimulates PAI-1 release by the transferable hydrophilic lipid(s), especially LPC, whereas Ox-LDL inhibits t-PA release by oxysterols or transferable lipid(s) other than LPC. Transferable lipid products in Ox-LDL may impair endothelial fibrinolysis.

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