Original Contributions

LysoPhosphatidylcholine in Oxidized Low-Density Lipoprotein Increases Endothelial Susceptibility to Polymorphonuclear Leukocyte–Induced Endothelial Dysfunction in Porcine Coronary Arteries

Role of Protein Kinase C

Seigo Sugiyama, Kiyotaka Kugiyama, Masamichi Ohgushi, Kazuteru Fujimoto, Hirofumi Yasue

Abstract We have shown that transferred lysophosphatidylcholine (lysoPC) from oxidized low-density lipoprotein (Ox-LDL) to endothelial surface membrane activates protein kinase C (PKC) in endothelial cells, suggesting that Ox-LDL could alter endothelial functions through PKC activation. The purposes of the present study were to examine whether the endothelial susceptibility to polymorphonuclear leukocytes (PMNs) may be altered in Ox-LDL–treated coronary arteries, which have properties closely resembling those observed in atherosclerotic arteries, and to determine the mechanism(s) by which Ox-LDL may affect the endothelial susceptibility to PMNs. Isolated porcine coronary arteries were cannulated and perfused with oxygenated culture medium with or without LDLs or lipids at a constant flow (37°C, pH 7.4). The treatment of porcine coronary arteries with Ox-LDL increased endothelial adhesiveness to PMNs and augmented PMN-induced impairment of endothelial-dependent arterial relaxation (EDR). Furthermore, Ox-LDL stimulated the expression of intercellular adhesion molecule-1 (ICAM-1) in the porcine coronary arterial endothelium. These effects of Ox-LDL were not mediated by the scavenger-receptor–mediated process but were attributed to lysoPC in Ox-LDL. Blocking of the PMN adhesion to endothelium by using anti-CD18 monoclonal antibody abolished the PMN-induced impairment of EDR. Coincubation with staurosporine or calphostin C, inhibitors of PKC, during treatment of the arteries with Ox-LDL or lysoPC attenuated the augmentative effects of Ox-LDL and lysoPC on endothelial ICAM-1 expression, endothelial adhesiveness to PMNs, and PMN-induced EDR impairment. Treatment of the arteries with phorbol 12-myristate 13-acetate, a potent stimulator of PKC, induced ICAM-1 expression and enhanced the endothelial adhesiveness to PMNs and PMN-induced EDR impairment, mimicking the effects of Ox-LDL. These results suggest that lysoPC in Ox-LDL induces endothelial ICAM-1 expression, which facilitates PMN adherence to endothelium and the subsequent augmentation of PMN-induced EDR impairment. PKC activation in endothelial cells by lysoPC in Ox-LDL may at least in part be involved in these effects of Ox-LDL. LysoPC in Ox-LDL increases endothelial susceptibility to PMN-induced endothelial dysfunction. (Circ Res. 1994;74:565–575.)

Key Words • lysophosphatidylcholine • oxidized low-density lipoproteins • protein kinase C • endothelium • intercellular adhesion molecule-1

Atherosclerosis is associated with the alteration of various endothelial functions.1–4 We3 and others4,5 have recently demonstrated that endothelium-dependent arterial relaxation (EDR) is impaired in human atherosclerotic coronary arteries. Oxidized low-density lipoprotein (Ox-LDL), an atherogenic lipoprotein that exists in the atherosclerotic arterial walls,6 has been shown to cause alterations of various endothelial functions.7–10 We have recently shown that Ox-LDL induced EDR impairment, which mimics that observed in human atherosclerotic arteries,7 suggesting that Ox-LDL may contribute to the atherosclerotic impairment of EDR. Furthermore, we have recently demonstrated that protein kinase C (PKC) activated by lysophosphatidylcholine (lysoPC), which is transferred from Ox-LDL to the endothelial surface membrane, may partly be involved in the mechanism(s) of the endothelial functional alterations by Ox-LDL.7,11,12

Polymorphonuclear leukocytes (PMNs), a major class of leukocytes, has an important role in the endothelial injury or dysfunction in many pathophysiological conditions, such as inflammation or ischemia/reperfusion in acute myocardial infarction,13–17 and PMN-induced endothelial injury could occur in the arteries already afflicted with atherosclerosis and other vascular diseases. It seems to be clinically important to elucidate the endothelial susceptibility to PMNs in atherosclerotic arteries. It has been shown that atherosclerosis enhances the endothelial adhesiveness to leukocytes,1,18 which suggests that the endothelial susceptibility to PMNs may increase in atherosclerotic arteries. However, the underlying mechanism(s) responsible for the enhanced endothelial adhesiveness to leukocytes in the atherosclerotic arteries remains unclear. One may expect that Ox-LDL may play a role in the altered interactions between leukocytes and endothelium in the
atherosclerotic arteries. Therefore, the purposes of the present study were to examine whether the endothelial susceptibility to PMNs may be altered in the Ox-LDL–treated coronary arteries, which have properties closely resembling those observed in the atherosclerotic arteries, and to determine the mechanism(s) by which Ox-LDL may affect the endothelial susceptibility to PMNs. We also examined whether PKC activation in endothelial cells plays a role in the effects of Ox-LDL.

Materials and Methods
Preparation of Porcine PMNs
Suspensions of PMNs were prepared from citrate-anticoagulated venous blood from domestic Yorkshire pigs by using the standard techniques of dextran sedimentation, sequential Ficoll-Hypaque gradient separation, and hypotonic lysis of erythrocytes with sodium chloride solution, as described in our previous study. The preparations were composed of >96% PMNs by Türk stain (0.01% of methylrosaniline chloride and 1.0% acetic acid), neutrophil alkaline phosphatase, and neutrophil esterase stain and of >98% viable cells by the trypan blue dye exclusion test. Isolated PMNs were suspended in serum-free DMEM at a concentration of 1 × 10⁵ cells per milliliter. In some experiments, PMNs were activated by opsonized zymosan at a concentration of 0.5 mg/mL just before usage. Opsonized zymosan was prepared as described previously.

Preparation of Porcine Coronary Arteries
The porcine hearts were freshly removed from normal domestic Yorkshire pigs within 10 minutes after death, and the left anterior descending coronary arteries were dissected (5-cm length) and immersed in the cold modified Krebs-Henseleit bicarbonate solution (KHS, pH 7.4) composed of (mmol/L) NaCl 118, KCl 5.9, NaHCO₃ 1.2, MgSO₄ 1.2, CaCl₂ 2.0, NaH₂PO₄ 25, and glucose 10. Siliconized polypropylene connectors were attached to both ends of the coronary arteries, nonvascular tissues adherent to the adventitial surface of the arteries were carefully removed, and all side branches were ligated carefully with silk suture. Special care was taken not to touch the endothelium. The arteries were placed in a bath filled with oxygenated KHS (300 mL) for 20 minutes at 37°C and then perfused with oxygenated (15% O₂, 5% CO₂, 80% N₂) serum-free DMEM with or without lipoproteins or lipids at a constant flow (0.5 mL/min) for 30 minutes at 37°C. To determine the possible involvement of PKC and the scavenger receptor–mediated mechanisms in the effects of Ox-LDL, some arteries were perfused for 5 minutes by serum-free DMEM with or without the PKC inhibitor or the scavenger receptor blocker and then perfused with solution containing Ox-LDL or lysophosphatidic acid, in which the PKC inhibitor or the scavenger receptor blocker remained present, by the same procedure described above. After the exposure to lipoproteins or lipids, the lumen of the coronary arteries was washed with serum-free DMEM at a constant flow (2.0 mL/min) for 10 minutes to avoid contamination of lipoproteins, lipids, or other additives with the next perfusate. The arteries were then perfused with serum-free DMEM containing autologous PMNs (1 × 10⁵ cells per milliliter) activated by the opsonized zymosan at a constant flow (0.5 mL/min) for 60 minutes at 37°C. In some experiments, the arteries were perfused with serum-free DMEM containing the activated PMNs pretreated with monoclonal antibody (MAb) against glycoprotein CD18 (MEM-48, 20 µg per 1 × 10⁵ PMNs) or nonactivated PMNs (1 × 10⁵ cells per milliliter). After perfusion with PMNs, the lumen of the arteries was washed again with serum-free DMEM at a constant flow (2.0 mL/min) for 10 minutes to remove nonadherent PMNs. The treated arteries were used for the organ chamber experiment and the experiment examining the endothelial adhesiveness to PMNs. Some of the arteries treated with lipoproteins or lipids before exposure to the PMNs were used for the immunohistochemical study.

PMN Adherence to Coronary Arterial Endothelium
To detach the adhered PMNs from the endothelium of the treated arteries, the lumen was filled with 0.05% trypsin/0.02% EDTA in Hanks’ balanced salt solution, and the end of the artery was clamped, and then the preparation was kept in the organ bath filled with DMEM for 10 minutes at 37°C. After the incubation, the lumen was flushed twice with 5 mL DMEM containing 15% fetal calf serum and trypsin inhibitor. The mixture was collected and centrifuged at 300g for 10 minutes. The harvested cells were suspended in the buffer and then stained with Türk stain, and the number of PMNs was counted in a hemocytometer. The cells were identified by neutrophil alkaline phosphatase and esterase stain. The arteries were cut and opened to measure the endothelial luminal surface area (in square millimeters). The number of the adhered PMNs is calculated as the ratio of the number of PMNs adhered to 1 mm² of endothelial luminal surface (PMNs per square millimeter) to the number of the PMNs adhered to the endothelium of the coronary arteries treated by the perfusate containing no lipoproteins or lipids (relative adhesion-to-control ratio). After the trypsinization, no PMNs were found on the endothelial luminal surface of the arteries by an examination with a stereoscopic microscope after staining with Türk solution.

Organ Chamber Experiments
The coronary arteries pretreated with lipoproteins or lipids and the subsequent exposure to the PMNs were cut into 3-mm-long ring segments, and the rings were suspended by stainless-steel hooks in the organ chambers filled with KHS. This solution was aerated with 15% O₂/5% CO₂/80% N₂ and maintained at 37°C. During these procedures, care was taken not to injure the luminal surface. The rings were then stretched to an optimum basal tension of 4 g, and the isometric tension was monitored using a force transducer (UL-20GR, Minewa, Tokyo, Japan) and a polygraph (SR6211, Graphtec, Tokyo, Japan) as described in our previous reports. After equilibration for 120 minutes, the rings were exposed to 60 mmol/L KCl to examine the contractile response of vascular smooth muscle. After washing, the rings were contracted with prostaglandin F₂₅ (PGF₂₅, 30 µmol/L) and tested with increasing concentrations of various vasodilators (0.001 to 1.0 µmol/L thrombin, 0.1 to 100 nmol/L bradykinin, and 0.01 to 100 µmol/L sodium nitroprusside). The extent of the maximum relaxation was expressed as the maximum percent decrease of the contraction elicited by PGF₂₅ (30 µmol/L). For relaxations, the effective concentrations of agonists causing 50% relaxation (EC₅₀) and 30% relaxation (EC₃₀) of the contraction by PGF₂₅ were calculated from each concentration-response curve.

Immunohistochemical Demonstration of Intercellular Adhesion Molecule-1 in Coronary Arterial Endothelium
The arteries treated with lipoproteins or lipids were immediately fixed with the Zamboni fixative at 4°C for 6 hours. They were then rinsed and embedded in O.C.T. compound (Miles Inc, Elkhart, Ind), quickly frozen, and stored at −80°C. Frozen tissue specimens were cut into 8-µm-thick sections. The sections were stained by using the immunoperoxidase method. Briefly, the sections were incubated with nonimmune horse and mouse sera at room temperature for 1 hour and then incubated with anti-intercellular adhesion molecule-1 (ICAM-1) MAb (ICAM-1 MAB-15.2, mouse IgG1, 10 µg/mL) at 4°C overnight. After washing, the sections were incubated with biotinylated anti-mouse IgG (host animal, horse) at 4°C overnight. Furthermore, they were incubated with Vectastain avidin-biotin complex (ABC) reagent at room temperature for 60 minutes. The final reaction was achieved by incubating with freshly prepared 3,3’,5-diaminobenzidine tetrahydrochloride solution with 0.01% hydrogen peroxide. The nuclei were counterstained with hematoxylin. The presence or absence of immunoreactive products
for anti-ICAM-1 MAb were judged by three independent observers.

**Lipoprotein Preparations**

Low-density lipoprotein (LDL; density, 1.019 to 1.063 g/mL) was isolated by ultracentrifugation from the pooled fresh normal human plasma with EDTA (1 g/L). Ox-LDL was prepared by the incubation of native LDL (N-LDL, 100 μg protein per milliliter) with 5 μmol/L Cu²⁺ in phosphate-buffered saline (PBS) under sterile conditions at 37°C for 24 hours as reported previously.²⁻,²² Thiobarbituric acid–reactive substances in the incubation mixture containing Ox-LDL averaged 4.2±0.4 nmol malondialdehyde equivalents per milliliter of incubation mixture, and the electrophoretic mobility of Ox-LDL relative to that of N-LDL was 3.1±0.2. Ox-LDL was reisolated from the incubation mixture using ultracentrifugation (density, 1.21 g/mL) for 24 hours at 4°C. To determine whether lysoPC is responsible for the biologic effects of Ox-LDL, some aliquots of the incubation mixture containing Ox-LDL before subjecting to the ultracentrifugation were used for preparing Ox-LDL depleted of lysoPC. The aliquots of the incubation mixture containing Ox-LDL were steriley incubated with the defatted albumin (10 mg protein per milliliter) or phospholipase B (4 U/mL) for 2 hours at 37°C,²² and then the treated Ox-LDLs (albumin-treated Ox-LDL [Alb-Ox-LDL] and phospholipase B–treated Ox-LDL [PlB-Ox-LDL]) were recovered from the supernatant after ultracentrifugation (density, 1.21 g/mL) for 24 hours at 4°C. The albumin incubated with Ox-LDL (“treated albumin”) was also recovered from the infranatant albumin fraction after the ultracentrifugation, as described previously.²¹ 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 gel electrophoresis. Acetylation of LDL (acyt-tyl-LDL) was performed by the method described by Basu et al.²³ N-LDL from storage and the treated LDLs were extensively dialyzed against PBS containing 20 μmol/L butylated hydroxytoluene and 50 μmol/L EDTA for 24 hours under a nitrogen stream at 4°C just before use in the bioassay experiments. Lipids were extracted from N-LDL, Ox-LDL, treated albumin, and untreated albumin (control albumin) with chloroform/methanol (2:1 [vol/vol]) and dried under N₂.²⁻²² Lipid extracts were used for the bioassay experiments after dispersion in PBS by sonication. Some aliquots of the extracted lipids were analyzed by thin-layer chromatography using silica gel G plates developed with a solvent mixture containing chloroform/methanol/water (25:10:1 [vol/vol/vol]).²¹,²² The lipid band representing lysoPC was eluted with a solvent mixture containing chloroform/methanol/water (5:5:1 [vol/vol/vol]), and phospholipid phosphorus in the eluates was measured by the Bartlett procedures²⁶ with a phospholipid assay kit (Wako Chemical, Osaka, Japan). Cholesterol content was measured by a calorimetric procedure, and protein content was determined by the method of Lowry using bovine serum albumin as a standard.²⁵ Endotoxin levels in the LDL preparations and lipids were <10 pg/100 μg LDL measured by the chromogenic limulus test.²¹

**Drugs**

Staurosporine and calphostin C were obtained from Kyowa Hakko, Tokyo, Japan. HA1004 was from Daiichi-Seikagaku, Tokyo, Japan. PGF₂α was from Ono Pharmaceuticals, Osaka, Japan. A mouse anti-human CD18 MAB (MEM-48, IgG) and mouse anti-ICAM-1 MAB-15.2, (IgG) were from Biodesigen Inc, Kennebunkport, Me. The Vectastain ABC kit was from Vector Laboratories, Burlingame, Calif. AA861 was from Wako Chemical. All reagents for cell culture were from Gibco, Grand Island, NY. Synthetic lysophosphatidylcholine (palmitoyl), phosphatidylcholine (dipalmityl), zymosan, fucoidin, polysinosinic acid (5‘) (Poly[I]), phospholipase B (P-8914), and other chemicals were from Sigma Chemical Co, St Louis, Mo. Phospholipids were dispersed in PBS by sonication just before usage. Phorbol 12-myristate 13-acetate (PMA), AA861, staurosporine, and calphostin C were dissolved in dimethyl sulfoxide, and the total volume of dimethyl sulfoxide as a drug vehicle in the final solution was <0.1% (vol/vol).

**Data Analysis**

Results are expressed as mean±SEM. Statistical evaluation of the data was performed by Student’s t test for unpaired observations. When more than two groups were compared, ANOVA was used. Values were considered to be statistically different at P<.05.

**Results**

**Endothelial Adhesiveness to PMNs**

The pretreatment of the coronary arteries with Ox-LDL (50 μg protein per milliliter) or its extracted lipid (50 μg cholesterol per milliliter) increased the number of the activated PMNs adhered to the luminal surface of the coronary arteries as compared with the arteries pretreated by perfusate without lipoproteins or lipids (control), whereas the pretreatment of the arteries with N-LDL or its extracted lipid did not significantly increase the number of the PMNs adhered to the endothelium (Fig 1).

During the oxidative modification of LDL, an intrinsic LDL–associated phospholipase A₂ hydrolyses phosphatidylcholine (PC) to lysoPC,²⁶ which is capable of transferring from Ox-LDL to defatted albumin and is degraded by phospholipase B²¹,²² (Table 1). To determine whether lysoPC is responsible for the action of Ox-LDL, the effects of Alb-Ox-LDL and PlB-Ox-LDL, both of which were depleted of lysoPC, were examined. The pretreatment of the arteries with Alb-Ox-LDL or PlB-Ox-LDL failed to increase the number of the PMNs adhered to endothelium (Fig 1). The pretreatment with the lipid extract from treated albumin, which accepted lysoPC from Ox-LDL (Table 1), increased the number of the PMNs adhered to endothelium, whereas the lipid extract from control albumin had no effect. The pretreatment of the arteries with lysoPC (5 μmol/L) but not PC increased the number of the PMNs adhered to endothelium (Fig 1). Pretreatment of the arteries with 100 μg protein per milliliter of Ox-LDL or 10 μmol/L of lysoPC also significantly increased the number of PMNs adhered to endothelium (relative adhesion ratio: medium-only perfusate, 1.0±0.1; Ox-LDL, 2.6±0.3; and lysoPC, 2.4±0.3; P<.01, Ox-LDL and lysoPC versus medium-only perfusate; n=6 or 7).

The adhesion of the nonactivated PMNs to endothelium was also augmented by pretreatment of the arteries with Ox-LDL (50 μg protein per milliliter) or lysoPC (5 μmol/L) but not with N-LDL (50 μg protein per milliliter) (relative adhesion ratio: medium-only perfusate, 1.0±0.1; N-LDL, 1.1±0.1; Ox-LDL, 2.1±0.2; and lysoPC, 2.0±0.2; P<.01, Ox-LDL and lysoPC versus medium-only perfusate; n=6 to 8). The increase of the endothelial adhesiveness to PMNs observed in the Ox-LDL–treated arteries was also confirmed by scanning electron microscopy (data not shown).

The results indicated that Ox-LDL increased the endothelial adhesiveness to PMNs, and the transferable hydrophilic lipid from Ox-LDL to albumin, especially lysoPC, was primarily responsible for the effects of Ox-LDL.
Role of Adhesion Molecule

Incubation of the coronary arteries with Ox-LDL (50 to 100 μg protein per milliliter) or lysoPC (5 to 10 μmol/L) but not with N-LDL increased ICAM-1 expression in the coronary arterial endothelium by the immunohistochemical study using anti–ICAM-1 MAb (Fig 2).

Table 1. Lysophosphatidylcholine in the Preparations

<table>
<thead>
<tr>
<th>Amount of Lysophosphatidylcholine, nmol/mg protein</th>
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<tbody>
<tr>
<td>Native LDL</td>
</tr>
<tr>
<td>22±10</td>
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<tr>
<td>Ox-LDL</td>
</tr>
<tr>
<td>510±40</td>
</tr>
<tr>
<td>Alb-Ox-LDL</td>
</tr>
<tr>
<td>25±13</td>
</tr>
<tr>
<td>PIB-Ox-LDL</td>
</tr>
<tr>
<td>28±15</td>
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<tr>
<td>Treated albumin</td>
</tr>
<tr>
<td>3.5±0.1</td>
</tr>
<tr>
<td>Control albumin</td>
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<tr>
<td>Not detectable</td>
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</table>

Ox-LDL indicates oxidized low-density lipoprotein (LDL); Alb-Ox-LDL, Ox-LDL treated with defatted albumin; PIB-Ox-LDL, Ox-LDL treated with phospholipase B; treated albumin, the treated albumin recovered from the infranatant fraction of the incubation mixture containing Ox-LDL and defatted albumin after ultracentrifugation (ratio of albumin to LDL protein, 100:1); and control albumin, the incubated albumin recovered from the lipoprotein-free incubation mixture containing defatted albumin with the same preparative manipulation as treated albumin. Values are mean±SEM; n=6 to 8.

The effects of LDL or lysoPC on ICAM-1 expression were confirmed to be consistent by four independent experiments using a different batch of Ox-LDLs and N-LDLs. The PMN adherence to endothelium of the nontreated arteries was prevented when the activated PMNs were pretreated with MAb MEM-48 (20 μg per 1×10⁶ PMNs), which is one of the adhesion molecules on PMNs and a ligand for ICAM-1 on the endothelial cells. Furthermore, the PMN adherence to arterial endothelium treated with Ox-LDL (50 μg protein per milliliter) or lysoPC (5 μmol/L) was also prevented when the activated PMNs were pretreated with MAb MEM-48 (relative adhesion ratio: arteries treated with medium alone plus nontreated PMNs, 1.00±0.08; Ox-LDL–treated arteries plus nontreated PMNs, 2.43±0.33; and Ox-LDL–treated arteries plus MEM-48–treated PMNs, 0.26±0.04 [P<.01 vs Ox-LDL–treated arteries plus nontreated PMNs]; n=10 or 11; and lysoPC–treated arteries plus nontreated PMNs, 2.18±0.18; and lysoPC–treated arteries plus MEM-48–treated PMNs, 0.22±0.04 [P<.01 vs lysoPC–treated arteries plus nontreated PMNs]; n=9 and 11, respectively).

PMN-Induced Impairment of Vasorelaxation

We previously demonstrated that endothelium-dependent relaxation of porcine coronary arteries in response to thrombin was impaired after incubation of the arteries with 100 μg protein per milliliter of Ox-LDL or 10 μmol/L of lysoPC in the organ chamber. In the present experiment, vasorelaxation in response to...
Thrombin was not significantly impaired when the arteries were perfused for 30 minutes with <75 μg protein per milliliter of Ox-LDL or 7.5 μmol/L of lysoPC (Table 2). Therefore, the concentrations of 0.5 mg protein per milliliter in Ox-LDL and 5.0 μmol/L in lysoPC were the subthreshold concentrations for the induction of the vasorelaxatory impairment in response to thrombin in this experiment. The exposure of the porcine coronary arteries to the activated PMNs (10^7 cells per milliliter) alone for 60 minutes significantly inhibited vasorelaxations in response to thrombin and bradykinin (Table 3). Vasorelaxation of the arteries in response to sodium nitroprusside was, however, completely preserved after the exposure to the activated PMNs alone. The preincubation of the PMNs (10^7 cells per milliliter) with MAb anti-CD18 (20 μg per 10^7 PMNs) abolished the PMN-induced impairment of the vasorelaxation in response to thrombin and bradykinin (maximum relaxation [%] and EC_{50} [μmol/L], respectively, for thrombin: activated PMNs without MAb anti-CD18, 58.7±2.5 and 35±5; and activated PMNs treated with MAb anti-CD18, 95.3±3.1 and 15±3 [P<.01 versus the respective relaxation and EC_{50} values in the activated PMNs without MAb anti-CD18]; n=10 and 11, respectively; maximum relaxation [%] and EC_{50} [nmol/L], respectively, for bradykinin: activated PMNs without MAb anti-CD18, 115.7±1.2 and 1.8±0.1; and for activated PMNs treated with MAb anti-CD18, 117.3±1.1 and 1.1±0.1 [P<.05 versus the EC_{50} value in the activated PMNs without MAb anti-CD18]; n=9 each). The study using scanning electron microscopy showed that the endothelial cell lining of the arteries was completely preserved, but there were a few endothelial blebs after exposure to the activated PMNs.

The pretreatment of the coronary arteries with Ox-LDL (50 μg protein per milliliter) for 30 minutes and the subsequent exposure to the activated PMNs (10^7 cells per milliliter) significantly augmented the PMN-induced impairment of vasorelaxation in response to thrombin as compared with the pretreatment of the arteries with medium alone and the subsequent exposure to the activated PMNs (Fig 3). Pretreatment of the arteries for 30 minutes with the extracted lipid from Ox-LDL (Ox-lipid, 50 μg cholesterol per milliliter), which alone did not affect the vasorelaxation, significantly augmented the PMN-induced impairment of the vasorelaxation in response to thrombin (Fig 4). On the other hand, pretreatment of the arteries with N-LDL (50 μg protein per milliliter) or its extracted lipid (N-lipid, 50 μg cholesterol per milliliter) for 30 minutes and the subsequent exposure to the activated PMNs did not significantly augment the impairment of the vasorelaxation as compared with the pretreatment of the arteries with medium alone and the subsequent exposure to the activated PMNs. Pretreatment with Alb-Ox-LDL or PIB-Ox-LDL failed to augment the PMN-induced impairment of the vasorelaxation. Pretreatment with lysoPC (5.0 μmol/L) but not PC (5.0 μmol/L) significantly augmented the PMN-induced impairment of the vasorelaxation in response to thrombin (Figs 3 and 4). The PMN-induced impairment of vasorelaxation in response
The vasorelaxation in response to sodium nitroprusside was completely preserved after the pretreatment of the arteries with Ox-LDL or lysoPC and the subsequent exposure to the activated PMNs (maximum relaxation [%] and E<sub>C50</sub> [μmol/L], respectively: medium-only perfusate plus PMNs, 112.4±1.8 and 0.34±0.06; Ox-LDL plus PMNs, 114.3±2.2 and 0.36±0.07; and lysoPC plus PMNs, 110.4±2.5 and 0.38±0.05; P=NS; n=8 to 10).

The exposure of the arteries for 60 minutes to the nonactivated PMNs (1×10<sup>6</sup> cells per millilitre) alone did not significantly inhibit the vasorelaxation in response to thrombin. However, the subsequent exposure of the arteries to the nonactivated PMNs (1×10<sup>6</sup> cells per millilitre) after pretreatment with Ox-LDL (50 μg protein per millilitre) or lysoPC (5.0 μmol/L) significantly impaired vasorelaxation in response to thrombin (maximum relaxation [%] and E<sub>C50</sub> [U/L], respectively: medium-only perfusate plus nonactivated PMNs 96.8±2.1 and 13±2; Ox-LDL plus nonactivated PMNs, 81.3±3.1 and 21±2; and lysoPC plus nonactivated PMNs, 80.5±3.2 and 20±2; P<.01 [maximum relaxation] and P<.05 [E<sub>C50</sub>] in Ox-LDL plus nonactivated PMNs and lysoPC plus nonactivated PMNs versus the respective values in the medium-only perfusate plus nonactivated PMNs; n=7 to 9). Coincubation with indomethacin or AA861 (5-lipoxygenase inhibitor) during the pretreatment with Ox-LDL did not change the augmentative effect of Ox-LDL on the PMN-induced impairment of vasorelaxation in response to thrombin (data not shown). The contractile responses to KCl (60 mmol/L) and PGF<sub>2α</sub> (30 μmol/L) were not significantly different among the treatments of the coronary arteries (KCl [μmol/L] and PGF<sub>2α</sub> [μmol/L], respectively: medium-only perfusate, 5.4±0.2 and 6.0±0.2; treatment with activated PMNs alone, 5.6±0.2 and 6.1±0.2; treatment with Ox-LDL alone, 5.5±0.2 and 6.1±0.2; and treatment with Ox-LDL and subsequent exposure to activated PMNs, 5.6±0.2 and 6.2±0.2; n=7 to 9).

Role of PKC Activation
Recently, we have shown that lysoPC in Ox-LDL is directly transferred to endothelial surface membrane and is capable of activating PKC in endothelial surface membrane. Therefore, to determine the possible role of PKC activation in the observed actions of Ox-LDL, the effects of staurosporine or calphostin C, PKC inhibitors, were examined. Coincubation with staurosporine or calphostin C, PKC inhibitors, were examined.

### Table 2. Relaxation of Porcine Coronary Arteries in Response to Thrombin After Perfusion With Oxidized Low-Density Lipoprotein or Lysophosphatidylcholine

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Maximum Relaxation, %</th>
<th>E&lt;sub&gt;C50&lt;/sub&gt;, U/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium-only perfusate</td>
<td>102.6±4.4</td>
<td>12±2</td>
</tr>
<tr>
<td>Ox-LDL, μg protein/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>100.5±3.3</td>
<td>12±3</td>
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<tr>
<td>50</td>
<td>100.1±5.8</td>
<td>13±2</td>
</tr>
<tr>
<td>75</td>
<td>92.3±5.1</td>
<td>14±3</td>
</tr>
<tr>
<td>100</td>
<td>86.2±5.0*</td>
<td>20±3*</td>
</tr>
<tr>
<td>150</td>
<td>75.4±5.2†</td>
<td>23±3†</td>
</tr>
<tr>
<td>LysoPC, μmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>101.1±3.7</td>
<td>12±3</td>
</tr>
<tr>
<td>5.0</td>
<td>100.2±6.2</td>
<td>12±2</td>
</tr>
<tr>
<td>7.5</td>
<td>91.4±4.2</td>
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<td>10.0</td>
<td>85.9±5.1†</td>
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<tr>
<td>15.0</td>
<td>78.1±4.9†</td>
<td>23±3†</td>
</tr>
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</table>

Ox-LDL indicates oxidized low-density lipoprotein; LysoPC, lysophosphatidylcholine. Values are mean±SEM; n=8 to 10.

Maximum relaxations are expressed as the maximum percent decrease of the contractions elicited by prostaglandin F<sub>2α</sub> (30 μmol/L). E<sub>C50</sub> was calculated as the concentration causing a 30% decrease of the contraction with prostaglandin F<sub>2α</sub>. The isolated porcine coronary arteries were perfused with various concentrations of Ox-LDL or lysoPC for 30 minutes. The isometric tension of the rings from the treated arteries was monitored in the organ chambers. The rings were contracted with prostaglandin F<sub>2α</sub> (30 μmol/L) and then relaxed with thrombin.

*P<.05, †P<.01 vs medium-only perfusate.

### Table 3. Relaxation of Porcine Coronary Arteries After Perfusion With or Without Activated Polymorphonuclear Leukocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thrombin</th>
<th>Bradykinin</th>
<th>Sodium Nitroprusside</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximum Relaxation, %</td>
<td>E&lt;sub&gt;C50&lt;/sub&gt;, U/L</td>
<td>Maximum Relaxation, %</td>
</tr>
<tr>
<td>No pretreatment (control)</td>
<td>102.6±4.4</td>
<td>12±2</td>
<td>112.5±2.1</td>
</tr>
<tr>
<td>Activated PMNs</td>
<td>58.7±2.8*</td>
<td>37±3*</td>
<td>109.4±2.6</td>
</tr>
</tbody>
</table>

PMNs indicates polymorphonuclear leukocytes. Values are mean±SEM; n=7 to 9.

Maximum relaxations are expressed as the maximum percent decrease of the contractions elicited by prostaglandin F<sub>2α</sub> (30 μmol/L). E<sub>C50</sub> and E<sub>C50</sub> were calculated as the concentrations causing 30% and 50% decreases, respectively, of the contraction with prostaglandin F<sub>2α</sub>. The porcine coronary arteries were exposed for 60 minutes to the PMNs (1×10<sup>6</sup> cells per millilitre) activated by opsonized zymosan. The isometric tension of the rings from the treated arteries was monitored in a manner similar to that in Table 2. The exposure of the arteries to the activated PMNs significantly inhibited vasorelaxation in response to thrombin and bradykinin. Vasorelaxation in response to sodium nitroprusside was completely preserved after the exposure to the activated PMNs.

*P<.01, †P<.05 vs the respective control value (no pretreatment).
rine or calphostin C during pretreatment of the coronary arteries with Ox-LDL or lysoPC suppressed the augmentative effects of Ox-LDL and lysoPC on the endothelial adhesiveness of the treated arteries to PMNs (Table 4). At the same time, the coincubation with staurosporine or calphostin C during pretreatment of the coronary arteries with Ox-LDL or lysoPC also attenuated the augmentative effects of Ox-LDL and lysoPC on the PMN-induced impairment of vasorelaxation in response to thrombin. Furthermore, coincubation with staurosporine or calphostin C during the treatment of the coronary arteries with Ox-LDL or lysoPC abolished the stimulatory action of Ox-LDL and lysoPC on the ICAM-1 expression in the coronary arterial endothelium using the immunohistochemical study (Fig 5). The suppressive effect of staurosporine and calphostin C on ICAM-1 expression was confirmed to be consistent by five independent experiments. Neither staurosporine nor calphostin C is a specific PKC inhibitor; they also inhibit cAMP-dependent and cGMP-dependent protein kinases (A and G kinases) at higher concentrations. Coincubation with HA1004, which has strong inhibitory actions on A and G kinases but only a weak action on PKC, had no effect on the augmentative actions of Ox-LDL for both the endothelial adhesiveness to PMNs and the PMN-induced impairment of vasorelaxation (Table 4). Incubation of the arteries with PMA, a potent stimulator of PKC, and the subsequent exposure to activated PMNs (1 x 10⁷ cells per milliliter) augmented the endothelial adhesiveness to PMNs in a dose-dependent manner (relative adhesion ratio: medium-only perfusate, 1.0±0.1; 0.05 nmol/L PMA, 1.2±0.2; 0.1 nmol/L PMA, 2.0±0.2; and 1.0 nmol/L PMA, 2.4±0.1; P<.01 for 0.1 and 1.0 nmol/L PMA versus medium-only perfusate; n=9 to 11). Pretreatment of the arteries with the concentration of 0.1 nmol/L PMA, which alone did not affect vasorelaxations, significantly augmented the PMN-induced impairment of vasorelaxation in response to thrombin (Table 4). Furthermore, the incubation of the arteries with PMA consistently induced ICAM-1 expression in the coronary arterial endothelium (Fig 2). Coincubation with staurosporine or calphostin C during the pretreatment of the arteries with PMA attenuated the augmentees.
TABLE 4. Effects of Protein Kinase C Inhibitors on the Actions of Oxidized Low-Density Lipoprotein, Lysophosphatidylcholine, or Phorbol 12-Myristate 13-Acetate on Endothelial Adhesiveness to Polymorphonuclear Leukocytes and Polymorphonuclear Leukocyte–Induced Impairment of Vasorelaxation

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Endothelial Adhesiveness to PMNs, relative ratio to control</th>
<th>Maximum Relaxation in Response to Thrombin, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.0±0.1</td>
<td>58.9±3.1</td>
</tr>
<tr>
<td>Ox-LDL</td>
<td>2.5±0.3*</td>
<td>38.9±3.1</td>
</tr>
<tr>
<td>Ox-LDL+STS</td>
<td>1.0±0.1</td>
<td>62.3±4.3</td>
</tr>
<tr>
<td>Ox-LDL+CPC</td>
<td>0.9±0.1</td>
<td>63.4±4.7</td>
</tr>
<tr>
<td>Ox-LDL+HA1004</td>
<td>2.4±0.4*</td>
<td>39.7±4.1*</td>
</tr>
<tr>
<td>lysoPC</td>
<td>2.2±0.3*</td>
<td>38.5±2.5*</td>
</tr>
<tr>
<td>lysoPC+STS</td>
<td>1.1±0.3</td>
<td>60.1±3.7</td>
</tr>
<tr>
<td>lysoPC+CPC</td>
<td>0.8±0.3</td>
<td>59.4±4.3</td>
</tr>
<tr>
<td>PMA</td>
<td>2.0±0.2*</td>
<td>39.1±3.5*</td>
</tr>
<tr>
<td>PMA+STS</td>
<td>0.8±0.3</td>
<td>61.1±5.1</td>
</tr>
<tr>
<td>PMA+CPC</td>
<td>0.9±0.3</td>
<td>60.3±4.2</td>
</tr>
</tbody>
</table>

PMNs indicates polymorphonuclear leukocytes; control, pretreatment with medium alone and subsequent exposure to activated PMNs; Ox-LDL, oxidized low-density lipoprotein; STS, staurosporine; CTC, calphostin C; lysoPC, lysophosphatidylcholine; and PMA, phorbol 12-myristate 13-acetate. Values are mean±SEM; n=7 to 9.

The porcine coronary arteries were pretreated with Ox-LDL (50 μg protein per milliliter), lysoPC (50 μmol/L), or PMA (0.1 μmol/L) for 30 minutes and subsequently perfused with PMNs (1×10⁷ cells per milliliter) activated by opsonized zymosan. In some experiments, the arteries were perfused with Ox-LDL, lysoPC, or PMN in combination with STS (50 μmol/L), CPC (50 μmol/L), or HA1004 (50 μmol/L) and subsequently exposed to activated PMNs. After the treatments, PMN adherence to endothelium and vasorelaxation were examined and expressed in the same manners as in Figs 1 and 4, respectively. STS and CPC but not HA1004 attenuated the augmentative actions of Ox-LDL, lysoPC, or PMA on both the endothelial adhesiveness to PMNs and the PMN-induced impairment of endothelium-dependent arterial relaxation.

*P<.01 vs control.

Discussion

The present study demonstrated that the treatment of coronary arterial endothelium with Ox-LDL but not with N-LDL increased the endothelial adhesiveness to PMNs. The effect of Ox-LDL was not mediated by the scavenger-receptor–dependent mechanism but was attributed to lysoPC in Ox-LDL. The enhanced endothelial adhesiveness to PMNs by Ox-LDL was associated with the augmentation of PMN-induced EDR impairment. The augmentative effect of Ox-LDL on the PMN-induced EDR impairment was also mediated by the scavenger-receptor–independent mechanism and was primarily caused by lysoPC in Ox-LDL. Thus, the results from the experiment examining the endothelial adhesiveness to PMNs were concordant with those from the experiment examining EDR in the organ chamber. It has been shown that PMNs play an important role in the mechanisms of inflammation and ischemia/reperfusion vascular injury. It is also known that PMN adherence to endothelium is required for the first step of the PMN-endothelium interactions. The present study demonstrated that anti-CD18 MAbs, which is an antibody against glycoprotein CD18, one of the adhesion molecules on PMNs, inhibited the PMN adherence to the coronary arterial endothelium. At the same time, the PMN-induced EDR impairment was attenuated by the pretreatment of PMNs with anti-CD18 MAbs, supporting the idea that the enhanced PMN adhesion to endothelium subsequently results in the augmentation of the PMN-induced EDR impairment. The precise mechanisms of the PMN-induced EDR impairment remain to be determined, but oxygen-derived free radicals or proteases released from PMNs may at least in part play a role, since coincubation of the coronary arteries with superoxide dismutase plus catalase or with a protease inhibitor (ulinastatin, Mochida Pharmaceuticals, Japan) during incubation with the activated PMNs was partially effective in the prevention of PMN-induced impairment of vasorelaxations (authors’ unpublished observation). Treatment with Ox-LDL or lysoPC also enhanced the endothelial adhesiveness to the nonactivated PMNs. Furthermore, pretreatment with Ox-LDL or lysoPC and subsequent exposure to the nonactivated PMNs also significantly impaired EDR. A previous report shows that the adhered PMNs could be activated on the endothelium by an adhesion molecule (CD11/CD18–ICAM-1)–dependent mechanism. Therefore, it is possible that the upregulation of ICAM-1 expression on the endothelium by Ox-LDL or lysoPC enhances the endothelial adhesiveness to the nonactivated PMNs, which could be activated through
the adhesion molecule–dependent mechanism after the adherence to endothelium and subsequently could cause EDR impairment. Thus, the present results suggest that lysoPC in Ox-LDL enhances endothelial adhesiveness to PMNs, resulting in the increased susceptibility of endothelium to the PMN-induced endothelial dysfunction.

A number of adhesion molecules on the surface of endothelial cells are involved in the endothelial adhesiveness to leukocytes. ICAM-1 is one of the endothelial adhesion molecules on endothelial cells binding to the adhesion molecules (CD11a/CD18 or CD11b/CD18) on leukocytes, and the ICAM-1 expression on endothelium plays an important role in the interaction with leukocytes in the inflammation and ischemia/reperfusion. In the present study, the treatment of PMNs with anti-CD18 MAb abolished the PMN adherence to endothelium, suggesting that the ICAM-1 expression on the endothelial cells could partly be involved in these interactions. A report recently showed that atherosclerosis is associated with the increased expression of ICAM-1 in the endothelium, which may account for the facility of leukocyte adherence to the endothelium of the atherosclerotic arteries. The report, however, did not clarify the mechanism(s) explaining the induction of ICAM-1 expression in the endothelium of the atherosclerotic arteries. The present immunohistochemical study demonstrated that lysoPC and Ox-LDL but not N-LDL increased ICAM-1 expression in the coronary arterial endothelium, suggesting that lysoPC and Ox-LDL could explain the mechanism(s) of the enhanced expression of ICAM-1 in the endothelium of the atherosclerotic arteries. Thus, the increased expression of ICAM-1 by lysoPC and Ox-LDL may participate in the enhancement of the endothelial adhesiveness to PMNs and the subsequent augmentation of PMN-induced endothelial dysfunction, presently shown in the adhesion experiments and the organ chamber experiments. Earlier studies have indicated that the upregulation of ICAM-1 expression takes 2 to 3 hours after the stimulations. However, a recent report clearly shows that the increases of steady-state mRNA and protein level of ICAM-1 in the endothelial cells occur within 30 minutes after the stimulations. The other study using canine carotid arteries also shows that PMN adherence to endothelium significantly increased after only 20-minute perfusion of the arteries, and the response was almost completely inhibited by a MAb against ICAM-1. Thus, it is not surprising that the increase of ICAM-1 expression on the arterial endothelium ex vivo could occur within 30 minutes after the stimulation with Ox-LDL or lysoPC in the present study. However, it is possible that conformational changes of the constitutive ICAM-1 present on the endothelial cell membrane by unmasking of new functional sites on the ICAM-1 molecule and other inducible adhesion molecules, such as E- or P-selectin or the immunoglobulin superfamily,
may also participate in the enhanced endothelial adhesiveness to PMNs by Ox-LDL or lysoPC.

Recently, we have shown that lysoPC in Ox-LDL is transferred and incorporated into endothelial surface membrane in an apoprotein-independent manner. The transferred lysoPC activates PKC in the endothelial cell surface membrane and modulates various endothelial functions. The present study showed that both staurosporine and calphostin C, PKC inhibitors, suppressed lysoPC-induced or Ox-LDL-induced expression of ICAM-1 in the endothelium, and at the same time, the augmentative effects of lysoPC or Ox-LDL on both the endothelial adhesiveness to PMNs and the PMN-induced EDR impairment were also attenuated by these PKC inhibitors. Furthermore, the present study showed that PMA, a specific PKC activator, induced the expression of ICAM-1 in the coronary arterial endothelium, in agreement with the previous reports. Moreover, PMA enhanced the endothelial adhesiveness to PMNs and the PMN-induced EDR impairment, closely mimicking the results obtained by lysoPC or Ox-LDL. These results indicated that PKC activation by lysoPC in Ox-LDL may at least in part be involved in the enhanced expression of endothelial ICAM-1, which caused the increase of the endothelial adhesiveness to PMNs and the subsequent augmentation of the PMN-induced endothelial dysfunction. A report recently showed that lysoPC induced ICAM-1 expression in cultures of endothelial cells, which is consistent with the present results. However, the report failed to demonstrate that ICAM-1 expression is also induced by Ox-LDL and is suppressed by PKC inhibitors, as shown in the present study using the coronary arteries ex vivo. The reasons for the discrepancies of the results between their study and the present study remain unclear, but their study was performed with cultured endothelial cells after passages in vitro, raising the possibility that the cultured endothelial cells may have obtained different properties. The possibility cannot be excluded that other cellular components of the arteries such as smooth muscle cells could indirectly participate in the stimulatory effect of Ox-LDL on the ICAM-1 expression in the coronary arterial endothelium.

Although N-LDL was inactive on the endothelial susceptibility to PMNs in the present study, a previous report suggests that the long incubation of cultured endothelial cells for 2 to 4 days with very high concentrations of N-LDL (1.6 to 2.4 mg cholesterol per milliliter) may stimulate leukocyte/endothelial cell adhesion interactions. However, its precise mechanisms remain unclear. Intraexperimental auto-oxidation of N-LDL could not be completely excluded in their study.

Our previous studies show that the incubation of the arteries with Ox-LDL impairs EDR, suggesting that Ox-LDL may directly participate in the impairment of EDR in the atherosclerotic arteries. The present study indicated that Ox-LDL may cause further impairment of EDR in atherosclerotic arteries in vivo by the increase of endothelial susceptibility to PMN-induced endothelial dysfunction via enhancement of the endothelial adhesiveness to PMNs.

In conclusion, lysoPC in Ox-LDL enhances endothelial adhesiveness to PMNs, leading to the increase of endothelial susceptibility to PMN-induced endothelial dysfunction. Ox-LDL and lysoPC cause ICAM-1 expression in endothelium, which may partly account for the Ox-LDL-induced and lysoPC-induced enhancement of the endothelial adhesiveness to PMNs, and PKC activation in the endothelium may at least in part participate in these effects of Ox-LDL. These results imply that the endothelial susceptibility to PMN-induced cellular dysfunction may be increased in the atherosclerotic arteries where Ox-LDL exists.

Acknowledgments

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References

5. Vita JA, Treasure CB, Nabel EG, McNelachan JM, Fish RD, Yeung AC, Vekshein VI, Selwyn AP, Ganz P. Coronary vasomotor


Lysophosphatidylcholine in oxidized low-density lipoprotein increases endothelial susceptibility to polymorphonuclear leukocyte-induced endothelial dysfunction in porcine coronary arteries. Role of protein kinase C.
S Sugiyama, K Kugiyama, M Ohgushi, K Fujimoto and H Yasue

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