High Density Lipoprotein Reverses Inhibitory Effect of Oxidized Low Density Lipoprotein on Endothelium-Dependent Arterial Relaxation

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We have recently reported that oxidized low density lipoprotein (ox-LDL) inhibits endothelium-dependent arterial relaxation through its increased lysophosphatidylcholine (LPC). In this study we examined whether high density lipoprotein (HDL) has any effect on the inhibition of endothelium-dependent relaxation by ox-LDL in isolated strips of rabbit thoracic aorta. Both low density lipoprotein (LDL) and HDL were isolated from normal human plasma, and LDL was oxidized by exposure to copper. Preincubation of arterial strips with ox-LDL (0.1–0.5 mg protein/ml) inhibited endothelium-dependent relaxation to acetylcholine (ACH) in a concentration-dependent manner. HDL (1 mg protein/ml) by itself had no effect on the relaxation to ACh. In the presence of HDL, the inhibition by ox-LDL was markedly reduced. Although synthetic 1-α-palmitoyl LPC (5 μg/ml) completely abolished a relaxation to ACh, the preincubation of arterial strips with HDL completely prevented the LPC-induced inhibition. Moreover, a relaxation to ACh was almost completely recovered when the strips were washed with buffer containing HDL even after LPC-induced inhibition had occurred. HDL markedly reduced the incorporation of [1-14C]palmitate–labeled LPC ([14C]LPC) into cultured bovine aortic endothelial cells and promoted the release of cell-incorporated [14C]LPC into the medium, resulting in a reduction of the remaining [14C]LPC in the cells. Agarose electrophoresis after incubation of a mixture of ox-LDL labeled with [14C]LPC and unlabeled HDL demonstrated a transfer of [14C]LPC from ox-LDL to HDL. These results indicate that HDL reverses the ox-LDL–induced impairment of endothelium-dependent relaxation by removing LPC from ox-LDL and preventing LPC from acting on the endothelium. Thus, HDL may have a salutary effect against the impairment of endothelium-mediated vasodilation in atherosclerotic arteries. (Circulation Research 1993;72:1103–1109)

KEY WORDS • endothelium • endothelium-dependent relaxation • high density lipoprotein • oxidized low density lipoprotein • lysophosphatidylcholine

Atherosclerotic arteries are known to exhibit hyperreactivity in response to specific agonists such as ergonovine and serotonin.1,2 In addition, endothelium-dependent relaxation is markedly reduced in atherosclerotic arteries.3–6 These two characteristic alterations in vascular responsiveness in atherosclerosis may cooperatively predispose atherosclerotic coronary arteries to coronary spasm.7,8 Oxidized low density lipoprotein (ox-LDL) has been implicated as an important cause of atherosclerosis, and its presence in atherosclerotic lesion has been demonstrated.9 One of the properties of ox-LDL is its increased lysophosphatidylcholine (LPC) content.10 LPC is an amphiphilic compound, and its concentration is increased manifold in atherosclerotic arteries.11,12 Recently, we13 and another group14 have demonstrated that ox-LDL inhibits endothelium-dependent arterial relaxation through its increased LPC. Since low density lipoprotein (LDL) is oxidatively modified in intima and ox-LDL accumulates in atherosclerotic lesions, it is speculated that increased LPC in ox-LDL acts on endothelium and inhibits endothelium-dependent arterial relaxation.

High density lipoprotein (HDL) is known to exert its antiatherogenic effect by promoting reverse cholesterol transport.15 In addition, HDL has been demonstrated to exhibit a protective effect against a variety of unfavorable actions of LDL and ox-LDL.16–18 Since HDL, like most of the plasma macromolecules, is able to pass through the vascular endothelium and reach the subendothelial space of the intima,19 there might be an interaction between HDL and ox-LDL. These findings lead us to carry out an investigation to examine whether HDL may have any protective effect against the inhibition of endothelium-dependent relaxation by ox-LDL and to determine its underlying mechanism, if any.

Materials and Methods

Preparation of Lipoproteins

LDL (density, 1.020–1.063 g/ml) and HDL (density, 1.063–1.210 g/ml) were isolated by sequential ultracen-
trifugation from fresh human plasma drawn from healthy normolipemic donors who had fasted for 14 hours according to the method of Havel et al.\textsuperscript{20}

Native LDL (1 mg protein/ml) was oxidatively modified by exposure to 5 \( \mu M \) CuSO\(_4\) in phosphate-buffered saline without Ca\textsuperscript{2+} and Mg\textsuperscript{2+} for 24 hours at 37°C according to the method of Quinn et al.\textsuperscript{21} Oxidative modification of LDL was confirmed by agarose gel electrophoresis, and mobility of ox-LDL was 2.7 times that of native LDL. In addition, measurement of inorganic phosphorus revealed that as much as 40% of phosphatidylcholine in LDL was converted to LPC during the oxidative modification.

**Measurement of Endothelium-Dependent Relaxation**

Japanese white rabbits (2.5–3.5 kg) were anesthetized with pentobarbital sodium (30 mg/kg body wt i.v.), and the descending thoracic aortas were isolated and cleaned of surrounding tissue. Aortic rings approximately 3 mm wide were cut and opened. For isometric force measurements, transverse aortic strips were suspended in 5-ml organ baths containing Krebs' bicarbonate buffer composed of (mM) NaCl 118, KCl 4.0, CaCl\(_2\) 1.5, MgSO\(_4\) 1.2, NaH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, and glucose 5 and were equilibrated at 37°C with a 95% O\(_2\)–5% CO\(_2\) gas mixture. Final pH was approximately 7.38. One end of the strip was attached to the bottom of the chamber, and the other end was attached to a Statham 4C-2 force transducer (Gould, Inc., Glen Burnie, Md.), which was connected to an amplifier/recorder system (Nihon Kohden, Tokyo). An initial preload of 1.5 g was applied, and the strips were allowed to stabilize for 90 minutes. A test contraction was induced by raising KCl concentration to 40 mM. When the developed tension attained its peak value, the strips were relaxed by rinsing with the buffer. Then the strips were precontracted with 0.3 \( \mu M \) \( \alpha \)-phenylephrine hydrochloride (PE) and subsequently relaxed by a cumulative addition of acetylcholine chloride (ACh). After washout and equilibration, the strips were preincubated with selected concentration of ox-LDL, HDL, or synthetic \( \alpha \)-palmitoyl LPC for 30 minutes, and then the contraction–relaxation cycle was repeated. In some experiments, after the contraction–relaxation cycle was performed in the presence of LPC, the bath solution was changed for Krebs' buffer containing HDL (1 mg protein/ml), and responses to ACh were again assessed after equilibration for 30 minutes. In other experiments, the contraction–relaxation cycle was produced by a cumulative addition of nitroglycerin. Relaxation values were expressed as percent decreases of the PE (0.3 \( \mu M \))–induced contractile tone.

**Incorporation of Radiolabeled LPC Into Endothelial Cells**

Cultured bovine aortic endothelial cells (BAECs) were prepared as described previously.\textsuperscript{22} Cultures used in the present study were from the third to 12th passage. Cultured endothelial cells showed typical morphology and homogeneous staining for factor VIII antigen.\textsuperscript{23} The cells were plated on a 35-mm dish. After 2 days, the cultured cells formed a confluent monolayer. The final cell density on the day of the study was 1 x 10\(^6\) cells per dish. The prepared cells were washed twice with serum-free Dulbecco's modified Eagle's medium (DMEM) and then incubated with 2 ml DMEM containing 5 \( \mu g/ml \) [\( ^{1-14} \)C]palmitate–labeled LPC ([\( ^{14} \)C]LPC; specific activity, 0.6 \( \mu Ci/ml \)) for 30 minutes at 37°C in the presence or absence of HDL (1 mg protein/ml) in the medium. After the incubation, the cells were washed three times with 2 ml DMEM to remove unincorporated [\( ^{14} \)C]LPC and then dissolved by adding 2 ml of 0.2% sodium dodecyl sulfate. Aliquots were used for measurement of radioactivity of cell-incorporated [\( ^{14} \)C]LPC.

**Removal of Radiolabeled LPC From Endothelial Cells by HDL**

In another series of experiments, after 5 \( \mu g/ml \) [\( ^{14} \)C]LPC was incorporated into endothelial cells as described above, [\( ^{14} \)C]LPC-incorporated cells were incubated in 2 ml fresh DMEM for another 30 minutes at 37°C in the presence or absence of HDL (1 mg protein/ml). At the end of the incubation, the medium was harvested, and aliquots were used for measurement of radioactivity of [\( ^{14} \)C]LPC released into the medium. The cells were dissolved by adding 2 ml of 0.2% sodium dodecyl sulfate, and aliquots were used for the measurement of radioactivity of [\( ^{14} \)C]LPC remaining in the cells.

**Preparation of Radiolabeled Lipoproteins**

[\( ^{14} \)C]LPC-labeled ox-LDL ([\( ^{14} \)C]LPC–ox-LDL) and [\( ^{14} \)C]LPC-labeled HDL ([\( ^{14} \)C]LPC–HDL) were prepared according to the method described by Albers et al.\textsuperscript{24} Approximately 1.5 \( \mu Ci \) [\( ^{14} \)C]LPC was dried under N\(_2\) and redissolved in 5 \( \mu l \) of 95% ethanol. This ethanolic [\( ^{14} \)C]LPC solution was slowly added beneath the surface of 5 ml ox-LDL (1 mg protein/ml) with gentle stirring. The [\( ^{14} \)C]LPC–ox-LDL mixture was incubated at 37°C for 2 hours to allow for the equilibration of the exogenous-labeled lipid with the lipoprotein and then dialyzed overnight against Krebs' bicarbonate buffer. The final preparation had an approximate specific activity of 1.000 dpm/\( \mu g \) protein.

[\( ^{14} \)C]LPC–HDL was prepared exactly as described above for [\( ^{14} \)C]LPC–ox-LDL, except that ethanolic [\( ^{14} \)C]LPC was added to 5 ml HDL (2 mg protein/ml). The final preparation had an approximate specific activity of 350 dpm/\( \mu g \) protein.

**Transfer of Radiolabeled LPC From ox-LDL to HDL**

A mixture of [\( ^{14} \)C]LPC–ox-LDL (1 mg protein/ml) and unlabeled HDL (2 mg protein/ml) was incubated in Krebs' buffer for 30 minutes at 37°C. At the end of the incubation, 5-\( \mu l \) aliquots were subjected to agarose electrophoresis according to the method of Noble\textsuperscript{25} with precast Agarose Universal Electrophoresis Film (Corning, Palo Alto, Calif.) in a Corning cassette electrophoresis cell filled with 0.05 M barbital buffer containing 0.035% EDTA, pH 8.6. To determine the position of migration of ox-LDL and HDL, [\( ^{14} \)C]LPC–ox-LDL (1 mg protein/ml) and [\( ^{14} \)C]LPC–HDL (2 mg protein/ml) were separately incubated for 30 minutes at 37°C and subjected to agarose electrophoresis. After electrophoresis, the gel was analyzed by Bio Image Analyzer BAS 2000 (Fuji Film, Tokyo).

**Materials**

The following drugs were used: PE, ACh, and \( \alpha \)-palmitoyl LPC (Sigma Chemical Co., St. Louis, Mo.); nitroglycerin (Nihonkayaku, Tokyo); and [\( ^{14} \)C]LPC (Du Pont/New England Nuclear, Wilmington, Del.). LPC,
stored in chloroform–methanol mixtures at \(-20^\circ\text{C}\), was dried under a stream of \(N_2\) gas, dissolved in 0.9% saline, and then sonicated just before use. The other drugs were dissolved in distilled water and then diluted in the buffer. All concentrations are expressed as final concentrations.

**Determinations**

Protein content of the lipoproteins was determined by the method described by Bradford\(^\text{26}\) with bovine serum albumin as a standard. Radioactivity of \([^{14}\text{C}]\)-labeled samples was determined with a liquid scintillation system (model LS8001, Beckman Instruments, Palo Alto, Calif.).

**Statistical Analysis**

Data are expressed as mean±SEM. The significance of the difference between group means was analyzed by one-way analysis of variance and the Bonferroni test for samples. Values of \(p<0.05\) were taken as statistically significant.

**Results**

**Effect of ox-LDL on Endothelium-Dependent Relaxation to ACh**

Representative tracings of the responses to ACh in rabbit aortic strips preincubated with ox-LDL (0.5 mg protein/ml) for 30 minutes are shown in Figure 1 (top panel). We confirmed previous observations that ox-LDL by itself altered neither the resting tension nor PE-elicted contraction. Strips showed reduced relaxation to ACh after preincubation with ox-LDL. Strips showed the same dilatation to ACh after preincubation with the buffer for 30 minutes as compared with the control run (data not shown). The preincubation of strips with ox-LDL (0.1 and 0.5 mg protein/ml) significantly inhibited endothelium-dependent relaxation to ACh in a dose-dependent manner (Figure 2). In contrast, endothelium-independent relaxation to nitroglycerin was not affected by preincubation with ox-LDL, as reported previously\(^\text{13}\) (data not shown).

**Effect of HDL on the Inhibition by ox-LDL of Endothelium-Dependent Relaxation to ACh**

Representative tracings of strips preincubated with ox-LDL in the presence of HDL are demonstrated in Figure 1 (bottom panel). In the presence of HDL (1 mg protein/ml), the inhibitory effect of ox-LDL (0.5 mg protein/ml) on the endothelium-dependent relaxation to ACh was markedly reduced. As shown in Figure 3, the inhibitory effect of ox-LDL (0.5 mg protein/ml) was slightly reduced by HDL (0.1 mg protein/ml) and significantly abolished by HDL (1 mg protein/ml). HDL (1 mg protein/ml) by itself had no effect on the resting tension, PE-induced contraction, or endothelium-dependent relaxation to ACh (data not shown).

**Effect of HDL on the Inhibition by LPC of Endothelium-Dependent Relaxation to ACh**

We previously reported that LPC inhibited endothelium-dependent relaxation and that the inhibitory effect of ox-LDL was due to increased LPC content.\(^\text{13}\) Therefore, we examined whether HDL had any effect on the action of LPC. Representative tracings of strips preincubated with LPC in the presence or absence of HDL are depicted in Figure 4. LPC alone did not alter the resting tension. Whereas endothelium-dependent relaxation to ACh disappeared completely after incubation with LPC (5 mg/ml), coexistence of HDL (1 mg protein/ml) almost completely prevented the inhibition by LPC. Representative tracings of the strips preincubated with LPC and subsequently washed with Krebs’ buffer con-
Effect of HDL on the Incorporation of Radiolabeled LPC Into Endothelial Cells and the Removal of Radiolabeled LPC From Endothelial Cells by HDL

During 30 minutes of incubation of cultured BAECs with DMEM containing \([^{14}C]\)LPC (5 \(\mu\)g/ml), 11.7% \([^{14}C]\)LPC added to the medium was incorporated into the cells. In the presence of HDL (1 mg protein/ml) in the medium, however, the incorporation of \([^{14}C]\)LPC into endothelial cells was decreased to 0.6% (Figure 7, left panel). After another 30-minute incubation of \([^{14}C]\)LPC-incorporated endothelial cells with fresh DMEM, only 4.7% of the initially incorporated \([^{14}C]\)LPC was released into the medium, and 95.3% remained in the cells. On the other hand, another 30-minute incubation of \([^{14}C]\)LPC-incorporated endothelial cells with DMEM containing HDL (1 mg protein/ml) promoted the release of the initially incorporated \([^{14}C]\)LPC into the medium up to 53.7% and reduced the remaining \([^{14}C]\)LPC in the cells to 46.3% (Figure 7, right panel). These results obtained by LPC...
transfer experiment with HDL were in accordance with those of the above vasorelaxation experiment.

Transfer of LPC From ox-LDL to HDL

Figure 8 shows an autoradiograph of agarose electrophoresis that was performed after a mixture of [14C]LPC-labeled ox-LDL (1 mg protein/ml) and unlabeled HDL (2 mg protein/ml) had been incubated for 30 minutes. ox-LDL and HDL were separated, and approximately half of [14C]LPC in ox-LDL migrated with HDL. This indicates that, during the incubation of ox-LDL with HDL, [14C]LPC in ox-LDL was transferred to HDL. When the concentration of unlabeled HDL in the mixture was increased, more of the [14C]LPC in ox-LDL was transferred to HDL (data not shown).

Discussion

In this study, HDL had a preventive effect on the ox-LDL–induced inhibition of endothelium-dependent relaxation in rabbit aortic strips. According to our previous study, the inhibitory action of ox-LDL resulted from its increased LPC during oxidative modification.13 HDL also completely prevented the inhibition by LPC and reversed it, even after LPC-induced inhibition had occurred. Thus, we examined the incorporation of radiolabeled LPC into cultured endothelial cells and found that HDL markedly inhibited the incorporation of LPC into the endothelial cells and removed the cell-incorporated LPC. Finally, we confirmed a transfer of LPC from ox-LDL to HDL during incubation of a mixture of ox-LDL and HDL. These observations lead us to speculate that HDL can prevent LPC in ox-LDL from acting on endothelium by removal of LPC from ox-LDL and that, even after LPC has been incorporated into endothelial cells, HDL can remove LPC from endothelial cells and attenuate its effect.

Previous studies have shown that ox-LDL inhibits endothelium-dependent relaxation.13,14,17-29 The mechanisms underlying the inhibitory effect of ox-LDL may involve the impairment of production and/or release of endothelium-derived relaxing factor (EDRF) and the promotion of inactivation of EDRF after it was released. We have shown that ox-LDL inhibits the production and/or release of EDRF from cultured BAECs by use of a bioassay experiment.30 In addition, we have recently reported that ox-LDL inhibits both inositol 1,4,5-trisphos-
enzymes,35-37 membrane fluidity.40pling.39integral around transmembrane ion content.13

LPC and/or HDL ameliorate the cytotoxic effect of ox-LDL on vascular smooth muscle and endothelial cells16 and prevent oxidative modification of LDL.17 Furthermore, HDL inhibits enhanced platelet aggregation evoked by ox-LDL.18 Thus, HDL possesses a protective effect against a variety of unfavorable actions of LDL and ox-LDL. The present study shows that HDL also has a protective effect on the impairment of endothelium-dependent relaxation by ox-LDL and LPC.

Since HDL, as well as LDL, readily passes through endothelium and accumulates in the subendothelial space of the intima,19 it is possible that HDL interacts with ox-LDL in the subendothelial space of atherosclerotic lesions. Although the real concentrations of ox-LDL accumulated in the vascular wall are unknown, we used the concentration of HDL compatible with that of normolipidemic human plasma HDL20 in this study.

Endothelium-dependent relaxations are shown to be impaired in coronary arteries from hypercholesterolemic or atherosclerotic pigs and humans.4,14-22,41 If ox-LDL is responsible for this impairment, HDL may play a beneficial role through the mechanism drawn from our results. Recently, Kuhn et al.44 have reported that there is a positive correlation between HDL cholesterol and normal ACh-induced coronary vasoreactivity in patients undergoing coronary arteriography, implying the relevance of our findings.

In conclusion, this study demonstrates that HDL reverses the ox-LDL–induced impairment of endothelium-dependent relaxation by removing LPC from ox-LDL and preventing LPC from acting on the endothelium. HDL, aside from its antiatherogenic potential, may have a salutary effect against the impaired endothelium-mediated vasorelaxation in the atherosclerotic artery and could contribute to reducing the susceptibility of atherosclerotic coronary arteries to vasospasm.

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