Oxidized Lipoproteins Inhibit Endothelium-Dependent Vasodilation
Effects of Pressure and High-Density Lipoprotein

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Abstract  Hypertension and atherogenic low-density lipoproteins cause attenuation of endothelium-dependent dilations in vivo. We investigated a potential interference of high transmural pressure with the effects of low-density lipoproteins on endothelium-dependent dilation in vitro. Furthermore, we determined whether high-density lipoproteins preserve endothelium function. Endothelium-intact rabbit renal arteries were isolated, placed in an organ bath, perfused intraluminally with Tyrode's solution, and exposed to different degrees of transmural pressure and native or oxidized low-density lipoproteins. In preconstricted arteries perfused under low-pressure conditions (30 mm Hg), acetylcholine dose-dependently elicited endothelium-dependent dilations that were not altered by increasing the perfusion pressure to 100 mm Hg for 90 minutes (high-pressure conditions). Incubation of the arteries with native or oxidized low-density lipoproteins (0.2 and 1 mg/mL for 60 minutes, respectively) under low-pressure conditions did not attenuate acetylcholine-induced dilations. However, under high-pressure conditions dilations were dose-dependently attenuated by oxidized but not by native low-density lipoproteins. Endothelium-independent dilations to glyceroltrinitrate (0.001 to 3 μmol/L) were not affected. Preincubation of the segments with high-density lipoproteins (0.5 mg/mL, 30 minutes) prevented attenuation of dilator responses. The attenuation of endothelium-dependent dilations by oxidized low-density lipoproteins under high-pressure conditions was accompanied by a transmural, dose-dependent infiltration of the vessel wall with lipoprotein, as detected by light microscopy of cryostat sections stained with Sudan III. This infiltration was prevented by high-density lipoprotein. Under low-pressure conditions no lipoprotein infiltration was visible. In segments incubated with native low-density lipoprotein, no lipoprotein infiltration was detectable. We suggest that the inhibitory effect of oxidized low-density lipoprotein on endothelium-dependent dilations is related to the arterial infiltration with lipid, which depends on the transmural pressure and is prevented by high-density lipoprotein. This mechanism may be important in patients with hypercholesterolemia and hypertension. (Hypertension. 1994;23:556-564.)

Key Words  • hypercholesterolemia • lipoproteins, LDL • endothelium-derived relaxing factor

Endothelial cells regulate vascular tone in part by releasing endothelium-derived relaxing factor (EDRF) and other vasoactive agents such as endothelin.2 Many studies have focused on the potential impairment of endothelial function under various pathophysiological conditions. Hypertension and hypercholesterolemia, which are independent risk factors for the development of arteriosclerosis,5,4 are associated with an attenuation of endothelial function. For example, inhibition of endothelium-dependent dilations has been observed in patients with essential hypertension,5,6 or in coronary arteries of hypercholesterolemic patients.7,8

Attenuation of endothelium-dependent dilations in hypercholesterolemia is likely to be related to high levels of low-density lipoprotein (LDL), especially to its more atherogenic form, the oxidatively modified LDLs (Ox-LDL), which accumulate in atherosclerotic arteries.9,10 Various in vitro investigations studying vascular effects of Ox-LDL have demonstrated decreased formation of EDRF,11-13 inactivation of EDRF,16,17 or interference with the EDRF effector pathway, the guanylate cyclase-stimulating cascade.18,19

The mechanisms responsible for attenuation of endothelium-dependent dilations in hypertension are much less understood. Whereas most investigations of endothelial function in hypertension refer to the chronic state of the disease, some animal studies indicate that an acute rise in blood pressure also attenuates dilator responses to acetylcholine, an agent that stimulates EDRF release.20

If a change in blood pressure can modulate endothelium-dependent dilations under certain conditions, it is of interest to know whether there is an interaction between the effects of lipoproteins and hypertension on vascular dilator responses, as hypercholesterolemia and high blood pressure frequently occur together. We hypothesized that an acute rise in transmural pressure enhances the deleterious effects of LDLs on endothelium-dependent dilations. To evaluate this hypothesis, we studied the influence of native LDL (N-LDL) and Ox-LDL on endothelium-dependent dilation of isolated rabbit arteries under various transmural pressure gradients. Furthermore, we investigated whether high-density lipoprotein (HDL) prevents the deleterious effects of LDL.

Methods

Drugs
Norepinephrine was purchased from Hoechst; glyceroltrinitrate from Merck; and indomethacin, L-arginine, and acetycholine from Sigma Chemical Co. Glyceroltrinitrate was dissolved in ethanol and indomethacin in ethanol/0.1 mol/L NaHCO3.
Preparation of Lipoproteins and Oxidation of LDL

For preparation of lipoproteins, plasma was separated from human blood, and EDTA (0.2 mmol/L), butylated hydroxytoluene (20 μmol/L), phenylmethylsulfonyl fluoride (1 mmol/L) (Sigma), and chloramphenicol (10 mg/dL, Boehringer Mannheim) were added.21 LDL and HDL were isolated by sequential ultracentrifugation22 and concentrated and desalted by gel filtration as described recently.18 Lipoproteins prepared by this method are referred to as native and were kept in the dark at 4°C for no longer than 3 weeks.

For oxidative modification of LDL, antioxidant-free LDL (0.3 mg protein/mL) were incubated with CuSO4 (5 μmol/L) for 15 to 20 hours at 20°C. The degree of oxidation was quantified by the absorption increase at 234 nm, indicating conjugated diene formation of fatty acids,23 and the increase in relative mobility on agarose gel (lithophor electrophoresis kits, IMMUNO), indicating an enhanced negative charge of Ox-LDL.24 The relative mobility of Ox-LDL was 1.3 compared with N-LDL when the increase in diene formation was 78±5% of its maximum. Finally, Ox-LDL were desalted, concentrated, and stored at −80°C. In total, 10 successive LDL preparations were used in this study.

Vessel Preparation and Diameter Determination

The experimental setup for measuring outer vascular diameter has been described recently.25 Briefly, segments of the renal artery (0.8 cm in length) were obtained from rabbits of either sex (New Zealand White rabbits, 4 to 5 months old, 2.5 to 3.5 kg, killed by decapitation, n=85). All procedures were carried out in accordance with the guidelines of the German Ministry of Agriculture for the use and care of laboratory animals. The intact segments were cannulated at both ends with steel cannulas and placed in an organ bath (37°C) containing oxygenated Tyrode’s solution (37°C, pH 7.4). Perfusion routes for bath perfusion and intraluminal perfusion were separate, and drugs could be administered to either route independently. Outer vascular diameters were recorded continuously by a photoelectric device.26 The transmural pressure was adjusted hydrostatically by elevating the outflow tubing of the intraluminal perfusion to either 30 mm Hg (low-pressure condition) or 100 mm Hg (high-pressure condition). The latter value was chosen because in preliminary experiments using lower transmural pressure gradients (65 mm Hg), the effects induced by incubation of the segments with Ox-LDL were less pronounced and less reproducible compared with the high transmural pressure of 100 mm Hg. When higher transmural pressure gradients (150 mm Hg) were used, the segments showed a deterioration of smooth muscle function within 1 to 2 hours and could not be kept at a stable level of preconstriction. The intraluminal pressure was measured by a pressure transducer (Statham, H Sachs) connected to the intraluminal perfusion proximally to the segments. Under low-pressure conditions resting diameters were similar to those in situ, whereas high-pressure conditions resting diameters were similar to those in situ, whereas high-pressure conditions resulting in a further increase in resting diameter (Table). After an initial equilibration period (60 minutes) in the organ bath under low-pressure conditions (bath perfusion rate, 30 mL/h), the intraluminal pressure was measured by a transducer (Statham, H Sachs) connected to the intraluminal perfusion until a preconstriction of 450 to 600 μm Hg was reached. The outflow tubing of the control segment was kept at the original level. After an equilibration period of 90 minutes, the segments were again preconstricted by addition of norepinephrine to the organ bath superfusion, and the acetylcholine-induced dose-response curve was repeated. Special care was taken to achieve similar levels of preconstriction in the control segment and in the segment incubated under high-pressure conditions, because it is known that the initial stretch influences the effects of EDRF on vascular tone.27

To study the influence of the lipoproteins on endothelium-dependent dilations, we incubated another series of preconstricted segments with 0.2 or 1 μmol/L of N-LDL or Ox-LDL for 60 minutes or their respective buffers as control (added to the intraluminal perfusion) before adding acetylcholine under low- or high-pressure conditions. It is noteworthy that the distension of the preconstricted vessels did not differ between low- and high-pressure conditions (Table). The lipoproteins were then present in the intraluminal perfusion throughout the experiment. Because Ox-LDL has an enhancing effect on norepinephrine-induced vasoconstrictions,28 the concentration of norepinephrine before addition of Ox-LDL was chosen to induce a vasoconstriction that was approximately 150 to 200 μmol/L lower than in the control segments. Then when Ox-LDL was added to the intraluminal perfusate, the vessels responded with a further decrease in diameter, resulting finally in an almost identical level of preconstriction compared with the control segments (see the Table).

To determine whether high-pressure conditions in the presence of Ox-LDL (1 mg/mL) affect the endothelium-independent dilator capacities of the isolated arteries, we used glyceroenrinate (0.001 to 3 μmol/L) as vasodilator instead of acetylcholine in four additional experiments, performed according to the above-mentioned protocol.

To determine the influence of HDL on endothelium-dependent vasodilations in the absence and presence of Ox-LDL, we preincubated arteries with HDL (0.5 mg/mL added to the intraluminal perfusion) starting 30 minutes before treatment with Ox-LDL or its buffer and acetylcholine as described above. Intraluminal HDL perfusion was continued throughout the experiment.

To exclude the possibility that acetylcholine induced the release of other endothelial dilating or contracting factors,29 we performed several experiments in the presence of indomethacin (10 μmol/L) added to the intraluminal perfusate. The effectiveness of the indomethacin treatment in terms of suppression of prostacyclin formation has been demonstrated in earlier studies by measurement of the stable prostacyclin metabolite 6-keto-prostaglandin F1α.30 Indomethacin did not significantly alter acetylcholine-induced dilations under low- and high-pressure conditions or in the presence of 1 mg/mL Ox-LDL (data not shown).

Lipoprotein Staining of Arterial Slice Preparations

For detection of arterial wall infiltration with lipoprotein, segments were incubated with N-LDL, Ox-LDL, Ox-LDL plus HDL, or buffer as control under low- and high-pressure conditions as described above (n=3–4 segments in each group). Then the segments were imbedded in OCP (Nieth)
Pressure from 30 to 100 mm Hg resulted in an increase in unstimulated vascular diameter to 2018±30 μm and in a higher sensitivity of the segments to norepinephrine: the EC₅₀ averaged 0.1 μmol/L under high-pressure conditions versus 0.3 μmol/L under low-pressure conditions. To achieve a comparable level of preconstriction, we preconstricted segments under high-pressure conditions with 0.2 μmol/L norepinephrine (contractile responses of 529±18 μm, n=40).

Effect of Transmural Pressure on Endothelium-Dependent Dilation

Acetylcholine induced concentration-dependent dilations in segments perfused under low-pressure conditions and in time-matched segments perfused under high-pressure conditions (Fig 1). The stimulus-response curves were not significantly different, indicating that raising the transmural pressure from 30 to 100 mm Hg for 90 minutes did not alter endothelium-dependent dilator responses.

Influence of N-LDL and Ox-LDL on Endothelium-Dependent Dilation Under Low- and High-Pressure Conditions

Under low-pressure conditions treatment of the isolated renal arteries with N-LDL or Ox-LDL (both were perfused intraluminally at 1 mg/mL for 60 minutes) had no influence on acetylcholine-induced dilator responses (data not shown). Under high-pressure conditions treatment of the arteries with N-LDL also did not affect dilations to acetylcholine (Fig 2). However, treatment of the arteries with N-LDL under high-pressure conditions dose dependently inhibited acetylcholine-induced dilations (Fig 3). Preconstriction levels did not differ between the lipoprotein-treated renal arteries and controls (Table). In preliminary experiments the segments were incubated with 1 mg/mL Ox-LDL at a transmural pressure of 65 mm Hg. At this transmural pressure acetylcholine-induced dilator responses were reduced by Ox-LDL; however, inhibition of dilator responses was less pronounced and less reproducible
compared with the high transmural pressure of 100 mm Hg (data not shown).

Endothelium-Independent Dilation in Ox-LDL-Treated Segments Under High-Pressure Conditions

To determine whether the smooth muscle dilator capacity is changed under high-pressure conditions in arteries treated with Ox-LDL (1 mg/mL), we induced endothelium-independent dilator responses by adding glycercoltrinitrate (1 nmol/L to 3 μmol/L) to the intraluminal lining instead of acetylcholine. Glyceroltrinitrate-induced dilations did not differ between the Ox-LDL-treated segments and control vessels (Fig 4).

Influence of HDL on Ox-LDL-Induced Inhibition of Endothelium-Dependent Vasodilations Under High-Pressure Conditions

To determine whether HDL prevents the Ox-LDL-induced attenuation of dilator responses to acetylcholine under high-pressure conditions, we preincubated segments with HDL (0.5 mg/mL) starting 30 minutes before treatment with Ox-LDL (1 mg/mL). Acetylcholine-induced dilations were fully preserved when the segments were preincubated with HDL before Ox-LDL treatment (Fig 5). Treatment of segments with HDL in the absence of Ox-LDL had no influence on acetylcholine-induced dilations compared with controls (n=7, Fig 5).

Arterial Lipoprotein Infiltration After Incubation With N-LDL and Ox-LDL Under Low- and High-Pressure Conditions

We investigated whether lipoprotein infiltration into the arterial wall of segments incubated with N-LDL (1 mg/mL) and Ox-LDL (0.2 and 1 mg/mL) is related to the transmural pressure. After incubation with lipoproteins or buffer (as control) under low- and high-pressure conditions, vessel segments were frozen in liquid nitrogen, and cryostat sections were prepared. Light microscopy of Hemalaun- and Sudan III-stained sections showed no fat staining in control arteries (not shown). Similarly, under low-pressure conditions incubation of the segments with N-LDL (not shown) or Ox-LDL (Fig 6, left) revealed no visible lipoprotein infiltration of the vascular wall. However, under high-pressure conditions sections of segments incubated with 0.2 (not shown) and 1 mg/mL Ox-LDL (Fig 6, right) showed uptake of lipoproteins throughout the vascular wall. Staining of segments incubated with 1 mg/mL Ox-LDL was more pronounced than that in segments incubated with the lower concentration. No lipid staining was visible after treatment of the arteries with N-LDL under high-pressure conditions (not shown).

Lipid Staining After Incubation With Ox-LDL Under High-Pressure Conditions in the Presence of HDL

When the segments were preincubated for 30 minutes with HDL (0.5 mg/mL) before treatment with Ox-LDL (1 mg/mL) under high-pressure conditions, no lipid staining was visible (not shown).

En Face Silver Staining of Endothelial Margins

Fig 7 shows endothelial cell margins of a representative vessel after incubation with Ox-LDL (1 mg/mL, 90 minutes) under high-pressure conditions, stained with silver nitrate and visualized by light microscopy. The typical formation of the cell margins demonstrates that the endothelial cell layer was still intact.

Discussion

In this study we investigated the interaction of increased transmural pressure and lipoproteins on endothelium-dependent dilations in isolated perfused rabbit renal arteries. The salient findings were that Ox-LDL but not N-LDL dose dependently attenuated acetylcholine-induced dilations under high-pressure conditions and that this attenuation was prevented by HDL. Conversely, Ox-LDL and N-LDL were without effect under low-pressure conditions. Histological sections revealed a marked transmural lipoprotein infiltration of the vascular wall of segments incubated with Ox-LDL under high-pressure conditions, which also was prevented by HDL. In view of the known ability of Ox-LDL to inactivate EDNF,16,17 we suggest that the infiltration of Ox-LDL into the vessel wall under high-pressure conditions is one factor responsible for inactivation of EDNF and attenuation of endothelium-dependent dilations.
Hypertension is another pathophysiological state in which attenuation of endothelium-dependent dilations occurs. 30-35 Information on disturbed endothelial function in hypertension has been obtained mainly from chronically hypertensive animals. However, several studies have indicated that an acute increase in transmural pressure also can impair endothelium-dependent dilations. 20-32 It has been suggested that hypertension induces morphological changes of the endothelium that increase its permeability for macromolecules. 32 One might assume that the access of LDLs to the vascular wall is facilitated under such conditions. 

In recent years several investigations have demonstrated inhibition of endothelium-dependent dilations by Ox-LDL in a variety of experimental models. 11-14,16-19 Proposed mechanisms responsible for this observation include the suppression of the formation of EDRF, 11-14 an accelerated inactivation of this labile compound, 16-17 and an attenuation of its effector pathway in the smooth muscle cells. 18-19

The experimental model used in this study allowed us to differentiate between the effects of transmural pressure and of lipoproteins on endothelium-dependent dilations. It is important to emphasize that the distension of the preconstricted arteries before addition of the lipoproteins did not differ between segments investigated under low- and high-pressure conditions. The lack of effect of lipoproteins under low-pressure conditions and the failure of high pressure alone to impair endothelium-dependent dilations enabled us to investigate the potential interaction of increased pressure and atherogenic lipoproteins. When Ox-LDL was applied under high-pressure conditions, endothelium-dependent dilations were attenuated, whereas endothelium-independent dilations were preserved, indicating an intact smooth muscle function. Attenuation of endothelial function by Ox-LDL and high pressure was accompanied by a marked transmural lipoprotein infiltration of the vessel wall. Furthermore, the degree of lipoprotein infiltration was dependent on the concentration of Ox-LDL, and no infiltration was detected under low-pressure conditions. Thus, attenuation of endothelial
Fig 6. Photomicrographs show representative transmural cryostat sections from rabbit renal arteries stained with Hemaireaun and Sudan III to detect lipoprotein infiltration after incubation with oxidized low-density lipoproteins (Ox-LDL, 1 mg/mL, 60 minutes) under low- or high-pressure conditions (magnification ×80, bar=80 μm). Above, Section obtained from an artery after incubation with Ox-LDL under low-pressure conditions. No lipid staining is visible. Below, Artery incubated with Ox-LDL under high-pressure conditions. Lipoprotein infiltration (appearing bright red) can be detected throughout the vascular wall.

function occurred only in the presence of a substantial Ox-LDL infiltration of the vessel wall. This infiltration could provide a mechanism for the inhibition of EDRF-mediated vasodilations. After its release from the endothelium, the labile EDRF, which is likely to be or closely related to nitric oxide, must reach the smooth muscle cell layers by diffusion. We and others previously have shown that Ox-LDL directly inactivates EDRF. Ox-LDL, present in the vascular wall, thus could inactivate EDRF during its transit from the endothelium to the smooth muscle cells. Therefore, we suggest that lipoprotein infiltration of the vessel wall after incubation with Ox-LDL under high-pressure conditions favors inactivation of EDRF.

The possibility that Ox-LDL inhibited formation of EDRF under high-pressure conditions cannot be excluded in our experiments. On the other hand, this possibility seems less likely because endothelium-dependent dilations were not attenuated in lipoprotein-treated arteries under low-pressure conditions. However, discrepancies among the different studies concerning the effect of Ox-LDL on EDRF formation have been found. This may be explained by different Ox-LDL preparations produced with various experimental protocols. LDLs, when highly oxidized at 37°C for 24 hours, contain a significant amount of lysocephatidylcholine, which is likely to be responsible for part of the effects of Ox-LDL on endothelial func-
When LDLs are only moderately oxidized (i.e., at 20°C for 15 to 20 hours), lysophosphatidylcholine content of Ox-LDL is relatively low (unpublished observations), and Ox-LDL inactivates EDRF without attenuation of EDRF formation. Thus, the lack of effect of Ox-LDL under low-pressure conditions in this study could be due to the relatively short exposure of the segments to Ox-LDL and the relatively mild degree of oxidation compared with preparations oxidized to a higher degree.

The idea that lipoprotein infiltration contributes to inactivation of EDRF provides an explanation for differences between this and other investigations using arterial ring or strip preparations. In an earlier study we could demonstrate that arterial ring preparations are more sensitive to Ox-LDL–induced attenuation of endothelium-dependent vasodilations than intact segments. In ring or strip preparations immersed in a lipoprotein-containing solution, the access of Ox-LDL to the vascular wall and the subsequent inactivation of EDRF may be easier than in intact segments, in which the lipoproteins have to first surmount the endothelial barrier to reach the subintimal space.

N-LDLs, which also have the capacity to inactivate EDRF directly, did not affect acetylcholine-induced dilations. However, fat staining of segments incubated with N-LDL revealed no significant infiltration of the vascular wall under low- or high-pressure conditions. We therefore assume that the intact endothelium can act as a barrier against N-LDL and prevent a significant infiltration and thus a direct inactivation of EDRF in the subintimal smooth muscle layers.

The resistance of the intact endothelium against a transendothelial convective transport of N-LDL is in accordance with a recently published study by Fry and coworkers. They reported that the transendothelial transport of LDL is insensitive to pressure as long as the endothelium is intact. However, in arteries with injured endothelium, transendothelial transport increased significantly with increasing pressure. Because Ox-LDLs are potentially cytotoxic for endothelial cells, one can speculate that incubation of the arteries with Ox-LDL can injure the endothelium, thus initiating a greater pressure-dependent transendothelial transport into the subintima and smooth muscle cell layers. Under these conditions lipoproteins might reach the vessel wall via transcellular and paracellular channels, as suggested recently. Binding of LDL to proteoglycans produced by arterial smooth muscle cells might contribute to accumulation of lipoprotein in the vessel wall.

The lipoprotein infiltration and attenuation of endothelial function were prevented by HDL. In a recent study it was shown that HDL can remove lysophosphatidylcholine from Ox-LDL and thereby prevent toxic effects of lysophosphatidylcholine on the endothelium. Such a removal of lysophosphatidylcholine from Ox-LDL thus could provide one mechanism as to how HDL prevents increased permeability and impairment of endothelium-dependent vasodilations under high-pressure conditions in the present study.

Study Limitations

Care must be taken in applying the results of this study directly to the situation of subjects with hypercholesterolemia and hypertension. It is not clear whether Ox-LDL occurs in vivo only in atheromatous lesions or also within the bloodstream. Reasons argue against the presence of Ox-LDL within the circulation. However, lipoproteins that resemble Ox-LDL have been isolated from human plasma. But even if Ox-LDL does not occur within the circulation, a pressure-dependent convective transport component should increase Ox-LDL in deeper layers of the vascular wall, because Ox-LDLs accumulate in the subendothelial, extracellular space. Furthermore, it is difficult to compare the acute alteration of transmural pressure under the constant-flow conditions chosen in this study with acute or chronic hypertension in patients. A luminal pressure of 30 mm Hg is certainly lower than the pressure occurring naturally in conduit arteries, and a mean pressure of 100 mm Hg does not correspond to excessive hypertension. In addition, arteries in vivo are exposed to pulsatile instead of constant pressure changes, and we cannot exclude the possibility that this difference contributed to part of the results. However, in our experimental setup, without fascia and tissue pressure surrounding the vessels,
a pressure of 30 mm Hg was sufficient to prestretch the isolated arteries to a diameter similar to that in situ, and raising the pressure to 100 mm Hg resulted in a significant increase in diameter. Thus, the prestretching of the arteries may reflect the in vivo situation and the effective transmural pressure.

In summary, we suggest that the inhibitory effect of oxidized LDL on endothelium-dependent dilations is related to the infiltration of Ox-LDL into the blood vessel wall, which depends on the transmural pressure. This mechanism may be important in patients with hypercholesterolemia and hypertension.

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