Inhibition of Cyclic AMP- and Cyclic GMP-Mediated Dilations in Isolated Arteries by Oxidized Low Density Lipoproteins

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We studied the effects of native (N) and oxidized (Ox) low density lipoproteins (LDLs) on adenosine 3',5'-cyclic monophosphate (cAMP)-mediated and on guanosine 3',5'-cyclic monophosphate (cGMP)-mediated dilator mechanisms in isolated, perfused human mammary and rabbit femoral arteries. Dilations were induced in preconstricted, deendothelialized segments by either forskolin (Fo) or sodium nitroprusside (SNP) (intraluminal or adventitial application). Lipoproteins (0.5 mg/ml) were administered to the segments from the intraluminal side. N-LDL had no effect on Fo-induced dilation and caused a weak attenuation of SNP-induced dilation only when SNP was also administered into the intraluminal perfuse. In contrast, Ox-LDL inhibited both Fo- and SNP-induced dilation, independent of the route of dilator application. The effects of Ox-LDL were specific for dilation mediated by cyclic nucleotides. Dilation elicited by the Ca²⁺ antagonist nitrendipine was inhibited neither by N-LDL nor by Ox-LDL. Determination of basal and stimulated (SNP, Fo) cGMP and cAMP content in rabbit femoral segments after preincubation with N-LDL and Ox-LDL revealed a significant decrease of stimulated vascular cGMP and cAMP content by Ox-LDL, whereas N-LDL had no effect. These data indicate that Ox-LDL selectively inhibits vascular smooth muscle relaxation elicited by increases in cyclic nucleotides. This inhibition might contribute to the attenuation of vasodilation in hypercholesterolemia and atherosclerosis. (Arteriosclerosis and Thrombosis 1992;12:180-186)

During the development of arteriosclerosis, changes in vascular reactivity, particularly the attenuation of dilator responses, have been observed. The heterogeneity of these changes implies the involvement of different underlying mechanisms. Impairment of endothelium-mediated dilation is frequently associated with early stages of the disease, whereas attenuation of dilation to endothelium-independent agonists has been observed in more advanced stages. The latter attenuation of vasodilation suggests that dilator mechanisms distal to the level of the endothelium are disturbed. This could involve either impaired diffusion of exogenously applied agonists or endothelium-derived dilators to the vascular smooth muscle or impairment of the smooth muscle itself.

Because native (N) and oxidized (Ox) low density lipoproteins (LDLs) accumulate in the arterial wall, they could interfere with the dilator mechanisms of endothelium-derived autacoids such as nitric oxide (NO) or prostacyclin at the level of the vascular smooth muscle. Both dilators act via increases in cyclic nucleotides: NO-induced dilation involves guanosine 3',5'-cyclic monophosphate (cGMP), whereas prostacyclin is mediated by adenosine 3',5'-cyclic monophosphate (cAMP). To analyze the possible influence of lipoproteins on cyclic nucleotide-mediated dilator mechanisms, we studied the effects of N-LDL and Ox-LDL on cGMP and cAMP content and of dilator responses induced by cyclic nucleotide-increasing agonists in isolated rabbit and human arteries. Forskolin (Fo) was used as a stimulus for adenylylate cyclase and sodium nitroprusside (SNP) as a stimulus for guanylate cyclase.

**Methods**

**Preparation and Oxidation of Low Density Lipoproteins**

Plasma was separated from freshly drawn human blood, and EDTA (0.2 mM), butylated hydroxytoluene (BHT, 20 μM), phenylmethylsulfonyl fluoride (PMSF, 1 mM) (all from Sigma, Munich, FRG), and chloramphenicol (10 mg/ml, Boehringer Mannheim, Mann-
hepatic digestion, and bacterial growth.\textsuperscript{14} LDLs (density, 1.019–1.063 g/ml) were isolated by sequential ultracentrifugation at 200,000g (modified from the method used by Havel et al\textsuperscript{15} to minimize preparation time). Thereafter, LDLs were concentrated by centrifugal ultrafiltration and desalted by gel filtration with a Sephadex G-75-120 column (Sigma). The column was equilibrated with Tyrode’s solution containing EDTA (0.2 mM) and BHT (20 \(\mu\)M). Stock solutions of LDL (8–12 mg protein/ml) were sterilized by filtration (Millipore, Millipore, FRG; pore size, 0.22 \(\mu\)m) and kept in the dark at 4°C for no longer than 3 weeks. LDLs prepared by this method are referred to as native LDL (N-LDL). Protein content was measured as described by Bradford.\textsuperscript{16} LDL homogeneity was tested by agarose gel electrophoresis (lipidophor electrophoresis kits from IMMUNO GmbH, Heidelberg, FRG), during which the LDL preparation migrated as a single, sharp band corresponding to the apolipoprotein B-100 fraction. In total, six different LDL preparations were used in this study.

For oxidative modification, N-LDLs were separated from EDTA and BHT by gel filtration on a Sephadex G-75-120 column equilibrated with 0.02 M NaH\textsubscript{2}PO\textsubscript{4} buffer (pH 7.4). Antioxidant-free LDLs (0.3 mg protein/ml) were incubated with CuSO\textsubscript{4} (5 \(\mu\)M) for 20–24 hours at 23°C. The degree of oxidation was quantified by three different methods: 1) the absorption increase at the 234-nm wavelength, indicating conjugated diene formation of fatty acids;\textsuperscript{17} 2) the increase in relative mobility on agarose gel, indicating an enhanced negative charge on Ox-LDL;\textsuperscript{18} and 3) sodium dodecyl sulfate–polyacrylamide gel electrophoresis, which demonstrated fractionation of apolipoprotein B-100.\textsuperscript{19} When the formation of conjugated dienes (as an index of lipid peroxidation)\textsuperscript{17} was about 80% of its maximum (\(E_{max}\)), the oxidative process was stopped by adding BHT (20 \(\mu\)M) and EDTA (0.2 mM) to the mixture. Therefore, we first determined in one sample of an LDL preparation the individual \(E_{max}\) and \(E_{max}\) of this preparation at 234 nm (\(E_{max}\), absorption before adding Cu\textsuperscript{2+} into the cuvette; \(E_{max}\), maximum absorption value, reached approximately 24 hours after initiating the oxidation). From these values, the degree of LDL oxidation as a percentage of maximum absorbance was determined by measuring the absorbance of samples repeatedly during oxidation of the entire preparation. The relative mobility of Ox-LDL on agarose gel electrophoresis as an index of lipoprotein oxidation\textsuperscript{18} was then 1.3 compared with that of N-LDL. Finally, Ox-LDLs were desalted, concentrated to the same volume as before starting the oxidation procedure, and stored in the same manner as N-LDL.

### Drugs

Fo (Sigma) was dissolved in dimethyl sulfoxide and further diluted in Tyrode’s solution of the following millimolar composition: Na\textsuperscript{+} 144, K\textsuperscript{+} 4, Ca\textsuperscript{2+} 1.6, Mg\textsuperscript{2+} 1.0, Cl\textsuperscript{−} 140, HCO\textsubscript{3}\textsuperscript{−} 11.9, H\textsubscript{2}PO\textsubscript{4}\textsuperscript{−} 0.4, Ca\textsubscript{Na\textsubscript{2}}EDTA 0.025, and glucose 11; PO\textsubscript{2}, 130 mm Hg; pH 7.4. SNP (Sigma) was dissolved in 1 mM sodium acetate and, as norepinephrine (Hoechst, Frankfurt, FRG) and acetylcholine (Sigma), further diluted in Tyrode’s solution. Nitrendipine (Bayer, Leverkusen, FRG) was dissolved in absolute ethanol and further diluted with Tyrode’s solution. To avoid inactivation of nitrendipine and SNP by light, the drugs and the stock solutions were kept in the dark, and the perfusion routes of the experimental setup were covered with aluminum foil. Zaprinast, provided by May & Baker Ltd., Dagenham, UK, was dissolved in 1N NaOH and further diluted in Tyrode’s solution. 3-Isobutyl-1-methylxanthine (IBMX), purchased from SERVA, Heidelberg, FRG, was dissolved in distilled water.

### Vessel Preparation

Intact segments of the femoral artery were obtained from rabbits of either sex (weight 2.5–3.5 kg). The segments were carefully cleaned from connective tissue, and the endothelium was removed mechanically by gently rubbing the segments over a rough steel cannula. Absence of endothelium was proven by the lack of a dilator response to acetylcholine.\textsuperscript{20} The segments were fixed between two steel cannulas and placed in an organ bath (37°C) containing oxygenated Tyrode’s solution (pH 7.4). The solution was perfused through the organ bath at a rate of 0.66 ml/min. In addition to superfusion in the organ bath, the segments were perfused intraluminally (Tyrode’s solution: PO\textsubscript{2} 130 mm Hg; PO\textsubscript{2} 28 mm Hg; pH 7.38; at a rate of 0.5 ml/min).

Side branches of human internal mammary arteries were obtained from patients undergoing internal mammary artery coronary bypass operations (Department of Thoracic and Cardiac Surgery, University Hospital of Freiburg, Freiburg, FRG). The human arteries were prepared in the same manner as described for the rabbit femoral arteries.

Outer vascular diameters were recorded continuously by a photoelectric device. The transmural pressure was adjusted hydrostatically to 40 mm Hg (isobaric conditions). Under these conditions, resting diameter of the femoral arteries was 1.657±27 \(\mu\)m (\(n=60\)), and of the human arteries 1.893±67 \(\mu\)m (\(n=8\)). Full details of this experimental setup have been published earlier.\textsuperscript{20}

Vasodilation was induced by adding the respective agonist either to the organ bath (SNP, Fo, nitrendipine) or to the intraluminal perfusate (SNP) of norepinephrine-constricted segments. The level of preconstriction induced by norepinephrine (0.01–0.1 \(\mu\)M) amounted to 19±4% of resting diameter. Effects of the lipoproteins on dilation induced by SNP, Fo, and nitrendipine were studied by comparing dilation before and after preincubation of the segments with N-LDL or Ox-LDL (0.5 mg/ml for 30 minutes added to the intraluminal perfusate). During determination of the dilator responses, the segments were continuously perfused intraluminally with the lipoproteins. To exclude a time-related impairment of dilation, a sec-
Results

Effects of Native and Oxidized Low Density Lipoproteins on cGMP-Mediated Vasodilation Induced by Sodium Nitroprusside

After preincubation of deendothelialized segments with N-LDL (added intraluminally), SNP-induced dilation remained unaffected when SNP was applied to the organ bath (Figure 1, left panel). However, when added together with N-LDL to the intraluminal perfusate, dilation was significantly reduced (Figure 1, right panel). Preincubation of the segments with Ox-LDL significantly inhibited SNP-induced dilation (Figure 2, left panel). This inhibition tended to be more pronounced when SNP was added together with Ox-LDL to the intraluminal perfusate (Figure 2, right panel).

Inhibition of the dilation by Ox-LDL and N-LDL was partly reversible 30 minutes after stopping the perfusion with Ox-LDL (data not shown). Ox-LDL inhibited SNP-induced dilation also in human internal mammary arteries (Figure 3, left panel; Ox-LDL and SNP added separately).

Inhibition of cAMP-Mediated Vasodilation by Native and Oxidized Low Density Lipoproteins

Fo-induced dilation of rabbit femoral segments was impaired only by Ox-LDL (Figure 4, left panel) but not by N-LDL (Figure 4, right panel). The mode of application of Fo (separate or combined with the lipoproteins) had no influence on the degree of inhibition. The inhibition was partly reversible 30 minutes after stopping the perfusion with Ox-LDL (data not shown). Fo-induced dilation also was inhibited by Ox-LDL in human internal mammary arteries (Figure 3, right panel).

Influence of Native and Oxidized Low Density Lipoproteins on Vascular Content of cGMP and cAMP

To investigate whether inhibition of the mechanical responses by the lipoproteins was associated with
a decrease in the tissue content of cyclic nucleotides, we determined cGMP and cAMP (basal and after stimulation with SNP or Fo) in deendothelialized femoral segments preincubated with N-LDL, Ox-LDL, or the respective buffer without lipoprotein. The concentration of the lipoproteins (0.5 mg/ml) and the duration of incubation (30 minutes) were chosen according to the protocol for diameter recordings. Figure 5 shows basal and SNP-stimulated tissue cGMP content before and after incubation with N-LDL or Ox-LDL. N-LDL had no influence on vascular cGMP content (Figure 5, right panel), whereas Ox-LDL significantly reduced the SNP-stimulated cGMP level (Figure 5, left panel). Similar results were obtained with regard to the vascular cAMP level after stimulation with Fo: the stimulated cAMP content (2.1-fold of basal cAMP) was reduced significantly only by Ox-LDL (by 21 ± 6%, n=4), whereas N-LDL had no influence.

Influence of Oxidized Low Density Lipoproteins on Dilation Induced by Nitrendipine

To clarify whether inhibition of dilation by lipoproteins was a general phenomenon or specific for cyclic nucleotides, we studied the influence of N-LDL and Ox-LDL on dilation induced by the Ca2+ antagonist nitrendipine. Preincubation of preconstricted femoral segments with Ox-LDL (0.5 mg/ml, for 30 minutes) had no influence on dilation elicited by 0.03 and 0.3 μM nitrendipine (Figure 6). N-LDL was also without effect (data not shown).

Discussion

The data presented in this study demonstrate that Ox-LDLs inhibit vasodilation mediated by cyclic nucleotides in isolated rabbit and human arteries. The inhibition of dilator responses was accompanied by an attenuation of the agonist-induced rise in tissue content of the cyclic nucleotides cAMP and cGMP. Dilation induced by the Ca2+ antagonist nitrendipine remained unaffected. N-LDL only caused a weak inhibition of SNP-induced dilation when the former could interact directly with this nitrovasodilator. These findings may provide additional understanding of the mechanisms responsible for the frequently described attenuation of dilation in atherosclerotic arteries,1-3 in which N-LDL and Ox-LDL accumu-
late.9–11 In addition to direct effects on the endothelium, the lipoproteins could decrease the efficacy of endogenous dilators and exogenously applied drugs either by direct inactivation of the dilators or by affecting the signal cascade in the smooth muscle cells. This study provides evidence for both mechanisms.

Inactivation by N-LDL or Ox-LDL may be the predominant mechanism for the attenuated effects of dilators that are identical with NO (endothelium-derived relaxing factor [EDRF])23 or that release NO (SNP).24 When SNP was applied together with the lipoproteins from the intraluminal side, dilation was inhibited to a greater extent compared with separate application of lipoproteins and the drug. This observation argues in favor of an interaction between the lipoproteins and the NO-releasing SNP. It is also in agreement with a previous study, in which direct inactivation of EDRF/NO by N-LDL and Ox-LDL was demonstrated.25 The higher degree of inhibition of dilation and rise in cGMP caused by Ox-LDL compared with N-LDL may result from a higher capacity of Ox-LDL to inactivate SNP or NO, respectively. Furthermore, Ox-LDL might interfere with the cGMP-forming enzyme guanylate cyclase. Inhibition of an isolated soluble guanylate cyclase by Ox-LDL has been demonstrated in vitro (Reference 26 and our own unpublished observations) and may also take place intracellularly. Yet, the lack of effect of Ox-LDL on basal levels of cGMP in this study makes this possibility less likely. The mode of application of Fo (separately or combined with the lipoproteins), however, had no influence on the degree of inhibition of vasodilation. A mechanism different from inactivation of the vasodilator by Ox-LDL may be responsible for the inhibition of Fo-induced dilation and rise in cAMP levels. Direct inactivation of the enzyme adenylate cyclase may play a minor role because—as in the case of guanylate cyclase—the basal vascular content of cAMP was not affected by Ox-LDL. Thus, the specific site of action of Ox-LDL

![Figure 4](image4)

**Figure 4.** Line plots of endothelium-independent vasodilation evoked by cumulative doses of forskolin (FO) in preconstricted (preconstr.) rabbit femoral segments before (control) and after preincubation (0.5 mg/ml, for 30 minutes) with oxidized low density lipoproteins (ox-LDL, left panel) or native low density lipoproteins (n-LDL, right panel) added to the intraluminal (intralum.) perfusate. FO was added to the extraluminal (extralum.) perfusate. Filled symbols indicate statistical significance vs. control, with p<0.05, n=9 segments in each series.

![Figure 5](image5)

**Figure 5.** Bar graphs of effects of native low density lipoproteins (n-LDL) and oxidized low density lipoproteins (ox-LDL) (0.5 mg/ml, for 30 minutes) on basal (control) and stimulated (sodium nitroprusside, SNP) guanosine 3',5'-cyclic monophosphate (cGMP) content (pmol/mg protein) in endothelium-denuded rabbit femoral segments. n-LDL was without inhibitory effect, whereas ox-LDL significantly reduced the stimulated cGMP content but not the basal cGMP content. n=6, *p<0.05 vs. SNP alone.

![Figure 6](image6)

**Figure 6.** Bar graph of effects of oxidized low density lipoproteins (ox-LDL, 0.5 mg/ml, for 30 minutes) on vasodilation elicited by the Ca²⁺ antagonist nitrendipine (0.03 and 0.3 μM) in preconstricted (preconstr.) rabbit femoral segments. ox-LDL had no inhibitory effect. n=8 segments.
on the attenuation of Fo-induced relaxation cannot be identified by the experiments in this study. One might speculate, however, that Ox-LDL affects the signal chain linking Fo to the membrane-bound adenylate cyclase by changing the fluidity of the cell membrane through excess cholesterol load. 

Because it is known that Ox-LDL may exert cytotoxic effects, we considered the possibility that Ox-LDL inhibited dilation by an unspecific attenuation of the dilator capability of isolated segments. However, two observations argue against this assumption: dilation induced by the Ca²⁺ antagonist nitrendipine was not affected, and inhibition of the SNP- and Fo-induced dilation was partly reversible. Therefore, a cytotoxic effect of Ox-LDL after 30 minutes' incubation seems unlikely.

In several studies, an enhanced responsiveness of atherosclerotic arteries to vasoconstrictors has been reported. Reduction of cGMP and cAMP levels, as described in this study, may also contribute to this phenomenon. A decreased level of tissue cyclic nucleotides could render arteries more sensitive to contractile agonists and also could explain the potentiation of contractile responses by Ox-LDL in vitro. However, in this study the unstimulated cAMP and cGMP levels were not affected after incubation of endothelium-denuded segments with N-LDL or Ox-LDL. Thus, decreased cyclic nucleotide levels in segments after incubation with Ox-LDL are unlikely to be the sole cause for potentiation of vasoconstrictors.

In summary, this study demonstrates attenuation of dilation mediated by cyclic nucleotides in human and rabbit arteries after incubation with LDLs. Likely mechanisms are inactivation of NO by N-LDL or Ox-LDL and interference of Ox-LDL with the adenylate cyclase-activating cascade. These mechanisms may contribute to the reduced dilator responses observed in atherosclerotic arteries.

Acknowledgments

We thank A. Zeiher (Department of Internal Medicine, University Hospital of Freiburg) for providing human mammary arteries, Ch. Kircher and I. Winter for skillful technical assistance, I. Krause for preparation of lipoproteins, and K. Großkop and R. Henn for secretarial help.

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**KEY WORDS** • cyclic nucleotides • vascular reactivity • oxidized low density lipoproteins • arteriosclerosis • vasospasm • human arteries
Inhibition of cyclic AMP- and cyclic GMP-mediated dilations in isolated arteries by oxidized low density lipoproteins.

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doi: 10.1161/01.ATV.12.2.180

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/12/2/180

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