Oxidized Low-Density Lipoprotein Inhibits Acetylcholine-Induced Vasorelaxation and Increases 5-HT-Induced Vasoconstriction in Isolated Human Saphenous Vein

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ABSTRACT

The present study determined the vasomotor effects of oxidized low-density lipoprotein (ox-LDL) in human saphenous veins and determined whether decreased availability of L-arginine was responsible for the impaired endothelial function. Human saphenous veins were obtained from white males undergoing coronary bypass surgery. We examined the effects of ox-LDL on ACh-induced endothelium-dependent relaxation, sodium nitroprusside-induced endothelium-independent relaxation and 5-HT-induced contraction. ACh-induced vasorelaxation in the presence of L-arginine and ox-LDL was also examined. In addition, we assessed the endothelial influence on the contractile response to 5-HT. ox-LDL significantly inhibited ACh-induced relaxation but did not affect sodium nitroprusside-induced relaxation. L-Arginine pretreatment did not prevent ox-LDL-induced impairment of the relaxation response to ACh. ox-LDL significantly potentiated 5-HT-induced contraction at concentrations between 3 × 10⁻⁶ M and 10⁻⁴ M, an effect that was endothelium-dependent. Denudation of endothelium also significantly enhanced the contractile response to 5-HT. These data suggest that ox-LDL impairs ACh-induced endothelium-dependent relaxation and enhances 5-HT-induced endothelium-dependent contraction in human saphenous vein. L-Arginine deficiency is not responsible for the endothelial dysfunction induced by ox-LDL in human saphenous vein.

Received for publication July 15, 1997.

ABBREVIATIONS: LDL, low-density lipoprotein; ox-LDL, oxidized low-density lipoprotein; EDRF, endothelium-derived relaxing factor; EDHF, endothelium-derived hyperpolarizing factor; 5-HT, serotonin; ADMA, asymmetrical dimethylarginine; EDCF, endothelium-derived contracting factor.
was involved in the impairment of endothelial function induced by ox-LDL. Because the L-arginine-EDRF pathway exists in the endothelial cells of human saphenous vein (Lüscher et al., 1988; Yang et al., 1991), this blood vessel represents a model with which to examine the vasoactive effects of ox-LDL in humans. Furthermore, because the human saphenous vein is used for coronary artery bypass, and bypass grafts are exposed to the actions of ox-LDL, it was of interest to examine the effects of ox-LDL on this vessel.

Materials and Methods

Drugs. Acetylcholine chloride, 5-HT, sodium nitroprusside, L-arginine, L-phenylephrine and indomethacin were purchased from Sigma Chemical Company (St. Louis, MO). Serotonin first was dissolved in a 1:1 solution of 1 M HCl and 1 M NaOH to make a 10^{-4} M solution and then was diluted in deionized water. Indomethacin was dissolved in 1% Na_{2}CO_{3} to make a 0.06 M stock solution. Other drugs were dissolved in deionized water.

Preparation of blood vessel. All studies were approved by the appropriate institutional review boards. Human saphenous veins were obtained from white males undergoing coronary bypass surgery from University Hospital and the Medical College of Georgia (Augusta, GA). After the vein was removed, it was immediately placed in cold Krebs buffer, previously aerated with 95% O_{2}–5% CO_{2}, and transported to our lab. Composition of the Krebs buffer solution was as follows (mM): NaCl 118.0, KCl 4.7, NaHCO_{3} 25.0, MgSO_{4} 1.2, KH_{2}PO_{4} 1.1, CaCl_{2} 2.5, EDTA 0.01, glucose 11.0. The final pH of the Krebs’ buffer was 7.4. The vessel was carefully cleaned of fat and connective tissue and cut into 3 to 5-mm rings. Each ring was suspended between stainless steel hooks in a 10-ml tissue bath containing Krebs buffer maintained at 37°C and continuously aerated with 95% O_{2}–5% CO_{2}. One hook was connected to a force transducer for recording the isometric tension. The rings were progressively stretched to the optimal resting tension (2 g) and were allowed to equilibrate for 45 min. The buffer was changed every 20 min. All rings were initially contracted twice with KCl (70 mM) to confirm the viability of vessels before the experimental protocols were performed.

Preparation of ox-LDL. Human LDL (5 mg/ml) was purchased from PerImmune Inc. (Rockville, MD) in NaCl (0.5 M) with 0.15% EDTA (pH 7.2) and stored at 4°C. Native LDL was oxidatively modified according to a modification of the method of Cox and Cohen (1996). Before oxidation, LDL (1–2 ml) was dialyzed at 4°C for 24 h against phosphate-buffered saline (PBS) to remove EDTA. Composition of the PBS was NaCl 137 mM, NaH_{2}PO_{4}·H_{2}O 10 mM, NaOH 7 mM, pH 7.2. Then LDL (5 mg/ml) was oxidized by incubating it with CuSO_{4} (5 µM) for 24 h at 37°C, followed by dialysis at 4°C for 24 h against PBS containing 0.01% EDTA to remove the copper ion. The lipid peroxide content of the copper-oxidized LDL was measured fluorometrically as thiobarbituric acid-reactive substances (Yagi, 1976). Lipid peroxidation was expressed as nanomoles of MDA (malonaldehyde) per milligram of LDL protein, using 1,1,3,3-tetraethoxypropane as the standard. The lipid peroxidation values of the LDL before and after incubation with CuSO_{4} (5 µM) for 24 h were 0 and 15.03 ± 3.47 nmol MDA/mg protein (P < 0.01), respectively. The ox-LDL sample was stored at 4°C.

Experimental protocols. After the vessel rings were contracted twice with KCl to confirm the viability of vessels, the presence of functional endothelium was determined. First, vessel rings were preconstricted with phenylephrine (10^{-5} M). After achievement of a stable contraction, a concentration-response curve to ACh (10^{-9} to 10^{-4} M) was constructed to check for the presence of functional endothelium. In human saphenous veins, EDRF production is less than that in arteries (Lüscher et al., 1988; Yang et al., 1991). The maximal values of ACh-induced endothelium-dependent relaxation at 10^{-5} M in human saphenous veins is about 20% in the absence of indomethacin and about 40% in the presence of indomethacin (Lüscher et al., 1988; Yang et al., 1991). In the vessel rings without endothelium, ACh does not produce relaxation. Because the saphenous veins were from patients undergoing coronary artery bypass, mechanical damage to endothelium may occur during surgery. In the present study, vessel rings with a relaxation response to ACh (10^{-6} M) of 15% or more were considered to have functional endothelium and were chosen to determine the effect of ox-LDL on ACh-induced relaxation. The first relaxation response to ACh was used as control. After washout and return to baseline, vessel rings were incubated with ox-LDL (100 µg protein/ml) or ox-LDL plus L-arginine (500 µM) for 45 min. Then the vessel rings were preconstricted with phenylephrine and exposed to cumulative concentrations of ACh as before.

The concentration 100 µg protein/ml of ox-LDL represents a pathophysiological concentration and has been reported to inhibit endothelium-dependent relaxation and to potentiate endothelium-dependent contraction in porcine coronary artery (Cox and Cohen, 1996; Tanner et al., 1991). Plasma L-arginine concentration in normal individuals is about 120 µM (Pasini et al., 1992). Kugiyama et al. (1990) demonstrated that the inhibitory effect of ox-LDL on endothelium-dependent relaxation could not be reversed by L-arginine (100 µM) in rabbit aorta. However, Tanner et al. (1991) showed that L-arginine (300 µM) restored the impaired endothelium-dependent relaxation induced by ox-LDL to control levels in porcine coronary artery. On the basis of the study of Tanner et al. (1991), we used L-arginine at 500 µM to determine whether it is capable of reversing the impairment of endothelium-dependent relaxation induced by ox-LDL.

Endothelium-independent relaxation responses to sodium nitroprusside (10^{-9} to 10^{-4} M), a nitric oxide donor, in the presence and absence of ox-LDL were also examined in vessel rings without endothelium to determine whether ox-LDL inhibited the activity of guanylyl cyclase. To determine the effect of ox-LDL on the contractile response to 5-HT, vessel rings both with or without endothelium were incubated with ox-LDL (100 µg protein/ml) for 45 min. Control rings were incubated with an equivalent volume of PBS for 45 min. Then the dose-response curves to 5-HT (10^{-9} to 10^{-4} M) were constructed. For each paired test, the vessel rings incubated with PBS or ox-LDL were from the same patient and were studied in parallel. In addition, we examined the effect of the endothelium on the contractile response to 5-HT by constructing 5-HT dose-response curves (10^{-9} to 10^{-4} M) in vessel rings with and without endothelium. The endothelium was removed by gently rubbing the interior surface of the lumen with a cotton swab. The absence of endothelium was confirmed by the absence of a relaxation response to ACh. All experiments were performed in the presence of the cyclooxygenase inhibitor indomethacin (6 µM) to exclude the influence of endothelium-dependent prostaglandins (Pearson et al., 1993; Yang et al., 1991).

Data analysis. Data are expressed as the mean ± S.E.M. 5-HT-induced contractions were expressed as a percentage of the maximal contraction evoked by KCl (70 mM). Relaxation responses were calculated as a percent decrease in tension of the stable contraction produced by phenylephrine. EC_{50} was defined as the concentration of an agonist at which 50% of the maximal response was obtained. Student’s t test was used to test the significance of the differences between the two groups. Comparisons among more than two groups were performed using one-way ANOVA. When differences between groups were indicated, a Newman-Keuls’ post-hoc comparison was used. The differences were considered to be significant when P < 0.05.

Results

Effect of ox-LDL on ACh-induced endothelium-dependent relaxation. Phenylephrine was used to preconstrict human saphenous veins. Preconstriction before and after ox-LDL treatment was not significantly different (1.7 ±
0.2 vs. 1.4 ± 0.1 g, control vs. ox-LDL group). ACh produced concentration-dependent relaxation in human saphenous vein with endothelium that was significantly attenuated by ox-LDL pretreatment at concentrations between 10^{-6} and 10^{-4} M (fig. 1). In preliminary studies, consecutive relaxation responses to ACh were elicited to ensure that ACh-induced relaxation did not decrease with time. ox-LDL alone produced either no or very weak contractions. Only 5 of 19 patients showed minimal contraction of 15 ± 3% of KCl (70 mM) contraction. Endothelium-independent relaxation to sodium nitroprusside was not significantly affected by preincubation with ox-LDL (fig. 2).

**Effect of ox-LDL on 5-HT-induced vasoconstriction.** ox-LDL preincubation increased the vascular contractile response to 5-HT in the presence of endothelium. The differences were significant at concentrations between 3 × 10^{-6} and 10^{-4} M (fig. 3). The maximal contractile response to 5-HT was significantly increased from 95 ± 9% of KCl (70 mM) contraction in the control group to 124 ± 12% of KCl contraction in the ox-LDL group (table 1). However, there was no significant difference between the EC_{50} values (table 1). In the absence of endothelium, ox-LDL did not produce a significant effect on 5-HT-induced contraction (data not shown). This suggests that ox-LDL may interfere with endothelial function and thus increase the vascular reactivity to 5-HT.

In order to determine whether the ox-LDL-enhanced contractile response to 5-HT was due to interference with endothelial function, we also examined the role of endothelium in the vascular contractile response to 5-HT. 5-HT elicited concentration-dependent contractions in vessel rings both with and without endothelium. The contractile responses evoked by 5-HT in the absence of endothelium were significantly increased compared with the responses in vessel rings with endothelium (fig. 4). The maximal contraction was increased from 89 ± 6% to 120 ± 6% of KCl contraction. However, the difference between EC_{50} values was not significant (0.6 ± 0.3 μM vs. 0.1 ± 0.03 μM, with endothelium vs. without endothelium). These data suggest that endothelium inhibited the contractile response to 5-HT. Endothelium removal released the inhibitory effect of the endothelium and thus enhanced the contractile response to 5-HT.

**Effect of ox-LDL plus L-arginine on ACh-induced endothelium-dependent relaxation.** Phenylephrine-induced preconstriction before and after ox-LDL or ox-LDL plus L-arginine treatment was not significantly different.
1.2 was incubated with ox-LDL plus L-arginine, endothelium-dependent relaxation. The impairment of ACh-induced endothelium-dependent relaxation produced by ox-LDL. There were no significant differences in the impairment of ACh-induced relaxation between ox-LDL-treated vessels and ox-LDL plus L-arginine-treated vessels. L-arginine alone had no effect on ACh-induced endothelium-dependent relaxation.

1.7 ± 0.2 g in control, 1.4 ± 0.1 g in the ox-LDL group and 1.2 ± 0.1 g in the ox-LDL plus L-arginine group. ox-LDL significantly impaired the ACh-induced relaxation between the concentrations of 3 × 10⁻⁸ and 10⁻⁴ M. When the vein was incubated with ox-LDL plus L-arginine, endothelium-dependent relaxation was still impaired (fig. 5). L-arginine did not improve the impairment of ACh-induced relaxation produced by ox-LDL. There were no significant differences in the impairment of ACh-induced endothelium-dependent relaxation between ox-LDL-treated vessels and ox-LDL plus L-arginine-treated vessels. L-arginine alone had no effect on ACh-induced endothelium-dependent relaxation.

Fig. 4. The contractile responses to 5-HT in human saphenous vein both with and without endothelium. Values represent the mean ± S.E.M. of vessels from 11 patients. * P < 0.05, ** P < 0.01, *** P < 0.001.

Fig. 5. Effect of ox-LDL (100 µg protein/ml) or ox-LDL plus l-arginine (500 µM) on the ACh-induced endothelium-dependent relaxation of human saphenous vein. The rings were preconstricted with 10 µM phenylephrine. Values represent the mean ± S.E.M. of vessels from 5 to 9 patients. * P < 0.05, ** P < 0.01 between control and ox-LDL group; † P < 0.05, ‡ P < 0.01 between control and ox-LDL plus l-arginine group.

Discussion

This is the first study to evaluate the effects of ox-LDL on endothelium-dependent relaxation and 5-HT-induced vasoconstriction in human saphenous veins. ox-LDL inhibited ACh-induced endothelium-dependent relaxation but did not affect endothelium-dependent relaxation to the NO donor sodium nitroprusside. L-arginine did not prevent the impairment of endothelium-dependent relaxation induced by ox-LDL. ox-LDL also enhanced the vasoconstrictor responses to 5-HT, which were endothelium-dependent.

Hypercholesterolemia, particularly elevated plasma LDL levels, represents one of the most important risk factors for the development of atherosclerosis. ox-LDL is thought to be the atherogenic form of LDL (Steinberg and Witzum, 1990). LDL must first undergo oxidative modification in order to be potentially cytotoxic for endothelial cells. It has also been shown to impair endothelium-dependent relaxation (Galle et al., 1994; Mangin Jr. et al., 1993; Plane et al., 1992; Tanner et al., 1991) and to potentiate agonist-induced vasoconstriction in animal blood vessels (Cox and Cohen, 1996; Galle et al., 1990; Simon et al., 1990). Recent investigations have demonstrated that oxidation of native LDL occurs in vivo and ox-LDL has been identified in human atherosclerotic arteries (Yla-Herttuala et al., 1989). Furthermore, antioxidant administration improves endothelial function and retards the progression of atherosclerosis (Anderson et al., 1995; Guarnieri et al., 1996; Keaney et al., 1993). And in addition, single LDL apheresis improved impaired endothelial function in hypercholesterolemic humans, which correlated with the decrease in plasma ox-LDL levels (Tamai et al., 1997). Collectively, these studies support the interpretation that ox-LDL is an important atherogenic factor.

In the present study, ox-LDL enhanced the contractile response to 5-HT, which was endothelium-dependent. Our results are consistent with the study of Cox and Cohen (1996) in pig coronary artery and suggest that ox-LDL may induce endothelial dysfunction to produce its vasomotor effect. We also examined the influence of endothelium on 5-HT-induced contraction. Endothelial removal significantly increased the vascular contractile response to 5-HT, an observation that is in agreement with the results reported by Valentijn et al. (1996) in rabbit saphenous vein. Thus the effect of ox-LDL on the vascular contractile response to 5-HT mimicked the response elicited by endothelial removal. Several studies have shown that ox-LDL inhibits 5-HT-induced EDRF-mediated relaxation in pig coronary artery (Cox and Cohen, 1996; Simon et al., 1990; Tanner et al., 1991). Data concerning human vessels have not been published. Golin et al. (1991) demonstrated that 5-HT had a vasodilating effect on normal human coronary arteries in vivo. However, the normal vasodilator response to 5-HT was reversed to vasoconstriction in atherosclerotic coronary arteries. This suggests that the enhanced vasoconstriction in response to 5-HT in human coronary artery may be associated with endothelial dysfunction. Therefore, it is possible that ox-LDL increases the contractile responsiveness to 5-HT in human saphenous vein by producing a functional impairment in 5-HT-induced endothelium-dependent relaxation. Another possibility is that ox-LDL also
interferes with the basal release of EDRF and thus increases the contractile response to 5-HT. However, there is very little EDRF release under basal conditions in human saphenous vein (Yang et al., 1991). This mechanism does not seem to be important, an interpretation supported by our finding in this study that ox-LDL alone causes no or minimal vasoconstriction.

Studies performed in animal vessels have shown the inhibitory effect of ox-LDL on EDRF-mediated relaxation produced by various agonists (Galle et al., 1994; Mangin Jr. et al., 1993; Plane et al., 1992; Tanner et al., 1991). In the present study, ox-LDL also inhibited the ACh-induced relaxation in human saphenous vein. Läscher et al. (1988) and Yang et al. (1991) indicated that ACh-induced endothelium-dependent relaxation in human saphenous vein was mediated by EDRF. Thus ox-LDL could conceivably interact with and inhibit the EDRF pathway.

Oxidation of LDL has been demonstrated to occur in vivo (Ylä-Herttuala et al., 1989). Serum LDL levels are about 1 mg/ml and 2 mg/ml in normal and hypercholesterolemic humans, respectively (Casino et al., 1994; Creager et al., 1992). About 5% of plasma LDL from monkeys and humans is modified (Cazzolato et al., 1991; Hodis et al., 1994). Thus 100 μg protein/ml (the concentration employed in the present study) of ox-LDL represents a pathophysiologically relevant concentration that may occur in human atherosclerotic lesions. The concentrations of ox-LDL in atherosclerotic lesions may be even higher. If the vasoactive effects of ox-LDL in vitro occur in coronary artery in vivo, then ox-LDL may contribute to the clinical events observed in atherosclerosis. Additionally, because saphenous veins are often used for coronary artery bypass grafts, decreased function of saphenous vein grafts associated with the development of atherosclerosis may be due to the vasoactive effects of ox-LDL.

The mechanisms responsible for the impaired endothelium-dependent relaxation are not yet clear. ox-LDL may interfere with multiple steps and hence have many effects, including decreased substrate (L-arginine) availability for EDRF formation (Tanner et al., 1991), altered transmembrane signaling transduction (Ohgushi et al., 1993), decreased expression of NO synthase (Liao et al., 1995), increased production of endothelium-derived contracting factors (Boulanger et al., 1992), increased degradation or inactivation of EDRF (Müggel et al., 1991; Ohara et al., 1993) and decreased response of vascular smooth muscle to EDRF (Schmidt et al., 1991).

A possible mechanism for reduced synthesis of EDRF is decreased availability of L-arginine, the natural substrate for EDRF synthesis. Impaired endothelium-dependent vasorelaxation can be improved by intravascular infusion or diet supplementation of L-arginine in hypercholesterolemic animals and humans (Böger et al., 1995; Creager et al., 1992; Girerd et al., 1990; Tanner et al., 1991), which suggests that endothelial dysfunction in hypercholesterolemia may be caused by a reduction in intracellular L-arginine availability or metabolism. ox-LDL may interfere with receptor-operated release of L-arginine from intracellular stores or with the synthesis of L-arginine (Tanner et al., 1991). However, other studies do not support the interpretation that L-arginine deficiency is responsible for the impaired endothelial function (Casino et al., 1994; Hayashi et al., 1995; Kugiyama et al., 1990; Pohl et al., 1995). Because administration of exogenous L-arginine does not affect endothelium-dependent relaxation in normal vessels, the amount of endogenous L-arginine appears to be sufficient for EDRF formation and is not a rate-limiting factor for EDRF synthesis in normal vessels (Creager et al., 1992; Girerd et al., 1990). Most authors found no difference in L-arginine concentrations between normal and hypercholesterolemic animals and humans (Bode-Böger et al., 1996; Hayashi et al., 1995; Pasini et al., 1992), although decreased plasma L-arginine levels in hypercholesterolemia have also been reported (Jeserich et al., 1992). Moreover, Minor et al. (1990) has suggested that NO synthesis is not impaired, but rather is actually increased, in diet-induced hypercholesterolemic and atherosclerotic rabbits.

If ox-LDL-induced impairment of endothelial function involves L-arginine deficiency, then administration of L-arginine would reverse the impairment. However, the results of the present study do not support this hypothesis. L-Arginine pretreatment did not prevent ox-LDL-induced impairment of endothelium-dependent relaxation. The present study evaluated short-term effects of ox-LDL in vitro, which did not fully reflect the condition in hypercholesterolemia. Recent investigations suggested that the plasma concentration of ADMA, an endogenous inhibitor of NO synthase (Vallance et al., 1992), is increased in hypercholesterolemic animals, resulting in decreased NO formation (Yu et al., 1994). The increased L-arginine/ADMA ratio produced by exogenous L-arginine supplementation would competitively overcome the inhibition of NO synthase produced by the increased ADMA level and enhance NO production in hypercholesterolemic rabbits (Bode-Böger et al., 1996). The different levels of ADMA may explain the various effects of L-arginine administration on impaired endothelium-dependent relaxation in hypercholesterolemia.

The inhibitory effect of ox-LDL on endothelium-dependent relaxation may involve alterations in muscarinic receptor(s) and/or in the receptor signal transduction pathway. This effect seems concentration-dependent. In rabbit aorta, low concentrations of ox-LDL (≤50 μg protein/ml) selectively inhibited endothelium-dependent relaxation evoked by the receptor-dependent agonist ACh. However, high concentrations of ox-LDL (>50 μg protein/ml) also inhibited endothelium-dependent relaxation evoked by the receptor-dependent agonist A23187 (Kugiyama et al., 1990). The effect of ox-LDL on NO synthase appears to be both time- and concentration-dependent (Hirata et al., 1995; Liao et al., 1995). Galle et al. (1991) showed that EDRF formation was not attenuated by a 1-h incubation with the potentially cytotoxic ox-LDL (1 mg protein/ml). ox-LDL seems less likely to influence NO synthase in our study because of the short incubation with ox-LDL. ox-LDL (100 μg protein/ml) had no effect on endothelium-independent relaxation in response to sodium nitroprusside in our study, so the capacity of vascular smooth muscle to relax in response to EDRF was unaffected by ox-LDL. Another possible explanation for the impaired endothelium-dependent relaxation is related to the release of EDCF, which could oppose the effects of EDRF. Because our buffer contained indomethacin, we can exclude the influence of vasoconstrictor prostanoids. Whether ox-LDL increases endothelin release is controversial (Boulanger et al., 1992; He et al., 1996). He et al. (1996) showed that different degrees of oxidation of LDL had different effects on endothelin production. Extensively oxidized LDL (24-h exposure to copper)
inhibited endothelial secretion from cultured endothelial cells. In our study, LDL is oxidized through a 24-h exposure to CuSO₄. Thus, increased endothelial release is unlikely to contribute to the vasomotor effects of ox-LDL.

Endothelium-dependent relaxation may be mediated by both EDRF and EDHF. Recently, Shoemaker et al. (1996) reported that EDHF was involved in ACh-induced relaxation in human saphenous vein. Therefore, the possibility that ox-LDL also inhibits EDHF-mediated relaxation cannot be totally excluded in our study. Another mechanism that cannot be excluded is the increased inactivation of EDRF by ox-LDL or superoxide anion. Hypercholesterolemia and ox-LDL have been reported to increase superoxide production (Maeba et al., 1995; Ohara et al., 1993). ox-LDL may also directly inactivate EDRF after its release from endothelium (Chin et al., 1992; Galle et al., 1991).

In summary, ox-LDL inhibited endothelium-dependent relaxation and increased endothelium-dependent contraction evoked by 5-HT in human vessels. ox-LDL-induced impairment of endothelium-dependent relaxation, at least in human saphenous vein, is probably not due to a deficiency of L-arginine for EDRF formation. Because ox-LDL does produce endothelial dysfunction in human saphenous vein, it may influence the consequences of coronary bypass surgery and saphenous vein graft function. These results also indicate that human saphenous vein can be used as a model to study the vasomotor effects of ox-LDL.

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