Oxidized Low Density Lipoproteins Cause Contraction and Inhibit Endothelium-dependent Relaxation in the Pig Coronary Artery

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Abstract

The direct vasoactive effects of native and oxidatively modified low density lipoproteins as well as their effects on endothelium-dependent relaxations to 5-hydroxytryptamine were studied in isolated rings of pig right coronary artery. Slowly developing contractions were caused by native low density lipoproteins (100 μ g protein/ml). The contractions were more pronounced in the absence than in the presence of the trace metal chelator, EDTA, and coincided with the formation of lipid peroxides during the response. The lipophilic antioxidant, butylated hydroxytoluene, prevented the oxidation of, and contraction to, native low density lipoproteins. Low density lipoproteins oxidized by exposure to copper contracted coronary arteries more rapidly with a threshold of only 1 μ g protein/ml, but with a similar maximal contraction at 100 μ g protein/ml. Superoxide dismutase inhibited the contraction to native low density lipoproteins, but not to oxidized low density lipoproteins. Catalase blocked contractions to both native and oxidized low density lipoproteins. Contractions to oxidized low density lipoproteins were unaffected by indomethacin, but were abolished by removal of the endothelium or by inhibitors of endothelium-derived relaxing factor. Oxidized low density lipoproteins but not native low density lipoproteins inhibited endothelium-dependent relaxations to 5-hydroxytryptamine. Thus, oxidized low density lipoproteins cause endothelium-dependent coronary artery contractions which are mediated by a hydroperoxide. Contractions to native low density lipoproteins are due to their oxidation in the organ chamber by the superoxide anion radical. Oxidized, but not native, low density lipoproteins impair normal endothelial cell vasodilator function in vitro. Oxidized low density lipoproteins, important in the pathogenesis of atherosclerosis, may directly contribute to the increased risk of vasospasm seen in hypercholesterolemia and atherosclerosis. (*J. Clin. Invest.* 1990; 86:75-79.) Key words: endothelium • low density lipoproteins • oxidation • vasospasm • coronary artery

Introduction

Coronary artery spasm may account for a variety of clinical syndromes such as variant angina, acute myocardial infarction,

and sudden cardiac death (1, 2). The mechanisms of vasospasm however are not entirely clear. Atherosclerosis and hypercholesterolemia have been implicated in the pathogenesis of vasospasm and a local hyperreactivity of the coronary artery may be involved. A reduced release of endothelium-derived relaxing factor(s) (EDRF)¹ may play an important role. In atherosclerotic vessels or those exposed to hypercholesterolemia, endothelium-dependent relaxations to several agonists are inhibited and contractions may be augmented (3-7).

LDLs, which are the major carriers of cholesterol in the blood, are known to be of primary importance in the development of coronary atherosclerosis, and recently it has been reported that in vitro exposure of rabbit aorta and pig coronary arteries to high concentrations of human LDL inhibited endothelium-dependent relaxations (8, 9). It was concluded that abnormal reactivity of atherosclerotic and hypercholesterolemic blood vessels could result from direct interactions of LDL with the endothelium.

LDL is oxidatively modified when incubated in vitro with three major cell types in the vascular wall: endothelial cells, vascular smooth muscle cells, and macrophages (10-13). This modification involves peroxidation of lipids measured as thiobarbituric acid reactive substances (TBARS) (14, 15) and is accompanied by a number of other changes in LDL. LDL particles show an increased negative charge causing higher electrophoretic mobility (10), increased density (10), formation of lysophosphatidylcholine (16), and a new configuration of the apoprotein B which leads to its recognition by the acetyl-LDL receptor (10, 17). Oxidized LDL (ox-LDL) is rapidly taken up by macrophages, thus transforming these cells into foam cells, one of the earliest signs of atherosclerosis (10, 11, 18). Recent studies suggest that oxidation of LDL occurs in vivo (19-21), and oxidatively modified LDL has been detected in atherosclerotic lesions of the rabbit and man (19).

It is not known if native LDL (n-LDL) or ox-LDL have direct vasoactive properties, which may contribute to abnormal vascular reactivity during hypercholesterolemia and atherosclerosis. The role of ox-LDL in impaired endothelium-dependent relaxation under these conditions has also not yet been determined. The purpose of this study was therefore to determine possible direct vasoactive effects of n-LDL and ox-LDL as well as their effects on endothelium-dependent relaxation in pig coronary arteries.

Methods

The right coronary artery was dissected from the heart of domestic pigs killed by exsanguination after anesthesia with ketamine (20 mg/kg

1. Abbreviations used in this paper: BHT, butylated hydroxytoluene; EDRF, endothelium-derived relaxing factor(s); 5-HT, 5-hydroxytryptamine; MDA, malondialdehyde; n-LDL, native LDL; ox-LDL, oxidized LDL; PSS, physiological salt solution; TBARS, thiobarbituric acid reactive substances.

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i.m.), acepromazine (0.8 mg/kg i.m.), and chloralose (75 mg/kg i.v.), and anticoagulation with sodium heparin (100 U/kg i.v.).

Organ chamber experiments. Rings of artery (6 mm long) were suspended from strain gauges for measurement of isometric circumferential force. The rings were placed in organ chambers filled with 7 ml of physiological salt solution (PSS) of the following millimolar composition: NaCl 118.3, KCl 4.7, MgSO₄ 0.6, KH₂PO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, Ca-EDTA 0.026, and glucose 11.0. The solution was maintained at 37°C and gassed with 20% O₂/5% CO₂/75% N₂. In some rings the endothelium was removed by inserting a stainless steel wire into the lumen and gently rolling the ring on wetted filter paper. The presence or absence of endothelial function was later confirmed by the presence or absence of relaxation caused by the endothelium-dependent vasodilator, bradykinin. The rings were progressively stretched over 1 h to the optimal tension for contraction, previously determined to be 12 g (22). To measure relaxation or contraction caused by n-LDL and ox-LDL, rings ($n = 71$) were contracted with the thromboxane A₂ mimetic U46619 to 8.6 ± 0.3 g, $35 \pm 2\%$ of their maximal potassium-induced contraction which averaged 24 ± 1 g. In some cases similar tone was provided by potassium. When the U46619-induced contraction stabilized, n-LDL or ox-LDL was added. Inhibitors were added 30 min before LDL. Indomethacin (10^{-5} M), SOD (120 U/ml), and catalase (1,200 U/ml) did not significantly alter the contraction caused by U46619 (8.1 ± 0.7 g, $n = 6$; 7.7 ± 0.6 g, $n = 7$; and 7.1 ± 1.1 g, $n = 9$, respectively). Methylene blue and LY83583 increased the U46619-induced tone from 7.7 ± 0.6 g ($n = 11$) to 10.3 ± 1.0 g ($n = 6$) and 10.6 ± 1.8 g ($n = 5$) which was statistically significant only for methylene blue.

Pilot experiments revealed that n-LDL caused slowly developing contractions that reached a plateau after ~ 1 h, whereas ox-LDL caused immediate contractions. The comparison between these responses suggested that the response to n-LDL may be due to its oxidation in the organ chamber during the experiment. n-LDL responses were therefore studied under different oxidizing conditions with simultaneous measurement of its degree of oxidation by removing 1 ml of the chamber solution after the response to n-LDL reached a plateau for measurement of lipid peroxides. To prevent foaming Antifoam B (10 ppm), containing nonionic emulsifiers, was added to the organ chamber before addition of LDL. A possible influence of the anti-foaming solution on the physical integrity of LDL was excluded by agarose gel electrophoresis of LDL incubated in the organ chamber for 1 h with antifoaming solution (50 ppm).

To determine the effects of LDL on endothelium-dependent relaxations to 5-hydroxytryptamine (5-HT) (7) rings were contracted with U46619. After contractions stabilized, n-LDL or ox-LDL (100 μ g protein/ml) were added and 10 min later a dose response curve to 5-HT was constructed. To block 5-HT receptors on the vascular smooth muscle, ketanserin (10^{-6} M) was added 30 min before addition of 5-HT. Relaxations are expressed as a percentage of the maximal relaxation caused by papaverine (10^{-5} M).

Lipoprotein preparation. LDL was isolated by sequential ultracentrifugation from citrated fresh human plasma drawn from healthy normolipemic donors. EDTA (1 mg/ml) was added to the plasma and its density was increased to 1.025 by adding solid KBr. After centrifugation for 18 h at 42,000 rpm, 15°C in an ultracentrifuge using a 55.2 ti rotor (Beckman Instruments, Inc., Fullerton, CA), the infranatant was collected. The pooled infranatant was then adjusted to density 1.063, centrifuged as before, and LDL ($d = 1.025$ – 1.063 g/ml) was collected from the supernatant. In some preparations, before centrifugation the lipophilic antioxidant, butylated hydroxytoluene (BHT, 20 μ M), was added directly to the plasma. The purity and integrity of LDL preparations were controlled by agarose gel electrophoresis and the protein concentration of LDL was determined by the method of Lowry (23). Before each experiment, LDL was dialyzed at 4°C for 24 h against three changes of PSS gassed with 95% N₂/5% CO₂. LDL was oxidized by exposure to 5 μ M CuSO₄ for 20 h in EDTA-free H₂O at room temperature followed by dialysis at 4°C for 24 h against three changes of PSS. Copper-oxidized LDL has previously been shown to be indis-

tinguishable from LDL which was biologically oxidized by endothelial cells in culture (14).

Determination of lipid peroxidation. The lipid peroxide content of n-LDL and copper-oxidized LDL was measured fluorometrically as TBARS (24). LDL (100 μ g protein) was mixed with 1.5 ml of 0.67% thiobarbituric acid and 1.5 ml of 20% trichloroacetic acid containing 1 mg/ml EDTA. After heating at 100°C for 30 min, fluorescent reaction products were assayed on a spectrofluorometer with an excitation wavelength of 515 nm and an emission wavelength of 553 nm. Freshly diluted tetramethoxypropane, which yields malondialdehyde (MDA), was used as a standard and results are expressed as nanomoles of MDA equivalents. Baseline values for TBARS were obtained immediately after preparation of n-LDL or ox-LDL for each experiment.

Drugs. The following pharmacological agents were used: bradykinin acetate, 5-HT creatinine sulfate, catalase (from bovine liver, 2,890 U/mg protein), SOD (from bovine erythrocytes, 3,050 U/mg protein), indomethacin, methylene blue, BHT, 2-thiobarbituric acid, Antifoam B (Sigma Chemical Co., St. Louis, MO), LY83583 (a gift from Eli Lilly Co., Indianapolis, IN), malonaldehyde bis(dimethyl acetal) (1,1,3,3-tetramethoxypropane) (Aldrich Chemical Co., Milwaukee, WI), U46619 (9,11-dideoxy-11 α ,9 α -epoxymethano-prostaglandin F_{2 α}) (The Upjohn Co., Kalamazoo, MI).

Data analysis. The data are expressed as means $\pm$ SEM. n refers to the number of different animals from which arteries were obtained for study. Statistical analysis was performed using Student's t test for unpaired observations, except for dose-response curves to 5-HT, which were compared by analysis of variance. Differences were considered to be statistically significant when the P -value was < 0.05 .

Results

Vasoactive effects of n-LDL and ox-LDL. In the presence of EDTA (26 μ M) in coronary artery rings with endothelium contracted by U46619, the addition of n-LDL (Fig. 1 A) resulted in slowly developing further contractions. The threshold for contraction was at concentrations of n-LDL of 10–100 μ g protein/ml. Contractions reached a plateau after ~ 1 h. ox-LDL (Fig. 1 B) also contracted rings with endothelium, however the response compared with n-LDL was more rapid and occurred at lower concentrations with a threshold of 0.1–1 μ g protein/ml. The maximal contraction to ox-LDL (100 μ g protein/ml) was significantly greater than that to n-LDL (100 μ g protein/ml) in the presence of EDTA (Table I). Rings without endothelium did not respond significantly to either n-LDL (-1.2 ± 0.6 g, $n = 9$) or ox-LDL (0.0 ± 0.9 , $n = 6$, Fig. 1). In rings contracted with potassium, ox-LDL also caused contractions, which were not significantly different from the contractions in U46619 contracted rings (4.3 ± 0.8 g, $n = 3$).

Responses to n-LDL under different oxidizing conditions. The contractions of rings with endothelium caused by n-LDL were significantly larger when EDTA was omitted from the PSS (Fig. 2, Table I). Under these conditions contractions to n-LDL (100 μ g protein/ml) averaged 5.3 ± 1.1 g (Table I). TBARS rose from a baseline value of 0.33 ± 0.2 to 8.2 ± 1.6 (nmol MDA/mg LDL protein) measured after 1 h when contractions had attained a plateau (Table I). In PSS with EDTA the contractions as well as the TBARS were significantly smaller than in the absence of EDTA (Fig. 2, Table I). No significant contractions were observed when LDL treated with BHT (20 μ M) was added to the organ chambers in the absence of EDTA (Fig. 2, Table I). There was also no significant increase in TBARS after 1 h incubation of BHT-treated LDL in the organ chamber (Table I).

Figure 1. Original tracings of responses of pig coronary artery rings with and without endothelium to increasing concentrations of n-LDL (A) or ox-LDL (B; 0.1–100 μ g protein/ml). All rings were first contracted with U46619 with EDTA present. n-LDL causes a slowly developing contraction in the ring with, but not without, endothelium. ox-LDL also contracts only the ring with endothelium, but the contraction is more rapid and occurs at lower concentrations.

The maximal contractions to ox-LDL (100 μ g protein/ml) in PSS with EDTA averaged 5.7 ± 0.8 g and were not significantly different from the maximal contractions to n-LDL in the absence of EDTA (Table I). ox-LDL had TBARS of 6.0 ± 1.0 (nmol MDA/mg protein) which was not significantly

Table I. Effect of LDL on Pig Coronary Artery Tone and Formation of Lipid Peroxides

	n-LDL	n-LDL	BHT-LDL	ox-LDL
EDTA	Without	With	Without	With
Change in tension (g)	5.3 ± 1.1	$2.2 \pm 0.5^*$	$-1.1 \pm 0.8^\ddagger$	5.7 ± 0.8
TBARS (nmole MDA/mg protein)	8.2 ± 1.6	$1.0 \pm 0.1^\ddagger$	$0.5 \pm 0.2^\ddagger$	6.0 ± 1.0
n	9	9	6	11

Values are means $\pm$ SEM. Rings with endothelium were contracted with U46619 and maximal responses to 100 μ g LDL protein/ml are indicated as either further contraction or relaxation. TBARS were measured after 1 h incubation in the organ chamber. Statistical comparison of data with that for n-LDL in PSS without EDTA: * $P < 0.05$, $^\ddagger P < 0.001$. n indicates the number of animals from which arteries were studied.

Figure 2. Original tracings of responses of coronary artery rings with endothelium to n-LDL (100 μ g protein/ml) under different oxidizing conditions. All rings were first contracted with U46619. n-LDL causes a slowly developing contraction which is larger in the absence than in the presence of EDTA. BHT-LDL causes no contraction.

different from that of n-LDL after 60 min incubation in PSS without EDTA (Table I).

Effect of inhibitors on n-LDL and ox-LDL responses. Indomethacin (10^{-5} M) had no significant effect on the maximal contractions of coronary artery rings with endothelium induced by n-LDL or ox-LDL (Table II). SOD (120 U/ml) significantly inhibited the contractions to n-LDL, but did not significantly affect the contractions to ox-LDL (Table II). SOD significantly decreased TBARS formation after the addition of n-LDL to PSS without EDTA (2.1 ± 0.9 nmol MDA/mg LDL protein, $P < 0.05$). In the presence of catalase (1,200 U/ml) contractions to both n-LDL and ox-LDL were abolished and relaxations occurred (Table II). Methylene blue (10^{-5} M) or LY83583 (10^{-5} M) also prevented contractions caused by ox-LDL and resulted in ox-LDL-induced relaxations (Table II).

Effect of n-LDL and ox-LDL on endothelium-dependent relaxations to 5-HT. In the presence of ketanserin (10^{-6} M) the endothelium-dependent agonist, 5-HT, caused concentration-dependent relaxations. After 10 min exposure to n-LDL (100 μ g protein/ml) in the presence of EDTA, the response to 5-HT

Table II. Effect of Inhibitors on n-LDL and ox-LDL-induced Contractions of Pig Coronary Artery Rings with Endothelium

Treatment	Change in tension (g)			
	n-LDL	n	ox-LDL	n
Control	5.3 ± 1.1	9	5.7 ± 0.8	11
Indomethacin (10^{-5} M)	6.4 ± 0.5	3	4.1 ± 1.2	3
SOD (120 U/ml)	$0.7 \pm 0.9^*$	4	5.7 ± 0.9	3
Catalase (1,200 U/ml)	$-1.4 \pm 0.5^\ddagger$	6	$-1.7 \pm 0.6^\ddagger$	3
Methylene blue (10^{-5} M)			$-5.0 \pm 1.0^\ddagger$	6
LY83583 (10^{-5} M)			$-4.3 \pm 0.8^\ddagger$	5

Values are means $\pm$ SEM. Inhibitors were added 30 min before the addition of n-LDL or ox-LDL. Rings were contracted with U46619 and values are the maximal change in tension in grams due to 100 μ g protein/ml n-LDL or ox-LDL. Experiments with n-LDL were performed in PSS without EDTA and those with ox-LDL in PSS with EDTA. n indicates the number of animals from which arteries were studied. Statistical comparisons to control data: * $P < 0.05$, $^\ddagger P < 0.005$, $^\ddagger P < 0.001$.

Figure 3. Effect of n-LDL (A; $n = 8$) and ox-LDL (B; $n = 7$) on endothelium-dependent relaxation evoked by 5-HT in intact pig coronary artery rings. Rings in PSS with EDTA were contracted with U46619, and LDL (100 $\mu\text{g/ml}$) was added 10 min before addition of 5-HT. Relaxations are expressed as the percentage of the maximal relaxation caused by papaverine (10^{-5} M). ox-LDL, but not n-LDL, at the same protein concentration of 100 $\mu\text{g/ml}$ caused a significant inhibition of relaxation. Comparisons were made by analysis of variance where $P < 0.05$ was considered significant.

was not significantly altered (Fig. 3). A 10-min exposure to ox-LDL (100 μg protein/ml) significantly inhibited 5-HT-mediated relaxations (Fig. 3).

Discussion

This study shows that oxidatively modified LDL, which may play a key role in the pathogenesis of atherosclerosis, have direct contractile effects in pig coronary arteries. Contractions to n-LDL are due to its oxidation in the organ chamber and ox-LDL, but not n-LDL, inhibit endothelium-dependent relaxations to 5-HT.

The LDL molecule is known to be unstable after its isolation from the blood. It is readily auto-oxidized in the presence of air and is highly sensitive to metal-catalyzed oxidation (25, 26). The aerated organ chambers to which n-LDL was added therefore represent a highly oxidizing environment. Oxidation of n-LDL under these conditions is dependent on trace metals in the buffer and the superoxide anion radical, as evidenced by the inhibitory effects of the trace metal chelator, EDTA, and SOD. Oxidation of n-LDL was confirmed by measuring increased levels of lipid peroxides at the end of the experiment. Oxidation and contractions were also prevented by the lipophilic antioxidant, BHT. The oxidation of n-LDL occurring under the experimental conditions in the present study was not dependent upon the coronary artery ring as demonstrated by similar TBARS measurements when the artery was omitted from the organ chamber (unpublished observations). This study demonstrates the instability of n-LDL when used in vitro unless it is protected from oxidation.

Recent studies provide evidence that oxidation of LDL occurs in vivo (19–21). Oxidation of LDL may not occur in the circulation, because plasma inhibits the oxidation (18), but could occur in the subintima of blood vessels. ox-LDL may therefore exert its vasoconstrictor effect within the vascular wall. Atherosclerotic lesions, both in man and in the rabbit, have been shown to contain oxidatively modified LDL (19), emphasizing the relevance of our findings. The real concentrations of ox-LDL in the vascular wall are unknown, but it is not unlikely that the low concentrations of ox-LDL causing contractions in vitro (0.1–100 $\mu\text{g/ml}$) occur in vivo. Further studies, however, will be necessary to determine if oxidation of LDL in vivo results in coronary artery contractions.

Contractions to ox-LDL were abolished by the removal of the endothelium, suggesting several possible mechanisms for

these contractile responses. The endothelium attenuates the spontaneous development of myogenic tone in the pig coronary artery, a phenomenon which is due to basal release of EDRF (22). Inhibition of the basal release of EDRF either by endothelium removal or by inhibitors of EDRF, methylene blue, LY83583, or hemoglobin (27–29) causes contractions and increases sensitivity of the pig coronary artery to contractile agents (7, 22). Thus, ox-LDL may act through a similar mechanism. It may block either the basal release of EDRF or interfere with its action on the vascular smooth muscle. As demonstrated by the inhibitory effect of catalase on ox-LDL responses, hydroperoxides associated with the lipid peroxides in ox-LDL are possible inhibitors of EDRF. The prevention of contraction by methylene blue and LY83583 makes it less likely that endothelium-derived vasoconstrictor substances such as endothelin (30) mediate the contractions caused by ox-LDL. Vasoconstrictor prostanoids were ruled out by the failure of the cyclooxygenase inhibitor indomethacin to block the contractions.

Preliminary studies showed that ox-LDL causes significant, but smaller, contractions of pig coronary arteries with endothelium which are not contracted with U46619 (1.1 ± 0.2 g, $n = 6$). Thus, the magnitude of contractions to ox-LDL depends upon preexisting tone which in this study was provided by the thromboxane A_2 -mimetic. Since contractile responses to ox-LDL were not significantly different when artery rings were contracted with potassium, it is unlikely that contractions to ox-LDL were due to some specific interactions with U46619. Nevertheless, contractile synergism may occur in vivo between ox-LDL and vasoactive factors such as thromboxane A_2 which may be released from platelets at sites of atherosclerosis.

Endothelium-dependent relaxations to 5-HT and other agonists are impaired in coronary arteries from hypercholesterolemic or atherosclerotic pigs and man (7, 31–33). Previous studies in rabbit aorta (8) and pig coronary artery (9) showed that LDL, under conditions shown to be oxidizing in the present study, impaired endothelium-dependent relaxation. The observation that ox-LDL, but not n-LDL, inhibits the response to 5-HT suggests a role of oxidatively modified LDL in the impaired endothelium-dependent vascular relaxation observed in arteries exposed to LDL in vitro and in vivo.

In conclusion, this study demonstrates that aside from its atherogenic potential, ox-LDL, by impairing normal endothelial vasodilator function, could directly contribute to the in-

creased risk of coronary vasospasm seen in hypercholesterolemia and atherosclerosis.

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