Native and oxidized low-density lipoproteins have different inhibitory effects on endothelium-derived relaxing factor in the rabbit aorta

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- 1 The effect of native low-density lipoproteins (LDL) and oxidized LDL (OXLDL) on the relaxations to endothelium-derived relaxing factor (EDRF) in isolated, intact aortic rings of the rabbit were investigated.
- 2 Native LDL induced a concentration-dependent reversible inhibition of the relaxations elicited by acetylcholine (ACh) or A23187, in rings pre-contracted by noradrenaline (NA), adrenaline (Ad) and 5-hydroxytryptamine (5-HT), but not phenylephrine (PE), which was not influenced by indomethacin.
- 3 The inhibition was surmountable in the rings pre-contracted with NA and Ad and only partially in those pre-contracted with 5-HT.
- 4 OXLDL induced an inhibition of the relaxations elicited by ACh and A23187 which was independent of the contractile agonist. The extent of inhibition and its reversibility varied with the LDL from individual donors, but was unaffected by indomethacin.
- 5 Native and oxidized LDL inhibited relaxations evoked by exogenous nitric oxide (NO) to the same extent. Higher concentrations of NO overcame the inhibition. The inhibition was independent of the contractile agonist and the preparation of LDL from individual donors.
- 6 Only OXLDL inhibited reversibly relaxations evoked by glyceryl trinitrate (GTN) and the inhibition was independent of the LDL preparation from individual donors.
- 7 This study demonstrates that native and OXLDL influence the response to EDRF in isolated aorta. We suggest that these lipoproteins may contribute to the attenuation of responses to EDRF found in isolated arteries from hypercholesterolaemic and atherosclerotic animals.

Introduction

Furchgott & Zawadzki, (1980) were the first to demonstrate that endothelium-dependent relaxation in the rabbit aorta is accounted for by the release of endothelium-derived relaxing factor (EDRF), which is now known to be nitric oxide (Moncada *et al.*, 1988). The attenuation of this response in arteries from cholesterol-fed animals (Verbeuren *et al.*, 1986; Shimokawa & Vanhoutte, 1989) and from human atherosclerotic arteries (Förstermann *et al.*, 1988) has led to the suggestion that a dysfunction in EDRF release is important in atherosclerosis. We have linked this observation to low-density lipoproteins (LDL) which at high plasma concentrations are known risk factors for coronary heart disease. In support of this claim, we have shown in our laboratory that LDL inhibit, in an irreversible manner, relaxations mediated by EDRF in rabbit aorta precontracted by noradrenaline or 5-hydroxytryptamine (Andrews *et al.*, 1987). Furthermore, LDL-modified by oxidation, which are known to be present in the intima of atherosclerotic arteries and thought to be the atherogenic form of LDL (Ylä-Herttuala *et al.*, 1989), behaved in a similar way to that of native LDL (Dunn *et al.*, 1988). In preliminary publications we have found that the extent of inhibition and its reversibility depends on several factors, including the degree of oxidation and the nature of the LDL of individual donors (Jacobs *et al.*, 1989; Plane *et al.*, 1989). Here, we describe in more detail the effects of LDL on EDRF-mediated relaxations and compare them to those of LDL modified by oxidation.

Methods

Preparation of lipoproteins

Native low-density lipoproteins Fresh plasma was obtained from apparently healthy human volunteers. Native LDL

(density, 1.019–1.063 g ml⁻¹) was prepared separately from the plasma of each donor by discontinuous density gradient ultracentrifugation in a Kontron vertical rotor (TFT 70.38) with a Centrikon T 2080 ultracentrifuge. To remove contaminating plasma proteins, LDL was recentrifuged for 10 h in a conventional rotor at 190,000 *g* (*r* = 6.75 cm) in buffer with density adjusted to 1.063 g ml⁻¹ (Andrews *et al.*, 1987). Buffers contained 0.3 mM EDTA to prevent oxidation. The purified LDL was finally dialysed for 24 h against three changes of 2 l of modified Tyrode buffer (composition (m): NaCl 0.13, NaHCO₃ 0.012, NaH₂PO₄ 0.0042, KCl 0.0027) containing 0.3 mM EDTA. LDL prepared by this procedure had no significant oxidation as measured by lipid peroxidation or fluorescence spectroscopy (excitation 350 nm, emission 420 nm), a method which correlates well with the production of thiobarbituric acid reactive substances as shown previously by Dunn *et al.* (1988).

Oxidized low-density lipoproteins The oxidative modification of LDL was performed by incubation of freshly prepared LDL with 5 μ M Cu²⁺ for 24 h at 18°C, followed by extensive dialysis against Tyrode buffer. Under these conditions, the LDL of individual donors was oxidized to a similar extent as determined by fluorescence spectroscopy. Cu²⁺-oxidized LDL have been shown to have similar properties to cell-modified LDL (Steinbrecher *et al.*, 1984).

Measurement of endothelium-dependent relaxation

The descending thoracic aortae were removed from 6 month-old New Zealand White rabbits and trimmed free of fat and adhering connective tissue. Two mm wide, transverse rings were cut and paired tissues were mounted under a resting tension of 2 g, in oxygenated Krebs-bicarbonate buffer containing 0.3 mM EDTA at 37°C, for isometric force measurements (Andrews *et al.*, 1987). Tissues were equilibrated for 90 min and pre-contracted with noradrenaline (NA), adrenaline (Ad), 5-hydroxytryptamine (5-HT), phenylephrine (PE) or

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prostaglandin $F_{2\alpha}$ to give 75% of the maximal contraction obtained with NA. They were then relaxed by graded doses of acetylcholine (ACh), A23187, nitric oxide solution (NO) or glyceryl trinitrate (GTN). After washout and equilibration, the tissues were preincubated with native LDL or oxidized LDL (OXLDL, made from the same native LDL preparation) at concentrations in the range of 0.5–2 mg protein ml^{-1} or Tyrode buffer (control) for 0–60 min and then the contraction/relaxation cycle was repeated. Control tissues were used to correct for time-dependent changes in the responses. In some experiments, LDL was added to the pre-contracted rings. Finally, after removal of the lipoproteins by washing, the EDRF responses of the tissues were again assessed to determine the reversibility of the effects. After the experiment, the integrity of the endothelium was assessed by silver staining (Poole *et al.*, 1958).

Statistical analysis

Comparisons were made by use of Student's *t* test for unpaired samples where $P < 0.05$ was considered significant.

Drugs and chemicals

NA, Ad and PE were from Sigma, U.K. 5-HT and all other chemicals were from B.D.H. (U.K.).

Results

Native LDL reversibly inhibits responses to EDRF

Figure 1a shows a typical trace of the endothelium-dependent relaxation evoked by graded doses of ACh in an aortic ring pre-contracted with NA, as described by Furchgott & Zawadzki (1980). These relaxations could be repeated in the presence of Tyrode buffer and after further washout, without

significant change in the sensitivity to ACh. LDL (2 mg protein ml^{-1}), caused a reduction in the relaxations evoked by ACh, compared to the initial relaxations, which were overcome at higher concentrations of ACh (Figure 1b). This decrease in sensitivity to ACh was reversed after washout. The threshold concentration for inhibition was about 0.5 mg protein ml^{-1} and rapidly increased to a maximum at 2 mg protein ml^{-1} (results not shown). Experiments were therefore routinely done at this latter concentration.

Similar inhibitory effects by LDL of endothelium-dependent relaxations elicited by ACh were found when rings were pre-contracted with Ad (not shown). In addition, pre-contraction with 5-HT (0.3 μM), showed a significant decrease in maximal relaxation in the presence of LDL (Figure 2). LDL, on the other hand, had no influence on relaxations elicited by ACh in rings pre-contracted with phenylephrine (Figure 1c) or prostaglandin $F_{2\alpha}$ (not shown), whereas if a subthreshold concentration of 5-HT (10 nM) which did not enhance contraction, was also present, an inhibition similar to that shown in Figure 2 was found. The lipoproteins themselves had no contractile effects in the absence or presence of a pre-contraction.

Prolonged pre-incubation with LDL for up to 1 h did not intensify the inhibition of relaxation, and the results were similar even if LDL was added to the pre-contracted ring immediately before the addition of ACh (not shown). The onset of the effect was therefore very rapid. Fifty μM indomethacin, 20 units ml^{-1} of superoxide dismutase or 10 μM ascorbic acid had no effect on the inhibition of relaxation. No significant differences were detected in the inhibition of relaxation by LDL obtained from the plasma of different donors. The inhibition by LDL of relaxations elicited by the Ca^{2+} ionophore, A23187 which act via a receptor-independent mechanism, was not significantly different (Figure 3a,b), showing that interference with receptor binding of the relaxant agent is not implicated in the inhibition. Furthermore, no visible loss of endothelium was found in the rings treated with LDL, as determined by silver staining.

Figure 1 Traces showing the inhibition of acetylcholine (ACh)-evoked endothelium-dependent relaxations by native low-density lipoproteins (LDL) in intact aortic rings of the rabbit. Tissues were precontracted with 0.03 μM noradrenaline (NA) (a and b) or 0.1 μM phenylephrine (PE) (c), and relaxed with cumulative doses of ACh (0.01–1 μM). The cycle of contraction-relaxation was repeated in the presence of (a) Tyrode buffer (control), (b and c) LDL (2 mg protein ml^{-1}) and then after removal of the LDL by washing. Addition of ACh is shown by — and cumulative concentrations are shown in —log unit.

Figure 2 Dose-response curves showing the effect of native low-density lipoproteins (LDL) on endothelium-dependent relaxations evoked by acetylcholine (ACh) in rings pre-contracted with $0.3 \mu\text{M}$ 5-hydroxytryptamine (5-HT). Experiments were performed as in Figure 1. Curves were constructed from responses in tissues before and after treatment with native LDL. Relaxations are expressed as the % relaxation of the initial maximum contraction. Each point is the mean of 5 preparations of native LDL from 5 different donors. Vertical bars indicate s.e.mean. Comparisons were made by Student's *t* test for unpaired samples, * $P < 0.05$. (●) Control; (■) + LDL; (□) after washout.

Inhibition of relaxation by OXLDL

Distinctive differences were found between the effect of LDL and oxidized LDL on endothelium-dependent relaxation. It was discovered that to achieve maximum inhibition with OXLDL, it was necessary to pre-incubate the rings with the lipoprotein at $2 \text{ mg protein ml}^{-1}$ for 30 min. In contrast to native LDL, inhibition occurred when PE as well as NA and 5-HT were used to pre-contrast the rings. In addition, the

Figure 3 Traces showing the inhibition of Ca^{2+} ionophore, A23187-evoked endothelium-dependent relaxations by low-density lipoproteins (LDL) and oxidized LDL (OXLDL). Tissues: (a) control, (b) treated with LDL, (c) treated with OXLDL. The experiment was performed as in Figure 1 except that in (c) rings were pre-incubated with OXLDL ($2 \text{ mg protein ml}^{-1}$) for 30 min before pre-contraction with noradrenaline (NA) or phenylephrine (PE).

inhibitory behaviour of OXLDL was found to fall into three major categories which were reproducible on different occasions of preparation from particular donors and were unaffected by $50 \mu\text{M}$ indomethacin.

Combined data from experiments using OXLDL prepared from 4 donors are shown in Figure 4a. As demonstrated by the dose-response curves, maximal relaxations evoked by ACh were inhibited by 80–90% in the presence of OXLDL and the inhibition persisted after washout. In contrast, OXLDL from 3 other donors, inhibited the response to ACh and partially reduced the maximal relaxation (Figure 4b). These effects were reversed after washout. Inhibition by OXLDL from 3 further donors, on the other hand, resembled in some respects that of native LDL and was reversible (cf. Figures 2 and 4c). However, a 30 min pre-incubation was necessary to produce the inhibition. Relaxations elicited by A23187 were also inhibited by OXLDL from these different donors in a similar

Figure 4 Dose-response curves showing the effect of oxidized low-density lipoproteins (OXLDL) on endothelium-dependent relaxations evoked by acetylcholine (ACh). Tissues were pre-contracted with $0.1 \mu\text{M}$ phenylephrine (PE). Experiments were performed as in Figure 1 except that tissues were pre-incubated for 30 min with OXLDL before contraction. Curves are constructed from the initial relaxations and those after treatment OXLDL ($2 \text{ mg protein ml}^{-1}$). OXLDL was prepared from the plasma of donor: (a) TH, $n = 4$, TB, $n = 4$, RR, $n = 4$, GW, $n = 3$; (b) MT, $n = 4$, GD, $n = 4$, RB, $n = 3$; (c) HP $n = 3$; FP $n = 3$; MJ, $n = 4$. Relaxations are expressed as the % relaxation of the initial maximum contraction. Each point is the mean of 10–15 experiments with preparations of OXLDL; vertical bars indicate s.e.mean. Significant differences were found between donors in groups (a–c) but not within the groups. (□) Control; (■) + OXLDL; (●) after washout. * $P < 0.05$, significantly different from control.

Figure 5 Dose-response curves showing the effects of lipoproteins on endothelium-independent relaxation by nitric oxide. Rings denuded of endothelium were pre-contracted with phenylephrine (PE) and relaxed by graded doses of nitric oxide (NO), the relaxation cycle was repeated in the presence of Tyrode buffer, native low-density lipoproteins (LDL) or oxidized LDL (OXLDL) (without pre-incubation) and after washout. Each point is the mean of 4 experiments with different preparations of lipoproteins from different donors; vertical bars indicate s.e.mean. (□) Control; (○) + LDL; (●) + OXLDL. * $P < 0.05$, significantly different from control.

Figure 6 Dose response curves showing the effects of lipoproteins on endothelium-independent relaxation by glyceryl trinitrate. Rings pre-contracted with $0.1 \mu\text{M}$ phenylephrine (PE) and relaxed by graded doses of glyceryl trinitrate (GTN), the relaxation cycle was repeated in the presence of Tyrode buffer, native low-density lipoproteins (LDL) or oxidized LDL (OXLDL) (without pre-incubation). Each point is the mean of 4 experiments with different preparations of lipoproteins from different donors; vertical bars show s.e.mean. (□) Control; (○) + OXLDL; (●) + LDL.

fashion to those of ACh. This is illustrated by the effect of one of the most potent LDL preparations (donor: TH; Figure 3a,c). No visible loss of endothelial cells was found after treatment with any of the preparations of OXLDL.

LDL and OXLDL inhibit relaxations by nitric oxide

Nitric oxide solutions evoked dose-dependent relaxations in endothelium-denuded aortic rings which were inhibited by both native LDL and OXLDL. The inhibitions by the two forms of LDL were not significantly different as shown by the dose-response curves in Figure 5 and were reversible after washout. Furthermore, the inhibitions were independent of the agonist used to contract the tissues and the preparation of LDL from the plasma of different donors. Pre-incubation of the tissues for up to 1 h with the lipoproteins did not alter the inhibitions.

The effect of LDL and OXLDL on relaxations evoked by glyceryl trinitrate

Figure 6 shows the dose-response curves obtained from the data of 4 experiments in which rings pre-contracted with PE and relaxed by the addition of cumulative concentrations of glyceryl trinitrate (GTN) were used. No significant shift in the curve was found when relaxations were performed in the presence of native LDL. When similar experiments were performed in the presence of OXLDL, a small but significant rightward shift in the dose-response curve was found compared with the control (Figure 6). This inhibition was reversed by removal of the lipoproteins. The inhibition by OXLDL was independent of the donor, the presence or absence of endothelium and the agonist used to pre-contrast the tissues.

Discussion

This study has revealed that native LDL at comparable concentrations to those found in hypercholesterolaemic plasma of human patients ($2\text{--}4 \text{ mg LDL protein ml}^{-1}$) and OXLDL inhibit endothelium-dependent relaxation elicited by receptor activation or by a receptor-independent pathway. The inhibitory effects of native LDL and OXLDL in other respects appear to be different and operate by distinctive mechanisms.

The reversibility and almost immediate effects of native LDL make it unlikely that the inhibition involves the endocytosis of the lipoproteins (Brown & Goldstein, 1979; Baker *et al.*, 1984) or their transcytosis into the subendothelial space (Simionescu, 1989), processes which are both time-dependent. We have found that the inhibition of ACh-evoked relaxations by both native or oxidised LDL takes place if non-atherosclerotic aortic rings from 2 month-old Watanabe hereditary hyperlipidaemic rabbits are used instead of those from normal rabbits (Bruckdorfer *et al.*, 1988 and unpublished results). Since endothelial cells of this strain of rabbits lack the functional high affinity receptors implicated in the endocytosis and degradation of native LDL (Baker *et al.*, 1984), it is most unlikely that such receptors are involved in the inhibition of endothelium-dependent relaxation. Furthermore, the high affinity receptors would be fully saturated at concentrations of LDL ($100 \mu\text{g ml}^{-1}$) lower than the threshold concentration for inhibition (0.5 mg ml^{-1}).

Several other mechanisms which may explain the effects of native LDL are also excluded. Inhibition is not mediated by cyclo-oxygenase products or by the generation of superoxide. Ascorbate, an inhibitor of NA oxidation does not prevent the effect of LDL. Furthermore, the rapidity of the inhibition rules out oxidation of LDL, which requires long exposures to transitional metal ions or endothelial cells. A simple binding of ACh or A23187 by LDL is unlikely because of the lack of inhibition with PE as a contractile agonist. The failure of native LDL to inhibit relaxations elicited by glyceryl trinitrate, a drug which is thought to release NO intracellularly

in the vascular smooth muscle, also rules out an action on soluble guanylate cyclase. An inhibition of the relaxations elicited by EDRF and not GTN by various inhibitors has similarly been found in bioassay experiments (Moncada *et al.*, 1986). Nevertheless, LDL inhibit relaxations elicited by NO which is added directly into the organ bath. This suggests that LDL are able to react with or sequester NO and this might be the mechanism by which the responses mediated by endogenous NO are inhibited. This mechanism is clearly not sufficient since it does not occur in rings pre-contracted with PE alone, although there was inhibition when subthreshold concentrations of 5-HT (1 nM) which do not enhance contraction, were also present. Further investigations are required to clarify whether at this low concentration, 5-HT receptors are implicated in the inhibition.

The modification of LDL during oxidation causes significant changes in the composition and structure of LDL. The changes include lipid peroxidation, the formation of conjugates between fragments of fatty acids and apolipoprotein B and the conversion of phosphatidylcholine to lysophosphatidylcholine (Steinbrecher *et al.*, 1984). As a result, the uptake of the modified lipoproteins by cells is no longer by receptor-mediated endocytosis, but by the so-called scavenger receptors; found on macrophages (Steinbrecher *et al.*, 1984) and also present on endothelial cells (Baker *et al.*, 1984). Subtle differences in these oxidation products may result from the variable composition of polyunsaturated fatty acids and antioxidants in the initial LDL from individual donors, as shown by Esterbauer *et al.* (1989). Thus the changes that occur on oxidation may explain many of the differences between native and OXLDL, including the variation in the inhibitory properties of the OXLDL from individual donors. Different constituents in OXLDL may account for the rapid inhibition of the relaxations evoked by NO and GTN compared with the time-dependent and variable inhibition of endothelium-dependent relaxation. Furthermore, the effect of many preparations of OXLDL on relaxations mediated by endogenous NO were more potent than those on the former relaxants, suggesting that the interaction of OXLDL with NO or soluble guanylate cyclase is unlikely to be the major mechanism. In addition, transcytosis of OXLDL (Simionescu, 1988) or uptake via the scavenger receptors may be required before these modified lipoproteins can influence events within the endothelial cells

or on the abluminal side of the endothelium. Recently, it was shown by Jialal & Chait (1989) that OXLDL or a lipid extract from the lipoproteins inhibit cholesterol esterification in cultured endothelial cells, independently of the receptor for native LDL or the scavenger receptor, and it was suggested that this might result from the translocation of a lipid component across the cell membrane. A similar time-dependent translocation of constituents of OXLDL into the endothelial cells or subendothelial space may be an alternative mechanism to account for the inhibition of endothelium-dependent relaxation.

Our observations on the different degrees of inhibition produced by OXLDL correlate with the various degrees of attenuation of EDRF responses obtained in isolated arterial preparations from cholesterol-fed animals (Verbeuren *et al.*, 1986; Harrison *et al.*, 1987; Shimokawa & Vanhoutte, 1989) and atherosclerotic human arteries (Forstermann *et al.*, 1988). An attenuation of responses of glyceryl trinitrate was also found in severely diseased arteries in some of these investigations. An exhaustive study in coronary arteries from hypercholesterolaemic pigs (Shimokawa & Vanhoutte, 1989), without signs of atherosclerosis, showed a moderate impairment in the responses to selective relaxants, whereas where atherosclerosis was present inhibition was more severe with all relaxants and EDRF release was inhibited (Shimokawa & Vanhoutte, 1989). In atherosclerotic arteries from monkeys (Harrison *et al.*, 1987), a surmountable inhibition of responses to A23187 were observed, not unlike that found with native LDL and some preparations of OXLDL.

In summary, we suggest that isolated non-atherosclerotic arteries of animals at the very early stages of hypercholesterolaemia which are exposed mainly to native LDL, might be expected to show only modest impairment of EDRF responses. An enhancement in platelet aggregation is predicted from the interaction of LDL with NO. OXLDL, on the other hand, represents the result of long-term hypercholesterolaemia, by which time oxidatively modified LDL will have accumulated in the intima and given rise to the continuum of inhibition of EDRF responses found in isolated atherosclerotic arteries.

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