

Rabbit aortic endothelial dysfunction by low-density lipoprotein is attenuated by L-arginine, L-ascorbate and pyridoxine

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1 We investigated the relative effectiveness of L-arginine, L-ascorbate and pyridoxine in preventing the impairment of endothelium-mediated vasorelaxation induced by native low-density lipoprotein (nLDL) from healthy subjects, oxidised LDL (oxLDL, formed by oxidation of nLDL) or nLDL from type II diabetic patients (dLDL).

2 Rabbit aortic rings were exposed to nLDL, dLDL or oxLDL (50–200 mg protein l⁻¹), or corresponding vehicle, following which they were constricted with noradrenaline 10⁻⁶ M; concentration–relaxation curves were determined to acetylcholine (ACh), A23187, or sodium nitroprusside (NP), in the absence or presence of L-arginine (10⁻⁵–10⁻³ M), L-ascorbate (10⁻⁵–10⁻³ M) and pyridoxine (0.5–2.0 mM).

3 nLDL, dLDL and oxLDL all inhibited relaxant responses to ACh and A23187, but not to NP, in a concentration-dependent manner (oxLDL > dLDL > nLDL).

4 In the presence of all LDL preparations, L-arginine, L-ascorbate or pyridoxine each improved ACh and A23187 responses, although none completely normalised endothelium-dependent relaxations. The maximal effect of L-arginine occurred at 10⁻⁴ M. The combination of L-arginine 10⁻⁴ M, L-ascorbate 10⁻⁵ M and pyridoxine 2.0 mM was equally effective as L-arginine 10⁻⁴ M alone.

5 Our results confirm that nLDL, dLDL and oxLDL exert inhibitory effects on endothelium dependent, but not endothelium independent, relaxation of rabbit aorta. ACh and A23187 responses in the presence of any LDL species can be ameliorated by supplementation with L-arginine, L-ascorbate or pyridoxine, either singly or in combination, with no agent or combination proving superior to L-arginine alone. Nevertheless, ACh and A23187 responses are not completely normalised with such supplements, suggesting that there also exists a component of LDL-induced inhibition of endothelium-mediated vasorelaxation that is independent of the nitric oxide system.

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Abbreviations: ACh, acetylcholine; dLDL, native low-density lipoprotein from type II diabetic subjects; EDHF, endothelium-derived hyperpolarising factor; LDL, low-density lipoprotein; nLDL, native low-density lipoprotein from healthy subjects; NO, nitric oxide; NOS, nitric oxide synthase; NP, sodium nitroprusside; oxLDL, oxidised nLDL; TBARS, thiobarbituric acid reactive substances

Introduction

The vascular endothelium produces a variety of vasoactive substances, which have important effects on vascular tone, haemostasis, leucocyte and platelet adhesion, vascular permeability, and atherogenesis (reviewed recently by Anderson, 2003). One of the most important of these mediators is nitric oxide (NO). NO is synthesised in vascular endothelial cells by the action of the enzyme nitric oxide synthase (NOS), which oxidises the amino-acid L-arginine to generate L-citrulline and NO. NO acts on vascular smooth muscle to promote vasorelaxation, on leucocytes and platelets to prevent adhesion to the endothelium and platelet aggregation, on endothelial

cells themselves to inhibit vascular permeability, and on a host of cellular components of the vascular wall to inhibit atherogenesis (Anderson, 2003). It reacts rapidly with superoxide anion (O₂⁻) to form peroxynitrite (Beckman & Koppenol, 1996), so that conditions associated with increased oxidative stress give rise to an accelerated scavenging and removal of NO.

Endothelial dysfunction is found in a variety of cardiovascular disease states, such as type II diabetes and hypercholesterolaemia as well as in established atherosclerosis, and studies suggest that the degree of endothelial dysfunction increases with the number of risk factors present in an individual (Vita *et al.*, 1990; Celermajer *et al.*, 1994). This dysfunction is characterised by reduced bioactivity of NO; this may be due either to suppression of NOS activity or to increased NO scavenging attributable to enhanced O₂⁻ production, since increased oxidative stress is a characteristic feature of these conditions (Cai & Harrison, 2000). Indeed, it has been

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suggested that endothelial dysfunction and impairment of NO availability may play a pathogenetic role in atherosclerosis (Kinlay & Ganz, 1997; Ross, 1999).

Cholesterol is mainly transported in the circulation in the form of low-density lipoprotein (LDL) particles, and increased LDL concentration is associated both with the stimulation of atherogenesis and impairment of vascular endothelium-dependent relaxation (Ludmer *et al.*, 1986; Chowienczyk *et al.*, 1992; Casino *et al.*, 1995). This impairment can largely be attributed to a decrease in NO availability, although the precise mechanism by which LDL exerts this effect is unclear at present. Additionally, LDL can itself undergo oxidation in the presence of reactive oxygen species; oxidised (including minimally oxidised) LDL exerts a greater impairment of endothelium-dependent relaxation than does nonoxidised LDL (Galle *et al.*, 1991; Chin *et al.*, 1992), as does glyco-oxidised LDL, as found for example in patients with type II diabetes (Galle *et al.*, 1998). Thus, qualitative as well as quantitative differences in LDL can be important as regards effects on vascular endothelial function. Recently, McNeill *et al.* (2000) have reported that incubation of rabbit aortic rings in the organ bath with LDL isolated from type II diabetic patients causes a greater reduction in acetylcholine (ACh)-induced vasorelaxation than does an equivalent concentration of LDL from healthy control subjects. Furthermore, Posch *et al.* (1999) have demonstrated that glycated LDL reduces the shear stress-induced increase in endothelial cell NO synthesis, by inhibition of shear stress-stimulated L-arginine uptake and NO bioactivity through increased endothelial cell superoxide anion release; and the same group has recently shown that both LDL taken from diabetic patients and LDL glycated *in vitro* increase endothelial cell apoptosis (Artwohl *et al.*, 2003).

Supplementation with L-arginine, the precursor for NO synthesis, has been shown to improve endothelium-dependent vasorelaxation in hypercholesterolaemic subjects *in vivo* (Drexler *et al.*, 1991; Creager *et al.*, 1992; Clarkson *et al.*, 1996). L-Ascorbate (vitamin C) can also improve endothelium-dependent relaxation, due to removal of reactive oxygen species (Fontana *et al.*, 1999). In addition, pyridoxine (vitamin B6) has been demonstrated in one study to ameliorate endothelial dysfunction *in vivo* in cardiac transplant recipients (Miner *et al.*, 2001), and this may be attributable to the essential role of pyridoxine as a cofactor in the metabolism of homocysteine, which is associated with the generation of oxidative stress *in vivo*. Therefore, L-arginine, L-ascorbate and pyridoxine are potentially promising as therapies in cardiovascular diseases associated with endothelial dysfunction and impaired NO availability.

The aim of the present study, therefore, was to evaluate the effectiveness of L-arginine, L-ascorbate and pyridoxine, administered either singly or in combination, in preventing the impairment of endothelium-mediated vasorelaxation induced by LDL, in rabbit aortic rings *in vitro*. We assessed the effectiveness of supplementation with these substances in aortic tissues incubated with native LDL (nLDL) taken from healthy subjects, LDL from the same healthy subjects following *in vitro* oxidation (oxLDL), and nLDL taken from subjects with type II diabetes (dLDL). To date, no studies have compared the effectiveness of L-arginine and L-ascorbate in healthy arteries exposed to LDL of different oxidation and/or glycation states, nor have any studies examined the effect of pyridoxine on LDL-induced endothelial dysfunction.

Methods

Animals and preparation of vascular rings

New Zealand White rabbits, weighing 2–2.5 kg, were from the Nanjing Experimental Animals Centre of Jiangsu Province, China. They were provided with food and water *ad libitum* and, on the day of experimentation, were anaesthetised with sodium pentobarbital (60 mg kg⁻¹); the descending thoracic aorta was dissected out carefully, following which the animal was killed by pentobarbital overdose. The aorta was trimmed of adherent connective tissue and fat, and then cut into 2-mm-long rings, which were continuously oxygenated with 95% O₂/5% CO₂.

Subjects and LDL preparation

Seven healthy control subjects (five male, two female) and 11 subjects with type II diabetes (eight male, three female) were recruited for these experiments. Their characteristics are shown in Table 1. Verbal consent was obtained for blood sampling in all cases, and the study was approved by the institutional review board for human studies of Nanjing Medical University and by the King's College London Research Ethics Committee. Venous blood (100 ml) was drawn from an antecubital vein and collected into EDTA (1.3 mM final concentration). Plasma was separated by centrifugation (500 × *g*, 10 min, room temperature), and nLDL (1.019–1.063 g ml⁻¹) isolated from the plasma by discontinuous density gradient ultracentrifugation as described previously (Chung *et al.*, 1980), followed by extensive dialysis with phosphate-buffered saline (PBS), pH 7.4, containing 0.3 mM EDTA (48 h, 4°C). Within each subject group, isolated LDL were pooled, in order to obtain sufficient LDL to carry out all the experiments outlined below. All LDL isolates were adjusted to a protein concentration of 0.5 mg ml⁻¹, as measured using the method of Bradford (1976), and were stored at 4°C and used within 1 month.

In order to obtain oxLDL, copper oxidation was performed on nLDL as follows. Prior to oxidation, a sample of nLDL was dialysed overnight at 4°C against 2000 volumes of PBS without EDTA; protein concentration was adjusted to 0.5 mg ml⁻¹, and CuSO₄ was added to a final concentration of 20 μM for 24 h at 37°C. EDTA was then added at a final concentration of 2.7 mM to stop the reaction. Again, the resultant oxLDL was extensively dialysed with PBS, pH 7.4,

Table 1 Subject characteristics

	Controls	Type II diabetic subjects
Number and sex	Five male, two female	Eight male, three female
Age (years)	46.7 ± 2.9	48.2 ± 7.3
Total cholesterol (mM)	5.5 ± 0.5	5.7 ± 0.9
HDL cholesterol (mM)	1.4 ± 0.5	1.1 ± 0.2
LDL cholesterol (mM)	3.2 ± 0.4	3.1 ± 0.8
Triglycerides (mM)	1.7 ± 0.2	2.1 ± 0.5
Glucose (mM)	4.9 ± 0.3	9.1 ± 1.6**
HbA1c (%)	4.9 ± 0.2	8.8 ± 1.8**

***P* < 0.01 as compared with control subjects.

containing 0.3 mM EDTA (48 h, 4°C), stored at 4°C and used within 1 month.

Oxidation and glycation status of isolated LDL

LDL oxidation status was determined by measuring the concentrations of thiobarbituric acid reactive substances (TBARS) in the different LDL samples according to the method of Ohkawa *et al.* (1979). In brief, 1.5 ml 20% acetic acid (pH 3.5) and 1.5 ml of a 0.8% thiobarbituric acid solution were added to a sample of the LDL, and the volume brought to 4.0 ml with distilled water. The mixture was shaken thoroughly and heated in an oil bath at 95°C for 60 min. After the mixture was cooled with tap water, 1.0 ml of distilled water and 5.0 ml of butyl alcohol and pyridine (15:1) were added, and the sample was shaken gently for 5 min. After centrifugation at $1500 \times g$ for 10 min, the butyl alcohol-pyridine phase containing the TBARS was separated and its absorbance was measured at 532 nm. The results were expressed as nmol equivalent malondialdehyde per mg protein, with malondialdehyde from tetramethoxypropane used as standard and double-distilled water as a control.

LDL glycation was quantified in our samples by competitive ELISA, using a proprietary assay kit (Glycacor), from which the percentage of total LDL in the glycated state was calculated.

Organ bath pharmacology

Rabbit aortic rings were mounted in 3 ml organ baths containing Krebs buffer of the following composition (mM): NaCl 125, KCl 4.8, NaHCO₃ 25, KH₂PO₄ 1.2, MgSO₄ 1.2, glucose 11, EDTA 0.3, CaCl₂ 2.5, pH 7.4 (gassed with 95% O₂/5% CO₂ at 37°C). Preliminary length-tension curves suggested that a resting tension of 2 g gave optimal contractile responses to KCl 45 mM, therefore this level of resting tension was used in all experiments.

Once stable and reproducible contractions were obtained with KCl 45 mM, rings were washed extensively and subsequently contracted with noradrenaline 10^{-6} M. Following attainment of plateau constriction, ACh 1 μ M was added; only rings which demonstrated $\geq 60\%$ relaxation to ACh were considered to have functional endothelium, and were used for further experiments.

Following further extensive washing, some rings were exposed to nLDL, oxLDL or dLDL (protein concentration 50, 100 or 200 mg l⁻¹), in the absence or presence of one or more of the following: L-arginine (10^{-5} , 10^{-4} or 10^{-3} M), sodium L-ascorbate (10^{-5} , 10^{-4} or 10^{-3} M), pyridoxine hydrochloride (0.5, 1.0 or 2.0 mM) or superoxide dismutase (SOD, 250 U ml⁻¹). After 15 min incubation, noradrenaline 10^{-6} M was reapplied and, following attainment of plateau of contraction, cumulative concentration-relaxation curves were performed to either ACh (concentration range 10^{-9} – 10^{-5} M, log-unit increments) or to the calcium ionophore A23187 (concentration range 10^{-9} – 10^{-7} M, half log-unit increments). For comparison, concentration-effect curves were also performed to the endothelium-independent NO-releasing vasodilator sodium nitroprusside (NP, concentration range 10^{-8} – 10^{-5} M, log-unit increments), following noradrenaline 10^{-6} M precontraction.

Materials and drugs

The Glycacor kit was from BioGnosis Ltd (Hailsham, East Sussex, U.K.). All other drugs and chemicals were from Sigma (Dorset, U.K.).

Statistical analysis

All results are presented as mean \pm s.e.m. of six experiments, and plotted using GraphPad Prism version 3.03. Data were analysed by repeated measures two-way ANOVA using StatView version 5.0.1. Statistical significance was taken as $P < 0.05$ (two-tailed).

Results

Oxidation and glycation state of LDL preparations

nLDL was prepared from plasma from healthy subjects, and a portion of this was subjected to Cu²⁺ oxidation, to yield oxLDL. dLDL was obtained from plasma taken from type II diabetic subjects. Controls and type II diabetic subjects were of similar age and sex distribution, and had comparable lipid profiles; however, both plasma glucose and HbA_{1c} were significantly higher in type II diabetic subjects than in controls (Table 1).

TBARS measurement revealed that dLDL was more oxidised than nLDL, and oxLDL even more so, as evidenced by an approximately 10- and 20-fold increase in malondialdehyde equivalents in dLDL and oxLDL, respectively, as compared with nLDL (nLDL: 2.7, dLDL: 28.1 and oxLDL: 58.5 nmol (mg protein)⁻¹). Storage of each of our LDL preparations for up to 1 month was not associated with an increase in measured TBARS (data not shown). Since LDL oxidation (including *in vitro* oxidation of nLDL) is associated with a shift towards a smaller denser profile, thus resulting in an electrophoretic mobility change (Sobal *et al.*, 2000), we also examined the electrophoretic profile of our LDL preparations. As seen in Figure 1, oxLDL had a greater mobility than did nLDL, with the mobility of dLDL being intermediate between the two.

Glycation state of our LDL preparations was assessed by competitive ELISA. nLDL and oxLDL were glycated to only a small degree (14.3 and 15.0% of total LDL, respectively), whereas dLDL exhibited a much greater degree of glycation (49.0% of total LDL).

Effect of LDL preparations on endothelium-dependent and endothelium-independent relaxation of rabbit aortic rings

Tensioned rings of rabbit aorta with intact endothelium were precontracted with noradrenaline in organ baths, following which endothelium-dependent relaxant responses were studied to either ACh or A23187. For comparison, endothelium-independent relaxation to NP was also examined. All three agents elicited concentration-dependent relaxation in aortic rings in the absence of LDL (Figures 2–4). nLDL, oxLDL and dLDL had no effect on NP-induced relaxation (Figure 4); by contrast, each induced a concentration-dependent inhibition of relaxation to both ACh (Figure 2) and A23187 (Figure 3).

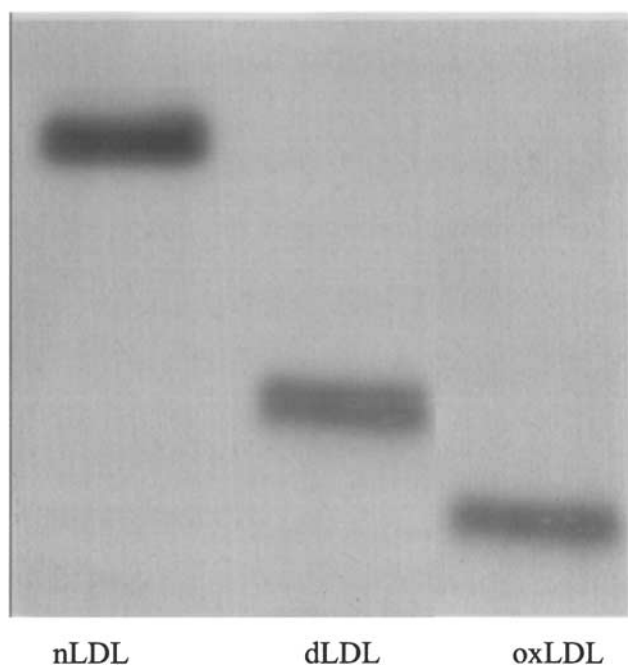


Figure 1 Nondenaturing polyacrylamide gradient gel electrophoresis of LDL samples, showing differences in mobility between nLDL, dLDL and oxLDL. oxLDL migrates fastest and nLDL slowest, and dLDL is intermediate.

None of these LDL species affected the level of precontraction to noradrenaline (Table 2). The inhibitory effects of the different LDL preparations on ACh-dependent responses were in the rank order: oxLDL > dLDL > nLDL (Table 3).

Effect of L-arginine, L-ascorbate, pyridoxine and SOD on endothelium-dependent relaxation of rabbit aortic rings

Since nLDL, dLDL and oxLDL were all found to inhibit endothelium-dependent responses in a concentration-dependent manner, in the range 50–200 mg protein^l⁻¹, an LDL concentration of 100 mg protein^l⁻¹ was used in all the following experiments, to assess whether L-arginine, L-ascorbate and/or pyridoxine could prevent the impairment of endothelium-dependent relaxation. Rabbit aortic rings were incubated with nLDL, dLDL or oxLDL (100 mg l⁻¹), in the absence or presence of different concentrations of L-arginine (10⁻⁵–10⁻³ M), L-ascorbate (10⁻⁵–10⁻³ M) or pyridoxine (0.5–2.0 mM). After 15 min incubation, rings were precontracted with noradrenaline, and concentration–relaxation responses determined to ACh or to A23187, as before. L-arginine, L-ascorbate and pyridoxine did not affect the degree of precontraction to noradrenaline, either in the absence or presence of different LDL species (Table 2). In the absence of LDL, L-arginine and L-ascorbate did not affect ACh- or A23187-dependent responses (Figure 5a, b, d and e). On the other hand, pyridoxine 0.5 mM significantly increased the relaxation to ACh 10⁻⁹ and 10⁻⁶ M ($P < 0.05$), and pyridoxine 1.0 mM significantly increased the relaxation to ACh 10⁻⁶ and 10⁻⁵ M ($P < 0.05$); furthermore, pyridoxine 2.0 mM significantly increased the relaxation to A23187 10⁻⁹ M, 5 × 10⁻⁹ M, 10⁻⁸ M and 5 × 10⁻⁸ M ($P < 0.05$ for each). While these increases to ACh and to A23187 responses in the presence of these

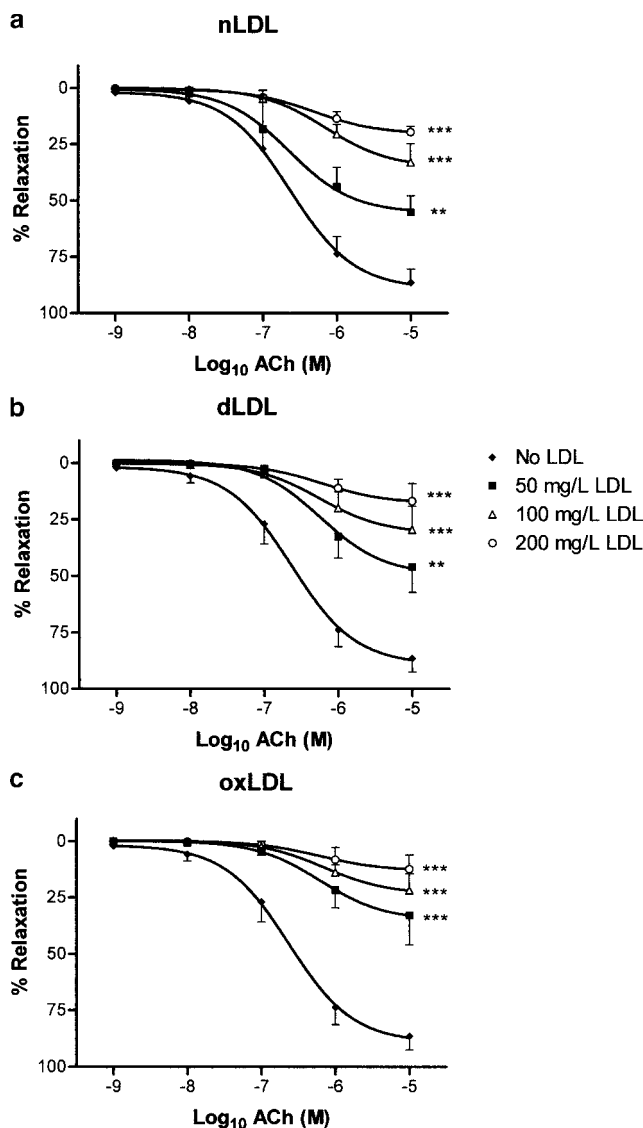


Figure 2 Effects of (a) nLDL, (b) dLDL and (c) oxLDL on relaxant responses to ACh. Responses are shown in the absence of LDL, and in the presence of 50, 100 and 200 mg protein^l⁻¹ of each type of LDL. **, *** $P < 0.01$ and < 0.001 as compared with the absence of LDL.

concentrations of pyridoxine reached statistical significance, as did the corresponding curves overall, these changes were small in magnitude (Figure 5c and f). By contrast, in the presence of any of our LDL preparations, L-arginine, L-ascorbate or pyridoxine each improved ACh- and A23187-dependent responses considerably, although none of these agents could restore ACh or A23187 maximal relaxations to those seen in the absence of LDL (Figures 6–8). The maximal effect of L-arginine was seen at 10⁻⁴ M, and higher concentrations had no additional effect; moreover, the effects of L-arginine on ACh- or A23187-induced relaxations in the presence of any of our LDL samples could be completely inhibited by coincubation with L-NAME 10⁻⁴ M (data not shown). All concentrations of L-ascorbate tested (10⁻⁵–10⁻³ M) improved ACh responses to the same degree in the presence of nLDL and oxLDL approximately, although in the case of dLDL 10⁻⁵ M L-ascorbate was less effective than the higher concentrations;

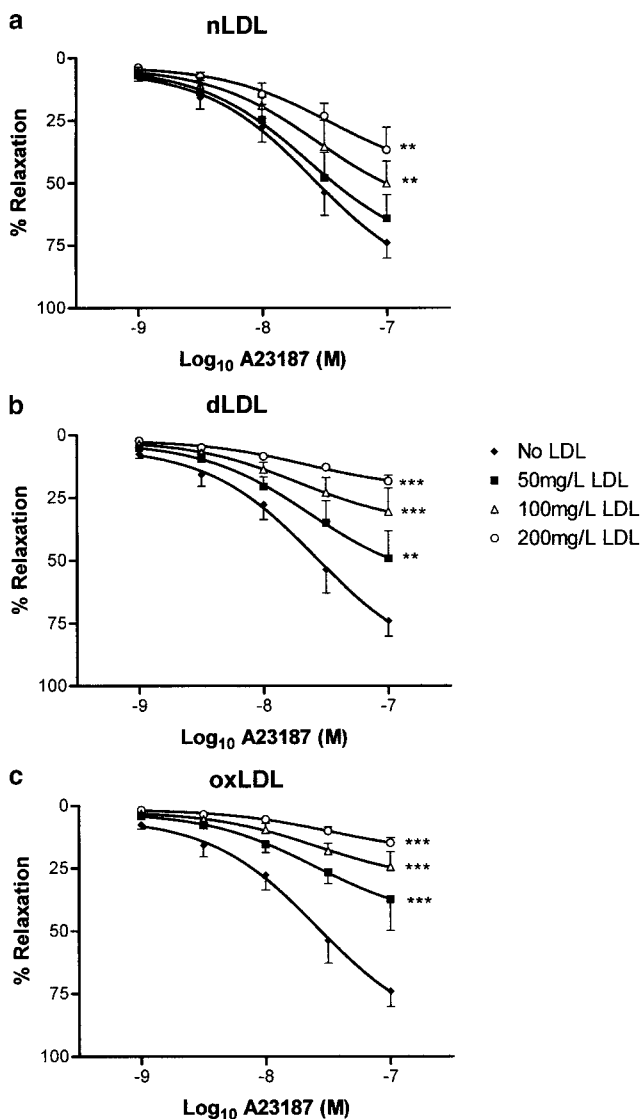


Figure 3 Effects of (a) nLDL, (b) dLDL and (c) oxLDL on relaxant responses to A23187. Responses are shown in the absence of LDL, and in the presence of 50, 100 and 200 mg protein⁻¹ of each type of LDL. **, *** $P < 0.01$ and < 0.001 as compared with the absence of LDL.

in the presence of nLDL or dLDL, all concentrations of L-ascorbate tested improved A23187 relaxant responses to a similar degree, but in the presence of oxLDL 10^{-5} M L-ascorbate was less effective than higher concentrations. On the other hand, the improvement in both ACh and A23187 relaxations by pyridoxine was progressive as the concentration of pyridoxine was increased (0.5, 1.0 and 2.0 mM). L-arginine, L-ascorbate and pyridoxine had no effect on NP-induced relaxant responses, either in the absence or presence of any LDL species (data not shown).

In order to confirm that the amelioration of endothelium-dependent responses by L-ascorbate in the presence of nLDL, dLDL and oxLDL was indeed due to scavenging of reactive oxygen species, we also tested the effect of SOD 250 U ml⁻¹ (a concentration that we have previously found to exert maximal effects on superoxide anion scavenging in rat aortic rings *in vitro*) on vasorelaxant responses to ACh and to A23187. We found that SOD had no significant effect on ACh or A23187

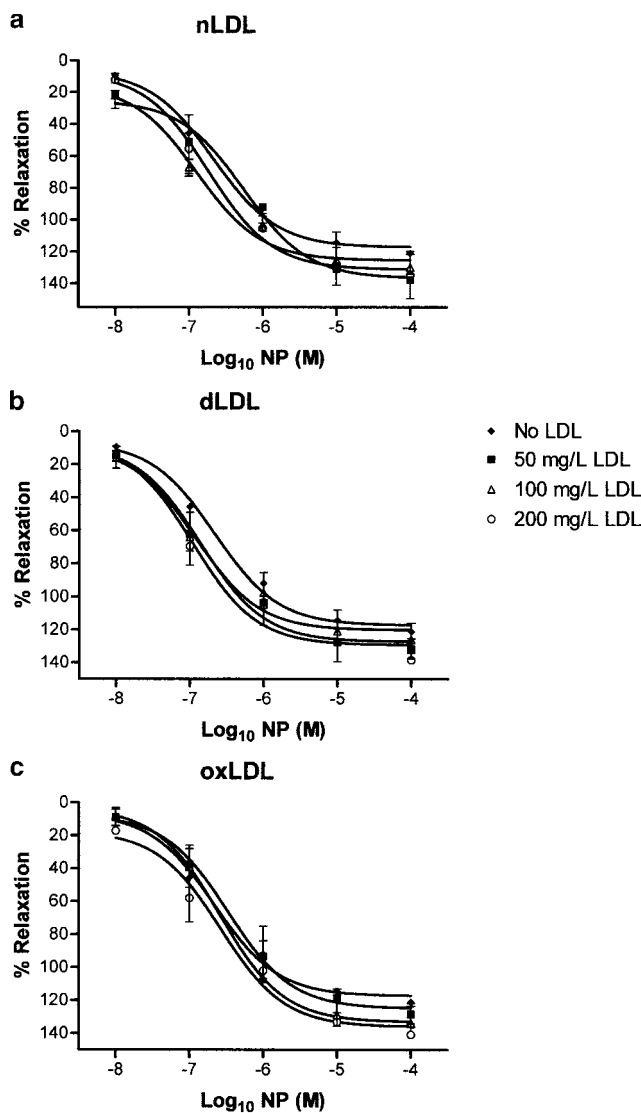


Figure 4 Effects of (a) nLDL, (b) dLDL and (c) oxLDL on relaxant responses to NP. Responses are shown in the absence of LDL, and in the presence of 50, 100 and 200 mg protein⁻¹ of each type of LDL.

Table 2 Magnitude of contractions to noradrenaline 10^{-6} M (expressed as developed tension (g)) in the absence and presence of nLDL, dLDL or oxLDL (each at 100 mg l⁻¹), and of L-arginine 10^{-4} M, L-ascorbate 10^{-5} M or pyridoxine 2.0 mM

	No LDL	nLDL	dLDL	oxLDL
Vehicle	7.6 ± 2.1	7.3 ± 2.7	7.6 ± 1.0	6.5 ± 2.1
L-arginine	7.5 ± 1.8	5.8 ± 1.2	8.6 ± 2.1	7.5 ± 0.6
L-ascorbate	5.6 ± 1.1	9.6 ± 3.5	5.6 ± 1.5	7.6 ± 3.1
Pyridoxine	7.0 ± 1.4	7.9 ± 1.9	7.9 ± 1.6	6.5 ± 1.5

responses in the absence of LDL. However, in the presence of any of these LDL species, SOD improved ACh and A23187 relaxations, to a similar degree as did L-ascorbate (Figure 9).

We also wished to determine whether coincubation of aortic rings with the combination of L-arginine 10^{-4} M, L-ascorbate 10^{-5} M and pyridoxine 2.0 mM gave rise to a greater improvement in ACh-mediated relaxations than L-arginine 10^{-4} M

alone. However we found that, in the presence of nLDL, dLDL or oxLDL (each at 100 mg protein^l), the EC₅₀ and E_{max} to ACh were in fact the same in the presence either of the combination or of L-arginine alone (Table 4), although both regimes improved E_{max} as compared with values in the absence of these supplements.

Table 3 Maximal relaxation (expressed as % relaxation of noradrenaline precontraction) to acetylcholine in the presence of different concentrations of nLDL, dLDL and oxLDL

	Maximal relaxation
nLDL 50 mg l ⁻¹	55.5 ± 4.4
nLDL 100 mg l ⁻¹	35.2 ± 3.6
nLDL 200 mg l ⁻¹	20.4 ± 1.5
dLDL 50 mg l ⁻¹	49.6 ± 3.3*
dLDL 100 mg l ⁻¹	31.2 ± 3.0*
dLDL 200 mg l ⁻¹	17.9 ± 1.2*
oxLDL 50 mg l ⁻¹	35.1 ± 1.5**, #
oxLDL 100 mg l ⁻¹	23.5 ± 2.3**, #
oxLDL 200 mg l ⁻¹	13.2 ± 1.2**, #

*,** *P* < 0.05 and < 0.01, respectively, as compared with the same concentration of nLDL.

#*P* < 0.05 as compared with the same concentration of dLDL.

Discussion

Previous studies have demonstrated that LDL exerts an inhibitory effect on endothelium-dependent vascular relaxation, and this is largely due to impairment of endothelial NO availability (Ludmer *et al.*, 1986; Chowienczyk *et al.*, 1992; Casino *et al.*, 1995), either due to decreased synthesis or to increased scavenging by reactive oxygen species. This is likely to be a major contributor to the endothelial dysfunction observed in patients with hypercholesterolaemia, and this endothelial dysfunction may in turn contribute to the pathogenesis of atherosclerosis (Kinlay & Ganz, 1997; Ross, 1999). LDL that has undergone *in vitro* oxidation or glyco-oxidation exerts a more marked inhibitory effect on endothelial function than does nonoxidised LDL (Galle *et al.*, 1991; 1998; Chin *et al.*, 1992), and indeed glycated LDL, such as that found in the circulation of type II diabetic patients, is more susceptible to oxidation than is nonglycated LDL (Sobal *et al.*, 2000), a finding confirmed in our study by high TBARS measurement in dLDL; furthermore, nLDL isolated from patients with type II diabetes also exerts a more marked inhibitory effect on endothelium-dependent vasorelaxation than does nLDL from healthy controls (McNeill *et al.*, 2000). These data are borne out by the results of the present study. nLDL, oxLDL and dLDL all inhibited endothelium-dependent relaxation of rabbit aortic rings to both ACh and A23187 in a concentration-dependent manner, whereas they

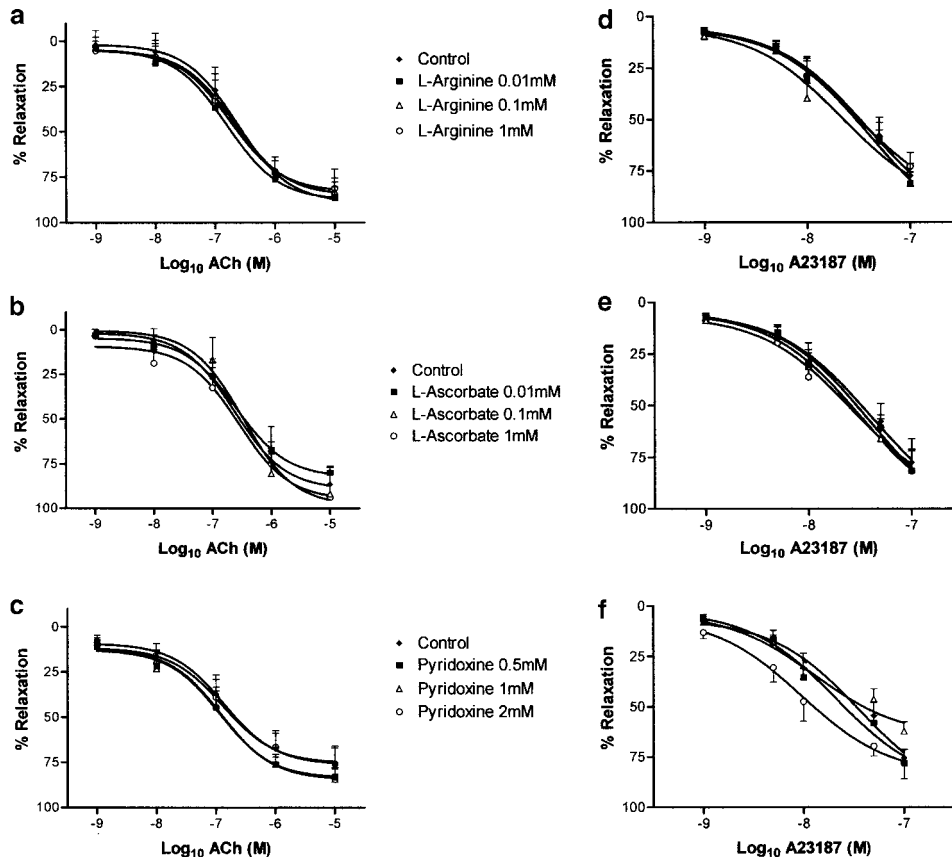


Figure 5 Effects of L-arginine, L-ascorbate and pyridoxine on relaxant responses to ACh (a–c) and to A23187 (d–f), in the absence of LDL. Responses are shown in the absence and in the presence of incremental concentrations of L-arginine (a and d), L-ascorbate (b and e) or pyridoxine (c and f). **P* < 0.05 as compared with control

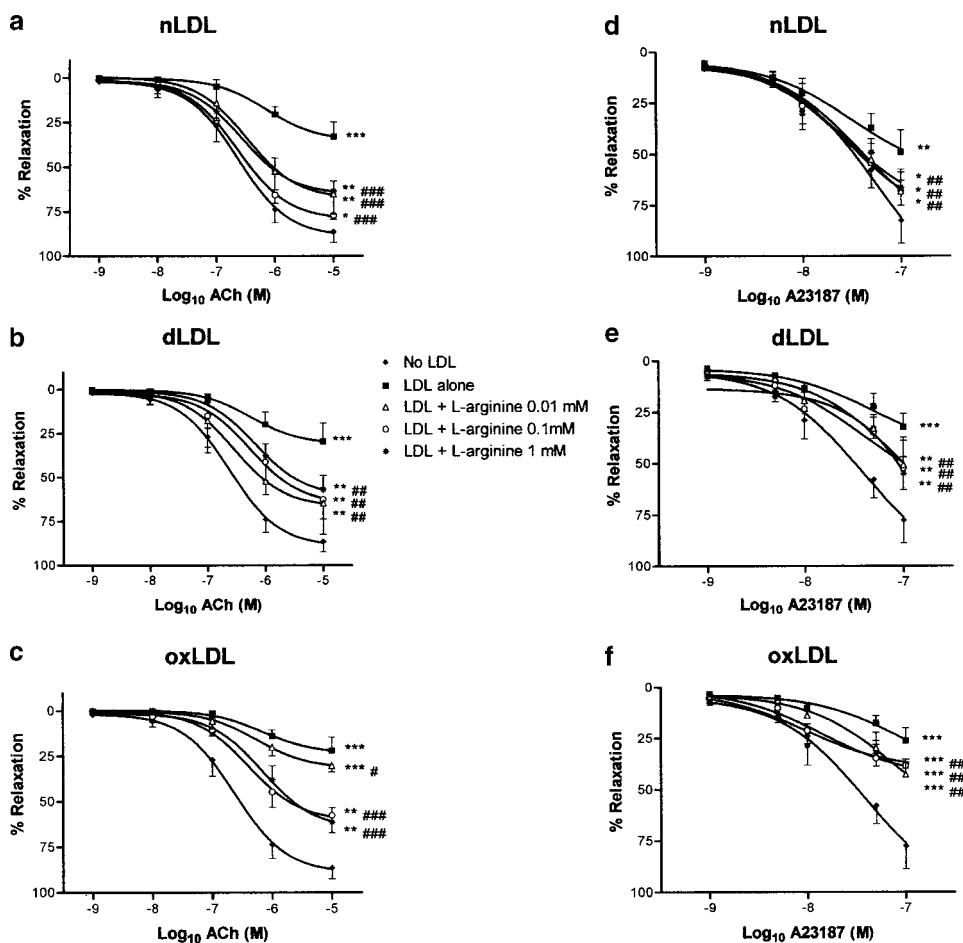


Figure 6 Effects of L-arginine supplementation on ACh (a–c) and on A23187 (d–f) relaxant responses in the presence of 100 mg protein⁻¹ nLDL (a and d), dLDL (b and e) and oxLDL (c and f). Responses are shown in the absence of LDL, and in the presence of LDL either alone or with L-arginine 10⁻⁵, 10⁻⁴ or 10⁻³ M. *, **, ****P* < 0.05, < 0.01 and < 0.001 as compared with the absence of LDL. #, ##, ###*P* < 0.05, < 0.01 and < 0.001 as compared with LDL alone.

had no effect on endothelium-independent relaxations to NP. The rank order of inhibitory effect, for a given LDL protein concentration, was oxLDL > dLDL > nLDL, and this was the same as the rank order of LDL lipid peroxidation (as determined from TBARS). Strategies to prevent the deleterious effects of LDL on vascular endothelial function may be expected to improve clinical end points in patients at risk of atherosclerosis, although this remains to be proven in clinical outcome studies.

In this study, we examined the effects of three therapies that might be expected to prevent the inhibitory effects of LDL on endothelium-dependent relaxation: L-arginine, a substrate for NOS and therefore a precursor of NO; L-ascorbate (vitamin C), an antioxidant vitamin that scavenges reactive oxygen species; and pyridoxine (vitamin B₆), a cofactor involved in homocysteine metabolism. We found that each of these gave rise to an improvement in rabbit aortic ACh and A23187 relaxant responses in the presence of nLDL, dLDL or oxLDL (100 mg protein⁻¹), although none restored ACh and A23187 responses to those seen in the absence of LDL. The effect of L-arginine was maximal at 10⁻⁴ M, and concentrations of L-ascorbate higher than 10⁻⁵ M similarly gave rise to no additional improvement in ACh responses (except in the case of dLDL coinubation). Although this action of L-ascorbate

can be explained by scavenging of reactive oxygen species, it can also exert other effects that may give rise to improvement in endothelial function independently of its antioxidant action, namely increasing the intracellular availability of the NOS cofactor tetrahydrobiopterin (Baker *et al.*, 2001) or catalysing the release of NO from endogenous *S*-nitrosothiols (Al-Sa'doni & Ferro, 2000). Nevertheless, since we also found that 250 U ml⁻¹ SOD (which removes O₂⁻) improved both ACh and A23187 responses, and did so to a similar degree as L-ascorbate, in the presence of all LDL species, it is likely that oxidative stress is indeed important in the causation of LDL-induced endothelial dysfunction, and also that the beneficial effect of L-ascorbate on endothelium-dependent responses is indeed due to scavenging of reactive oxygen species. Our data therefore support the notion that nLDL, dLDL and oxLDL exert their inhibitory effects on endothelial function both through suppression of NO synthesis and enhancement of oxidative stress.

In the case of pyridoxine, a progressive improvement in both ACh and A23187 responses was seen in the concentration range 0.5–2.0 mM. The highest concentration of pyridoxine improved ACh and A23187 responses to a similar degree as did 10⁻⁴ M L-arginine or L-ascorbate, suggesting that homocysteine may play an important role in LDL-induced

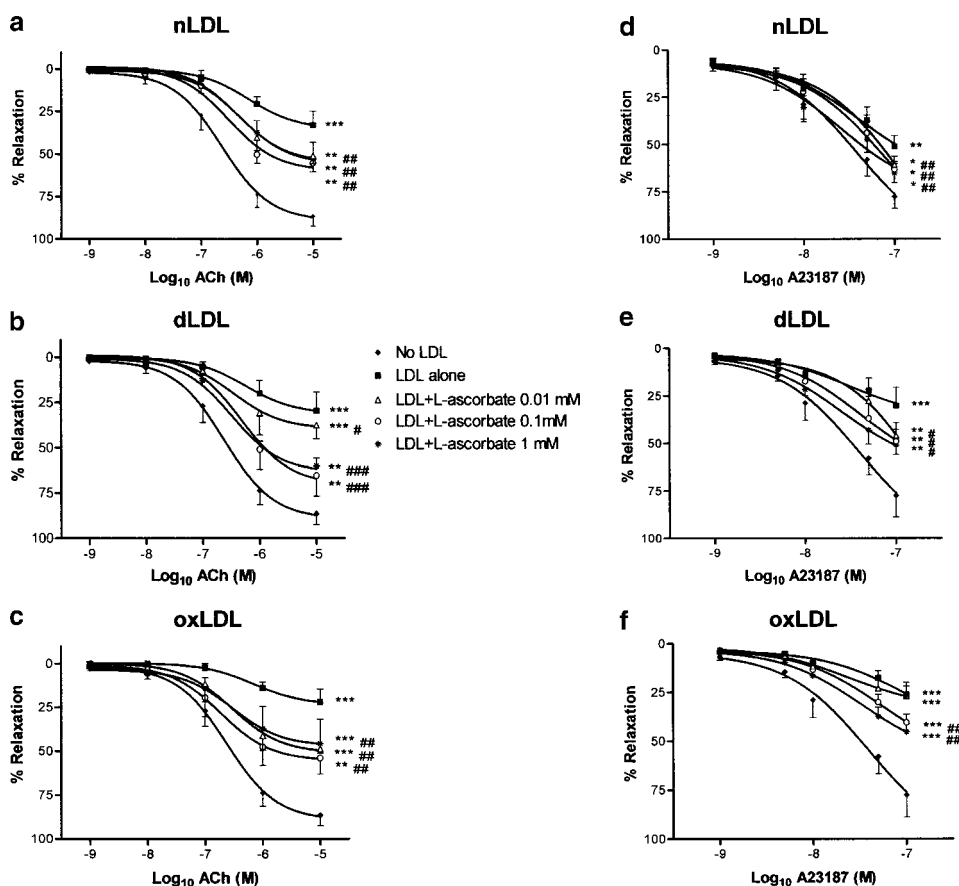


Figure 7 Effects of L-ascorbate supplementation on ACh (a–c) and on A23187 (d–f) relaxant responses in the presence of 100 mg protein⁻¹ nLDL (a and d), dLDL (b and e) and oxLDL (c and f). Responses are shown in the absence of LDL, and in the presence of LDL either alone or with L-ascorbate 10⁻⁵, 10⁻⁴ or 10⁻³ M. ** ****P* < 0.01 and < 0.001 as compared with the absence of LDL. #, ##, ### *P* < 0.05, < 0.01 and < 0.001 as compared with LDL alone.

endothelial dysfunction. On the other hand, pyridoxine has been previously demonstrated to exert other effects that may improve endothelial function, namely inhibition of O₂⁻ formation and of lipid peroxidation (Jain & Lim, 2001), and scavenging of singlet oxygen (Ohta & Foote, 2002). The precise mechanism by which pyridoxine improves endothelium-dependent vasorelaxation in our model therefore remains to be determined.

The combination of L-arginine 10⁻⁴ M, L-ascorbate 10⁻⁵ M and pyridoxine 2.0 mM was no more effective in improving ACh responses, in the presence of nLDL, dLDL or oxLDL, than was L-arginine 10⁻⁴ M alone. This suggests that oxidative stress plays a minor role in LDL-induced endothelial dysfunction when the NOS substrate L-arginine is present in excess. Indeed, it has been previously shown that LDL can uncouple endothelial cell NOS from NO generation, such that it generates O₂⁻, and that the enzyme can be recoupled to NO formation by supplementation with L-arginine (Pritchard *et al.*, 1995). Our experiments therefore lend support to the view that nLDL, oxLDL and dLDL uncouple endothelial NOS in this way, and that L-arginine supplementation both increases NO generation and decreases O₂⁻ production. Furthermore, since pyridoxine was as effective in restoring vascular ACh and A23187 responses as L-arginine or L-ascorbate, in the presence of any of our LDL preparations, it is possible that LDL induces accumulation of homocysteine, which is predomi-

nantly acting through suppression of NO production rather than through generation of reactive oxygen species. In agreement with this, hyperhomocysteinemia has been previously demonstrated to reduce both L-arginine uptake and NOS activity in vascular endothelial cells (Jin *et al.*, 2001). On the other hand, pyridoxine may be exerting antioxidant effects, as discussed above. It is worth noting that previous reports have suggested that pyridoxine analogues may chelate metal ions (Kesel *et al.*, 1999), such as Ca²⁺ (an essential cofactor for the endothelial isoform of NOS), Fe²⁺ (essential for soluble guanylyl cyclase function) and Zn²⁺ (essential for the activity of endogenous cytosolic Cu–Zn SOD), and that L-arginine and pyridoxine may exert mutually antagonistic effects in a mouse model of nociception (Abacioglu *et al.*, 2001); such effects may contribute to the lack of additive action by pyridoxine seen in the present study.

LDL may exert additional inhibitory effects on endothelial function that are unrelated to NO generation or oxidative stress, particularly when lipid peroxidation is prominent. The major component of oxidised forms of LDL that inhibits endothelial function is believed to be lysophosphatidylcholine, since removal of lysophosphatidylcholine with phospholipase B or albumin renders oxLDL inactive in this respect (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990; Mangin *et al.*, 1993), and conversely direct treatment of arterial rings with lysophosphatidylcholine mimics the inhibitory effect of

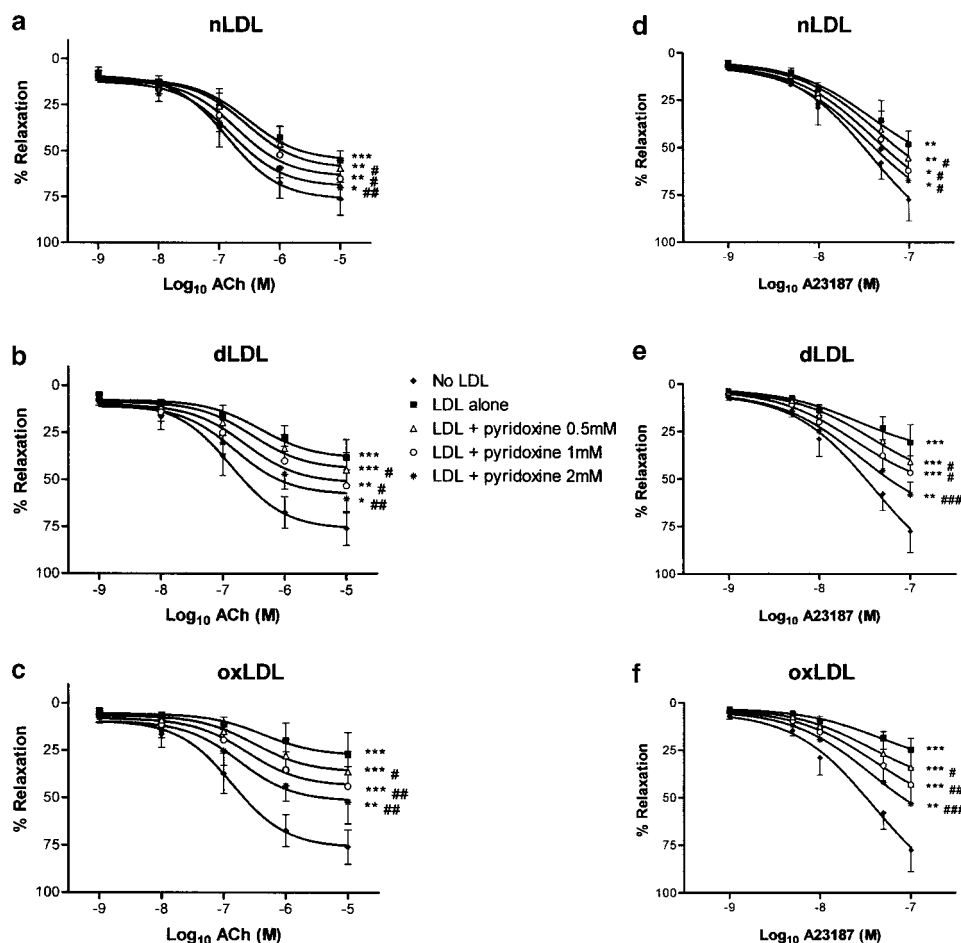


Figure 8 Effects of pyridoxine supplementation on ACh (a–c) and on A23187 (d–f) relaxant responses in the presence of 100 mg protein⁻¹ nLDL (a and d), dLDL (b and e) and oxLDL (c and f). Responses are shown in the absence of LDL, and in the presence of LDL either alone or with pyridoxine 0.5, 1.0 or 2.0 mM. *, **, ****P* < 0.05, < 0.01 and < 0.001 as compared with absence of LDL. #, ## *P* < 0.05 and < 0.01 as compared with LDL alone.

oxLDL on endothelium-dependent relaxation (Kugiyama *et al.*, 1990; Yokoyama *et al.*, 1990; Tanner *et al.*, 1991; Mangin *et al.*, 1993). Although it is generally held that endothelium-derived hyperpolarising factor (EDHF) is of lesser importance in mediating endothelium-dependent vasorelaxation in large arteries as compared with resistance vessels, lysophosphatidylcholine has been previously demonstrated to inhibit relaxation not only to endothelium-derived NO but also to EDHF in rabbit aorta (Cowan & Steffen, 1995). Our results are consistent with these findings, since L-arginine was capable of restoring endothelium-dependent relaxations almost to normal in the presence of nLDL, whereas it was much less effective in this regard in the presence either of dLDL or of oxLDL, both of which exhibit a greater degree of lipid peroxidation. It is likely, therefore, that nLDL, dLDL and oxLDL all inhibit endothelial NO-dependent relaxation of rabbit aorta, which is reversible by L-arginine, pyridoxine and L-ascorbate, but that they additionally inhibit EDHF-dependent relaxation, especially in the case of dLDL and oxLDL.

Our study is the first to demonstrate similar effects of L-arginine and of L-ascorbate on endothelial function in arterial rings exposed to nLDL, dLDL or oxLDL. It is also the first to show that pyridoxine can favourably affect LDL-induced

Table 4 Potency (expressed as $-\log_{10}EC_{50}$) and maximal relaxation (E_{max} , expressed as a percentage of noradrenaline precontraction) to acetylcholine in the presence of nLDL, dLDL and oxLDL (each at 100 mg l⁻¹), and L-arginine 10⁻⁴ M either alone or in combination with L-ascorbate 10⁻⁵ M and pyridoxine 2.0 mM

	$-\log_{10}EC_{50}$	E_{max}
nLDL	6.6 ± 0.2	35.2 ± 3.6
nLDL + L-arginine	6.6 ± 0.2	58.1 ± 3.8***
nLDL + L-arginine + L-ascorbate + pyridoxine	6.8 ± 0.1	57.9 ± 2.7**
dLDL	6.4 ± 0.2	31.2 ± 3.0
dLDL + L-arginine	6.9 ± 0.1	73.3 ± 1.8**
dLDL + L-arginine + L-ascorbate + pyridoxine	6.8 ± 0.1	73.4 ± 2.2**
oxLDL	6.4 ± 0.2	23.5 ± 2.3
oxLDL + L-arginine	6.5 ± 0.1	61.3 ± 2.1***
oxLDL + L-arginine + L-ascorbate + pyridoxine	6.5 ± 0.1	53.9 ± 1.8**

, **P* < 0.01 and < 0.001, respectively, as compared with the relevant LDL species alone.

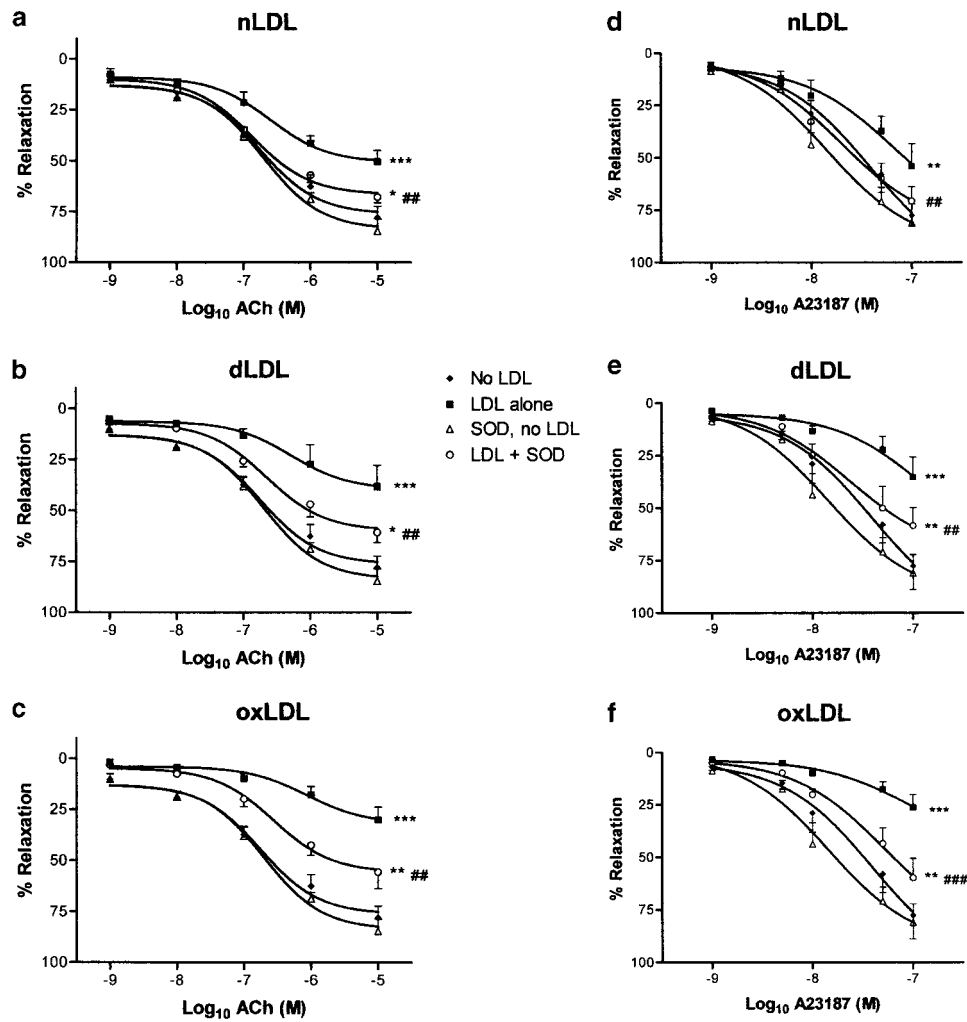


Figure 9 Effects of supplementation with SOD 250 U ml⁻¹ on ACh (a–c) and on A23187 (d–f) relaxant responses, in the absence and presence of 100 mg protein L⁻¹ nLDL (a and d), dLDL (b and e) and oxLDL (c and f). *, **, ****P* < 0.05, < 0.01 and < 0.001 as compared with absence of LDL. ###, ###*P* < 0.01 and < 0.001 as compared with LDL alone.

endothelial dysfunction. Our results suggest that combining L-arginine with L-ascorbate and pyridoxine is unlikely to produce additional benefit on endothelial function as compared with L-arginine alone; if this is borne out by future studies of combinations of such supplements on endothelial function *in vivo*, this will have important implications for therapy in patients with vascular disease and endothelial dysfunction.

In conclusion, our experiments confirm that nLDL, dLDL and oxLDL exert inhibitory effects on endothelium-dependent, but not endothelium-independent, relaxation of rabbit aorta, and that these effects are more marked for oxLDL and dLDL than for nLDL. Their inhibitory effects on endothelium-mediated vasorelaxation are largely

mediated through a decrease in NO availability, both through suppression of NO synthesis and augmentation of NO scavenging by reactive oxygen species, and can be ameliorated by supplementation with L-arginine, L-ascorbate or pyridoxine, either singly or in combination. In addition, there exists a component of inhibition of endothelium-mediated vasorelaxation that is not ameliorated by these agents, particularly in the presence of dLDL and oxLDL. Whether the favourable effects of L-arginine, L-ascorbate and pyridoxine on endothelial function translate into clinical benefit remains to be proven.

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