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European Journal of Pharmacology 424 (2001) 141–149

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Endothelial dysfunction induced by oxidized low-density lipoproteins in isolated mouse aorta: a comparison with apolipoprotein-E deficient mice

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Received 11 January 2001; received in revised form 16 May 2001; accepted 12 June 2001

Abstract

We characterized the acute effects of oxidized low-density lipoproteins (oxidized-LDL) on vascular reactivity in isolated aorta from wild-type C57BL/6J mice, and compared these with the chronic alterations in vascular function observed in apolipoprotein-E gene knockout [ApoE(−/−)] mice fed a high-fat diet, which results in hyperlipidemia and atherosclerosis. In the abdominal (but not thoracic) aorta, oxidized-LDL (100 μg/ml) reduced relaxations induced by acetylcholine (10^{-9} M–10^{-5} M), which are mediated entirely by nitric oxide (NO). The relaxations induced by the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10^{-8} M–10^{-4} M), the cyclic GMP analogue 8-bromo cyclic GMP (100 μM) and the nonspecific vasodilator papaverine (100 μM) were not changed by oxidized-LDL. Native LDL had no effect on vasorelaxations. The attenuation of endothelium-dependent relaxations caused by oxidized-LDL mimicked the endothelial dysfunction found in ApoE(−/−) mice. These results are consistent with the suggestion that oxidized-LDL has an important role in the pathogenesis of endothelial NO dysfunction associated with hyperlipidemia and atherosclerosis in these mice.

Keywords: Apolipoprotein-E; Atherosclerosis; Endothelial dysfunction; Hyperlipidemia; Nitric oxide (NO); LDL (low-density lipoproteins, oxidized)

1. Introduction

Endothelial cells have a pivotal role in maintaining a normal vascular tone by releasing several vasorelaxing factors including nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) (Siegel et al., 1989; Moncada et al., 1991; Feletou and Vanhoutte, 1999). Moreover, endothelium-dependent vasorelaxation is attenuated by hyperlipidemia and in atherosclerotic vessels in both animals and humans (Ludmer et al., 1986; Verbeuren et al., 1986; Jayakody et al., 1987; Golino et al., 1991; Dusting et al., 1995). The causes of endothelial dysfunction in these disorders are complex and the pathogenic mechanisms have not yet been fully elucidated. Several lines of evidence suggest that oxidized low-density lipoproteins (oxidized-LDL) might have a causative role in the development of the endothelial dysfunction in these pathological conditions (for review, see Cox and Cohen, 1996). This suggestion is based on several in vitro studies showing that treatment with oxidized-LDL, but not unmodified, native low-density lipoproteins (LDL), compromised endothelium-dependent vasorelaxations; this impairment of endothelial function is similar to that observed in vessels from animals and humans with hyperlipidemia or atherosclerosis (Jayakody et al., 1987; Shimokawa and Vanhoutte, 1989; Jacobs et al., 1990; Tanner et al., 1991; Kamata et al., 1996).

Recently, apolipoprotein-E gene knockout [ApoE(−/−)] mice, which lack the gene encoding apolipoprotein-E (Plump et al., 1992), have been shown to develop hyperlipidemia and atherosclerosis similar to humans (Nakashima et al., 1994). Moreover, these mice develop hypertension and show endothelial dysfunction (Plump et al., 1992; Barton et al., 1998; Yang et al., 1999). However, the importance of oxidized-LDL in the pathogenesis of endothelial dysfunction in ApoE(−/−) mice has not been clarified. We hypothesized that in vitro treatment of normal mouse vessels with oxidized-LDL should pharmacologically mimic the endothelial dysfunction found in the vessels from chronically hyperlipidemic and atherosclerotic animals. Furthermore, the effect of oxidized-LDL on vascular reactivity has not previously been studied in mouse blood vessels. We cannot find a study that systemi-

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0014-2999/01/$ - see front matter
PII: S0014-2999(01)01140-2

cally characterizes and compares the vascular functional changes induced by oxidized-LDL with those that occur in hyperlipidemia or atherosclerosis in the same species and tissue using common experimental protocols. Therefore, we set out to characterize the acute vascular effects of oxidized-LDL in mouse isolated aorta, and compare these vascular functional changes with those found in ApoE(−/−) mice. All experiments were carried out under the same experimental settings.

2. Materials and methods

2.1. Animals and tissue preparations

ApoE(−/−) mice with >99% C57BL/6J background were purchased from the Animal Resource Centre (Western Australia, Australia) when they were 4–5 weeks old. To accelerate the development of hyperlipidemia and atherosclerosis, they were immediately transferred to a standardized high-fat chow containing 0.15% cholesterol (Harlan Teklad, Madison, WI, USA). C57BL/6J mice were used as wild-type control. All animals were killed at 28–30 weeks of age. For the study on the effects of LDL or oxidized-LDL, C57BL/6J mice of around 15 weeks old were used.

On the day of experimentation, animals were anesthetized by isoflurane inhalation and killed by decapitation. This procedure was approved by the Animal Experimentation Ethics Committee of the University of Melbourne. Blood samples were collected for plasma lipid assay. Thoracic and abdominal aorta were removed to a physiological salt solution at room temperature in a Petri dish and the adventitial fat tissues were cleaned off. Segments of 1.5 mm long were obtained from both thoracic and abdominal aorta avoiding the bifurcations of major arteries. For functional studies in ApoE(−/−) mice, vessel segments were used immediately after dissection; whereas to study the effects of oxidized-LDL, the segments were incubated overnight in Dulbecco's modification of Eagle's medium (DMEM, CSL Biosciences, Melbourne, Australia), which was supplemented with 0.1% foetal calf serum and contained either saline (vehicle control) or oxidized-LDL, in a 95% O_2, 5% CO_2 environment at 37 °C. The concentration of oxidized-LDL used was 100 μg/ml. Higher concentrations severely impaired the contraction responses which would complicate analysis of vasorelaxation responses. This concentration was used by most previous studies (Cox and Cohen, 1996).

2.2. Vascular functional studies

Aortic segments were transferred to a Mulvany myograph (Model 610M, J.P. Trading, Denmark). Segments were mounted onto two parallel stainless steel pins through the lumen. One pin was fixed to a displacement micrometer and the other one was connected to an isometric tension transducer. The tissue was placed in a chamber containing 6 ml physiological salt solution, which was maintained at 36 °C ± 1 and gassed with 5% CO_2 and 95% O_2. The isometric tension was displayed and recorded with a PowerLab data recording system (AD Instruments, Australia). After a 20 min period of equilibration, the resting tension was raised stepwise to 5 mN, then a further equilibration period of 20 min was given.

Contractility of the vessels was initially tested with 123 mM high-K^+ physiological salt solution. Because K^+ did not produce maximal contractions in these vessels, then the tissues were maximally contracted with the thromboxane analogue 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$ (U46619, 1 μM). Concentration–response curves were obtained by cumulative addition of stock solutions of various drugs into the chamber. The relaxations to acetylcholine (10^{-9}–10^{-5} M) and *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10^{-8}–10^{-4} M) and contractions to 5-hydroxytryptamine (5-HT, 10^{-8}–10^{-5} M) were studied sequentially in a single tissue. Relaxations were induced after the tone had been raised with U46619 to 40 − 50% of the maximum. Tissues were washed thoroughly and at least 20 min of equilibration was allowed between two curves. In some experiments, the tissues were preincubated with the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) for 30–40 min before contraction.

2.3. Preparation of oxidized-LDL

Native LDL (5 mg protein/ml) purchased from Sigma was dialysed against phosphate-buffer saline (pH 7.2) at 4 °C for 24 h and then oxidized by incubation with 10 μM $CuSO_4$ at room temperature overnight. Then the reaction was stopped by 1 mM EDTA and the mixture was dialysed against phosphate-buffer saline again. The oxidation of LDL was determined by the thiobarbituric acid reactive substances assay (Yokode et al., 1988; Hein and Kuo, 1998) using a luminescence spectrometer (Perkin-Elmer) with excitation at 515 nm and emission at 550 nm. $CuSO_4$ incubation increased the thiobarbituric acid reactive substances content from 1.6 to 5.8 nmol malondialdehyde equivalent/mg protein. Protein content was determined by Lowry's assay (Lowry et al., 1951).

2.4. Quantification of atherosclerotic lesions

After the functional experiments in which the abdominal aortae from ApoE(−/−) mice were tested, the vessel segments were cut open and stained with oil red O (0.5% in 60% isopropyl alcohol) for 10 min at room temperature. The *en face* image of the lumen was pictured. The pictures were digitised and the lesion area (red stained) was quanti-

fied using MCID imaging analysis software (Imaging Research, Canada). The results were expressed as % of the total luminal surface area.

2.5. Measurement of plasma lipid levels

Heparinized blood samples were centrifuged at 14000 rpm for 10 min. Plasma was removed to 1.5 ml Eppendorf vials and stored at −80 °C to be assayed in 2 months. Plasma total cholesterol, low-density lipoproteins (LDL), high-density lipoproteins (HDL) and triglycerides were measured with commercial biochemical assay kits from Genzyme (MA, USA) (for LDL) or Beckman Instruments (CA, USA) (for other lipids).

2.6. Histological examination of atherosclerotic lesions

Freshly dissected tissues were fixed in 4% paraformaldehyde for 24 h. Then the tissue blocks were embedded in paraffin and transverse sections of 4 μm in thickness were cut serially. Tissue sections were stained with haematoxylin-eosin and examined by light microscope.

2.7. Drugs and solutions

The following drugs were used: acetylcholine perchlorate, 8-bromo guanosine 3′:5′-cyclic monophosphate (sodium salt, 8-bromo cyclic GMP), 5-hydroxytryptamine (creatinine sulfate complex, 5-HT), N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME), *S*-nitroso-*N*-acetylpenicillamine (SNAP), papaverine hydrochloride, 9,11-dideoxy-9α,11α-methanoepoxyprostaglandin $F_{2\alpha}$ (U46619). Acetylcholine was from BDH Chemicals (England); U46619 was from Calbiochem (CA, USA); other drugs were from Sigma (St. Louis, MO, USA).

Stock solutions of the drugs were made by dissolving them in distilled water, except for SNAP and U46619, which were dissolved in dimethylsulphoxide (DMSO) and diluted in physiological salt solution. The physiological salt solution had the following composition (mM): NaCl 118.0, KCl 4.7, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5 and D-glucose 5.0. The isotonic high-K^+ (123 mM) physiological salt solution was prepared by replacing NaCl in normal physiological salt solution with equal molar KCl.

2.8. Data and statistical analysis

The tension of the vessel wall was measured in mN. Relaxations were expressed as percentage reductions of U46619-produced tone. Contractions to 5-HT were expressed as percentages of 123 mM high-K^+ physiological salt solution-induced contractions. The EC_{50} (which is the concentration required to produce 50% of the maximal response) values were calculated using a GraphPad Prism software. Data were presented as mean ± standard error of the mean (S.E.M.). The mean data were analysed with one-way analysis of variance followed by Tukey's test. A value of $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Hyperlipidemia and atherosclerosis in ApoE(−/−) mice

At 28–30 weeks of age, ApoE(−/−) mice demonstrated significantly elevated plasma levels of total cholesterol, LDL, HDL and triglycerides, as compared to wild-type C57BL/6J of the same age (Table 1). In C57BL/6J mice, there was no atherosclerotic lesion detectable anywhere in the aorta. In ApoE(−/−) mice, most of the lesions, identified as areas stained red by oil red O, were found at the aortic sinus, the lesser curvature of the aortic arch and the upper half of the abdominal aorta. Other lesions were mainly located at the sites of bifurcations of arteries. Light microscopic examination of transverse sections demonstrated that all pathological phases of atherosclerosis, namely fatty streaks, fibrofatty lesions and fibrous plaques, could be found along the aorta.

3.2. Contractile responses in isolated aorta

In both thoracic and abdominal aorta, the contractions induced by 123 mM high-K^+ physiological salt solution were significantly decreased in ApoE(−/−) mice comparing with C57BL/6J control (Fig. 1). Similarly, the maximal contractions to U46619 (1 μM) were also attenuated in ApoE(−/−) mice (Fig. 1). In contrast, the contractions to 5-HT (10^{-8} M–10^{-5} M), which were expressed as % of high-K^+ physiological salt solution-in-

Table 1
Plasma lipid levels in normal C57BL/6J mice (control) and apolipoprotein-E gene knockout mice [ApoE(−/−)]

	Total cholesterol (mM)	HDL (mM)	LDL (mM)	Triglycerides (mM)
Control ($n = 14$)	2.80 ± 0.087	2.70 ± 0.082	undetectable	1.14 ± 0.11
ApoE(−/−) ($n = 23$)	27.1 ± 1.24[a]	5.79 ± 0.26[a]	6.92 ± 0.38	3.79 ± 0.42[a]

HDL, high-density lipoprotein cholesterol; LDL, low-density lipoprotein cholesterol. LDL levels less than 0.3 mM were undetectable with the assay kit used in the present study. Data are mean ± standard error of the mean (S.E.M.).

[a] $P < 0.001$ vs. control, one-way analysis of variance.

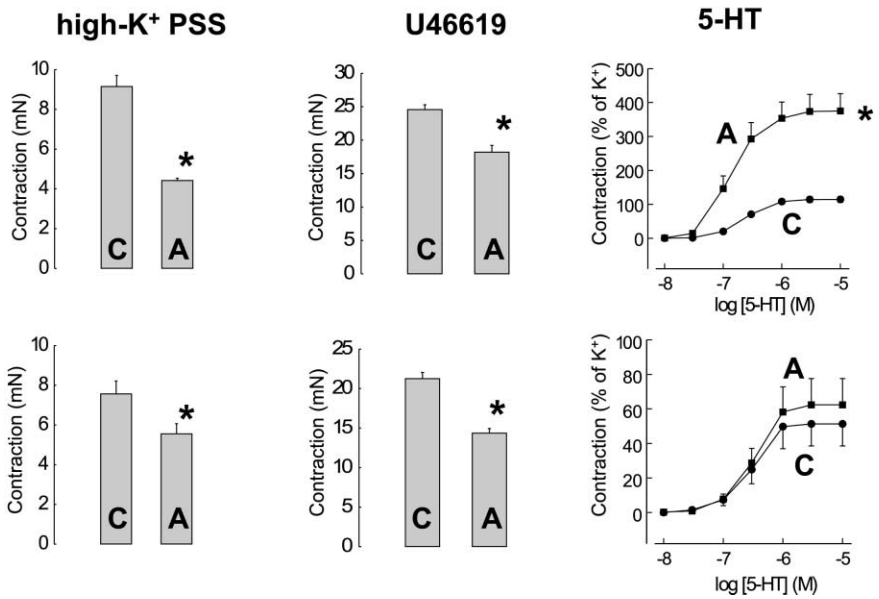

Fig. 1. Contractile responses induced by high-K^+ (123 mM) physiological salt solution (PSS), U46619 (1 μM) and 5-hydroxytryptamine (5-HT, 10^{-8}–10^{-5} M) in the thoracic (upper panel) and abdominal (lower panel) aorta from normal C57BL/6J mice (C) and apolipoprotein-E gene knockout [ApoE(−/−)] mice (A). Contractions to high-K^+ physiological salt solution and U46619 are expressed in mN; contractions to 5-HT are expressed as % of those induced by high-K^+ physiological salt solution. Data are mean ± standard error of the mean (S.E.M.). $^*P < 0.05$, one-way analysis of variance, $n = 4$–20.

duced contractions, were markedly enhanced in the thoracic, but not abdominal, aorta in ApoE(−/−) mice (Fig. 1). The EC_{50} and R_{max} (maximal response) values of 5-HT-induced contractions were given in Table 2.

In tissues obtained from C57BL/6J mice, incubation with oxidized-LDL (100 μg protein/ml) had no effects on the contractile responses to K^+ (123 mM) or U46619 (1 μM) in either thoracic or abdominal aorta (Fig. 2). Also, oxidized-LDL treatment did not change the contractions to 5-HT (10^{-8} M–10^{-5} M) (Fig. 2 and Table 3).

3.3. Relaxation responses in isolated aorta

In the thoracic and abdominal aorta from both control and ApoE(−/−) mice, acetylcholine (10^{-9} M–10^{-5} M) produced endothelium-dependent relaxations, which were blocked by pretreatment with the NO synthase inhibitor L-NAME (100 μM) (Fig. 3). In the abdominal aorta, relaxations to acetylcholine were attenuated in ApoE(−/−) as compared to control mice (Fig. 3). In contrast, there were no differences between control and

Table 2
EC_{50} and R_{max} values of the relaxations to acetylcholine and SNAP, and the contractions to 5-HT in thoracic and abdominal aorta from normal C57BL/6J (control) and ApoE(−/−) mice

	Thoracic aorta		Abdominal aorta	
	EC_{50} (μM)	R_{max} (%)	EC_{50} (μM)	R_{max} (%)
ACh				
Control	0.074 ± 0.022 (8)	66.1 ± 4.3 (8)	0.038 ± 0.006 (8)	88.9 ± 1.0 (8)
ApoE(−/−)	0.15 ± 0.054 (8)	77.7 ± 6.4 (8)	0.08 ± 0.012[a] (8)	71.8 ± 4.1[b] (8)
SNAP				
Control	0.55 ± 0.25 (7)	97.0 ± 1.0 (7)	0.38 ± 0.13 (12)	96.6 ± 0.6 (12)
ApoE(−/−)	0.45 ± 0.24 (8)	95.6 ± 0.9 (8)	0.48 ± 0.17 (12)	93.6 ± 1.3 (12)
5-HT				
Control	0.23 ± 0.013 (4)	114.4 ± 8.5 (4)	0.34 ± 0.041 (8)	51.3 ± 12.8 (8)
ApoE(−/−)	0.15 ± 0.026[c] (5)	375.3 ± 51.1[b] (5)	0.34 ± 0.047 (8)	62.3 ± 15.2 (8)

EC_{50}, the concentration required to produce 50% of the maximal response; R_{max}, maximal response; ACh, acetylcholine; SNAP, *S*-nitroso-*N*-acetylpenicillamine; 5-HT, 5-hydroxytryptamine. Data are mean ± S.E.M.

[a] $P < 0.005$ vs. control, one-way analysis of variance. Figures in brackets are the numbers of experiments.

[b] $P < 0.01$ vs. control, one-way analysis of variance. Figures in brackets are the numbers of experiments.

[c] $P < 0.05$ vs. control, one-way analysis of variance. Figures in brackets are the numbers of experiments.

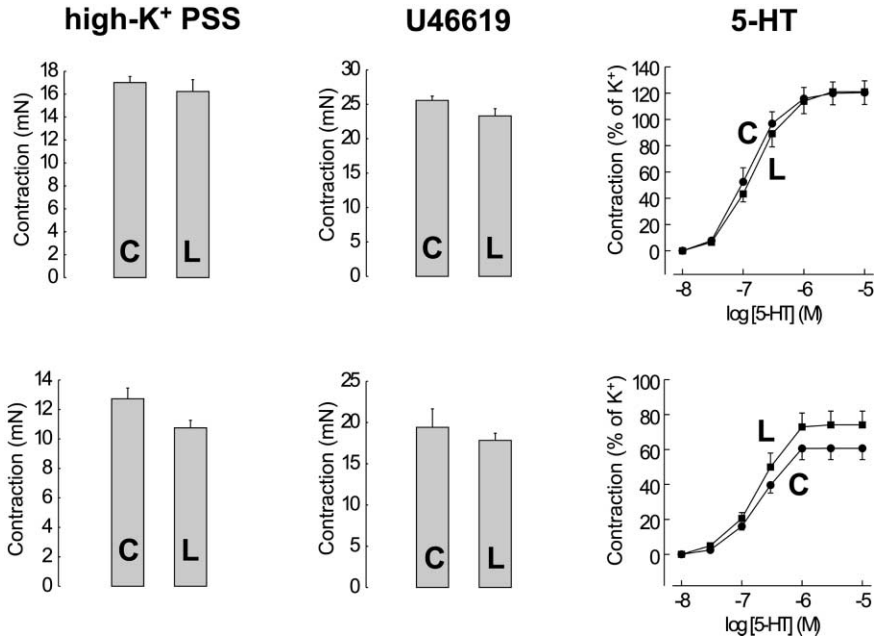

Fig. 2. Contractile responses induced by high-K^+ (123 mM) physiological salt solution (PSS), U46619 (1 μM) and 5-HT (10^{-8}–10^{-5} M) in the thoracic (upper panel) and abdominal (lower panel) aorta from wild-type C57BL/6J mice incubated overnight with either saline (C) or oxidized low-density lipoprotein (oxidized-LDL, 100 μg protein/ml) (L). Contractions to high-K^+ physiological salt solution and U46619 are expressed in mN; contractions to 5-HT are expressed as % of those induced by high-K^+ physiological salt solution. Data are mean ± S.E.M. $^*P < 0.05$, one-way analysis of variance, $n = 5$–20.

ApoE(−/−) in the relaxations produced by the NO donor SNAP (10^{-8} M–10^{-4} M) (Fig. 3), the cell permeable cyclic GMP analogue 8-bromo cyclic GMP (100 μM) [52.5% ± 4.3 in ApoE(−/−) vs. 55.1% ± 6.3 in control, $P > 0.05$, $n = 3$–6] or the nonspecific vasodilator papaverine (100 μM) [97.7% ± 1.0 in ApoE(−/−) vs. 98.0% ± 0.2 in control, $P > 0.05$, $n = 3$–6]. In the thoracic aorta, on the other hand, acetylcholine-induced responses in control and ApoE(−/−) did not differ (Fig. 3). Nor was there any difference between SNAP-induced relaxations in the thoracic aorta (Fig. 3). The EC_{50} and R_{max} values of acetylcholine-and SNAP-induced relaxations in these tissues were summarized in Table 2.

In tissues from wild-type C57BL/6J, incubation with oxidized-LDL (100 μg protein/ml) significantly reduced the relaxations to acetylcholine in the abdominal aorta while it had no effect in the thoracic aorta (Fig. 4 and Table 3). However, the relaxations to SNAP were not changed by oxidized-LDL (Fig. 4 and Table 3). Also, oxidized-LDL did not change the relaxations produced by

Table 3
EC_{50} and R_{max} values of the relaxations to acetylcholine and SNAP, and the contractions to 5-HT in the aorta from C57BL/6J mice incubated overnight with either saline (control) or oxidized-LDL

	Thoracic aorta		Abdominal aorta	
	EC_{50} (μM)	R_{max} (%)	EC_{50} (μM)	R_{max} (%)
ACh				
Control	0.049 ± 0.007 (7)	74.4 ± 4.1 (7)	0.16 ± 0.097 (9)	84.4 ± 4.6 (9)
Oxidized-LDL	0.072 ± 0.023 (7)	59.4 ± 6.2 (7)	0.11 ± 0.025 (11)	56.6 ± 3.7[a] (11)
SNAP				
Control	0.46 ± 0.15 (4)	98.3 ± 1.2 (4)	0.39 ± 0.12 (6)	89.3 ± 3.3 (6)
Oxidized-LDL	0.41 ± 0.11 (4)	99.0 ± 1.4 (4)	0.41 ± 0.19 (6)	93.7 ± 0.8 (6)
5-HT				
Control	0.12 ± 0.015 (5)	120.4 ± 9.0 (5)	0.21 ± 0.03 (10)	60.7 ± 6.5 (10)
Oxidized-LDL	0.16 ± 0.02 (5)	121.0 ± 9.2 (5)	0.21 ± 0.02 (10)	74.1 ± 7.8 (10)

Oxidized-LDL, oxidized low-density lipoprotein (100 μg protein/ml). Data are mean ± S.E.M.
[a] $P < 0.001$ vs. control, one-way analysis of variance. Figures in bracket are the number of experiments.

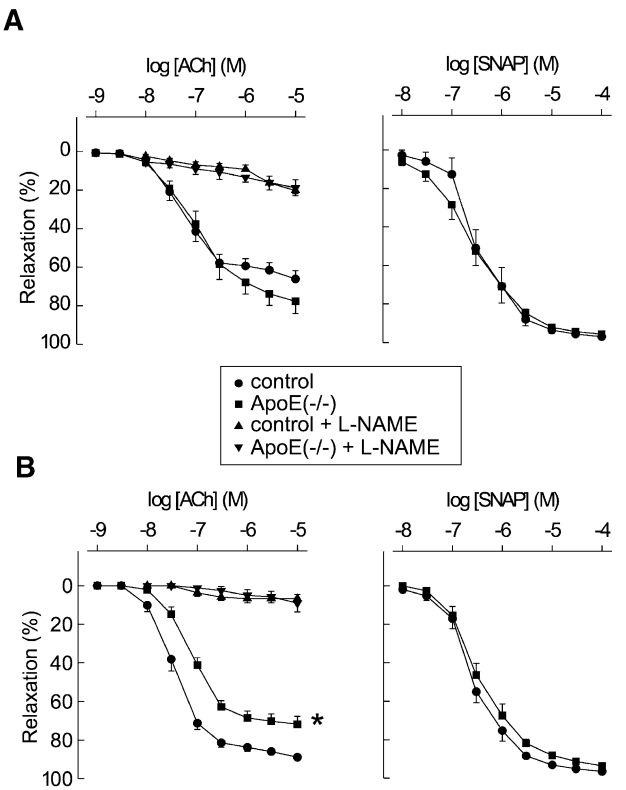

Fig. 3. Relaxations induced by acetylcholine (ACh, 10^{-9} M–10^{-5} M) and the nitric oxide (NO) donor *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10^{-8}–10^{-4} M) in the thoracic (A) and abdominal (B) aorta from normal C57BL/6J (control) and ApoE(−/−) mice. Acetylcholine-induced relaxations were blocked by the NO synthase inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME, 100 μM) in all of these tissues. Relaxations are expressed as % reductions of U46619-produced tone, which is equivalent to 40–50% of the maximal response induced by U46619. Data are mean ± S.E.M. $^{*}P < 0.005$, one-way analysis of variance followed by Tukey's test, $n = 3$–8.

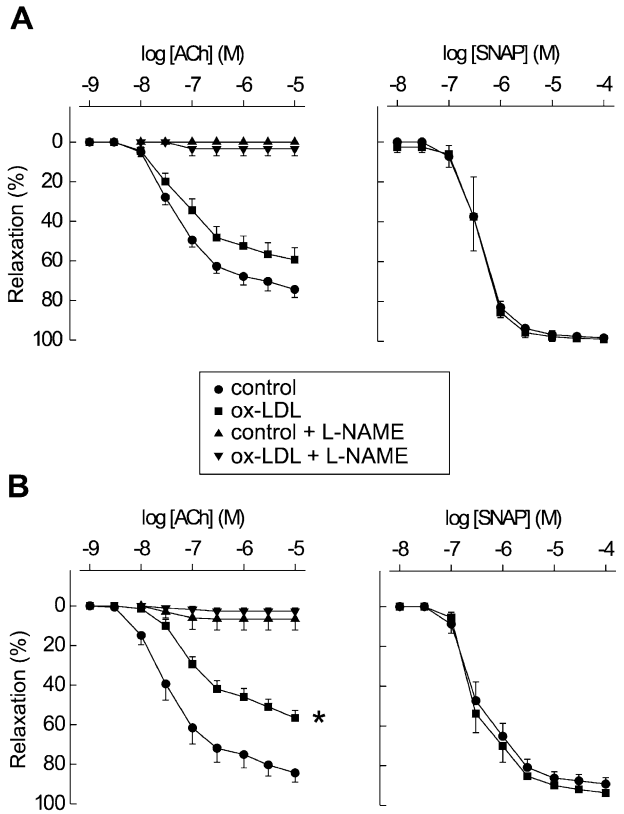

Fig. 4. Relaxations induced by acetylcholine (ACh, 10^{-9} M–10^{-5} M) and the NO donor SNAP (10^{-8}–10^{-4} M) in the thoracic (A) and abdominal (B) aorta from normal C57BL/6J mice incubated overnight with either saline (control) or oxidized-LDL (ox-LDL, 100 μg protein/ml). Acetylcholine-induced relaxations were blocked by L-NAME (100 μM) in all of these tissues. Relaxations are expressed as % reductions of U46619-produced tone, which is equivalent to 40–50% of the maximal response induced by U46619. Data are mean ± S.E.M. $^{*}P < 0.001$, one-way analysis of variance followed by Tukey's test, $n = 4$–11.

8-bromo cyclic GMP (27.8% ± 1.0 in oxidized-LDL-treated vs. 28.1% ± 4.5 in control tissues, $P > 0.05$; $n = 4$) or papaverine (96.4% ± 0.4 in oxidized-LDL-treated vs. 96.5% ± 2.3 in control tissues, $P > 0.05$, $n = 4$). In all of these tissues, acetylcholine-induced relaxations were abolished by L-NAME (Fig. 4). Unlike oxidized-LDL, native LDL at the same concentration did not significantly change acetylcholine-induced relaxations in the abdominal aorta (Fig. 5).

3.4. Relationship between endothelium-dependent relaxations and atherosclerotic lesion area

To determine whether the extent of atherosclerosis has a significant impact on endothelium-dependent relaxations induced by acetylcholine in the abdominal aorta from ApoE(−/−) mice, the EC_{50} of acetylcholine and the maximal relaxation were plotted against the total lesion area for each individual preparation, and the results were analysed by linear regression. There was no correlation

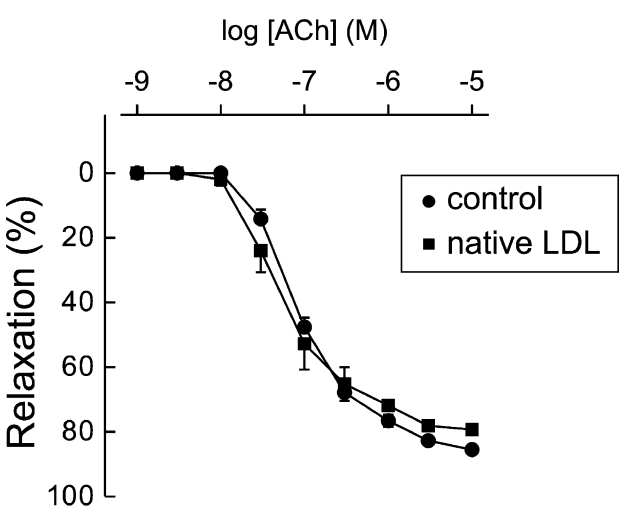

Fig. 5. Effect of overnight incubation with native low-density lipoprotein (LDL, 100 μg protein/ml) on acetylcholine (ACh)-induced relaxations in the abdominal aorta from normal C57BL/6J mice. Relaxations are expressed as % reductions of U46619-produced tone. Data are mean ± S.E.M., $n = 4$.

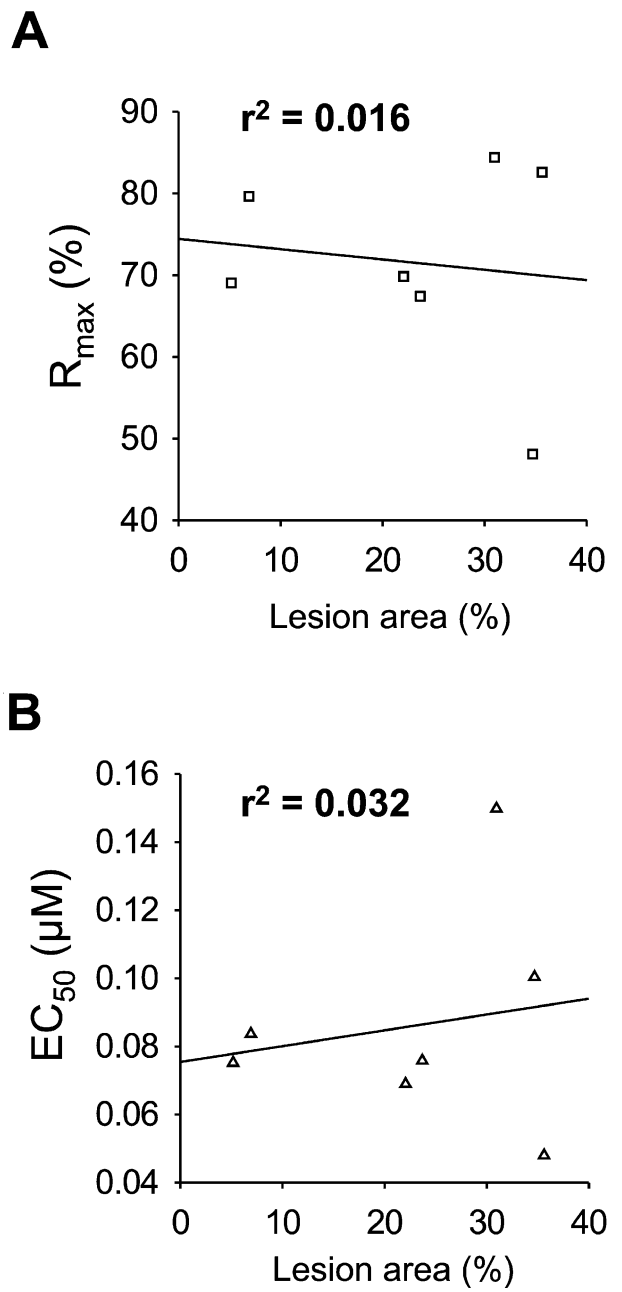

Fig. 6. Relationship between (A) the R_{max} (maximal response) and (B) EC_{50} (the concentration required to produce 50% of the maximal response) values of acetylcholine-induced relaxations and the atherosclerotic lesion areas in abdominal aortic rings from ApoE(−/−) mice. Lesion area is expressed as % of the total endothelial surface. There is not a significant correlation between the R_{max}/EC_{50} values and the lesion areas ($P > 0.05$, $n = 7$).

between EC_{50} or R_{max} and atherosclerotic lesion area (Fig. 6).

4. Discussion

This study characterized the acute changes in vascular function induced by oxidized-LDL in isolated mouse aorta, and compared with those found in hyperlipidemic and atherosclerotic ApoE(−/−) mice. Our results demonstrate that in vitro treatment with oxidized-LDL mimicked the endothelial NO dysfunction observed in ApoE(−/−) mice. This is based on the following observations: (1) oxidized-LDL produced a specific reduction of endothelium-dependent relaxations, which was similar to that observed in ApoE(−/−) aorta, whereas the NO donor SNAP-and the nonspecific vasodilator papaverine-induced endothelium-independent relaxations were not altered in either situation; (2) the endothelial dysfunction in ApoE(−/−) mice exhibited an obvious distributional variation (i.e. it was observed in abdominal aorta, but not in the thoracic aorta), and similar heterogeneity was also found with the effect of oxidized-LDL; (3) the effect of oxidized-LDL on endothelium-dependent relaxations could not be reproduced by native LDL at the same concentration; (4) there was no correlation between endothelium-dependent relaxations and the extent of atherosclerotic lesions; and (5) although the plasma concentration of oxidized-LDL has not been determined in atherosclerotic mice, the concentration of oxidized-LDL used in this study is in the range of the level of circulating oxidized-LDL estimated in human plasma in vivo (Cox and Cohen, 1996). However, it should be noted that the endothelial dysfunction in hyperlipidemia and atherosclerosis is a multiple factorial process, and the present study does not exclude the involvement of other pathogenic factors (Barton et al., 1998).

The abdominal aorta appeared to be more susceptible to the impact of oxidized-LDL and hyperlipidemia and atherosclerosis than the thoracic aorta. Such regional heterogeneity of endothelial dysfunction has not been observed in other hyperlipidemic or atherosclerotic models. In ApoE and LDL-receptor double knockout mice, Bonthu et al. (1997) demonstrated that the relaxation to acetylcholine was reduced to a greater degree in the proximal segment than in the distal segment of thoracic aorta. It was suggested that this difference might be due to the different lesion density in these two regions. However, this appears unlikely in our study because the lesion area did not correlate with the impairment of acetylcholine-induced relaxations in ApoE(−/−) abdominal aorta, suggesting that this endothelial dysfunction is not simply a result of a physical barrier effect. The mechanisms responsible for this regional variation are unknown. However, we have recently observed that in the abdominal aorta of ApoE(−/−) mice, superoxide anion ($O_2^{\cdot -}$) production is significantly higher than in the thoracic aorta, suggesting that there is a difference of oxidative stress status between these two regions.

Our results showed that in control, atherosclerotic and oxidized-LDL-treated vessels, acetylcholine-induced relaxations were abolished by the NO synthase inhibitor L-NAME, indicating that in mouse aorta this endothelium-dependent response is mediated by NO in both physio-

logical and pathophysiological situations, while other endothelium-derived mediators, such as EDHF (Feletou and Vanhoutte, 1999) or prostacyclin (Siegel et al., 1989) are unlikely to be involved. Brandes et al. (1997) have demonstrated that the relaxation mediated by endothelium-dependent hyperpolarizing mechanisms in renal arteries from hyperlipidemic rabbits was increased, and they suggested the increased EDHF component may compensate for the impaired relaxation mediated by NO and contribute to the maintenance of normal vascular function. However, this mechanism clearly has a minimal role in mouse aorta because hyperpolarizing mechanisms are not involved in the endothelium-dependent relaxation in either hyperlipidemia or oxidized-LDL treatment. This is consistent with the observation that EDHF has little role in large conduit arteries, but may contribute more in resistance vessels (Garland et al., 1995).

Precisely how oxidized-LDL suppresses endothelial NO-mediated relaxations has not been well defined. However, it has been shown that oxidized-LDL stimulated protein kinase C activity in endothelial cells (Li et al., 1998). Protein kinase C, in turn, may activate a vascular NAD(P)H oxidase, which has been identified as one of the major sources of $O_2^{\cdot -}$ in vascular tissues, leading to an overproduction of $O_2^{\cdot -}$ and a decreased bioavailability of NO (Heitzer et al., 1999; Munzel et al., 1999). Moreover, oxidized-LDL may stimulate phospholipase D activity in vascular tissues, an effect which could also activate NAD(P)H oxidase activity (Natarajan et al., 1995; Cox and Cohen, 1997; Touyz and Schiffrin, 1999). In separate experiments, we demonstrated that the inhibition of acetylcholine-induced relaxations by oxidized-LDL was diminished by incubation with the superoxide dismutase mimetic Mn(III)tetrakis(4-benzoic acid)porphyrin (data not shown), suggesting that increased $O_2^{\cdot -}$ generation may be involved in this process. This assumption is supported by the finding that oxidized-LDL had no effect on 8-bromo cyclic GMP-induced relaxations, indicating that the efficiency of the signal transduction pathway of the NO/cyclic GMP system (see Moncada et al., 1991) was not affected by oxidized-LDL.

The effect of hyperlipidemia and atherosclerosis on vascular contractile responses has been controversial (Wroblewski and Witanowska, 1982; Freiman et al., 1986; Ibengwe and Suzuki, 1986; Galle et al., 1991). In the present study, it was shown that in both thoracic and abdominal aorta from ApoE(−/−) mice, the contractions to K^+ were significantly reduced as compared to wild-type C57BL/6J mice, suggesting that the contractility of vascular smooth muscle cells was compromised in hyperlipidemia and atherosclerosis. Likewise, the contractions to U46619 were also reduced in ApoE(−/−) mice. This might be due to the upregulation of inducible NO synthase expression in atherosclerotic arteries (Arthur et al., 1997; Wilcox et al., 1997). On the other hand, the contractile responses to 5-HT were selectively increased in the thoracic aorta in ApoE(−/−) mice. Similar results were also obtained in rabbit arteries with experimental hyperlipidemia or atherosclerosis (Dusting et al., 1990; Galle et al., 1991; Sobey et al., 1991). The mechanisms of the enhanced reactivity to 5-HT probably relate to the upregulation of 5-HT receptor expression or the increase in receptor-mediated Ca^{2+} mobilization (Miwa et al., 1994).

In summary, in vitro treatment with oxidized-LDL specifically attenuated NO-mediated relaxations induced by acetylcholine in the abdominal aorta from wild-type C57BL/6J mice. This impairment of endothelial NO function mimicked the endothelial dysfunction found in ApoE(−/−) mice fed a high-fat diet. These results support the hypothesis that oxidized-LDL has an important role in the pathogenesis of endothelial NO dysfunction in hyperlipidemia and atherosclerosis.

Acknowledgements

This work was supported by an institute block grant (No. 983001) from the National Health and Medical Research Council of Australia. We also would like to thank Ms. Faye Docherty (Department of Anatomy and Cell Biology, the University of Melbourne) for the histological tissue processing and staining.

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