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Endothelium dysfunction in LDL receptor knockout mice: a role for H_2O_2

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1 In this study, the role of endogenous H_2O_2 as an endothelium-dependent relaxant factor was characterised in aortas from C57BL/6J and LDL receptor-deficient mice ($LDLR^{-/-}$).

2 Aortic rings from $LDLR^{-/-}$ mice showed impaired endothelium-dependent relaxation to acetylcholine (ACh; 0.001–100 μM) and to the Ca^{2+} ionophore A23187 (0.001–3 μM) compared with aortic rings from control mice. Endothelium-independent relaxation produced by the NO donor, 3-morpholino-sydnonimine (SIN-1) was not different between strains.

3 Pretreatment of vessels with L-NNA (100 μM) or L-NNA (100 μM) plus L-NAME (300 μM) plus haemoglobin (10 μM) markedly decreased, but did not abolish the relaxation to ACh in control mice. In the aortas from $LDLR^{-/-}$ mice treated with L-NNA (100 μM), ACh induced a contractile effect. Catalase (800 and 2400 U ml^{-1}) shifted to the right the endothelium-dependent relaxation to ACh in aortas from control but not from $LDLR^{-/-}$ mice. Aminotriazole (50 mM), which inhibits catalase, abolished its effect on control mice. Treatment of vessels with L-NNA and catalase abolished vasorelaxation induced by ACh. Indomethacin (10 μM) did not modify the concentration–response curve to ACh. Superoxide dismutase (300 U ml^{-1}) did not change ACh-induced relaxation in both strains.

4 Exogenous H_2O_2 produced a concentration-dependent relaxation in endothelium-denuded aortic rings, which was not different between strains.

5 It is concluded that H_2O_2 greatly contributes to relaxation to ACh in aorta from control mice. Endothelial-dependent relaxation to ACh is impaired in $LDLR^{-/-}$ mice. Reduced biosynthesis or increased inactivation of H_2O_2 is the possible mechanism responsible for endothelial dysfunction in aortas of atherosclerosis-susceptible $LDLR^{-/-}$ mice.
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Keywords: Atherosclerosis; LDL receptor-deficient mice; endothelium; hydrogen peroxide

Abbreviations: ACh, acetylcholine; ANOVA, two-way analysis of variance; eNOS, endothelial NO synthase; H_2O_2, hydrogen peroxide; $LDLR^{-/-}$, LDL receptor-deficient mice; L-NAME, N^{ω}-nitro-L-arginine-methyl-ester; L-NNA, N^{ω}-nitro-L-arginine; NO, nitric oxide; s.e.m., standard error; SIN-1, 3-morpholino-sydnonimine; SOD, superoxide dismutase

Introduction

Atherosclerosis is a progressive disease characterised by the accumulation of lipids and fibrous elements in the large arteries (Ludewig *et al*., 2002). In Westernised societies, it is the underlying cause of about 50% of all deaths.

Endothelium-derived vasoactive factors play an important regulatory role in vascular homeostasis and pathogenesis of atherosclerosis. The endothelium synthesises and releases several relaxing factors, including nitric oxide (NO), endothelium-derived hyperpolarising factor (EDHF) and prostacyclin (PGI_2). Endothelium-derived NO not only modulates the tone of the underlying vascular smooth muscle, but also inhibits several proatherogenic processes, including smooth muscle cell proliferation and migration, platelet aggregation, oxidation of low-density lipoproteins (LDL), monocyte and platelet adhesion, and synthesis of inflammatory cytokines, thus exhibiting important antiatherogenic effects (Shimokava, 1999). Chemical inactivation and reduced biosynthesis of NO have been described as the key mechanisms responsible for endothelial dysfunction in aortas of atherosclerosis-susceptible apo-E-deficient mice (D'Uscio *et al*., 2001).

Recently, hydrogen peroxide (H_2O_2) was described as a new endothelium-derived relaxant factor in mice and human mesenteric arteries (Matoba *et al*., 2000, 2002). Furthermore, H_2O_2 has been shown to elicit both hyperpolarisation and vasodilation of peripheral as well as cerebral arteries (Beny & von der Weid, 1991; Iida & Katusic, 2000; Matoba *et al*., 2002) and to activate Ca^{2+}-activated K^+ channels on vascular smooth muscle cells (Barlow & White, 1998; Bychkov *et al*., 1999; Barlow *et al*., 2000) and a nonselective cation channel in endothelial cells (Ji *et al*., 2002). In mice aorta Matoba *et al.* (2000), based on experiments where N^{ω}-nitro-L-arginine (L-NNA) inhibited approximately 80% of acetylcholine (ACh)-induced relaxation, proposed that NO is the major component responsible for relaxation in this vessel.

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Mice homozygous for the inactivated LDL receptor gene ($LDLR^{-/-}$) provide a model of atherosclerosis. These animals develop hypercholesterolaemia characterised by moderate levels of LDL and aortic atherosclerosis (Ishibashi *et al*., 1994). Vascular reactivity in this model of atherosclerosis is largely unknown. In this work, we describe for the first time the mechanism of impaired endothelium-dependent relaxation to ACh in the aorta from $LDLR^{-/-}$ mice and a role for endogenous H_2O_2 in this altered endothelial function.

Methods

Animals

We used 12- to 14-week-old male homozygous LDL receptor-deficient mice ($LDLR^{-/-}$; 26.5 ± 2.2 g) and age-matched wild-type C57BL/6J (28.0 ± 2.6 g) control mice. $LDLR^{-/-}$ were originally obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A. and bred in our animal facilities at the Federal University of Minas Gerais. C57BL/6J were obtained from CEBIO/ICB (UFMG, Brazil). The animals were maintained in collective cages in an appropriate room with controlled temperature and with a 12-h light cycle. Blood cholesterol, triglycerides and glucose levels in our $LDLR^{-/-}$ mice were $240 \pm 5.7\ \mathrm{mg\,dl^{-1}}$ ($P<0.001$); $120 \pm 8.2\ \mathrm{mg\,dl^{-1}}$ and $150 \pm 8.9\ \mathrm{mg\,dl^{-1}}$, respectively. The values for C57Bl/6 control mice were, respectively, 104 ± 8.9, 130 ± 21.6 and $128 \pm 13.47\ \mathrm{mg\,dl^{-1}}$. $LDLR^{-/-}$ mice showed higher levels of atherogenic lipoprotein LDL and IDL and serum LDL oxidised as compared to C57Bl/6 control mice. Twelve-week-old LDL receptor knockout animals developed a moderate fatty streak (intimal thickening with foam cells and apparent smooth muscle cell infiltration) in the aorta. There was no visible anatomic or morphologic endothelial damage caused by manipulation or by their transgenic status in the experimental conditions described here. The animal feeding and treatment protocol were reviewed and approved by the Animal Care Committee of the Instituto de Ciências Biológicas, UFMG, Brazil. Mice were killed by cervical dislocation and exsanguination and tissues were rapidly removed.

Mice aortic rings preparation and mounting

Rings (2–3 mm) from the proximal and distal thoracic aorta, free of fat and connective tissue were set up in gassed (95% O_2 and 5% CO_2) Krebs–Henseleit solution ($\mathrm{mmol\,l^{-1}}$): NaCl 110.8, KCl 5.9, $NaHCO_3$ 25.0, $MgSO_4$ 1.07, $CaCl_2$ 2.49, NaH_2PO_4 2.33 and glucose 11.51, at 37°C, under a tension of $0.5 \times g$, for 1 h equilibration period. During this period, the incubation medium was changed every 15 min. After the equilibration period, two contractile responses were evoked by submaximal concentrations of phenylephrine (0.1 μM) to elicit reproducible responses. The presence of a functional endothelium was assessed by the ability of ACh (10 μM) to induce more than 50% relaxation of vessels precontracted with phenylephrine (0.1 μM). When necessary, the endothelium was removed by rubbing the intimal surface with a wooden stick. ACh, A23187, SIN-1 and exogenous H_2O_2 were added in increasing cumulative concentrations, once the response to 0.1 μM phenylephrine had stabilised. There was no difference in the concentration–response curve to phenylephrine between strains (not shown) and for this reason we choose the same concentration of phenylephrine to precontract the vessels in control or $LDLR^{-/-}$ mice. When necessary, after 60 min washing, the vessels were incubated for 20 min with different drugs, as indicated, and a second cumulative concentration–response curve for ACh was constructed and compared with the first one. Aortic rings were incubated with aminotriazole (50 mM) for a period of 60 min. In some experiments, aminotriazole was incubated together with catalase in solution for 40 min and then the tissues were treated with these drugs for a further 20 min. In experiments performed in the presence of L-NNA or N^{ω}-nitro-L-arginine-methyl-ester (L-NAME), vessels were precontracted with (0.03 μM) phenylephrine, to achieve the same tension level as the others. The other inhibitors did not affect the contraction induced by 0.1 μM phenylephrine (not shown). Mechanical activity recorded isometrically by a force transducer (World Precision Instruments, Inc., Sarasota, FL, U.S.A.) was fed to an amplifier-recorder (Model TBM-4; World Precision Instruments, Inc.) and to a personal computer equipped with an analogue-to-digital converter board (AD16JR; World Precision Instruments, Inc.), using CVMS data acquisition/recording software (World Precision Instruments, Inc.).

Drugs

ACh, aminotriazole, catalase, indomethacin, L-NNA, phenylephrine, L-NAME, (3-morpholino-sydnonimine) (SIN-1), and superoxide dismutase (SOD) were purchased from Sigma (St Louis, U.S.A.) H_2O_2 from Merck (Darmstadt, Germany) and haemoglobin from Calbiochem (San Diego, U.S.A.). Indomethacin was dissolved in 0.5% $\mathrm{w\,v^{-1}}$ sodium bicarbonate and the other drugs were dissolved in distilled water at a concentration of 10 mM. All subsequent dilutions were made with Krebs–Henseleit solution just before use.

Statistical analysis

Results are expressed as means ± s.e.m. Two-way analysis of variance (ANOVA) with Bonferroni multiple comparison post-test was used to compare the concentration–response curves obtained in aortic rings. All statistical analyses were considered significant when $P<0.05$.

Results

Vascular relaxation in control and $LDLR^{-/-}$ mice

In vessels from control mice, ACh (0.001–100 μM) and the Ca^{2+}-ionophore A23187 (0.001–3 μM) produced endothelium-dependent relaxation of the sustained contractions induced by phenylephrine (Figure 1a, b). In $LDLR^{-/-}$, endothelium-dependent relaxation to ACh (Figure 1a) and A23187 (Figure 1b) was markedly impaired as compared with control mice. Endothelium-independent relaxation to the NO donor SIN-1 (0.001–10 μM) was not different between strains (Figure 1c).

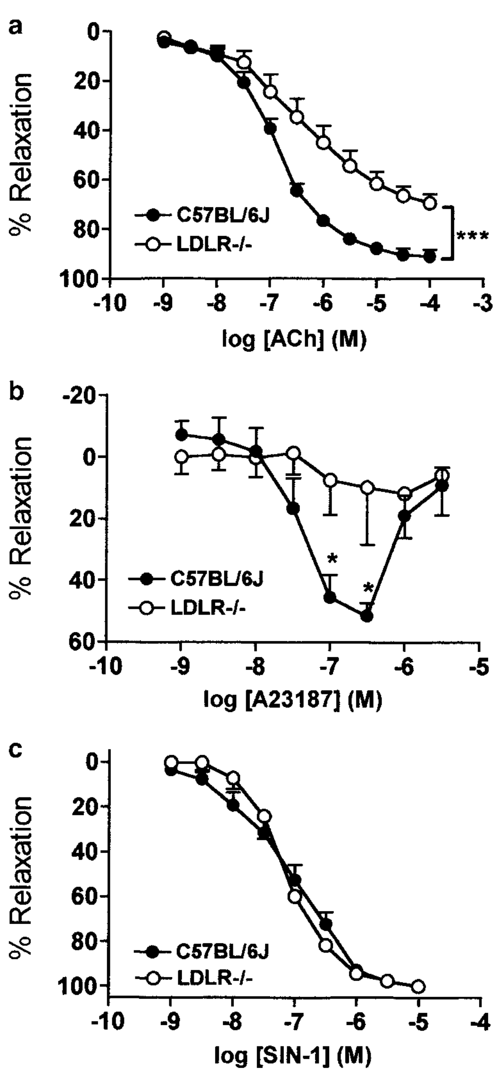

Figure 1 Concentration–response curves to acetylcholine (**a**), A23187 (**b**) and SIN-1 (**c**) in the aortas from control and $LDLR^{-/-}$ mice containing (**a** and **b**) or not containing (**c**) functional endothelium. The values are mean ± s.e.m. from eight experiments. *$P<0.05$, ***$P<0.001$, two-way ANOVA with Bonferroni multiple comparison post-test.

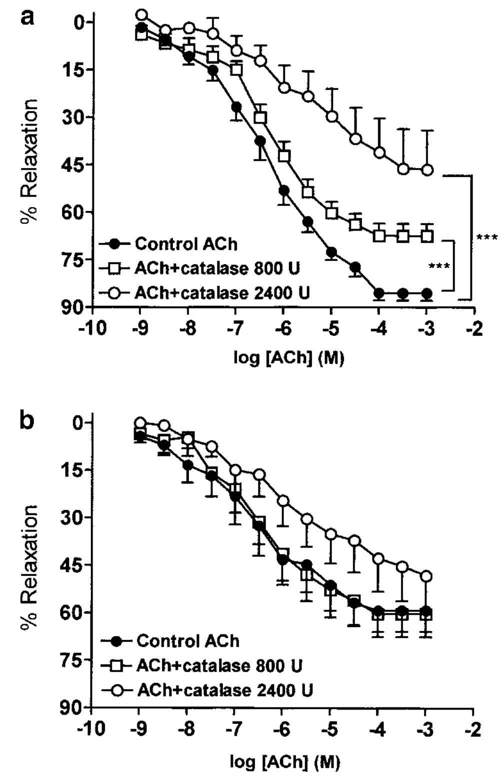

Figure 2 Catalase inhibits endothelium-dependent vasodilator effect of acetylcholine in aortas from control (**a**) but not from $LDLR^{-/-}$ (**b**) mice. The values are mean ± s.e.m. from at least four experiments. ***$P<0.001$, two-way ANOVA with Bonferroni multiple comparison post-test.

Effect of catalase, L-NNA and indomethacin on endothelium-dependent relaxation to acetylcholine

Incubation of endothelium-intact aortic rings from control mice with catalase (800 and 2400 U ml^{-1}) markedly inhibited the concentration–response curve to ACh (Figure 2a). In aortas from $LDLR^{-/-}$ mice, relaxation to ACh in the presence of catalase (800 and 2400 U ml^{-1}) was not significantly different from $LDLR^{-/-}$ controls (Figure 2b). Aminotriazole, an inhibitor of catalase (Margoliash & Novogrodsky, 1957), potentiated endothelium-dependent relataxion to ACh in both strains (Figure 3a, b). However, this potentiation was more pronounced in control animals (Figure 3a, b). In addition, aminotriazole abolished the inhibitor effect of catalase on ACh-induced relaxation in control animals, but did not modify relaxation to ACh in $LDLR^{-/-}$ mice (Figure 3a, b).

Indomethacin had little effect on the concentration–response curve to ACh (Figure 4a). L-NNA (100 μM) alone or L-NNA (100 μM) plus L-NAME (300 μM) plus haemoglobin (10 μM) or L-NNA plus indomethacin (10 μM) reduced, but did not abolish ACh-induced relaxation (Figure 4a). When control animals were treated simultaneously with L-NNA (100 μM) and catalase (2400 U ml^{-1}), ACh-induced relaxation was abolished (Figure 4a). In aortic rings from $LDLR^{-/-}$ mice, relaxation was abolished by L-NNA (100 μM) and ACh elicited contraction instead of relaxation (Figure 4b).

Effect of SOD on endothelium-dependent relaxation to acetylcholine

Endothelium-dependent relaxation to ACh of the sustained contractions induced by phenylephrine was not modified by SOD (300 U ml^{-1}) in aortic rings from both control and $LDLR^{-/-}$ mice (Figure 5a, b).

Vasorelaxant effect of exogenous H_2O_2 in endothelium-denuded aortic rings

Vasoactive properties of exogenous H_2O_2 were examined in endothelium-denuded aortic rings from control and $LDLR^{-/-}$ mice. H_2O_2 concentration dependently produced relaxation of the sustained contractions induced by phenylephrine in both control and $LDLR^{-/-}$ aortic rings, which was not significantly different between strains (Figure 6).

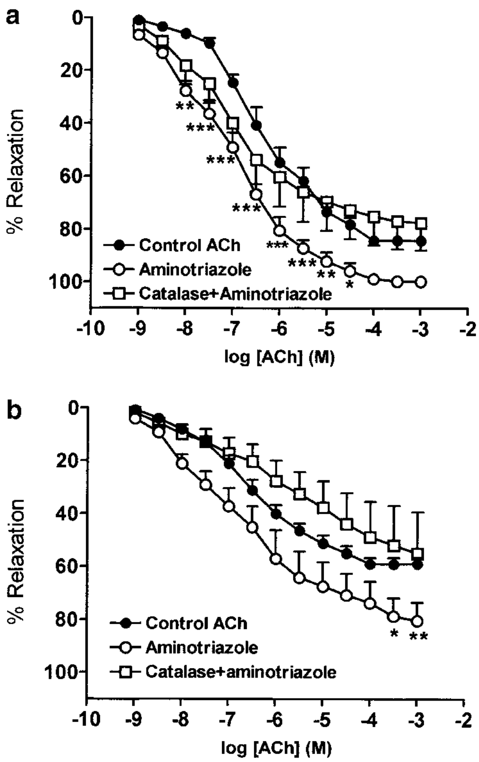

Figure 3 Effect of aminotriazole (50 mM) on endothelium-dependent relaxation to ACh in the presence of catalase (2400 U ml^{-1}) in control (**a**) and LDLR$^{-/-}$ (**b**) mice. The values are mean$\pm$s.e.m. from at least four experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared to control values. Two-way ANOVA with Bonferroni multiple comparison post-test.

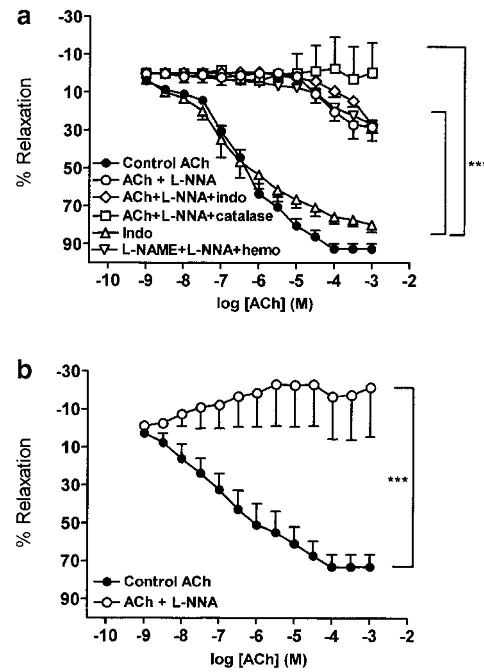

Figure 4 Effect of L-NNA (100 μM), L-NNA (100 μM) + L-NAME (300 μM) + haemoglobin (hemo; 10 μM), indomethacin (Indo; 10 μM), L-NNA plus indomethacin, and L-NNA plus catalase (2400 U ml^{-1}) on the vasodilator effect of acetylcholine in aortas from control mice (**a**) and the effect of L-NNA alone (100 μM) in aortas from LDLR$^{-/-}$ mice (**b**). The values are mean$\pm$s.e.m. from at least four experiments. ***$P<0.001$, two-way ANOVA with Bonferroni multiple comparison post-test.

Discussion

The major findings of this work are that H_2O_2 is an endothelium-derived relaxant factor in aortas of mice and that the impairment of its production or decrease in half-life is implicated in the endothelium dysfunction found in LDLR$^{-/-}$ mice.

Consistent with previous works (Matoba *et al.*, 2000; D'Uscio *et al.*, 2001), we found that L-NNA reduced but did not abolish and indomethacin had little effect on ACh-induced relaxation in control mice aorta. When we treated the vessels with L-NNA together with L-NAME and haemoglobin to be sure that NO synthase activity was completely blocked, we found the same extent of inhibition as L-NNA alone. This result suggests that an NO/prostanoid-independent component contributes to vasorelaxation induced by ACh. Increasing evidences support the idea that endogenous H_2O_2 is an endothelium-dependent relaxant factor in mice (Matoba *et al.*, 2000) and humans (Matoba *et al.*, 2002). To examine the role of H_2O_2 as a relaxant factor in the aorta of control mice, we used catalase, an enzyme that specifically decomposes H_2O_2 into oxygen and water (Deisseroth & Dounce, 1970). We found that catalase markedly inhibited ACh-induced relaxation, suggesting that H_2O_2 importantly contributes to relaxation mediated by ACh. Catalase also inhibited endothelium-dependent H_2O_2-mediated relaxation in human (Matoba *et al.*, 2002) and mice (Matoba *et al.*, 2000) mesenteric arteries, as well as relaxation induced by exogenous

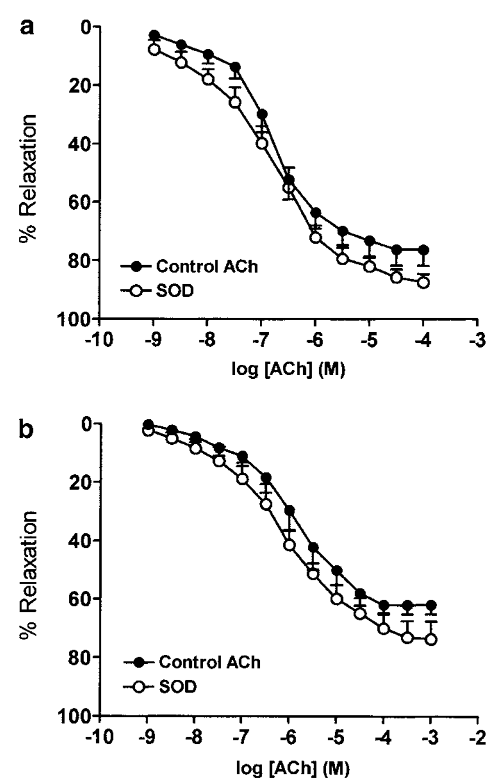

Figure 5 Effect of SOD (300 U ml^{-1}) on concentration–response curves to acetylcholine in control (**a**) and LDLR$^{-/-}$ (**b**) mice. The values are mean$\pm$s.e.m. from six experiments.

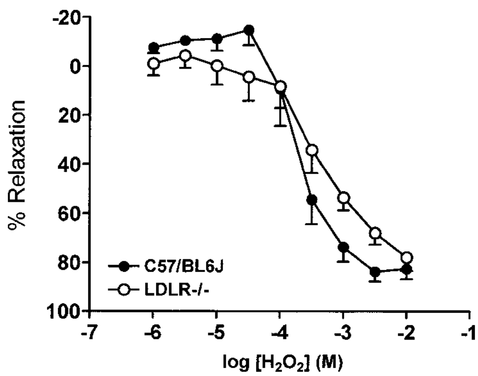

Figure 6 Concentration–response curves to H_2O_2 in endothelium-denuded aortas from control and LDLR$^{-/-}$ mice. The values are mean ± s.e.m. of five experiments.

application of H_2O_2 in canine cerebral arteries (Iida & Katusic, 2000). Inhibition of endogenous catalase by aminotriazole potentiated vasorelaxation to ACh reinforcing the hypothesis that endogenous H_2O_2 is an endothelial vasorelaxant factor in the aorta of control mice. Aminotriazole also abolished the inhibitor effect of catalase on ACh-induced relaxation. Pretreatment with both L-NNA and catalase abolished relaxation to ACh in the aortas of control mice. Together, our results support the idea that H_2O_2 greatly contributes with endothelium-dependent relaxation to ACh in the aorta of control mice. As L-NNA strongly inhibited relaxation to ACh, it is likely that endothelial NO synthase (eNOS) could be the major source of H_2O_2 as reported for mice mesenteric artery (Matoba *et al.*, 2000). However, the relaxation resistant to L-NNA found in the present study supports the idea that H_2O_2 could also be formed by an alternative pathway.

Atherosclerosis is a chronic process, which can be triggered by cardiovascular risk factors such as hypercholesterolaemia, ageing, hypertension, and diabetes mellitus (Lusis, 2000). Endothelial dysfunction is a hallmark of cardiovascular risk factors and has been implicated in the pathogenesis of atherosclerosis (Ross, 1993). It is now well accepted that NO, a potent vasodilator formed in endothelial cells from L-arginine by eNOS, plays a central role in endothelial dysfunction induced by hypercholesterolaemia and inhibits several components of the atherogenic process, such as vascular smooth muscle cells contraction and proliferation, platelet aggregation and monocyte adhesion (Busse & Fleming, 1996; Kojda & Harrison, 1999).

As reported in the apolipoprotein E-deficient mice (Deckert *et al.*, 1999) and humans (Oemar *et al.*, 1998), we found in this work that endothelium-dependent relaxation to ACh is impaired in the aorta of LDLR$^{-/-}$ mice. Endothelium-dependent relaxation to the calcium ionophore A23187 was also impaired, indicating that reduced Ca^{2+}-dependent NOS activity may be, at least in part, responsible for endothelial dysfunction in LDLR$^{-/-}$ aorta. This is in agreement with a previous study that showed that endothelial dysfunction in LDLR$^{-/-}$ mice is reversed by combined administration of L-arginine and tetrahydrobiopterin (BH4) (Jiang *et al.*, 2000). Vascular responsiveness to endothelial NO was not altered since endothelium-independent relaxation to the NO donor SIN-1 was not different between strains. L-NNA at the same concentration used for control animals completely abolished ACh-induced relaxation in LDLR$^{-/-}$ mice, and most importantly inhibition by catalase was not significant. Exogenous H_2O_2 elicit concentration-dependent relaxation in the aorta of control and LDLR$^{-/-}$ mice, which was not different between strains, ruling out the possibility that differences in smooth muscle responsiveness to H_2O_2 would account for the decreased participation of H_2O_2 in vasorelaxation induced by ACh in LDLR$^{-/-}$ mice. Even though the concentration of H_2O_2 we used was relatively high, it has been reported that vascular endothelial cells are able to produce approximately 0.8 mm of H_2O_2 (Suematsu *et al.*, 1993). Moreover, the functionality of our arteries was unaffected with concentrations of H_2O_2 up to 1 mM (data not shown). Together, our results suggest that in aortas from LDLR$^{-/-}$ mice, endothelial H_2O_2-dependent relaxations are impaired.

Recently, Cosentino *et al.* (2001) reported that the vasodilator effect of ACh in C57BL/6J mice was not modified in the presence of catalase (1200 U ml^{-1}). Possible differences between their results and ours may be because of the maximal vasodilator effect achieved by ACh in their work ($\sim$30 *versus* $\sim$90% in our work). This remarkable difference in the functionality of the endothelium could be the main reason for the absence of the effect of catalase described by Cosentino *et al.* (2001). Furthermore, they used different experimental conditions, such as the presence of indomethacin in addition to catalase, an incubation time of only 5 min and a catalase that was not thymol-free.

The exact mechanism of impaired endothelial H_2O_2-dependent relaxation is unknown. However, as endothelial response to A23187 was also impaired, it is possible that there is a decreased production of H_2O_2 *via* eNOS. SOD did not modify vasorelaxation to ACh in both strains. Therefore, it is possible that an increased enzymatic inactivation by catalase is decreasing the availability of H_2O_2. In support of this hypothesis, it has been reported that lipid peroxides induce the expression of catalase (Meilhac *et al.*, 2000), suggesting that oxidative stress, as seen in atherosclerosis (Kojda & Harrison, 1999) could induce cellular antioxidant protective response. Our results do not permit us to distinguish among these possibilities and further investigations are required. However, as we showed that aminotriazole was less effective in potentiating relaxation to ACh in LDLR$^{-/-}$ as compared to control mice, it is interesting to speculate that an overexpression of catalase as proposed by Meilhac *et al.* (2000) would be involved in the impaired relaxations to H_2O_2 in LDLR$^{-/-}$ mice.

Altogether, our results strongly suggest that lack of H_2O_2, an endothelium-dependent relaxant factor in the aorta of mice, contributes to endothelial dysfunction in a mice model of atherosclerosis. The present work may open a new research field in the participation of H_2O_2 in the pathogenesis and pathophysiology of atherosclerosis and other cardiovascular diseases.

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