

Reduced progression of atherosclerosis in apolipoprotein E-deficient mice treated with lacidipine is associated with a decreased susceptibility of low-density lipoprotein to oxidation

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Summary

A study has been carried out in the apolipoprotein (apo) E-deficient mouse to investigate the activity of lacidipine (a calcium antagonist with antioxidant properties) in inhibiting the development of atherosclerotic lesions; of particular interest were changes in the susceptibility of low-density lipoproteins (LDL) to oxidation. Mice receiving a Western-type diet to accelerate the development of atherosclerosis were treated orally with vehicle or lacidipine at 3 or 10 mg/kg/day for 8 weeks. Lacidipine treatment (at 3 or 10 mg/kg) had no effect on the plasma lipid profile. However, a significant ($P < 0.01$) dose-related reduction of 43 and 50% of the aortic lesion area in respect to vehicle-treated mice was observed. Moreover, the resistance of mouse plasma LDL to undergo lipid peroxidation was significantly ($P < 0.01$) increased in apo E-deficient mice treated with lacidipine. The native LDL-like particle, derived from apo E-deficient mice treated with lacidipine, contained significantly lower concentrations of malonyldialdehyde than the vehicle-treated control group ($P < 0.01$). After exposure to human umbilical vein endothelial cells, LDL-like particle vitamin E levels (expressed as area under the curve; AUC), were significantly higher ($P < 0.01$) in both the 3 and 10 mg/kg lacidipine-treated groups, in comparison with the vehicle-treated control animals. We conclude that lacidipine reduced the extent of the atherosclerotic area in hypercholesterolaemic apo E-deficient mice, and that this reduction may be associated with the capacity of the drug to decrease the susceptibility of LDL to oxidation.

Keywords

antioxidant, apo E-deficient mice, atherosclerosis, dihydropyridine, high-fat diet, lacidipine, oxidized LDL

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Several lines of evidence from both *in vitro* and *in vivo* studies suggest that the oxidation of low-density lipoprotein (LDL) plays an important role in the pathogenesis of atherosclerosis,

although the reasons for this are not fully understood (Avogaro *et al.* 1988; Boyd *et al.* 1989; Steinberg *et al.* 1989; Rosenfeld 1991; Wiklund *et al.* 1991). Oxidatively modified

LDL is taken up by macrophages at an increased rate, in comparison to native LDL, and thus promotes cellular cholesterol accumulation and foam cell formation, which are the hallmarks of early atherosclerotic lesions (Steinberg 1997). Moreover, the oxidation process can induce the expression of adhesion molecules and facilitate transcription factor expression, mechanisms which play an important role in the further development of atherosclerosis (Cominacini *et al.* 1997a). LDL oxidation, occurring inside the artery wall, can be inhibited by many defence systems such as antioxidants in plasma, if these molecules are preserved, and able to carry out such activity (Steinberg *et al.* 1989). Because oxidized LDL cannot be detected in the circulation, considerable research has recently involved the measurement of the susceptibility of isolated LDL particles to *in vitro* oxidation (Esterbauer *et al.* 1987; Cominacini *et al.* 1991a). Antioxidant supplementation has been shown to reduce the progression of atherosclerotic lesions in Watanabe hyperlipidaemic rabbits (Mao *et al.* 1991) and to increase the resistance of LDL to oxidation in both healthy and diabetic subjects (Cominacini *et al.* 1991b; Dieber-Rotheneder *et al.* 1991; Babiy *et al.* 1992).

1,4-dihydropyridine (1,4-DHP) calcium channel blockers (CCBs), although markedly varied in their chemical structures and antihypertensive effects, contain aromatic rings capable of stabilizing oxygen radicals, and a hydrogen-donating reaction may also contribute to their antioxidant activity (Napoli *et al.* 1999). Of the several DHP CCBs, lacidipine has been demonstrated to have antioxidant properties in models of biological membranes by illustrating an activity comparable to the reference antioxidant compound vitamin E (van Amsterdam *et al.* 1992). In addition, lacidipine has been shown to reduce the extent of atherosclerotic lesions in cholesterol-fed hamsters (Cristofori *et al.* 2000a), in apolipoprotein (apo) E-deficient mice that fed on a conventional rodent diet (Cristofori *et al.* 2000b) and in man (Zanchetti *et al.* 2002). Apo E-deficient mice are characterized by a spontaneous and very pronounced hyper-

cholesterolaemia and by several atherosclerotic features which are typical of lesions found in other animal models of atherosclerosis (Nakashima *et al.* 1994; Reddick *et al.* 1994). Furthermore, the LDL of apo E-deficient mice has been demonstrated to be highly susceptible to oxidation (Hayek *et al.* 1994). The apo E-deficient mouse model could therefore prove to be invaluable in assessing the atherogenic relevance of factors involved in the oxidative modification of lipoprotein (Breslow 1993).

The aim of the present study was to further investigate the effect of lacidipine on the development of atherosclerotic lesions in the apo E-deficient mouse challenged with a high fat (Western-type) diet and to evaluate the associated susceptibility of LDL to oxidation under conditions of oxidative stress.

Materials and methods

Animals and animal husbandry

Homozygous, female apo E-deficient mice (GlaxoSmithKline Research and Development, Ware, UK), approximately 6 weeks old at the beginning of the experiments, were used. This colony was established from animals purchased from the Jackson Laboratory, which originated from apo E-deficient mice first engineered at the University of North Carolina in the laboratory of Dr Nobuyo Maeda (Chapel Hill, NC, USA) (Piedrahita *et al.* 1992; Zhang *et al.* 1992). The mice were randomly allocated to three groups of 20 animals each (one vehicle-treated control and two lacidipine-treated groups).

Animals were housed three per cage on wood shavings and fed a Western-type diet (Adjusted Calories Diet, Harlan Tekland TD88137, Madison, WI, USA, containing 42% fat from milk fat and 0.15% cholesterol). Diet and drinking water were available *ad libitum*. Animals were observed daily, and body weights and food intake were determined weekly. The research complied with national legislation and was performed under a Project Licence obtained according to

Table 1 Plasma lipid profile in control (vehicle-treated) apo E-deficient mice and in mice treated with lacidipine at 3 and 10 mg/kg for 8 weeks

Dose level of lacidipine (mg/kg)	Total cholesterol* (mmol/l)	Triglyceride* (mmol/l)	VLDL† cholesterol (mmol/l)	LDL† cholesterol (mmol/l)	HDL† cholesterol (mmol/l)
Control	36.88 ± 0.28	0.87 ± 0.03	10.00 ± 0.07	1.52 ± 0.02	0.22 ± 0.02
3	35.20 ± 0.31	1.23 ± 0.07	11.30 ± 0.05	1.72 ± 0.01	0.25 ± 0.02
10	35.20 ± 0.22	1.18 ± 0.04	12.00 ± 0.03	1.43 ± 0.03	0.33 ± 0.01

*Values are expressed as means (±SEM); *n* = 20 mice in each group.

†VLDL, very low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. The level of total cholesterol in the three main lipoprotein classes (VLDL, LDL and HDL) was quantified from six pooled plasma samples taken from mice in each dose-level group. Each pooled sample was the result of taking plasma from three or four mice.

Italian law (art.7, Legislative Decree number 116, 27 January 1992) which acknowledges European Directive 86/609/EEC. The studies are also in accord with the GlaxoSmithKline policy on the Care and Use of Animals, and also complied with related codes of practice.

Administration of lacidipine

Mice were orally dosed, by gavage, once daily for 8 weeks, with lacidipine (GlaxoSmithKline) at 3 or 10 mg/kg body weight, or vehicle (0.5% methylcellulose; methocel, Sigma Chemical Co., Milan, Italy) at a standard dose volume of 10 ml/kg body weight. In apo E-deficient mice, the 3 mg/kg lacidipine dose level induced plasma drug levels comparable to that achieved with the human therapeutic dose of 4 mg/kg (Zanchetti *et al.* 2002). The 10 mg/kg dose was chosen in this study to maximize any effect of the treatment.

Biochemical measurements

After the 8-week lacidipine treatment period, mice were fasted overnight and sacrificed under pentobarbital anaesthesia (60 mg/kg, i.p.) by withdrawal of blood from the vena cava. Blood was anti-coagulated with 1 mM tripotassium ethylenediaminetetraacetic acid (EDTA) and centrifuged at 1900 g for 10 min at 4 °C. Total plasma cholesterol and triglyceride levels were determined on fresh plasma samples using an automated enzymatic technique (Boehringer Mannheim GmbH, Monza, Milan, Italy). Separation of total lipoprotein was performed by sequential density ultracentrifugation with a TLA-100.2 rotor (Optima TL Ultracentrifuge, Beckman Instruments, Palo Alto, CA, USA) in NaBr solution at a final density of 1.019, 1.063 and 1.210 for very low-density lipoprotein (VLDL), LDL and high-density lipoprotein (HDL), respectively, with a total centrifugation time of 5 h at 16 °C (Cristofori *et al.* 2000b).

LDL-like particles ($d = 1.006\text{--}1.063$) were isolated from pooled plasma by sequential-density ultracentrifugation, as previously described (Hayek *et al.* 1994). In particular, for each dose-level group, six pooled samples of 2.5 ml each were used. Each pooled sample was the result of mixing the plasma from three or four mice. To minimize LDL oxidation during the isolation process, all solutions were deoxygenated by bubbling with argon during the isolation. LDL was stored in the dark under nitrogen at 4 °C, under sterile conditions, and used within 3 days. Immediately before the oxidation incubations, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25 M gel (Pharmacia, Uppsala, Sweden) in 0.01 mol/l phosphate-buffered saline at pH 7.4.

LDLs derived from apo E-deficient mice were oxidized either with copper ions or with human umbilical vein endothelial cells (HUVECs). The method for LDL oxidation with Cu^{2+} and for the evaluation of LDL susceptibility to oxidation (i.e. evaluation of the length of time of the lag phase) was based on development of fluorescence during copper-catalysed LDL oxidative modification, as previously described (Cominacini *et al.* 1991a). The hydroperoxide content of native LDL was determined by evaluating the level of malonyldialdehyde (MDA), as reported by Carbonneau *et al.* (1991). The extent of LDL oxidation was determined as the thiobarbituric acid adduct, using the high-performance liquid chromatography (HPLC) method of Carbonneau *et al.* (1991). Analytical separations were performed with a Hewlett-Packard 1050 HPLC connected to a reverse phase C18, 15×0.46 cm column (Bio-Rad, Hercules, California, USA); the system included a guard column (Microguard System, Bio-Rad) with a Hewlett-Packard spectrophotometric UV-VIS detector at 532 nm. An MDA standard was prepared by dissolving 220.3 mg of 1,1,3,3-tetraethoxypropane (Aldrich-Chemie, Steinheim, Germany) in 100 ml of deionized water to give a 10 mmol/l stock solution. Measurement of protein was carried out using the Pierce BCA protein assay reagent (Smith *et al.* 1985).

HUVECs were isolated from human umbilical veins as described by Cominacini *et al.* (1996) and used at passage 2–4. LDL oxidation was carried out by adding 1.5 ml of serum-free F-12 medium (Gibco, Life Technology, Rockville, MD, USA) containing 200 µg/ml protein to each well of HUVECs and incubating for 24 h at 37 °C as previously described (Cominacini *et al.* 1996). Vitamin E was measured by HPLC with fluorescence detection as previously described (Cominacini *et al.* 1991a).

Tissue preparation and quantification of atherosclerotic lesions

At the end of the 8-week experimental period, blood was collected and the animals were perfused with 10% buffered formalin for 10 min. The hearts were rapidly dissected and sectioned according to the method of Tangirala *et al.* (1995a). Using this technique, the heart was sectioned transversely just below the level of the atria, postfixed in 10% buffered formalin overnight and embedded in paraffin. Sequential 7-µm sections were cut from the apex towards the base of the heart until the aortic valve leaflets appeared. From this point, 17 sections of the aortic root for each animal, representing every second serial section, over a distance of about 240 µm, were taken and stained with haematoxylin and eosin (H&E). The extent of the atherosclerotic lesions were measured using a computerized image analysis system

made up of the image analysis software and board, as described previously (Cristofori *et al.* 2000b), and a black and white video camera (Jai; 711.00-CV) mounted on a Leitz Diaplan microscope (original magnification, $\times 40$). Results are expressed as the mean (in μm^2) of the data (\pm SEM) from the 17 sections analysed for each animal. All assessments were carried out by the same operator in a 'blind' fashion.

Statistical analysis

Results are expressed as the mean (\pm SEM.) ANOVA one-way analysis following Dunnett's test was used to compare the differences between the lacidipine groups and the vehicle-treated (control) group, and $P < 0.05$ was considered significant.

Results

During the 8-week period of lacidipine treatment at 3 and 10 mg/kg, the drug was well tolerated at both dose levels; there were no clinical signs in the animals attributable to the compound. Body weight increase in apo E-deficient vehicle control mice was the same as in the lacidipine-treated groups. The administration of lacidipine did not affect either the plasma total cholesterol triglyceride levels, or the cholesterol distribution in the different lipoprotein fractions (Table 1). Mean levels of total cholesterol were 36.88 ± 0.28 mmol/l in the apo E-deficient control group, and 35.20 ± 0.31 mmol/l and 35.20 ± 0.22 mmol/l in apo E-deficient mice treated with lacidipine at 3 and 10.0 mg/kg/day, respectively.

Quantification of atherosclerotic lesions

In control apo E-deficient mice, severe atherosclerosis developed during the 8-week period of the study and covered almost the entire aortic surface at the level of the aortic sinus (Figure 1). When the extent of the atherosclerosis was quantified at the aortic root of the heart, a significant dose-related reduction in aortic lesion development was found in mice that were given lacidipine at 3 and 10 mg/kg ($P < 0.01$ at both dose levels). The average lesion area was $305.5 \pm 8.8 \times 10^3 \mu\text{m}^2$ in the apo E-deficient control group (Figure 2), $173.7 \pm 6.7 \times 10^3 \mu\text{m}^2$ in animals treated at 3 mg/kg lacidipine and $152.6 \pm 7.6 \times 10^3 \mu\text{m}^2$ in the 10 mg/kg lacidipine group (reductions of 43.1% and 50.1% of the control lesion area, respectively). All the results were expressed as mean \pm SEM. Histological assessment of the aortic arch demonstrated advanced atherosclerotic disease with plaques showing foamy macrophages, calcification and fibrous caps, a morphology which closely resembles human atherosclerosis. No significant differences in the histological appearance were noted.

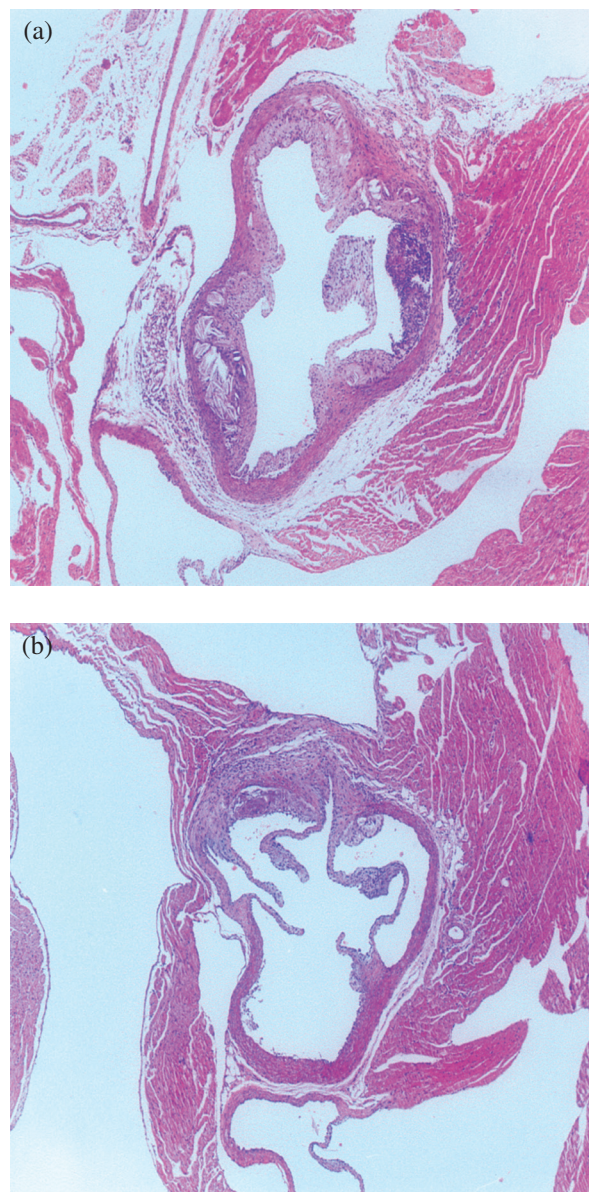


Figure 1 Atherosclerotic lesions in sections of the aortic root of the heart in a control apo E-deficient mouse (a), and in an apo E-deficient mouse receiving 3 mg/kg lacidipine (b) (haematoxylin and eosin-stained; original magnification, $\times 40$). In the control mouse (a) extensive, advanced lesions with a well-defined fibrous cap and a necrotic core with nodular deposits of calcification are present. The volume of the lesions in apo E-deficient mouse treated with lacidipine at 3 mg/kg (b) appears markedly reduced, and the lesion is less complex.

Susceptibility of mouse LDL to undergo lipid peroxidation

After 8-week treatment with lacidipine at 3 and 10 mg/kg, the susceptibility of mouse LDL to undergo lipid peroxidation following incubation with CuSO_4 was significantly reduced

Figure 2 Effect of lacidipine at 0 (control), 3 and 10 mg/kg on atherosclerotic lesions measured in the aortic root of apo E-deficient mice after 8-week treatment. Results are expressed as mean \pm SEM of the lesion area ($\times 10^3 \mu\text{m}^2$) measured in 17 cross-sections through the aortic root of each mouse, over a distance of 240 μm $n = 20$ in each group; ** $P < 0.01$, in lacidipine-treated animals compared with the controls.

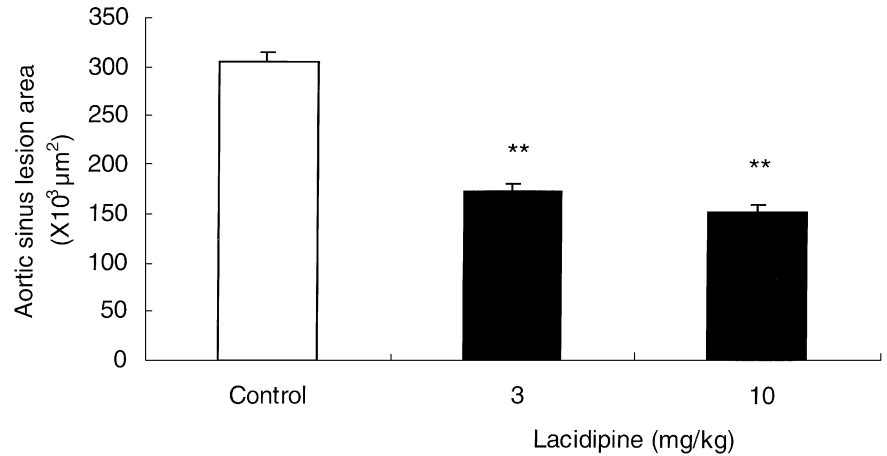


Figure 3 The susceptibility of the mouse low-density lipoprotein (LDL) to undergo lipid peroxidation following incubation with CuSO_4 . LDL-like particles were derived from plasma of apo E-deficient mice after 8-week treatment with vehicle (control) or lacidipine at 3 or 10 mg/kg. Results are expressed as mean \pm SEM of the lag phase (min); $n = 6$ pooled samples in each dose-level group; * $P < 0.05$ in comparison with the 3 mg/kg lacidipine group; ** $P < 0.01$ in comparison with the control group.

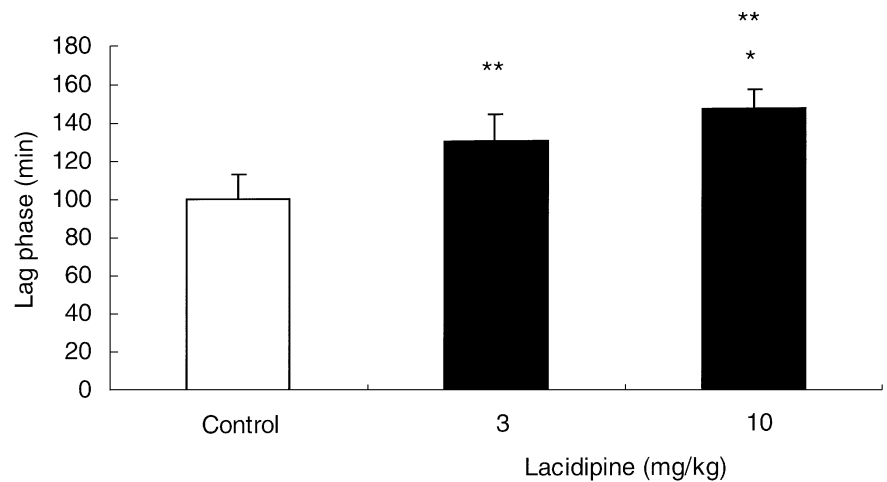
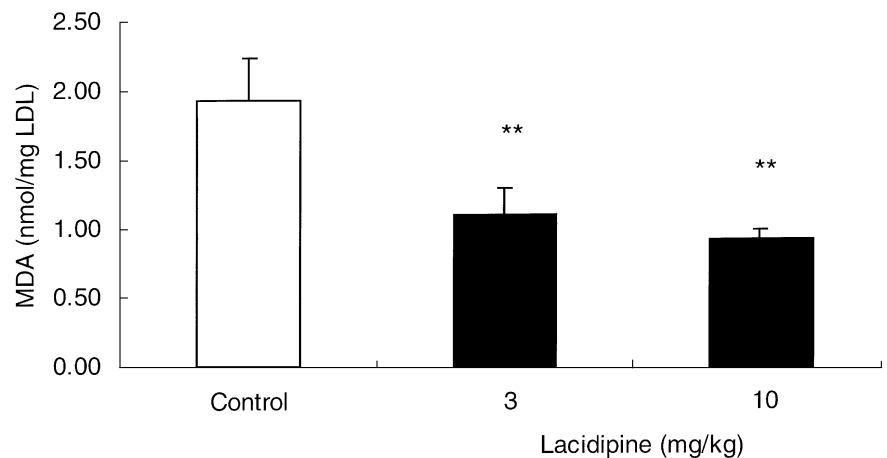


Figure 4 Effect of lacidipine on the hydroperoxide content of native low-density lipoprotein (LDL). Malondialdehyde (MDA) content in LDL-like particles derived from apoE-deficient mice after 8-week treatment with vehicle (control) or lacidipine at 3 or 10 mg/kg. The results are expressed as mean \pm SEM; $n = 6$ pooled samples in each dose-level group; ** $P < 0.01$ in comparison with the control group.



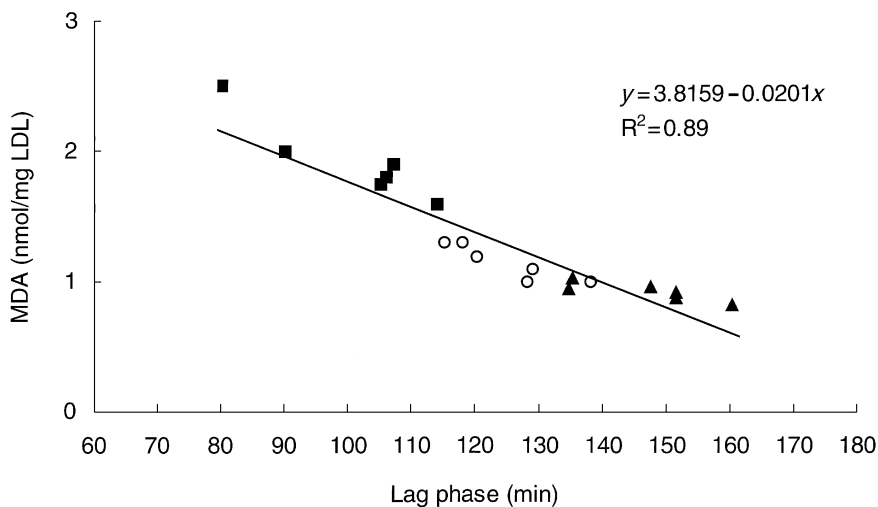


Figure 5 Relationship between the hydroperoxide content of native low-density lipoprotein (LDL) [measured as malonyldialdehyde (MDA) in LDL-like particles] and the resistance to oxidation of LDL-like particles (expressed as lag phase in minutes) from the plasma of individual apoE-deficient mice treated with vehicle (control; solid squares) or lacidipine at 3 mg/kg (open circles) or 10 mg/kg (solid triangles). $n = 6$ pooled samples in each dose-level group.

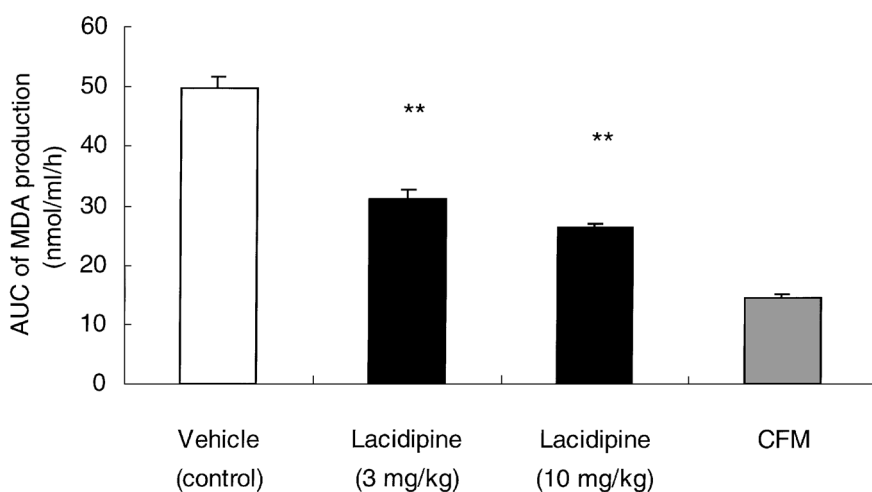


Figure 6 HUVEC-mediated oxidation of low-density lipoprotein (LDL)-like particles, expressed as the area under the curve (AUC, in nmol/ml/h; mean \pm SEM) of malonyldialdehyde (MDA) production during a 24-h incubation period. LDL-like particles were derived from the plasma of apo E-deficient mice after 8-week treatment with vehicle, or lacidipine at 3 and 10 mg/kg. A dose-related reduction in AUC values was observed at both dose levels of lacidipine. $**P < 0.01$ in comparison with the cell-free medium (CFM) and the vehicle control; $n = 6$ for vehicle control; $n = 11$ for lacidipine at 3 mg/kg; $n = 6$ for lacidipine at 10 mg/kg and $n = 8$ for CFM.

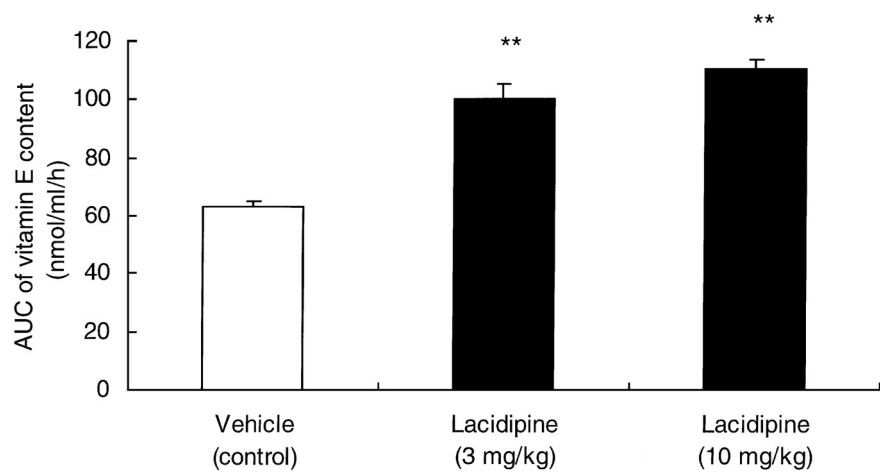
($P < 0.01$) in comparison with the control group. In the vehicle-treated control group, the lag time required for the initiation of CuSO_4 -induced LDL oxidation was 100.3 ± 12.7 min (Figure 3). In contrast, in the two lacidipine-treated groups, the time required for LDL oxidation was increased, being 125.8 ± 15.2 min with lacidipine at 3 mg/kg and 146.8 ± 10.0 min at 10 mg/kg. This result paralleled the significantly ($P < 0.01$) lower concentrations of hydroperoxide content (MDA), detected in the LDL-like particles, derived from both lacidipine-treated groups, compared with the vehicle-treated controls (Figure 4). The mean MDA values, measured in vehicle and lacidipine-treated groups at 3 and 10 mg/kg, were 1.93 ± 0.31 , 1.21 ± 0.30 and 0.93 ± 0.07 nmol/mg LDL, respectively. By plotting the lag-phase values detected in individual pooled samples from apo E-deficient mice treated with lacidipine or vehicle with the corresponding MDA values

(Figure 5), a significant relationship was identified ($R^2 = 0.89$; $P < 0.01$).

HUVEC-mediated oxidation of LDL-like particles

Changes in MDA production and vitamin E content of LDL-like particles derived from apo E-deficient mice were also monitored and quantified during a 24-h incubation period with HUVECs. The amount of MDA formation in the cell-free medium (CFM) over the 24-h period was less than 1 nmol/ml. However, with HUVECs incubated with LDL-like particles from apo E-deficient mice treated with vehicle, a significant increase in MDA production was detected ($P < 0.01$). The formation of MDA was significantly ($P < 0.01$) reduced by lacidipine treatment, because lower quantities of MDA were detected from the fourth to the 24th hour of the incubation period. Expressing

Figure 7 Vitamin E content, expressed as the area under the curve (AUC, in nmol/ml/h; mean \pm SEM) during a 24-h incubation period with human umbilical vein endothelial cells (HUVECs). Low-density lipoprotein (LDL)-like particles were derived from the plasma of apo E-deficient mice after 8-week treatment with vehicle or lacidipine at 3 or 10 mg/kg. Dose-related increases in the AUC values were observed at both dose levels of lacidipine in comparison with the vehicle-treated control group; ** $P < 0.01$; $n = 6$ for vehicle control; $n = 11$ for lacidipine at 3 mg/kg and $n = 6$ for lacidipine at 10 mg/kg.



these results as area under the curve (AUC), calculated by using the mean values of MDA production at various time intervals, during the 24-h incubation, a significant ($P < 0.01$) dose-related reduction in the lacidipine-treated mice was detected (Figure 6).

Vitamin E content of LDL-like particles

No difference was detected in the basal LDL-like particle concentrations of vitamin E between apo E-deficient mice treated with vehicle or lacidipine (5.43 ± 0.09 $\mu\text{g}/\text{mg}$ LDL in control mice *vs.* 5.85 ± 0.29 and 5.86 ± 0.16 $\mu\text{g}/\text{mg}$ LDL protein in the 3 and 10 mg/kg lacidipine-treated groups, respectively). All the results were expressed as mean \pm SEM. However, after 24-h exposure to HUVECs, a significant decrease in the LDL-like particle concentration of vitamin E in the vehicle (control) group was observed as, at the end of the incubation period, a value below 15 nmol/ml/h was measured. Nevertheless, in apo E-deficient animals treated with lacidipine at 3 and 10 mg/kg, levels of vitamin E were maintained with concentrations significantly ($P < 0.01$) higher in both lacidipine-treated groups in comparison with the vehicle-treated control mice. Expressing the data as AUC, calculated as the mean values of vitamin E content, at specific time points during the 24-h incubation period, a significant ($P < 0.01$) and dose-related preservation of vitamin E levels in the lacidipine-treated mice was demonstrated (Figure 7).

Discussion

The present study demonstrates that lacidipine treatment significantly reduced the progression of aortic lesions in apo E-deficient mice that fed on a high-fat diet and that this effect was paralleled by a marked decrease in the propensity for the

oxidation of plasma LDL, induced by different forms of oxidative stress. However, the anti-atherosclerotic activity of lacidipine in apo E-deficient mice was not caused by a reduction in plasma cholesterol levels. Therefore, the present results tend to reinforce the concept that mode of action of lacidipine on the atherosclerotic processes may be related to the known antioxidant properties of the compound (van Amsterdam *et al.* 1992). There is now growing evidence of a relationship between the susceptibility of LDL to oxidation and atherosclerotic risk (Regnstrom *et al.* 1992; Cominacini *et al.* 1993, 1994a, 1994b; Sasahara *et al.* 1994; Cominacini *et al.* 1997b; Sobal *et al.* 2001). Oxidatively modified LDL is taken up by macrophages at an increased rate, in comparison to native LDL, and this process can therefore promote cellular cholesterol accumulation and foam cell formation, which are the hallmarks of early atherosclerotic lesions (Steinberg 1997).

Several studies have demonstrated the important role of the inhibition of LDL oxidation in reducing the atherosclerotic process. Angiotensin-converting enzyme (ACE) inhibiting drugs such as captopril (Hayek *et al.* 1998) and fosinopril (Hayek *et al.* 1999) have been reported to inhibit LDL oxidation and the atherosclerotic process in apo E-deficient mice. Other potent antioxidative agents, such as vitamin E, are also reported to attenuate atherosclerosis in this animal model (Maor *et al.* 1997). Similarly, Tangirala *et al.* (1995b) also demonstrated anti-atherogenic effects in apo E-deficient mice using the antioxidant N,N'-diphenyl-1,4-phenylenediamine (DPPD).

Lacidipine belongs to the class of 1,4-DHP drugs, and an analysis of structural-functional relationships on the oxidative modification of LDL suggests an important role for the 2-substitution of the phenyl ring (Napoli *et al.* 1999). In addition, calcium antagonists of the DHP type are highly lipophilic and therefore their antioxidative effects could also

be explained by hypothesizing a direct interaction with plasma lipoproteins (Rojstaczer & Triggle 1996).

Vitamin E is the major lipid-soluble antioxidant present in blood. It acts synergistically with other circulating and cellular antioxidants to protect cells from damage and lysis induced by oxidative stress. Most of the vitamin E in blood plasma is present in the LDL fraction, and hence vitamin E is optimally placed to prevent free-radical-mediated modification of this lipoprotein. In the present study, the amount of vitamin E in apo E-deficient mice treated with lacidipine, and in vehicle-treated control groups, was measured. Significant differences in the basal LDL-like particle concentrations of vitamin E were found between the apo E-deficient mice treated with lacidipine and the vehicle-treated control group. The levels of vitamin E were maintained in animals treated with lacidipine at both the 3 and 10 mg/kg dose levels, because the vitamin E concentrations were significantly ($P < 0.01$) higher in both lacidipine-treated groups in comparison with the controls. This could mean that lacidipine may have reduced the progression of aortic atherosclerosis by lengthening the LDL-lag phase and working as a radical scavenger. This concept is consistent with the classic kinetic model proposed by Niki for lipid peroxidation (Niki 1987), where the length of the lag phase is directly related to the amount of antioxidant contained in the lipoprotein. Another possibility in explaining the effect of lacidipine on LDL-lag phases in apo E-deficient mice derives from the scheme proposed by Esterbauer *et al.* (1989) and by Yoshida & Niki (1992). According to these authors, the role of the copper in the oxidative process of LDL is to catalyse the conversion of trace amounts of preformed lipid hydroperoxides to alkoxy and peroxy radicals, which in turn start another lipid peroxidation reaction. In our study, hydroperoxides were reduced in the LDL-like particles of apo E-deficient mice treated with lacidipine, in comparison with the vehicle-treated control group, and there was a direct correlation between the hydroperoxide concentrations in LDL and the length of lag phase. Therefore, lacidipine may have lengthened the LDL-lag phase also by reducing the LDL content of the lipid hydroperoxides.

In the present study, the antioxidative properties of lacidipine were also assessed in the *in vitro* model of LDL oxidation obtained by incubation with HUVECs, and interesting results were obtained. It is well known that the process of LDL modification induced by such endothelial cells *in vitro* closely resembles the corresponding process *in vivo*. Although cell-mediated oxidative modification of LDL has been the subject of several studies in recent years, the mechanism by which cells initiate LDL oxidation still remains unclear. Both extracellular superoxide radicals (Cathcart *et al.* 1989) and lipoxygenase activity (Cathcart *et al.* 1991; Chamulitrat *et al.* 1991) have

been proposed. Attention has also focused on the reaction of superoxide with nitric oxide (Bruckdorfer 1993). Nitric oxide can react with superoxide to form the peroxynitrite anion. Decomposition of the peroxynitrite anion generates a strong oxidant with reactivity similar to the hydroxyl radical, which has been shown to initiate lipid peroxidation (Bruckdorfer 1993). Whichever mechanism of oxidation may be involved, this led to the initial hypothesis that the action of modifying cells is to accelerate the formation of lipid peroxides within the LDL particle. In the present study, the reduction in the content of hydroperoxides found in LDL-like particles of apo E-deficient mice treated with lacidipine may therefore also be the result of the action of the drug on endothelial cells. This conclusion is consistent with the demonstrated inhibitory effect of lacidipine on the generation of reactive oxygen species induced by pro-oxidant stimuli in endothelial cells (Cominacini *et al.* 1998). The results of the present study also show that LDL vitamin E consumption during HUVEC oxidation was reduced in apo E-deficient mice treated with lacidipine, in comparison with the vehicle-treated control group. Although the mechanism underlying this effect of lacidipine on vitamin E is not clear, one hypothesis was proposed by Thomas *et al.* (1995) which was that lacidipine acts as co-antioxidant.

In conclusion, in the present study, it has been demonstrated that lacidipine reduces the extent of the atherosclerotic lesion in the hypercholesterolaemic apo E-deficient mouse with a decrease in the susceptibility of LDL to oxidation. These effects of lacidipine may be related to the reducing property of the molecule, as well as the capacity of the drug to interfere in the cellular production of peroxidative products.

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