British Journal of Pharmacology (1996) 118, 215-219

Effect of lacidipine on fatty and proliferative lesions induced in hypercholesterolaemic rabbits

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1 The *in vivo* antiatherogenic activity of the calcium antagonist, lacidipine, was investigated in two different types of atherosclerotic lesions (proliferative and fatty lesions) induced in rabbits.

2 The proliferative lesion was obtained by positioning a hollow silastic collar around one carotid artery, while aortic fatty lesions were induced by cholesterol feeding. Cholesterol (1%) and lacidipine (1, 3, and 10 mg kg^{-1}) were given daily mixed with standard diet for 8 weeks to White New Zealand rabbits. The intimal hyperplasia (proliferative lesion) was induced 6 weeks after dietary and drug treatment started.

3 The neointimal formation was determined by measuring cross sectional thickness of intimal (I) and medial (M) tissue of fixed arteries. In untreated animals ($n=5$), 14 days after collar positioning an intimal hyperplasia was clearly detectable: the arteries with no collar (sham) showed an I/M tissue ratio of 0.03 ± 0.02, whereas in the carotid with collar the ratio was 0.62 ± 0.12. In lacidipine-treated animals a significant and dose-dependent effect on proliferative lesions at all three doses tested, was observed. I/M ratios were 0.47 ± 0.02, 0.40 ± 0.09, 0.32 ± 0.02 for doses 1, 3, and 10 mg kg^{-1} day^{-1}, respectively ($P<0.05$).

4 The fatty lesion extent was significantly reduced by lacidipine at the 10 mg kg^{-1} day^{-1} dose, although a trend was also observed with lower dosage.

5 These results suggest a direct antiatherosclerotic effect of lacidipine, independent of modulation of risk factors such as hypercholesterolaemia and/or hypertension. Furthermore, the proliferative lesions are apparently more sensitive to lacidipine than are lipid-rich lesions.

Keywords: Calcium antagonists; lacidipine, atherosclerosis; animal model; smooth muscle cell proliferation; proliferative lesion; fatty lesion

Introduction

With increasing knowledge on the pathogenesis of atherosclerosis it appears that prevention of cardiovascular disease will involve not only the correction of risk factors such as dyslipidaemia or elevated arterial blood pressure but also the direct pharmacological control of atherogenic processes occurring in the arterial wall (Ross, 1993). While the former approach is now definitely accepted in man, the latter still represents a 'therapeutic hope' which requires experimental and clinical confirmation. Recently, a great effort has been made in evaluating the direct effect of drug therapy on the arterial wall (Jackson & Schwartz, 1992). Abnormalities or dysfunctions of the vessel wall can favour vascular smooth muscle cell migration from the media and proliferation within the intima, and increase lipid deposition or reduce lipid clearance from infiltrating monocytes. These processes trigger a cascade of events leading to the development of vascular disease (Popma *et al.*, 1991; Jackson & Schwartz, 1992; Ross, 1993).

Therapeutic interventions which interfere with early stages of atherosclerosis may improve chances halting and slowing the progression of the disease. Among drugs currently available for therapy of vascular disease, calcium antagonists have been investigated extensively as antiatherogenic agents in a variety of *in vitro* and *in vivo* experimental models (Lichtor *et al.*, 1989; Bernini *et al.*, 1989; 1993a; Henry, 1990; Jackson & Schwartz, 1992). In *in vivo* models, calcium antagonists protect against lesions induced by cholesterol feeding, endothelial injury, and experimental calcinosis (Catapano *et al.*, 1988; Bernini *et al.*, 1989; Weinstein & Heider, 1989; Keogh & Schroeder, 1990; Kunjara-Na-Ayudhya *et al.*, 1994; Soma *et al.*, 1995b). The *in vitro* effects of these drugs on processes which play a role in the development of atherosclerotic lesions might help in explaining these results. For instance, several calcium antagonists inhibit the migration of smooth muscle cells (SMC) (Nomoto *et al.*, 1988), the uptake of lipids by macrophages (Daugherty *et al.*, 1987; Stein & Stein, 1987; Schmitz *et al.*, 1988; Bernini *et al.*, 1991), and the production of collagen, elastin, and proteoglycans (Waters *et al.*, 1990). However, few attempts have been made to relate these *in vitro* effects to the complex changes which occur in the vessel wall. Recently, a model of carotid neointimal hyperplasia, mainly dependent on SMC migration and proliferation, has been developed (Soma *et al.*, 1993; 1995a). Using this model in cholesterol-fed rabbits we have investigated the *in vivo* effect of lacidipine, a second generation dihydropiridine, on two different vascular lesions: one mainly characterized by migration and proliferation of SMC (proliferative lesion), induced by collar insertion around a carotid artery, and the other by lipid deposition in arterial macrophages (aortic fatty lesion), induced by a cholesterol-rich diet.

Methods

New Zealand male rabbits ($n=100$) (2-2.5 kg, Charles River, Calco, Italy) were used in this study. Animals were divided into the following groups and maintained in identical experimental conditions: (1) Control group ($n=25$): cholesterol-rich diet; (2) low dose lacidipine-treated group ($n=25$): cholesterol-rich diet + lacidipine 1 mg kg^{-1} day^{-1}; (3) intermediate dose lacidipine-treated group ($n=25$): cholesterol-rich diet + lacidipine

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3 mg kg^{-1} day^{-1}; (4) high dose lacidipine-treated group ($n=25$): cholesterol-rich diet + lacidipine 10 mg kg^{-1} day^{-1}.

All animals received 1% cholesterol-rich diet daily and the drug-treated animals received lacidipine mixed with food pellets. The daily doses of cholesterol as well as lacidipine were given in the morning (at 08 h 00 min) each mixed in 20 g of food pellets. Normal chow, up to 150 g, was added after all medicated diet was eaten (usually within 30 min). Records of food intake did not show any significant difference among the groups or within the groups. High performance liquid chromatography (h.l.p.c.) analysis confirmed drug stability in the diet up to 3 months (Ratti *et al.*, personal communication). The doses used here did not affect arterial blood pressure. Animals had free access to water and were kept in a 12 h light-dark cycle. Blood was drawn from the central ear artery at days 0, 29 and 56 after treatment started. Total serum cholesterol was measured by an enzymatic procedure (Catapano *et al.*, 1988), HDL cholesterol was determined by the same method after VLDL + LDL precipitation with phosphotungstic acid (Soma *et al.*, 1995b). During treatment body weight did not differ significantly between the groups throughout the study. Of the animals included in the study, 3 died (1 in the cholesterol-fed group, 1 in the lacidipine 1 mg kg^{-1} day^{-1}, and 1 in the lacidipine 10 mg kg^{-1} day^{-1}). Autopsy was performed in two cases: no evidence for presence of liver and/or intestinal involvement was detached. At the end of the treatment the animals were killed by an overdose of sodium pentobarbitone (65 mg kg^{-1}, i.v.).

Fatty lesions

Aortae were immediately retrieved, cleaned from blood and adherent tissue, and fixed in buffered formaldehyde (10%) for 24 h at 4°C. Aortic lipids were stained with Sudan IV as previously described (Catapano *et al.*, 1988). The extension of aortic atherosclerotic plaques, determined as Sudan stainable areas, was measured by planimetry by two independent operators and expressed as a percentage of the aortic inner surface covered by plaques: data are the means of two evaluations; coefficient of variation was less than 5%. For the determination of aortic cholesterol, the aortae were carefully minced and lyophilized. Lipids were extracted with chloroform-methanol as described and total cholesterol determined with commonly available enzymatic kits.

Proliferative lesions

For the induction of proliferative lesions, intimal hyperplasia was mechanically induced in one carotid artery of five rabbits from each group, six weeks after dietary and drug treatment started. Rabbits were anaesthetized by intramuscular injection of 5 mg kg^{-1} xylazine and 35 mg kg^{-1} ketamine. Animals were then placed in dorsal recumbency. A midline neck incision was made and both carotid arteries were surgically exposed. A non-occlusive, biologically inert, soft, and hollow Silastic collar was positioned around both carotids. The collar was 1.5 cm in length and it touched the artery circumference at two points 1 cm apart. In the sham arteries the collar was removed just before carotids were repositioned and the wounds were sutured. Animals were killed 2 weeks after the collar placement. All animals were given a lethal dose of sodium pentobarbitone, and the vasculature was perfused with 0.1 mol l^{-1} phosphate-buffered-saline (PBS) for 10 min. Animals were then perfusion-fixed for another 20 min with 3% glutaraldehyde buffered with 0.1 mol l^{-1} PBS at a pressure of 100 mmHg (Soma *et al.*, 1993, 1995a). Both carotid arteries were removed, paraffin embedded and stained with hematoxylin/eosin. At least 600 cross sections (5 μm) were cut for each artery. Neointimal formation was measured by light microscopy and expressed as the ratio between the cross-sectional thickness of intimal and medial tissue. Intimal/Medial (I/M) ratios were calculated to normalize the data. The value of I/M obtained from each animal is the mean of at least 120 measurements performed on at least 60 sections (Soma *et al.*, 1993; 1995a).

Statistics

Group data are expressed as means ± s.d. Statistical comparison of all numerical data between groups was performed by ANOVA. A P value <0.05 was taken as statistically significant. Where statistically significant differences were established between groups, further comparison for homogeneity means was made by using a Post Hoc Duncan's multiple range test. All statistical calculations were made with CSS Software (Statsoft, U.S.A.).

Results

There were no significant differences in the baseline body weight and serum cholesterol values between the groups of animals examined. Serum cholesterol values increased with cholesterol feeding in a time-dependent manner. Lacidipine had no significant effect on serum cholesterol levels with the possible exception of the high dose group (Table 1 at day 56). However, plasma cholesterol levels of the latter group were higher at 4 and 6 weeks, thus the overall exposure to cholesterol (calculated as the area under the curve) did not differ among the cholesterol-fed groups (Figure 1).

Effect of lacidipine on fatty lesions

The area of the aorta covered by plaques was analysed by computer-assisted analysis. The data are given in Table 2. Upon cholesterol feeding the area of the aorta covered by plaques was 30 ± 18.7%. Lacidipine at a dose of 1 or 3 mg kg^{-1} day^{-1} did not reduce the area of the aorta covered by plaque (28.6 ± 19.8 and 29.3 ± 14.0, respectively, NS). At a dose of 10 mg kg^{-1} day^{-1}, however, lacidipine effectively reduced the lesion area by 40% to 18.5 ± 8.9 ($P<0.01$). When aortic cholesterol was determined, a significant decrease was also detected in samples from animals treated with the

Table 1 Plasma cholesterol (Chol), triglycerides (TG), and HDL cholesterol (HDL) in the experimental groups (mmol l^{-1})

	Day 0			*Day 29*		*Day 56*	
	Chol	*HDL*	*TG*	*Chol*	*Chol*	*HDL*	*TG*
HC	1.95 ± 0.4	0.9 ± 0.1	1.0 ± 0.4	13.31 ± 6.5	45.6 ± 15.4	4.6 ± 5.7	0.8 ± 0.4
Lacidipine 1 mg kg^{-1} day^{-1}	1.92 ± 0.6	0.9 ± 0.2	1.0 ± 0.4	17.5 ± 8.5	46.9 ± 19.9	6.2 ± 6.9	1.0 ± 0.9
Lacidipine 3 mg $l kg^{-1}$ day^{-1}	2.35 ± 0.4	0.9 ± 0.2	1.4 ± 0.8	19.5 ± 7.0	49.3 ± 21.5	7.9 ± 6.7	0.9 ± 0.6
Lacidipine 10 mg kg^{-1} day^{-1}	1.99 ± 0.6	0.9 ± 0.3	1.0 ± 0.6	23.0 ± 12.5*	37.3 ± 14.2*	7.8 ± 6.8	0.5 ± 0.4

Values are mean ± s.d. *$P<0.05$ vs HC.

10 mg kg^{-1} day^{-1} dosage but only a trend to reduction was observed with the lower dosage.

Effect of lacidipine on proliferative lesions

The effectiveness of the periarterial insertion of the collar in inducing intimal carotid thickening was assessed in 5 animals in each group. In the control group a marked increase in intimal thickness was evident in the carotids with collar (Figure 2a), whereas the sham-operated arteries did not show thickening of the intima either in positive control or drug-treated rabbits (Figure 2b). The mean value of the I/M ratio in the collar arteries of the control group ($n=5$) was twenty fold greater (0.62 ± 0.12) than in the sham operated group ($n=20$) (0.03 ± 0.02). Neointimal formation in hypercholesterolaemic rabbits was thus greater than that observed in normocholesterolaemic animals (Soma *et al.*, 1993), suggesting a detrimental effect of hypercholesterolaemia on this type of lesion. The intimal thickening was mostly cellular but abundant extracellular matrix and lipid deposition were also present. Light microscopic observation of the sham tissue confirmed the absence of intimal thickening or lipid deposits (Figure 2b). Figure

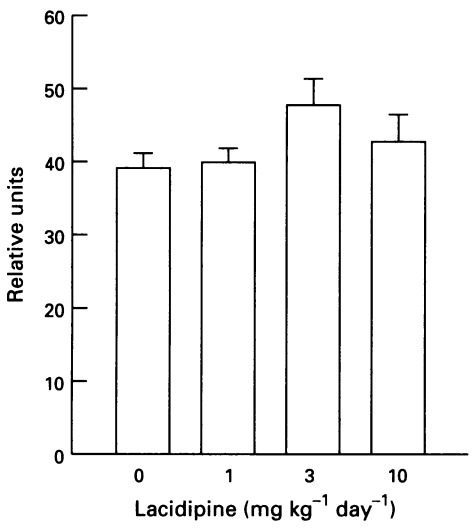

Figure 1 Cumulative exposure to cholesterol of rabbits from different treatment group throughout the study. Plasma cholesterol from each animal was determined before, at 4 wks, 6 wks, and 8 wks after cholesterol-diet and drug-treatment started. The area under the curves were calculated for each animal (relative units) from each group. Rabbits received either cholesterol-rich diet alone (HC) or cholesterol-rich diet plus lacidipine at the doses of 1 mg kg^{-1} day^{-1}, 3 mg kg^{-1} day^{-1}, 10 mg kg^{-1} day^{-1} respectively. Cholesterol and lacidipine were administered for 8 wks mixed with food.

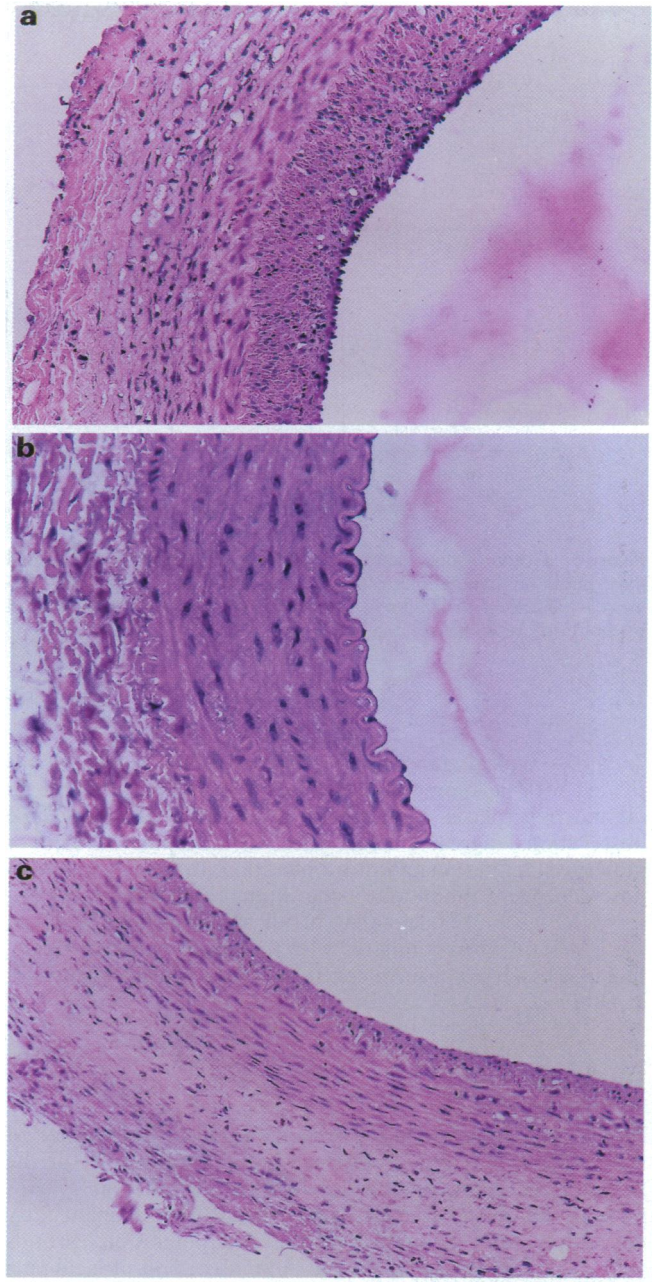

Figure 2 Microscopic views of transverse sections (5 μm thick) of carotid arteries in hypercholesterolaemic rabbits 15 days after collar placement. (a) Collar carotid artery from a control rabbit showing a marked neointimal hyperplasia (b). Sham-operated artery without neointimal thickening. (c) Collar carotid artery from a lacidipine-treated rabbit (high dose) showing fewer cellular layers. Magnification in (a) and (c) = × 100; in (b) = × 200.

Table 2 Percentage of aortic surface covered by plaques total and esterified cholesterol in the different experimental groups

	Aortic area covered by plaques	*Total cholesterol (mg g^{-1} dry tissue) (n = 10)*
Hypercholesterolaemic (24)	30.0 ± 18.7	9.7 ± 1.2
Lacidipine (24) day^{-1} 1 mg kg^{-1}	28.6 ± 19.8	8.3 ± 0.9
Lacidipine (25) 3 mg kg day^{-1}	29.3 ± 14.0	7.9 ± 1.3
Lacidipine (24) 10 mg kg day^{-1}	18.5 ± 8.9*	3.1 ± 1.0*

Values are mean ± s.d. *$P<0.01$ vs hypercholesterolaemic.
Numbers in parentheses represent the animals in each group.

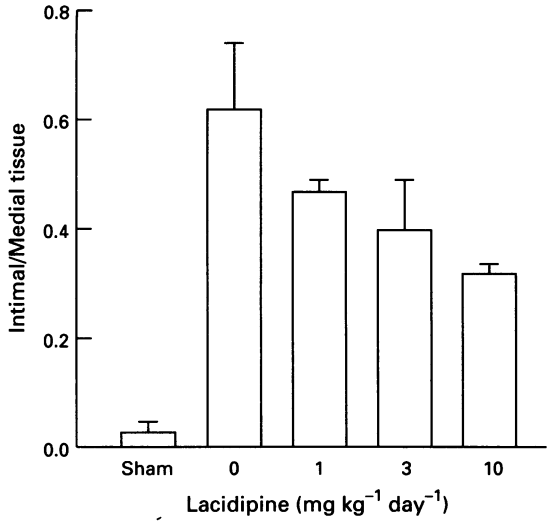

Figure 3 Graph of dose-dependent effect of lacidipine on neointimal hyperplasia in hypercholesterolaemic rabbits. Diet and drug treatment is described for Figure 1. Neointimal hyperplasia was induced by position the collar for the last 2 weeks.

2c shows typical transverse sections obtained from animals treated with lacidipine (higher dose). The inhibitory effect on intimal hyperplasia consisted of fewer layers of cells. These observations suggest a direct effect of lacidipine on smooth muscle cell migration and/or proliferation. In treated animals a dose-dependent inhibition of the intimal hyperplasia was observed (Figure 3). Lacipidine, at all concentrations, reduced the I/M ratio when compared with the positive-control-group. Differences versus positive control were statistically significant at all doses.

Discussion

In the present investigation we quantitated the influence of lacidipine, a new dihydropiridine calcium antagonist of the second generation, on the development of different atherosclerotic lesions, characterized by either a predominant fatty deposition (aorta) or smooth muscle cell proliferation (carotids with collar), in the same animal. Administration of lacidipine resulted in a profound and differential response to these lesions. The most dramatic results were obtained in the proliferative lesions where a dose-dependent inhibitory effect on neointimal hyperplasia was observed. Fatty lesions were also affected but only at high doses of lacidipine (10 mg kg^{-1} day^{-1}). These findings are consistent with the view that lacidipine inhibits *in vivo* both migration/proliferation of smooth muscle cells and lipid uptake by cells (possibly macrophages).

The increasing knowledge of the pathogenesis of atherosclerosis has prompted investigations into the possibility of direct pharmacological control of the pathological processes occurring in the arterial wall. Calcium antagonists are well established in the treatment of a number of cardiovascular disorders (Nayler, 1993; Waters & Lesperance, 1994). Besides evidence that they reduce blood pressure, experimental and clinical data support the concept that Ca^{2+} antagonists may protect against structural changes occurring in the vessel wall during the progression of atherosclerosis (Lichtlen *et al.*, 1987; Parmley, 1987; Jackson & Schwartz, 1992; Nayler, 1992). Several calcium-dependent processes contribute to atherogenesis, including lipid infiltration and oxidation, endothelial injury, chemotactic and growth factors activity, smooth muscle cell migration and proliferation (Nayler, 1993).

Calcium antagonists modulate LDL cholesterol metabolism (Bernini & Allorio, 1988) and affect SMC migration and proliferation *in vitro* (Nomoto *et al.*, 1988; Jackson & Schwartz, 1992; Corsini *et al.*, 1993; 1994). Several Ca^{2+} antagonists effectively reduce the extent of atherosclerotic lesion in animals fed a cholesterol-rich diet by interfering with cholesterol esterification (Bernini *et al.*, 1993b). However, fewer data are available on the effect of calcium antagonists on intimal hyperplasia due to SMC migration and proliferation (Jackson & Schwartz, 1992). In this study we aimed to discover whether lacidipine, a dihydropyridine calcium antagonist, could affect different types of atherosclerotic lesions. In this model aortic lesions induced by cholesterol feeding are mainly due to lipid accumulation by macrophages, while the predominant cell type in cuffed-carotid arteries are SMC with scarce macrophages (Soma *et al.*, 1993; 1995a). Thus, it is possible to investigate in the same animal the effect of a drug on two different types of lesions, a fatty lesion vs a proliferative lesion. Lacidipine effectively reduced the extent of fatty lesions at the highest dose used (10 mg g^{-1} day^{-1}). This finding was not related to a cholesterol-lowering effect since the calculated arterial exposure to cholesterol, from data obtained at 4, 6, and 8 weeks, was similar in all experimental groups (Figure 1). At 10 mg lacipidine also decreased the cholesterol content of aortae, these data directly correlate with the extent of aortic lesions in cholesterol-fed rabbits (Table 2), suggesting a lacidipine effect on cholesterol accumulation in arterial walls. Lacidipine also inhibited in a dose-dependent manner the hyperplasia acutely induced in carotid of hypercholesterolaemic rabbits (Figure 3); at doses as low as 1 mg kg^{-1} day^{-1} lacidipine inhibited neointimal formation by about 30%.

These results can be explained by several *in vitro* observations. Lacidipine possesses antioxidant properties *in vitro*, protecting LDL from oxidative modification (Bernini *et al.*, 1989). Several *in vivo* studies with antioxidants (including probucol and vitamin E) indicate that these compounds effectively reduce the extent of 'fatty lesions' in aortae of cholesterol-fed and Watanabe rabbits (Kita *et al.*, 1987; Carew *et al.*, 1987; Erikson *et al.*, 1988; Daugherty *et al.*, 1989). Furthermore, lacidipine directly affects intracellular cholesterol homeostasis by inhibiting cholesterol esterification in macrophages, thus resulting in a decreased cellular accumulation of cholesteryl esters (Bernini *et al.*, 1993b; Soma *et al*, 1994). These two hypotheses are currently being addressed *in vivo*. Furthermore, the results on the proliferative lesions strongly support the *in vitro* observation that lacidipine can effect directly SMC migration and/or proliferation, a property shared with other calcium antagonists (Bernini *et al.*, 1993b; Soma *et al.*, 1994).

Overall, in this model, proliferative lesions appeared to be more sensitive to lacidipine than fatty lesions (Table 2 and Figure 3). We do not have an explanation for this differential effect but one possibility could be a different sensitivity of the mechanisms underlying the two different types of lesion to lacidipine: cholesterol homeostasis by macrophages for the fatty lesion and SMC migration and/or proliferation for the proliferative lesion. *In vitro* the inhibition of cholesterol esterification in macrophages required concentrations five times higher than those necessary to inhibit SMC proliferation (Bernini *et al.*, 1993b; Soma *et al.*, 1994). It seems unlikely that lacidipine might distribute differently among areas of the vascular bed.

In conclusion, the present *in vivo* findings show that lacidipine possesses antiatherosclerotic activity at early stages of experimental atherosclerosis. This direct antiatherosclerotic activity of lacidipine offers new therapeutic directions for this calcium antagonist, that require further study to be fully understood.

We are truly indebted to Drs G. Gaviraghi, E. Ratti, and Pastorino, for fruitful discussion and support during the whole study. We also would like to thank Mrs. Laura Mozzarelli for her skill and speed in typing the manuscript. This research was partially supported by MURST (Italy), MPI (Italy), CNR target project 'Aging' and two research grants of the European Community (PL931790 and PL950329).

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(Received October 31, 1995
Revised December 4, 1995
Accepted January 16, 1995)