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Effect of lercanidipine and its (R)-enantiomer on atherosclerotic lesions induced in hypercholesterolemic rabbits

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1 The *in vivo* antiatherogenic activity of the calcium antagonist lercanidipine and its (R)-enantiomer was investigated in two different types of atherosclerotic lesions (hyperplastic and fatty-streak lesions) in rabbits.

2 Lercanidipine (0.3, 1, and 3 mg kg^{-1} $week^{-1}$) as well as its (R)-enantiomer at 3 mg kg^{-1} $week^{-1}$ were given by subcutaneous injection for 10 weeks to White New Zealand rabbits, with cholesterol feeding beginning at week 2. The hyperplastic lesion was obtained by positioning a hollow silastic collar around one carotid artery, while aortic fatty streak lesions were induced by cholesterol feeding. In untreated animals ($n=5$), 14 days after collar positioning an intimal hyperplasia was clearly detectable: the arteries without collar showed a intima/media (I/M) ratio of 0.03 ± 0.02, whereas in carotids with a collar the ratio was 2 ± 0.42. In lercanidipine-treated animals a significant and dose-dependent effect on intimal hyperplasia was observed. I/M ratios were 0.73 ± 0.4, 0.42 ± 0.1, 0.32 ± 0.1 for 0.3, 1, and 3 mg kg^{-1} $week^{-1}$, respectively ($P<0.05$). The lercanidipine enantiomer (3 mg kg^{-1} $week^{-1}$) was as effective as the racemate (0.41 ± 0.11). Proliferation of smooth muscle cells, assessed by incorporation of BrdU into DNA, was reduced by about 50%, 70%, 85%, and 80% by lercanidipine (0.3, 1, and 3 mg kg^{-1} $week^{-1}$) and its (R)-enantiomer, respectively.

3 The area of fatty-streaks in the aorta ($n=11-15$) was significantly reduced by lercanidipine (3 mg kg^{-1} $week^{-1}$, 16% vs 27%, $P<0.05$), a trend was observed also with lower doses. When different segments of the aorta were considered (arch, thoracic, abdominal) a significant and dose-dependent effect in the thoracic and abdominal aorta was observed also at lower doses. The (R)-enantiomer was as effective as lercanidipine.

4 These results suggest a direct antiatherosclerotic effect of lercanidipine, independent of modulation of risk factors such as hypercholesterolemia and/or hypertension as demonstrated by the absence of stereoselectivity.

Keywords: Calcium antagonists; lercanidipine; atherosclerosis; animal model; smooth muscle cell proliferation; fatty-streaks; intimal hyperplasia

Introduction

With the 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 dyslipidemia 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 endothelial cells 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 (Ross, 1993; Jackson & Schwartz, 1992; Popma *et al*., 1991).

Therapeutic interventions which interfere with early stages of atherosclerosis may improve chances of halting or slowing the progression of the diseases. Among drugs currently available for therapy of vascular diseases, calcium antagonists have been investigated extensively experimentally as anti-atherogenic agents in a variety of *in vitro* (Bernini *et al*., 1989, 1993a; Lichtor *et al*., 1989) and *in vivo* (Bernini *et al*., 1989; Jackson & Schwartz, 1992; Henry, 1990) experimental models. In *in vivo* models calcium antagonists protect against lesions induced by cholesterol feeding, endothelial injury, and experimental calcinosis (Bernini *et al*., 1989; Keogh & Schroeder, 1990; Weinstein & Heider, 1989; Catapano *et al*., 1988). 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 (Bernini *et al*., 1989; Catapano *et al*., 1988; Lichtor *et al*., 1989). 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; Bernini *et al*., 1991; Schmitz *et al*., 1988; Stein & Stein, 1987), and the production of collagen, elastin, and proteoglycans (Waters *et al*., 1990) and in several *in vivo* experiments the 'antiatherosclerotic' properties of Ca^{2+} antagonists have been substantiated (Catapano, 1997). The question whether these effects can be

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extrapolated to human being however is still unsolved and studies on the beneficial effects of Ca^{2+} antagonist on myocardial infarction morbility and mortality are so far negative (Cleland & Krikler, 1993).

Whether the effects of calcium antagonist on experimental atherosclerosis are linked to the action on the L-type channel block still remains unclear. 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, 1995). Lercanidipine, a new dihydropyridine calcium antagonist, presents with a chiral centre that produces two enantiomers. The (R)-enantiomer is about 2–3 orders of magnitude less effective as a ligand to the calcium channel and in lowering blood pressure, thus offering a unique opportunity to evaluate whether Ca^{2+} antagonism plays a role in determining the antiatherosclerotic activity of the 1,4-dihydropyridine calcium antagonists. Lercanidipine has been shown to effectively reduce smooth muscle cell proliferation *in vitro* (Corsini *et al*., 1996) and to be an effective antioxidant for low density lipoprotein (LDL) both in copper and endothelial cell mediated oxidation (Catapano *et al*., manuscript in preparation).

Using this model in cholesterol-fed rabbits we investigated the *in vivo* effect of lercanidipine, and its enantiomer, on two different vascular lesions: one mainly characterized by intimal SMC hyperplasia, induced by collar insertion around a carotid artery, and the other by lipid deposition in arterial macrophages (aortic fatty-streak lesion), induced by feeding the animals with 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 ($n=20$): cholesterol-rich diet; (2) low dose lercanidipine-treated group ($n=20$): cholesterol-rich diet + lercanidipine 0.3 mg kg^{-1} $week^{-1}$; (3) intermediate dose lercanidipine-treated group ($n=20$): cholesterol-rich diet + lercanidipine 1 mg kg^{-1} $week^{-1}$; (4) high dose lercanidipine-treated group ($n=20$): cholesterol-rich diet + lercanidipine 3 mg kg^{-1} $week^{-1}$; (5) high dose (R)-lercanidipine ($n=20$): cholesterol-rich diet + lercanidipine enantiomer (3 mg kg^{-1} $week^{-1}$).

All animals received a daily cholesterol supplement for 8 weeks after a 2 week period of subcutaneous, once a week, pretreatment with the indicated doses of the drug. The daily doses of cholesterol (1.6 g) were given in the morning (at 08.00 h) each mixed in 20 g of food pellets. Normal chow, up to 150 g, was added after all the cholesterol-rich diet was eaten (usually within 30 min). Lercanidipine and (R)-lercanidipine (hydrochloride salt, Recordati, Milano, Italy) were administered subcutaneously as solutions in 50% propylene glycol. The doses of lercanidipine and its (R)-enantiomer utilized here did not affect arterial blood pressure, at the dose of 3 mg kg^{-1} $week^{-1}$. Systolic blood pressure was 95 + 3.5, 93 + 3.1, and 96 + 6.3 mm Hg^{-1} in controls, lercanidipine and (R) lercanidipine treated animals respectively after 24 h from the administration of the drug. 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 day 29 after treatment started in order to monitor the lercanidipine plasma level at 24 h post-dose. A stereospecific analytical method was utilized (Farina *et al*., 1994). The doses of lercanidipine used were determined from preliminary kinetic studies. In rabbits subcutaneous administration of lercanidipine 3 and 1 mg kg^{-1} resulted in plasma levels of 3.2 and 0.5 mg ml^{-1}, after 7 days from the administration.

Total serum cholesterol was measured by an enzymatic procedure (Catapano *et al*., 1988), at collar insertion and sacrifice. High density lipoprotein (HDL) cholesterol was determined by the same method after of very low density and low density lipoproteins (LDL) precipitation with phosphotungstic acid (Catapano *et al*., 1988). At the end of the treatment the animals were sacrificed by an overdose of sodium pentobarbital (65 mg kg^{-1}, i.v.).

Fatty streaks

Aortas 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 per cent of the aorta inner surface covered by plaques. Data are the means of two evaluations, the coefficient of variation was less than 5%.

Intimal hyperplasia

Intimal hyperplasia was mechanically induced by a silastic collar (Dupont, Milano, Italy) placed around one carotid artery of five separate rabbits from each group, 6 weeks after dietary 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 neck midline 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 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 sacrificed 2 weeks after the collar placement. All animals were given a lethal dose of sodium pentobarbital, 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 mm Hg^{-1} (Soma *et al*., 1993, 1995). Both carotid arteries were retrieved, paraffin embedded and stained with hematoxylin/eosin. At least 600 cross sections (5 μm) were cut for each artery. Intimal thickness formation was measured by light microscopy and expressed as the ratio between the cross-sectional thickness of intimal and medial tissue. Intima/media (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, 1995).

Cell proliferation assay

Proliferation index was assessed by injecting collared rabbits with 5-bromo-2′-deoxyuridine (BrdU) (40 mg kg^{-1}, i.v.), 3 h before sacrifice. BrdU incorporation was revealed by using a monoclonal antibody antiBrdU (dilution 1 : 100), after a DNA denaturation with in 2N HCl, a buffering with $Na_2B_4O_7$ and a digestion with pepsin 0.05% in 20 mM HCl at 37°C for 20 min in PFA-fixed samples. All nuclei were stained with the DNA

intercalating dye Hoechst 33528 1 μg ml^{-1} in PBS and labelling index (LI) was calculated as the per cent of BrdU-positive vs total nuclei.

Image analysis system

Total and BrdU-positive cells and the cross-sectional thickness of the intima and underlying media were analysed by an image analysis system interfaced to a Zeiss Axioscope microscope. The image-analysis system consisted of a Macintosh IIx computer (Apple) equipped with a Frame Grabber Card (QuickCapture, Data Translation), a Sony high-resolution video camera, and a Trinitron SuperMac 21-in colour monitor. All measurements were performed using National Institutes of Health Software Image, version 1.47 (Dr Wayne Rasband, NIH, Bethesda, MD, U.S.A.).

Statistics

Group data are expressed as means ± s.d. Statistical comparison of all numerical data between groups were 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 performed by CSS Software (Statsoft, U.S.A.).

Results

Of the animals included in the study five died (two in the cholesterol-fed group, one in the lercanidipine 0.3 mg kg^{-1} week^{-1}, and two in the lercanidipine 3 mg kg^{-1} week^{-1}). Autopsy was performed in two cases: no evidence for presence of liver and/or intestinal involvement was detected. Five other animals were excluded because of loss of the samples (two lercanidipine 0.3 mg kg^{-1} week^{-1}, one lercanidipine 1 mg kg^{-1} week^{-1}, two lercanidipine 3 mg kg^{-1} week^{-1}).

Records of food intake did not show any significant difference among the groups or within the groups. 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. Lercanidipine had no significant effect on serum total cholesterol levels (Tables 1 and 2), and HDL cholesterol and body weight (from 2.8 – 3.0 kg in the different groups). Exposure of the animals to lercanidipine was confirmed by the plasma levels of the drug (Table 3). The enantioselective method used showed that plasma concentration of lercanidipine enantiomers increased linearly with the dose in the range tested.

Effect of lercanidipine on fatty streaks

Upon cholesterol feeding the area of the aorta covered by plaques was 27 ± 13% (Table 1). Lercanidipine at the doses of 0.3 and 1 mg kg^{-1} week^{-1} did not reduce the plaque area (23 ± 17% and 19 ± 12%, respectively, P = n.s.). At a dose of 3 mg kg^{-1} week^{-1}, however, lercanidipine effectively reduced the lesion area by 40% to 16 ± 9 ($P<0.01$). The (R)-enantiomer (3 mg kg^{-1} week^{-1}) showed effects similar to those of the high dose lercanidipine (17 ± 9%, $P<0.01$). When three different areas of the aorta were evaluated the effect of the treatment was clearly detectable in the thoracic and abdominal segments with a clear dose-response effect (Table 1). The high dose of the enantiomer was as effective as the high dose lercanidipine. No statistically significant effects were observed in the arch of the aorta, after treatment either with lercanidipine or its (R)-enantiomer.

Table 1 Plasma cholesterol and percentage of the aorta covered by plaques in the different experimental groups

	Plasma cholesterol at sacrifice (mg dl^{-1} ± s.d.)	*Total aorta* (X ± s.d.)	*Arch* (X ± s.d.)	*Thoracic* (X ± s.d.)	*Abdominal* (X ± s.d.)
Controls (13)*	1754 ± 313	27 ± 13	49 ± 12	30 ± 21	27 ± 19
Lercanidipine (12)* 0.3 mg kg^{-1} week^{-1}	1695 ± 307	23 ± 17	39 ± 17	20 ± 13**	22 ± 7
Lercanidipine (14)* 1 mg kg^{-1} week^{-1}	1631 ± 313	19 ± 12	47 ± 12	19 ± 9**	13 ± 8**
Lercanidipine (11)* 3 mg kg^{-1} week^{-1}	1716 ± 251	16 ± 9**	41 ± 13	12 ± 8**	14 ± 7**
(R)-enantiomer (15)* 3 mg kg^{-1} week^{-1}	1495 ± 207	17 ± 9**	47 ± 10	13 ± 6***	13 ± 6**

*Animals used; **$P<0.05$ vs controls; ***$P<0.01$ vs controls.

Table 2 Effect of lercanidipine racemate and (R)-enantiomer on intimal hyperplasia, BrdU labelling of neointimal cells, and plasma cholesterol in hypercholesterolemic rabbits, ($n=5$)

Treatment	I/M ± s.d.	*Labelling index* ± s.d.	*Plasma cholesterol at surgery* (mg dl^{-1} ± s.d.)	*Plasma cholesterol at sacrifice* (mg dl^{-1} ± s.d.)
Controls	2.0 ± 0.42	7.3 ± 0.66*	1415 ± 343	1645 ± 418
Lercanidipine 0.3 mg kg^{-1} week^{-1}	0.73 ± 0.43*	3.7 ± 0.7*	1527 ± 588	1764 ± 630
Lercanidipine 1 mg kg^{-1} week^{-1}	0.42 ± 0.07*	2.3 ± 0.61*	986 ± 318	1088 ± 352
Lercanidipine 3 mg kg^{-1} week^{-1}	0.32 ± 0.08*	1.0 ± 0.30*	1216 ± 211	1356 ± 268
(R)-Lercanidipine 3 mg kg^{-1} week^{-1}	0.41 ± 0.11*	1.7 ± 0.35*	1297 ± 255	1451 ± 248

*$P<0.001$ vs controls.

Effect of lercanidipine on collar-induced intimal hyperplasia

In the control group a marked increase in intimal thickness was evident in the carotids with collar (Table 2 and Figure 1), whereas the sham-operated arteries did not show thickening of the intima either in positive control or drug-treated rabbits. The mean value of the I/M ratio in the collared arteries of the control group ($n=5$) was more than 60 fold greater (2 ± 0.42) than in the sham group ($n=20$) (0.03 ± 0.02). The media thickness was not affected either by the positioning of the collar nor by lercanidipine treatment (151 ± 11 μm in the sham carotid, vs 147 ± 12 μm in cholesterol-fed animals and 156 ± 13 μm in the lercanidipine treated animals, $n=5$, $P=$ n.s.).

The intimal hyperplasia was mostly cellular but extracellular matrix and lipid deposition were also present. Light microscopic observation of the sham tissue confirmed the absence of intimal hyperplasia or lipid deposits in spite of the severe hypercholesterolemia in these animals. Lercanidipine, at all doses, reduced the I/M ratio dose-dependently when compared with the control-group. Differences versus the control were statistically significant at all doses including the 3 mg kg^{-1} week^{-1} of the (R)-enantiomer (Table 2 and Figure 1). In animals treated with lercanidipine the inhibitory effect on intimal hyperplasia consisted of fewer layers of cells (Figure 1). As for cholesterol feeding, lercanidipine or its enantiomer had no effect on the media thickness of the carotid artery, therefore the effect of the drug on the ratio is due to a reduction of the intimal thickness.

Cell specific antibodies were used to evaluate the composition of intimal and medial cell population in carotid arteries with and without collar by immunostaining techniques. The medial tissue of sham-operated arteries and collar-applied arteries of all animals showed a strong reactivity for α-smooth muscle (SM)-actin-positive cells (Figure 2a). The lesion induced by collar positioning contained predominantly α-SM actin-positive cells (>90% of the whole cell population) (Figure 2a). Also a few macrophages, indentified by a positive reaction with the specific antibody RAM11 (Figure 2b) were detected in the lesion but not in the media. Direct *in vivo* evaluation of cell proliferation was performed by measuring the incorporation of BrdU, a thymidine analogue, into replicating DNA. Double-immunofluorescence labelling techniques indicates that at any time point more than 95% of the BrdU-positive cells were smooth muscle cells. An average of 7% of total cells in control animals were BrdU-positive (Figure 2c and Table 2). Pretreatment with lercanidipine racemate resulted in a significant dose-dependent inhibition of smooth muscle cell proliferation (Table 2). The (R)-enantiomer (3 mg kg^{-1} week^{-1}) showed effects similar to those of the high dose lercanidipine (Table 2).

Table 3 Lercanidipine enantiomers mean plasma concentrations measured on day 29, 24 h after subcutaneous administration of lercanidipine ($n=5$)

Drug	*Dose*	*Mean concentration* (ng ml^{-1})±s.d. *(R)-enantiomer*	*(S)-enantiomer*
Lercanidipine	0.3	3.4±3.1	2.6±2.1
Lercanidipine	1	8±4.4	6.4±3.5
Lercanidipine	3	25±7.6	20±5.9

Discussion

The increasing knowledge on 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). There are three subclasses of calcium channel antagonists: the phenylalkylamine derivatives (e.g., verapamil), the benzothiazepines (e.g., diltiazem), and the dihydropyridines (e.g., nifedipine). All three subclasses modify calcium entry into cells by interacting with specific binding sites on the α_1 subunit of the L-type voltage-dependent calcium channel (Nayler, 1993). The short-acting dihydropyridines, such as nifedipine, should be used with caution for long-term therapy since they have been reported to increase the incidence of acute coronary events. This is not the case for verapamil, diltiazem, or long-acting dihydropyridines such as lercanidipine.

Besides evidence that calcium antagonists 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 (Jackson & Schwartz, 1992; Nayler, 1992; Lichtlen *et al*., 1987; Parmley, 1987). Several calcium-dependent processes contribute to atherogenesis, including lipid infiltration and oxidation, endothelial injury, chemotactic and growth factor activities, smooth muscle cell migration and proliferation (Nayler, 1993; Catapano, 1997).

Calcium antagonists modulate LDL cholesterol metabolism (Bernini & Allorio, 1988) and affect SMC migration and proliferation *in vitro* (Jackson & Schwartz, 1992; Nomoto *et*

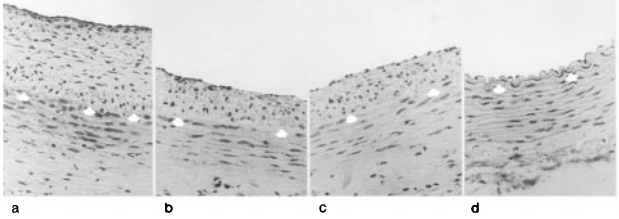

Figure 1 Representative sections of collared (a–c) and sham-operated (d) carotid arteries from cholesterol fed rabbits. (a) control; (b) lercanidipine 3 mg kg^{-1} week^{-1}; (c) (R)-enantiomer 3 mg kg^{-1} week^{-1}; (d) sham. Arrows indicate the internal elastic lamina. Sections were stained with ematotoxylin-eosin for nuclei (original magnification × 40).

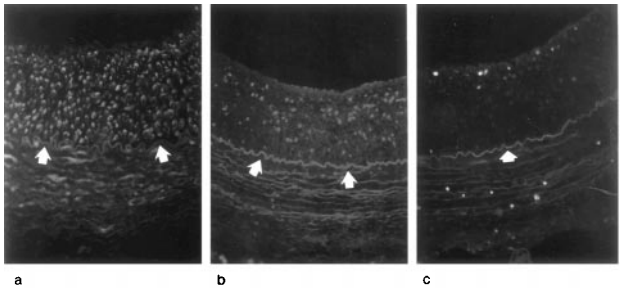

Figure 2 Representative sections of collared arteries from cholesterol fed rabbits. (a) staining for α actin (α-SM1); (b) staining for macrophages (RAM 11); (c) BrdU staining. For experimental details see Methods. Arrows indicate the internal elastic lamina (original magnification × 40).

al., 1988; Corsini *et al*., 1993, 1994). Several Ca^{2+} antagonists effectively reduce the extent of fatty-streak 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 cell migration and proliferation (Jackson & Schwartz, 1992; Soma *et al*., 1996). Lercanidipine has been shown *in vitro* to interfere with SMC cell proliferation and migration (Corsini *et al*., 1996), to modulate acyl cholesterol acyl transferase activity (Bernini *et al*., personal communication) and to act as antioxidant for LDL in copper and endothelial cell-mediated oxidation (Catapano *et al*., manuscript in preparation).

In this study we aimed at addressing whether lercanidipine, a dihydropyridine calcium antagonist, could affect two different types of atherosclerotic lesions. The aortic fatty-streak 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 (Figure 2a,b) (Soma *et al*., 1993, 1995, 1996). The mechanisms by which the latter lesion is induced are matter of debate. Among many inflammatory responses, changes in blood flow velocity, loss of perivascular innervation, kinking of the artery have been advocated (De Meyer *et al*., 1997). Thus, it is possible to investigate in the same animal the effect of a drug on two different types of lesions, a fatty-streak lesion versus an intimal hyperplastic lesion. Lercanidipine effectively reduced the extent of fatty lesions in a dose-dependent manner and the effect was particularly clear in the abdominal and thoracic aorta (Table 1). The fact that, in analogy with other 1,4-DHPs (Catapano, 1992, 1997), no significant effect was noted in the arch, might relate to the fact that the extent of lesions is too large to be affected by the treatment. Alternatively one could speculate that the surface area covered by lesions is not affected while the thickness of the lesions is reduced. Our evaluation procedure does not allow detection of this effect.

Lercanidipine also inhibited in a dose-dependent manner the hyperplasia acutely induced in carotid of hypercholesterolemic rabbits; at doses as low as 0.3 mg kg^{-1} $week^{-1}$ lercanidipine inhibited intimal hyperplasia by about 60%. Notably, the hyperplasia in hypercholesterolemic rabbits was greater than that observed in normocholesterolemic animal (Soma *et al*., 1993) suggesting a detrimental effect of hypercholesterolemia on this lesion. The mechanisms responsible for the inhibitory effect of lercanidipine and its (R)-enantiomer on the development of intimal lesions demonstrated here remains to be clarified. The systemic exposure of wall tissues to both the racemate and (R)-enantiomer greatly reduced active DNA synthesis of the smooth muscle cells, indicating an effect on myocyte proliferation (Table 2 and Figure 2c). An effect on smooth muscle cell migration cannot be excluded. This is in contrast with the results by Üstünes *et al*. (1996) who recently observed no effect on intimal thickening by verapamil in normolipidemic rabbits. The apparent discrepancy could depend on at least two reasons: (a) as reported for clinical effects, the relative specificity of calcium antagonists from different classes stems from the fact that each class has a unique binding site on the α_1 calcium channel subunit; (b) the antiproliferative effect shown by lercanidipine in this study may be mediated by its antioxidant properties. Lercanidipine, in fact, possesses antioxidant properties *in vitro*, protecting LDL from oxidative modification (Catapano *et al*., manuscript in preparation); (c) the presence of hypercholesterolemia determines the response to lercanidipine. The latter possibility is unlikely since preliminary data from our laboratory in normocholesterolemic animals indicate that lercanidipine and its enantiomer effectively reduce the intima/media ratio in the carotid.

Several *in vivo* studies with antioxidants (including probucol and vitamin E) indicate that these compounds effectively reduce the extent of fatty-streak lesions in aortas of cholesterol-fed and Watanabe rabbits (Kita *et al*., 1987; Carew *et al*., 1987; Daugherty *et al*., 1989; Erikson *et al*., 1988). Furthermore, lercanidipine directly affects intracellular cholesterol homeostasis by inhibiting cholesterol esterification in macrophages, an effect resulting in a decreased cellular accumulation of cholesteryl esters (Bernini *et al*., 1993b; Soma *et al*., 1994). Finally, the results on the hyperplastic lesions strongly support the *in vitro* observation that lercanidipine can affect directly SMC cell migration and/or proliferation (Corsini *et al*., 1996), a property shared with other calcium antagonists (Bernini *et al*., 1993b; Soma *et al*., 1994).

Overall, in this model, hyperplasia of the intima appeared to be more sensitive to lercanidipine than fatty-streak lesions. 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 lercanidipine: cholesterol homeostasis by macrophages for the fatty lesion and SMC migration and/or proliferation for the intimal hyperplasia. *In vitro* the inhibition of cholesterol esterification in macrophages required in fact concentrations higher than those necessary to inhibit SMC proliferation (Corsini *et al*., 1996). Alternatively, lercanidipine might distribute differently among areas of the vascular bed.

The effect of the lercanidipine (R)-enantiomer is of interest for two reasons: (1) the enantiomer shows at least two orders of magnitude lower affinities than lercanidipine at the Ca^{2+} channel (Leonardi *et al*., 1997). This is an indication that the calcium antagonist activity might not be essential to the antiatherosclerotic activity, in agreement with the effect of the enantiomer at 3 mg kg^{-1} $week^{-1}$ and to the fact that blood pressure was not lowered by either treatment; (2) the (R)-enantiomer shares antioxidant properties with the racemate. The latter may contribute, at least in part, to the antiatherosclerotic activity, since it is known that several antioxidants are effective in reducing atherosclerotic lesions in cholesterol-fed-rabbits (Kita *et al*., 1987; Carew *et al*., 1987; Soma *et al*., 1996). Whether the effects on fatty streaks and intimal hyperplasia are linked to the antioxidant activity of lercanidipine or to other properties, however, remains to be addressed.

In conclusion the present *in vivo* findings show that lercanidipine possesses antiatherosclerotic activities at early stages of experimental atherosclerosis that appear to be independent of the Ca^{2+} antagonist activity. This direct antiatherosclerotic activity of lercanidipine offers new therapeutic directions for this calcium antagonist, that require further studies to be fully understood and may pave the way to the use of antiatherosclerotic drugs not acting on risk factors but directly interfering with the early steps of atherosclerotic lesion formation.

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References

BERNINI, F. & ALLORIO, M. (1988). Calcium antagonists and low density lipoprotein receptors. *Ann. N.Y. Acad. Sci.,* **522,** 390–398.

BERNINI, F., BELLOSTA, S., DIDONI, G. & FUMAGALLI, R. (1991). Calcium antagonists and cholesteryl ester metabolism in macrophages. *J. Cardiovasc. Pharmacol.,* **18** (suppl. 10), S42–S45.

BERNINI, F., CATAPANO, A.L., CORSINI, A., FUMAGALLI, R. & PAOLETTI, R. (1989). Effects of calcium antagonists on lipids and atherosclerosis. *Am. J. Cardiol.,* **64,** 1291–1341.

BERNINI, F., CORSINI, A., MAZZOTTI, M., BELLOSTA, S., SOMA, M.R. & PAOLETTI, R. (1993a). Lacidipine and atherosclerosis. In: *Calcium Antagonists: Pharmacology and Clinical Research*. (eds.). Godfraind, T., Govoni, S., Paoletti, R. & Vanhoutte, P.M. pp. 157–164. Dordrecht: Kluwer Academic Publishers.

BERNINI, F., CORSINI, A., RAITERI, M., SOMA, M.R. & PAOLETTI, R. (1993b). Effects of lacidipine on experimental models of atherosclerosis. *J. Hypertens.,* **11** (suppl. 3), S61–S66.

CAREW, T.E., SCHWENKE, D.C. & STEINBERG, D. (1987). Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 7725–7729.

CATAPANO, A.L. (1992). The antiatherogenic potential of calcium antagonists: experimental evidences. *J. Cardiovasc. Pharmacol.,* **20** (suppl. A), S44–S50.

CATAPANO, A.L. (1997). Calcium antagonists and atherosclerosis: experimental evidence. *Eur. Heart. J.,* **18** (suppl. A), A80–A86.

CATAPANO, A.L., MAGGI, F.M. & CICERANO, V. (1988). The antiatherosclerotic effect of anipamil in cholesterol-fed rabbits. *Ann. N.Y. Acad. Sci.,* **522,** 519–521.

CLELAND, J.G.F. & KRIKLER, D.M. (1993). Modification of atherosclerosis by agents that do lower cholesterol. *Br. Heart J.,* **69** (suppl.), S54–S62.

CORSINI, A., BONFATTI, M., QUARATO, P., ACCOMAZZO, M.R., RAITERI, M., SARTANI, A., TESTA, R., NICOSIA, S., PAOLETTI, R. & FUMAGALLI, R. (1996). Effect of the new calcium antagonist Lercanidipine and its enantiomers on the migration and proliferation of arterial myocytes. *J. Cardiovasc. Pharmacol.,* **28,** 687–694.

CORSINI, A., QUARATO, P., RAITERI, M., PAUBERT-BRAQUET, M., NGUYEN, G., PICQUOT, S., BOURGAIN, R.H., FUMAGALLI, R. & PAOLETTI, R. (1993). Effect of nifedipine-atenolol association on arterial myocyte migration and proliferation. *Pharm. Res.,* **27,** 299–307.

CORSINI, A., RAITERI, M., DIMITRI, V., DONETTI, E., SOMA, M.R., BERNINI, F., FUMAGALLI, R. & PAOLETTI, R. (1994). Vascular smooth muscle and atherosclerosis: role of isoprenoids and calcium antagonists. *J. Vasc. Med. Biol.,* **5,** 111–119.

DAUGHERTY, A., RATERI, D.L. & SCHONFELD, S.B. (1987). Inhibition of cholesteryl ester deposition in macrophages by calcium entry blockers: an effect dissociable from calcium entry blockade. *Br. J. Pharmacol.,* **91,** 113–118.

DAUGHERTY, A., ZWEIFEL, B.S. & SCHONFELD, G. (1989). Probucol attenuates the development of aortic atherosclerosis in cholesterol-fed rabbits. *Br. J. Pharmacol.,* **98,** 612–618.

DE MEYER, G.R.Y., VAN PUT, D.J., KOCKX, M.M., VAN SCHIL, P., BOSMANS, R., BULT, H., BUYSSENS. N., VANMAELE, R., HERMAN, A.G. (1997). Possible mechanisms of collar-induced intimal thickening. *Arterioscler. Thromb. Vasc. Biol.,* **17,** 1924–1930.

ERIKSON, U., NILSSON, S. & STENPORT, G. (1988). Probucol quantitative regression swedish trial: new angiographic technique to measure atheroma volume of the femoral artery. *Am. J. Cardiol.,* **62,** 44B–47B.

FARINA, P., TARGA, G., TAJANA, A. & LEONI, B. (1994). Enantiomeric LC determination of lercanidipine in plasma. *Proc Fifth International Symposium on Chiral Discrimination*. Stockholm, Sweden. *ACC,* **118,** 83.

HENRY, P.D. (1990). Calcium channel blockers and atherosclerosis. *J. Cardiovasc. Pharmacol.,* **16,** S12–S15.

JACKSON, C.L. & SCHWARTZ, S.M. (1992). Pharmacology of smooth muscle cell replication. *Hypertension,* **20,** 713–736.

KEOGH, A.M. & SCHROEDER, S. (1990). A review of calcium antagonists and atherosclerosis. *J. Cardiovasc. Pharmacol.,* **16,** S28–S35.

KITA, T., NAGANO, Y., YOKODE, M., ISHII, K., KUME, N., OOSHIMA, A., YOSHIDA, H. & KAWAI, C. (1987). Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familial hypercholesterolemia. *Proc. Natl. Acad. Sci. U.S.A.,* **84,** 5928–5931.

LEONARDI, A., POGGESI, E., TADDEI, C., GUARNERI, L., ANGELICO, P., ACCOMAZZO, M.R., NICOSIA, S. & TESTA, R. (1997). In vitro calcium antagonist activity of lercanidipine and its enantiomers. *J. Cardiovasc. Pharmacol.,* **29** (suppl. 1), S10–S18.

LICHTLEN, P.R., NELLESSEN, U., RAFFLENBLEU, W. & HECKER, H. (1987). International Nifedipine Trial on Antiatherosclerotic Therapy (INTACT). *Cardiovasc. Drugs Ther.,* **1,** 71–79.

LICHTOR, T., DAVIS, H.R., VESSELINOVITCH, D. & MULLAN, S. (1989). Suppression of atherogenesis by nifedipine in the cholesterol-fed rhesus monkey. *Appl. Pathology,* **7,** 8–18.

NAYLER, W.G. (1992). Calcium channels and their involvement in cardiovascular disease. *Biochem. Pharmacol.,* **43,** 39–46.

NAYLER, W.G. (1993). Pharmacological aspects of calcium antagonism. Short term and long term benefits. *Drugs,* **46,** 40–47.

NOMOTO, A., MUTOH, S., HAGIHARA, H. & YAMAGUCHI, I. (1988). Smooth muscle cell migration induced by inflammatory cell products and its inhibition by a potent calcium antagonist, nilvadipine. *Atherosclerosis,* **72,** 213–219.

PARMLEY, W.W. (1987). Calcium channel blockers and atherogenesis. *Am. J. Med.,* **82,** 3–8.

POPMA, J.J., CALIFF, R.M. & TOPOL, E.J. (1991). Clinical trials of restenosis after coronary angioplasty. *Circulation,* **84,** 1426–1436.

ROSS, R. (1993). The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature,* **362,** 801–809.

SCHMITZ, G., ROBENEK, H., BEUCK, M., KRAUSE, R., SCHUREK, A. & NIEMANN, R. (1988). Ca^{++} antagonists and ACAT inhibitors promote cholesterol efflux from macrophages by different mechanisms. *Arteriosclerosis,* **8,** 46–56.

SOMA, M.R., BERNINI, F., CORSINI, A. & PAOLETTI, R. (1994). Calcium antagonists in atherosclerosis: focus on lacidipine. *Res. Clin. Forums,* **16,** 47–51.

SOMA, M.R., DONETTI, E., PAROLINI, C., MAZZINI, G., FERRARI, C., FUMAGALLI, R. & PAOLETTI, R. (1993). HMG CoA reductase inhibitors: in vivo effects on carotid intimal thickening in normocholesterolemic rabbits. *Arterioscl. Thromb.,* **13,** 571–578.

SOMA, M.R., DONETTI, E., SEREGNI, R., BARBERI, L., FUMAGALLI, R., PAOLETTI, R. & CATAPANO, A.L. (1996). Effect of lacidipine on fatty and proliferative lesions induced in hypercholaesterolemic rabbits. *Br. J. Pharmacol.,* **118,** 215–219.

SOMA, M.R., DONETTI, E., PAROLINI, C., SIRTORI, C.R., FUMAGALLI, R. & FRANCESCHINI, G. (1995). Recombinant apolipoprotein A-I Milano dimer inhibits carotid intima thickening induced by perivascular manipulation in rabbits. *Circ. Res.,* **76,** 405–411.

STEIN, O. & STEIN, Y. (1987). Effect of verapamil on cholesteryl ester hydrolysis and reesterification in macrophages. *Arteriosclerosis,* **7,** 578–584.

ÜSTÜNES, L., YASA, M., KERRY, Z., OZDEMIR, N., BERKAN, T., ERHAN, Y. & OZER, A. (1996). Effect of verapamil on intimal thickening and vascular reactivity in the collared carotid artery of the rabbit. *Br. J. Pharmacol.,* **118,** 1681–1688.

WATERS, D. & LESPÉRANCE, J. (1994). Calcium channel blockers and coronary atherosclerosis: from the rabbit to the real world. *Am. Heart J.,* **128,** 1309–1316.

WATERS, D., LESPÉRANCE, J., THEROUX, P., FRANCETICH, M., REITMAN, M. & HAVEL, R.J. (1990). Prevention of progression of minimal coronary lesions with a calcium channel blocker. *J. Am. Coll. Cardiol.,* **15,** 116A.

WEINSTEIN, D.B. & HEIDER, J.G. (1989). Antiatherogenic properties of calcium antagonists: state of the art. *Am. J. Med.,* **86** (suppl. 4A), 27–32.

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