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Effect of lacidipine on cholesterol esterification: *in vivo* and *in vitro* studies

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1 Cholesterol esterification and accumulation in the arterial wall is a hallmark of atherogenesis. Several preclinical studies suggest that calcium antagonists may exert antiatherosclerotic activity by directly affecting atherogenesis in the arterial wall. We investigated the effect of the second generation dihydropyridine calcium antagonist lacidipine on cholesterol metabolism *in vivo* in the aortic arch of cholesterol fed rabbits, and *in vitro* in mouse cultured peritoneal macrophages.

2 Treatment of cholesterol-fed rabbits with 1, 3 and 10 mg kg^{-1} day^{-1} of lacidipine for two months reduced, in a dose-dependent manner, cholesterol esterification in the aortic arch: 24 ± 6, 30 ± 12, and 41 ± 8% inhibition, respectively ($P < 0.001$ vs HC control). Concomitantly, drug treatment reduced total cholesterol content of the vessel wall. Lacidipine 3 and 10 mg kg^{-1} day^{-1} reduced cholesterolaemia (~ 20%); no effect was observed at the lowest dose used (1 mg kg^{-1} day^{-1}). These results suggest that the action of lacidipine on cholesterol esterification in the arterial wall involves, at least in part, a direct effect on cellular cholesterol metabolism. Inhibition of cholesterol esterification in the arterial wall was observed also in a reference group of animals treated with the specific ACAT inhibitor CI-976.

3 To evaluate the action of lacidipine on intracellular cholesterol metabolism we performed *in vitro* experiments with murine macrophages, the main cell type that accumulates cholesterol in the arterial wall. Lacidipine almost completely inhibited cholesterol esterification in cholesterol loaded macrophages in culture. The effect was observed independently of esterification stimuli and in cell free homogenates. The drug modified intracellular cholesterol distribution, doubling the free- to esterified sterol ratio, but did not influence the cellular rate of cholesteryl ester hydrolysis in the cell. All together these results indicate an inhibitory effect of lacidipine on cholesterol esterification catalyzed by the enzyme ACAT in murine macrophages.

4 We concluded that lacidipine influences cellular cholesterol metabolism. This effect may contribute to the potential antiatherosclerotic activity of this drug.

Keywords: Atherosclerosis; calcium antagonists; ACAT; macrophages

Introduction

In the last decade the pharmacological treatment of atherosclerosis has greatly improved, thanks to the introduction in therapy of new hypolipidaemic drugs. However, only about a third of the individuals treated benefits from this therapeutic regimen (Scandinavian Simvastatin Survival Study Group, 1994; Shepherd *et al*., 1995). It is therefore important to identify new agents active on different risk factors, or able to influence directly the atherogenic processes occurring in the arterial wall.

Several experimental studies suggest that calcium antagonists (CA) may inhibit atherogenesis in animal models without affecting blood pressure or cholesterolaemia, but act on atherogenic processes at the cellular level (Paoletti *et al*., 1995).

Major events in atherogenesis involve arterial smooth muscle cells (SMC) and infiltrated macrophages. The former mainly provide the fibrotic component of the lesion, the latter contribute to its lipid content (Basha & Sowers, 1995). Cholesterol metabolism and accumulation in the macrophages present in the atheroma is of particular importance not only in lesion development, but also in determining their stability and therefore the thrombogenic potential (Falk *et al*., 1995).

Several studies have demonstrated that various CA may inhibit SMC migration and proliferation both in *in vitro* and *in vivo* models (Corsini *et al*., 1994). On the other hand, not all CA affect cellular lipid metabolism and the results obtained are sometimes contradictory or suggest different effects depending on the specific molecule considered (Daugherty *et al*., 1987; Bernini *et al*., 1989; 1991). Nifedipine stimulates neutral hydrolases reducing the amount of cellular esterified cholesterol, without influencing cholesterol esterification (Etinjin & Hajjar, 1985); verapamil inhibits the hydrolysis of lipoprotein-derived cholesterol esters in lysosomes (Stein & Stein, 1987). The latter effect results in the accumulation of esterified cholesterol in lysosomes, reducing the delivery of free cholesterol to cytoplasm where it undergoes esterification by the enzyme acyl-coenzyme A cholesterol acyltransferase (ACAT). In previous studies we observed slight or no effects of verapamil and nifedipine on the esterification of cytoplasmic cholesterol (Bernini *et al*., 1991). It follows that both drugs indirectly influence the rate of cholesteryl ester formation in cells. A recent study demonstrated that the new dihydropyridine derivative lacidipine reduces proliferative and fatty lesions in hypercholesterolaemic rabbits independently of cholesterol lowering (Soma *et al*., 1996). A preliminary observation in our laboratory indicated that lacidipine inhibits the *in vitro* incorporation of [^{14}C]-oleate into cholesteryl esters in macrophages incubated with acetylated low-density lipoproteins (AcLDL). In the same experimental conditions, only verapamil showed an inhibitory effect on this process, while nifedipine was inactive (Bernini *et al*., 1993a). Since lacidipine is structurally related to nifedipine and not to verapamil, it can be hypothesized that this dihydropyridine CA may have a distinctive ability to influence cholesterol metabolism in cells.

In the present study we evaluated the effect of oral administration of lacidipine on cholesterol esterification in the aortic arch of hypercholesterolaemic rabbits. In addition, a detailed study on the effect of lacidipine on cholesterol metabolism in

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cultured macrophages, the main cell type involved in lipid accumulation in the arterial wall (Ross, 1993), was performed.

Methods

Animal treatment

New Zealand male rabbits (2–2.5 kg, Charles River, Calco, Italy) were used in this study. All groups of animals ($n=7$) except one were fed 1% cholesterol-rich diet for two months and the drug-treated animals received lacidipine (1,3,10 mg kg^{-1} day^{-1}) or the ACAT-inhibitor CI-976 (2,2-dimethyl-N-(2,4,6-trimethoxyphenyl)-dodecanamide; 5 mg kg^{-1} day^{-1}) mixed with food pellets. In a previous study, high performance liquid chromatographic analysis revealed that lacidipine mixed in the diet was stable for up to 3 months (Soma *et al*., 1996). At the end of the treatment the animals were killed by an overdose of sodium pentobarbitone (65 mg kg^{-1}, i.v.).

Lacidipine was kindly provided by Glaxo Wellcome (Verona, Italy) and CI-976 by Parke-Davis (Ann Arbor, MI, U.S.A.).

Plasma lipids

Plasma cholesterol levels were measured the day of death. Blood was obtained from the marginal ear vein. Plasma was prepared by centrifugation at 3000 *g* for 20 min, with EDTA as an anticoagulant. Cholesterol determination was carried out by use of an enzymatic kit (Boehringer Mannheim, Germany) (Roschlau *et al*., 1974).

Tissue preparation

After the animals had been killed, the aortic arches were quickly excised, rinsed in chilled 0.9% saline, opened longitudinally and stripped of adventitial tissue. The tissue was weighed and homogenized in 0.25 M sucrose/0.02 M Tris buffer, pH 7.4. The homogenate was centrifuged for 20 min at 9000 *g*, at 4°C; aliquots of the resulting supernatant were used for lipid and protein analysis and for the determination of ACAT activity.

ACAT assay and cholesterol content in aortic arch

ACAT activity was determined essentially by the method of Helgerud (Helgerud *et al*., 1981). The reaction mixture consisted of 360 μg of homogenate in Tris/sucrose buffer and [^{14}C]-oleoyl coenzyme A (0.5 μCi per sample) complexed with bovine serum albumin, in 0.1 M potassium phosphate buffer, pH 7.4 (final volume 0.5 ml). After incubation for 2 h at 37°C, the reaction was stopped by the addition of 5 ml of chloroform/methanol (2:1 v/v), and lipids were extracted. After centrifugation, the chloroform layer was dried under N_2 flux. For the determination of cholesterol content in the aortic arch, we followed the same procedure except for the addition of [^{14}C]-oleoyl coenzyme A in the reaction mixture. The extracted lipids were separated by thin layer chromatography (t.l.c.) (isooctane/diethyl ether/acetic acid, 75:25:2, v/v/v) (Bernini *et al*., 1993b). Cholesterol radioactivity in the spots was determined by liquid scintillation counting (Insta-Fluor, Packard, Groningen, The Netherlands) while cholesterol mass content in the spots was determined by an enzymatic method (Roschlau *et al*., 1974). We tested the linearity of this method between 1.5 and 50 μg of cholesterol ($r^2=0.99$). In every determination, [^{3}H]-cholesterol was added as internal standard with a recovery of more than 90%.

ACAT assay in cell free homogenate of murine macrophages

Cells were incubated with AcLDL (50 μg protein ml^{-1}) for 24 h. The monolayer was then removed, homogenized and incubated with lacidipine or the ACAT inhibitor CI-976 and [^{14}C]-oleoyl CoA for 1 h at 37°C, in 0.1 M potassium phosphate buffer, pH 7.4. Lipids were extracted with 4 ml of chloroform/methanol (2:1 v/v) and the radioactivity associated with cholesteryl esters was determined as described above.

Cells

Mouse peritoneal macrophages (MPM) were obtained from mice (BALB/c, Charles River, Calco, Italy) after intraperitoneal injection of thioglycollate. Cells (3×10^6 in 35 mm Multidish Nunclon, Nunc, Roskilde, Denmark) were plated and maintained in Dulbecco's minimum essential medium (DMEM, Gibco, Paisley, U.K.) with 10% foetal calf serum (FCS, Mascia Brunelli, Milano, Italy). Unattached cells were eliminated by washing with phosphate-buffered saline (PBS) 3 h after plating.

Cell viability was evaluated as described by Marks *et al*. (1956). Briefly, after incubation with drugs cells were washed with PBS and MTT (Sigma Chemical Co. St Louis, MO) was added at a concentration of 10 μg ml^{-1} in the culture medium. Following 90 min of incubation, the supernatants were decanted, the formazan precipitates were solubilized by the addition of 100% DMSO (Mercks) and placed on a plate shaker for 10 min. Absorbance was evaluated at 620 nm.

Preparation of LDL, AcLDL and [^{3}H]-CE-AcLDL

Human low-density lipoproteins (LDL; d = 1.019–1.063 g ml^{-1}) were isolated from plasma of healthy volunteers by sequential ultracentrifugation (Beckman L5-50, Palo Alto, CA) (Havel *et al*., 1955). For acetylation, LDL were dialyzed against 0.15 M NaCl, pH 7.4, diluted with an equal volume of saturated Na-acetate and treated with acetic anhydride, according to Basu *et al*. (1976).

AcLDL were radiolabelled with [^{3}H]-cholesteryl linoleate (Amersham, Buckinghamshire, U.K.) ([^{3}H]-CE-AcLDL) by incubation with serum containing the cholesteryl ester transfer protein (Nagelkerke & Van Berkel, 1986). All lipoproteins were sterile filtered.

Cholesterol esterification in cell culture

Macrophages were treated with lacidipine for 2 or 24 h. After additional 4 h of incubation in the presence of the drug and of AcLDL (50 μg protein ml^{-1}) or 25-hydroxycholesterol (5 μg ml^{-1}), cholesterol esterification was evaluated as the incorporation of radioactivity into cellular cholesteryl esters after addition of [1-^{14}C]-oleic acid (Amersham, Buckinghamshire, U.K.; 0.68 μCi per sample, 54 mCi $mmol^{-1}$) albumin complex (Brown *et al*., 1980). In another set of experiments, cells were preloaded with cholesterol via incubation for 8 h with AcLDL (50 μg protein ml^{-1}), followed by a second incubation for 16 h with lipoprotein-free medium. The cells were then incubated for a further 2 h with lacidipine and for another 5 h with lacidipine and [^{14}C]-oleate-albumin complex, without addition of lipoproteins. At the end of incubation cells were washed with PBS and lipids were extracted with hexane/isopropanol (3:2). The extracted lipids were separated by t.l.c. (isoctane/diethyl ether/acetic acid, 75:25:2, v/v/v). Cholesterol radioactivity in the spots was determined by liquid scintillation counting (Insta-Fluor, Packard, Groningen, The Netherlands).

Intracellular distribution of lipoprotein-derived cholesterol

MPMs were incubated for 24 h with [^{3}H]-CE-AcLDL (40 μg protein ml^{-1}) or AcLDL (50 μg protein ml^{-1}) and lacidipine. The medium was subsequently discarded, cells were washed with PBS and the lipids were extracted with hexane:isopropanol (3:2, v/v). Free and esterified cholesterol were partitioned by t.l.c. and the radioactivity or mass of the spots were

determined by liquid scintillation counting or by the enzymatic method, respectively.

Cellular cholesterol efflux

MPMs were incubated with [^{3}H]-CE-AcLDL (40 μg protein ml^{-1}) for 24 h. After the cholesterol loading period, the cell monolayers were washed and incubated for an additional 24 h in a lipoprotein-free medium containing 0.2% essential fatty acid free albumin (EFAF). The cells were then incubated for up 24 h in DMEM containing 0.2% EFAF and lacidipine. The medium was then recovered and centrifuged at 3000 r.p.m. for 10 min. An aliquot was counted for [^{3}H]-cholesterol (Formula 989, Packard, Groningen, The Netherlands).

Cholesteryl ester hydrolysis

This process was studied in cells loaded with cholesteryl esters by exposure for 24 h to a medium containing 50 μg protein ml^{-1} AcLDL and 0.5 μCi ml^{-1} [1,2-^{3}H]-cholesterol (Amersham, Buckinghamshire, U.K.). After the loading period, in which the radiolabelled cholesterol is incorporated and esterified, the cell monolayers were washed and incubated for additional 24 h in a lipoprotein-free medium containing 0.2% EFAF to allow the intracellular pools of labelled cholesterol to equilibrate. To quantitate cholesteryl ester hydrolysis, the loaded cells were incubated for up to 24 h in DMEM containing the tested drugs, 0.2% EFAF, and an ACAT inhibitor. The inhibition of ACAT prevents the re-esterification of any free cholesterol generated by cholesteryl ester hydrolysis and, thus, allows the assessment of the hydrolase activity, evaluated as the decrease of radiolabelled cholesteryl esters (Harrison *et al*., 1990).

Cell protein content was measured according to Lowry *et al.* (1951).

Results

Cholesterol feeding induced a clear-cut increase of blood cholesterol and of cholesterol esterification in the rabbit aortic arch (Figures 1 and 2). Lacidipine influenced cholesterol esterification at all tested doses showing a dose-dependent inhibitory effect ranging from 24% to 41% of the average value observed in cholesterol-fed animals (Figure 2). The ACAT inhibitor CI-976 completely prevented the increased rate of sterol esterification elicited by cholesterol feeding. The reduced esterification of cholesterol was paralleled by a significant drop of the sterol ester content at all doses of lacidipine, while the decreased total cholesterol content reached statistical significance only at the highest dose used (10 mg kg^{-1} day^{-1}) (Table 1). A 20% reduction of cholesterolaemia was observed with 3 and 10 mg kg^{-1} day^{-1} lacidipine; the decrease reached 70% with CI-976. The lowest dose of the calcium antagonist did not significantly affect the plasma cholesterol. (Figure 1).

The possible interference of lacidipine with cholesterol metabolism at the cellular level was explored in murine macrophages. Incubation of cells for 6 h or 28 h with the drug at concentrations up to 20 μM did not induce negative effects on cell viability (Table 2). Lacidipine reduced cholesterol esterification elicited in cells by AcLDL in a concentration-dependent manner (Figure 3a), the lowest effective concentration being 10 μM. The cellular esterification activity dropped to 30% of the control value in the presence of 20 μM lacidipine. In this experiment macrophages were exposed for 2 h to the drug before the addition of AcLDL and a further incubation of 4 h. When the incubation time was extended to 24 h (Figure 3b), the lowest effective concentration observed was 3 μM ($P<0.01$). To test the effect of lacidipine on cellular cholesterol content and partition between the free and esterified fractions, cells were exposed to the drug and either AcLDL or AcLDL labelled with [^{3}H]-cholesteryl ester. The drug reduced the amount of both cellular cholesterol mass and radioactivity in the esterified fraction (Table 3). In another series of experiments ACAT activity was stimulated by incubation of MPM with 250-hydroxycholesterol instead of AcLDL. In this experimental condition lacidipine inhibited cholesterol esterification at a concentration as low as 5 μM ($P<0.05$) (Figure 4). Because ACAT activation by AcLDL in the absence of a cholesterol acceptor in the medium is not reversible (Brown *et*

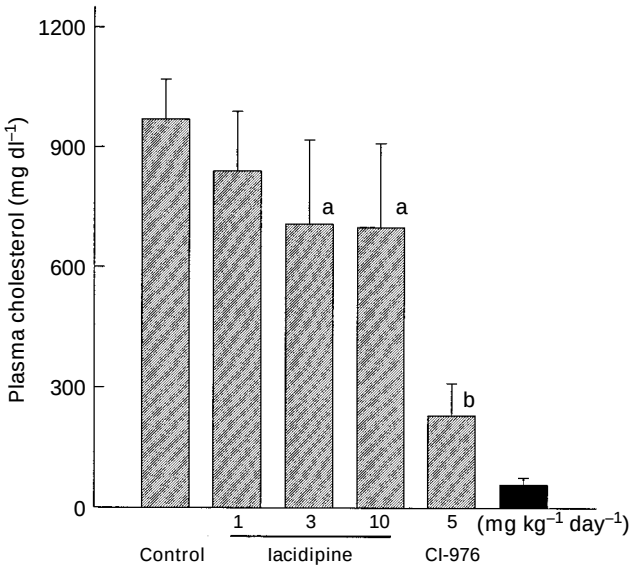

Figure 1 Rabbit plasma cholesterol at death. Blood was obtained from the marginal ear vein of hypercholesterolaemic (HC; hatched columns) or normocholesterolaemic (solid column) rabbits. Plasma was prepared as described in Methods. Data are the mean$\pm$s.d. for each group ($n=7$). Tukey test: $^aP<0.05$ vs HC; $^bP<0.0001$ vs HC and lacidipine 1-3-10 mg kg^{-1} day^{-1}.

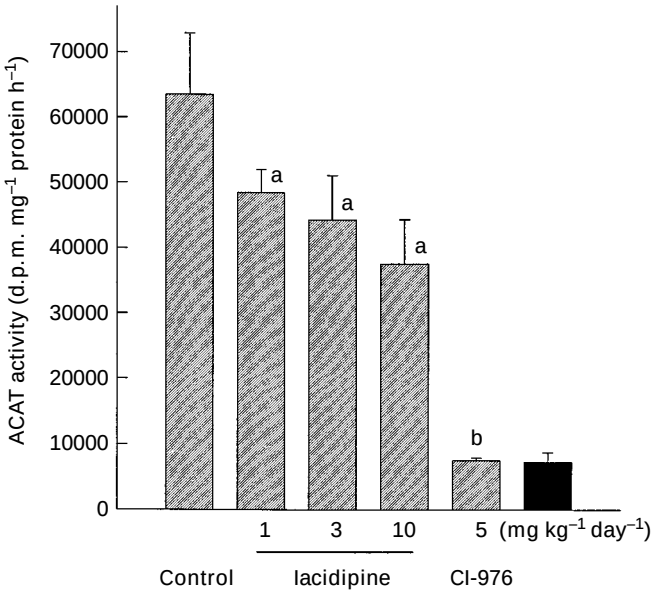

Figure 2 Effect of lacidipine on cholesterol esterification in the aortic arch homogenate of the hypercholesterolaemic rabbit. Hypercholesterolaemic animals received 1% cholesterol diet daily and the indicated compounds mixed with food pellets. ACAT activity was measured in the aortic arch after incubation of aortic homogenate with [^{14}C]-oleoyl CoA (0.5 μCi/sample). Data are the mean$\pm$s.d. for each group ($n=7$). Hypercholesterolaemic (HC; hatched columns); normocholesterolaemic (solid column). Tukey test: $^aP<0.001$ vs HC; $^bP<0.0001$ vs HC and lacidipine 1-3-10 mg kg^{-1} day^{-1}.

Table 1 Effect of lacidipine on cholesterol content in the aortic arch of hypercholesterolaemic (HC) rabbits

Group	*Free chol* (μg ml^{-1} protein)	*Esterified chol* (μg ml^{-1} protein)	*Total chol* (μg mg^{-1} protein)
Normocholesterolaemic	11.3±2.0	2.3±2.2	13.6±1.7
Hypercholesterolaemic	42.5±8.0	46.2±8.6	88.7±16.7
Lacidipine 1 mg kg^{-1}	31.2±3.3	32.6±3.4[b]	63.8±6.7
Lacidipine 3 mg kg^{-1}	36.9±13.0	32.8±11.6[b]	69.7±15.4
Lacidipine 10 mg kg^{-1}	25.4±4.7[a]	33.8±6.3[b]	59.0±10.6[c]
CI-976 5 mg kg^{-1}	5.8±0.8[c]	5.8±0.8[c]	11.6±1.7[c]

Lipids were extracted from the aortic homogenate by use of chloroform/methanol (2:1) and total, free and esterified cholesterol contents were determined with an enzymatic kit after t.l.c. separation. Data are the mean±s.d. for each group ($n=7$). Tukey test: [a]$P<0.01$; [b]$P<0.05$ vs HC. [c]$P<0.0005$ vs HC and lacidipine 1, 3 or 10 mg kg^{-1} day^{-1}.

Table 2 Effect of lacidipine on the viability of murine macrophages

Drug (μM)	*Inhibition 2+4 h* (%)	*Inhibition 24+4 h* (%)
Lacidipine (1)	<1	<1
Lacidipine (5)	2.7±1.1	<1
Lacidipine (10)	4.5±1.3	2.1±1.7
Lacidipine (20)	4.8±2.8	3.2±1.0
Lacidipine (50)	68.5±4.8	89.4±5.3

The effect of lacidipine on MPM viability was assessed by use of the colorimetric MTT assay which relies on the ability of viable cells to metabolize a tetrazolium dye actively. Cells were treated with lacidipine at the indicated concentrations for 2 or 24 h, then with lacidipine and AcLDL 50 μg ml^{-1} for 4 h. Data are expressed as % inhibition of absorbance observed in the controls. Data are the mean±s.d. of triplicate samples.

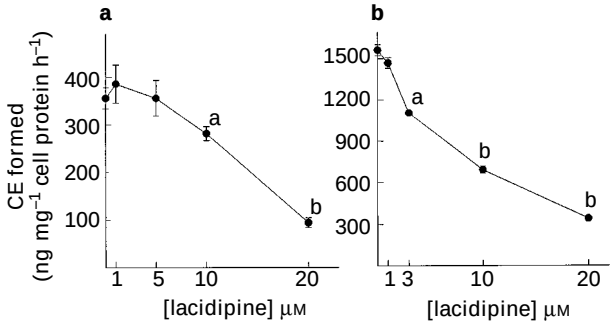

Figure 3 Effect of lacidipine on cholesterol esterification in murine macrophages in the presence of AcLDL. Cells were preincubated in DMEM+EFAF 0.2% and lacidipine for 2 h (a) or 24 h (b). Monolayers underwent a second incubation (4 h) in the presence of AcLDL (50 μg protein ml^{-1}), [1-^{14}C]-oleic acid albumin complex and lacidipine at the indicated concentrations. Data are the mean of triplicate samples; vertical lines show s.d. Student's t test: [a]$P<0.01$; [b]$P<0.001$ vs control.

al., 1980), we tested the effect of lacidipine on pre-loaded MPM after removal of AcLDL, i.e. after AcLDL-derived cholesterol had already been released from lysosomes to the substrate pool of ACAT. In this experiment we compared the effect of the drug with results obtained by simultaneous incubation with AcLDL. Results in Table 4 show that lacidipine was equally active in both experimental conditions. We next investigated whether lacidipine could influence the neutral cholesteryl ester hydrolase or cellular cholesterol efflux of MPM. To investigate the former variable, we performed experiments with cells pre-loaded with radioactive esterified cholesterol in the presence of a specific ACAT inhibitor. The blockade of intracellular re-esterification of cholesterol in pre-loaded cells allowed us to assess the rate of hydrolysis of esterified cholesterol stored in the cytoplasm (Harrison *et al*., 1990). In these conditions about 50% of cholesteryl esters were hydrolyzed in control cells; the addition of lacidipine only slightly influenced this cellular activity (Figure 5). Cellular cholesterol efflux was evaluated in similar experimental conditions, but in the absence of the ACAT inhibitor, by counting the amount of radioactive cholesterol released in the culture medium. Lacidipine did not affect this parameter (control: $400.5\pm15\times10^3$; lacidipine 20 μM: 373.2×10^3 d.p.m. mg^{-1} protein). Taken together the results suggest that lacidipine may influence cholesterol metabolism in macrophages by specifically inhibiting ACAT activity without affecting other processes involved in cholesterol metabolism. Consistent with this hypothesis, we observed that the drug could inhibit cholesterol esterification by more than 80% when added to cell-free homogenates. As expected complete inhibition of ACAT activity was observed with the ACAT inhibitor (Table 5).

Discussion

The calcium antagonists represent one of the first class of drugs that, at the experimental level, have demonstrated antiatherosclerotic effects independently deleterious side effects. Evaluation of the individual molecules has revealed that most of them can exhibit this effect in one or more animal models (Corsini *et al*., 1994). However, the study of the possible mechanisms of action has highlighted numerous differences not

Table 3 Effect of lacidipine on cholesterol distribution in murine macrophages incubated with AcLDL or [^{3}H]-CE-AcLDL

Drug (μM)	*Free Chol*	*Chol ester*	*Total Chol*	*FC/CE ratio*
		(μg mg^{-1} cell protein)		
Control	51.4±3.2	24.7±1.0	81.0±5.1	2.1
Lacidipine 20	50.1±2.4	18.2±2.2[b]	71.1±3.0[a]	2.8
		(d.p.m. mg^{-1} cell protein×10^{-3})		
Control	563±39	424±28	986±47	1.3
Lacidipine 20	607±32	257±12[c]	862±39	2.4

Macrophages were incubated with AcLDL (50 μg protein ml^{-1}) or AcLDL reconstituted with [^{3}H]-cholesteryl linoleate (40 μg protein ml^{-1}) and lacidipine for 24 h. Data are the mean±s.d. of triplicate samples. Students t test: [a]$P<0.05$; [b]$P<0.01$; [c]$P<0.001$ vs control.

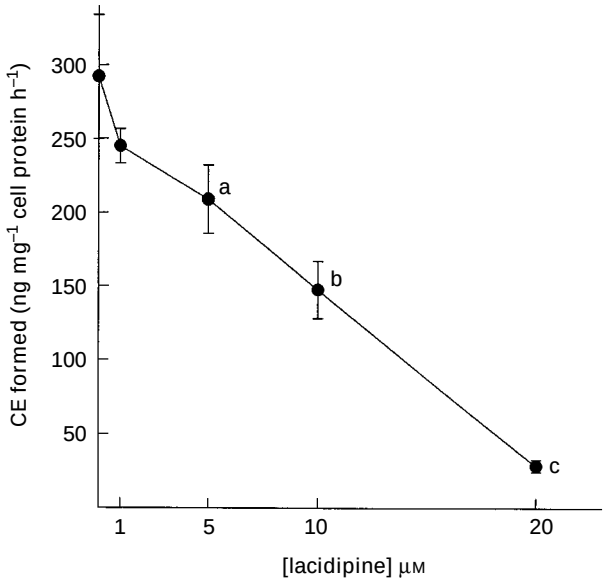

Figure 4 Effect of lacidipine on cholesterol esterification in murine macrophages incubated with 25-hydroxycholesterol. Cells were preincubated in DMEM + EFAF 0.2% and lacidipine for 2 h. Monolayers underwent a second incubation (4 h) in the presence of 25-hydroxycholesterol (5 μg ml^{-1}), [1-^{14}C]-oleic acid albumin complex and lacidipine. Data are the mean of triplicate samples; vertical lines show s.e.mean. Student's t test: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$ vs control.

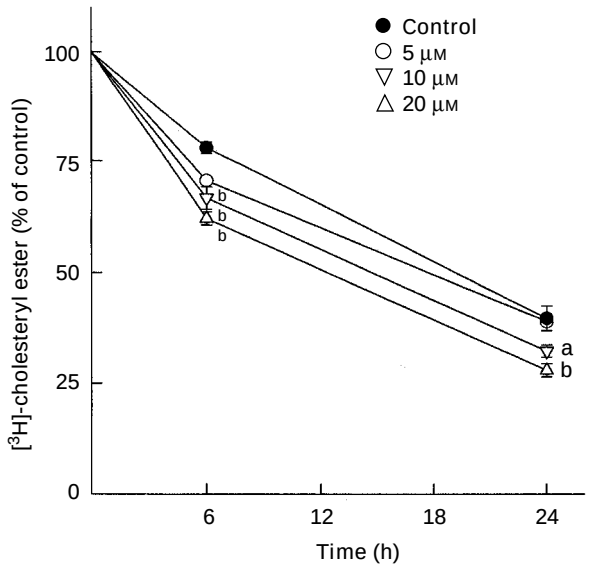

Figure 5 Effect of lacidipine on cholesterol ester hydrolysis in mouse peritoneal macrophages. Cells were incubated in 24 h with AcLDL and [^{3}H]-cholesterol (0.5 μCi/well), followed by 24 h incubation in a lipoprotein free medium. Then cells were incubated with the ACAT inhibitor alone (control) or with lacidipine 5 μM, 10 μM or 20 μM for 24 h. Data are the mean of triplicate samples; vertical lines show s.d. Student's t test: $^{a}P<0.05$; $^{b}P<0.001$ vs the ACAT inhibitor alone.

Table 4 Comparative effect of lacidipine on cholesterol esterification in preloaded and non-preloaded murine macrophages

	Cholesteryl esters formed (% of inhibition)	
Drug (μM)	*Pre-loaded*	*Non pre-loaded*
Lacidipine (10)	24.7 ± 4.0^{a}	19.7 ± 3.5^{b}
Lacidipine (20)	76.2 ± 1.9^{b}	73.5 ± 2.5^{b}

Pre-loaded = addition of lacidipine after AcLDL incubation; non pre-loaded = addition of lacidipine before AcLDL incubation. Macrophages were pre-loaded as described in Methods. Control values (ng cholesteryl esters mg^{-1} cell protein h^{-1}): pre-loaded 1695 ± 92; non pre-loaded 1944 ± 97. Student's t test: $^{a}P<0.01$; $^{b}P<0.001$.

Table 5 Effect of lacidipine on cholesterol esterification in cell free homogenate of murine macrophages

Drugs (μM)	*[^{14}C]-chol ester* (pmol/dish h^{-1})
Control	457.1 ± 47.0
Lacidipine (10)	280.5 ± 15.0^{a}
Lacidipine (20)	66.5 ± 1.5^{b}
CI-976 (2.5)	18.5 ± 6.5^{b}
CI-976 (25)	7.5 ± 1.0^{b}

Cells were incubated with AcLDL (50 μg protein ml^{-1}) for 24 h. The monolayer was then removed, homogenized and incubated with lacidipine or the ACAT inhibitor CI-976 and [^{14}C]-oleoyl CoA for 1 h at 37°C. Data are the mean $\pm$ s.d. of triplicate samples. Student's t test: $^{a}P<0.01$; $^{b}P<0.001$ vs control.

only among calcium antagonists belonging to different classes, but also among compounds of the same class, especially the dihydropyridine derivatives (Bernini *et al*., 1991; Paoletti & Bernini, 1990).

In this study, we have evaluated the effect of the new dihydropyridine calcium antagonist lacidipine on the metabolism of cholesterol in experimental models of atherogenesis, both *in vivo* in the aortic arch of rabbits fed hypercholesterolemic diet, and *in vitro* in cholesterol rich mouse peritoneal macrophages, a widely accepted model for studying foam cell formation (Hussain *et al*., 1992). Our results show that the rate of cholesterol esterification in the aortic arch was reduced in a dose-dependent manner in the animals treated with lacidipine. Consistent with the inhibitory effect of the drug on cholesterol esterification was the concomitant reduction of esterified cholesterol content observed in the aortic wall. The doses of lacidipine used in our study, although substantially higher than those used therapeutically in man, were selected for two reasons: firstly, pharmacokinetic studies in rabbits have indicated that lacidipine in the range of doses from 1 to 18 mg kg^{-1} give C_{max} from 5 ng ml^{-1} to 120 ng ml^{-1} (M. Pellegatti Glaxo SpA, Verona, Italy, personal communication), comparable to those observed in man (median range from 0.9 to 19.7 ng ml^{-1}, with doses from 4 to 8 mg, Squassante *et al*., 1994); secondly, the same doses in hypercholesterolaemic rabbits proved to be active on cellular processes in the arterial wall (inhibition of myocyte proliferation in the carotid artery, Soma *et al*., 1996). The effect of the drug on the vessels was already present at the lowest dose in the absence of any hypocholesterolaemic activity. Lowering of aortic total cholesterol content independently of a hypocholesterolaemic effect in rabbits with lacidipine 10 mg kg^{-1} day^{-1} has been demonstrated (Soma *et al*., 1996), as well as a reduction of plasma cholesterol in cholesterol fed rabbits by lacidipine (10 mg kg^{-1} day^{-1}) (Soma *et al*., 1996). The mechanism of the latter effect is not known; it has been speculated that, in addition to the arterial wall, the drug might interfere with cholesterol esterification activity in other body compartments, such as the intestinal epithelium, thus interfering with sterol absorption from a cholesterol-rich diet. An alternative explanation is suggested by the observations of Block *et al*. (1991) that calcium channel blockers may decrease the expression of HMG-CoA reductase and increase LDL receptor activity. A marked reduction of cholesterol esterification was observed in the aorta of rabbits treated with CI-976, a potent and specific ACAT inhibitor, which also strongly counteracted the diet-

induced cholesterolaemia. However, the rate of esterification measured in the presence of CI-976 was similar to that observed in animals fed a normal diet, despite the finding that cholesterolaemia in this group was only 25% of that in the group treated with CI-976. This observation is consistent with the ability of this compound to interact directly with the arterial wall (Bocan *et al*., 1991).

The above *in vivo* results suggest that lacidipine may influence cholesterol metabolism at the cellular level and our studies in murine cultured macrophages are consistent with this hypothesis. Lacidipine inhibited intracellular cholesterol esterification with a parallel increase in the free to esterified cholesterol ratio. This effect was observed in cells in which esterification activity was stimulated either by lipoprotein derived cholesterol or by 25-hydroxycholesterol, as well as in cholesterol pre-loaded cells, i.e. after removal of the esterification stimulus from cultured medium. These results demonstrate that lacidipine acts by a mechanism that is independent of the way the esterification was induced, indicating the ability of the drug to interfere with intracellular processes regulating the formation and accumulation of esterified cholesterol. Since our *in vitro* studies were performed in the absence of extracellular acceptors of cholesterol (i.e. HDL), the net amount of esterified cholesterol formed within the cells depends on the rate of its synthesis and/or hydrolysis. The results demonstrate that lacidipine did not affect the cellular hydrolysis of esterified cholesterol. On the contrary, the capacity of the drug to inhibit the esterification of cholesterol in cell-free homogenates indicates a direct effect on the synthesis of cholesteryl esters through the inhibition of ACAT, the enzyme that catalyzes such a process. A similar effect was observed with the ACAT inhibitor. It was noted that in the cell culture experiments lacidipine reduced cellular cholesteryl ester content without affecting free cholesterol. In contrast the *in vivo* data indicated a significant reduction of aortic free cholesterol content. This apparent discrepancy may be explained by the hypothesis that, in the *in vivo* situation, the tissue cholesterol content is influenced by cholesterol efflux and the entire reverse cholesterol transport which is not present in a cell culture system. Robenek & Schmitz (1988) have shown that the calcium channel blocker nifedipine induces the formation of membrane-surrounded 'lamellar bodies' in cholesterol-loaded macrophages which are released from the cells in an HDL-independent fashion. Such a mechanism might contribute to the *in vivo* effect of lacidipine. However, we did not observe an increased cholesterol efflux from the cultured macrophages, which suggests that lacidipine is not able to induce the secretion of cholesterol containing particles.

Elucidation of the molecular mechanism of the effect of lacidipine was not the purpose of the present study. However, we suggest a hypothesis based on the physico-chemical properties of the drug. Lacidipine is a highly lipophilic compound and binds in a prolonged way to lipid membranes (Herbette *et al*., 1993). Since the activity of ACAT is sensitive to variations in the lipophilicity and composition of the biophase (Suckling & Stange, 1985), the presence of the drug could alter the availability of the substrate (i.e. cholesterol) for the enzyme. This mechanism is consistent with the inhibitory activity of the drug observed in the whole cells and in the cell-free homogenate. Such a mechanism has been proposed to explain the ability of oxysterols to induce the cellular esterification of cholesterol: these molecules could modify the biophase facilitating the access of cholesterol to the catalytic site of the ACAT enzyme (Suckling & Stange, 1985).

The results obtained in our *in vitro* studies demonstrate that lacidipine may influence the intracellular cholesterol metabolism by reducing the formation of esterified cholesterol, the storage form of this sterol. This activity appears to be peculiar to lacidipine, since previous studies have demonstrated that nifedipine and verapamil do not display this effect (Bernini *et al*., 1991). This observation also suggests that the inhibitory activity of lacidipine on ACAT is not due to a block of the L type calcium channels, and is consistent with a mechanism of action involving the physico-chemical properties of the drug.

Experiments in cultured macrophages were performed with relatively high concentrations of lacidipine, exceeding those observed in clinical use (Squassante *et al*., 1994). However, similar concentrations have been found to be active on *in vitro* myocyte proliferation (Bernini *et al*., 1993a) and the same doses used in our *in vivo* experiments have been shown to be active in inhibiting cellular proliferation in the rabbit carotid (Soma *et al*., 1996). As mentioned before these doses induce plasma concentrations of lacidipine similar to those observed in man. Since the drug is highly lipophilic and rapidly distributed in tissues (Pellegatti *et al*., 1990); it is possible that lacidipine may accumulate in the vessel walls, thus achieving active concentrations similar to those effective *in vitro*.

In conclusion, lacidipine inhibits cholesterol esterification *in vivo* in rabbit aortic arch and *in vitro* in macrophages in culture. These results support a unique effect of lacidipine on cellular metabolism of cholesterol which may contribute to the potential antiatherosclerotic effect of this drug.

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