

Lacidipine Modulates the Secretion of Matrix Metalloproteinase-9 by Human Macrophages

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ABSTRACT

Activated macrophages within the arterial wall secrete matrix-degrading metalloproteinases (MMPs) that weaken the atherosclerotic plaque and contribute to its fissuration. Preclinical studies have shown that calcium antagonists may reduce atherogenesis in the arterial wall. In the present study we evaluated the effect of lacidipine on 92-kDa gelatinase B (MMP-9) expression in human macrophages in cultures. Cells were treated for 24 h with lacidipine and the conditioned media were analyzed. Lacidipine (1–20 μ M) significantly reduced, in a dose-dependent manner, MMP-9 potential gelatinolytic capacity up to 50%. When MMP-9 expression was stimulated by treatment with phorbol esters or tumor necrosis factor- α , lacidipine was able to inhibit this enhanced gelatinolytic capacity up to 50 and 60%, respectively. Western blot analysis and enzyme-linked immunosorbent assay showed a reduction of MMP-9 protein

actually released by cells. The addition of lacidipine in the incubation media determined no significant variation in Ca^{2+} concentration. The drug did not affect MMP-9 mRNA levels, but it effectively reduced the amount of both active and total free MMP-9 secreted by human macrophages. Lacidipine reduced also the secretion of the tissue inhibitor of metalloproteinase-1 (TIMP-1); however we observed an overall reduction of the gelatinolytic activity of the cells. Finally, peritoneal macrophages, obtained from mice treated with lacidipine, showed a reduced secretion of MMP-9. Together, our data indicate that lacidipine may potentially exert an antiatherosclerotic activity by modulating the secretion of MMP-9 by macrophages. This, in addition to the previously demonstrated inhibition of cholesterol esterification, may contribute to increase plaque stability.

The composition and vulnerability of plaque is a major determinant of atherosclerosis complications. Remodeling of the arterial extracellular matrix occurs during all phases of human atherosclerosis and abnormal extracellular matrix metabolism may contribute to vascular remodeling during the development and complication of human atherosclerotic lesion (Galis et al., 1994). Plaque disruption with superimposed thrombosis is the main cause for the acute coronary syndromes of unstable angina, myocardial infarction, and sudden death (Falk et al., 1995).

Plaque instability, manifesting as ulceration of the fibrous cap, plaque rupture, and intraplaque hemorrhage, is characteristic of plaques with a high content of lipid and an excess of macrophages in the cap (Fernandez-Ortiz et al., 1994). The size and consistency of the atheromatous core, which is rich in extracellular lipid (i.e., cholesterol and its esters), are

critical for the stability of individual lesions. The central, lipid-rich core of the typical lesion contains many lipid-laden macrophage foam cells derived from blood monocytes (Libby, 1995). Such lesional foam cells can produce large amounts of tissue factor, a powerful procoagulant that potently stimulates thrombus formation when in contact with blood (Wilcox et al., 1989). Thus, the integrity of the fibrous cap overlying this lipid-rich core determines the stability of an atherosclerotic plaque and protects the blood compartment in the arterial lumen from potentially disastrous contact with the underlying thrombogenic lipid core (Libby, 1995). Macrophages, and other cells present in the vessel wall, are capable of degrading extracellular matrix by phagocytosis or by secreting proteolytic enzymes, in particular, a family of matrix metalloproteinases (MMPs) (Dollery et al., 1995). At least 23 different MMPs have been identified (Massova et al., 1998) and their number is still increasing. MMPs act extracellularly at physiological pH and require activation from proenzyme precursors to attain enzymatic activity (Libby, 1995).

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ABBREVIATIONS: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PMA, phorbol-12-myristate-13-acetate; TNF- α , tumor necrosis factor- α ; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; ELISA, enzyme-linked immunosorbent assay.

The 92-kDa gelatinase B or metalloproteinase-9 (MMP-9) is expressed by virtually all activated macrophages and, through the degradation of the basement membrane, it facilitates macrophage extravasation. MMP-9 has been shown to be common in atherectomy materials from unstable angina (Brown et al., 1995) and abdominal aortic aneurysm (Newman et al., 1994). Ubiquitous inhibitors known as tissue inhibitors of metalloproteinases (TIMPs) hold the activity of these enzymes in check under usual circumstance (Libby, 1995). MMPs can therefore perform their biological function only when a local excess over endogenous inhibitors prevails.

Stabilizing plaques may obtain clinical benefits even in the absence of regression (Brown et al., 1993b). Several lipid-lowering trials with angiographic follow-up have shown that stability of coronary plaques over the short term is associated with a good long-term prognosis. Experimental observations indicate that lipid-lowering, obtained by dietary intervention, may stabilize vulnerable plaques by reducing expression and activity of enzymes that degrade the arterial extracellular matrix (Aikawa et al., 1998). Besides lipid-lowering therapies, plaque stabilization could be achieved also by a direct inhibition of MMPs in the arterial wall. Along with transforming growth factor- β , corticosteroids, and heparin, several synthetic inhibitors have been investigated, namely, tetracyclines, antracyclines, and synthetic peptides, although without much luck (Dollery et al., 1995). A number of MMPs inhibitors have now entered development. To date, however, no compound has reached the marketplace and only a relatively small number are known to be in clinical trials (Beckett and Whittaker, 1998). We have shown that statins, a class of drug that can achieve relatively large reductions in plasma cholesterol levels and represent an established treatment of hypercholesterolemia (Brown and Goldstein, 1991; Havel and Rapaport, 1995; Corsini et al., 1999), reduce MMP-9 secretion by mouse and human macrophages (Bellosta et al., 1998), and recently Ikeda et al. (2000) have shown that fluvastatin is effective in inhibiting also MMP-1 expression in human vascular endothelial cells.

In our laboratory we previously demonstrated that lacidipine, a third-generation dihydropyridine calcium antagonist, may directly interfere with major processes of atherogenesis occurring in the arterial wall (Bernini et al., 1993). The drug inhibits smooth muscle cells migration and proliferation and, through the inhibition of acyl-CoA:cholesterol acyltransferase activity, prevents in vitro cholesterol accumulation in macrophages and in vivo in the aorta of hypercholesterolemic rabbits (Bernini et al., 1997).

In the present study, we evaluated the effect of lacidipine on in vitro MMP-9 expression by human macrophages and ex vivo by peritoneal macrophages obtained from lacidipine-treated mice.

Materials and Methods

Drugs. Lacidipine was kindly provided by Glaxo Wellcome (Verona, Italy). Nifedipine was purchased from Sigma-Aldrich (Milan, Italy).

Cell Culture. Circulating human monocytes were isolated from blood of healthy donors as previously described (Bellosta et al., 1998). The monocytes were collected, washed, resuspended in serum-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies, San Giuliano Milanese, Italy) and plated at a density of 3×10^6 cells in 35-mm dish. After 2 h, cell monolayers were washed twice and the

adherent cells were incubated for 10 to 14 days with DMEM containing 10% human AB serum and 8 μ g/ml insulin, to allow for differentiation in macrophages. To generate the conditioned media, cells were incubated for 24 h at 37°C with DMEM, supplemented with 0.2% bovine serum albumin (BSA; Sigma-Aldrich) and the indicated concentrations of lacidipine or nifedipine, in the absence or presence of phorbol esters [phorbol-12-myristate-13-acetate (PMA), 50 ng/ml] or TNF- α (20 ng/ml) (Sigma-Aldrich). At the end of the incubation, the conditioned media were collected and the gelatinolytic capacity of secreted MMP-9 analyzed by zymography. Cellular protein content was measured according to Lowry et al. (1951). Cellular toxicity caused by the drug was assessed using the dimethylthiazoldiphenyltetrazolium bromide assay as described (Mosmann, 1983).

Mice Treatment. CD-1 mice have been treated with lacidipine (1 or 3 mg kg⁻¹ day⁻¹; 10 animals/group) given by gavage for 14 days. Peritoneal macrophages were collected by peritoneal lavage with phosphate-buffered saline (PBS), 3 days after an intraperitoneal injection of 4% thioglycollate in water. Cells were pelleted, washed twice with serum-free DMEM, and plated at a density of 3×10^6 cells/35-mm dish. After 2 h, plates were washed three times with DMEM to remove nonadherent cells, and incubated overnight in DMEM containing 10% fetal bovine serum. Cells were then washed and incubated for 24 h with DMEM containing 0.2% BSA alone and MMP-9 gelatinolytic capacity measured by gelatin zymography.

SDS-Polyacrylamide Gel Electrophoresis Zymography. MMP-9 gelatinolytic capacity has been evaluated as described previously (Bellosta et al., 1998). Briefly, samples (5 μ l of conditioned medium per lane) underwent electrophoresis at 4°C on 7.5% polyacrylamide gels containing 10% SDS and gelatin (1 mg/ml) under nonreducing conditions and without boiling. After electrophoresis, SDS was removed from gels in two washes with 2.5% Triton X-100 (Sigma-Aldrich) at room temperature. After washes, the gels were incubated overnight at 37°C with gentle shaking in 50 mM Tris, pH 7.5, containing 150 mM NaCl, 10 mM CaCl₂, and 1 μ M ZnCl₂, to activate the metalloproteinase ability to digest the substrate. For inhibition studies and to confirm the identity of MMP-9, identical gels have been incubated in the above-described buffer containing either 20 mM EDTA, an inhibitor of MMPs, or 1 mM PMSF, an inhibitor of serine proteases. The addition of PMSF did not alter the MMP-9 gelatinolytic capacity, whereas the treatment with EDTA completely abolished it (data not shown). At the end of the incubation, the gels were stained with a solution of 0.1% Coomassie brilliant blue R-250 (Sigma-Aldrich) in 25% methanol and 7% acetic acid. Clear zones against the blue background indicated the presence of proteolytic activity. It has been described that, in this type of SDS-containing gel, both the latent form of MMP-9, the pro-MMP-9, and the activated gelatinase develop gelatinolytic activity (Kleiner and Stetler-Stevenson, 1994), therefore, we used the word "activity" to indicate the potential total gelatinolytic capacity measured in the conditioned media. We have previously shown (Bellosta et al., 1998) that, in our experimental conditions, the gelatinolytic capacity is due entirely to the 92-kDa pro-MMP-9, as assessed by zymography, by Western blot analysis with a specific antibody against human MMP-9. Finally, after activation of the protease by incubation for 2 h at 37°C with 2 mM 4-aminophenylmercuric acetate the propeptide is cleaved to the active <90-kDa form (data not shown).

Western Blot Analysis. Aliquots of the conditioned media (40 μ l/lane) were run on 10% polyacrylamide gel containing SDS, under nonreducing conditions (Bellosta et al., 1998). The proteins were blotted to nitrocellulose membranes (Bio-Rad Laboratories, Milan, Italy) and incubated with a 3% solution of defatted dried milk in PBS containing 0.1% Tween 20. Then a mouse monoclonal antibody anti-human MMP-9 (clone 6-6B; Amersham Pharmacia Biotech Italia, Cologno Monzese, Italy) diluted in PBS-1% Tween 20 (2 μ g/ml) was added and the incubation continued for 1 h (Ramos-DeSimone et al., 1993). The bound primary antibody was detected using an anti-mouse secondary antibody conjugated to horseradish peroxidase

(Sigma-Aldrich) and the enhanced chemiluminescence kit (Amersham Pharmacia) according to the manufacturer's instructions.

Northern Blot Analysis. RNA was extracted (RNAzol B; Tel-Test Inc., Friendswood, TX) from human monocyte-derived macrophages treated with and without lacidipine for 24 h before harvest. Equal amounts of total RNA (10 μ g) were transferred to GeneScreen Plus membranes by slot-blot. A human MMP-9 probe (generously provided by Dr. H. Sato, Kanazawa University, Kanazawa, Japan) was labeled with [32 P]dCTP by random priming and incubated with the blots in ExpressHyb (Clontech Laboratories, Palo Alto, CA) for 1 h at 42°C. After exhaustive washings, blots were exposed for autoradiography (X-OMAT AR; Kodak, Rochester, NY). After autoradiography to detect MMP-9 mRNA, the blot was stripped and reprobed for the actin gene to control gel loading.

Measurement of Intracellular Ca^{2+} . Macrophages were incubated for 24 h at 37°C with DMEM supplemented with 0.2% BSA and in the absence or presence of 20 μ M lacidipine. Intracellular Ca^{2+} was monitored using the fluorescent probe fura-2 as described (Malagoli et al., 1987). Suspensions of macrophages (1×10^7 cells ml^{-1}) were incubated with 2 μ M fura-2 (Sigma-Aldrich) for 30 min at 37°C. Cells were washed free of extracellular probe, resuspended at 5×10^6 ml^{-1} , and allowed to equilibrate for 10 min at 37°C. They were then transferred to the thermostatically controlled cuvette compartment of a spectrofluorimeter (Shimazu RF 5000; Shimazu Corp., Kyoto, Japan). All the incubations were carried out in the dark. Calcium concentrations were determined from the ratio of fura-2 fluorescence at 340-nm excitation and 380-nm excitation. To avoid interferences, the autofluorescence derived from the cells and from the incorporated lacidipine was subtracted. At the end of each experiment the maximum fluorescence was determined after permeabilization of the cells with 10^{-6} M ionomycin (Sigma-Aldrich), as described (Iredace and Dickenson, 1995). Minimum fluorescence was determined by lowering the free Ca^{2+} with 10^{-2} M EGTA (Sigma-Aldrich) in the same cell suspension. The reproducibility in the determinations was better than $\pm 10\%$ for cells from the same day and identical processing. Data represent the mean \pm S.D. of five experiments performed in duplicate.

ELISA. The amount of secreted MMP-9 and TIMP-1 protein was quantified using the highly specific Biotrak Matrix Metalloproteinase-9 or TIMP-1 ELISA systems (Amersham Pharmacia), respectively. The MMP-9 assay uses two antibodies directed against different epitopes of human MMP-9 and does not show detectable cross-reactivity with MMP-1, -2, and -3, and TIMP-1 and -2 (Amersham Pharmacia). The TIMP-1 assay uses an antibody that recognizes total human TIMP-1 (i.e., both free TIMP-1 and that complexed with MMPs) and does not cross-react with TIMP-2.

The Biotrak MMP-9 activity assay system (Amersham Pharmacia) was used to quantify the amount of both active and latent forms of free (not complexed with TIMP-1) MMP-9 in the macrophage-conditioned media. This assay recognizes the pro and active forms of MMP-9 and does not cross-react with other MMPs or TIMPs. Aliquots of conditioned media were analyzed as suggested by the manufacturer.

Collagen Degradation. The gelatinolytic activity present in the conditioned media was determined by measuring the release of radioactive peptides from denatured labeled collagen, following the method previously described (Liotta et al., 1980; Murphy and Crabbe, 1995). Briefly, human macrophages were incubated with the indicated compounds for 24 h. Then media were collected, added with 1 mM PMSF, and the released pro-MMP-9 activated by incubation with 2 mM 4-aminophenylmercuric acetate for 2 h at 37°C. [^3H]Collagen (NEN Life Science Products, Cinisello Balsamo, Italy; specific activity 0.193 mCi/mg; purity 98.9%), after denaturation at 60°C for 20 min in serum-free DMEM, was added to the media (0.25 μ Ci/ml) and incubated in the absence of cells for further 24 h. At the end of the incubation, undigested collagen was precipitated with ice-cold trichloroacetic acid (final 15%). The mixture was centrifuged and the

radioactivity present in the supernatant was measured by scintillation counting.

Statistical Analysis. For quantitation of zymograms, densitometric scanning was performed using a system incorporating a video camera and a computer analysis package (NIH Image 1.52 image analysis software). Each experiment was performed at least three times with different preparations of cells. Background was set for each gel and each lane was analyzed sequentially. Results were normalized by cellular protein content and expressed as optical density units. To validate the method, a linear response of optical density units versus dilution was obtained for different serial dilutions on two separate samples (data not shown). To standardize conditions between gels, an aliquot of a standard sample was loaded on each gel, and values for each band were then normalized to the value of the band of the reference sample run on the same gel. Data are presented as the mean \pm S.D. and analyzed using Dunnett's test.

Results

In the present study, we concentrated our attention on MMP-9, the most prevalent form of gelatinase secreted by macrophages. To study the effect of calcium channel blockers on MMP-9 expression, we incubated human monocyte-derived macrophages for 24 h with 20 μ M lacidipine and 50 μ M nifedipine. The conditioned media were then collected and analyzed by gelatin zymography to evaluate the potential gelatinolytic capacity of MMP-9. As shown in Fig. 1, lacidipine inhibited MMP-9 gelatinolytic capacity. It is worthwhile to notice that nifedipine, the prototype of the dihydropyridine family, did not show any inhibitory effect even at a concentration as high as 50 μ M. The analysis of the data obtained in three different experiments showed that the addition of lacidipine inhibited in a dose-dependent manner MMP-9 gelatinolytic capacity; the effect starts at a concentration of 5 μ M and becomes significantly different at 10 μ M (Fig. 2A). Then

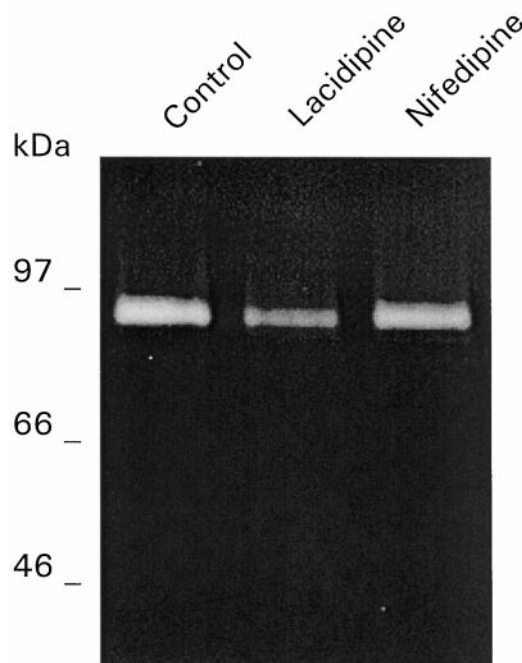


Fig. 1. Representative gelatin zymogram showing the effect of lacidipine (20 μ M) and nifedipine (50 μ M) on MMP-9 gelatinolytic capacity of human macrophages. Cells were incubated with the indicated compounds for 24 h.

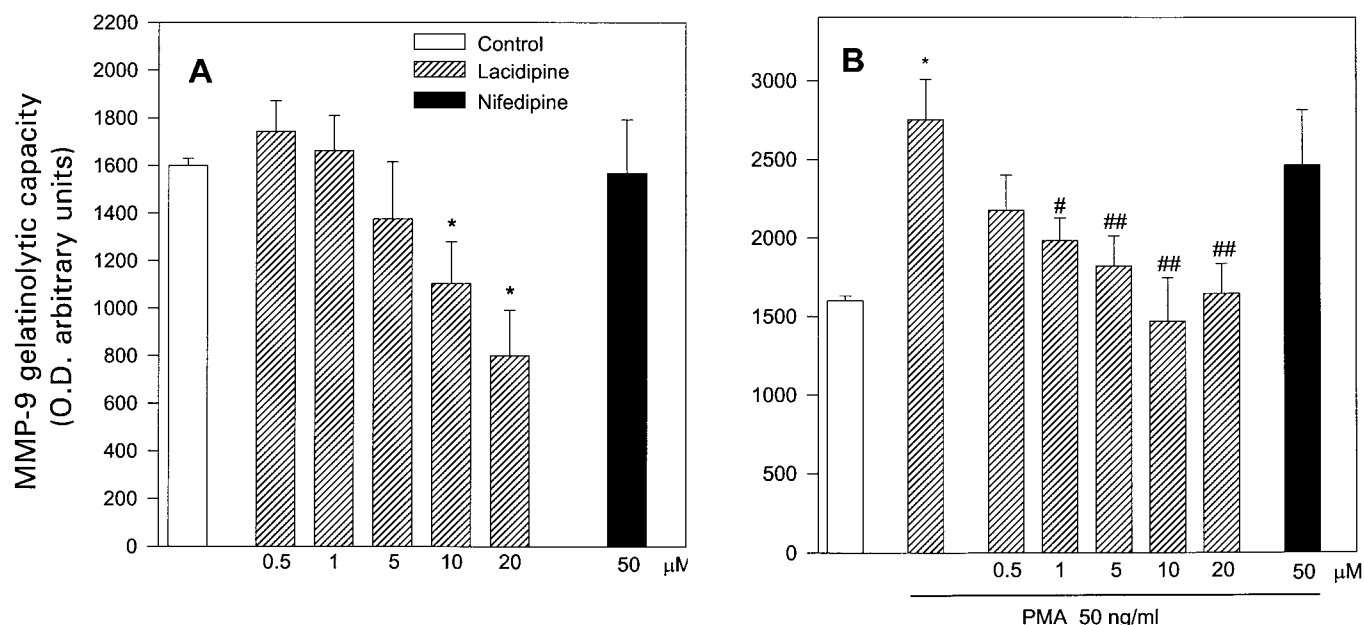


Fig. 2. Lacidipine inhibits MMP-9 gelatinolytic capacity in human macrophages. Cells were incubated for 24 h with DMEM containing increasing concentration of lacidipine in the absence (A) or presence (B) of phorbol esters (PMA, 50 ng/ml). Then media were collected and MMP-9 potential gelatinolytic capacity measured by gelatin zymography. Data were quantified by densitometric scanning and expressed as the mean \pm S.D. of three experiments performed in duplicate. * $P < 0.01$ versus control (not treated cells); # $P < 0.05$, ## $P < 0.01$ versus PMA alone.

we stimulated MMPs expression by incubating the cells with phorbol ester (PMA, 50 ng/ml). The treatment with PMA increased MMP-9 gelatinolytic capacity (70%, average stimulation obtained in three separate experiments) and lacidipine was still effective in inhibiting the enhanced lysis of the substrate by the protease by almost 50% (Fig. 2B). Inhibitory effects from 40 to 50% were observed also in mouse peritoneal macrophages.

The time course of the effect was monitored by incubating the cells with the drug and analyzing the gelatinolytic activity of MMP-9 released into the conditioned media at different time points (1–48 h). The inhibitory activity of lacidipine became evident after 2 h of incubation (data not shown). In another series of experiments, lacidipine inhibitory effect was maintained up to 48 h after the removal of the drug (data not shown).

To exclude possible drug toxicity as the reason of its inhibitory effect, human macrophages were incubated with lacidipine from 1 to 20 μ M and then cell viability was assessed using the dimethylthiazoldiphenyltetrazolium bromide assay. Lacidipine did not cause any appreciable cellular toxicity, even at the highest concentration used (20 μ M; data not shown).

It has been reported that TNF- α (a cytokine known to be present in atherosclerotic plaques) is able to stimulate the secretion of MMP-9 from human macrophages, without affecting TIMP-1 production (Saren et al., 1996). Therefore, we incubated human macrophages with TNF- α (20 ng/ml) and lacidipine (10 and 20 μ M) and, after 24 h, measured MMP-9 gelatinolytic capacity in the incubation media. As shown in Fig. 3, lacidipine maintained its inhibitory effect (up to 60%) also in the presence of TNF- α .

To ascertain how lacidipine could interfere with MMP-9 gelatinolytic potential, we performed a Western blot analysis of the media collected from human monocyte-derived macrophages incubated with the drug. As shown in Fig. 4, the

addition of lacidipine 20 μ M reduced the amount of MMP-9 protein actually released into the incubation media.

The above-mentioned data indicate that, in vitro, lacidipine treatment reduced the total gelatinolytic capacity of MMP-9 by inhibiting its secretion by human monocyte-derived macrophages. Since the final net level of proteinase activity depends on several factors such as the relative concentrations of active enzymes and specific inhibitors (i.e., TIMPs), we measured the amount of MMP-9 and of TIMP-1, its natural inhibitor secreted by human macrophages, using two specific ELISA systems. As shown in Table 1, the addition of lacidipine (10 and 20 μ M) dose dependently inhibited the secretion of both MMP-9 and TIMP-1 protein. We used, therefore, a different ELISA assay that allows the specific and quantitative measurement of the levels of MMP-9 not complexed with TIMP-1 released into the media. As expected, the data indicate that the amount of active, free MMP-9 released by human macrophages in culture is much less (about 1000-fold less) than the zymogen form, probably because of the absence of activating mechanism in this in vitro system. Lacidipine reduces free MMP-9, both in the active and the total (active plus pro-MMP-9) form (Table 2).

Next, we wanted to see whether the overall inhibitory effect of lacidipine on MMP-9 secretion was effectively paralleled by a reduction of the potential gelatinolytic capacity of the cells. The data reported in Fig. 5 indicate that lacidipine inhibited the actual amount of radioactive denatured collagen degraded by human macrophages.

We evaluated by Northern blot analysis whether the reduced levels of secreted MMP-9 in lacidipine-treated human monocyte-derived macrophages could reflect a reduction in MMP-9 gene expression. As shown in Fig. 6, Northern blots of human macrophages treated for 24 h with lacidipine demonstrated no appreciable reduction in the level of MMP-9 expression.

Then we analyzed the effect of lacidipine treatment on

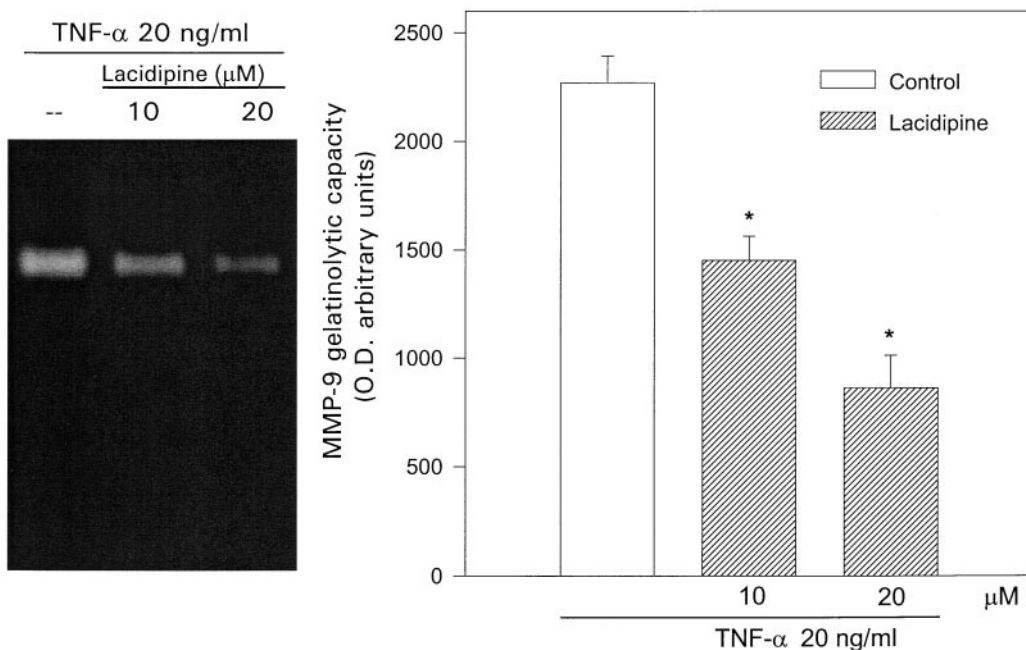


Fig. 3. Lacidipine inhibits MMP-9 gelatinolytic capacity in human macrophages incubated with TNF- α . Cells were incubated with DMEM containing TNF- α and lacidipine for 24 h. Then media were collected and MMP-9 potential gelatinolytic capacity measured by gelatin zymography. Data were quantified by densitometric scanning and expressed as mean \pm S.D. of three experiments performed in duplicate. * $P < 0.05$ versus TNF- α alone.

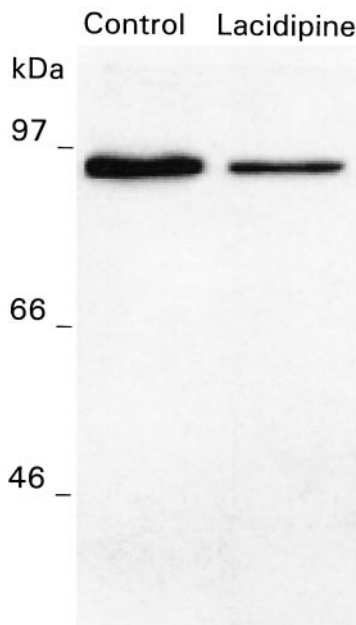


Fig. 4. Representative Western blot analysis of MMP-9 secretion by human macrophages. Cells were incubated for 24 h with DMEM containing or not 20 μ M lacidipine, and MMP-9 secretion assessed by Western blot analysis. Similar data were obtained in three separate experiments.

intracellular calcium concentrations. Human macrophages loaded with the fluorescent Ca^{2+} -sensitive probe fura-2 gave an intracellular Ca^{2+} basal concentration of 119.3 ± 19.2 nM. The addition of lacidipine in the incubation media determined no significant variation in Ca^{2+} concentration (112.3 ± 18.7 nM; $P = \text{N.S.}$).

Finally, to validate in an in vivo model our data, we measured ex vivo the gelatinolytic capacity of MMP-9 secreted by peritoneal macrophages obtained from CD-1 mice that had been treated for 14 days with lacidipine (1 or 3 mg kg^{-1} day $^{-1}$), given by gavage. These doses give a lacidipine-plasma level similar to those observed in humans (M. Quar-

TABLE 1

Effect of lacidipine on MMP-9 and TIMP-1 secretion by human macrophages

Human macrophages were incubated with DMEM containing the indicated concentrations of lacidipine for 24 h. Then the amount of secreted MMP-9 and TIMP-1 was measured by ELISA. Data are the mean \pm S.D. of two experiments performed in triplicate.

Additions	Secreted Protein	
	MMP-9	TIMP-1
μM	$\text{ng/ml medium} \pm \text{S.D.}$	
No addition	1620 \pm 28.8	94.1 \pm 1.1
Lacidipine (10)	1365 \pm 12.3*	62.3 \pm 2.3*
Lacidipine (20)	1140 \pm 32.4*	35.5 \pm 5.7*

* $P < 0.01$ versus control (not treated cells).

TABLE 2

Effect of lacidipine on the secretion of active and total free MMP-9 by human macrophages

Human macrophages were incubated as described in Table 1. Then media were collected and the amount of active and total free MMP-9 released by cells was measured by ELISA, as described under *Materials and Methods*. Data are the mean \pm S.D. of two experiments performed in triplicate.

Additions	Active Free MMP-9	
	Active Free MMP-9	Total Free MMP-9
μM	$\text{ng/ml medium} \pm \text{S.D.}$	
No addition	2.45 \pm 0.01	2720 \pm 68
Lacidipine (10)	1.60 \pm 0.01*	1968 \pm 38*
Lacidipine (20)	0.90 \pm 0.03*	1760 \pm 87*

* $P < 0.05$ versus control (not treated cells).

taroli, Glaxo Wellcome S.p.A., Verona, Italy, unpublished data). The data reported in Fig. 7 show that lacidipine significantly inhibited MMP-9 expression also in these experimental conditions.

Discussion

In this study, we have demonstrated that the in vitro incubation of human macrophages with lacidipine, a calcium antagonist with antiatherosclerotic properties, reduced the secretion of MMP-9 (gelatinase B). The experiments showed

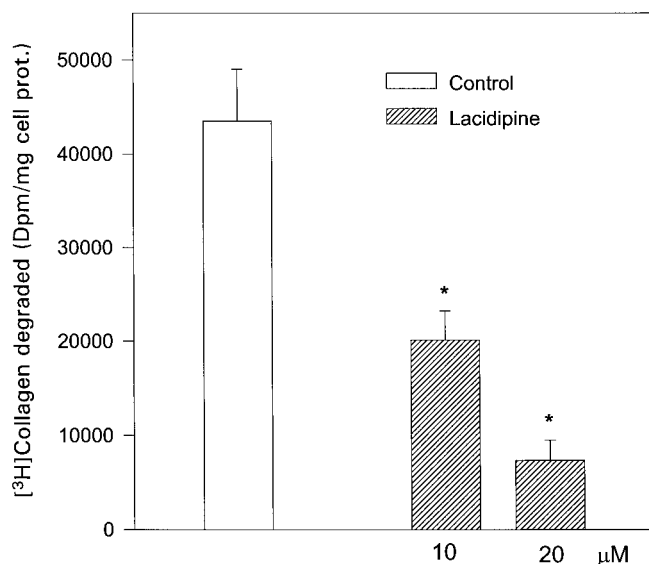


Fig. 5. Lacidipine reduces the degradation of collagen by human macrophages. Cells were incubated for 24 h with DMEM containing the indicated concentrations of lacidipine. Then media were collected, added with [³H]collagen (0.25 μCi/ml), and incubated in the absence of cells for 24 h. At the end, the amount of collagen degraded was measured by scintillation counting. Data are expressed as mean ± S.D. of three experiments performed in triplicate. **P* < 0.01 versus control (not treated cells).

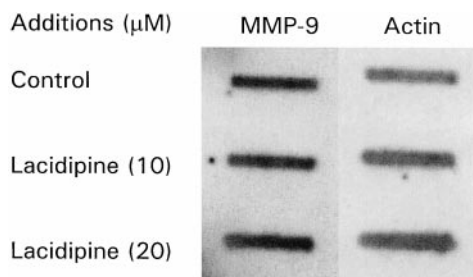


Fig. 6. Representative Northern blot analysis of the effect of lacidipine on MMP-9 mRNA levels in human macrophages. Cells were incubated with lacidipine (10 or 20 μM) for 24 h and RNA purified as described under *Materials and Methods*. Northern blot were prepared using 10 μg of total RNA and probed for MMP-9 expression. After exposing on X-ray film, the blot was stripped and reprobed for actin expression. Similar data were obtained in three separate experiments.

that lacidipine reduced MMP-9 total potential gelatinolytic capacity in gelatin zymography and that the effect of the drug was maintained also when MMP-9 production was induced by the coinubation with PMA or TNF-α, known inducers of MMPs gene transcription. Moreover, the ex vivo data showed that lacidipine also is effective in inhibiting MMP-9 gelatinolytic potential in peritoneal macrophages obtained from mice treated for 14 days with the drug. As evaluated by Western blot analysis and by ELISA, lacidipine reduced the amount of MMP-9 protein actually released by the cells, suggesting a direct effect on the secretion process.

Lacidipine reduces also the secretion of TIMP-1, the endogenous inhibitor of MMP-9 and of other MMPs. This effect could counteract the inhibitory action of lacidipine on MMP-9 final activity. However, our data show that the drug effectively reduces the amount of free MMP-9, not complexed with TIMP-1, both in the active and in the latent form. Consistently, the treatment of the cells with lacidipine resulted in an overall reduction of the gelatinolytic activity of the cells, even higher than that expected from the reduction of MMP-9

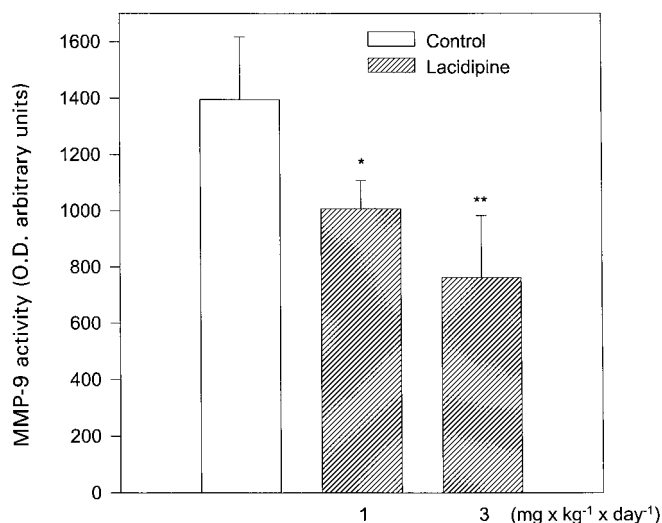


Fig. 7. Lacidipine reduces MMP-9 gelatinolytic capacity in mouse peritoneal macrophages. CD-1 mice (10 animals/group) received by gavage the indicated doses of lacidipine for 14 days, and then peritoneal macrophages were collected as described under *Materials and Methods*. Cells were incubated for 24 h with DMEM and MMP-9 potential gelatinolytic capacity measured by gelatin zymography. **P* < 0.05, ***P* < 0.01 versus control (macrophages obtained from mice treated with vehicle alone).

secretion, as judged by zymography, Western blot, and ELISA. The more striking inhibitory effect observed on collagen degradation could be due to the different sensitivity of the various detection methods used. Otherwise, lacidipine could have inhibited also the secretion of other MMPs with collagenolytic activity, such as MMP-2, MMP-8, and MMP-13 (Ikeda et al., 2000). New experiments are currently under way to further elucidate this matter.

The reduced levels of MMP-9 secreted into the media by human macrophages could also be the result of a lacidipine-mediated decrease of MMP-9 gene expression. Our data clearly indicate that this is not the case in that mRNA levels did not appreciably change in treated cells. This would suggest a mechanism of action of the drug affecting post-transcriptional processes of MMP-9. Otherwise, lacidipine treatment could cause a reduced translational efficiency combined with normal secretion of the translated product, but we do not have any evidence for such a mechanism.

Our data show that the treatment with the calcium antagonist lacidipine interferes with the gelatinolytic capacity of human macrophages in culture. Recent reports demonstrated an influence of intracellular calcium levels on the gelatinolytic activity of MMP-2 (Kohn et al., 1994), indicating that lower calcium levels lead to decreased MMP-2 activity. In contrast, Lohi and Keski-Oja (1995) found that higher calcium levels lead to decreased MMP-2 activity in human fibrosarcoma cells. Analogously, Roth et al. (1996) reported that lower intracellular calcium levels cause an increased proteolytic activity of MMP-2 and an inhibition of TIMP-2 transcription and of collagen deposition. In our experiments, nifedipine, the prototype dihydropyridine with high affinity with calcium channel, is completely inactive. In addition, the presence of lacidipine in the incubation medium did not affect intracellular Ca²⁺ levels, indicating that the effect of the drug on MMP-9 secretion process was independent of the variation in intracellular Ca²⁺ concentration.

The effect of lacidipine might be related to its physicochem-

ical properties. Unlike nifedipine, lacidipine is a highly lipophilic compound and binds in a prolonged way to lipid membranes (Herbette et al., 1993). This elevated lipophilicity allows its concentration in cellular membrane up to 800-fold (Herbette et al., 1993). Therefore, the presence of lacidipine could alter the composition of the biophase, thus interfering with MMP-9 secretion process. A similar hypothesis was proposed for explaining its effect on cholesterol esterification (Bernini et al., 1997).

Increased MMPs activity has been associated with a wide variety of pathological conditions such as arthritis, cancer, multiple sclerosis, and atherosclerosis (White, 1997). The potential utility of MMPs inhibitors in these diverse disease states is evident and several MMP inhibitors have now entered clinical development (Beckett and Whittaker, 1998). Among these, the orally active Marimastat (BB-2516) has reached phase III, although there are some reports of musculoskeletal syndrome characterized by severe joint pain and stiffness (Wojtowiczpraga et al., 1998). Inhibition of MMP activity has demonstrated a direct effect in reducing tumor cells invasion and angiogenesis (Brown et al., 1993a). While limiting our attention to the atherosclerosis field, the MMP inhibitor GM 6001 has been shown to block smooth muscle cell migration (Bendeck et al., 1996). The synthetic MMP inhibitor BB-94 (Batimastat) inhibits gelatinases A and B at nanomolar concentrations (Botos et al., 1996), and is able to reduce intimal thickening after arterial injury by decreasing both smooth muscle cells migration and proliferation (Zempo et al., 1996). These data support the conclusion that MMPs play a significant role in regulating intimal thickening in injured arteries and therefore in atherogenesis. Thus, MMP inhibition might play a beneficial role in the antiatherosclerotic therapy.

The concentrations of lacidipine active on MMP secretion used in our study are higher than those reported in plasma of treated patients. However, as mentioned before, lacidipine concentrates severalfold in arterial cells and its duration of action in vivo is unrelated to its plasma half-life (Herbette et al., 1993). Consistently, in our experimental conditions lacidipine retained its fully inhibitory activity up to 48 h after washout. In previous studies similar in vitro concentrations of lacidipine inhibited macrophage acyl-CoA:cholesterol acyltransferase activity and myocyte proliferation in cell culture systems (Bernini et al., 1993, 1997). These inhibitory effects were confirmed in vivo in rabbits at doses capable of inducing plasma concentrations of the drug similar to those observed in humans (Soma et al., 1996; Bernini et al., 1997). In our present study, mice were treated with doses of drug that give a lacidipine-plasma level similar to that observed in humans (M. Quartaroli, Glaxo Wellcome S.p.A., unpublished data). Macrophages obtained from these animals showed a reduced ex vivo ability to secrete MMP-9. These results support the concept that lacidipine, in vivo, at plasma levels of clinical relevance, may accumulate in the vessel wall and achieve concentrations active on cellular functions involved in atherogenesis.

We have previously shown that lacidipine inhibits cholesterol esterification and accumulation in the aortic wall. Our present data further characterize the potential beneficial effects of lacidipine in stabilizing the atherosclerotic plaque by demonstrating the inhibitory effect of the drug on MMP-9 secretion by macrophages.

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Correction to “Lalsoacidipine modulates the secretion of matrix metalloproteinase-9 by human macrophages”

In the above article (Bellosta S, Canavesi M, Favari E, Cominacini L, Gaviraghi G, Fumagalli R, Paoletti R and Bernini F (2001) *J Pharmacol Exp Ther* **296**:736–743), a printing error occurred in the title. “Lalsoacidipine” should be “Lacidipine”. We apologize for any inconvenience caused by this error.

Correction to “Pharmacokinetics and immunological effects of exogenously administered recombinant human B lymphocyte stimulator (BLyS) in mice”

In the above article (Parry TJ, Riccobene TA, Strawn SJ, Williams R, Daoud R, Carrell J, Sosnovtseva S, Miceli RC, Poortman CM, Sekut L, Li Y, Fikes J and Sung C (2001) *J Pharmacol Exp Ther* **296**:396–404), the authors regret that the axis label of Fig. 2D (page 400) is incorrectly labeled “Serum IgG Conc. (mg/dL)”. The correct label is “Serum IgG Conc. (ng/mL)”.