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Dihydropyridine Calcium Antagonist Modulates Cholesterol Metabolism and Eicosanoid Biosynthesis in Vascular Cells

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Abstract Recent clinical studies have shown that calcium channel blockers can retard and possibly reduce the angiographic progression of coronary artery disease. Calcium channel blockers also inhibit dietary-induced atherosclerosis in animal models of this disease. In this study, we delineate potential cellular and molecular mechanisms by which nicardipine, a dihydropyridine calcium antagonist, may alter lipoprotein and cholesterol trafficking, affect the regulatory signal transduction pathways involved in accelerating cholesteryl ester (CE) catabolism in vascular smooth muscle cells, and modulate cell-cell interactions of vascular and inflammatory cells. We demonstrate in arterial smooth muscle cells that nicardipine increases 1) LDL binding, uptake, and degradation, 2) RNA transcript levels for the LDL receptor, 3) CE catabolic activity, 4) PGI_2 release, and 5) RNA transcript levels for cyclooxygenase. Furthermore, nicardipine blocked cytokine-induced monocyte adhesion to endothelial cells and smooth muscle cells. Taken together, these findings support the hypothesis that nicardipine may function as an anti-atherosclerotic agent by promoting CE catabolism and cholesterol clearance and by reducing monocyte adhesion to the activated endothelium.

Key words: calcium channel blocker, atherosclerosis, LDL, LDL-receptor, vascular smooth muscle, PGI_2, cyclic AMP, cyclooxygenase

Calcium channel blockers can suppress the development of experimental atherosclerosis in animals (Henry et al., 1981; Watanabe et al., 1987), and clinical studies have demonstrated that these agents can retard the angiographic progression of coronary artery disease (Waters et al., 1990; Lichtlen et al., 1990). Calcium antagonists can modulate several important processes in the genesis of atherosclerosis and have been shown to: 1) inhibit platelet deposition and aggregation (Moore et al., 1985), 2) reduce intracellular lipid accumulation in vascular smooth muscle cells by enhancing lipolysis and lipid efflux (Etingin et al., 1985), 3) inhibit smooth muscle cell excitation-contraction coupling (Morgon et al., 1990), 4) inhibit smooth muscle cell migration and proliferation (Stein et al., 1987; Jackson et al., 1988), and 5) alter the binding of LDL to monocyte/macrophages and smooth muscle cells (Stein et al., 1985; Paoletti et al., 1988), the two types of progenitor cells which are converted to foam cells during atherogenesis.

In animal and in vitro models of vascular disease, calcium channel blockers reduce cholesterol accretion (Willis et al., 1985; Etingin et al., 1985, 1990). The mechanism(s) by which calcium channel blockers enhance lipolysis and eventual cholesterol efflux involves, in part, the cyclic AMP-dependent protein kinase cascade (Etingin et al., 1985). Phosphorylation by protein kinase A activates cytoplasmic cholesteryl ester (CE) hydrolase, promoting increased CE catabolic activity and cholesterol egress (Hajjar, 1986). Eicosanoids activate cyclic AMP dependent pathways in the cell, which in turn promote intracellular cholesterol turnover (Hajjar, 1986; Hajjar et al., 1982). Similar findings have been reported in vivo in vascular tissue derived from patients treated with calcium antagonists (Etingin et al., 1990).

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Calcium channel blockers also modulate LDL metabolism in vascular cells (Stein et al., 1987; Mori et al., 1988). We determined whether or not cholesterol delivery to the cell was altered in vascular smooth muscle cells treated with nicardipine by measuring LDL binding, internalization, degradation, and LDL-receptor gene expression. Since enhanced LDL receptor surface expression can lead to subsequent CE internalization, we also assessed intracellular metabolism of cholesterol. To evaluate the signal transduction mechanisms documented to regulate the CE cycle, we examined eicosanoid synthesis and cyclooxygenase gene expression since a major cyclooxygenase product, PGI_2, elevates CE metabolism by the protein kinase A pathway (Hajjar, 1986; Hajjar et al., 1982, 1983). Finally, monocyte adhesion to the activated endothelium in the presence of this calcium antagonist was assessed to determine if nicardipine alters this initial step in the pathway of monocyte infiltration into the vessel wall. Inhibition of monocyte adhesion to the vessel wall could potentially prevent their entry into the subendothelial space and subsequent accumulation of free and esterified cholesterol, a characteristic feature of atherosclerosis (Territo et al., 1984; Navab et al., 1988).

MATERIALS AND METHODS

Materials

Nicardipine was provided by Syntex (Palo Alto, CA). [1-^{14}C]cholesteryl oleate (55 mCi/mmol sp activity), [1-^{14}]oleic acid (40mCi/mmol sp act, ^{125}I-cAMP radioimmunoassay (RIA) kits, and Aquasol-2 liquid scintillation fluid were obtained from New England Nuclear, Boston, MA. Unlabeled CE and egg lecithin were obtained from Supelco, Inc., Bellefonte, PA. Fatty acid-free bovine serum albumin (BSA), neutral alumina (WN-3), 4-methoxyethanol, and sodium taurocholate were obtained from Sigma Chemical Company, St. Louis, MO. Organic solvents were obtained from Mallinckrodt, Inc. Science Products Division, St. Louis, MO. TNF was a gift from Dr. R. Nachman, Cornell University Medical College, New York, NY.

Disposable tissue culture materials were purchased from Corning Glass Works, Corning, NY. Tissue culture plates (Linbro) were purchased from Flow Laboratories, Inc., McLean, VA. Dulbecco's modified Eagle's medium (MEM) and Fungizone (250 μg/ml) were purchased from Flow Laboratories, Inc. L-glutamine (200 mM), penicillin (5,000 IU/ml), streptomycin (5 mg/ml), and fetal bovine serum were purchased from Gibco Laboratories, Grand Island, NY.

Tissue Culture

Rat aortic smooth muscle cells were isolated from thoracic aortae after the removal of adventitial tissue and the endothelium (Ross, 1971). Cells were confirmed to be smooth muscle cells by their growth pattern as observed by phase contrast microscopy and immunohistochemical staining of smooth muscle cell specific actin. Human smooth muscle cells were derived by explant from saphenous veins. Endothelial cells were isolated from human umbilical veins (Jaffe et al., 1973).

ACEH, NCEH, and ACAT Assays

To assess CE metabolic activity in response to nicardipine, smooth muscle cells were plated in wells (35 × 10 mm^2) at a density of 2 × 10^5 cells/well in Dulbecco's MEM with 10% fetal calf serum. The cells were allowed to adhere for 24–48 h. They were then washed twice with Dulbecco's MEM without serum. Isobutylmethylxanthine (MIX), a phosphodiesterase inhibitor, was also added to cells at a final concentration of 0.1 mM for 20 min before nicardipine addition, in order to maximize intracellular cAMP levels. MIX was prepared by dilution in Hepes-Hanks buffer and sonicated on ice for 15 min to enhance dissolution. Nicardipine (10 μM) was added to smooth muscle cells for 1 h at 37°C before harvesting.

For enzyme activity assays, cells were harvested using a rubber policeman after aspiration of the incubation medium and the subsequent addition of 2.0 ml ice-cold isotonic sucrose buffer. Cell preparations were briefly sonicated over ice for 30 s and aliquots were taken for the assay of lysosomal (acid) CE hydrolase (ACEH) activity (Haley et al., 1980), cytoplasmic (neutral) CE hydrolase (NCEH) activity (Hajjar et al., 1983), and CE synthetic (acyl coenzyme A: cholesterol o-acyltransferase (ACAT) activity. Activity of ACAT was assayed by measuring the synthesis of cholesteryl oleate from radioactive oleoyl coenzyme A (CoA) and exogenous free cholesterol (Hajjar et al., 1983). Oleoyl CoA and cholesterol were prepared as unilamellar liposomes as described by Hajjar et al. (Hajjar et al., 1983). Protein determinations were done by the method of Lowry et al. (Lowry et al., 1951).

Assay of Intracellular cAMP

Smooth muscle cells were incubated in media or media with nicardipine (0.1 μM) for 2 h. After supernatant removal, cells were harvested in isotonic sucrose buffer consisting of 250 mM sucrose, 10 mM Tris-HCl, and 0.1 mM EDTA (pH 7.3). cAMP was assayed by RIA (Hajjar et al., 1982).

Isolation and labeling of LDL. LDL (d = 1.019–1.063 g/ml) was prepared from human plasma and was isolated by ultracentrifugation (Havel et al., 1955). The LDL was dialyzed against HEPES-buffered saline with EDTA, sterilized by filtration through a 0.22 μm filter, and stored under nitrogen at 4°C. LDL preparations were routinely screened for peroxides (Marshall et al., 1985) and were used within 2 weeks after isolation. LDL was iodinated by the method of Bilheimer et al. (1972) as described by Goldstein et al. (1983).

LDL binding, internalization, and degradation. The binding, internalization, and degradation of LDL was performed according to the methods of Goldstein et al. (1983), using [^{125}I]-LDL. Smooth muscle cells were cultured in serum-free media alone (control) or media containing nicardipine (0.01–25 μM) for 19 h prior to the addition of [^{125}I]-LDL (10 μg/ml) for an additional 5 h at 37°C. Supernatants were assayed for trichloroacetic acid (TCA) precipitable and non-iodine TCA soluble radioactivity. The cells were washed 3 times with ice-cold phosphate buffered saline (PBS) containing 2 mg/ml of bovine serum albumin (BSA), then 2 times with PBS. The monolayer was incubated for 1 h with PBS containing 4 mg/ml dextran sulfate (Sigma). Dextran-releasable radioactivity was quantitated by gamma spectroscopy as a measure of receptor-bound LDL. The cells were then solubilized in 0.2 M NaOH as a measure of internalized LDL and for protein determination.

RNA isolation and Northern analysis. Total cellular RNA was isolated by the method of Chirgwin et al. (1979). Cells were harvested in 4.0 M guanidinium isothiocyanate (GIT buffer) containing 25 mM Na-acetate and 0.1% β-mercaptoethanol and centrifuged through 5.7 M CsCl containing 25 mM Na-acetate (pH 6.0). The RNA was solubilized in 0.3 M Na-acetate, precipitated in 70% ethanol, and stored at −70°C. RNA was quantified by UV spectrophotometry at 260 and 280 nm. Total cellular RNA was electrophoresed in a 1% denaturing agarose gel containing formaldehyde and ethidium bromide and was transferred to a nylon membrane (Zeta-probe). The filter was washed in 10 × SSC buffer (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate), air dried, and baked in a vacuum oven for 2 h at 80°C. The membranes were prewashed in 0.1 × SSC and 0.5% SDS for 1 h at 65°C, followed by pre-hybridization in 50% formamide, 0.25 M $NaHPO_4$ (pH 7.2), 0.2 M NaCl, 7% SDS, and 1.0 mM EDTA for 5 min at 43°C. The cDNA for the LDL receptor was purchased from ATCC (Rockville, MD). The 2.7 kb Sac II/Sma I fragment of the LDL receptor cDNA was used. The cDNAs for cyclooxygenase (constitutive-form of the enzyme) and GAPDH were a gift from Dr. David Dewitt (Michigan State University, E. Lansing, MI). Probes were labeled with [^{32}P]-dCTP by the method of random hexamer primer extension. The membranes were hybridized with the labeled probe for 16–24 h at 43°C in a shaking water bath. The filters were washed under high stringency conditions with a final wash in 0.1 × SSC and 0.1% SDS at 65°C, air dried, and exposed to Kodak XAR-5 film with intensifying screens at −70°C. The amounts of RNA were quantified by laser densitometry, and normalized by comparison to GAPDH.

PGI_2 levels. Smooth muscle cells were incubated in serum-free media (control) or serum-free media containing nicardipine (1 μM and 10 μM). PGI_2 levels in smooth muscle cell conditioned media were measured by radioimmunoassay of its stable hydrolysis product, 6-keto $PGF_{1\alpha}$ (Pomerantz et al., 1984).

Monocyte Adhesion Studies. U-937 monocytic cell lines were purchased from ATCC. Human umbilical vein endothelial cells were subpassaged 1–3 times prior to use and confirmed to be endothelial cells by immunofluorescent staining with von Willebrand factor antiserum. All cells in a single experiment were obtained from the same umbilical cord. Mononuclear cell adhesion to endothelial cells was measured as described by DiCorleto and de la Motte (DiCorleto et al., 1985), using ^{51}chromium-labeled U-937 cells. In each experiment, monocytes were used at greater than 10-fold excess over the number of endothelial cells. Labeled monocytes (1 × 10^6/well) were added to monolayers of endothelial cells or smooth muscle cells in 24-well plates and incubated at 4°C following a 2 h incubation with TNF and/or nicardipine. After 2 h culture me-

dia containing nonadherent mononuclear cells was removed, and the wells were gently rinsed twice with culture media. The cells were solubilized in 0.2% NaOH for 1 h prior to counting in a gamma counter. Nonspecific adherence of monocytes to blank wells ranged from 1–4%.

Statistical analyses. All data are expressed as mean ± SEM, and analyzed by either Students t-test or analysis of variance. Differences with $P < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Calcium channel blockers diminish the accumulation of arterial cholesterol and cholesteryl esters in dietary-induced atherosclerosis in animal models and alter cholesterol metabolism in cultured vascular cells. We evaluated possible mechanisms by which nicardipine, a drug in the class of dihydropyridine calcium antagonists, could alter several critical processes related to smooth muscle cell lipid metabolism and monocyte/endothelial cell interactions that are central to the pathogenesis of atherosclerosis.

Lipid accretion within the vessel wall may result from changes in the delivery or intracellular processing of LDL-cholesterol. To determine the potential mechanisms by which nicardipine could alter lipid accretion in the vessel, we evaluated the effects of nicardipine on cholesterol delivery processes. Nicardipine increased the binding, internalization, and degradation of ^{125}I-LDL in vascular smooth muscle cells in a dose-dependent manner (Fig. 1). The maximum effects were observed at a 25 μM concentration of nicardipine. Binding was increased four-fold, cell-associated LDL increased almost three-fold, and degradation increased by 50%. These results are in agreement with previous studies which evaluated the effects of verapamil on LDL binding and degradation in vascular smooth muscle cells (Stein et al., 1985). In contrast, Mori et al. (1988) reported that nicardipine increased smooth muscle cell associated LDL, had no effect on LDL binding, and decreased LDL degradation. The reason(s) for this difference is unclear.

To determine the mechanism by which nicardipine exerts its effect on LDL receptor cell surface expression, we next evaluated LDL receptor gene expression by Northern analysis. Arterial smooth muscle cells treated with nicardipine for 24 h had a 3.5-fold increase in the steady state levels of LDL receptor mRNA (Fig. 2), when compared with a housekeeping gene,

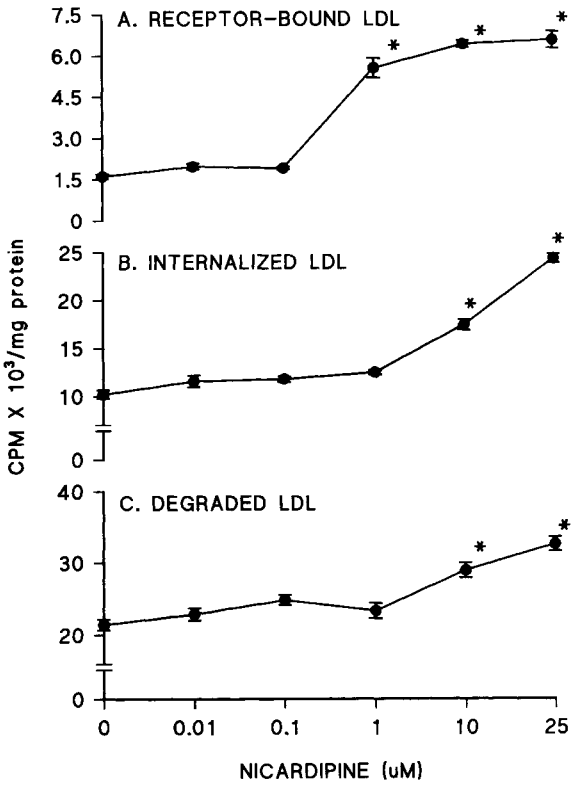

Fig. 1. Effect of nicardipine on LDL binding, uptake, and degradation. Smooth muscle cells were grown in 24 mm 12-well cluster plates in serum-free media or serum free-media with increasing concentrations of nicardipine for 19 h prior to the addition of 10 μg/ml ^{125}I-LDL. After 5 h at 37°C, degraded LDL was assayed in the supernatant, surface bound LDL was assessed after release by 4 mg/ml dextran sulfate in PBS, and cell associated LDL was determined after cell monolayers were solubilized in 0.2 M NaOH. The data are expressed as cpm/mg cell protein ± SEM; each treatment performed in quadruplicate. Nicardipine stimulated LDL binding and degradation in vascular smooth muscle cells. * = $P < 0.05$ vs. control.

GAPDH. Thus, nicardipine increases LDL receptor surface expression by upregulating expression of the gene encoding the receptor. The molecular mechanism by which this occurs is unclear, but most agents and second messengers which increase LDL-receptor mRNA steady state levels act at the transcriptional level (Auwerx et al., 1989; Auwerx et al., 1989; Mazzone et al., 1990).

Normally, lipoprotein bound cholesteryl ester (CE) which enters the cell by receptor-mediated endocytosis is delivered to the lysosome, where it is hydrolyzed by the lysosomal enzyme, acid cholesteryl ester hydrolase (ACEH) (Brown et al., 1986). The liberated free cholesterol is then re-esterified with fatty acids by ACAT to form cytoplasmic CE, which is hydrolyzed by the cytoplasmic (neutral) CE hydrolase (NCEH). The

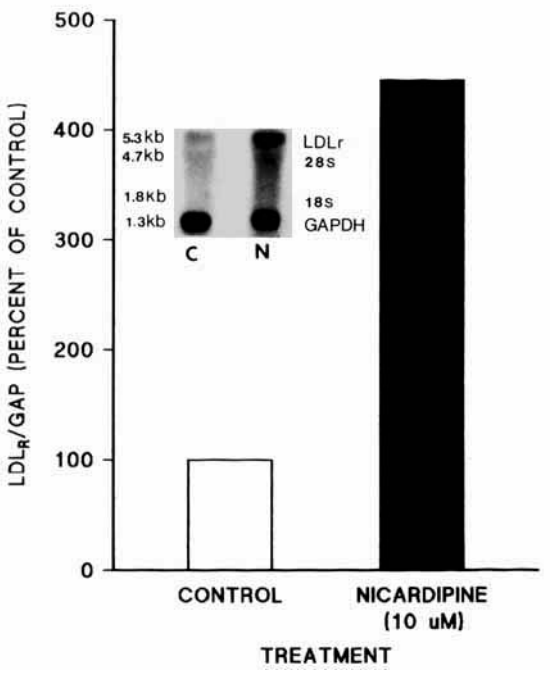

Fig. 2. Nicardipine increases LDL receptor mRNA steady state levels in human smooth muscle cells. Human saphenous vein smooth muscle cells were grown in media containing lipoprotein deficient serum (5 mg/ml) with or without 10 μM nicardipine for 24 h. Nicardipine treated smooth muscle cells had a 3.5-fold increase in LDL-receptor mRNA steady state levels when compared with GAPDH.

activities of ACEH and NCEH are increased by cyclic AMP (cAMP) and protein kinase A (Hajjar, 1986). We tested the hypothesis that nicardipine may also enhance lysosomal and cytoplasmic CE hydrolytic activity. NCEH activity was significantly increased (three-fold) in nicardipine treated smooth muscle cells and ACEH activity was significantly increased by 20% (Fig. 3). We have previously shown that nifedipine and verapamil also increased CE hydrolytic activity commensurate with increased cyclic AMP levels (Etingin et al., 1985). In this regard, we found a four-fold increase in cyclic AMP in nicardipine treated smooth muscle cells (Fig. 4), which is two-fold greater than the effects observed with nifedipine or verapamil.

The increase in cyclic AMP in response to nicardipine is related, in part, to its effect on PGI_2, a major vasodilator, and platelet disaggregator. ACEH and NCEH activities are increased in response to exogenous and endogenous eicosanoids, including PGI_2 (Hajjar et al., 1982, 1983; Hajjar, 1986). To determine if nicardipine could influence cyclic AMP-dependent CE hydrolytic activity by increasing the production of PGI_2, we evaluated the effect of nicardipine on PGI_2 synthesis in smooth muscle cells. Increased PGI_2 production by vascular cells in response to nifedipine has been previously demonstrated in aortic rings in vitro (Weiss et al., 1989). We found that release of PGI_2 was increased two-fold in response to nicardipine treatment (Fig. 5). The increase was paralleled by a similar increase in cyclooxygenase mRNA steady state levels (Fig. 6). Cyclooxygenase is the enzyme which converts phospholipid-derived arachidonic acid to various prostanoids, including PGI_2. This suggests that nicardipine is act-

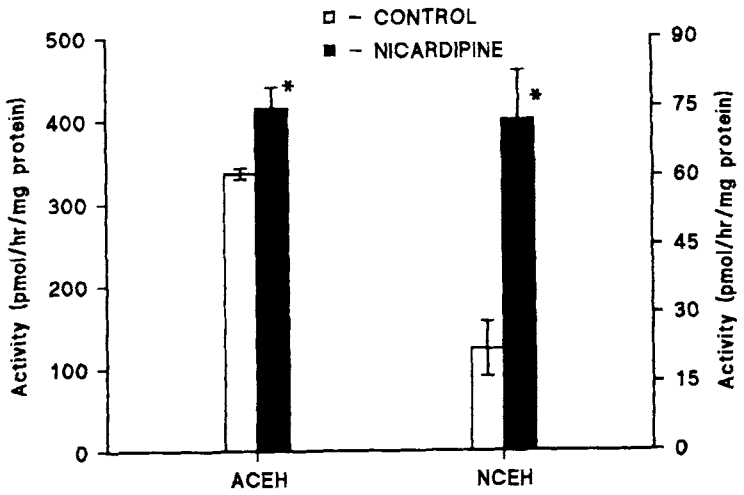

Fig. 3. Effect of nicardipine on SMC CE hydrolytic activities. Acid and neutral CE hydrolase activity was assayed in aortic SMC cultured in media alone or media containing 10 μM nicardipine, in 35 mm 6-well plates. ACEH activity was increased by 20% and NCEH activity was increased three-fold in nicardipine treated cells. The data are expressed as mean ± SEM; each treatment was performed in triplicate, n = 4. * = $P < 0.05$ vs. control.

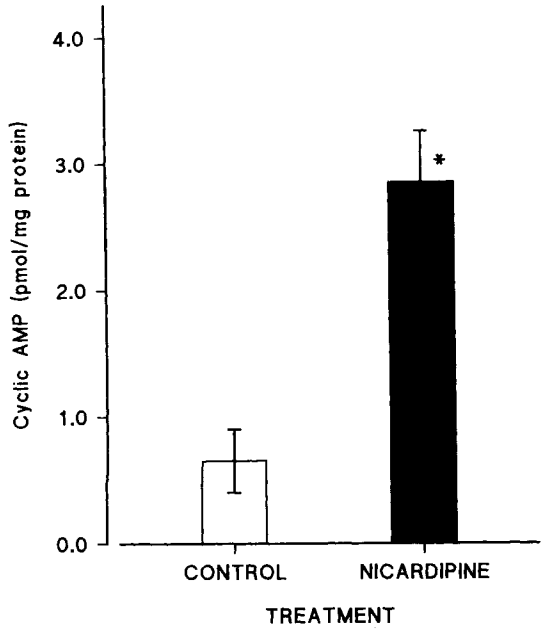

Fig. 4. Effect of nicardipine on intracellular cyclic AMP production by human smooth muscle cells. Cyclic AMP was assayed by RIA in cellular homogenates of SMC grown in media or in media with 0.1 μM nicardipine (mean ± SEM, each treatment performed in triplicate). Nicardipine produced a four-fold increase in cyclic AMP production in these cells. * = $P < 0.05$ vs. control.

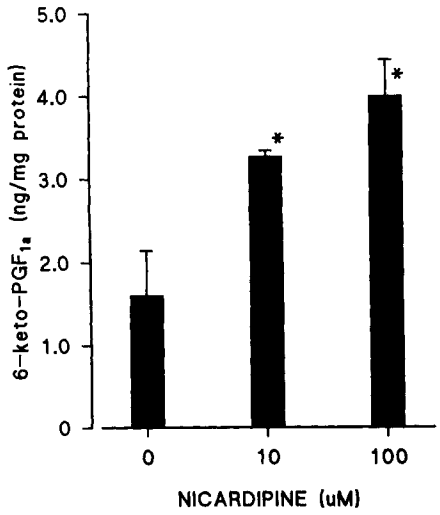

Fig. 5. Dose effects of nicardipine on PGI_2 production by rat arterial smooth muscle cells: Rat smooth muscle cells grown to confluent density in 35 mm 6-well cluster plates were incubated in serum-free M-199 containing media alone (control), 10 μM nicardipine, and 100 μM nicardipine. After 24 h incubation at 37°C, 6-keto-$PGF_{1\alpha}$ was determined in supernatants by radioimmunoassay. Data is expressed as ng 6-keto-$PGF_{1\alpha}$/mg protein (mean ± SEM, each treatment performed in triplicate). * = $P < 0.05$ from control.

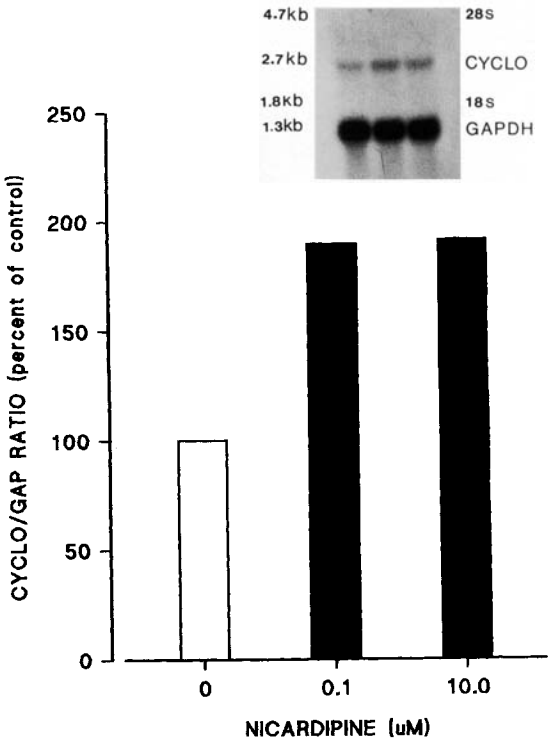

Fig. 6. Nicardipine induces a two-fold increase in cyclooxygenase mRNA steady state levels. Total cellular RNA was extracted from rat aortic smooth muscles cells grown in media alone or media containing 0.1 and 10.0 μM nicardipine for 24 h. There is a two-fold induction of cyclooxygenase mRNA in both groups of nicardipine treated cells when compared with control cells (0) and normalized to GAPDH, a constitutively expressed gene.

ing by increasing transcription of the cyclooxygenase gene or by increasing the half-life of the message. In addition to the implications of increased PGI_2 on cholesterol metabolism, an increase in PGI_2 in vivo by nicardipine could block platelet deposition and activation, which are thought to be early pro-atherosclerotic events in the genesis of vascular lesions.

ACAT (acyl-coenzyme A cholesterol acetyltransferase) is the major intracellular enzyme which esterifies free cellular cholesterol after it is hydrolyzed in the lysosome. Nicardipine had no significant effect of ACAT activity (data not shown).

Lastly, we evaluated the effect of nicardipine on monocyte adhesion to cultured endothelial cells. One of the earliest events in animal models of dietary-induced atherosclerosis is the binding of monocytes to the vascular endothelium. These monocytes are thought to be the progenitors of lipid-enriched macrophage foam cells. Cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), enhance monocyte adhe-

TABLE I. Effects of Nicardipine on Monocyte Adhesion to Vascular Endothelial Cells and Smooth Muscle Cells*

Treatment	No. monocytes/ EC	No. monocytes/ SMC
Control	0.3 ± 0.1^{a}	1.7 ± 0.1^{c}
TNF (50 ng/ml)	$1.5 \pm 0.1^{a,b}$	$3.4 \pm 0.2^{c,d}$
Nicardipine (0.1 μM)	0.5 ± 0.1	2.2 ± 0.3
TNF and nicardipine	0.9 ± 0.1^{b}	2.1 ± 0.1^{d}

*Effects of nicardipine on monocyte adhesion to cytokine activated endothelial cells (EC) and smooth muscle cells (SMC). Human umbilical vein endothelial cells and human saphenous vein smooth muscle cells in 24-well plates were incubated for 2 hr at 37°C in media alone, with TNF (50 ng/ml), with 0.1 μM nicardipine, or TNF and nicardipine. ^{51}Cr labeled U-937 monocytes were added and allowed to adhere for 2 hr at 4°C. The data are expressed as monocytes/EC or monocytes/SMC ± SEM, each treatment performed in quadruplicate. Groups which were significantly different from each other ($P < 0.05$) are indicated by the superscripted letters.

sion to endothelial cells by increasing the surface expression of adhesion molecules for monocytes (Pober et al., 1990). In Table I we show that nicardipine decreased the binding of U-937 cells (a human monocyte-like cell line) to cytokine (TNF) activated endothelial cells and smooth muscle cells. The effect of calcium channel blockers in modulating monocyte/endothelial cell interaction has not been previously addressed. The mechanism by which nicardipine modulates monocyte/endothelial binding is unclear, but implies that expression of cell surface adhesion molecules for monocytes may be dependent on intracellular calcium levels or transmembrane Ca^{++} flux. Thus, in cytokine activated endothelial cells and smooth muscle cells, nicardipine would have a beneficial effect by inhibiting monocyte adhesion, an early event in atherosclerosis.

Taken together, these studies address several potential mechanisms by which nicardipine may impact on the atherosclerotic process. We demonstrate that treatment of vascular smooth muscle cells with nicardipine results in increased binding, clearance, and metabolic processing of LDL-CE, and that this increase reflects an increase in mRNA transcript levels of the LDL receptor. Increased signal transduction responses in the form of PGI_2 and cyclic AMP production in cells treated with nicardipine results in upregulation of the activity of enzymes (ACEH and NCEH) which hydrolyze cholesteryl ester. The effect of nicardipine on PGI_2 is mediated through its effect on cyclooxygenase mRNA steady state levels. Finally, we demonstrate that nicardipine inhibits agonist (TNF)-induced monocyte adhesion to endothelial cells and smooth muscle cells. Our results delineate potential mechanisms by which nicardipine can act as an anti-atherosclerotic agent by altering critical pathophysiological processes associated with atherosclerosis.

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