Nifedipine Increases Cholesteryl Ester Hydrolytic Activity in Lipid-laden Rabbit Arterial Smooth Muscle Cells

A Possible Mechanism for its Antiatherogenic Effect

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Abstract

Calcium and cholesterol (CHOL) accumulation are characteristic features of human atherosclerotic plaques. Calcium channel blockers have been shown to increase calcium levels in myocardial cells and suppress free and esterified CHOL deposition in arteries of CHOL-fed animals.

To test the hypothesis that Nifedipine alters CHOL metabolism, thereby decreasing free and esterified CHOL accumulation in smooth muscle cells (SMC), we cultured arterial SMC from rabbits fed a normal or egg-supplemented diet for 6 mo. Cultured cells were treated with 0.1 mg/liter Nifedipine every 3 d during a 1-wk experiment. Although Nifedipine significantly increased lysosomal and cytoplasmic cholesteryl ester (CE) hydrolase activity in normal SMC via increased levels of intracellular cyclic AMP, no change in total CHOL content was observed after 1 wk of Nifedipine treatment. Contrary to these observations, lipid-laden SMC demonstrated a significant 50% loss in CHOL and CE after treatment with Nifedipine, due in part to the observed increase in CE hydrolytic activities. These data support our hypothesis that Nifedipine decreases CHOL and CE accumulation in arterial SMC by increasing arterial CE hydrolysis.

Introduction

Hypocalcemic agents, calcium channel blockers, and other calcium antagonists have been shown to reduce arterial lipid accumulation in animals without altering serum cholesterol (CHOL)¹, blood pressure, or heart rate (1). Conversely, agents that promote calcium deposition, such as vitamin D₃, have been shown to enhance atherogenesis (2).

The role of calcium in atherogenesis and the mechanisms leading to the reduction of cellular lipid by calcium antagonists

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1. *Abbreviations used in this paper:* β -Gal, β -galactosidase; ACAT, acyl coenzyme A cholesterol o-acyltransferase; ACEH, lysosomal (acid) cholesteryl ester hydrolase; CE, cholesteryl ester; CHOL, cholesterol; CoA, coenzyme A; MEM, modified Eagle's medium; MIX, isobutylmethylxanthine; NCEH, cytoplasmic (neutral) CE hydrolase; Neut-glu, neutral- α -glucosidase; SMC, smooth muscle cells.

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have yet to be elucidated. Although studies have recently shown that Nifedipine treatment did not alter atherogenesis in either rabbits fed a very high CHOL diet (2%) (3) or Watanabe heritable hyperlipidemic rabbits (4), we report a cellular mechanism that could explain the previous *in vivo* findings of several investigators (1, 4-8), that the treatment of animals, fed lower concentrations of CHOL, with calcium antagonists suppresses atherogenesis without reducing hypercholesterolemia.

To elucidate the mechanism(s) responsible for the reduction of cholesteryl ester (CE) accumulation in arteries after Nifedipine treatment, we examined the effects of Nifedipine on arterial CE hydrolysis, CE synthesis, and cyclic AMP (cAMP) levels in cultured arterial smooth muscle cells. Our results show that (a) pharmacological doses of Nifedipine can significantly enhance intracellular cAMP levels in cultured arterial smooth muscle cells, and (b) this enhancement of cAMP causes a concomitant increase in the activities of lysosomal and cytoplasmic CE hydrolases, thus contributing to a decrease in CHOL and CE in lipid-laden cells over 1 wk. In this study, we have identified for the first time a possible cellular mechanism for the antiatherogenic effect of Nifedipine.

Methods

Materials. [1-¹⁴C]cholesteryl oleate (55 mCi/mmol sp act), [1-¹⁴C]oleic acid (40 mCi/mmol sp act), ¹²⁵I-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. Acetylsalicylic acid, fatty acid-free bovine serum albumin (BSA), 1-methyl-3-isobutylmethylxanthine, neutral alumina (WN-3), 4-methylumbelliferyl- β -D-galactopyranoside monohydrate, 4-methoxyethanol, and sodium taurocholate were obtained from Sigma Chemical Company, St. Louis, MO. Thin-layer silica gel chromatoplates (K6; 250- μ m thick) were obtained from Whatman, Inc., Clifton, NJ. Nanograde quality organic solvents were obtained from Mallinckrodt, Inc., Science Products Div., St. Louis, MO. SQ-22536 was a gift from Dr. D. Harris of Squibb Corp., Princeton, NJ; and Nifedipine was a gift from Dr. E. Weiss of Pfizer Inc., 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 μ g/ml) were purchased from Flow Laboratories, Inc. L-glutamine (200 mM), penicillin (5,000 IU/ml), streptomycin (5 mg/ml), and fetal bovine serum (heat inactivated) were purchased from Gibco Laboratories, Grand Island, NY.

Experimental animals. Young New Zealand white female rabbits weighing 2.5-3.0 kg were used as a source of cultured thoracic arterial smooth muscle cells (SMC). Rabbits were fed commercial rabbit chow (Ralston Purina Co., St. Louis, MO) or an egg-supplemented diet (one egg per 60 g rabbit chow). Those rabbits fed a normal rabbit chow or an egg-supplemented diet for 6 mo had average serum cholesterol levels of 65 $\pm$ 4 mg/dl and 291 $\pm$ 9 mg/dl (mean $\pm$ SE), respectively.

Bovine arterial SMC were obtained from bovine thoracic arteries supplied by a local abattoir.

Tissue culture. Freshly isolated rabbit SMC were removed from thoracic arteries by the method of Haley et al. (9). Cells cultured from thoracic arteries were confirmed to be SMC by their growth pattern as observed by phase contrast microscopy and ultrastructural characteristics using transmission electron microscopy. Bovine SMC were cultured from arterial explants after the removal of adventitial tissue according to modification of the techniques of Ross (10).

Biochemical methods. To assess CE metabolic activity in response to Nifedipine, SMC were plated in wells ($35 \times 10 \text{ mm}^2$) at a density of 2×10^5 cells/well in Dulbecco's MEM with 10% fetal calf serum. Cells were allowed to adhere for 24–48 h. Cells were then washed twice with Dulbecco's MEM without serum. Various concentrations of Nifedipine were added to SMC for 2 h at 37°C before harvesting and assay of enzyme activities. Bovine arterial SMC used in these experiments were subpassaged 2–6 times. Rabbit arterial SMC used in these experiments were subpassaged only once. When required, SQ-22536, an adenylate cyclase inhibitor, was added to wells at a final concentration of 0.1 mM for 30 min before Nifedipine addition to inhibit cAMP production.

Isobutylmethylxanthine (MIX), a phosphodiesterase inhibitor, was also added to cells at a final concentration of 1.0 mM for 20 min before Nifedipine addition to maximize intracellular cAMP levels. MIX was prepared by dilution in Hepes-Hanks' buffer and sonicated on ice for 15 min to enhance dissolution.

Experiments were also conducted to determine if the activities of various cell marker enzymes were altered by adding Nifedipine. Activities of neutral- α -glucosidase, a microsomal marker enzyme, and β -galactosidase, a lysosomal marker enzyme, were assayed similar to the general experimental design described for the assay of CE metabolic activities (11, 12). Nifedipine was prepared in 10 mM Na_2CO_3 after initial dilution in 5 μl chloroform. Buffer controls consisted of the organic solvent appropriately diluted in 10 mM Na_2CO_3 buffer without Nifedipine.

Assay of intracellular cAMP. 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). Assays of intracellular levels of cAMP in the arterial SMC were done by the method of Hajjar et al. (13) with RIA kits (14). Recoveries averaged 80%.

Cell preparation for metabolic studies. 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 from the assay of lysosomal (acid) CE hydrolase (ACEH) activity, cytoplasmic (neutral) CE hydrolase (NCEH), CE synthetic (acyl coenzyme A: cholesterol o -acyltransferase; ACAT) activity, marker enzyme activities, and protein. Protein determinations were done by the method of Lowry et al. (15). BSA was used as a standard.

ACEH activity. ACEH activity in arterial SMC was assayed at pH 3.9 as described by Haley et al. (9).

NCEH activity. Cholesteryl [$1\text{-}^{14}\text{C}$]oleate was used as substrate in a mixed micelle of egg-lecithin/taurocholate/cholesteryl oleate (12). NCEH activity in arterial SMC was assayed by methods described previously by Hajjar et al. (12).

ACAT activity. Activity of ACAT was assayed by measuring the synthesis of cholesteryl oleate from radioactive oleoyl coenzyme A (CoA) and exogenous free cholesterol (16). Oleoyl CoA and cholesterol were prepared as unilamellar liposomes as described by Hajjar et al. (16). All assay conditions for ACAT in arterial SMC were optimized.

Marker enzymes. Neutral- α -glucosidase (cytoplasmic) and β -galactosidase (lysosomal) activities were assayed in aliquots of SMC homogenates as previously described by Peters et al. (17).

Units of activity. For lysosomal and cytoplasmic CE hydrolase and ACAT, 1 U of activity corresponds to 1 nmol substrate hydrolyzed or esterified per min. For marker enzymes, 1 U of activity corresponds

to 1 nmol of substrate hydrolyzed per hour. Activities were expressed as units per milligram protein.

Effect of Nifedipine on CHOL and CE accumulation. The effects of Nifedipine on CHOL and CE accumulation were assessed in cultured rabbit arterial SMC over 1 wk. Freshly isolated arterial cells were plated into culture dishes as described previously on the day of enzymatic removal from arteries of rabbits fed a normal or CHOL supplemented diet. These experiments were not done with bovine SMC since it was not possible to obtain freshly isolated lipid-laden bovine SMC.

On days 0, 3, and 6 of the experiment, Nifedipine (100 $\mu\text{g}/\text{liter}$) was added to each well containing serum, 0.1% BSA, and confluent cells (ca 3.0×10^5 cells/well). Serum albumin and high density lipoproteins (HDL) in the serum served as cholesterol acceptors. Cells were washed before adding Nifedipine at days 0, 3, and 6. Cells were harvested at days 0, 4, and 7 to assess their CHOL and CE content. Control groups of cells were maintained using Nifedipine buffer and culture medium only. Cell viability and ultrastructural characteristics were examined. No changes were seen after adding Nifedipine.

Before the cells were harvested, they were washed twice with serum-free medium, and their lipids were extracted twice in situ with hexane/isopropanol, 3:2 (vol/vol) (15). Lipid extracts were stored at -70°C under N_2 (g) until lipid analyses were performed (within 1 wk). Cells were removed from wells after lipid extraction by adding 1 ml 0.2N NaOH for 1 h for protein determination (18).

Lipid analysis. Analysis of CHOL and CE in SMC was done after evaporating samples to dryness under N_2 (g) by methods described extensively elsewhere. Samples were resuspended in 100–200 μl chloroform/methanol (2:1, vol/vol). The extract and a lipid standard containing CHOL and CE (1 $\mu\text{g}/\mu\text{l}$) were fractionated separately by thin layer chromatography (19). A two-solvent system was used to separate CHOL and CE (20). These lipids were quantitated by scanning microfluorometric analysis (21).

Statistical analysis. Mean ACEH, NCEH, ACAT, and marker enzyme activities after the addition of increasing concentrations of Nifedipine were compared with the use of single factor analysis of variance (22). Activity of ACEH in the presence of the SQ-22536 inhibitor was compared with the use of a two-factor analysis of variance, as was the lipid quantitation data.

Results

Effects of varying concentrations of Nifedipine on lysosomal and cytoplasmic CE hydrolase activities in cultured bovine arterial SMC are shown in Table I. At pharmacologic concentrations, 20–100 $\mu\text{g}/\text{liter}$ Nifedipine significantly increased ($P < 0.005$) lysosomal ACEH activity by twofold compared with cells treated with buffer alone. NCEH activity was modestly but significantly increased ($P < 0.05$) after the addition of 50–

Table I. Effect of Nifedipine on CE Metabolic Activities in Intact Cultured Bovine Arterial SMC

	ACEH activity	NCEH activity	ACAT activity
	U/mg protein	U/mg protein	U/mg protein
Untreated cells	34.3 $\pm$ 0.9	4.2 $\pm$ 0.3	15.0 $\pm$ 0.7
Nifedipine, 20 $\mu\text{g}/\text{liter}$	63.2 $\pm$ 2.6	3.4 $\pm$ 0.5	16.4 $\pm$ 0.9
Nifedipine, 50 $\mu\text{g}/\text{liter}$	69.5 $\pm$ 4.7	6.3 $\pm$ 0.3	16.5 $\pm$ 0.7
Nifedipine, 100 $\mu\text{g}/\text{liter}$	50.4 $\pm$ 6.0	5.5 $\pm$ 0.2	16.5 $\pm$ 2.1

Cells were incubated for 2 h in the presence of 0–100 $\mu\text{g}/\text{liter}$ Nifedipine. Range indicates mean $\pm$ SD for four separate analyses.

100 $\mu\text{g/liter}$ Nifedipine (Table I). Regarding CE synthetic activity, ACAT activity was not altered by adding variable amounts of Nifedipine (Table I). In both normal and lipid-laden rabbit arterial SMC, the activities of ACEH and NCEH are similarly increased in the presence of Nifedipine, as shown in Table II. ACAT activity was not altered by the presence of Nifedipine. Consistent with our previous *in vivo* reports, ACAT activity is increased in aortic SMC by diet-induced hypercholesterolemia (23).

When we assayed the activity of the lysosomal marker enzyme β -galactosidase (β -Gal) or the microsomal marker enzyme neutral- α -glucosidase (Neut-glu), their activities were not altered in either normal or lipid-laden rabbit SMC by adding Nifedipine. β -Gal (controls): 0.40 ± 0.08 U/mg protein, 20 $\mu\text{g/liter}$; Nifedipine: 0.42 ± 0.09 U/mg protein; Neut-glu (controls): 1.97 ± 0.14 U/mg protein; 20 $\mu\text{g/liter}$ Nifedipine: 2.20 ± 0.20 U/mg protein. Similar results were obtained in lipid-laden SMC. β -Gal (lipid-laden controls): 0.42 ± 0.10 , 20 $\mu\text{g/liter}$; Nifedipine: 0.42 ± 0.14 ; Neut-glu (lipid-laden controls): 2.1 ± 0.15 ; 20 $\mu\text{g/liter}$ Nifedipine: 2.4 ± 0.30 .

Intracellular levels of cAMP in cultured bovine arterial SMC were increased by adding 20–100 $\mu\text{g/liter}$ Nifedipine by two- to threefold (Table III). Moreover, the phosphodiesterase inhibitor (MIX) increased base-line cAMP levels, which could then be significantly augmented by adding 20–50 $\mu\text{g/liter}$ Nifedipine (Table III).

To determine if the increase of CE hydrolytic activities by Nifedipine depends on the enhancement of cAMP, an adenylate cyclase inhibitor, SQ-22536, was added to the cultured cells before adding Nifedipine. Rabbit SMC pretreated with SQ-22536 were assayed for ACEH and NCEH activities in response to 0–50 $\mu\text{g/liter}$ Nifedipine (Table IV). In the 0–50

Table II. Effect of Nifedipine on CE Hydrolase Activities in Normal and Lipid-laden Rabbit Arterial SMC

	ACEH activity	NCEH activity	ACAT activity
	U/mg protein	U/mg protein	U/mg protein
Normal cells, untreated	16.7 ± 3.5	0.9 ± 0.1	15.0 ± 0.7
Normal cells, 20 $\mu\text{g/liter}$ Nifedipine	29.2 ± 6.9	2.1 ± 0.5	16.1 ± 1.8
Normal cells, 50 $\mu\text{g/liter}$ Nifedipine	22.4 ± 0.6	2.1 ± 0.8	15.4 ± 2.0
Normal cells, 100 $\mu\text{g/liter}$ Nifedipine	21.6 ± 0.9	2.3 ± 0.9	17.4 ± 1.3
Lipid-laden cells, untreated	13.4 ± 3.0	0.9 ± 0.2	23.8 ± 3.0
Lipid-laden cells, 20 $\mu\text{g/liter}$ Nifedipine	22.6 ± 1.7	2.3 ± 0.1	28.1 ± 3.2
Lipid-laden cells, 50 $\mu\text{g/liter}$ Nifedipine	20.4 ± 3.3	1.3 ± 0.6	28.1 ± 2.2
Lipid-laden cells, 100 $\mu\text{g/liter}$ Nifedipine	19.9 ± 1.8	2.2 ± 0.2	28.5 ± 6.2

Freshly isolated cells were incubated for 2 h in the presence of 0–100 $\mu\text{g/liter}$ Nifedipine. Normal cells contained 23.3 ± 1.5 μg CHOL/mg protein and 6.6 ± 1.5 μg CE/mg protein. Lipid-laden cells contained 49.0 ± 5.0 μg CHOL/mg protein and 19.1 ± 2.4 μg CE/mg protein. Range indicated mean $\pm$ SD for four separate analyses.

Table III. Effect of Nifedipine on cAMP Levels in Bovine Arterial SMC in the Presence or Absence of a Phosphodiesterase Inhibitor, MIX

	Intracellular cAMP levels	
	–MIX	+MIX
	pmol/mg protein	pmol/mg protein
Untreated cells	1.2 ± 0.2	5.4 ± 0.1
Nifedipine, 20 $\mu\text{g/liter}$	4.0 ± 0.2	6.1 ± 0.2
Nifedipine, 50 $\mu\text{g/liter}$	3.5 ± 0.3	5.6 ± 0.2
Nifedipine, 100 $\mu\text{g/liter}$	2.8 ± 0.2	5.3 ± 0.2

Cells were incubated for 2 h in the presence of 0–100 $\mu\text{g/liter}$ Nifedipine after pretreatment with 1 mM MIX or buffer. Range indicates mean $\pm$ SD for four separate analyses.

$\mu\text{g/liter}$ range, the enhancement of CE hydrolytic activity seen with Nifedipine is significantly inhibited by the adenylate cyclase inhibitor, indicating that this enhancement depends on levels of intracellular cAMP. Similar data was obtained in bovine SMC (data not shown).

Finally, SMC cultured from rabbits fed normal or egg-supplemented diets were examined for CHOL and CE content after 1 wk of Nifedipine treatment to assess whether increased CE hydrolytic activity could alter the cellular CE content. Fig. 1 depicts the differential CHOL and CE levels in these SMC before Nifedipine treatment. After 1 wk of Nifedipine treatment, SMC from animals fed normal diets showed no significant difference in CHOL or CE content. However, lipid-laden cells from CHOL-fed animals, which contained twice the amount of CHOL and three times the amount of CE before Nifedipine treatment, showed a significant 50% loss in free and esterified CHOL as a result of Nifedipine treatment ($P < 0.005$). No significant reduction in CHOL or CE content was observed over 1 wk in normal or lipid-laden cells treated with buffer alone.

Discussion

Based on these results, we have identified for the first time a possible mechanism by which Nifedipine works at the cellular

Table IV. Effect of Nifedipine on CE Hydrolase Activities in the Presence or Absence of an Adenylate Cyclase Inhibitor, SQ22536

	–SQ22536	+SQ22536
ACEH activity (U/mg protein)		
Untreated cells	16.7 ± 3.5	16.0 ± 4.1
Nifedipine, 20 $\mu\text{g/liter}$	29.2 ± 6.9	16.8 ± 3.1
Nifedipine, 50 $\mu\text{g/liter}$	22.4 ± 0.6	16.2 ± 3.1
NCEH activity (U/mg protein)		
Untreated cells	0.9 ± 0.1	0.9 ± 0.2
Nifedipine, 20 $\mu\text{g/liter}$	2.1 ± 0.5	0.6 ± 0.2
Nifedipine, 50 $\mu\text{g/liter}$	2.1 ± 0.8	0.9 ± 0.1

Cultured rabbit arterial SMC were incubated for 2 h in the presence or absence of 0.1 mM SQ22536. CE hydrolase activities were measured as described in Methods. Range indicates $\pm$ SD for four separate analyses.

Figure 1. Effect of Nifedipine on CHOL and CE accumulation in arterial SMC from rabbits fed either a normal or egg-supplemented diet for 6 mo. Normal (□) and lipid-laden (▨) SMC were challenged three times with 100 μ g/liter Nifedipine during the 1-wk experiment (see Methods). Range bars indicate mean $\pm$ SD for four separate analyses.

level to reduce CE levels in lipid-laden cells. We have shown that the loss of CE may be due in part to increase in lysosomal and cytoplasmic CE hydrolase activities. It seems that this enhancement effect using pharmacological concentrations of Nifedipine is mediated by intracellular levels of cAMP since this enhancement effect was abolished when cAMP production was inhibited.

Various types of cellular injury such as mechanical (24), chemical (24), and immunological injury (25) have been proposed to lead to arterial lipid accretion. Many agents have been shown in vivo to protect the arterial wall against this lipid accretion such as calcium channel blockers (Nifedipine, Verapamil) (26), lipid resin binders (27), and anti-platelet agents (28). The postulated mechanisms for exerting a protective effect against intracellular lipid accumulation by these agents include (a) prevention of local cellular necrosis and decreased release of locally active proteases by enhancing membrane stability (27), (b) decreased intimal SMC proliferation, thereby limiting intracellular lipid accretion in areas of intimal thickening (1), and (c) decreased lipoprotein binding to SMC (29).

Altered lipoprotein binding to arterial SMC can lead to increased intracellular lipid accumulation, particularly since the CE and CHOL endocytosed by low density lipoprotein can serve as a substrate for the lysosomal CE hydrolase and

ACAT enzymes, respectively (16). Binding of various matrix molecules to cells, such as glycosaminoglycans, which may trap LDL-CE, or very low density lipoproteins, which carry triacylglycerols and CE to extrahepatic tissues, have been shown to be calcium-dependent (29). Calcium channel blockers may reduce this binding, thereby limiting intracellular lipid accretion. Ranganathan et al. (30) have shown that calcium channel blockers also inhibit CHOL synthesis in cultured human fibroblasts, possibly by altering LDL metabolism without altering 3-hydroxy-3-methylglutaryl-CoA reductase activity. This accumulation of undegraded LDL can further increase CHOL and CE accumulation.

It is widely known that cyclic nucleotides, which have been shown to play a major role in the release of hormones, also regulate CE metabolism in various cell types, such as arterial SMC (12, 13), macrophages (31), adrenocortical cells (32), and ovarian cells (33). cAMP has also been shown to enhance the mobilization and excretion of CE from cultured SMC of atherosclerotic arteries (34). In addition, Adelstein et al. (35) have shown that cAMP modulates the activity of myosin light-chain kinase in turkey gizzard SMC, thereby modulating excitation-contraction coupling. Since Nifedipine is known to decrease excitation-contraction coupling (36), its enhancement effect on intracellular levels of cAMP in SMC may explain both its antiatherogenic effect (by increasing CE hydrolase activities) and its vasodilatory or antispasm effect (by decreasing the activity of myosin light-chain kinase). Finally, with regard to whole body metabolism, it has been shown by Henry (5) that although calcium channel blockers may have multiple actions on SMC, their effects on lipid accumulation are not dependent on alterations in blood pressure or heart rate (5). These results suggest that the antianginal effect of Nifedipine in vivo is not solely responsible for its antiatherogenic effect, and that other cellular mechanisms may have a role in the decrease of arterial lipid accumulation.

Since calcium and CE accumulation are independently characteristic features of the development of human atherosclerotic plaques, the mechanism proposed herein may explain heretofore disparate effects of calcium channel blockers in vascular tissue. With widespread use of these agents in cerebrovascular spasm and cardiovascular disease, further investigation into their use of antiatherogenic agents may be warranted.

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