Calcium Channel Blockers Enhance Cholesteryl Ester Hydrolysis and Decrease Total Cholesterol Accumulation in Human Aortic Tissue

Orli R. Etingin and David P. Hajjar

Calcium channel blockers (CCBs), which are used clinically for treatment of angina and hypertension, are known to inhibit calcium influx into arterial smooth muscle cells and thereby decrease smooth muscle cell contraction. In addition, they prevent cholesteryl ester (CE) accumulation, the hallmark of human atherosclerosis, in arteries of cholesterol-fed animals by cellular mechanisms that remain undefined. To assess whether CCBs enhance CE hydrolysis and reduce CE accumulation in human arterial cells, we measured activities of the CE metabolic cycle in aortic tissues that were stripped of endothelial cells and adventitia from 35 patients undergoing coronary artery bypass surgery. Patients who were treated with either nifedipine or diltiazem ($n=23$) for several months demonstrated a threefold increase in arterial CE hydrolytic activities compared with untreated patients. This difference was independent of serum cholesterol levels, age, or treatment with other medications. No effects were observed on CE synthetic activity. Cyclic AMP levels in the aortic tissue of patients treated with CCBs were also significantly elevated twofold to threefold. In addition, both free and esterified cholesterol were significantly reduced in aortic tissue from patients taking CCBs compared with untreated patients. These data are the first to show that CCBs can increase CE hydrolysis in human aortic tissue by increasing intracellular cyclic AMP with resultant decrease in CE accumulation. Collectively, these findings support the hypothesis that CCBs can act as antiatherosclerotic agents in human tissue by mobilizing stored CE in the arterial wall. (Circulation Research 1990;66:185–190)

The precise role of calcium channel blockers (CCBs) in the treatment of myocardial infarction and post-myocardial infarction patients is not fully understood. Several clinical studies have demonstrated a prophylactic effect by CCBs in recurrent myocardial infarction for some patient populations. On a cellular level, CCBs have also been shown to inhibit smooth muscle excitation-contraction coupling and platelet aggregation in vitro. This is thought to contribute to their observed therapeutic effects.

The role of CCBs as antiatherosclerotic agents has not been defined. Most studies have demonstrated an inhibitory effect of CCBs on atherogenesis in cholesterol-fed rabbits. Although one report showed no effect on atherogenesis by CCB administration, the animals used in this study were fed a diet containing very large quantities of cholesterol. Despite some differences in results, the majority of the reports to date have shown that CCBs are efficacious as antiatherosclerotic agents in animal models of atherosclerosis. As yet, no studies have the reported effects of CCBs on the atherogenic process, including intracellular cholesterol metabolism, in human arterial tissue.

We previously demonstrated that pharmacological doses of nifedipine, a widely used CCB, increases cholesteryl ester (CE) catabolism and cholesterol efflux in cultured rabbit arterial smooth muscle cells by increasing intracellular levels of cyclic AMP. The increase in cyclic AMP enhances cyclic AMP–dependent protein kinase in these animal cells, thereby activating the CE hydrolytic enzymes and

From the Departments of Medicine (O.R.E.), Pathology, and Biochemistry and the National Institutes of Health Specialized Center of Research in Thrombosis, Cornell University Medical College, New York, New York.

Supported by the National Institutes of Health grants HL-18828, HL-01687, and HL-39701. D.P.H. is an Established Investigator of the American Heart Association, New York Affiliate.

Address for reprints: Orli R. Etingin, Department of Medicine (Hematology-Oncology), Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

Received February 21, 1989; accepted August 7, 1989.
cholesterol efflux, which may explain decreased CE accumulation and, hence, its antiatherosclerotic effect.

In the present study, we examined the effect of CCBs on cholesterol metabolism in transmural specimens of aortic tissue that were obtained from 35 patients undergoing coronary artery bypass grafts. Twenty-three of these patients were treated with standard doses of CCBs. The purpose of this investigation was to determine whether CCB treatment could alter human arterial CE catabolism to reduce lipid accumulation in vessels susceptible to atherosclerosis.

Subjects and Methods

At the time of bypass surgery, portions of uninvolved areas of aortas were obtained from 35 patients (27 men and 8 women). Mean age of patients receiving CCB treatment (17 men and 6 women) was 65±10 years. Mean age of patients who did not receive CCB treatment (10 men and 2 women) was 58±13 years (mean±SD). Doses of CCBs were as follows: nifedipine (10 mg three times daily), diltaiazem (30–60 mg three to four times daily), and verapamil (120 mg twice daily). Similar proportions of patients from each group were simultaneously receiving nitrate or β-blocker therapy, but no patients were receiving any agents that are known inhibitors of cyclic AMP phosphodiesterase (e.g., methyloxanthines or dipyridamole). Patients in the CCB group had been taking CCBs for a minimum of 3 weeks and had received an additional dose of CCB within 6 hours of surgery. Arterial samples were provided by the Departments of Cardiothoracic Surgery and Pathology at The New York Hospital. Laboratory use of these tissues was approved by the Human Rights Committee at the hospital. Aortic specimens measured approximately 0.3 cm×0.3 cm. Two to four specimens were obtained per patient. At the time of surgery, specimens were placed in ice-cold isotonic sucrose buffer (250 mM sucrose, 10 mM Tris-HCl–1 mM EDTA, pH 7.3) and immediately placed at 4°C.9,10 Within 2 hours, samples were stored at −70°C. Samples were kept frozen for a maximum of 2 weeks for enzyme assays. Previous results in our laboratory have demonstrated that CE enzyme activities, cyclic AMP, and lipid are not altered or oxidized after this freezethawing procedure. After thawing, adventitial tissue was trimmed, and the endothelial surface was gently removed by scraping. The remaining layer (intima media) was homogenized with the use of a tissue chopper and resuspended in sucrose buffer.10 The activities of the enzymes involved in the CE cycle, intracellular cyclic AMP levels, and lipid content were assayed together in one aortic homogenate. Generally, three to four separate analyses were performed in this manner. The accuracy of adventitial and endothelial removal was verified by tissue sectioning and microscopic examination. In addition, we determined microscopically whether the aortic tissue contained large numbers of macrophages or T lymphocytes in the patient samples studied. The arterial tissue examined contained primarily smooth muscle cells with few macrophages.

CE hydrolytic activities were assessed in homogenates from human aortas as described extensively in previous reports.9,10 Lysosomal (acid cholesteryl ester hydrolase [ACEH]) and cytoplasmic (neutral cholesteryl ester hydrolase [NCEH]) CE hydrolytic activities were measured by quantitation of released radioactive oleic acid from cholesteryl oleate labeled with carbon 14, which was added to the arterial homogenates. Substrates were prepared to measure CE catabolic activity as either unilamellar phospholipid liposomes (ACEH) or phospholipid–bile salt micelles (NCEH).9,10 CE synthetic activity (acyl coenzyme A:cholesterol acyltransferase [ACAT]) was assayed by measuring the synthesis of cholesteryl oleate from radioactive oleoyl coenzyme A and exogenous free cholesterol in the form of unilamellar liposomes.11 The substrate presentation in micellar form does not precisely mimic that in vivo. Activities of the enzymes involved in the CE cycle were expressed on a milligram protein basis. Protein was quantitated by methods described elsewhere.12 Cyclic AMP was measured with the use of a commercially available radioimmunoassay kit.13,14 Cyclic AMP was extracted from tissue by standard methods previously outlined.13,14 The free and esterified cholesterol content of aortic tissue homogenates was measured after extraction of lipid in situ by the method of Folch et al.15 Free and total cholesterol levels were determined by automated gas chromatography.16

Finally, we tested the hypothesis that diltaiazem would reduce cholesterol accumulation in lipid-laden arterial smooth muscle cells as was demonstrated with nifedipine.8 We exposed lipid-laden smooth muscle cells enzymatically harvested from aortas of cholesterol-fed New Zealand White rabbits8 to diltaiazem for a 1-week period. On days 0, 3, and 6 of the experimental period, 100 μg/l diltaiazem was added to each well containing serum, 0.1% bovine serum albumin, and confluent rabbit arterial smooth muscle cells (approximately 3.0×10⁶ cells/well).8 Serum albumin and high density lipoproteins in the serum served as cholesterol acceptors.8 Cells were harvested at days 0, 4, and 7 to assess their cholesterol and CE content. Control groups of cells were maintained in culture medium that contained no diltaiazem. Arterial lipids from cultured cells were extracted twice in situ with hexane/isopropanol (3:2 vol/vol).17 Lipid extracts were stored at −70°C under N₂ (gas) until lipid analyses were performed (within 1 week). The extract and a lipid standard containing cholesterol and CE were fractionated separately by thin-layer chromatography as described previously.18 These lipids were quantitated by scanning microfluorometric analysis.18 Statistical analysis of the effects of CCBs on ACEH, NCEH, and ACAT activities and cyclic AMP levels were compared by use of single-factor analysis of variance on unpaired samples.19 Lipid quantitation data was compared with the use of a
Results

Aortic smooth muscle cells from 23 patients who had received CCBs within 6 hours of surgery (nifedipine, n=8; diltiazem, n=14; or verapamil, n=1) demonstrated a threefold increase \((p<0.05)\) in lysosomal and cytoplasmic CE hydrolytic activities compared with that of aortic cells from patients receiving no CCB therapy (Figure 1). Baseline serum cholesterol levels were not affected by drug treatment (200±54 mg/dl in CCB patients vs. 182±50 mg/dl in controls). Arterial CE synthetic activity was not altered in patients on CCBs (Figure 1).

Cyclic AMP levels were also measured in human arterial cells to determine whether increased CE hydrolysis was accompanied by increased cyclic AMP levels, since activation of arterial CE hydrolyase occurs only if cyclic AMP levels are elevated.\(^{19,20}\) We observed a threefold increase in intracellular cyclic AMP levels in arterial cells obtained from patients on CCBs as compared with untreated patients (0.75±0.15 pmol/mg protein in controls vs. 2.8±0.80 pmol/mg protein in CCB patients, mean±SD, \(p<0.05\)). There was a positive correlation between arterial cyclic AMP levels and CE hydrolytic activity in CCB-treated patients as shown in Figure 2.

Consistent with the increase in arterial CE hydrolytic activity in CCB-treated patients, both the free and esterified cholesterol content of aortic tissue was significantly reduced in patients taking CCBs compared with untreated patients (Figure 3).

Finally, we also observed that diltiazem (which most patients in this study were given) can also decrease free and esterified cholesterol levels in lipid-laden cultured arterial smooth muscle cells (Figure 4), as seen with nifedipine.\(^8\) The mechanism responsible for this increased mobilization of lipid from these cells appears to be associated with

one-tailed \(t\) test. Differences were considered significant at a value of \(p<0.05\).

Figure 1. Bar graphs showing cholesterol ester metabolic activities in aortic tissue from calcium channel blocker (CCB)-treated patients. Arterial cholesterol ester hydrolytic activities (acid cholesterol ester hydrolyase [ACEH] and neutral cholesterol ester hydrolyase [NCEH]) were significantly increased \((p<0.05)\) in CCB patients \((n=23)\) compared with controls \((n=12)\). Cholesterol ester synthetic activity (acyl coenzyme A:cholesterol acyltransferase [ACAT]) was not significantly altered in CCB patients compared with controls. Range bars indicate mean±SD for three separate analyses of each aortic tissue homogenate; each analysis was done in triplicate.

Figure 2. Plot showing acid cholesterol ester hydrolytic (ACEH) activity and cyclic AMP levels in aortic tissue from patients treated with calcium channel blockers. The level of ACEH activity was strongly correlated \((r=0.87, p<0.001; y=3.64x+2.22)\) with tissue concentration of cyclic AMP when all tissue samples were considered. When untreated patients \((\circ)\) were examined separately, there was no correlation between ACEH activity and cyclic AMP. However, ACEH and cyclic AMP were correlated in patients treated with calcium channel blockers \((\bullet)\) when analyzed separately \((r=0.56, p<0.01; y=2.49±5.45)\).
an increase in CE hydrolytic activities in these cells (Table 1). No effects of diltiazem were observed regarding cholesterol or CE accumulation in normal cells (Figure 4).

Discussion

In this study, we have shown for the first time that various CCBs can stimulate CE hydrolysis in human arterial cells derived from patients undergoing coronary artery bypass surgery (Figure 1) and that CCBs can reduce the actual mass of free and esterified cholesterol from human aortic tissue (Figure 3) and in lipid-laden smooth muscle cells in vitro (Figure 4). The enhanced hydrolysis is accompanied by increased levels of intracellular cyclic AMP (Figure 2) that is similar to that observed in vitro. These findings could explain mechanistically the CCB-induced enhancement of lipolysis. Hence, pharmacological levels of CCBs used in this study may promote an antiatherosclerotic response in the vessel wall. Similar activation of arterial CE hydrolytic activity was observed in patients on the three different CCBs.

Various types of cell perturbation, including mechanical,20 chemical,20 or immunological injury21 are thought to lead to arterial lipid accretion. Several compounds have been proposed as protective agents against arterial lipid accretion in vivo including CCBs (nifedipine and verapamil),22 lipid resin binders,23 and antiplatelet aggregatory agents.24 The postulated mechanisms by which these substances exert a protective effect against intracellular lipid accumulation include 1) prevention of cell necrosis and decreased release of locally active proteases by enhancing membrane stability,25 2) decreased intimal smooth muscle cell proliferation, thereby limiting intracellular lipid accretion in areas of intimal thickening,25 and 3) decreased lipoprotein binding to smooth muscle cells.26 Increased lipoprotein uptake in arterial cells can lead to increased intracellular lipid accumulation. This can occur due to endocytosis of low density lipoprotein CE, which can serve as a substrate in the CE cycle. Binding of matrix molecules to cells, such as glycosaminoglycans, can result in trapping of low density lipoprotein CE extracellularly, thereby reducing intracellular CE hydrolysis.20 Since this binding phenomenon is calcium dependent, CCBs may reduce it, thereby limiting intracellular lipid accretion.27 Most recently, Schmitz and his colleagues28 have shown that calcium antagonists (nifedipine) promote cholesterol efflux from macrophages by high density

![Figure 3](image1.png)

**Figure 3.** Bar graph showing the levels of cholesterol and cholesteryl ester in aortic tissue from calcium channel blocker (CCB)-treated patients. Free and esterified cholesterol was measured in aliquots of human aortic homogenates as described in "Subjects and Methods." A twofold to threefold reduction in cholesterol and cholesteryl ester was observed in those aortic homogenates prepared from CCB-treated patients as compared with those tissues prepared from untreated patients (p<0.05). Range bars indicate mean±SEM for four separate analyses of each aortic homogenate; each analysis was done in duplicate.

![Figure 4](image2.png)

**Figure 4.** Bar graphs showing effect of diltiazem on cholesterol and cholesteryl ester mass in normal and lipid-laden rabbit aortic cells. Rabbit arterial smooth muscle cells (SMC) were treated on days 0, 3, and 6 with 100 μg/l diltiazem. On day 7, cellular lipids were extracted and quantitated by thin layer chromatography. Lipid-laden cells, isolated from hypercholesterolemic rabbits (serum cholesterol levels, 300±9 mg/dl, mean±SEM) were prepared as described in "Subjects and Methods." When the cells were exposed to diltiazem, they demonstrated a 60% decrease in cholesterol and cholesteryl ester (p<0.05). Normal cells treated with diltiazem demonstrated no significant change in cholesterol or cholesteryl ester content. Normal cells that were maintained for 1 week in culture medium without diltiazem (see text) demonstrated no significant change in cholesterol or cholesteryl ester content (day 0: cholesterol, 17.0±3.1 μg/mg protein; cholesteryl ester, 6.2±0.8 μg/mg protein; day 7: cholesterol, 19.3±1.8 μg/mg protein; cholesteryl ester, 7.1±2.4 μg/mg protein). Range bars indicate mean±SD for four separate analyses; each was done in triplicate.
lipoprotein–independent mechanisms. They propose that CCBs work to enhance cholesterol release at the level of membrane-associated sterols in the lysosomal route; that is, cholesterol is released from the cell after lysosomal hydrolysis by ACEH before it can be reesterified (ACAT) or further hydrolyzed by NCEH. Further experimental work is needed, however, to clarify whether the enhancement of CE hydrolysis by calcium antagonists is coordinately regulated with cholesterol efflux.

It is also known that cyclic nucleotides can regulate lipid metabolism in various cell types, such as arterial smooth muscle cells, macrophages, and ovarian cells. Cyclic AMP has also been shown to enhance the mobilization and excretion of CE from cultured smooth muscle cells derived from atherosclerotic arteries. Interestingly, decreased levels of arterial cyclic nucleotide levels have been correlated with the severity of atherosclerosis. In addition, Adelstein et al have reported that cyclic AMP modulates the activity of myosin light-chain kinase in turkey gizzard smooth muscle, thereby influencing excitation-contraction coupling. Since nifedipine is known to decrease excitation-contraction coupling, its enhancement effect on intracellular levels of cyclic AMP may explain its antiatherogenic effect (by increasing CE hydrolase activities) and its vasodilatory or “antispasm” effect (by decreasing the activity of myosin light-chain kinase). Furthermore, elevation of plasma cyclic AMP levels is a unique feature of CCBs among antiangiial drugs, not attributable to nitrates or β-blockers.

Finally, with regard to cardiovascular metabolism, it has been shown by Henry and Bentley that, although CCBs may have multiple actions on arteries, their effects on lipid accumulation are not dependent on alterations in blood pressure or heart rate. This suggests that the antiatherogenic effect of nifedipine in vivo is not directly responsible for its antiatherogenic effect and that other cellular mechanisms may control the decrease of arterial lipid accumulation.

This study provides evidence for the first time that smooth muscle cells from arteries of patients receiving CCB therapy exhibit increased CE catabolic activities and reduction in cholesterol and CE mass. The data reported herein provides the basis for evidence that these agents, which are used widely in clinical practice, may also be useful as antiatherosclerotic agents in humans. The effects we report herein are independent of serum cholesterol levels or blood pressure, suggesting that our observations, in fact, are due to a direct cellular metabolic response to these agents.

Because a number of drugs administered to patients can affect calcium metabolism, the potential clinical implications of these studies are evident. There is, however, a paucity of studies related to the potential regression of atherosclerosis in humans by modulation of calcium flux over long periods of time. Specifically, there are no previous studies with calcium-entry blockers. Thus, it will be important to determine whether these agents that interfere with calcium entry can also be effective in the process of atherosclerosis regression.

Acknowledgments

We wish to thank Ms. Barbara Summers for excellent technical assistance, Dr. Kenneth Pomerantz for assistance in lipid analyses, and Dr. Ralph L. Nachman for his critical review of the manuscript.

References


**KEY WORDS** • atherosclerosis • calcium channel blockers • cholesterol metabolism • cyclic AMP
Calcium channel blockers enhance cholesteryl ester hydrolysis and decrease total cholesterol accumulation in human aortic tissue.
O R Etingin and D P Hajjar

Circ Res. 1990;66:185-190
doi: 10.1161/01.RES.66.1.185

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/66/1/185

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/