Alterations in Basal and Serotonin-Stimulated Calcium Permeability and Vasoconstriction in Atherosclerotic Aorta

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Abstract  Hypersensitivity to vasoactive stimuli, a common finding in atherosclerotic arteries, is thought to play an important role in the pathology of arterial and coronary vasospasm and may be a factor in myocardial ischemia and infarction. While this phenomenon is well documented, the underlying mechanism is unknown. The present study used isometric force measurements coupled with \(^{45}\)Ca\(^{2+}\) and Fura 2-AM techniques in aortic smooth muscle to probe transmembrane calcium movements and cytosolic calcium levels in an attempt to determine their relation to altered vasomotion in a rabbit model of dietary atherosclerosis. Following 10 weeks of cholesterol feeding (2%), basal (unstimulated) calcium influx was augmented 1.5-fold in atherosclerotic segments with no change in basal calcium efflux. Serotonin-stimulated calcium uptake was increased 1.9-fold in atherosclerotic segments and was accompanied by a fivefold increase in serotonin vasoconstrictor sensitivity and a 1.4-fold increase in serotonin-stimulated calcium efflux. Endothelial denudation did not alter either force generation or \(^{45}\)Ca\(^{2+}\) movements in serotonin-stimulated segments. In arterial smooth muscle cells dispersed from atherosclerotic vessels, basal and serotonin-stimulated cytosolic calcium levels were augmented approximately 2.3-fold and twofold, respectively. These findings contribute to our understanding of the cellular defects in calcium metabolism, which may ultimately explain the cellular basis of serotonin hypersensitivity in atherosclerotic arteries and certain arterial vasospastic syndromes in this disease state. (Arterioscler Thromb. 1994;14:1854-1859.)

Key Words • arterial smooth muscle • calcium influx • cytosolic calcium • cholesterol • vasospasm • coronary spasm

The object of this study was to characterize \(^{45}\)Ca\(^{2+}\) movements and cytosolic calcium levels along with vasoconstrictor responses in aortic segments obtained from atherosclerotic rabbits. Enhanced vasoconstrictor activity of vascular smooth muscle is well documented in atherosclerotic and hypercholesterolemic states. Increased sensitivity to vasoactive stimuli has been demonstrated to a number of agonists in a variety of animal models of hyperlipidemic disorders, including human coronary arteries in vivo. This increased responsiveness has been implicated as an important factor in the pathogenesis of coronary vasospasm, an augmented vasoconstrictor phenomenon thought to be important in Printzmetal's angina, and myocardial infarction as well as claudication and intermittent claudication in other vascular beds. Although a number of vasoactive agents have been proposed as spasmogens mediating this effect, the most commonly reported is serotonin, a biogenic amine concentrated in platelet aggregates with atherosclerotic plaques at autopsy suggesting that platelet-derived mediators, including serotonin, may play a role in vasospasm. Despite the extensive demonstration of arterial hyperreactivity in atherosclerotic vessel disease, the underlying mechanism remains to be clarified. One potential explanation of vascular hyperreactivity in atherosclerosis is related to transmembrane calcium movements. Calcium is widely held to be a central factor in regulating vasomotor activity. Alterations in regulation of arterial smooth muscle calcium handling may result in alterations in contractile behavior similar to those seen in atherosclerosis. This study tested this hypothesis by measuring transmembrane \(^{45}\)Ca\(^{2+}\) movements in segments of thoracic aorta freshly obtained from atherosclerotic rabbits. We found that dietary atherosclerosis increased arterial smooth muscle calcium influx under both basal and serotonin-stimulated conditions. The increased calcium influx was paralleled by increases in cytosolic calcium levels and vasoconstrictor sensitivity to serotonin in atherosclerotic vessels.

Methods

Animal Model

This study used a model of dietary atherosclerosis in which male New Zealand White rabbits (aged 3 to 4 months) were fed a calibrated diet containing 2% cholesterol (Purina) for 10 weeks. Control animals were housed on site in the same room with diet animals and fed the same calibrated diet but without the added cholesterol. Food and water was provided ad libitum to all animals. Blood was drawn from the marginal ear vein before the initiation of the diet for control serum cholesterol determinations and again at the time of experimentation.

Tissue Preparation

The thoracic aorta was removed following euthanasia by overdose with sodium pentobarbital (100 mg/kg IV). The vessels were dissected free of fat and connective tissue and cut into ring segments approximately 4 mm in width. The dissection was performed in an oxygenated physiological saline...
solution (PSS) with the following composition (in mmol/L): 2.5 HEPES, 2.5 NaHEPES, 140 NaCl, 4.5 KCl, 1.0 MgCl₂, 1.5 CaCl₂, and 10.0 glucose and was maintained at 37°C and pH 7.4. The aortic segments were equilibrated for 60 to 90 minutes before experimentation.

**Calcium Influx**

Calcium influx was measured by using a radioactive tracer ([^45]Ca²⁺) technique. For assay, Ca²⁺ uptake medium was prepared with PSS by adding[^45]Ca²⁺ (2.5 μCi/mL). The ring segments were incubated at 37°C for 30 seconds to 30 minutes. Uptake was found to be linear over a 5-minute period, which permitted a 3-minute pulse-labeling protocol in selected experiments. After the[^45]Ca²⁺ incubation period, an aliquot of the calcium uptake medium was obtained to determine specific activity. Ring segments were blotted lightly, and uptake was terminated by transferring the segments to pre-chilled, calcium-free PSS containing 2 mmol/L EGTA followed by vigorous agitation with a stream of 100% oxygen for 60 minutes. This wash was established from preliminary experiments in which a 60-minute wash period yielded a stable[^45]Ca²⁺ tissue content. The segments were then weighed to within 10 μg on a Mettler AE 163 balance followed by digestion overnight in 7.5 mmol/L EGTA. Liquid scintillation cocktail was added to aliquots of uptake medium and to tissue samples for counting in a Beckman LS 5000 TD scintillation spectrometer. Calcium uptake was determined from counts derived from the tissue[^45]Ca²⁺ fraction divided by the specific activity of the uptake media (counts per minute per mg) and normalized to the wet weight of the ring. Calcium influx was determined from the slope of each uptake curve over time and was expressed as micromoles per kilogram wet weight per minute.

**Calcium Efflux**

Measurement of calcium efflux was made by using a[^45]Ca²⁺ technique for isotopic tracer analysis in arterial tissue. Aortic ring segments were equilibrated to isotopic equilibrium (4 hours) in PSS containing 4.0 μCi/mL[^45]Ca²⁺ (loading medium). Following this[^45]Ca²⁺ loading procedure, the segments were mounted on stainless steel hooks connected to glass capillary tubes, and each ring segment was then passed through a series of tubes containing nonradioactive PSS (efflux medium) maintained at 37°C. The efflux media were vigorously aerated to prevent the formation of an unstirred water layer, which can affect the characteristics of[^45]Ca²⁺ efflux. Media changes were made at 5-minute intervals from 5 to 40 minutes to remove extracellular and loosely-bound[^45]Ca²⁺ and 2-minute intervals from 40 to 60 minutes to resolve basal and activated efflux. A agonists were added at 50 minutes and remained in the efflux media until termination of the experiment. Following the efflux protocol, the tissues were collected, weighed, and prepared for counting as described above. Aliquots of the loading medium were collected to determine specific activity. Efflux curves were generated by adding the activity in the tissues to the activity in the wash tubes in reverse order. Efflux rate constants at each time point were determined from the following rate equation:

\[ k = \frac{1}{1 + 2 - (1) \times (\ln A1/A2)} \]

where A1 and A2 are the tissue counts at time (t) 1 and 12, respectively.

**Cytosolic Calcium**

The concentration of intracellular free calcium in dispersed arterial smooth muscle cells was determined by using Fura 2-AM according to Gleason et al. Briefly, arterial smooth muscle cells were enzymatically dispersed from the medial smooth muscle layer and plated at high density (5 x 10⁶/cm²) in primary culture in minimum essential medium plus 10% fetal calf serum for 2 days. This procedure permits the reexpression of surface receptors for agonist stimulation without significant cell growth or fundamental alterations in cell characteristics. The cells were released with trypsin, suspended in calcium-free PSS, incubated for 60 minutes with Fura 2-AM (2 μmol/L, Molecular Probes, Inc) supplemented with 0.1% bovine serum albumin and pluronic F-127 (0.03%; Molecular Probes). The cells were washed and pelleted three times and resuspended in calcium containing PSS followed by equilibration at 37°C for 5 minutes. Aliquots (1 mL) of suspended cells containing 1 x 10⁶ cells were placed in a temperature-regulated (37°C) cuvette, and fluorescence intensity was monitored at 505 nm in a spectrofluorimeter (Jasco) by using alternating excitation wavelengths of 340 and 380 nm. The intensity ratio (R) at 340/380 nm was used for calculating steady-state cytosolic calcium levels. The cells were then permeabilized with 10% Triton X-100 to determine the maximum fluorescence (Rmax), after the addition of 5 mmol/L EGTA, minimum fluorescence (Rmin) was determined. The intracellular free calcium concentration [Ca²⁺], was calculated by using the following equation:

\[ [Ca^{2+}] = K_d \times \frac{(R - R_{min})}{(R_{max} - R)} \times (F2/B2) \]

in which a dissociation constant (K_d) of 224 nmol for Ca²⁺ binding to Fura 2 at 37°C was used. F2 represented the 380-nm emission intensity with EGTA, and B2 corresponded to the 380-nm emission intensity with Triton X-100.

**Force Development**

To measure vasoconstrictor activity, ring segments were mounted for isometric tension studies on a wire myograph. The rings were mounted horizontally in separate muscle chambers on two stainless steel pins (127 μm radius; one pin was attached to a movable support and the other to a stationary support. The water-jacketed chambers were filled with PSS (10 mL) that was maintained at 37°C and continuously aerated with 100% oxygen. Before experimentation, each ring was stretched to its predetermined optimal passive tension (5 g) and equilibrated in PSS for approximately 1 hour. Preliminary studies indicated that the optimal passive tension was not significantly different between diet and control aortic segments. Isometric force was measured with Grass FT.03 force transducers and recorded on a Grass model 7D polygraph. Contractile force was normalized to the width of the segment and expressed in grams per millimeter width to account for ring-to-ring width differences. Drug studies were performed by cumulative additions of drugs to the organ bath. After each drug application, force development was permitted to reach a peak level before the next drug addition was added. Concentration-response curves were constructed by taking the force developed at each dose and expressing it as a percent of the maximum force developed to the drug itself.

**Lipid Determinations**

Serum and tissue lipid samples were extracted for lipid measurement according to the method of Bligh and Dyer. For tissue samples, segments were homogenized in a 1:1 chloroform/methanol mixture by using a Dounce homogenizer prior to extraction. The chloroform phase was then obtained and dried under nitrogen. Total and free (unesterified) cholesterol was determined by gas-liquid chromatography with cholesterol methyl ether as an internal standard. Serum cholesterol content was normalized as milligrams per deciliter, and tissue cholesterol was normalized to the wet weight of the sample.

**Statistics**

Statistics used were Student's t tests for peak-to-peak comparisons with Bonferroni corrections as needed.
Drugs and Chemicals
All buffer components were products of Fisher Scientific. 5-Hydroxytryptamine (serotonin) was purchased from Sigma Chemical. Serotonin was prepared as a 1-mmol/L stock in distilled water supplemented with 10⁻⁷ mol/L EDTA.

Results
Calcium Influx
Basal calcium uptake was linear through 5 minutes in both control and atherosclerotic animals. As illustrated in Fig 1, calcium influx was 1.5-fold greater in atherosclerotic than in control ring segments (22.2±2.1 versus 14.3±1.1 μmol·kg wet wt⁻¹·min⁻¹; n=7, P≤.03). In addition, to account for any differential washout between control and atherosclerotic tissues, segments were incubated for 2 hours in uptake medium and then submitted to a 0°C washout protocol in selected experiments. After this 2-hour protocol atherosclerotic segments demonstrated a 25% higher calcium content than control segments (176±21 versus 141±16 μmol/kg wet wt, respectively). This difference in ⁴³Ca²⁺ content likely represents a stable cellular pool of calcium in atherosclerotic aortic segments.

Calcium uptake in response to 10 μmol/L serotonin was measured as described by Flaim et al by using a uniform pulse of 3 minutes with ⁴⁵Ca²⁺ after preincubation with the drug for varying times. Serotonin stimulated a biphasic increase in calcium uptake (Fig 2). Uptake increased to a peak value within approximately 3 minutes in control and 5 minutes in atherosclerotic ring segments. Peak calcium uptake was 1.9-fold higher in atherosclerotic compared with control segments at 1 μmol/L serotonin (0.035±0.003 versus 0.025±0.002 min⁻¹; n=5, P≤.03). Serotonin activated calcium efflux in a concentration-dependent manner, reaching a maximal efflux at 10 μmol/L. In ring segments from atherosclerotic animals, serotonin increased calcium efflux k values at lower concentrations than in segments from controls, but both groups reached the same value at maximum serotonin concentration. Taken as percent of maximum efflux response, serotonin-stimulated calcium efflux in atherosclerotic segments yielded a fivefold increase in sensitivity to serotonin (Fig 5).

Cytosolic Calcium
Arterial smooth muscle cells derived from the atherosclerotic aorta and maintained 5 days in primary culture demonstrated a doubling of basal [Ca²⁺] relative to cells isolated from the control aorta (182.8±26.9 versus 90.3±4.3 nmol/L, respectively, P<.02) (Fig 6). No differ
Atherosclerosis Alters Calcium Metabolism in Arterial Smooth Muscle

Fig 4. Line graph of the effect of atherosclerosis on serotonin-stimulated calcium efflux in aortic ring segments shows an increase in peak serotonin-stimulated (1 μmol/L) calcium efflux in aortic ring segments of atherosclerotic versus normal animals. (n=5).

Difference in R_max or R_m1 was observed between the two groups. Serotonin (10 μmol/L) elicited an increase in [Ca^{2+}], in cells isolated from both control and atherosclerotic vessels, with peak [Ca^{2+}] increasing 1.5-fold in cells derived from control aorta (90.3±4.3 versus 138.7±9.2 nmol/L) and 2.3-fold in cells derived from atherosclerotic aorta (182.8±26.9 versus 423.2±26.2 nmol/L; P<.01).

**Forced Development**

As observed with calcium uptake, serotonin (1 μmol/L) also elicited a biphasic contraction in aortic ring segments taken from control and atherosclerotic animals. There was a rapid increase in tension that peaked at 2 to 4 minutes followed by a reduced increase phase. However, there was no difference in maximum force development (ie, at 100 μmol/L serotonin) over time between atherosclerotic and control animals (83.1±9.7 versus 75.4±8.1 g/mm, respectively). However, in the atherosclerotic animals, 1 μmol/L serotonin elicited a 1.5-fold increase in peak force development compared with the controls (0.81±0.09 versus 0.55±0.03 g/mm; n=4, P<.01) (Fig 7). Force was elevated at each time point in the atherosclerotic segments. Concentration-response analysis revealed a leftward shift in the atherosclerotic compared with the control ring segments (Fig 7), reflected in a fivefold increase in sensitivity in the atherosclerotic group (ED_50=8.13±0.93×10^{-7} versus 42.1±3.8×10^{-7} mol/L, respectively; n=5, P<.04). To determine whether these changes in atherosclerosis are related to dysfunctional endothelial responses, the endothelium was removed in selected experiments. Removal of the endothelium failed to significantly affect calcium uptake (ED_50=45.6±4.3×10^{-7} versus 55.7±7.6×10^{-7}, intact versus denuded; n=3, P>.05) or contractile sensitivity (43.4±0.4×10^{-7} versus 38.9±0.5×10^{-7}, intact versus denuded; n=3, P>.05) in response to serotonin.

**Lipid Measurements**

Serum total cholesterol was measured in animals at the beginning of cholesterol feeding and just prior to death. Tissue total cholesterol was measured on a randomly chosen segment of aorta from each animal. Serum total cholesterol increased in the cholesterol-fed animals from 51±6.7 to 1083±158 mg dL (P<.01,

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Fig 5. Line graph of the effects of atherosclerosis on the sensitivity of calcium efflux (k) to serotonin in aortic ring segments shows a concentration-dependent increase in calcium efflux in response to serotonin, which is fivefold greater in atherosclerotic than in control segments.

**Fig 6. Bar graph showing the effects of atherosclerosis on basal (unstimulated) and serotonin-stimulated cytosolic calcium levels in aortic smooth muscle cells isolated from rabbit thoracic aorta. Basal calcium levels were increased twofold in cells isolated from atherosclerotic animals, and serotonin-stimulated calcium was increased 2.3-fold (n=4). *P<.05 basal atherosclerotic vs basal control; **P<.001 serotonin-stimulated atherosclerotic vs serotonin-stimulated control.

**Fig 7. Line graphs. Top, Effects of atherosclerosis on contractile sensitivity to serotonin in aortic ring segments demonstrating a fivefold increase in sensitivity to serotonin in atherosclerotic aortic ring segments (n=5). Bottom, Biphasic force development over time (1 μmol/L serotonin), which was greater at all points in the atherosclerotic than in the control vessels (P<.01).
n=10) by the end of the feeding period. Tissue total cholesterol increased from 0.7±0.05 to 4.5±1.4 μg/mg wet wt (P<.01, n=10). Grossly visible atherosclerotic lesions were present on the internal surface of all aortas from the cholesterol-fed animals, but none were present in the control animals. Smooth muscle cells were also isolated by enzymatic dispersion and subjected to cholesterol assay. In this subset, unesterified (free) cholesterol increased from 15 to 24 μg/μg protein (P<.05, n=4) in the atherosclerotic versus control groups. Taken together, these data demonstrate well-developed serum hypercholesterolemia and atherosclerosis as well as enrichment of the aortic smooth muscle cells with cholesterol.

Discussion

Atherosclerosis is a widely prevalent disorder in industrialized cultures. One of its principle consequences is the predisposition of affected vessels to undergo vasospasm, a condition that may predispose subjects to myocardial ischemia and myocardial infarction. Many investigators have hypothesized that hyperactivity of blood vessels may contribute to spasm and have demonstrated hypersensitivity, particularly to serotonin, in a number of preparations. While serotonin hypersensitivity is well documented, the underlying basis remains unknown. Like most vasoconstrictors, serotonin acts by way of specific membrane receptors and uses extracellular calcium as a second messenger. In addition, calcium channel antagonists are valuable as antiatherosclerotic agents and as effective in the treatment of vasospastic syndromes. We therefore pursued the hypothesis that alterations in calcium permeability by arterial smooth muscle mediate changes in contractile responsiveness to serotonin in atherosclerotic vessels.

The first principal finding of this study was that basal calcium influx and cytosolic calcium levels are altered in atherosclerosis. Aortic segments taken from atherosclerotic animals demonstrated an almost twofold increase in basal calcium influx with little to no change in basal calcium efflux. Furthermore, in a tertiary culture, smooth muscle cells derived from the atherosclerotic aorta also demonstrated a twofold increase in cytosolic calcium levels relative to cells derived from control aortas under similar conditions. These data suggest that the increase in \([Ca^{2+}]\) results from the imbalance between calcium influx and efflux as reflected in the \[^{45}\text{Ca}^{2+}\] flux experiments. However, the observation of increased calcium influx without a concomitant increase in calcium efflux was unexpected. By definition, calcium influx and efflux would be expected to be equal under the steady-state conditions of this study. Notwithstanding, the mechanism responsible for the increase in calcium influx may be explained on the basis that the smooth muscle plasma membrane in which calcium channels and calcium extrusion proteins reside is altered by exposure to the cholesterol gradient between cholesterol-rich lipoproteins and the arterial smooth muscle cell membrane in the atherosclerotic animal. Under these conditions, cholesterol exchange occurs between low-density lipoproteins and arterial smooth muscle membranes. Increased cholesterol content of plasma membranes is associated with increased calcium uptake in vascular smooth muscle cells. Gleason et al have demonstrated that calcium influx was increased with cholesterol enrichment of the cell membrane in cultured rabbit aortic smooth muscle cells, a phenomenon that resulted in increased cytosolic calcium levels. Bialecki et al report an increase in basal calcium influx associated with acute increases in cellular cholesterol content in cultured rat pulmonary arterial smooth muscle and intact rabbit carotid arteries perfused in vitro. Zhou et al treated vascular smooth muscle cells with cholesterol-rich phospholipid dispersions and demonstrated an increased cholesterol content and increased calcium uptake and a potentiation of these effects with oxysterols. Each of these studies argues for increased calcium influx associated with enrichment of smooth muscle with free cholesterol. It has also been shown that an increase in the cholesterol content of sarcoplasmic reticulum membranes reduces Ca\(^{2+}\)-ATPase activity, a phenomenon that may be involved in suppressing calcium extrusion from the cell. Finally, Chen et al have demonstrated an increase in the cholesterol content of highly purified plasma membrane preparations freshly isolated from aortic smooth muscle cells dispensed from the atherosclerotic rabbit aorta. In their studies, this increase was associated with marked changes in the membrane bilayer structure. Given that the membrane lipid bilayer is altered in dietary atherosclerosis, we suggest that cholesterol enrichment of the plasma membrane may be an underlying factor in increased calcium influx, increased intracellular calcium levels, and the associated hyperreactivity to serotonin.

However, it remains to be determined what role, if any, increased basal calcium influx plays in smooth muscle function. Strickberger et al using \[^{45}\text{Ca}^{2+}\] efflux data, predicted from mathematical modeling that an increased calcium influx would occur in atherosclerotic aorta and that this increase would exceed calcium efflux, thereby resulting in calcium accumulation in various intracellular compartments. Thus, Ca\(^{2+}\) accumulation could lead to increased cytosolic calcium levels, increased smooth muscle excitability, and eventually to cell death by calcium overload. This could explain the necrosis of the arterial wall cells and dystrophic calcification of typical lesions in atherosclerosis. It is also likely that increased Ca\(^{2+}\) influx contributes to many of the alterations in smooth muscle cell function such as cell proliferation, increased vasoreactivity, and increased collagen synthesis. Given the putative role of calcium channel blocking drugs in the prevention and/or regression of atherosclerosis, it is likely that augmented calcium uptake and intracellular calcium levels in the unstimulated (ie, basal) state in arterial smooth muscle play an important role in the overall atherogenic process.

The second important finding of this study was an increase in force development to serotonin stimulation that was paralleled by increases in calcium uptake and intracellular calcium in atherosclerotic vessels. Furthermore, an increased vasoconstrictor sensitivity to serotonin was observed in atherosclerotic aorta, an observation that has been previously documented. The increased influx in response to serotonin cannot be attributed solely to increased basal calcium uptake since subtracting the basal, unstimulated uptake from each serotonin-stimulated calcium uptake still yields a significant increase in calcium uptake. The increased calcium...
uptake to serotonin was accompanied by an increased calcium efflux, a response that may represent calcium efflux secondary to increased cytosolic calcium or, alternatively, a direct stimulation of efflux pathways by serotonin, or a combination of the two processes.

While this study demonstrated that serotonin-stimulated transmembrane calcium movements and arterial hypersensitivity are altered in parallel in atherosclerosis, the degree to which these alterations in arterial smooth muscle contribute to vasospastic syndromes needs to be clarified. Some studies have suggested that the endothelium is dysfunctional in atherosclerosis or that endothelium-mediated arterial relaxation is impaired by oxidized lipoproteins. Our studies do not support this notion in our model, since removal of the endothelium failed to significantly alter calcium uptake or contractile sensitivity in response to serotonin. However, the etiology of arterial vasospasm in general is likely multifactorial with both an endothelial defect as well as a smooth muscle defect, with our model showing primarily the latter.

In conclusion, the results of this study demonstrated substantial alterations in transmembrane calcium movements and cytosolic calcium concentrations in the basal and activated states in atherosclerotic smooth muscle that are associated with contractile dysfunction. We hypothesize that the changes in calcium permeability, secondary to arterial smooth muscle membrane bilayer lipid changes, play an important role in the altered contractile sensitivity to serotonin in atherosclerotic vessels. We further suggest that these alterations may play a pivotal role overall in the etiology of various vasospastic syndromes, and perhaps also in the etiology of atherosclerotic lesions.

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