Effect of Early and Advanced Atherosclerosis on Vascular Responses to Serotonin, Thromboxane A₂, and ADP

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In monkeys with early and advanced atherosclerosis, we examined responses to the three major vasoactive agonists that are released when platelets aggregate. Measurements were obtained in normal cynomolgus monkeys and in monkeys fed an atherogenic diet for 4±1, 9±1, and 19±1 months (mean±SEM). Morphometry of femoral and iliac arteries indicated that 4 months of atherogenic diet produced only slight intimal thickening, 9 months produced early lesions, and 19 months produced approximately 5–10-fold greater intimal proliferation than did 9 months of atherogenic diet. Serotonin and adenosine 5'-diphosphate (ADP), which are endothelium-dependent agonists, and adenosine and phenylephrine, which are endothelium-independent agonists, were injected intra-arterially into the perfused hind limb. Thromboxane A₂ analogue U46619 also was studied. Vasoconstrictor responses to serotonin were potentiated, and vasodilator responses to ADP were impaired by early and advanced atherosclerosis. In contrast, vasoconstrictor responses to phenylephrine and vasodilator responses to adenosine were similar in all groups. Vasoconstrictor responses to U46619 were potentiated by advanced atherosclerosis. Thus, vascular responses to serotonin, ADP, and thromboxane A₂ are altered by atherosclerosis in a direction that would favor vasoconstriction when platelets aggregate. Furthermore, because responses to endothelium-dependent agonists are altered, these data suggest that endothelium is dysfunctional in early atherosclerosis. These findings may explain, in part, the propensity for exaggerated vasoconstriction even in arteries with minimal atherosclerotic lesions. (Circulation 1989;79:698–705)

Recent observations in patients¹ and experimental animals in vitro²⁻⁴ and in vivo⁵,⁶ suggest that atherosclerosis predisposes to vasospasm. Spasm often occurs at sites with only modest lesions as defined by angiography.⁷,⁸ Moreover, although coronary arteriograms may not reveal segmental narrowing due to atherosclerosis in vessel segments prone to spasm, there may be evidence at autopsy of minimal atherosclerosis in the segment of vessel at which spasm occurred.⁹

Mechanisms by which atherosclerosis predisposes to vasospasm are not clear. Several groups⁶,¹⁰⁻¹² have suggested that platelets may adhere to atherosclerotic lesions, aggregate, and release serotonin and other vasoactive substances. We showed previously that vasoconstrictor responses to serotonin are greatly potentiated in monkeys with advanced atherosclerosis.⁶ Thus, we have shown that serotonin that is released by platelets may be an important mediator of vasospasm. Platelets, however, also release substantial quantities of two other vasoactive substances, thromboxane and adenosine diphosphate (ADP).¹³ It is not known whether or not vascular responses to thromboxane and ADP are altered by atherosclerosis.

In this study, we tested two hypotheses. First, we tested the hypothesis that responses to the three major platelet products, ADP, thromboxane, and serotonin are altered by advanced atherosclerotic lesions in a direction that would favor vasoconstriction. Second, we tested the hypothesis that even minimal atherosclerotic lesions may alter vascular responses.
Methods

Monkeys

Four groups of adult male Malaysian cynomolgus monkeys were studied. Eight normal monkeys were fed commercial laboratory chow (Purina Monkey Chow, Ralston Purina, Richmond, Indiana). Eight monkeys were fed atherogenic diet, which contained cholesterol (1 mg/calorie) and fat (41% of total calories) for 4±1 months (mean±SEM) (hypercholesterolemic group). Ten monkeys were fed atherogenic diet for 9±1 months (early atherosclerotic group). Nine were fed an atherogenic diet for 19±1 months (advanced atherosclerotic group). The monkeys weighed 6.5±0.3 kg in the normal group, 6.1±0.4 kg in the hypercholesterolemic group, 6.2±0.2 kg in the early atherosclerotic group, and 5.9±0.4 kg in the advanced atherosclerotic group. None of the monkeys described in this study has been described in other papers. Two monkeys that were fed atherogenic diet for 9 and 12 months did not have lesions on histologic and morphometric analysis and thus were included in the hypercholesterolemic group. At intervals of 3–4 months, the monkeys were sedated with ketamine HCl (10 mg/kg i.m.), and a venous blood sample was obtained. Total cholesterol and triglycerides were determined with the method used by the Lipid Research Clinics Protocol for the Autoanalyzer II (Technicon Instruments, Tarrytown, New York).

Hemodynamic Studies

At the time of study, the monkeys were sedated with ketamine (15 mg/kg i.m.) and anesthetized with chloralose (100 mg/kg i.v.). A tracheostomy was performed, and the monkeys were intubated and ventilated with room air and supplemental oxygen. Gallamine triethiodide (5 mg/kg i.v.) was given for paralysis of skeletal muscles, and heparin sodium (500 units/kg i.v.) was given for anticoagulation. Arterial blood gases and pH were monitored during each study and maintained at normal levels by adjustment of ventilation or injection of small amounts of sodium bicarbonate. Rectal temperature was maintained at 37–38°C with a heating pad.

A polyethylene catheter was inserted through the right brachial artery for measuring aortic pressure and to obtain blood samples. Catheters were inserted into the right and left brachial veins for injecting fluids and drugs.

Through a laparotomy, the bifurcation of the abdominal aorta and the proximal left iliac artery were exposed and isolated. The left dorsal pedal artery also was exposed and a PE-50 catheter was inserted retrogradely to measure pressure. A calibrated Harvard Model 1210 pulsatile perfusion pump (South Natick, Massachusetts) was used to perfuse the left iliac artery at constant flow with blood from the abdominal aorta, and iliac perfusion pressure was measured continuously. When the pump was stopped, perfusion pressure decreased rapidly to 10–15 mm Hg, which indicates that vascular isolation was adequate. Baseline perfusion pressure of the hind limb was established by adjusting blood flow so that the perfusion pressure was similar to the animal’s mean arterial pressure. The difference between iliac pressure and dorsal pedal pressure, at constant flow, indicates resistance of large arteries in the limb. The method has been described in detail previously.6,14,15

Maximal vasodilator responses of the hind limb were produced by infusion of papaverine hydrochloride (Sigma Chemical, St. Louis, Missouri) at 2.5 mg/min i.a. Perfusion pressure was measured during baseline flow and 40% below baseline flow. Infusion of higher doses of papaverine (5 mg/min) usually did not produce a further decrease in perfusion pressure, which indicates that the vessels were dilated maximally to the stimulus. In those experiments in which further vasodilation was observed, pressure-flow determinations were repeated.

We studied effects of adenosine, ADP (adenosine 5’-diphosphate from equine muscle), phenylephrine hydrochloride, serotonin (5-hydroxytryptamine creatine sulfate complex), all from Sigma Chemical, St. Louis, Missouri, and the stable analogue of thromboxane A2, U46619 (5Z,9α,11α,13αE155S)-5-hydroxy-11,9-[epoxymethano]prosta-5,13-DIEN-1-OIC acid from Upjohn, Kalamazoo, Michigan). Several lines of evidence suggest that thromboxane A2 and U46619 produce similar physiologic effects by activation of the same receptor.16 Adenosine (5×10-7 and 5×10-7 mol), ADP (5×10-10 and 5×10-9 mol), serotonin (1×10-8 and 3×10-8 mol), and U46619 (1×10-9 and 3×10-9 mol) were injected as a 0.1 ml bolus into the iliac perfusion tubing. Injection of 0.1 ml of vehicle for all of the agonists had minimal hemodynamic effects. Maximal changes in perfusion pressure and pressure in dorsal pedal artery were measured after bolus injections.

Morphologic Studies

The monkeys were killed with intravenous potassium chloride. The iliac and femoral artery vessels were removed, examined for gross lesions, and fixed by immersion in 10% buffered formalin. Specimens were taken at standardized sites from the proximal and midssegment of the iliac artery and proximal and midssegment of the femoral artery as described previously.14 Histologic study was carried out on paraffin-embedded sections. Sections were stained with hematoxylin and eosin and Verhoeff-Van Gieson. The size of the intima and media were morphometrically determined with an image analyzer as described previously.14

Mean values were analyzed with an analysis of variance general linear models procedure from Statistical Analysis Systems (SAS). Tukey’s studentized range test was used to determine which pairs of means were significantly different. The pressure-flow slopes fulfilled the conditions of parallelism. Nonparametric one-way analysis of variance (Kruskal-Wallis test)
Results

Plasma Lipids

Plasma total cholesterol level was 107 ± 5 mg/dl in normal monkeys, 584 ± 37 mg/dl in hypercholesterolemic monkeys, 589 ± 57 mg/dl in early atherosclerotic monkeys, and 637 ± 35 mg/dl in atherosclerotic monkeys. Plasma triglyceride levels were less than 40 mg/dl in all four groups.

Morphologic Changes

Five of eight monkeys that were fed atherogenic diet for 4 months (hypercholesterolemic group) had minimal intimal thickening confined to proximal iliac artery. The three remaining monkeys in the hypercholesterolemic group, and the normal monkeys, were normal on histological analysis. In animals fed atherogenic diet for 9 months (early atherosclerotic group), the intimal lesions were predominantly fatty streak lesions. All animals in this group had involvement of the iliac artery, and all but two animals had lesions in the femoral artery. In atherosclerotic monkeys, morphologic changes were similar to those described previously.14,17 There was dense fibrofatty intimal thickening with focal intimal necrosis of the iliac arteries and the proximal part of the femoral arteries. Intimal thickening in the midportion of the femoral arteries consisted largely of fatty streak lesions.

Morphometry showed increases in intimal area in the iliac and femoral arteries of both groups of atherosclerotic monkeys (Table 1). Intimal area was much smaller in monkeys fed atherogenic diet for 9–12 months (early atherosclerotic group) than in monkeys fed atherogenic diet for 19 months. Medial area was not significantly different between groups.

Hemodynamic Studies

Baseline values. Total hind limb vascular resistance and large artery resistance were not significantly different between normal, hypercholesterolemic early atherosclerotic, and advanced atherosclerotic monkeys (Table 2), although values for resistance tended to be increased in atherosclerotic monkeys.

Pressure-flow curves. Pressure-flow relations during maximal vasodilatation are shown in Figure 1. During maximal vasodilatation, perfusion pressure tended to be higher in atherosclerotic monkeys than in normal monkeys and early atherosclerotic monkeys, but differences in slope and intercept did not achieve statistical significance (p > 0.05).

Responses to serotonin. Serotonin produced vasodilatation in the limb (reduction in iliac perfusion pressure) in normal and hypercholesterolemic monkeys (Figure 2). The dilator response to serotonin was impaired in monkeys with early atherosclerosis (p < 0.05 vs. normal monkeys). In monkeys with

![Figure 1. Plot of pressure-flow relation in the perfused hind limb after maximal vasodilatation with papaverine (3 mg/min) in seven normal, five early atherosclerotic (AS), and six advanced AS monkeys. Papaverine was not infused in hypercholesterolemic monkeys. Values are in total hind limb (left) and in large artery segment from iliac artery to dorsal pedal artery (right). Resistance tended to be higher in monkeys with advanced AS than in normal and early AS monkeys (p > 0.05). Values are mean ± SEM.](http://circ.ahajournals.org/lookup/suppl/doi:10.1161/01.CIR.79.3.776/-/DC1/figure1.pdf)

![Figure 2. Bar graphs of responses of limb to intra-arterial injections of serotonin in eight normal, eight hypercholesterolemic, 10 early atherosclerotic (AS), and nine advanced AS monkeys. Increases in perfusion pressure indicate vasoconstriction, and decreases indicate vasodilatation. Responses to serotonin were different in early AS and advanced AS monkeys compared with normal and hypercholesterolemic groups (p < 0.05). Values are mean ± SEM.](http://circ.ahajournals.org/lookup/suppl/doi:10.1161/01.CIR.79.3.776/-/DC1/figure2.pdf)
advanced atherosclerosis, the normal vasodilator response to serotonin was reversed to vasoconstriction ($p<0.05$ vs. normal monkeys) (Figure 2).

Serotonin produced minimal constriction of the large artery segment in the limb of normal and hypercholesterolemic monkeys (Figure 3). The response to serotonin was potentiated in monkeys with early atherosclerosis ($p<0.05$ vs. normal monkeys). There was striking potentiation of constrictor responses in the group with advanced atherosclerosis ($p<0.05$ vs. normal monkeys).

**Responses to phenylephrine.** In contrast to augmented vasoconstrictor responses to serotonin in atherosclerotic monkeys, responses to phenylephrine tended to be less in hypercholesterolemic, early atherosclerotic, and atherosclerotic monkeys than in normal monkeys (Figure 4). Phenylephrine produced modest constriction of large arteries of the limb in all groups, and responses were not significantly different (Figure 5).

**Responses to thromboxane.** Vasoconstrictor responses to the thromboxane analogue were augmented in atherosclerotic monkeys (Figure 6). Thromboxane analogue produced constriction of large arteries of the limb, and this response was potentiated in monkeys with advanced atherosclerosis (Figure 7). Responses to thromboxane were not measured in hypercholesterolemic monkeys.

**Responses to ADP and adenosine.** ADP produced vasodilatation in the limb of normal monkeys (Figure 8). Vasodilator responses to ADP were significantly impaired in hypercholesterolemic monkeys and monkeys with early atherosclerosis and in advanced atherosclerosis (Figure 8) ($p<0.05$ vs. normal monkeys). ADP had minimal effects on resistance of large arteries of the limb in all groups (data not included).

To test the specificity of impaired vasodilator responses to ADP, effects of adenosine were examined. Vasodilator responses to adenosine in the limb were not significantly different in monkeys that were normal, hypercholesterolemic, early atherosclerotic, and in monkeys with advanced atherosclerosis (Figure 9). Adenosine had minimal effects on resistance of large arteries of the limb in all groups (data not included).

**Discussion**

These data indicate first that, atherosclerosis affects vascular responses to the three major vasoactive products that are released by platelets in profoundly different ways: vasoconstrictor responses to serotonin and thromboxane are potentiated, and vasodilator responses to ADP are impaired. Second, even very early atherosclerotic lesions produce marked alterations in vascular responses. Third, ADP produces dilatation of small vessels but not of large arteries. Thus, impairment of dilator responses to ADP by atherosclerosis provides evidence that atherosclerosis impairs endothelium-dependent responses of small vessels, even though small vessels do not develop atherosclerotic lesions.
Pathogenesis of Vasospasm

The mechanism by which atherosclerosis predisposes to vasospasm is not clear, but platelets have been implicated in several studies. Platelets may adhere to atherosclerotic lesions, aggregate, and release vasoactive substances, primarily serotonin, thromboxane, and ADP. The concentrations of adenine nucleotides (adenosine 5'-triphosphate and ADP) and serotonin released by human platelets are approximately 18.5 and 0.9 μM per 10⁶ platelets, respectively. Serotonin is released at concentrations 16-fold greater than thromboxane. In a model of experimental stenosis of coronary arteries, the concentrations of serotonin and thromboxane are markedly elevated at the site of stenosis. Thus, high local concentrations of these vasoactive substances in atherosclerotic arteries may initiate abnormal vasoconstriction responses.

Endothelium appears to have a critical role in modulation of vascular responses to vasoactive substances that are released from aggregating platelets. Adenine nucleotides account for vasodilator response to aggregation of platelets in vitro, whereas serotonin and thromboxane account for vasoconstriction in canine arteries. In a provocative experiment, arterial injury produced by angioplasty of the common carotid artery led to vasoconstriction, and there was a correlation between platelet deposition and degree of vasoconstriction. Atherosclerosis does not lead to loss of endothelium, but it nevertheless appears to increase responsiveness to serotonergic vasoconstrictor responses in vivo and impair vasodilator responses to aggregation of platelets probably in part by producing a functional defect in the endothelium. Thus, it seems reasonable that platelets may have an important role in the pathogenesis of vasospasm.

Considerations of Previous Studies

We have shown previously that vasoconstrictor responses to serotonin in the limb are augmented by atherosclerosis. In the previous experiments, the atherosclerotic lesions were moderately advanced because they consisted predominantly of fibrous plaques. In this study, we examined monkeys with similar moderately advanced lesions and also monkeys with early atherosclerosis in which the lesions consisted predominantly of fatty streaks.

Effects of thromboxane A₂ or its stable analogue U46619 have not been compared in previous studies in the normal or atherosclerotic limb. A study in vitro of coronary vessels from pigs and dogs suggests that removal of endothelium does not alter constrictor responses to U46619. A recent report in coronary arteries of rabbits, however, suggests that endothelium may modulate constrictor responses to the stable thromboxane A₂ analogue 9,11-epithio-11,12-methano-TxA₂(S₅A₂). In the present study, phenylephrine, an endothelium-independent agonist, produced similar constrictor responses in the hind limb in normal monkeys and in monkeys with advanced atherosclerosis. The finding that vasoconstrictor responses to thromboxane are potentiated in monkeys with advanced atherosclerosis...
sis, but that responses to phenylephrine are not altered by atherosclerosis, indicates that augmentation of vasoconstrictor response to thromboxane is somewhat selective.

Vascular responses to ADP in the hind limb have not been evaluated previously in vivo. Studies in isolated canine femoral arteries indicate that ADP is a potent endothelium-dependent vasodilator.\textsuperscript{31} In the present study, vasodilator responses to ADP were impaired by hypercholesterolemia and atherosclerosis. Because vasodilator responses to adenine, which are not endothelium-dependent,\textsuperscript{32} were preserved in atherosclerotic monkeys, endothelial dysfunction, which has been shown with a bioassay for endothelium-derived relaxing factor in atherosclerotic rabbits,\textsuperscript{28} probably has an important role in impaired vasodilator responses to ADP in atherosclerotic monkeys.

ADP had minimal effects on large arteries in this study. Thus, impairment of vasodilator responses to ADP by hypercholesterolemia and atherosclerosis must occur in small vessels, which do not have atherosclerotic lesions. The calculation of resistance of small vessels indicates that resistance is distal to the dorsal pedal artery, but calculation does not allow for differentiation between resistance of small arteries, arterioles, and venules. Impairment by atherosclerosis of dilator responses to ADP in small vessels presumably occurs by impairment of endothelium-dependent relaxation in these vessels. This finding extends our previous studies, which suggest that responses of large arteries are altered by atherosclerosis,\textsuperscript{6,15} and it provides evidence of impaired endothelium-dependent responses in small vessels.\textsuperscript{33}

Shimokawa et al\textsuperscript{4,5} used a model of balloon denudation to evaluate responses of regenerated endothelium in vitro and after accelerating the development of atherosclerosis in vivo. Endothelium-dependent relaxation to aggregating platelets and serotonin is depressed in porcine coronary arteries with regenerated endothelium. Responses to platelet products in vivo were not examined in those studies. Our study extends the findings of the previous studies\textsuperscript{4,5} because we induced atherosclerosis by cholesterol feeding, and not by balloon injury, and examined responses to products of platelet aggregation in vivo.

Mechanisms of Altered Vascular Responses

We considered the possibility that alteration of clearance of serotonin, by several mechanisms, may contribute to alteration of responses to serotonin in atherosclerotic monkeys. Platelets take up serotonin, but differences in inactivation of serotonin by platelets probably did not have a role in alteration of vascular responses to serotonin. Inactivation of serotonin by blood is relatively slow, with a half-life of 60–120 seconds.\textsuperscript{34} In contrast, the half-life of serotonin in vivo is about 7 seconds because serotonin is metabolized primarily by the pulmonary endothelium.\textsuperscript{35} During transit of a bolus through the lung, uptake of serotonin by platelets is negligible.\textsuperscript{36} Also, aggregation of platelets by ADP, serotonin, or thromboxane probably does not occur during bolus injections, because the predicted max-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7}
\caption{Bar graphs of effects of intra-arterial injections of thromboxane A\textsubscript{2} analogue U46619 on large artery segment in eight normal, six early atherosclerotic (AS), and seven AS monkeys. Responses were not examined in hypercholesterolemic monkeys. Constrictor responses to thromboxane analogue were augmented in monkeys with advanced atherosclerosis (p<0.05). Values are mean±SEM.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8}
\caption{Bar graphs of responses to intra-arterial injections of ADP on total limb (iliac) perfusion pressure in eight normal, six hypercholesterolemic, 10 early atherosclerotic (AS), and nine AS monkeys. Responses to ADP were less in hypercholesterolemic, early AS, and advanced AS than in normal monkeys (p<0.05). Values are mean±SEM.}
\end{figure}
Implications

Arterial spasm or increased vasoconstriction often occurs at sites with only modest atherosclerotic lesions defined by angiography. We have shown that responses to several vasoactive substances that are released by platelets are altered by atherosclerosis, even in vessels with modest lesions. These altered responses may explain, at least in part, the susceptibility to vasospasm that occurs in arteries with minimal lesions.

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References


Figure 9. Bar graphs of responses to intraarterial injections of adenosine on total limb (iliac) perfusion pressure. Responses to adenosine were not significantly different in normal, hypercholesterolemic, early atherosclerotic (AS), and advanced AS monkeys.


**KEY WORDS** • thromboxane analogue • platelets • peripheral circulation • cynomolgus monkeys
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