Atherosclerosis Impairs Endothelium-Dependent Vascular Relaxation to Acetylcholine and Thrombin in Primates

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SUMMARY. To test the hypothesis that atherosclerosis impairs endothelium-dependent vascular relaxation, we examined the effect of the endothelium-dependent vasodilators acetylcholine and thrombin and the endothelium-independent vasodilator nitroglycerin on iliac arteries from normal cynomolgus monkeys and cynomolgus monkeys with diet-induced atherosclerosis. Rings of iliac artery were suspended in organ chambers at their optimal length for generating tension. After preconstriction with prostaglandin F2 alpha, cumulative concentration-response curves to acetylcholine, thrombin, and nitroglycerin were examined. The presence of endothelium was confirmed in each vessel by scanning electron microscopy. Atherosclerotic vessels showed morphologic evidence of moderate to severe atherosclerosis. Acetylcholine produced a maximal relaxation of 65 ± 10% in the normal group and 27 ± 10% in atherosclerotic vessels (P < 0.05). Thrombin (10 U/ml) produced relaxation of 39 ± 9% in the normal group and 13 ± 7% in atherosclerotic iliac arteries (P < 0.05). Nitroglycerin relaxed both normal and atherosclerotic blood vessels to an equal extent; maximal relaxation was 92 ± 4% in normal vessels and 98 ± 2% in atherosclerotic vessels. To determine if hypercholesterolemia alone produces an abnormality in endothelium-dependent relaxation, we performed two additional studies. First, because veins are exposed to hypercholesterolemia, but do not develop atherosclerosis, we studied relaxation responses to acetylcholine and thrombin in veins from normal monkeys and monkeys with diet-induced atherosclerosis. Veins from normal and atherosclerotic monkeys relaxed to a similar extent upon exposure to the endothelium-dependent vasodilators acetylcholine and thrombin. Second, we studied relaxation responses to acetylcholine, thrombin, and nitroglycerin in left circumflex coronary arteries from normal dogs and dogs fed a hypercholesterolemic diet for 4-5 weeks when serum cholesterol levels were elevated (serum cholesterol 442 ± 14 mg/dl), but before the onset of atherosclerosis. The endothelium-dependent vasodilators acetylcholine and thrombin produced equivalent degrees of relaxation in arteries removed from normal and hypercholesterolemic dogs. These studies demonstrate that atherosclerosis impairs endothelium-dependent relaxation in primate iliac arteries, and that this impairment is not due to a generalized defect in the endothelium caused by hypercholesterolemia, but requires the presence of atherosclerosis (Circ Res 58: 783-789, 1986)

CLINICAL and experimental observations suggest that atherosclerosis alters vascular reactivity. Coronary vessels with atherosclerosis are more susceptible to spasm induced by ergonovine (Schroeder et al., 1978; Waters et al., 1983) and may be predisposed to spontaneous coronary vasospasm (Schroeder et al., 1977; Maseri et al., 1978; Waters et al., 1983). Recent studies have demonstrated enhanced vasoconstrictor responses to serotonin and norepinephrine in the hindlimb of atherosclerotic monkeys (Heistad et al., 1984). Hypersensitivity of atherosclerotic rabbit aortas to ergonovine has also been demonstrated (Henry and Yokoyama, 1980). Enhanced constrictor responses to serotonin and histamine have been observed in coronary arteries following focal denudation of endothelium and hypercholesterolemia (Shimowawa et al., 1983; Kawachi et al., 1984).

The vasodilator actions of many agents, including acetylcholine and thrombin, depend upon release of endothelium-derived relaxing factor (Furchgott and Zawadski, 1980a, 1980b; Furchgott 1981; DeMey et al., 1982; Ku, 1982; Furchgott, 1984). In addition to promoting vasodilation, the endothelium attenuates vasoconstrictor effects of several agents such as platelets, serotonin, and norepinephrine in vitro (Cohen et al., 1983; Cocks and Angus, 1983) and in vivo (Brum et al., 1984; Lamping et al., 1985). Recently, it has been reported that the endothelium produces a vasoconstrictor factor in response to hypoxia (Rubanyi and Vanhoutte, 1985) and in spontaneously hypertensive rats (Luscher and Vanhoutte, 1985).

Atherosclerosis thus might contribute to increased
sensitivity to vasoconstrictor stimuli by producing a functional defect in the endothelium. Hypercholesterolemia causes morphological (Trillo and Pritchard, 1979; Ingerman-Wojensky et al., 1983) and perhaps functional (Henriksen et al., 1979) alterations of the endothelium. Furthermore, intimal thickening in atherosclerosis might cause a barrier to diffusion of endothelium-dependent relaxing factor (EDRF) from endothelium to vascular media.

We tested the hypothesis that atherosclerosis impairs responses to endothelium-mediated vasodilators. We examined effects of two endothelium-dependent vasodilators, acetylcholine and thrombin, and an endothelium-independent vasodilator, nitroglycerine, on iliac arteries from normal and atherosclerotic cynomolgous monkeys. This experimental primate model of diet-induced atherosclerosis closely resembles human atherosclerosis (Wissler and Vesselinovich, 1974; Armstrong et al., 1980).

To separate effects of hypercholesterolemia from effects of atherosclerosis, we performed two additional studies. First, we studied relaxation responses in veins from monkeys with diet-induced atherosclerosis. Veins as well as arteries are exposed to hypercholesterolemia, but veins do not develop atherosclerotic lesions. Second, we examined the effect of short-term hypercholesterolemia on the endothelium-dependent relaxation responses to acetylcholine and thrombin in circumflex arteries from dogs, an animal model that is relatively resistant to the development of atherosclerosis. Using these two approaches, we sought to determine whether alterations of endothelium-dependent relaxation in atherosclerotic arteries are due to a diffuse abnormality of endothelial function produced by hypercholesterolemia, or if the presence of atherosclerosis is required.

**Methods**

**Source of Normal and Atherosclerotic Blood Vessels**

Atherosclerosis was induced in a group of cynomolgous monkeys (n = 12) by feeding them an atherogenic diet (semisynthetic diet containing 40% fat and 0.74% cholesterol) for 18 months. A control group of monkeys (n = 13) was maintained on commercial laboratory chow (Purina Monkey Chow, Ralston Purina Company). The mean serum cholesterol at the end of 18 months was 622 ± 56 mg/dl in the atherosclerotic group vs. 99 ± 7 mg/dl in the control group (P < 0.005).

On the day of the study, normal or atherosclerotic monkeys were sedated with ketamine (15 mg/kg iv) and anesthetized with chloralose (100 mg/kg iv). The iliac arteries were isolated and excised.

**Isolated Vascular Ring Preparation**

Vessels were cut into 5-mm ring segments and were suspended in a vertically oriented organ bath in 25 ml of Kreb's buffer (composition in mM: NaCl, 118.3; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.2; KH2PO4, 1.2; NaHCO3, 25; EDTA- Ca, 0.026; glucose, 11.1; pH 7.40) aerated with 95% O2, 5% CO2, and maintained at 37°C. All studies were performed in the presence of propranolol (10−6 M). Tension was recorded with a linear force transducer (Grass FT03c) on an oscillographic recorder.

In some experiments, the endothelium was removed from the segment by rubbing the intimal surface with the tip of a closed hemostat.

Over 2 hours, the resting tension of the vascular ring was gradually increased until the optimal tension for generating force during isometric contraction was reached. At each tension, the vessel was exposed to KCl (100 mM), and the tension generated was recorded. After each KCl dose, the baths were washed with fresh buffer. The resting tension was increased until additional doses of KCl failed to increase further the constrictor response. The vessels were left at this optimal resting tension throughout the remainder of the study.

**Determining Preconstricting Dose of Prostaglandin F2α (PGF2α)**

To study vasodilator responses, we preconstricted the vascular rings with PGF2α. To establish the concentration of PGF2α that would give a submaximal constriction, we determined a complete PGF2α concentration-response relationship for each vessel. A dose of PGF2α that produced 30%–50% of the maximal constriction was used in subsequent experiments to preconstrict the vessel before the vasodilator drugs were added.

**Drug Preparation**

Drugs used in the study were prostaglandin F2α (Tris salt, Sigma), acetylcholine (Sigma), and nitroglycerine (American Critical Care). Bovine thrombin was generously supplied by Dr. Whyte Owen. All drug dilutions were prepared with distilled water. Drugs were diluted so that less than 0.1 ml was added for each dose.

**Protocols**

We examined cumulative relaxation responses to acetylcholine (10−9 to 10−4 M), thrombin (0.1, 1.0, 10.0 U/ml), and nitroglycerine (10−6 to 10−4 M). Before each concentration-response curve, vessels were preconstricted with PGF2α to an ED50 to ED10. Between each concentration-response curve, the vessels were washed at least three times with fresh buffer and were allowed to reequilibrate for at least 30 minutes.

**Studies in Veins**

In seven normal and five atherosclerotic monkeys, segments of jugular veins were removed and studied in a fashion identical to that used for iliac arteries. Responses to acetylcholine (10−9 to 10−4 M), thrombin (0.1 U/ml, 1.0 U/ml, and 10.0 U/ml) and nitroglycerine (10−6 to 10−4 M) were examined, each after preconstriction with PGF2α.

**Studies in Dogs**

Mongrel dogs of either sex (n = 5), weighing 19–30 kg, were fed a high-cholesterol diet deficient in essential fatty acids consisting of 5% cholesterol, total fat 21% (wt/wt) (Ehrhart Atherogenic Test Diet, Teklad). At the end of 4–5 weeks, serum cholesterol was 442 ± 14 (n = 5). Eight mongrel dogs (serum cholesterol 85 ± 12) were used as controls. On the day of the study, dogs were anesthetized with sodium pentobarbital (30 mg/kg, iv), the chest was opened by left thoracotomy, and the pericardium was opened. The heart was electrically arrested and removed. The left circumflex coronary artery...
was dissected free and excised. Vascular rings were prepared and studied in a manner identical to that used for monkey iliac arteries.

Quantification of Endothelium

All vascular rings were examined by scanning electron microscopy. At the completion of the experiment, each vascular segment was immersed in fixative (2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for approximately 5 minutes, while still mounted on the isolated ring apparatus. The vessel was then removed from the apparatus and maintained in fixative at 4°C for at least 24 hours. After fixation, the vessel was cut longitudinally to expose the intimal surface, mounted, and prepared for scanning electron microscopy. Each vessel segment was examined at 600× magnification, and a visual estimate of the percent surface area covered by endothelium was made. Despite efforts to minimize damage to the endothelium, some loss of endothelium did occur. Only those vessels with more than 30% endothelial coverage were included in the subsequent data analysis for acetylcholine and thrombin. The average endothelial coverage determined by this method was 46 ± 4% in normal monkeys and 63 ± 5% in the atherosclerotic monkeys.

To confirm the accuracy of our visual estimate of endothelial coverage as determined by scanning electron microscopy, six normal and six atherosclerotic vessels were chosen at random and examined by transmission electron microscopy at 2800X. From each vessel, 60–80 photographs of the intimal surface were obtained from randomly chosen sites. The area covered by endothelial cells and the total surface of the intimal surface were planimetered with a linear digitizer (MOP-3, Zeiss). The area covered by endothelium was expressed as the amount of intimal surface covered by endothelium divided by the total intimal surface. By this method, the normal vessels had 53 ± 8% endothelial coverage, and atherosclerotic vessels were covered with endothelium over 51 ± 3% of their surface (P > 0.5).

Assessment of Atherosclerosis

After fixation, a 1-mm section was cut from the end of each monkey iliac artery vascular ring and stained with hematoxylin and eosin or hematoxylin and orcein. Gross and histological examination showed moderate to severe atherosclerosis in the iliac arteries of monkeys fed an atherogenic diet for 18 months. All vessels in the atherosclerotic group had histological evidence of atherosclerosis. Atherosclerotic lesions were not present in iliac arteries from normal monkeys.

Data Analysis and Statistics

Relaxation responses to acetylcholine and nitroglycerin were expressed as the percent relaxation from the amount of preconstriction produced by PGF$_{2\alpha}$. Thrombin, when administered to a preconstricted normal vessel, typically caused a biphasic response, initial relaxation followed by a return to the preconstricted tension. Based on preliminary observations from our laboratory and by others (Ku, 1982; DeMey et al., 1982), only the relaxation portion of the thrombin response is endothelium dependent. Thus, for thrombin, we compared the relaxation responses in normal and atherosclerotic monkeys. This response was recorded as the cumulative relaxation response at each dose, expressed as a percentage of the initial preconstricted tension. The jugular veins relaxed to lower concentrations of acetylcholine, but often began to contract at higher concentrations. In preliminary experiments, we found the relaxation response was absent after removal of the endothelium. The ED$_{50}$ was calculated as the concentration of acetylcholine that produced half-maximal relaxation.

Student's t-test for unpaired comparisons was used to compare concentration-response curves at each drug concentration. Data are expressed as the mean ± SEM. The level of confidence chosen for statistical significance was P < 0.05.

Results

Baseline Characteristics

The optimal resting tensions were not different in normal and atherosclerotic vessels, 5.5 ± 0.4 g and 5.9 ± 0.6 g, respectively (P > 0.50). The response to KCl (100 mM) was greater in the normal vessels, 4.7 ± 0.6 g vs. 2.1 ± 0.4 g in the atherosclerotic vessels (P < 0.05). Similarly, the peak response to PGF$_{2\alpha}$ was greater in the normal vessels, 8.7 ± 0.6 g, compared to the atherosclerotic group, 4.7 ± 0.7 g (P < 0.05).

Responses to Acetylcholine

Responses to acetylcholine in atherosclerotic and normal vessels are shown in Figures 1 and 2. In all normal vessels with endothelium, acetylcholine produced concentration-dependent relaxation. In normal vessels denuded of endothelium, the response to acetylcholine was markedly attenuated, averaging 12% relaxation. Only one of four denuded vessels exhibited any response to acetylcholine. In contrast to the normal vessels, only seven of 12 atherosclerotic vessels with endothelium present relaxed to acetylcholine. The average total relaxation to acetyl-
Acetylcholine in all atherosclerotic vessels was less than half that observed in the normal vessels. The ED$_{50}$ for relaxation to acetylcholine could not be calculated for the atherosclerotic vessels that failed to respond; however, in the remaining atherosclerotic vessels, the ED$_{50}$ was not different from that of the normal vessels.

Responses to Thrombin

Typical responses to thrombin are shown in Figure 3. In normal vessels, thrombin produced initial vascular relaxation, often followed by either vasoconstriction or return to the baseline preconstricted tension. In atherosclerotic vessels, the relaxation response was markedly diminished, while contraction was preserved. The relaxation response was greater in normal vessels than atherosclerotic vessels (Table 1). Thrombin did not cause relaxation in normal vessels denuded of endothelium ($n = 4$).

Responses to Nitroglycerin

Figure 4 shows the cumulative relaxation responses to nitroglycerin observed in normal and atherosclerotic vessels. The maximal relaxation response and ED$_{50}$ for nitroglycerin were similar in normal and atherosclerotic vessels (Table 1).

Responses to Acetylcholine, Thrombin, and Nitroglycerin in Jugular Veins of Normal and Atherosclerotic Monkeys

Relaxation responses to acetylcholine, thrombin, and nitroglycerin in jugular veins removed from atherosclerotic and normal animals are summarized in Table 2. These three agents produced similar degrees of relaxation in veins from atherosclerotic and normal monkeys.

Responses to Acetylcholine, Thrombin, and Nitroglycerin in Circumflex Coronary Arteries from Normal and Hypercholesterolemic Dogs

The endothelium-dependent agonists acetylcholine and thrombin and the endothelium-independent agonist nitroglycerin produced similar degrees of relaxation in circumflex coronary arteries from normal and hypercholesterolemic dogs (Table 2).
Acetylcholine

Max% relaxation  Thrombin  Nitroglycerin

Max% relaxation  Thrombin  Nitroglycerin

Normal monkeys  65  4 ± 1 x 10^-7 M  Normal monkeys  13  31  39  Normal monkeys  92  2 x 10^-8 M
(n = 13)  ± 10  (n = 10)  ± 5  ± 8  ± 9  (n = 9)  ± 4
Atherosclerotic  27  9 ± 3 x 10^-7 M†  Atherosclerotic  4  12  13  Atherosclerotic  98  5 ± 2 x 10^-8 M
monkeys  ± 10*  (n = 9)  ± 3*  ± 6*  ± 7*  (n = 9)  ± 1
(n = 12)

Results are expressed as mean ± SE
* P < 0.05 compared to normal monkeys
† Calculation based only on vessels that responded to acetylcholine. % Relaxation = percent relaxation from preconstricted tension, Max% relaxation = maximal percent relaxation

Discussion

The major finding in this study is that vascular relaxation to endothelium-dependent vasodilator stimuli is impaired in atherosclerotic blood vessels. This defect in relaxation was observed in response to endothelium-dependent vasodilator agonists, acetylcholine and thrombin, but not to the endothelium-independent vasodilator nitroglycerin. Furthermore, the defect in relaxation observed in atherosclerotic blood vessels apparently was not produced by a diffuse alteration in the endothelium caused by hypercholesterolemia, since veins from atherosclerotic monkeys and coronary arteries from hypercholesterolemic dogs relaxed normally in response to endothelium-dependent agonists.

All vessels used in this study were examined by scanning electron microscopy to confirm the presence of endothelium. Despite efforts to minimize endothelial injury, some degree of endothelial denudation occurred in virtually all vessels during the course of an experiment. To assess the effect of partial denudation on the response to vasodilators, we measured the percentage of the surface area covered by endothelium. Our results showed that the intimal surface covered by endothelium was similar in normal and atherosclerotic vessels. Thus, impairment of endothelium-dependent vascular relaxation in atherosclerotic vessels could not be ascribed to greater denudation of endothelium.

Vessels from hypercholesterolemic monkeys with atherosclerotic lesions may show impaired relaxation in response to endothelium-dependent vasodilators for several reasons. First, hypercholesterolemia itself may contribute to the abnormality. Abundant evidence suggests that elevated serum cholesterol can produce morphological alterations of the endothelium (Trillo and Prichard, 1979; Ingerman-Wojensky et al., 1983). Thus, in damaged endothelium, synthesis of endothelium-derived relaxing factors (EDRF) may be impaired. Second, the atherosclerotic process may produce a barrier between endothelium and vascular smooth muscle so that EDRF, although present, is unable to reach the site of its action. This barrier might take several forms. Because EDRF has an extremely short half-

FIGURE 4. Relaxation responses to nitroglycerin in normal (○) and atherosclerotic (●) monkey iliac arteries. Increasing concentrations of nitroglycerin are shown along the abscissa, and percent relaxation is shown along the ordinate. At each concentration of nitroglycerin, normal and atherosclerotic monkey iliac arteries relaxed to the same extent.
life (Griffith et al., 1984; Rubanyi et al., 1985). Intimal thickening and increased diffusion distance might lead to diminution of the EDRF-mediated response. Alternatively, increases in lipids in the intima might adsorb lipophilic substances and impair diffusion of EDRF to the vascular smooth muscle. Finally, cellular elements in the advanced atherosclerotic plaque might lead to increased degradation of EDRF. A third factor in alteration of vascular responses in atherosclerosis might be that changes in membrane lipid composition and fluidity alter affinity of vascular muscle for EDRF or alter EDRF release (Lurie et al., 1985). This possibility is unlikely because responses were not impaired in hypercholesterolemic monkey veins or canine arteries. Fourth, the endothelium in atherosclerotic vessels might produce a vasoconstrictor agent (DeMey and Vanhoutte, 1982). Recent work in hypertensive rats has shown that endothelium is capable of producing a constrictor substance (Luscher and Vanhoutte, 1985; Rubanyi and Vanhoutte, 1985). Atherosclerosis may increase synthesis of an endothelium-derived vasoconstrictor.

Previous investigations have demonstrated that vasoconstrictor responses to serotonin and platelets are enhanced in vessels denuded of endothelium (Cohen et al., 1983; Cocks and Angus, 1983). These findings suggest that the endothelium attenuates responses to certain vasoconstrictor agents. This observation was extended by the recent observation that endothelial denudation and hypercholesterolemia can be used to produce coronary artery spasm in miniature swine and dogs (Shimowawa et al., 1983; Kawachi et al., 1984). Recent preliminary reports by Habib et al. (1984) and Herman et al. (1985) indicate that, after hypercholesterolemia induces intimal changes, endothelium-dependent vascular relaxation is impaired in rabbit aorta. This finding suggests that hypercholesterolemia and/or atherosclerosis impair endothelium-dependent vascular responses. Our results in monkey veins and hypercholesterolemic dog arteries suggest that the defect in endothelial function is produced by atherosclerosis and not by hypercholesterolemia per se.

Recently, a preliminary study by Bossaller et al. (1985) showed that responses to acetylcholine were abnormal in cholesterol-fed rabbits, whereas responses to A23187 were normal. These data suggest a defect in the muscarinic receptor rather than an inability of the endothelium to produce the relaxing factor. In our studies, we found abnormal responses to both acetylcholine and thrombin. Thus, in this primate model of atherosclerosis, abnormalities of endothelium-dependent relaxation are not solely related to an alteration of the muscarinic receptor, but involve either multiple receptors or some aspect of the underlying effector mechanism.

Our findings may explain in part the clinical observation that patients with coronary atherosclerosis are susceptible to coronary spasm and are more sensitive to the vasoconstrictor effects of ergonovine (Schroeder et al., 1977; Maseri et al., 1978; Waters et al., 1983). We speculate that a defect in endothelium-dependent vasodilation may be important in the pathogenesis of vascular spasm, and in clinical disorders in which vasospasm is associated with atherosclerosis.

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