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

Paul C. Freiman, Gordon G. Mitchell, Donald D. Heistad, Mark L. Armstrong, and David G. Harrison

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 F2α, 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 (100 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. 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.

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 cynomolgus monkeys. This experimental primate model of diet-induced atherosclerosis closely resembles human atherosclerosis (Wisler and Vesselinovitch, 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 cynomolgus 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 im) 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 (10⁻⁷M). 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 10⁻⁴M), thrombin (0.1, 1.0, 10.0 U/ml), and nitroglycerine (10⁻⁴ to 10⁻⁶M). Before each concentration-response curve, vessels were preconstricted with PGF₂α to an EDC₅₀ to EDC₅₀. 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 10⁻⁴M), thrombin (0.1 U/ml, 1.0 U/ml, and 10.0 U/ml) and nitroglycerine (10⁻⁴ to 10⁻⁶M) 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 600X 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 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**

Responses to acetylcholine and nitroglycerin were expressed as the percent relaxation from the amount of preconstriction produced by PGF$_2$α. 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.

**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$_2$α 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 acetylcholine.
choline 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 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).
TABLE 1  
Vascular Relaxation of Normal and Atherosclerotic Iliac Arteries

<table>
<thead>
<tr>
<th></th>
<th>Acetylcholine</th>
<th>Thrombin</th>
<th>Nitroglycerin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Max% relaxation</td>
<td>ED50</td>
<td>Max% relaxation</td>
</tr>
<tr>
<td>Normal monkeys</td>
<td>65 ± 10 U/ml</td>
<td>13 ± 5</td>
<td>Normal monkeys</td>
</tr>
<tr>
<td>(n = 13)</td>
<td>9 ± 10 U/ml</td>
<td>10 ± 3</td>
<td>(n = 10)</td>
</tr>
<tr>
<td>Atherosclerotic monkeys</td>
<td>27 ± 10*</td>
<td>4 ± 3*</td>
<td>Atherosclerotic monkeys</td>
</tr>
<tr>
<td>(n = 12)</td>
<td>9 ± 3 × 10⁻⁷ M</td>
<td>4 ± 3*</td>
<td>(n = 9)</td>
</tr>
</tbody>
</table>

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.
Jugular vein response to serotonin and platelets

\[ \text{EDR} \text{F release} \text{Lurie et al., 1985) This possibility is extended by the recent observation.} \]

Coronary artery response to serotonin and platelets

<table>
<thead>
<tr>
<th></th>
<th>Max% relaxation</th>
<th>ED50</th>
<th>% Relaxation</th>
<th>Nitroglycerin</th>
<th>Max% relaxation</th>
<th>ED50</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 1</td>
<td>1 0 10 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jugular vein</td>
<td></td>
<td></td>
<td>U/ml U/ml U/ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal monkey</td>
<td>39</td>
<td>±11</td>
<td>±2 ± 10⁻⁸ M</td>
<td></td>
<td>Normal monkey</td>
<td>38</td>
</tr>
<tr>
<td>Atherosclerotic</td>
<td>36</td>
<td>±14*</td>
<td>±2 ± 10⁻⁸ M</td>
<td></td>
<td>Atherosclerotic</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>monkey (n = 6)</td>
<td>40</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>48</td>
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<td></td>
<td></td>
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<td></td>
<td>100</td>
</tr>
</tbody>
</table>

| Coronary artery| Normal dog      | 100   | 7 ± 3 ± 10⁻⁸ M |               | Normal dog      | 38    | 91 ± 92 ± 92 ± 92 |
|                | Hypercholestero| 95    | ±5‡         | Hypercholes- | Hypercholes- | 51    | 93 ± 93 ± 93 ± 93 |
|                | lemic dog       | (n = 5)|            | terolemic dog| terolemic dog| 48    | 16* ± 16* ± 16* ± 16* |
|                |                 |       |              | (n = 8)       | (n = 8)       |       | ±6* ±6* ±6* ±6* |
|                |                 |       |              |               |               |       | ±0 ±0 ±0 ±0 ±0 ±0 |

Data are expressed as mean ± se. % Relaxation = percent relaxation from preconstucted tension Max% relaxation = maximal percent relaxation *P > 0.5, ‡P > 0.2, †P > 0.1 Hypercholesterolemic vs normal vessels

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Luscher TF, Vanhoutte PM (1985) Endothelium-dependent contractions to acetylcholine in the aorta of spontaneously hypertensive rats Abstract presented at the annual meeting of the Council for High Blood Pressure Research, September


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