Impaired Endothelium-Dependent Relaxation to Aggregating Platelets and Related Vasoactive Substances in Porcine Coronary Arteries in Hypercholesterolemia and Atherosclerosis

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Vasoconstrictor responses are augmented in porcine coronary arteries in hypercholesterolemia and atherosclerosis, leading to an occurrence of coronary vasospasm in the latter condition. The role of the endothelium in the vascular hyperreactivity in hypercholesterolemic and atherosclerotic coronary arteries was examined, particularly in response to aggregating platelets and related vasoactive substances. Male Yorkshire pigs underwent balloon endothelial denudation of the left anterior descending coronary artery (LAD) and 2% high-cholesterol feeding for 10 weeks. Electron microscopic examination demonstrated a full lining of endothelial cells in the LAD and the left circumflex coronary artery (LCX). Endothelium-dependent responses were examined in vitro. In cholesterol-fed animals, endothelium-dependent relaxations to aggregating platelets, serotonin, ADP, bradykinin, thrombin, and the calcium ionophore A23187 were depressed in LAD (atherosclerosis), while the relaxations to aggregating platelets, serotonin and ADP were depressed in LCX (hypercholesterolemia). Serotonin-induced contractions were endothelium-dependently augmented in atherosclerotic LAD; the endothelium-dependent component of the contractions was inhibited by blockers of cyclooxygenase. Bioassay studies demonstrated a depressed release of endothelium-derived relaxing factor(s) from the atherosclerotic LAD in response to serotonin. These experiments indicate that the endothelium-dependent relaxations to aggregating platelets and related vasoactive substances are severely impaired in atherosclerosis and moderately impaired in hypercholesterolemia. Since coronary atherosclerosis was induced by a combination of balloon endothelial injury (and regeneration) and high-cholesterol feeding in this study, the combined effects of those factors must account for the severely impaired responses in atherosclerosis. The depressed release of the endothelium-derived relaxing factor(s) and the concomitant release of vasoconstrictor product(s) of cyclooxygenase appear to be responsible for the impaired relaxations. (Circulation Research 1989;64:900–914)

The endothelial cells play an important role in modulating the responsiveness of underlying vascular smooth muscle. Among the possible pathophysiological roles of the endothelium, its protective action against platelet aggregation appears to be important. Impaired endothelium-dependent relaxations in atherosclerosis have been reported in the rabbit aorta, monkey iliac artery, pig coronary artery, and human coronary artery in vitro and in vivo. In contrast, no information is available concerning the endothelium-dependent relaxations to aggregating platelets in atherosclerosis. Augmented vasoconstrictor responses have been reported in hypercholesterolemia and atherosclerosis. In particular, in a pig model of coronary atherosclerosis induced by a combination of balloon endothelial injury and high-cholesterol feeding, coronary vasospasm with myocardial ischemia can be provoked in vivo. Aggregating platelets are an important source of vasoconstrictor substances and have been regarded as one of the most possible causes for coronary vasospasm. The present experiments were designed to examine whether or not the protective role of the endothelium against aggregating platelets is impaired in...
hypercholesterolemic and atherosclerotic porcine coronary arteries. When it appeared that this was the case, we attempted to determine the mechanism(s) involved.

Materials and Methods

Fifty-four male Yorkshire pigs, 6–8 weeks of age (18.2±0.6 kg), were randomly divided into two groups. The control group (n=23) was fed a regular chow (Hog Finisher, Bedike Brothers Feed and Seed Co, Dover, Minnesota) with 0.09% cholesterol; the cholesterol group (n=31) was fed a diet with 19% lard and 2% cholesterol (TD86019, Teklad, Madison, Wisconsin). After 2 weeks of feeding, pigs in the cholesterol group underwent coronary endothelial denudation of the left anterior descending coronary artery (LAD) to allow the rapid development of atherosclerosis. 15,16,18–20 Animals in the control group underwent coronary angiogram only. After this, they were fed for 8 more weeks. In this model, the effects of atherosclerosis in the LAD and hypercholesterolemia in the left circumflex coronary artery (LCX) can be evaluated in the same animals in the cholesterol group by comparing the data with those from the control group. 15,16,19 The serum concentrations of lipids (enzymatic method), 21 heart rate, and blood pressure (Mayo Medical Engineering, Rochester, Minnesota) were determined before and 10 weeks after the feeding. The pigs were housed individually in temperature-controlled animal quarters. To prevent excessive weight gain, the daily food intake was limited to an amount equal to 3% of the body weight per day. 16,22 In vivo experiments were performed after 10 weeks of feeding, using 42 pigs (n=18 in the control group and n=24 in the cholesterol group). Two pigs from the cholesterol group, in which small apical myocardial infarction had occurred, were excluded from the study in order to avoid any possible influence of the infarction on vascular reactivity. The other 10 pigs (n=5 in each group) were used for scanning and transmission electron microscopic examinations.

Coronary Endothelial Denudation

The details were reported previously. 22 The pigs were anesthetized with 300 mg i.m. ketamine hydrochloride and 12.5 mg/kg i.v. sodium pentobarbital. After collecting antologous blood (300 ml) from the left carotid artery, they were exsanguinated and the heart was removed. The coronary arteries were removed and immersed in cold modified Krebs-Ringer bicarbonate solution of the following millimolar composition: NaCl 118.3, KCl 4.7, MgSO4 1.2, KHPO4 1.2, CaCl2 2.5, NaHCO3 25.0, CaEDTA 0.016, and glucose 11.1 (control solution). The experiments were performed on 3- to 4-mm long rings of proximal LAD and LCX. Since endothelial denudation was performed in the first 4 cm of the proximal portion of the LAD from the left coronary orifice, this portion of the artery was used for organ chamber experiments. The comparable portion of the LCX was used as control. The rings were numbered from proximal to distal, and rings with the same number taken from the LAD and LCX were studied in parallel. The rings were cleaned of loose connective tissue, with special care taken not to touch the luminal surface. In some of the rings, the endothelium was removed deliberately by rubbing the luminal surface gently with a cotton swab wetted with control solution. 22

The rings were mounted horizontally in organ chambers filled with 25 ml of control solution (37° C, pH 7.4) gassed with 95% O2-5% CO2. The preparations were attached to a strain gauge (model UC2, Gould Statham, Oxnard, California), and isometric tension was recorded. The rings were progressively stretched until the contractile response evoked by 20 mM potassium chloride was maximal (optimal tension). 22 They were allowed to equilibrate for 30 minutes before the experiments.

Protocol

After 30 minutes of equilibration, bradykinin (concentration response curve [10^-10 to 10^-5 M] or one dose [10^-7 M]) was given to all rings during a contraction evoked by prostaglandin F2a (2×10^-6 M) to confirm the presence or absence of functional endothelial cells. After this, relaxations were examined during a contraction caused by prostaglandin
F\textsubscript{2\alpha} (2\times10^{-6} \text{ M}) and contractions were examined in quiescent rings in the following order:

Set A (relaxation): 1) serotonin (10^{-7}-3\times10^{-8} \text{ M}), 2) platelets (70,000/\mu \text{l}), and 3) thrombin (0.1 units/ml)

Set B (relaxation): 1) adenosine diphosphate (ADP) (10^{-5}-10^{-4} \text{ M}), 2) sodium nitroprusside (10^{-5}-10^{-6} \text{ M}), and 3) the calcium ionophore A23187 (10^{-5}-10^{-4} \text{ M})

Set C (contraction): 1) serotonin (10^{-6}-10^{-5} \text{ M}), 2) platelets (70,000/\mu \text{l}), and 3) KCl (5-100 mM)

In organ chamber experiments with isolated rings of blood vessels, unlike under physiological conditions, or when examining perfused segments of arteries, the administered drugs can reach directly the vascular smooth muscle from the adventitial sides and the cut surfaces. It is appropriate to inhibit the direct effects of the drugs on the vascular smooth muscle to examine their effects on the endothelium. Therefore, when determining relaxations to aggregating platelets and serotonin, the rings first were incubated with 10^{-5} \text{ M} ketanserin for 40 minutes. Previous experiments demonstrated that the \text{S}_{2}-serotnergic antagonist unmasks the endothelium-dependent responses to serotonin\textsuperscript{21,22} and platelets\textsuperscript{22} by inhibiting the \text{S}_{2}-serotnergic activation of vascular smooth muscle cells. Likewise, the rings were treated with the \text{P}_{1}'-purinergic blocker theophylline (10^{-4} \text{ M}) when studying relaxations to ADP since previous observations demonstrated that this drug inhibits the relaxing effect of the adenine nucleotide on smooth muscle cells without affecting the endothelium-dependent component of the action of ADP.\textsuperscript{22}

In most experiments of endothelium-dependent relaxations, all rings were treated with 10^{-5} \text{ M} indomethacin for 40 minutes before inducing contraction with 2\times10^{-5} \text{ M} prostaglandin \text{F}_{2\alpha} to prevent the synthesis of endogenous prostanoids. To examine the effects of indomethacin on the relaxations, rings were treated with either 10^{-3} \text{ M} indomethacin or 10^{-5} \text{ M} Na\textsubscript{2}CO\textsubscript{3} (solvent of indomethacin) only and were examined in parallel.

Since endothelium-dependent contractions were noted in response to serotonin in the atherosclerotic LAD from cholesterol-fed pigs, the effects of blockers of cylooxygenase (indomethacin and meclophenamate), thromboxane synthetase (dazoxiben), thromboxane receptor (SQ 29548), and lipooxygenase (nordihydroguaiaretic acid and BAY G6575) on those contractions were examined. In addition, in some cases 2.0-ml samples of fluid were withdrawn from the chambers before and 5 minutes after the completion of cumulative concentrations of serotonin (10^{-10}-10^{-3} \text{ M}) and frozen for later analysis. The concentrations of prostaglandins in the bath solution were determined by radioimmunoassay (Advanced Magnetics Inc, Cambridge, Massachusetts)\textsuperscript{26}; the detection level of prostaglandins in the present study was 20 pg/ml for 6-keto-prostaglandin \text{F}_{1\alpha} and thromboxane \text{B}_{2} and 5 pg/ml for prostaglandin \text{F}_{2\alpha} and prostaglandin E\textsubscript{2}. Cross-reactivity of the antisera for 6-keto-prostaglandin \text{F}_{1\alpha} to prostaglandin \text{F}_{2\alpha} was 2.2\%, that for prostaglandin E\textsubscript{2} to prostaglandin \text{F}_{2\alpha} was 1.3\%, and those the rest of the combinations of the four prostaglandins were less than 1\%.

Bioassay

The bioassay method used has been described in detail previously.\textsuperscript{27} After removal from the hearts, side branches of 4-cm segments of the LAD were tied. The segments were treated gently to minimize damage to the endothelium. The segments were tied to stainless steel canulas and placed into an organ chamber maintained at 37° C and filled with 12 ml aerated (95% O\textsubscript{2}-5% CO\textsubscript{2}) control solution. The segments were perfused at constant flow (2 ml/min) by means of a multichannel roller pump (Minipuls 2, Gilson Medical Electronics, Inc, Middleton, Wisconsin) with control solution maintained at 37° C. A stainless steel tube was also placed in the organ chamber, through which control solution was pumped at the same rate. A ring of the LAD from the same animal, from which the endothelium had been removed by rubbing the intimal surface (bioassay ring), was suspended directly below the organ chamber by means of two stainless steel stirrups passed through its lumen. One stirrup was fixed and the other connected to an isometric force transducer (model FT03C, Grass Instrument Co, Quincy, Massachusetts), and changes in isometric tension were recorded. The assembly of bioassay ring, stirrups, and force transducer could be moved freely below the organ chamber, allowing the preparation to be superfused with the perfusate either from the coronary segment with endothelium (endothelial superfusion, endothelial line) or from the stainless steel tube (direct superfusion, direct line); there was no difference in the wet weight of the bioassay ring between the two groups (14±1 mg in both).

The bioassay ring was first superfused (for 60 minutes) with control solution passing through the stainless steel canula. During this interval it was stretched in a stepwise manner with repetitive exposure to 20 mM KCl until the basal tension reached approximately 8-9 g, the optimal tension for active contraction of rings of isolated porcine coronary arteries, as determined in preliminary organ chamber studies. The bioassay ring was also treated with 10^{-6} \text{ M} ketanserin throughout the experiments by adding the antagonist directly to the ring using a separate infusion line located below the donor segment. To detect the basal release of endothelium-derived relaxing factor, the bioassay ring was contracted with prostaglandin \text{F}_{2\alpha} (2\times10^{-6} \text{ M}) under direct superfusion and then positioned beneath the endothelial line (containing 2\times10^{-6} \text{ M} prostaglandin \text{F}_{2\alpha}). Serotonin was chosen as the agonist in the bioassay experiment for the detection not only of endothelium-derived relaxing factor(s) but also of possible release of indomethacin-sensitive, endothelium-derived contracting factor(s) as suggested by the organ chamber experiments. Serotonin (3\times10^{-4} \text{ M}) was infused by means of infusion pumps.
The concentrations of serotonin and thromboxane B2 were determined by reverse-phase high pressure liquid chromatography and by radioimmunoassay, respectively.

**Morphology**

The hearts were divided into five horizontal blocks and examined macroscopically for the presence or absence of myocardial infarction.

The rings used in the organ chamber study were examined histologically by hematoxylin-eosin staining for determination of endothelial lining and general observation, by van Gieson's elastic staining for determination of the thickness of the intima and the media, and by Sudan IV staining for confirmation of lipid deposition in the blood vessel wall.

Scanning and transmission electron microscopic examinations were performed in five pigs in each group. The hearts of these pigs were fixed in situ with 2% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate (pH 7.25) (fixation solution) at a perfusion pressure of 110 mm Hg for 15 minutes. The coronary arteries were then removed and kept in the fixation solution until analysis. Longitudinal specimens were cut from each coronary artery, coated with carbon and gold-palladium alloy, and examined with a scanning electron microscope (Etec Autoscan, Hayward, California). To avoid possible anatomical differences in numbers of endothelial cells, 20 scanning electron micrographs (magnification, ×1,000) were taken randomly from each coronary specimen (Figure 1), and numbers of the cells were counted without knowledge and corrected for per square millimeter. Selected specimens were thin-sectioned (600–700 Å), mounted on a 200-mesh copper grid, and stained with uranyl acetate and lead citrate. Sections were examined with a Philips 201 transmission electron microscope (Philips Electronic Instruments, Inc., Mahwah, New Jersey).

**Calculations and Statistical Analysis**

Results are expressed as means ± SEM. Unless otherwise specified, n refers to the number of animals. In rings contracted with prostaglandin F2α, dilator responses are expressed as percent changes from the contracted levels, and in quiescent rings constrictor responses are expressed as percent of the maximal response to KCl (100 mM). For relaxations, the negative logarithm of the effective concentration of agonist causing 50% inhibition [IC50] of the contractions to prostaglandin F2α was calculated for each concentration-response curve and the means of these values are presented. For contractions evoked by serotonin, the effective concentration producing 40% of the maximal response to KCl (ED40) was calculated. The correlations between the cross-sectional area of the intima or the media and endothelium-dependent relaxations were examined...
Figure 1. Scanning electron micrographs of the endothelium of proximal coronary artery of the pig in control (A,B) and the cholesterol (C,D) groups (original magnification, ×1,000). LCX, left circumflex coronary artery; LAD, left anterior descending artery. Direction of flow is from top to bottom. In control group, in both left circumflex (A) and left anterior descending coronary arteries (B), endothelial cells were flat and arranged parallel to direction of blood flow. In cholesterol group, endothelial cells were elongated and the number of the cells had increased in both left circumflex (C) and left anterior descending coronary arteries (D). In the latter, size and shape of the nuclei were irregular, and in several regions adhesion of platelets and other blood cells were noted.

Results

Baseline Data

Body weight increased significantly in both groups (48.6±1.3 kg in the control and 50.3±1.2 kg in the cholesterol group) after 10 weeks of feeding. Heart rate and blood pressure were unchanged in both groups during the experimental period (data not shown). The serum concentration of cholesterol significantly increased in the cholesterol (604±40 mg/dl) but not in the control group (105±4 mg/dl); mainly the fractions of low density and very low density lipoproteins increased (data not shown). In contrast, the concentration of triglyceride was unchanged in both groups (36±4 mg/dl in the control and 48±5 mg/dl in the cholesterol group).

Changes During Denudation Procedure

Immediately after the denudation, the mean diameter of the denuded LAD was significantly reduced in the cholesterol group (3.10±0.07 mm before and 2.68±0.08 mm after denudation, n=29). In contrast, the mean diameter of the control, nondenuded LCX was unchanged (3.05±0.07 mm before and 3.08±0.08 after denudation, n=29). Heart rate and arterial blood pressure were not significantly different before and after the denudation (data not shown). The animals in the control group (n=23) had the same size coronary arteries as the cholesterol group (3.07±0.08 mm in the LADs and 3.02±0.06 mm in the LCXs).

Morphology

Macroscopically visible small regions of myocardial infarction, which were limited to the apex, were present in two of the 31 hearts in the cholesterol group and in none of the 23 hearts in the control group.

Under light microscopy, in all rings used for organ chamber experiments, the presence or absence of the endothelium was confirmed histologically. In the control group, no intimal thickening was noted and there
TABLE 1. Data From Light Microscopic Examination of Cross-Sectional Area of the Intima and Media of Porcine Coronary Arteries

<table>
<thead>
<tr>
<th></th>
<th>Intima (mm²)</th>
<th>Media (mm²)</th>
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<tbody>
<tr>
<td><strong>Control group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCX</td>
<td>0.03±0.01 (40)</td>
<td>1.21±0.06 (40)</td>
</tr>
<tr>
<td>LAD</td>
<td>0.02±0.01 (40)</td>
<td>1.20±0.06 (40)</td>
</tr>
<tr>
<td><strong>Cholesterol group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCX</td>
<td>0.04±0.01 (47)</td>
<td>1.28±0.05 (47)</td>
</tr>
<tr>
<td>LAD</td>
<td>0.48±0.05* (62)</td>
<td>1.86±0.08* (62)</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM.
LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery. Numbers in parentheses are the numbers of rings with endothelium tested in each group.
*p<0.05 compared with LAD in the control group or p<0.05 with LCX in the cholesterol group.

was no difference in cross-sectional area of the media between the LAD and the LCX (Table 1). In contrast, in the cholesterol group eccentric myointimal thickening with lipid deposition (atheroma) had occurred along the denuded LAD with concomitant medial thickening, whereas no intimal or medial thickening was noted in the LCX (Table 1). The endothelium covered the inner surface of the blood vessel including the atheromatous plaque of the LADs, in which no thrombus or hemorrhage was observed.15,16

Scanning electron microscopy showed that in the control group (n=5) the endothelial cells were flat and arranged parallel to the direction of blood flow in both the LCXs and the LADs (Figures 1A and

**FIGURE 2.** Transmission electron micrographs of the endothelium of the proximal coronary artery of the pig in the control (A, B) and the cholesterol (C, D) groups (original magnification, ×2,500). E, endothelial cells; IEL, internal elastic lamina; SMC, smooth muscle cells. In control group, endothelial cells were flat with well-formed junctional complexes in both left circumflex (A) and left anterior descending coronary arteries (B). In contrast, in left circumflex coronary arteries of the cholesterol group (C), endothelial cells had become rounded or cuboidal with lipid deposition. Note the absence of intimal thickening in this region. In left anterior descending coronary arteries of the cholesterol group (D), in addition to the same appearance of endothelial cells as in (C), some of them are under degeneration, suggesting the process of cell death. Multiple layers of modified smooth muscle cells with lipid deposition and interstitial tissue formed underlying intimal thickening (atheroma).
### Table 2. Characteristics of Porcine Coronary Smooth Muscle From Organ Chamber Experiments

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Cholesterol group</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>LCX</td>
<td>LAD</td>
</tr>
<tr>
<td>Optimal tension (g)</td>
<td>8.6±0.2 (86)</td>
<td>8.8±0.2 (86)</td>
</tr>
<tr>
<td>Developed tension to 2x10^-6 M PGF$_2$ (g)</td>
<td>5.9±0.3 (86)</td>
<td>6.1±0.3 (86)</td>
</tr>
<tr>
<td>Relaxation to sodium nitroprusside (n=6)</td>
<td>IC$_{50}$ (-log M)</td>
<td>7.67±0.09</td>
</tr>
<tr>
<td>Max. relaxation (%)</td>
<td>122±3</td>
<td>123±5</td>
</tr>
<tr>
<td>Contraction to KCl (n=6)</td>
<td>ED$_{50}$ (mM)</td>
<td>9.72±0.75</td>
</tr>
<tr>
<td>Max. contraction (g)</td>
<td>9.6±0.6</td>
<td>9.1±0.5</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM.

LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery; PGF$_2$, prostaglandin F$_2$; IC$_{50}$, effective concentration causing 50% inhibition of the contractions to prostaglandin F$_2$ (2x10^-6 M); Max. relaxation, maximal relaxation in percent of the response to prostaglandin F$_2$ (2x10^-6 M); ED$_{50}$, effective concentration producing 50% of the maximal response to KCl; Max. contraction, maximal contraction to KCl.

Numbers in parenthesis are the number of rings tested in each group. Since there was no difference in optimal tension or developed tension to PGF$_2$ between rings with and without endothelium in both control and cholesterol groups, the combined data are presented. For the relaxation to sodium nitroprusside or contraction to KCl, the data were obtained in rings without endothelium.

1B). In the cholesterol group (n=5), the endothelial cells were elongated and irregularly oriented in the LCXs (Figure 1C), and these tendencies were more pronounced in the LADs (Figure 1D). The number of the endothelial cells (x10^6/mm^2) in the control group was 4.2 (±0.5) in the LCXs and 4.1 (±0.4) in the LADs. In the cholesterol group, the number had increased significantly in both the LCXs (6.9±0.4) and the LADs (8.0±0.4).

Transmission electron micrography showed that in the control group endothelial cells were flat with well-formed junctional complexes in both LCXs and LADs (Figure 2A and B). In contrast, in the cholesterol group, endothelial cells were rounded to

### Table 3. Responses to Various Agonists in Endothelium-Dependent Relaxations of Porcine Coronary Arteries

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Control group</th>
<th>Cholesterol group</th>
<th>Control group</th>
<th>Cholesterol group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (-log M)</td>
<td>Max. relaxation (%)</td>
<td>IC$_{50}$ (-log M)</td>
<td>Max. relaxation (%)</td>
</tr>
<tr>
<td>Bradykinin</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>LCX</td>
<td>8.78±0.15</td>
<td>110.8±2.4</td>
<td>117.9±2.5</td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>8.85±0.14</td>
<td>116.3±3.6</td>
<td>104.1±6.7</td>
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<tr>
<td>Serotonin</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>LCX</td>
<td>7.14±0.25</td>
<td>76.3±5.6</td>
<td>55.3±10.9</td>
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</tr>
<tr>
<td>LAD</td>
<td>7.38±0.30</td>
<td>77.8±6.6</td>
<td>24.7±8.9</td>
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<tr>
<td>ADP</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>LCX</td>
<td>6.12±0.06</td>
<td>104.9±2.5</td>
<td>99.0±4.0</td>
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<tr>
<td>LAD</td>
<td>6.15±0.08</td>
<td>108.3±3.3</td>
<td>67.6±5.8</td>
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</tr>
<tr>
<td>A23187</td>
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<td></td>
</tr>
<tr>
<td>LCX</td>
<td>7.78±0.06</td>
<td>120.9±5.4</td>
<td>121.6±4.9</td>
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<tr>
<td>LAD</td>
<td>7.75±0.02</td>
<td>121.4±3.2</td>
<td>116.7±3.3</td>
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<tr>
<td>Thrombin (0.1 units/ml)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LCX</td>
<td></td>
<td>105.6±3.7</td>
<td>98.5±4.4</td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td></td>
<td>106.9±2.5</td>
<td>45.7±12.0</td>
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</table>

Data are expressed as means±SEM.

Each group consists of six experiments on rings with endothelium except for bradykinin (n=12, control group; n=14, cholesterol group). All rings were treated with indomethacin (10^-5 M). In the experiments with serotonin and ADP, rings were treated with ketanserin (10^-6 M) and theophylline (10^-4 M), respectively, to inhibit the direct effects on vascular smooth muscle.

IC$_{50}$, effective concentration causing 50% inhibition of the contractions to prostaglandin F$_2$ (2x10^-6 M); Max. relaxation, maximal relaxation in percent of the response to prostaglandin F$_2$ (2x10^-6 M); ADP, adenosine diphosphate; A23187, Ca$^{2+}$-ionophore; LCX, left circumflex coronary artery; LAD, left anterior descending coronary artery. IC$_{50}$ was not calculated for serotonin in the cholesterol group because the response did not attain the IC$_{50}$ level.

*p<0.05 compared with control.

†p<0.05 compared with LCX.
in the cholesterol-fed group, all of these relaxations were impaired in the LAD, while relaxations to serotonin and ADP were depressed in the LCX, compared with those obtained in the control group.

Aggregating platelets (70,000/μl) caused comparable, endothelium-dependent relaxations in the LCXs and LADs in the control group (Figure 5). In the cholesterol group these relaxations were depressed in the LCXs and impaired further in the LADs (Figure 5). The amount of serotonin released from aggregating platelets was not significantly different in the two groups (217±18 ng/ml in the control and 186±21 ng/ml in the cholesterol group). Similarly, there was no statistically significant difference in thromboxane B2 levels between the cholesterol group (179±32 pg/ml) and the control group (356±84 pg/ml).

In quiescent rings of control LCXs or LADs, aggregating platelets (70,000/μl) caused comparable, large contractions in the absence but comparable, small contractions in the presence of endothelium (Figure 6). In the cholesterol group, platelets caused significantly greater contractions in rings of both LCXs and LADs compared with blood vessels from the control group (Figure 6). Especially in rings with endothelium of the LAD, the induced contractions were comparable to those in rings without endothelium (Figure 6). In the cholesterol group, rings without endothelium showed contractions comparable to those in the control group (Figure 6).

In the first series of endothelium-dependent relaxations, the responses to various agonists were examined in the presence of 10^-3 M indomethacin. As shown in the Table 3 and Figures 3 and 4, in the control group, there was no difference in the endothelium-dependent relaxations to bradykinin, serotonin, ADP, the calcium ionophore A23187, and thrombin between the LCXs and LADs. In contrast,
In quiescent control rings of both LCXs and LADs, serotonin (10^{-9}–10^{-5} M) caused comparable contractions, which were significantly smaller in preparations with endothelium than in those without endothelium (Figure 7). In the cholesterol group, the contractions in rings without endothelium in both arteries were comparable to those in the control group. However, serotonin caused significantly larger contractions in rings with endothelium of both LADs and LCXs than in those of the normal group (Figure 7). At higher concentrations, these contractions were larger in rings with endothelium of LCX than those obtained in acutely denuded rings of the same artery (Figure 7). These augmented contractions were inhibited by 10^{-5} M indomethacin or 10^{-4} M meclofenamate but not by 10^{-4} M dazoxiben (a thromboxane synthetase blocker), 10^{-6} M SQ 29548 (a thromboxane receptor blocker), 2×10^{-5} M NDGA, or 1(T 5 M BAY G 6575 (both lipoxygenase blockers) (Table 4). The contractile properties of the smooth muscle (rings without endothelium) were unchanged by these blockers (Table 4). In the cholesterol group, a decreased release of prostacyclin in response to 10^{-5} M serotonin (from both the endothelium and the smooth muscle) was noted, while the amount of prostaglandin F_{2a} or prostaglandin E_{2} released was not statistically different between the two groups (Table 5). The levels of 6-keto-prostaglandin F_{1a} measured by radioimmunoassay may be slightly different from the values that would have been obtained by gas chromatography/mass spectrometry. However, this difference does not affect the direction of the reported change or its level of significance. The release of thromboxane B_{2} could not be detected. In contrast, in the LCXs no endothelium-dependent contractions were observed even though the contractions to the monoamine in rings with endothelium also were significantly augmented compared to the control group (Figure 7).

Since indomethacin-sensitive, endothelium-dependent contractions were observed in response to serotonin in the atherosclerotic LAD of the cholesterol group, endothelium-dependent relaxations to serotonin and ADP were reexamined in both groups in either the absence or the presence of indomethacin (10^{-5} M). The relaxations were significantly larger in the atherosclerotic LADs in the presence than in the absence of indomethacin, while they were comparable in both arteries in the control group or in the LCX in the cholesterol group (Figures 8 and 9). In contrast, the relaxations to sodium nitroprusside in rings without endothelium were comparable in the absence or presence of indomethacin in both groups (data not shown).

Bioassay Experiments

The basal release of endothelium-derived relaxing factor(s), which could be evaluated by measuring the degree of relaxations that occurred when endothelial superfusion was initiated, was significantly less in the cholesterol group than in the control group (Figure 10). Stimulated release of

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**Figure 5.** Relaxations to aggregating platelets (70,000/μl) during a contraction evoked by prostaglandin F_{2a} (2×10^{-6} M) in control and cholesterol groups with (+) and without (−) endothelium. Left: Relaxations in the left circumflex coronary artery (LCX). Right: Relaxations in the left anterior descending coronary artery (LAD). The responses are expressed as percent change in tension from the contraction level evoked by prostaglandin F_{2a}. Data shown as means±SEM. *Significant difference (p<0.05) compared with control; †, significant difference (p<0.05) compared with LCX.

**Figure 6.** Contractions evoked by aggregating platelets (70,000/μl) in quiescent rings from control and cholesterol groups, with (+) and without (−) endothelium. Left: Contractions in the left circumflex coronary artery (LCX). Right: Contractions in the left anterior descending coronary artery (LAD). Responses are expressed as percent increase in tension from resting level compared with maximal contractions to KCl (100 mM). *Significant difference (p<0.05) compared with control; †, significant difference (p<0.05) compared with LCX.
Correlations

In rings with endothelium from the atherosclerotic LADs, a significant correlation was noted between the cross-sectional area of the intima and the IC$_{50}$ values to bradykinin ($r=0.72$, $n=62$), ADP ($r=0.79$, $n=12$), and the calcium ionophore A23187 ($r=0.75$, $n=6$). A similar significant correlation also was noted between the cross-sectional area of the intima and the percent maximal relaxations to serotonin ($r=-0.82$, $n=12$) or the percent relaxations to thrombin (0.1 units/ml) ($r=-0.94$, $n=6$) and aggregating platelets (70,000/µl) ($r=-0.91$, $n=6$). In contrast, there was no significant correlation between the cross-sectional area of the media and the IC$_{50}$ values or percent maximal relaxations to those agonist ($r<±0.2$ for any agonist).

Discussion

The major findings in the present study were 1) the endothelium-dependent relaxations to bradykinin, serotonin, ADP, thrombin, and the calcium ionophore A23187 are impaired in coronary atherosclerosis, while the relaxations to serotonin and ADP are depressed in hypercholesterolemia, 2) the endothelium-dependent relaxations to aggregating platelets also are depressed in hypercholesterolemia and more severely impaired in atherosclerosis, 3) these impairments occur at a time that the ability of the underlying smooth muscle to relax or contract is unchanged, 4) the impaired endothelium-dependent relaxations in atherosclerosis are partly due to the depressed release of endothelium-derived relaxing factors from the endothelium, and 5) concomitant release of vasoconstrictor prod-

Table 4. Effects of Various Blockers on Contractions of the Atherosclerotic Porcine Left Anterior Descending Coronary Artery Evoked by Serotonin

<table>
<thead>
<tr>
<th>Blockers</th>
<th>n</th>
<th>With endothelium</th>
<th>Without endothelium</th>
<th>ED$_{50}$</th>
<th>Max. contraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>8</td>
<td>6.61±0.10</td>
<td>6.69±0.10</td>
<td>75.8±2.2</td>
<td>63.1±2.7</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>6</td>
<td>6.11±0.10*</td>
<td>6.52±0.10</td>
<td>60.1±5.0*</td>
<td>67.2±5.4</td>
</tr>
<tr>
<td>Meclofenamate</td>
<td>6</td>
<td>6.25±0.10*</td>
<td>6.53±0.15</td>
<td>57.8±3.8*</td>
<td>61.8±5.0</td>
</tr>
<tr>
<td>Dazoxiben</td>
<td>4</td>
<td>6.35±0.14</td>
<td>6.71±0.16</td>
<td>72.3±4.9</td>
<td>62.6±2.7</td>
</tr>
<tr>
<td>SQ 29548</td>
<td>4</td>
<td>6.41±0.17</td>
<td>6.51±0.21</td>
<td>76.3±6.2</td>
<td>62.4±5.0</td>
</tr>
<tr>
<td>NDGA</td>
<td>4</td>
<td>6.40±0.16</td>
<td>6.51±0.16</td>
<td>75.2±2.0</td>
<td>60.2±2.0</td>
</tr>
<tr>
<td>BAY G6575</td>
<td>4</td>
<td>6.30±0.17</td>
<td>6.65±0.17</td>
<td>74.4±3.3</td>
<td>62.6±3.7</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM.
ED$_{50}$, effective concentration of serotonin (-log M producing 40% of the maximal response to KCl; Max. contraction, maximal contraction to serotonin as expressed in percent of the maximal contraction to KCl in the same ring. Concentrations: indomethacin (10$^{-5}$ M), meclofenamate (10$^{-6}$ M), dazoxiben (10$^{-4}$ M), SQ 29548 (10$^{-6}$ M), nordihydroguaiaretic acid (NDGA) (2×10$^{-3}$ M), and BAY G6575 (10$^{-3}$ M).

*p<0.05 compared with no treatment.
TABLE 5. Concentrations (pg/ml) of Prostaglandins Released From the Porcine Left Anterior Descending Coronary Artery

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th></th>
<th>Cholesterol group</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With</td>
<td>Without</td>
<td>With</td>
<td>Without</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>5</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>6-keto-PGF(_1)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Basal</strong></td>
<td>36±9</td>
<td>35±8</td>
<td>47±10</td>
<td>35±7</td>
</tr>
<tr>
<td><strong>Stimulated</strong></td>
<td>79±20</td>
<td>33±8</td>
<td>27±9*</td>
<td>11±3*</td>
</tr>
<tr>
<td><strong>PGF(_2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Basal</strong></td>
<td>20±3</td>
<td>15±3</td>
<td>21±10</td>
<td>14±7</td>
</tr>
<tr>
<td><strong>Stimulated</strong></td>
<td>17±8</td>
<td>4±2</td>
<td>53±20</td>
<td>5±3</td>
</tr>
<tr>
<td><strong>PGE(_2)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Basal</strong></td>
<td>15±2</td>
<td>18±2</td>
<td>14±5</td>
<td>13±3</td>
</tr>
<tr>
<td><strong>Stimulated</strong></td>
<td>16±10</td>
<td>7±4</td>
<td>11±5</td>
<td>4±3</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM.
6 keto PGF\(_1\), 6 keto-prostaglandin F\(_1\); PGF\(_2\), prostaglandin F\(_2\); PGE\(_2\), prostaglandin E\(_2\); Basal, basal release of prostaglandins (during 40-minute incubation). Stimulated release of prostaglandins evoked by serotonin (10\(^{-5}\) M). The concentrations of released thromboxane B\(_2\) were below the detection level (20 pg/ml) in both groups.

*p<0.05 compared with control group.

uct(s) of cyclooxygenase may depress further the impaired relaxations in atherosclerosis.

In the present model, coronary atherosclerosis was induced in the LADs by a combination of balloon endothelial denudation and hypercholesterolemia\(^{12,15,16,18-20}\); the LCXs were affected only by hypercholesterolemia. Therefore, it is reasonable to consider that both endothelial recovery (regeneration) after the injury and hypercholesterolemia play causative roles in the impaired endothelial functions. Under atherogenic conditions, such as hyperlipidemia,\(^{32}\) hypertension,\(^{33}\) and increases in shear stress,\(^{34}\) the endothelial turnover rate is accelerated in response to the continuous injury by these factors. Under these conditions, regenerated endothelial cells seem to have an important role in mediating the interaction between blood components and vascular smooth muscle cells.\(^{35,36}\) In fact, the morphological appearances of endothelial cells in both LCXs and LADs from cholesterol animals are consistent with those of regenerated endothelial cells reported in previous studies.\(^{37,38}\) In porcine coronary arteries with regenerated endothelial cells (4 weeks after endothelium removal), endothelium-dependent relaxations to serotonin and aggregating platelets are reduced.\(^{22}\) This finding could explain partly the augmented vasoconstrictor responses to serotonin that have been described in animals with hypercholesterolemia,\(^{14}\) hypertension,\(^{39}\) and atherosclerosis.\(^{12,14-18}\) In recent study, the time course of the impairment of endothelium-dependent relaxations was examined up to 24 weeks after the balloon endothelium removal.\(^{40}\) After 4 weeks, the relaxations to serotonin and aggregating platelets were impaired as reported previously\(^{22}\) and remained so throughout the follow-up period. After 8 weeks the relaxations to thrombin and ADP were impaired, and after 16 weeks those to bradykinin were, while those to the calcium ionophore A23187 were well maintained.\(^{40}\) Another important factor is hypercholesterolemia itself. Low-density lipoproteins, when administered acutely, inhibit endothelium-dependent relaxations to acetylcholine, adenosine triphosphate, and the calcium ionophore A23187 in the rabbit aorta.\(^{41}\) In addition, lipid deposition was noted in the thickened intima, including endothelial cells. Thus, the above-mentioned two factors should be involved in the impaired endothelial functions. However, the endothelial denudation and repair alone cannot explain the impaired relaxations to bradykinin and the calcium ionophore A23187 or the severe impairments of the relaxations to thrombin and ADP observed in atherosclerotic coronary arteries in the present study. Similarly, the increase in low-density lipoproteins fails to

FIGURE 8. Cumulative concentration-response curves to serotonin in rings with endothelium during a contraction evoked by prostaglandin F\(_{2\alpha}\) (2×10\(^{-6}\) M) in presence of 10\(^{-6}\) M ketanserin. Relaxation responses are expressed as percent decrease in tension of contraction evoked by prostaglandin F\(_{2\alpha}\). Data shown as means±SEM. Left: Relaxations in the left circumflex coronary artery (LCX). Right: Relaxations in the left anterior descending coronary artery (LAD). Control rings, without indomethacin (●), were treated with 10\(^{-5}\) M Na\(_2\)CO\(_3\) (solvent for indomethacin) only. O, control rings with indomethacin. *Significant difference (p<0.05) between rings with (○) and without (●) indomethacin in the cholesterol group.
explain why the relaxations to bradykinin and the calcium ionophore A23187 were well maintained in the hypercholesterolemic porcine coronary arteries.

The depressed endothelium-dependent relaxations in atherosclerosis could be due to a number of factors.

First, the production and/or release of the endothelium-derived relaxing factor(s) may be decreased. This possibility is most likely because the depressed release of the factor(s) in both basal and stimulated conditions was confirmed in bioassay experiments. Similar observations have been reported in the atherosclerotic rabbit aorta.42,43

Second, since the half-life of the endothelium-derived relaxing factor(s) is extremely short,44 the thickened intima with lipid deposition may reduce the diffusion of the factor(s). In fact, there is a significant correlation between the impairment of endothelium-dependent relaxation and the degree of intimal thickening. However, the intraluminal release of the factor(s) in response to serotonin is decreased (bioassay experiment) and endothelium-dependent relaxations also are depressed in the hypercholesterolemic LCX that does not exhibit intimal thickening. The intraluminal release of the factor(s) in response to bradykinin also is depressed in the atherosclerotic porcine coronary arteries in the same model as used in the present study.45

Dietary treatment of atherosclerosis in monkeys restores endothelium-dependent relaxations to acetylcholine and thrombin even in the presence of intimal thickening.46 The thickening of the intima after balloon injury may have other consequences, such as inflammation47 and lipid deposition (in the presence of hyperlipidemia), which also may alter blood-vessel wall interactions. Therefore, the role of the atheroma, including possibly to constitute a "functional" (but not "distance") barrier, remains to be examined in further studies.

Third, the increase in the amount of the medial smooth muscle may result in a relative shortage of the endothelium-derived relaxing factor(s) to cause relaxations. This is unlikely to be the cause because no significant correlation exists between the degree of medial mass and the impairment of the relaxations.

Fourth, the characteristics of smooth muscle cells and, in particular, their sensitivity to endothelium-derived relaxing factor(s) may be changed. This
possibility seems less likely because in rings without endothelium the relaxations to sodium nitroprusside (which induces relaxations through activation of guanylate cyclase as does endothelium-derived relaxing factor(s)), and the contractions to potassium chloride (activating voltage-dependent Ca\textsuperscript{2+}-channels) and serotonin (receptor-operated Ca\textsuperscript{2+}-channels) were unaltered. Whether or not the relaxations to nitrovasodilators are impaired in atherosclerosis is controversial; it is impaired in the aorta of the rabbit fed a 0.3% cholesterol diet for 16 weeks\textsuperscript{8} but not in the same species fed a 1% cholesterol diet for 6 weeks\textsuperscript{9} or in the iliac artery of the monkey fed a 0.74% cholesterol diet for 18 months.\textsuperscript{11} These discrepancies could be due to the degree of atherosclerosis (early or advanced) and/or the anatomical type of blood vessels, that is, elastic (aorta) or muscular (femoral and coronary).

Finally, in coronary atherosclerosis, vasoconstrictor product(s) of cyclooxygenase may be released together with a smaller amount of endothelium-derived relaxing factor(s).\textsuperscript{48-51} This interpretation is supported by the following observations: 1) higher concentrations of serotonin induced endothelium-dependent contractions, which were inhibited by blockers of cyclooxygenase but not of lipoxygenase and 2) the endothelium-dependent relaxations to serotonin (organ chamber and bioassay experiments) and ADP (organ chamber experiments) were greater in the presence than in the absence of indomethacin. The release of the indomethacin-sensitive, endothelium-derived contracting factor(s) have been reported in the saphenous vein\textsuperscript{48} and the basilar artery\textsuperscript{51} of the dog and in the aorta of the spontaneously hypertensive rat.\textsuperscript{50} Since inhibition of thromboxane receptors (SQ 29548) or thromboxane synthetase (dazoxiben) did not antagonize the endothelium-dependent contractions, which were inhibited by blockers of cyclooxygenase but not of lipoxygenase and an endoperoxide intermediate of cyclooxygenase pathway may be responsible for the contractions. The same conclusions were reached for the endothelium-dependent contractions to arachidonic acid in canine saphenous veins.\textsuperscript{48} The pathological importance of the significant decrease in prostacyclin release during endothelium-dependent responses is unknown.

The mechanisms of the depressed endothelium-dependent relaxations in hypercholesterolemia appears easier to understand. Since no intimal or medial thickening, no impairments in the relaxations to sodium nitroprusside, or no endothelium-dependent contractions were reached for the endothelium-dependent relaxations to sodium nitroprusside, or no endothelium-dependent relaxations in hypercholesterolemia can be explained by the impaired endothelium-dependent responses to serotonin and ADP.

Among the endothelium-dependent relaxations to various factors derived from platelets and the coagulation system, those to serotonin are impaired in the chronic regenerated state (a very early stage of atherosclerosis).\textsuperscript{22} Combining this finding together with the present experiments, it is tempting to suggest that during the process of atherosclerosis, endothelium-dependent responses are impaired first to serotonin, then to ADP, and finally to other vasoactive agents. Since both serotonin and ADP are the major platelet-derived vasoactive substances, the endothelium-dependent responses to aggregating platelets are impaired, beginning from the early stage of atherosclerosis and progressively worsening during the process.

Endothelium-derived relaxing factor inhibits platelet aggregation.\textsuperscript{52-55} In human coronary arteries, aggregating platelets cause endothelium-dependent relaxations.\textsuperscript{30} If the endothelial dysfunction to aggregating platelets and their products observed in the present study also occurs during coronary atherosclerosis in humans, the impaired interactions between platelets and the blood vessel wall would favor the occurrence of platelet aggregation and platelet-induced contractions of coronary smooth muscle, leading to ischemic events such as coronary vasospasm and coronary thrombosis.

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Key Words • endothelium-derived relaxing factor • serotonin • adenosine diphosphate • thrombin
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