Endothelium-Dependent Inhibition of Ergonovine-Induced Contraction Is Impaired in Porcine Coronary Arteries With Regenerated Endothelium

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The inhibitory effects of the endothelium against ergonovine-induced contraction were examined in isolated porcine coronary arteries under normal conditions and after endothelial regeneration. Endothelium-dependent responses were examined in vitro in normal Yorkshire pigs (n = 16) and in pigs that had undergone balloon endothelium removal of the left anterior descending coronary artery (LAD) 4 weeks before the study (n = 10). The presence of a complete endothelial lining was confirmed histologically. In rings from normal arteries contracted with prostaglandin F2α, the presence of indomethacin and ketanserin (a 5-HT1 receptor blocker), ergonovine caused endothelium-dependent relaxations. They were attenuated by rauwolscine (an α1-adrenergic blocker), inhibited by methiothepin (a combined 5-HT1 and 5-HT2 receptor blocker) or by pertussis toxin (an inhibitor of several G proteins) and abolished by oxyhemoglobin (a selective inactivator of endothelium-derived relaxing factor). In quiescent rings from normal arteries, ergonovine caused contractions that were inhibited by the presence of the endothelium; this endothelium-dependent inhibition was abolished by oxyhemoglobin. The direct contractions were not affected by prazosin (an α1-receptor blocker), rauwolscine, 6-hydroxydopamine (an agent causing chemical sympathetectomy), or diphenhydramine (an H1-receptor blocker) but were inhibited by ketanserin. In rings with regenerated endothelium contracted with prostaglandin F2α, the endothelium-dependent relaxations to ergonovine were reduced significantly and were not inhibited by pertussis toxin. In quiescent rings with regenerated endothelium, the endothelium-dependent inhibition of ergonovine-induced contraction was less. Oxyhemoglobin caused endothelium-dependent contractions in quiescent rings (an indirect index of basally released endothelium-derived relaxing factor) that were reduced significantly in quiescent rings with regenerated endothelium. These results indicate that 1) the endothelium exerts its inhibitory action against ergonovine-induced contractions by the release of endothelium-derived relaxing factor under basal conditions and upon stimulation by ergonovine, 2) endothelium-dependent relaxations to ergonovine are mediated mainly by 5-HT1 receptor blockers, whereas the direct contractions are mediated by 5-HT2 receptor blockers with little contribution of α-adrenoceptors, 3) the inhibitory role of the endothelium is impaired significantly in the regenerated state because of the reduced ability to release the relaxing factor, and 4) endothelial pertussis toxin-sensitive G protein may be involved in the synthesis of the relaxing factor upon stimulation by ergonovine, and dysfunction of the G protein may account partly for the dysfunction of regenerated endothelium. (Circulation 1989;80:643–650)

Angina pectoris and occasionally acute myocardial infarctions can be exacerbated or caused by spasm of coronary arteries, but the underlying mechanism(s) is uncertain. Ergonovine is an ergot alkaloid that is used to test the susceptibility of patients to coronary vasospasm. In
canine and rabbit coronary arteries, ergonovine causes contractions of the smooth muscle mainly by activation of serotonergic receptors with little contribution of α-adrenoceptors.10–14 Ergonovine also may activate serotonergic receptors on the endothelium to trigger the release of endothelium-derived relaxing factor.15–18 After balloon injury of the endothelium in porcine coronary arteries, the regenerated endothelium has a reduced ability to form the relaxing factor in response to serotonin.18 The present study was designed to examine the ability of the normal and regenerated endothelium to inhibit ergonovine-induced contractions in porcine coronary arteries.

Methods
Animal Preparations
The present study consisted of two series of experiments. In a first series, the effects of ergonovine were examined in rings of left anterior descending (LAD) and left circumflex (LCx) coronary arteries from 16 normal male Yorkshire pigs (36.8±1.2 kg). Previous work showed that there is no difference in responsiveness between these two arteries.18 In a second series, 10 similar pigs (25.6±1.0 kg) underwent balloon endothelium-removal along the initial 4 cm of the proximal LAD.18 Four weeks later, the responses to ergonovine were examined in parallel in the LAD and LCx. The presence of complete endothelial regeneration was confirmed histologically.18 The used technique of endothelium-removal causes only minimal damage of the vascular smooth muscle and no medial scar.18 The body weight of the pigs after 4 weeks of maintenance (36.2±1.4 kg) was not significantly different from that of the pigs used in a first series of experiments.

Organ Chamber Experiments
The pigs were anesthetized with ketamine hydrochloride (300 mg i.m.) followed by sodium pentobarbital (12.5 mg/kg i.v.).18 The hearts were removed, and the LAD and LCx were dissected free and immersed in cold modified Krebs-Ringer bicarbonate solution of the following composition (mM): NaCl 118.3, KCl 4.7, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, NaHCO3 25.0, Ca-EDTA 0.016, and glucose 11.1 (control solution). The rings (3–4 mm long) were cleaned of loose connective tissue, with care taken not to touch the luminal surface. In some rings, the endothelium was removed by rubbing the luminal surface gently with a cotton swab wetted with control solution.18 The rings were suspended horizontally between two stainless steel stirrups in organ chambers filled with 25 ml control solution (37°C, pH 7.4) gassed with 95% O2-5% CO2. One of the stirrups was anchored in the organ chamber, and one was connected to a strain gauge (UC2, Gould Statham, Oxnard, California) for the recording of isometric tension. The rings were then stretched progressively until the contractile response evoked by potassium chloride (20 mM KCl) was maximal (optimal tension, 8–9 g18).

Drugs
The following drugs were used: diphenhydramine, ergonovine maleate, 6-hydroxydopamine, indomethacin, pertussis toxin, prazosin, prostaglandin F2α (all from Sigma Chemical, St. Louis, Missouri), ketanserin tartrate (Janssen Pharmaceutica, Piscataway, New Jersey), methiothepin (Hoffmann-LaRoche, Nutley, New Jersey), and rauwolscine hydrochloride (Carl Roth, Karlsruhe, FRG).

Oxyhemoglobin
Bovine hemoglobin (Type 1, Sigma Chemical, St. Louis, Missouri) contains a mixture of oxyhemoglobin and the oxidized derivative, methemoglobin. Pure hemoglobin (oxyhemoglobin) was prepared by adding to a 1-mM solution of commercial hemoglobin in distilled water, a 10-fold molar excess of the reducing agent sodium dithionite (Na2S2O4). Sodium dithionite was then removed by dialysis in 15 liters of distilled water (containing 0.001% ethylenediaminetetraacetic acid [EDTA]) for 2 hours at room temperature. The percentage of oxyhemoglobin was determined spectrophotometrically.19

Protocol
After achieving optimal tension, the rings were incubated with control solution in the presence or absence of pharmacologic inhibitors; the incubation time was 60 minutes for pertussis toxin,20 15 minutes for oxyhemoglobin, and 40 minutes for the others. The inhibitors remained in contact with the arterial rings during their subsequent exposure to contracting and relaxing agents. Preliminary experiments confirmed the inhibitory effects of prazosin (10−5 M), rauwolscine (10−7 M), ketanserin (10−6 M), and diphenhydramine (10−5 M) on contractions in response to phenylephrine, UK 14304 (a selective α2-adrenergic agonist), serotonin, and histamine, respectively (n=6 each, data not shown). Similarly, the inhibitory effects of methiothepin (10−6 M) and rauwolscine (10−7 M) on endothelium-dependent relaxations to serotonin and UK 14304 were confirmed, respectively (n=6 each, data not shown). When the effects of ergonovine were examined, a single concentration-response curve was obtained in each ring by cumulative addition of the alkaloid. When endothelium-dependent relaxations to ergonovine were determined, all rings were treated with indomethacin (10−5 M) and ketanserin (10−6 M) for 40 minutes to prevent the formation of endogenous prostaglandins and to inhibit the direct serotonergic activation of vascular smooth muscle by the ergot alkaloid, respectively.18 Preliminary experiments had shown that ketanserin unmask the endothelium-dependent relaxations to ergonovine (n=5, data not shown).
Data Analysis

The results are expressed as mean±SEM. Unless otherwise specified, n refers to the number of animals. When determining relaxations in rings contracted with prostaglandin F$_{2a}$ (10$^{-6}$ M), responses are expressed as percent changes from the contracted levels. When determining contractions in quiescent rings, responses are expressed as percentage of the maximal response to KCl (80 mM). The extent of the inhibition by pertussis toxin was expressed as percent inhibition of the area delineated by the concentration-response curves or of the maximal relaxation in response to ergonovine. The data were evaluated by Student's t test for paired observations. When more than two means were compared, an analysis of variance was used. If a significant F value was found, Scheffe's test for multiple comparisons was used to identify differences among groups. Values were considered to be statistically different when p was smaller than 0.05.

Results

Normal Coronary Arteries

Endothelium-dependent relaxations to ergonovine. In rings contracted with prostaglandin F$_{2a}$ (10$^{-6}$ M), ergonovine caused endothelium-dependent relaxations (Figures 1 and 2); these relaxations were slightly but significantly attenuated by rauwolscine (10$^{-7}$ M, a selective α$_2$-adrenergic blocker), inhibited by methiothepin (10$^{-6}$ M, a combined 5-HT$_1$- and 5-HT$_2$-serotonergic blocker), and abolished by a combination of the two blockers or by oxyhemoglobin (5×10$^{-6}$ M, a selective inactivator of endothelium-derived relaxing factor) (Figures 2 and 3). Rauwolscine, methiothepin or oxyhemoglobin did not significantly affect the contractions to prostaglandin F$_{2a}$ (10$^{-6}$ M).

Pertussis toxin (100 ng/ml) inhibited significantly the endothelium-dependent relaxations to ergonovine (Figure 4). The inhibition by pertussis toxin averaged 72±6% when considering the area under the concentration-response curve and 65±4% when considering the maximal relaxation.

Contractions. In quiescent rings, ergonovine caused dose-dependent contractions that were attenuated significantly by the presence of the endothelium (Figure 5). The endothelium-dependent attenuation of the ergonovine-induced contraction was prevented by oxyhemoglobin (5×10$^{-6}$ M) (Figure 5). Prazosin (10$^{-5}$ M), rauwolscine (10$^{-7}$ M), 6-hydroxydopamine (10$^{-6}$ M), or diphenhydramine (10$^{-6}$ M) had no significant effect on the ergonovine-induced contractions of the smooth muscle, but they were inhibited significantly by ketanserin (Figure 6).

FIGURE 1. Tracing of endothelium-dependent relaxation to ergonovine during a contraction evoked by prostaglandin F$_{2a}$ (10$^{-6}$ M) (PGF$_{2a}$) in an isolated porcine coronary artery. The rings were treated with indomethacin (10$^{-5}$ M) and ketanserin (10$^{-6}$ M).

FIGURE 2. Cumulative concentration-response curves to ergonovine during contractions of isolated porcine coronary arteries evoked by prostaglandin F$_{2a}$ (10$^{-6}$ M) in the presence of indomethacin (10$^{-5}$ M) and ketanserin (10$^{-5}$ M). The responses were obtained in parallel. The contractions evoked by prostaglandin F$_{2a}$ averaged 6.5±0.6 g in control rings with endothelium, 6.6±0.6 g in control rings without endothelium, and 8.4±1.3 g in rings with endothelium treated with oxyhemoglobin (5×10$^{-5}$ M). *Statistically significant difference (p<0.05) between control rings with endothelium and control rings without endothelium or oxyhemoglobin-treated rings with endothelium. Data are mean±SEM.
Denuded significantly were comparable with and without pertussis toxin (100 ng/ml) averaged 6.4±1.1 and 6.1±1.0 g, respectively. *Statistically significant difference (p<0.05) between rings with and without pertussis toxin. Data are mean±SEM.

Oxyhemoglobin (5×10⁻⁶ M) caused contractions in rings with endothelium, which were significantly smaller in rings without endothelium (Figure 7).

Previously Denuded Coronary Arteries

Endothelium-dependent relaxations. In the control LCx, ergonovine caused endothelium-dependent relaxations that were significantly inhibited by pertussis toxin (Figure 8). The ergonovine-induced relaxations (IC₃₀ [-log M] 7.56±0.12 and maximal percent relaxation 61±5%) and the inhibition by pertussis toxin of the relaxations (area under the curve; 77±3%, and maximal relaxation: 69±2%) were comparable to those observed in normal animals. In the previously denuded LAD, the endothelium-dependent relaxations to ergonovine were significantly attenuated (maximal relaxation 20±3%) compared with those in the control LCx, and pertussis toxin no longer affected the ergonovine-induced relaxations (Figure 8). The contractions evoked by prostaglandin F₂a₁₀⁻⁶ M) were comparable in the control LCx and the previously denuded LAD.

Contractions. Ergonovine caused comparable contractions in rings without endothelium of the LCx and in the previously denuded LAD (Figure 9). These contractions were significantly attenuated by the presence of the endothelium in the LCx, whereas in the LAD with regenerated endothelium, there was no significant difference in the contractions between rings with and without endothelium (Figure 9).

In the LCx, oxyhemoglobin caused contractions of rings with endothelium, but in the LAD with regenerated endothelium, the endothelium-dependent contractions were attenuated significantly (Figure 7). The contractions to oxyhemoglobin were minimal in rings without endothelium of both LCx and LAD (Figure 7).

Discussion

The present study shows that in porcine coronary arteries 1) the endothelium inhibits the contractions evoked by ergonovine, 2) this inhibition is impaired in arteries with regenerated endothelium, and 3) a dysfunction of the endothelial pertussis toxin-sensitive G protein may account in part for the impairment.
Ergonovine and Normal Endothelium

The endothelium-dependent relaxations to ergonovine were observed in the presence of indo-
methacin and were abolished by oxyhemoglobin, a selective inactivator of endothelium-derived relaxing factor.19 Thus, the relaxations can be attributed to the release of this relaxing factor.15-19 The endothelium-dependent response was attenuated by the selective α-adrenergic blocker, rauwolscine, inhibited by the combined 5-HT₃ and 5-HT₄-serotonergic blocker, methiothepin, and was unaffected by ketanserin, a 5-HT₄-serotonergic blocker. These results indicate that the endothelium-dependent relaxations to ergonovine are achieved mainly by activation of 5-HT₁-receptor with small contributions of α₂-adrenergic receptors.15,21 Endothelium-dependent relaxations to ergonovine have been reported in the rabbit aorta;22 however, in that preparation, the response was variable, and not blocked by 10⁻⁵ M methysergide.22 Methysergide has a relatively weak selectivity for the 5-HT₁-receptors and has an agonistic action at high concentrations.23

The contractions induced by ergonovine were inhibited significantly by ketanserin but not by prazosin (an α₁-adrenergic blocker), rauwolscine, 6-hydroxydopamine (an agent causing chemical sympathectomy), or diphenhydramine (an H₁-
histaminergic blocker). These results indicate that ergonovine-induced contractions of vascular smooth muscle are mediated mainly by 5-HT₂-serotonergic mechanisms with little contribution of α-adrenergic receptors.10-13 The ergonovine-induced contrac-
tions were inhibited significantly by the endothelium. The endothelium also releases relaxing factor under basal conditions as evidenced by the experiments with oxyhemoglobin.24 Because oxyhemoglobin abolished the endothelium-dependent inhibition of ergonovine-induced contractions, it seems logical to assume that both basal and stimulated release of endothelium-derived relaxing factor contribute to the inhibition of contractions to ergonovine in porcine coronary arteries.

Pertussis toxin is an irreversible blocker of several G proteins that modulate a variety of intracellular events.25-27 Pertussis toxin (100 ng/ml, 60 minutes) completely ADP-ribosylates the toxin-sensitive G proteins in intact endothelial cells.28 A previous study showed that this toxin practically abolishes endothelium-dependent relaxations to se-
rotonin (in the presence of ketanserin) and the α₁-adrenergic agonist, UK 14304, in porcine coro-

nary arteries, suggesting that endothelial 5-HT₁-receptor and α₁-adrenergic receptors may be coupled to the pertussis toxin-sensitive G protein (most likely Gₛ protein).20 The inhibitory effect of pertussis toxin is selective because the toxin does not inhibit endothelium-dependent relaxations to bradykinin, the calcium ionophore A23187, or nitric
KCl. The maximal contraction and 
ies. The significant difference (p<0.05) compared respectively. *Statistically significant difference (tension compared or those from LCx in the chronic study. †Statistically significant difference (p<0.05) between rings with and without endothelium. Data are mean±SEM. LCx, control left circumflex coronary arteries; LAD, previously denuded left anterior descending coronary arteries.

oxide (the putative endothelium-derived relaxing factor^{29-31}).^20 Thus, the toxin does not interfere in a nonspecific manner with the release or the ability of vascular smooth muscle to respond to endothelium-derived relaxing factor. The present results suggest that this pertussis toxin-sensitive G protein also is involved in the endothelium-dependent relaxations to ergonovine; this conclusion is in line with the fact that the ergot alkaloid activates endothelial 5-HT_1-serotonergic and α2-adrenergic receptors.

**Ergonovine and Regenerated Endothelium**

In coronary artery rings with regenerated endothelium, endothelium-dependent relaxations to ergonovine and hemoglobin-induced, endothelium-dependent contractions were reduced. Thus, less endothelium-derived relaxing factor, both under basal conditions and upon stimulation by ergonovine, may account for the impaired endothelium-dependent inhibition of ergonovine-induced contractions in a regenerated state. Endothelium-dependent contractions to ergonovine were not evident in the present study.

The present results are consistent with the previous findings in the same model that regenerated endothelium has a reduced ability to release endothelium-derived relaxing factor in response to serotonin^{18} and to UK 14304 (a selective α2 adrenergic agonist).^{32} In contrast, the ability of coronary smooth muscle to relax to sodium nitroprusside^{18} or nitric oxide^{32} is unaltered. The results with pertussis toxin indicate that a loss or dysfunction of the toxin-sensitive G protein in the regenerated cells may account for the diminished endothelial response to ergonovine. A dysfunction of the pertussis toxin–sensitive G protein during cell growth and regeneration has been noted also in rat hepatocytes.^{33}

**Clinical Implications**

Augmented contractions to ergonovine have been reported in the atherosclerotic rabbit aorta^{34,35} and in the atherosclerotic coronary artery of dogs^{36} and miniature pigs. The present study shows that endothelial dysfunction can contribute to the predominant vasoconstrictor responses to ergonovine. Obviously, the presence of a coronary stenosis may accelerate further the hemodynamic deterioration induced by ergonovine.^{38}
Ketanserin does not prevent spontaneous or ergonovine-induced anginal attacks in patients with variant angina.\textsuperscript{39–41} The contractions of canine coronary arteries evoked by serotonin also are resistant to the 5-HT\textsubscript{2}–serotonergic blocker,\textsuperscript{42,43} although they were blocked by methiothepin (a combined 5-HT\textsubscript{1} and 5-HT\textsubscript{2}–serotonergic blocker). Thus, the subtypes of serotonergic receptors in human coronary smooth muscle may be similar to those in dogs but different from those in pigs. However, of importance, in canine, porcine, and rabbit coronary arteries, the ergonovine-induced contractions are achieved mainly by serotonergic mechanisms.\textsuperscript{10–14}

If endothelial cells inhibit the contractions evoked by serotonin or ergonovine in humans, endothelial damage or dysfunction could markedly augment the contractile responses of the coronary smooth muscle, resulting in the occurrence of coronary vasospasm at the site of endothelial lesions. Thus, the present study implies that the ergonovine test could be regarded as a clinical tool to assess endothelial function and that serotonergic mechanisms may be important in the pathogenesis of coronary vasospasm.

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**Key Words**: coronary vasospasm • endothelium-derived relaxing factor • G protein • ketanserin • pertussis toxin • serotoninergic receptors
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