Endothelial Vasodilator Function Is Preserved at the Spastic/Inflammatory Coronary Lesions in Pigs

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Background—The question of whether or not endothelial vasodilator function in the spastic coronary artery is preserved is still controversial. We recently developed a porcine model in which long-term and local treatment with interleukin-1β (IL-1β) from the adventitial site causes coronary arteriosclerotic changes and vasospastic responses to autacoids. The aim of this study was to examine the endothelial vasodilator function in our new porcine model of the spasm both in vivo and in vitro.

Methods and Results—A segment of the porcine coronary artery was aseptically wrapped with cotton mesh that held absorbed IL-1β-bound microbeads. Two weeks after the procedure, intracoronary administration of serotonin caused coronary vasospasm at the IL-1β-treated site (n=10). Coronary vasodilatation to bradykinin, substance P, or an increase in coronary blood flow was preserved at the spastic site. Vasodilator responses to 3-morpholinosydnonimine (an NO donor) and nitroglycerin also were comparable between the 2 sites. The vasoconstricting response to NG-monomethyl-L-arginine and the extent of the augmentation of the serotonin-induced vasoconstriction were comparable between the 2 sites. Organ chamber experiments showed that endothelium-dependent relaxations to bradykinin, the calcium ionophore A23187, and even the vasospastic agonist serotonin were preserved at the spastic site, whereas contractions to serotonin were augmented at the spastic site regardless of the presence or absence of the endothelium (n=6). Endothelium-independent relaxations to sodium nitroprusside were also preserved at the spastic site.

Conclusions—These results indicate that endothelial vasodilator function is preserved at the spastic site and that the spasm is caused primarily by smooth muscle hypercontraction in our porcine model. (Circulation. 1999;100:1432-1437.)

Key Words: vasospasm ■ endothelium ■ serotonin ■ muscle, smooth ■ interleukins

Coronary artery spasm plays an important role in the pathogenesis of a wide variety of ischemic heart disease.1–4 However, the pathogenesis of the spasm is still unknown, and the elucidation of its mechanism remains an important clinical issue. Indeed, it is still controversial whether or not endothelial vasodilator function in the spastic coronary artery is preserved in humans.5–8 We previously developed a porcine model of coronary spasm in which atherosclerotic coronary lesions were induced by a combination of balloon removal of the endothelium and high-cholesterol feeding.9–11 In this model, endothelial dysfunction is inevitable because of endothelial regeneration per se.12,13 Although this finding may explain the coronary hypercontraction to serotonin in the human coronary artery after PTCA,14 most patients with vasospastic angina do not undergo such coronary intervention with massive removal of the endothelium before the appearance of the coronary vasospastic activity. We recently developed a porcine model in which long-term and local treatment with interleukin-1β (IL-1β) from the adventitial site causes coronary arteriosclerotic changes and vasospastic responses.15–19 These findings in our new porcine model confirmed the importance of the adventitial inflammation in the pathogenesis of coronary spasm in humans.20,21 Because this model does not require removal of the endothelium and thus the endothelial lining is preserved,18 it is a more suitable model, from a clinical point of view, to examine the endothelial vasodilator function at the spastic site. Thus, the aim of this study was to examine whether or not endothelial vasodilator function is altered at the spastic site in our new porcine model both in vivo and in vitro.

Methods

Animal Preparation

Ten male domestic pigs (2 to 3 months old and weighing 25 to 30 kg, Nihon Crea Inc, Tokyo) were used. The animals were housed individually under a controlled room temperature. They were sedated with ketamine hydrochloride 12.5 mg/kg IM and anesthetized with sodium pentobarbital 20 mg/kg IV. They were then intubated and mechanically ventilated with a respirator. Under aseptic conditions,
a left thoracotomy was performed, and the proximal segments of the left anterior descending and circumflex coronary arteries were carefully dissected. The dissected segments of the coronary arteries were aseptically wrapped with cotton mesh that held absorbed IL-1β 2.5 μg.15–19

This experiment was reviewed by the Ethics Committee on Animal Experiment at the Kyushu University School of Medicine and was carried out in accordance with the Guidelines for Animal Experiment at the Kyushu University School of Medicine and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Preparation of IL-1β Beads
IL-1β beads were prepared as previously reported.15–19 Briefly, 1 g sepharose microbeads (CNBr-activated sepharose 4B, 45 to 165 μm in diameter, Pharmacia), which bind to the amino-residues of proteins, including cytokines, was added to 50 mL of 1 mmol/L HCl solution and centrifuged 4 times at 1200 rpm for 5 minutes each time. The beads were then resuspended in 20 mL of NaHCO3/NaCl solution and centrifuged 4 times at 1200 rpm for 5 minutes, the supernatant was measured by an ELISA. The cytokine-bound beads overnight. After centrifugation at 1200 rpm for 5 minutes, the supernatant was measured by an ELISA. The cytokine-bound beads in the suspension was

were finally washed and resuspended so that the final concentration of cytokine was 50 μg/mL. The number of cytokines or control beads in the suspension was 70 μL. All of the above procedures were performed under sterile conditions.15–19

Because in our bead preparation most of the IL-1β molecules are bound inside the beads by a covalent bond at the amino-residues of the protein, ≤1.2% of the IL-1β molecules are actually bound to surface of the beads and biologically active.15 Thus, when 2.5 μg of IL-1β that is bound to the beads is applied to the coronary artery, ≤30 ng of IL-1β is biologically active.15 In addition, we previously confirmed that the treatment with control beads alone causes minimal intimal thickening and no hyperconstrictive responses.15

In Vivo Experiment
Two weeks after the operation, animals were anesthetized and ventilated as described above, and selective coronary arteriography was performed. A preshaped Judkins catheter was inserted into the right coronary artery and coronary arteriography in a left anterior oblique view was performed. Heparin 3000 U IV bolus was administered every 60 minutes. ECGs in leads I, II, III, V1, and V6 were recorded. The arterial pressure was measured with a pressure transducer (Gould Inc) connected to the Kifa catheter. The arterial pressure, heart rate, and ECGs were continuously monitored and recorded on a pen recorder (NEC San-Ei Polygraph System).15–19

Coronary arteriography was performed with the Toshiba cineangiography system (DG-15GB/CAS-CA, Toshiba Medical Inc). The angiograms were recorded on 35-mm cine film (Varicath I; VARIX) at 48 frames per second. The angle of the projection, the posture of the animal, and the distance from the x-ray focus to the animal and that from the animal to the image intensifier were all carefully kept constant during each experiment.15–19

The cineangiograms were projected on a screen with a cine projector (ELK-35CB, Nishimoto Sangyo Inc), and an end-diastolic image was selected. The coronary luminal diameters were measured with a caliper.15–19 With this technique, excellent correlations between repeated measurements (r=0.99) and between different observers (r=0.98) were confirmed in the range of the coronary diameter from 0.98 to 5.58 mm.15–19 The degree of the vasoconstricting response was expressed as the percent decrease in the luminal diameter from the control level.

The following protocols were examined in the coronary angiographic study in vivo. First, coronary arteriography was performed under control conditions (n=10). Second, coronary vasoconstricting responses to intracoronary serotonin 10 μg/kg were examined (n=10). Coronary arteriography was performed 2 minutes after intracoronary administration of serotonin.15–19 Third, coronary vaso-dilating responses were examined in response to intracoronary administration of bradykinin 0.1 μg/kg, substance P 10 μg/kg, 3-morpholinosydnonimine (SIN-1, an ONO donor) 10 μg/kg,20 and nitroglycerin 10 μg/kg (n=6). Fourth, coronary vasodilating responses to an increase in coronary blood flow caused by intracoronary infusion of adenosine 60 μg · kg⁻¹ · min⁻¹ for 2 minutes were examined (n=4). A slightly higher dose of adenosine was required to obtain flow-dependent dilation of the coronary artery compared with that observed in the previous clinical study in patients with variant angina (40 μg · kg⁻¹ · min⁻¹ for 2 minutes).8 This is probably because in the present study, the porcine coronary artery tended to be dilated under basal conditions because of the effect of general anesthesia. In this protocol, a small catheter for intracoronary infusion of adenosine was advanced into the coronary artery beyond the IL-1β-treated site, and the coronary vasodilating responses to the increase in flow (but not to adenosine) were examined. Fifth, changes in basal coronary diameter and vasoconstricting responses to intracoronary serotonin 10 μg/kg before and after intracoronary infusion of A²α-monomethyl-L-arginine (L-NMMA) 1 mg/kg over 10 minutes23 were examined (n=4).

Coronary arteriography was performed 2 minutes after intracoronary administration of these agents, when the vasodilator or vasoconstrictor effect of each agent peaked. Each dose of drugs was diluted with 1 mL of physiological saline and was injected into the left coronary artery.

In Vitro Experiment
One day after the in vivo experiment, the animals were sedated with ketamine hydrochloride 12.5 mg/kg IM, euthanized with a lethal dose of sodium pentobarbital, and exsanguinated, and then the heart was excised. The coronary arteries at the IL-1β-treated and control sites were carefully dissected, cleaned of any perivascular tissue, and cut into rings ≤4 mm long. In some of the rings, the endothelium was removed by gentle rubbing of the luminal surface with a cotton swab.12–15 The rings were fixed vertically between hooks in an organ bath of 20-mL capacity containing Krebs-Henseleit solution, which was maintained at 37°C and aerated with a mixture of 95% O₂-5% CO₂. The hook anchoring the upper end of the strip was connected to the lever of a force transducer (Nihon-Kohden Kogyo). The resting tension was adjusted to 5 g.19 KCl solution (62 mmol/L) was applied every 15 to 20 minutes until the amplitude of the contraction reached a constant value. The developed tension was expressed as a percentage of the tension attained in the last preconstriction with 62 mmol/L KCl. The presence or absence of the endothelium was confirmed by the presence or absence of the relaxation to bradykinin 10⁻⁵ mol/L during a contraction evoked by prostaglandin F₂α (PGF₂α). Endothelium-dependent relaxations to serotonin, bradykinin, and the calcium ionophore were examined in rings from the spastic and control sites in parallel during a contraction evoked by PGF₂α 10⁻⁶ mol/L.12–13 The endothelium-dependent relaxations to serotonin were examined in the presence of ketanserin 10⁻⁶ mol/L, a 5HT₂a-serotonergic receptor antagonist, to inhibit the direct vasoconstricting effect of the monoamine on the vascular smooth muscle.12,13

Drugs
The following drugs were used: recombinant human IL-1β (Otsuka Pharmaceutical Co), adenosine, 5-hydroxytryptamine (serotonin), histamine, bradykinin, substance P, the calcium ionophore A23187, SIN-1, L-NMMA (Sigma Chemical Co), nitroglycerin (Nihon-Kayaku Pharmaceutical Co), and PGF₂α (Otsu Pharmaceutical Co).

Data Analysis
All results are expressed as the mean ± SEM. Statistical analysis was performed by ANOVA followed by Fisher’s test for multiple comparisons. Paired data were analyzed by Student’s t test. A probability of <0.05 was considered to be statistically significant.

Results
In Vivo Experiment
Figure 1 shows coronary arteriograms taken 2 weeks after the application of IL-1β. After intracoronary administration of...
nitroglycerin, mild organic stenosis was noted at the IL-1β-treated site (Figure 1A). Intracoronary administration of serotonin (Figure 1B) repeatedly induced coronary hyperconstriction at the IL-1β-treated site, whereas it caused only mild vasoconstriction at the control (untreated) site. The difference in the extent of the coronary vasoconstriction to serotonin was highly significant between the control (13±3%) and the IL-1β-treated (43±3%) sites (n=10, P<0.01).

Endothelium-dependent vasodilatation to intracoronary bradykinin, substance P, or an increase in coronary blood flow was comparable between the IL-1β-treated and the control sites (Figure 2). Endothelium-independent vasodilatation to intracoronary nitroglycerin or SIN-1 was also comparable between the 2 sites, although the vasodilation tended to be greater at the IL-1β–treated sites than at the control sites (Figure 2). When the coronary vasodilating responses to bradykinin, substance P, or an increase in coronary blood flow were corrected by the increased coronary tone (ratio of the endothelium-dependent relaxation to each stimulus to the endothelium-independent relaxation to SIN-1 or nitroglycerin), the vasodilating responses to bradykinin, substance P, or an increase in coronary blood flow were still comparable between the 2 sites (data not shown). The extent of the L-NMMA–induced coronary vasoconstriction under basal conditions was comparable between the IL-1β–treated (14±5%) and the control (13±6%) sites, and the extent of the L-NMMA–induced augmentation of the serotonin-induced coronary vasoconstriction was also comparable between the 2 sites (Figure 3).

**In Vitro Experiment**

In organ-chamber experiments, serotonin-induced contractions were significantly greater in the IL-1β–treated coronary segments than in the control segments regardless of the presence or absence of the endothelium (Figure 4). The serotonin-induced contractions tended to be inhibited by the presence of the endothelium to the same extent at both sites (Figure 4). Endothelium-dependent relaxations to serotonin, bradykinin, and the calcium ionophore A23187 were all comparable between the control and the IL-1β–treated coronary segments (Figure 5). Furthermore, endothelium-independent relaxations to sodium nitroprusside were also comparable between the 2 sites (Figure 6).

![Figure 1. Coronary arteriograms 2 weeks after application of IL-1β after intracoronary administration of nitroglycerin 10 μg/kg (A) and serotonin 10 μg/kg (B). Intracoronary serotonin induced hyperconstriction at IL-1β–treated site (black arrow) but only mild constriction at control site (white arrow).](image1)

![Figure 2. Quantitative analysis of coronary vasodilating responses to bradykinin (BK) 0.1 μg/kg IC (n=6), substance P (SP) 10 μg/kg IC (n=6), increase in coronary blood flow caused by adenosine 60 μg·kg⁻¹·min⁻¹ IC for 2 minutes (n=4), SIN-1 10 μg/kg IC (n=6), and nitroglycerin (NG) 10 μg/kg IC (n=6) at control (open bar) and IL-1β–treated (black bar) sites. Vasodilating responses to these endothelium-dependent and -independent stimuli were preserved at spastic site.](image2)

![Figure 3. Vasoconstricting responses to serotonin before and after intracoronary L-NMMA at control (open bar) and IL-1β–treated (black bar) sites. Augmenting effect of L-NMMA on serotonin-induced vasoconstriction was comparable between control and spastic sites.](image3)
Discussion

The major findings of the present study were that (1) endothelium-dependent coronary vasodilations to pharmacological agents (bradykinin, substance P, the calcium ionophore A23187, and even the vasospastic agent serotonin) as well as to physiological stimulus (increase in flow) were all preserved at the spastic site and (2) vascular smooth muscle hypercontraction appears to play a central role in our porcine model of coronary spasm.

Animal Model of Coronary Spasm Without Removal of the Endothelium

It has been controversial in clinical studies whether or not endothelial vasodilator functions in the spastic coronary artery are preserved. Clinical studies with coronary angiography may have fundamental limitations, because it is difficult in practice with this in vivo methodology alone to clearly dissect the reduced endothelial vasodilator function and the enhanced vasoconstrictor response of the smooth muscle. Thus, this point needed to be addressed in animal models from which isolated spastic blood vessels are available. Because our previous porcine model of coronary spasm required a combination of balloon endothelium removal and high-cholesterol feeding to induce atherosclerotic coronary lesions, endothelial dysfunction due to endothelial regeneration after the balloon injury was inevitable. In contrast, our present porcine model with an inflammatory cytokine is unique and important because the spasm can be induced by the adventitial inflammation without removal of the endothelium. The preserved endothelium-dependent coronary vasodilatation to bradykinin 1 day after the IL-1β application further supports the preserved integrity of the endothelium after the procedure. Thus, the present porcine model enables us to examine the endothelial vasodilator function of the spastic coronary artery without the effect of removal/regeneration of the endothelium.

Figure 4. Contractions to serotonin in rings from control and IL-1β-treated sites in vitro. Serotonin-induced contractions were significantly greater at IL-1β-treated site than at control site regardless of presence or absence of endothelium.

Figure 5. Endothelium-dependent relaxations in rings from control and IL-1β-treated sites in response to serotonin (in presence of 10^-6 mol/L ketanserin) (left), bradykinin (middle), and calcium ionophore A23187 (right) in vitro. Endothelium-dependent relaxations to these agents were comparable between 2 sites.

Figure 6. Relaxations to sodium nitroprusside (SNP) in rings without endothelium from control and IL-1β-treated sites. Relaxations were comparable between 2 sites.
In Vivo Evaluation of Endothelial Vasodilator Function

To evaluate endothelium-dependent coronary vasodilatation in vivo, we examined the endothelium-dependent vasodilatation to bradykinin, substance P, or an increase in coronary blood flow and the endothelium-independent vasodilatation to SIN-1 or nitroglycerin in vivo. The present results showed that coronary vasodilatations to all these stimuli were preserved at the spastic site in vivo. Vasodilating responses to SIN-1 and nitroglycerin tended to be increased at the IL-1β-treated sites, suggesting the increased basal tone of the coronary artery at the spastic site. However, even after the correction of the increased basal tone, the vasodilator responses to bradykinin, substance P, or an increase in coronary blood flow were preserved at the spastic site. Thus, the present in vivo findings are consistent with the previous clinical reports that endothelium-dependent coronary vasodilations are fairly preserved at the spastic site in patients with vasospastic angina in response to substance P,5,24 histamine,25 or bradykinin.26 Furthermore, the present study demonstrated that the basal release of NO is also preserved at the spastic site, which is consistent with our previous in vivo findings.6,23

In Vitro Evaluation of Endothelial Vasodilator Function

Organ chamber experiments further demonstrated that endothelium-dependent relaxations to serotonin, bradykinin, or A23187 were comparable between the control and the IL-1β-treated sites. Moreover, the contractions to serotonin were significantly inhibited by the presence of the endothelium to a similar degree at both the control and the IL-1β-treated sites. Endothelium-independent relaxations to sodium nitroprusside were unaltered at the spastic site. These results further indicate that endothelial vasodilator function is preserved in the spastic porcine coronary artery. The present study further demonstrated that endothelium-dependent relaxations even to the vasospastic agonist serotonin are preserved not only at lower concentrations but also in a wide range of concentrations if the direct vasocontracting effect of the monoamine is inhibited. A contribution of endothelium-derived contracting factors (eg, endothelin)27 to the occurrence of coronary spasm is unlikely, because there was no component of endothelium-dependent contractions in the serotonin-induced coronary vasoconstrictions in the present study.

However, we do not deny the possible importance of endothelial dysfunction for the development of coronary arteriosclerotic lesions27 in which coronary spasm due to endothelial dysfunction for the development of coronary arteriosclerotic lesions27 may play a central role in the occurrence of the spasm in the present porcine model.18 The increased contractility of the spastic coronary artery is not due to the increased muscle mass, because there is no significant increase in the mass in our present model.15 Although the molecular mechanism for the smooth muscle hypercontraction remains to be clarified, we recently demonstrated that the rho A/rho-kinase–mediated signaling pathway for vascular smooth muscle contraction plays an important role in the pathogenesis of coronary spasm.29,30

Light chain chain phosphorylation plays a central role in the occurrence of the spasm in the present porcine model.19 The increased contractility of the spastic coronary artery is not due to the increased muscle mass, because there is no significant increase in the mass in our present model.15 Although the molecular mechanism for the smooth muscle hypercontraction remains to be clarified, we recently demonstrated that the rho A/rho-kinase–mediated signaling pathway for vascular smooth muscle contraction plays an important role in the pathogenesis of coronary spasm.29,30

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