

# Nitric oxide inhibition unmasks ischemic myocardium-derived vasoconstrictor signals activating endothelin type A receptor of coronary microvessels

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**Takahashi, Katsuaki, Tatsuya Komaru, Satoru Takeda, Kouichi Sato, Hiroshi Kanatsuka, and Kunio Shirato.** Nitric oxide inhibition unmasks ischemic myocardium-derived vasoconstrictor signals activating endothelin type A receptor of coronary microvessels. *Am J Physiol Heart Circ Physiol* 289: H85–H91, 2005. First published March 4, 2005; doi:10.1152/ajpheart.00667.2004.—NO plays an important role in the compensatory increase in coronary flow conductance against myocardial ischemia, and NO bioavailability is impaired in various diseases. We tested the hypothesis that, when NO production is inhibited, vasoconstrictor signals from the ischemic myocardium are unmasked. We investigated the involvement of endothelin type A (ET<sub>A</sub>) receptors in the transduction of the constrictor signal. To detect coronary vasoactive signals derived from ischemic myocardium, we used a bioassay system in which an isolated rabbit coronary microvessel (detector vessel, DV) was placed on beating myocardium perfused by the left anterior descending coronary artery (LAD) of an anesthetized open-chest dog ( $n = 38$ ). The DV was pressurized to 60 cmH<sub>2</sub>O throughout the experiment and observed with an intravital microscope equipped with a floating objective. After the intrinsic tone of the DV was established, vehicle ( $n = 7$ ), *N*<sup>ω</sup>-nitro-L-arginine (L-NNA, 100 μmol/l;  $n = 13$ ), L-NNA + BQ-123 (a selective ET<sub>A</sub> receptor blocker, 1 μmol/l;  $n = 7$ ), or BQ-123 alone (1 μmol/l;  $n = 7$ ) was superfused onto the DV. Thereafter, the LAD of the beating heart was occluded. Coronary occlusion produced significant dilation of the DV by  $10 \pm 4\%$ . When L-NNA was applied, the DV significantly constricted by  $12 \pm 5\%$  in response to LAD occlusion, and BQ-123 abolished the vasoconstriction. Pretreatment with BQ-123 alone produced an enhancement of the ischemia-induced dilation. We conclude that ischemic myocardium releases transferable vasomotor signals that produce coronary microvascular constriction during the blockade of NO production and the constrictor signal is mediated by ET<sub>A</sub> receptors.

coronary circulation; ischemia; microcirculation; vasoconstriction; cross talk

DURING MYOCARDIAL ISCHEMIA, the coronary arterial microvessels relax, resulting in a decrease in coronary resistance as a compensatory defense mechanism. Although ischemic myocardium releases various vasoactive substances (22), it is uncertain which substances are relevant to the regulation of the coronary microvascular tone.

Research on the cross talk between the myocardium and the coronary microvessels has been hampered because of methodological difficulties. Because the microvascular tone is regulated by many factors besides myocardium-derived factors, such as myogenic tone, shear stress, and neurohumoral factors,

it is difficult to selectively evaluate the role of myocardium-derived factors. In the case of ischemia, decreases in perfusion pressure and flow rate per se result in changes in myogenic and shear stress-related tone. Furthermore, neurohumoral factors modify the microvascular diameter. To overcome this dilemma, we recently developed (30, 31) a novel bioassay method in which isolated coronary microvessels are placed on the beating heart. This system enables us to control beating hearts and microvessels separately.

Early studies showed that cardiac NO generation is enhanced during myocardial ischemia and that the released NO participates in the regulation of coronary flow (12, 25). Therefore, it is conceivable that NO plays an important role in mediating the cross talk between ischemic myocardium and coronary resistance vessels. However, it is not certain whether the signals that cause ischemia-induced NO production derive from the myocardium or the vascular lumen.

Because NO bioavailability is impaired in various diseases (10, 27), it is of interest to determine how the cross talk between the ischemic myocardium and coronary microvessels is modified when NO production is impaired. As some vasoconstrictors such as endothelin (ET)-1 (18, 33) are elevated in cardiac ischemia, it is possible that blockade of NO production unmasks microvascular constrictions. Accordingly, we tested the hypothesis that when NO production is inhibited the vasoconstriction caused by ischemic myocardium-derived factor is unmasked and that the ET type A (ET<sub>A</sub>) receptor is responsible for the signal transduction.

## METHODS

In the bioassay method we used for the detection of myocardium-derived vasomotor signals, we combined an isolated microvascular preparation with an in vivo beating heart (31). The present study was approved by the institutional ethical committee for animal experiments and conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Pub. No. 85–23, revised 1996).

### Preparation of Detector Microvessels

Male Japanese White rabbits ( $n = 38$ ; body wt  $2.16 \pm 0.03$  kg, 10–13 wk old) were anesthetized with pentobarbital sodium (50 mg/kg iv) and heparinized (1,000 U iv). After the rabbits were killed by bleeding from the carotid artery, the hearts were quickly excised and immersed in chilled Krebs solution (in mmol/l: 120.0 NaCl, 4.7 KCl, 2.0 CaCl<sub>2</sub>, 1.17 MgSO<sub>4</sub>, 1.20 NaH<sub>2</sub>PO<sub>4</sub>, 0.02 calcium disodium EDTA, 25.0 NaHCO<sub>3</sub>, 5.0 glucose, pH adjusted

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to 7.40 by bubbling with 95% O<sub>2</sub>-5% CO<sub>2</sub>). A coronary arterial microvessel perfusing the left ventricle (100–300 μm) was carefully isolated and used as a detector vessel (DV). One end of the vessel was cannulated to a polyethylene micropipette filled with filtered Krebs solution, the tip of which was tapered (<100 μm), and tied with a monofilament of silk suture thread (diameter 2 μm). The micropipette was connected to a polyethylene tube and to a pressure reservoir. The other end of the vessel was completely ligated, and the intraluminal pressure was monitored with a strain gauge transducer (TC0406S01; Baxter) to detect vascular leaks. Vessels with any leakage were not used for further experiments. The DV was incubated in warm (38°C) Krebs solution bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> until use.

#### Beating Heart Preparation

Mongrel dogs of either sex ( $n = 38$ ;  $6.9 \pm 0.5$  kg) were used and prepared as in our previous studies (1, 31). Briefly, the dogs were anesthetized with  $\alpha$ -chloralose (60 mg/kg; Wako Chemicals) and artificially ventilated. The right jugular vein was cannulated for the administration of anesthetics. A catheter was placed in the ascending aorta for measurement of the aortic pressure. A thoracotomy was performed, and the pericardium was cut to expose the heart surface. The left anterior descending coronary artery (LAD) was dissected, and a snare was placed for coronary occlusion.

The heart rate was kept constant at 100–120 beats/min by left atrial pacing after suppression of the sinoatrial node with an injection of formaldehyde. Two 24-gauge stainless steel needles were inserted horizontally (5–7 mm apart) into the midmyocardium of the left ventricle. Both ends of each needle were fixed to a needle holder. This apparatus limits excessive movement of the heart, thereby keeping the region of interest within the observation field.

#### Bioassay Method

The incubated DV was gently placed on the LAD perfusion area of the beating left ventricle of the dog and pressurized to 60 cmH<sub>2</sub>O to produce intrinsic tone. Only one DV was used for each experiment. The DV and the heart surface were kept moist throughout the experiment by continuous superfusion of Krebs solution (38°C) at a rate of 1–1.5 ml/min with an infusion pump (model STC-521; Terumo, Tokyo, Japan).

For visualization of the DV placed on the beating left ventricle, an intravital microscope system equipped with a floating objective and a charge-coupled device camera (IGS; Nikon) was used. This optical system was originally developed in our laboratory (1) for the visualization of epimyocardial coronary microvessels of the beating heart throughout the cardiac cycle. The microscope objective was a Leitz model PL-fl ( $\times 10$ ; numerical aperture 0.30). An objective lifter was used to carefully adjust the distance between the objective and the vessels, so as not to compress the DV. The distance between the floating objective and the heart surface was  $\sim 0.5$  mm, and that space was filled with Krebs solution, which was superfused as described above.

Epi-illumination with a mercury lamp was applied to obtain the image of the DV. A polarizing filter (IGS; Nikon) was used to minimize reflected light from the surface of the heart. Obtained images were recorded with a digital video recorder (DSR-20; Sony). The spatial resolution of this optical system was 2 μm.

The images of the DV were captured on a PC monitor, and the inner diameters were measured with Scion Image (version  $\beta 4.0.2$ ; Scion). On the monitor screen, one cursor was set on the vessel wall of interest and another cursor was set on the nearest point of the other side of the vessel wall. Vascular diameters were measured at least three times during the end-diastolic phase.

#### Experimental Protocols

Experiments were performed  $\sim 1$  h after the DV was placed, when all hemodynamic variables and blood gas parameters had become stable and the intrinsic tone of the DV had developed.

**Protocol 1 (vehicle group,  $n = 7$ ).** After the intrinsic tone of the DV attained stability, the baseline hemodynamic variables were measured and the LAD of the beating heart was occluded. Images of the DV were collected at 2, 3, 5, and 10 min after the induction of ischemia. Hemodynamic variables were also collected after 10 min of ischemia.

**Protocol 2 (L-NNA group,  $n = 13$ ).** After the intrinsic tone of the DV was established, N<sup>ω</sup>-nitro-L-arginine (L-NNA, 100 μmol/l) was superfused onto the DV until the end of the experiments to block NO production. Twenty minutes after the pretreatment, the LAD was occluded. DV images and hemodynamic data were collected in the same fashion as in *protocol 1*.

**Protocol 3 (L-NNA+BQ-123 group,  $n = 7$ ).** After the intrinsic tone of the DV was confirmed, we superfused BQ-123 (a selective ET<sub>A</sub> receptor blocker, 1 μmol/l) in combination with L-NNA (100 μmol/l) onto the DV until the end of the experiment. Twenty minutes after the superfusion of L-NNA and BQ-123, the LAD occlusion was started. Vascular images and hemodynamic data were collected in the same fashion as in *protocol 1*.

**Protocol 4 (BQ-123 group,  $n = 7$ ).** After the intrinsic tone of the DV was confirmed, we superfused BQ-123 (1 μmol/l) alone onto the DV until the end of the experiment. Twenty minutes after the superfusion of BQ-123, the LAD was occluded. Data collection was performed as in *protocol 1*.

**Protocol 5 (ECE-I group,  $n = 4$ ).** We superfused FR901533 (100 μmol/l), a selective ET-converting enzyme (ECE) inhibitor (ECE-I), to investigate the role of ECE in the ischemia-induced ET release. Twenty minutes after the superfusion of FR901533, the LAD was occluded and data collection was performed as in *protocol 1*.

In each protocol, ischemia of the myocardium on which the DV was placed was confirmed by the dyskinetic wall motion and cyanotic color changes of the heart. At the end of each experiment, sodium nitroprusside (100 μmol/l) was superfused for 5 min to confirm the preserved vasodilator capacity.

#### Measurement of Plasma Concentration of ET-1

In another set of experiments ( $n = 9$ ), we measured plasma ET levels in the aorta and in the coronary vein draining from the ischemic area to investigate whether myocardial ischemia for 10 min enhances ET release into the vascular lumen. Blood samples (3.0 ml) were collected before and 10 min after LAD occlusion and immediately centrifuged at 3,000 rpm for 10 min at 4°C. The plasma fraction was stored at  $-20^{\circ}\text{C}$  until analysis. Plasma ET was quantitated with radioimmunoassay. Rabbit anti-ET-1 serum (Peninsula Laboratories) and <sup>125</sup>I-labeled ET-1 (PerkinElmer) were used for this purpose.

#### Drugs

L-NNA (Sigma) and sodium nitroprusside (Wako Chemicals) were freshly dissolved in physiological salt solution (PSS) to the desired concentrations. BQ-123 (Sigma), a selective ET<sub>A</sub> receptor blocker, was diluted with PSS to 100 μmol/l as a stock solution, and FR901533 (Fujisawa Pharmaceutical, Osaka, Japan) was dissolved with PSS to 50 mmol/l as a stock solution. These stock solutions were stored at  $-20^{\circ}\text{C}$  until use and freshly diluted to the desired concentration for the experiments.

#### Data Analysis

All variables are expressed as means  $\pm$  SE. The responses of the DV diameters are expressed as percent change in diameter. When the intrinsic tone of the DV was not observed (baseline diameter  $>90\%$  of the maximal diameter), the vessel was discarded. The maximal

diameters were defined as the greatest DV diameter among passive diameters right after isolation and after 100  $\mu\text{mol/l}$  of sodium nitroprusside at the distending pressure of 60  $\text{cmH}_2\text{O}$ . The aortic pressure and vascular diameters were statistically analyzed with one-way ANOVA for repeated measures and Student's *t*-test for paired samples, with Bonferroni correction applied to detect the time point when significant changes occurred. At  $P < 0.05$ , differences were accepted as significant.

## RESULTS

### Blood Gases and Aortic Pressure

Blood pH, blood gases, and aortic pressure before and after ischemia are shown in Table 1. Blood pH and blood gases were kept within the physiological range. There were no differences in mean aortic pressure between measurements before ischemia and at the end of ischemia in any of the protocols.

### Detector Vessel Responses

The DV sizes for each protocol were  $227 \pm 11$  (protocol 1),  $222 \pm 17$  (protocol 2),  $183 \pm 16$  (protocol 3),  $199 \pm 20$  (protocol 4), and  $191 \pm 10$  (protocol 5)  $\mu\text{m}$  at their maximal diameters. There were no statistically significant differences in vascular sizes among the five protocols. The intrinsic tone of DV before ischemia was defined as the DV diameter before ischemia normalized by the maximal diameter in each protocol as follows: protocol 1:  $76 \pm 5\%$ , protocol 2:  $83 \pm 2\%$ , protocol 3:  $83 \pm 3\%$ , protocol 4:  $77 \pm 3\%$ , protocol 5:  $77 \pm 7\%$ . There were no differences in intrinsic vascular tone among the protocols.

In protocol 1 (vehicle group), the DV began to dilate in response to ischemia in 2 min and the dilation attained statistical significance in 5 min. The magnitude of the DV dilation was  $10 \pm 4\%$  at 10 min after the induction of ischemia ( $P < 0.05$  vs. baseline; Figs. 1, A and B, and 2).

In protocol 2 (L-NNA group), L-NNA superfusion per se did not alter DV diameter [before L-NNA:  $191 \pm 13$   $\mu\text{m}$ , after L-NNA 20 min:  $185 \pm 15$   $\mu\text{m}$ ; not significant (NS)]. The DV began to constrict significantly 2 min after the induction of ischemia (% diameter change:  $-7 \pm 3\%$ ;  $P < 0.05$  vs. baseline). The microvascular constriction continued until 10 min after the induction of ischemia (% diameter change:  $-12 \pm 5\%$ ,  $P < 0.05$  vs. baseline; Figs. 1, C and D, and 2). The difference in vascular responses between protocol 1 and protocol 2 was statistically significant ( $P < 0.05$ ). These observations indicated that the blockade of NO production unmasked the ischemic myocardium-derived vasoconstrictor signals.

In protocol 3 (L-NNA+BQ-123 group), the combined pretreatment with BQ-123 and L-NNA per se did not alter the diameter of the DV (before:  $156 \pm 13$   $\mu\text{m}$ , after:  $146 \pm 11$   $\mu\text{m}$ ; NS). Myocardial ischemia did not produce significant diameter changes in the DV (Figs. 1, E and F, and 2). These results indicate that the vasoconstriction observed in protocol 2 was mediated by the activation of  $\text{ET}_A$  receptors in coronary arterial microvessels.

In protocol 4 (BQ-123 group), the pretreatment with BQ-123 did not change the DV diameter (before:  $139 \pm 14$   $\mu\text{m}$ , after:  $150 \pm 15$   $\mu\text{m}$ ). Myocardial ischemia produced significant dilation of DV, and the magnitude of the dilation was greater than that in the vehicle group (Figs. 1, G and H, and 2). BQ-123 induced an enhancement of the dilator responses, indicating that myocardial ischemia activates the vasoconstrictor signal mediated by ET even in the absence of L-NNA.

In protocol 5 (ECE-I group), FR901533 did not change the DV diameter (before:  $130 \pm 7$   $\mu\text{m}$ , after:  $147 \pm 14$   $\mu\text{m}$ ). Myocardial ischemia produced significant dilation of DV, but the magnitude of the dilation was not different from that of the vehicle group (% diameter changes: 2 min,  $8 \pm 2\%$ ; 3 min,  $10 \pm 1\%$ ; 5 min,  $12 \pm 2\%$ ; 10 min,  $13 \pm 2\%$ ).

There were no differences in the dilator responses of DV to sodium nitroprusside (100  $\mu\text{mol/l}$ ) among the five protocols.

### Plasma ET-1

Figure 3 shows the group data of plasma ET measurements before and after 10-min ischemia. In neither the coronary vein nor the aorta did the plasma ET-1 level change. The arteriovenous difference in the plasma ET-1 concentration, which represents ET-1 production in the coronary circulation, did not significantly increase with myocardial ischemia (Fig. 3C).

## DISCUSSION

In the present study, we found the following: 1) ischemic myocardium releases the transferable vasodilator signal; 2) when NO release is impaired, the ischemic myocardium-derived vasoactive signal that produces coronary microvascular constriction is unmasked; 3) the vasoconstrictor signal is mediated by  $\text{ET}_A$  receptors; 4) a  $\text{ET}_A$  receptor blocker enhances ischemia-induced coronary microvascular dilation, suggesting that ischemic myocardium simultaneously releases signals activating NO release and those activating ET release; 5) ECE-I does not enhance ischemia-induced dilation, suggesting that ET released during acute myocardial ischemia derives from mature ET pools rather than de novo synthesis.

Table 1. Blood gas analysis and aortic pressure data of dogs in vehicle, L-NNA, L-NNA+BQ-123, BQ-123, and FR901533 groups

	Vehicle (n = 7)	L-NNA (n = 13)	L-NNA + BQ-123 (n = 7)	BQ-123 (n = 7)	FR901533 (n = 4)
pH	$7.41 \pm 0.02$	$7.41 \pm 0.01$	$7.39 \pm 0.01$	$7.39 \pm 0.01$	$7.40 \pm 0.02$
Aortic $\text{PO}_2$ , mmHg	$97.5 \pm 4.1$	$115.6 \pm 9.3$	$99.0 \pm 5.8$	$101.1 \pm 6.4$	$91.9 \pm 3.9$
Aortic $\text{PCO}_2$ , mmHg	$30.2 \pm 1.2$	$30.2 \pm 1.1$	$34.9 \pm 1.4$	$33.0 \pm 1.1$	$35.5 \pm 3.0$
Mean aortic pressure, mmHg					
Before ischemia	$122 \pm 5$	$109 \pm 6$	$100 \pm 9$	$116 \pm 4$	$116 \pm 11$
After ischemia	$111 \pm 6$	$108 \pm 9$	$98 \pm 11$	$114 \pm 7$	$115 \pm 11$

Values are means  $\pm$  SE. L-NNA, N<sup>o</sup>-nitro-L-arginine.

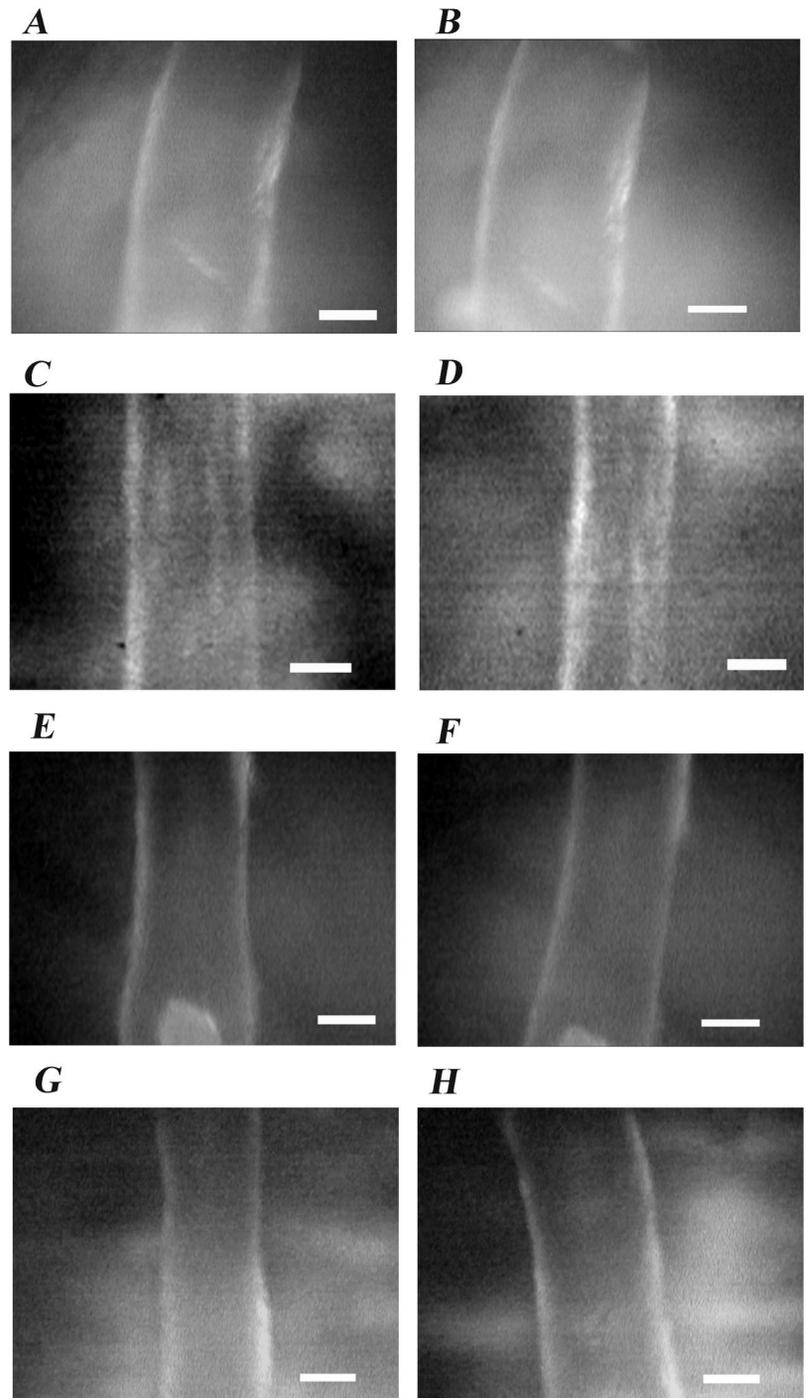


Fig. 1. Representative images of detector vessels at baseline (A, C, E, G) and 10 min after ischemia (B, D, F, H) in vehicle (A, B), *N*<sup>ω</sup>-nitro-L-arginine (L-NNA; C, D), L-NNA+BQ-123 (E, F), and BQ-123 (G, H) groups. Bar: 100  $\mu$ m.

#### Methodological Consideration

Our primary interest was to detect the vasomotor signals derived from ischemic myocardium. In our bioassay system, the distending pressure (60 cmH<sub>2</sub>O) and flow state (stop flow) of the DV were kept constant throughout the experiment irrespective of the myocardial condition. Therefore, we did not have to take myogenic factors and shear stress into account in the ischemia-induced microvascular responses. Furthermore, because the DVs were separated from the bloodstream and nervous controls, the DV responses to myocardial ischemia were unlikely to be influenced by systemic neurohumoral

changes. Thus we could specifically focus on the cross talk between the myocardium and the coronary microvessels. The magnitude of the dilation of the DV by myocardial ischemia was almost identical to that in our previous observation (30, 31), confirming the reproducibility of our method.

The diameters of the DVs were  $\sim$ 200  $\mu$ m at their maximal relaxations, and they were 150–200  $\mu$ m when intrinsic tone developed. It could be argued whether or not microvessels of this size contribute to vascular resistance. Because Chilian et al. (5) previously showed that 20% of vascular resistance resides in microvessels  $>$ 200  $\mu$ m in diameter, it is reasonable

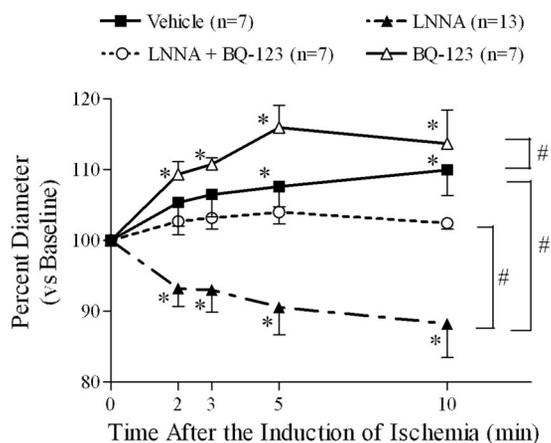


Fig. 2. Detector vessel responses to myocardial ischemia. In *protocol 1* (vehicle group), the vessels significantly dilated in response to myocardial ischemia. In contrast, in *protocol 2* (L-NNA group), they constricted significantly. In *protocol 3* (L-NNA+BQ-123 group), the constriction of the detector vessel was abolished. In *protocol 4* (BQ-123 group), the myocardial ischemia-induced dilation of the detector vessel was significantly enhanced. Difference in responses of detector vessels between protocols: # $P < 0.05$ , \* $P < 0.05$  compared with baseline diameter.

to consider that the DVs we used are vascular that bear significant coronary resistance.

To keep the heart surface moist and warm, we superfused warm Krebs solution throughout the experiment at the rate of 1–1.5 ml/min. Accordingly, the space between the floating objective and the heart surface (0.5 mm apart) was always filled with the solution. The Krebs solution remaining in that space probably diluted the signal from the myocardium. Therefore, it is possible that the magnitude of the vasomotor signals evaluated in our bioassay method was underestimated to some extent. In other words, the cross talk between the myocardium and the microvessel was very intense in terms of the vasomotor responses.

#### Vasodilator Signals Derived From Ischemic Myocardium

There have been earlier studies suggesting that NO release is enhanced during myocardial ischemia (12, 25). The present results are in line with those data, and we elucidated that the signal that stimulates NO production originates from the ischemic myocardium.

L-NNA abolished the dilator signal from the ischemic myocardium, demonstrating that the L-arginine-NO pathway is the main signaling system that mediates microvascular relaxation during myocardial ischemia. However, there could be some other possible mediators such as the activation of ATP-sensitive K ( $K_{ATP}$ ) channels and the release of adenosine (14). Vessel size specificity of vasodilatory mechanisms may explain this issue. Coronary microvascular responses to various interventions are strikingly heterogeneous among vessel sizes (14). We used relatively large microvessels ( $\sim 200 \mu\text{m}$ ), and in this size class of microvessels the L-arginine-NO pathway is a dominant mechanism, whereas adenosine and  $K_{ATP}$  channel activation is dominant in smaller microvessels (15, 16, 19).

We previously reported that vasodilator signals released from ischemic myocardium are mediated by pertussis toxin-sensitive G proteins ( $G_{PTX}$ ) located in the vascular wall (31)

and that microvascular  $G_{PTX}$  is functionally linked with the NO pathway in coronary microvessels  $>130 \mu\text{m}$  in diameter (17). In addition,  $G_{PTX}$  is known to exist in vascular endothelial cells (7). On the basis of those observations, it is conceivable that myocardial vasodilator signals act on the endothelial  $G_{PTX}$  of DV that activate endothelial NO synthase leading to NO production. We cannot draw conclusions about the mediator responsible for the NO release during myocardial ischemia from our studies, but we speculate that myocardial and/or interstitial acidosis is one plausible mediator. Kitakaze et al. (13) showed that cellular acidosis modulates the production of endogenous NO in canine ischemic myocardium, and the possibility that proton activates  $G_{PTX}$  was shown by Ishizaka et al. (11).

#### Vasoconstrictor Signals Derived From Ischemic Myocardium

When vessel diameters decrease in response to a decrease in the perfusion pressure, there could be two possibilities, passive collapse or active contraction. The present studies have clearly shown that when NO production is impaired myocardial ischemia produces active constriction by the stimulation of  $ET_A$  receptors without apparent elevation of plasma ET-1.

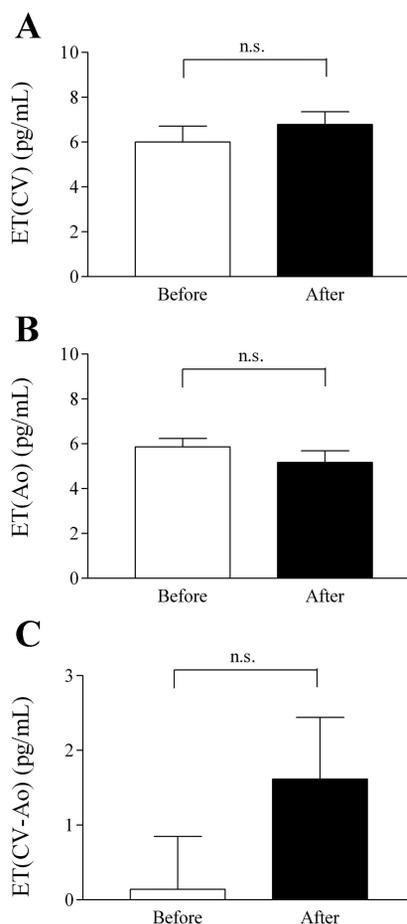


Fig. 3. Plasma endothelin (ET)-1 levels before and after myocardial ischemia in the coronary vein (CV) draining from the ischemic area (A) and in the aorta (Ao; B) ( $n = 9$ ). There were no significant differences (n.s.) in the plasma ET-1 level at either sampling point. The change in arteriovenous difference (CV – Ao) in the plasma ET-1 concentration caused by myocardial ischemia did not reach statistical difference (C).

The plasma ET-1 level in the present experiments was 5–7 pg/ml (~2 pmol/l), and there was no increase in either the aorta or the coronary vein in the ischemic region, demonstrating that no systemic or local increase in intravascular ET resulted from the 10-min ischemia. Although an elevation of plasma ET has been shown in myocardial ischemia in both humans (24) and animals (2, 35), an ischemic period of >1 h is required for the increase. Previous data from our group (36) and others (23) have shown that the threshold concentration of ET for coronary microvascular constriction is 10–100 pmol/l. Accordingly, the plasma ET in the vascular lumen during the 10-min ischemia was much lower than the vasoactive concentration.

ECE in the heart is localized to vascular endothelial cells and endocardial endothelial cells but not to cardiac myocytes in animals and humans (6, 32). Therefore, it is unlikely that the ischemic myocardium itself released ET in the present experiment. Endocardial endothelial cells are also an unlikely source of ET, which can account for the DV constriction, because the plasma ET was not increased by ischemia. It is conceivable that some signals derived from ischemic myocardium activate the ET release from vascular endothelium. Winegrad et al. (37) demonstrated that isolated cardiac myocytes do not release ET but do release angiotensin I, leading to ET release from vascular endothelium.

Because ET-mediated constriction of the DV took place without an increase in plasma ET-1, it is likely that the ET released from the microvascular endothelium constricts the microvascular smooth muscle in a paracrine manner. Wagner et al. (34) demonstrated that the secretion of ET is polarized, that is, the majority of ET is released into the basolateral compartment (abluminal side). These observations indicate that ET is a local paracrine regulator of vascular tone. Wang et al. (35) showed that the major portion of ET released during ischemia-reperfusion is trapped within the heart, with only small amounts released into the vascular lumen.

There are two distinct secretory pathways in the process of ET-1 release in the coronary vasculature (28, 29). One is a constitutive pathway, which is modulated at the level of protein synthesis and involves the basal release of ET-1, and the other is a regulated pathway that involves rapid, stimulated degranulation of ET-1-containing storage granules. In the latter pathway, ET is quickly released in response to various stimulations (8, 21) and exerts biological effects. The storage granules have been detected in endothelial cells, and big ET-1 and ECE have been found to colocalize with the storage granules located beneath the plasma membrane (29). As the vasoconstriction we observed was an immediate response, ET is likely to be released in the regulated manner.

It is possible that myocardial ischemia stimulates the release of a factor(s) that stimulates NO and ET-1 release simultaneously. Another possibility is that NO inhibits ET release and that inhibition of NO accelerates the release. In the present studies, BQ-123 augmented the ischemia-induced DV dilation even in the absence of NO blockade. Accordingly, ischemic myocardium is likely to release signals that stimulate NO production and stimulate ET release simultaneously. As ECE-I did not significantly enhance the DV dilation, the ET release in response to acute ischemia may derive from mature ET pools.

### Effect of NO on ET System

At first, NO was known to inhibit the production of ET in cultured endothelial cells (4) and in intact vessels (3). However, the vasoconstrictions we observed were immediate responses, and so the released ET must be the stored type, not produced de novo. To our knowledge, there is no evidence that NO inhibits ET release from storage granules.

The impact of NO on ET signal transduction was investigated in earlier studies, and it was shown that the suppression of NO production generally unmasks or magnifies ET-dependent effects (20), indicating that NO plays a role as a braking mechanism for the ET action. Goligorsky et al. (9) showed that NO plays a role in the physiological termination of ET-1 signaling at the receptor level (displacement of bound ET-1 from its receptor) and at the postreceptor level (intracellular calcium mobilization). These mechanisms may be involved in the phenomenon by which NO blockade unmasks DV constriction.

### Clinical Implications

NO bioavailability is limited in various diseases such as hypertension (27), hypercholesterolemia (27), diabetes mellitus (10), and atherosclerosis (26). It is plausible that under such disease conditions, acute myocardial ischemia results in a greater decrease in coronary flow. Very recently, we demonstrated (30) that the cross talk between coronary microvessels and the ischemic myocardium is impaired in hypercholesterolemia. Accordingly, ET<sub>A</sub> receptor blocker may be beneficial for the amelioration of decreased coronary microvascular blood flow and ischemic myocardial damage, especially in such disease conditions.

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