

Bradykinin mediates cardiac preconditioning at a distance

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Schoemaker, Regien G., and Caroline L. van Heijningen. Bradykinin mediates cardiac preconditioning at a distance. *Am J Physiol Heart Circ Physiol* 278: H1571–H1576, 2000.—Preconditioning the heart by brief coronary (CAO) or mesenteric artery occlusion (MAO) can protect against damage during subsequent prolonged CAO and reperfusion. The role of bradykinin (BK) in remote cardiac preconditioning by MAO is investigated by antagonizing the BK B₂ receptor [Hoechst 140 (HOE-140)] or simulating local BK release by mesenteric intra-arterial infusion. Anesthetized male Wistar rats ($n = 6-8$) were treated with HOE-140 or saline before starting the preconditioning protocol, CAO, MAO, or non-preconditioned control. Infarct size related to risk area [ratio of infarct area to area at risk (IA/AR)] was determined after 3 h of reperfusion following a 60-min CAO. IA/AR was $62 \pm 5\%$ in controls and not affected by HOE-140 ($58 \pm 6\%$). CAO as well as MAO significantly protected the heart (IA/AR, 37 ± 3 and $35 \pm 5\%$), which was prevented by HOE-140 (IA/AR, 71 ± 6 and $65 \pm 7\%$, respectively). Brief intramesenteric BK infusion mimicked MAO (IA/AR, $26 \pm 3\%$). Pretreatment with hexamethonium could abolish this protection (IA/AR, $67 \pm 4\%$). These data indicate an important role for BK in remote preconditioning by MAO. Results support the hypothesis that remote preconditioning acts through sensory nerve stimulation in the ischemic organ.

sensory nerves; mesenteric vascular bed

ISCHEMIC PRECONDITIONING has been described for the myocardium (13) as well as for many other organs, including brain (8), kidney (23), liver (10), and skeletal muscle (12), indicating a rather general phenomenon. However, within the heart, brief local ischemia not only preconditioned that same area but also adjacent myocardium was protected (15). Moreover, we showed that even brief ischemia in organs other than the heart was able to protect the heart (3) (also termed as remote preconditioning). This has been confirmed by others (14, 20). The mechanism within the heart showed similarities with the classical preconditioning (14, 20). Outside the heart we showed that for the phenomenon of remote cardiac preconditioning reperfusion of the stimulated organ is required before coronary artery occlusion (CAO) and that protection can be abolished by ganglion blockade with hexamethonium, thus indicat-

ing a neurogenic pathway that is activated during reperfusion (3). We hypothesized that reperfusion of the ischemic organ locally stimulates sensory nerves, which project to efferent nerves on the myocardium. One of the endogenous substances that is released during ischemia-reperfusion and capable of activating sensory nerves is bradykinin (BK). We herein investigate the role of BK in remote cardiac preconditioning by mesenteric artery occlusion (MAO). To inhibit the effects of endogenous BK, the BK B₂ receptor, responsible for afferent nerve stimulation, is blocked by its antagonist Hoechst 140 (HOE-140). To mimic the effects of local BK release, the effects of intramesenterically infused BK (7) to locally stimulate afferent nerves in the absence or presence of ganglion blockade were investigated.

MATERIALS AND METHODS

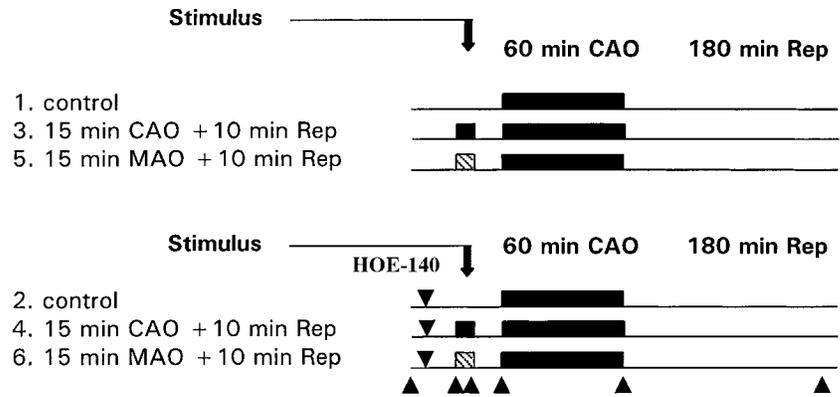
At all stages the experiments conform with the *Guide for the Care and Use of Laboratory Animals*. Male Wistar rats (Harlan; Zeist, The Netherlands) weighing 280–380 g at time of the experiment were used. Animals were housed in groups of two or three, placed on a 12:12-h light-dark cycle, and given food and water ad libitum.

Surgical and experimental preparation. Rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and intubated (PE-240) for positive pressure ventilation (Harvard Rodent Ventilator; Hilliston, MA) with room air (60–70 strokes/min, tidal volume 3 ml). The abdominal aorta was cannulated via the femoral artery to register mean arterial blood pressure (MAP) and heart rate (HR). A catheter was placed in the femoral vein for infusion of pentobarbital sodium to maintain anesthesia ($0.15 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$) and injection of HOE-140 or saline. An additional catheter was positioned in the right atrium via the right jugular vein for infusion of Haemacell (Behringwerke; Marburg, Germany) to compensate for cardiac effects of (surgery associated) fluid loss by maintaining right ventricular filling pressure at 5 mmHg. Then, the fourth intercostal space was opened, and a 6-0 silk suture was looped under the left descending coronary artery for later induction of CAO (3, 16). The abdominal cavity was opened, the anterior mesenteric artery was freed from surrounding tissue, and a loose suture was placed around the artery to facilitate later MAO with an atraumatic clamp (see Ref. 3 for details). Core temperature was monitored continuously using an electronic thermometer (Electromedics) and was maintained between 36.5 and 37.5°C by either heating pads or ice-filled packages.

Experimental protocol BK blockade. After surgical preparation, rats were allowed 30 min for stabilization before baseline hemodynamics were recorded. Rats were subdivided to undergo one of six protocols (Fig. 1). All protocols included a 60-min CAO, followed by 180 min of reperfusion. This was preceded by either no preconditioning (*protocols 1 and 2*),

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Fig. 1. Schematic presentation of 6 protocols in which effect of Hoechst 140 (HOE-140; ▼) on risk area and infarct size was studied. ▲, Measurements of heart rate and blood pressure. CAO, coronary artery occlusion; MAO, mesenteric artery occlusion; Rep, reperfusion.



classic preconditioning by a 15-min CAO followed by a 10-min reperfusion (protocols 3 and 4), or by remote preconditioning by a 15-min MAO followed by a 10-min reperfusion (protocols 5 and 6). The experiments were performed in the absence (protocols 1, 3, and 5) or presence (protocols 2, 4, and 6) of B₂ receptor blockade by HOE-140 (300 µg/kg) administered intravenously 10 min before preconditioning. The 15-min CAO that is used as preconditioning stimulus, by itself induces only negligible necrosis of $3 \pm 1\%$ (3). HR and MAP were recorded continuously and analyzed at the time points depicted in Fig. 1. Rats that fibrillated during ischemia or reperfusion were allowed to complete the protocol when conversion to normal sinus rhythm occurred spontaneously or when manual resuscitation was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified by appearance and disappearance of myocardial or small intestinal cyanosis.

Experimental protocol BK stimulation. Surgical preparation was slightly adjusted to include intramesenteric infusion of BK, in that no suture was placed around the mesenteric artery but one of the side branches was cannulated with the catheter tip positioned at the edge of the mainstream of the mesenteric artery (7). After surgical preparation, rats were allowed 30 min for stabilization before baseline hemodynamics were recorded. Rats were subdivided for three protocols (Fig. 2). All protocols again included 60 min of CAO, followed by 180 min of reperfusion. This was preceded by 5 min of intramesenteric infusion of saline or BK, followed by 5 min of rest. BK was infused at 1 µg/min, which is reported to stimulate sensory neurons without direct systemic hemodynamic effects (7). Whether the effects of intramesenteric BK infusion on myocardial infarct size could indeed be attributed to nerve stimulation rather than to circulating BK was further verified by blockade of the nervous pathway by hexamethonium (20 mg/kg iv) 15 min before the intramesenteric BK infusion. HR and MAP were recorded continuously and analyzed at the time points shown in Fig. 2.

Measurement of area at risk and infarcted area. At the end of each experiment the heart was quickly removed and placed in ice-cold saline before it was mounted on a modified Langendorff apparatus and perfused with cold saline via the aorta to wash out the blood. After the coronary artery ligature was retied, the heart was perfused with trypan blue (0.4%,

Sigma Chemical) to stain the perfused myocardium blue, whereas the area at risk (AR) remains unstained. The heart was placed at -80°C for 10 min before it was cut in slices (1 mm thickness) from apex to base. From each slice, the right ventricle was removed and the left ventricle (LV) including the septum was divided into the AR and the remaining LV. The AR was then incubated for 10 min in 37°C nitro blue tetrazolium (Sigma Chemical; 1 mg/ml Sørensen buffer, pH 7.4), which stains vital tissue purple but leaves irreversibly damaged tissue unstained [the infarct area (IA)]. After the IA was separated from the noninfarcted area, the right ventricle as well as the different areas of the LV were dried and weighed separately.

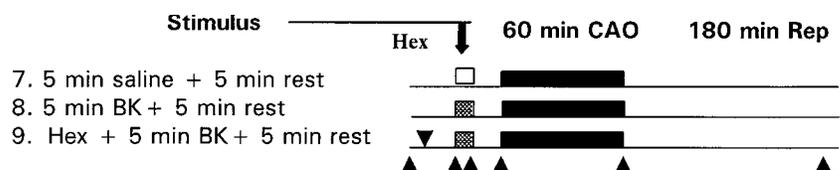
Evaluation of dose of HOE-140. To effectively inhibit the effects of BK during the experimental protocol, the dose and duration of HOE-140 were evaluated in separate rats. For that, rats were anesthetized with pentobarbital sodium and catheters were implanted in the femoral artery as well as the femoral vein (for detailed description see *Surgical and experimental preparation*). The former was connected to a pressure transducer for registration of MAP, whereas the latter was used for intravenous administration. After at least 30 min of stabilization, rats were injected with BK (4 µg/kg iv) and blood pressure decrease was measured. This procedure was repeated 5 min later to examine possible tachyphylaxis. HOE-140 (300 µg/kg) was then injected and the blood pressure response to BK was followed for 4 h.

Data analysis and presentation. Data are presented as means \pm SE unless indicated otherwise. Correlation between AR and IA was evaluated by linear regression analysis. Differences in the IA-to-AR ratios (IA/RA) between groups were analyzed using one-way ANOVA followed by Bonferroni's *t*-tests for multiple group comparisons. HR and MAP for the different groups were analyzed by two-way ANOVA for repeated measurements. Differences were regarded statistically significant at $P < 0.05$.

RESULTS

Mortality. A total of 71 rats entered the study. Fifty-two of these rats completed their protocol and have been included in the final analysis. The main cause of exclusion was premature death (17 rats) due to

Fig. 2. Schematic presentation of 3 protocols in which effect of intramesenteric bradykinin (BK) in absence or presence of hexamethonium (Hex, ▼) on risk area and infarct size was studied. ▲, Measurements of heart rate and blood pressure.



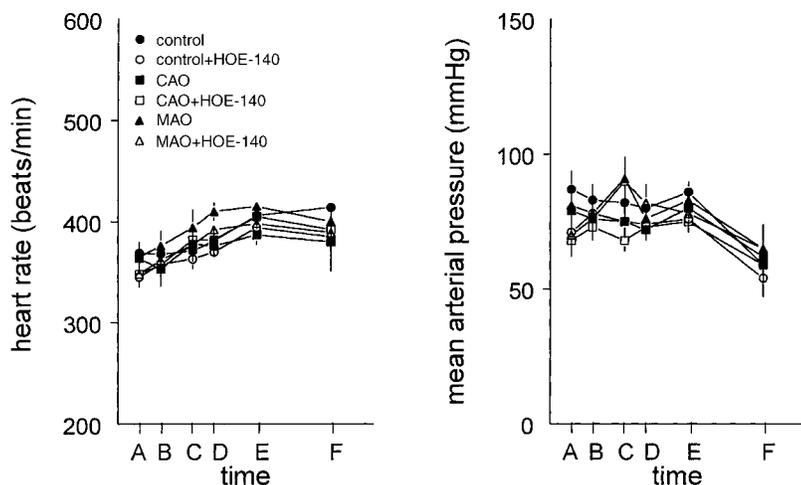


Fig. 3. Heart rate and blood pressure at different time points in protocols described in Fig. 1: A, after 30 min of stabilization; B, 10 min after injection of saline or HOE-140; C, at end of 15-min control, coronary or mesenteric artery occlusion; D, after 10 min of reperfusion; E, at end of 60-min CAO; F, at end of 3-h coronary reperfusion.

sustained ventricle fibrillation and/or hypotension during the 180-min reperfusion period.

Evaluation of dose of HOE-140. BK, administered intravenously at a dose of 4 $\mu\text{g}/\text{kg}$, decreased MAP by 23 ± 3 mmHg. The second administration 5 min later induced similar MAP reduction, indicating no tachyphylaxis. HOE-140 by itself had no effect on MAP or HR but completely blocked the response to BK for at least 90 min. At 120 min the response was still inhibited by 70%, whereas at 210 min the response to BK was fully restored.

BK blockade. Hemodynamics were registered continuously by means of MAP and HR. Significant differences were not observed for MAP or for HR among the protocols at any time point (Fig. 3). Moreover, administration of HOE-140 or ischemia and reperfusion did not cause significant changes in HR and MAP.

Without preconditioning, a significant correlation between AR and IA is observed (regression line, IA =

0.54; RA, +6.25; $r = 0.894$). Pretreatment with HOE-140 does not change that (Fig. 4), resulting in similar IA/AR as presented in Table 1.

Remote preconditioning by MAO resulted in pronounced cardiac protection, as indicated by the position of the points with respect to the regression line of control hearts in Fig. 4. This cardiac protection was completely abolished by HOE-140 (Fig 4). Accordingly, IA/AR was significantly lower after remote preconditioning and normalized by HOE-140 (Table 1).

Classic preconditioning by CAO resulted in similar protection as remote preconditioning and was also completely abolished by HOE-140. This was substantiated by the IA/AR (see Table 1).

BK stimulation. Saline infusion in the mesenteric vascular bed did not affect MAP, HR, or infarct ratio, and was similar to the control group of *protocol 1*. Therefore, data of intramesenteric BK infusion were compared with the control data of *protocol 1*. Although

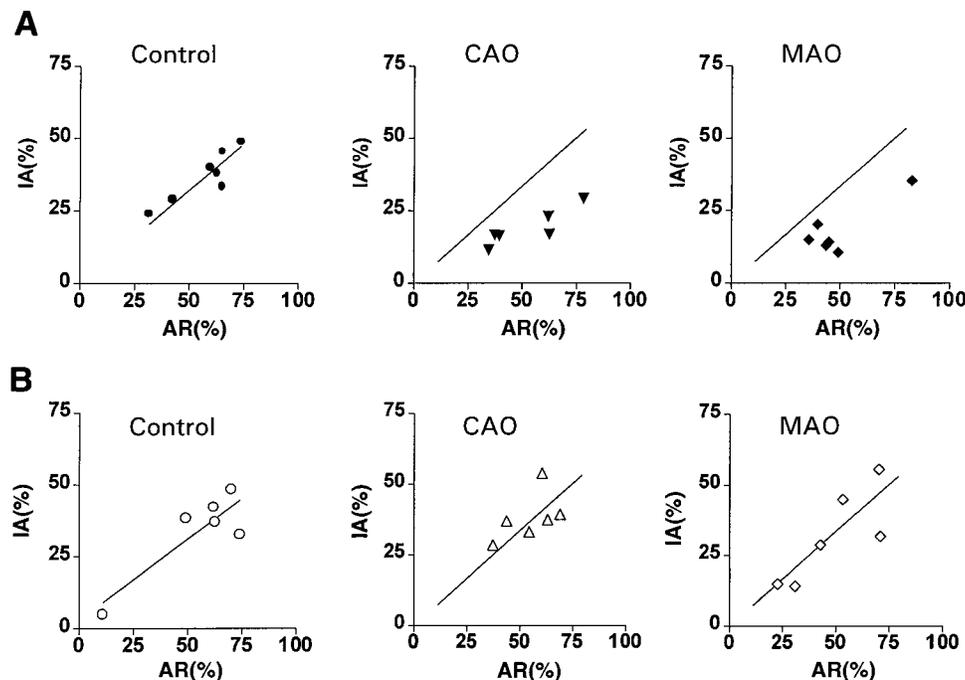


Fig. 4. Infarct area (IA) plotted against area at risk (AR) as percentage of left ventricular weight for different protocols. Regression line for controls is depicted in left panel of A. Because HOE-140 did not change regression line for controls in remaining panels, combined regression line for controls is displayed. A: without HOE-140 treatment. B: with HOE-140 treatment.

Table 1. IA-to-AR ratio for different protocols

	<i>n</i>	AR, %LV	IA/AR
Control	8	60 ± 5	62 ± 5
Control + HOE-140	6	50 ± 9	58 ± 6
CAO	6	52 ± 7	37 ± 3*
CAO + HOE-140	6	55 ± 5	71 ± 6†
MAO	6	52 ± 7	35 ± 5*
MAO + HOE-140	6	49 ± 8	65 ± 7†
Saline	3	53 ± 14	52 ± 4
Bradykinin	7	55 ± 5	26 ± 3*
Bradykinin + Hex	6	50 ± 5	67 ± 4†

Values are means ± SE; *n* = no. of rats studied. AR, area at risk; IA, infarct area; CAO, classic preconditioning by coronary artery occlusion; MAO, remote preconditioning by mesenteric artery occlusion; HOE-140, Hoechst 140 pretreatment; Hex, hexamethonium pretreatment. *Significant effect compared with control. †Significant effect of HOE-140 or Hex pretreatment on respective cardiac protection.

transiently BK infusion caused a slight increase in HR and MAP, steady-state hemodynamics after 5 min of infusion were not significantly altered. Pretreatment with hexamethonium caused a significant reduction in HR and MAP, which was not further altered by BK infusion (Fig. 5).

Whereas intramesenteric infusion of saline had no effect on infarction of the heart, 5 min of intramesenteric BK infusion, followed by 5 min of rest before the 60-min CAO resulted in significant cardiac protection (Table 1). Protection was abolished by pretreatment with the ganglion blocker hexamethonium.

DISCUSSION

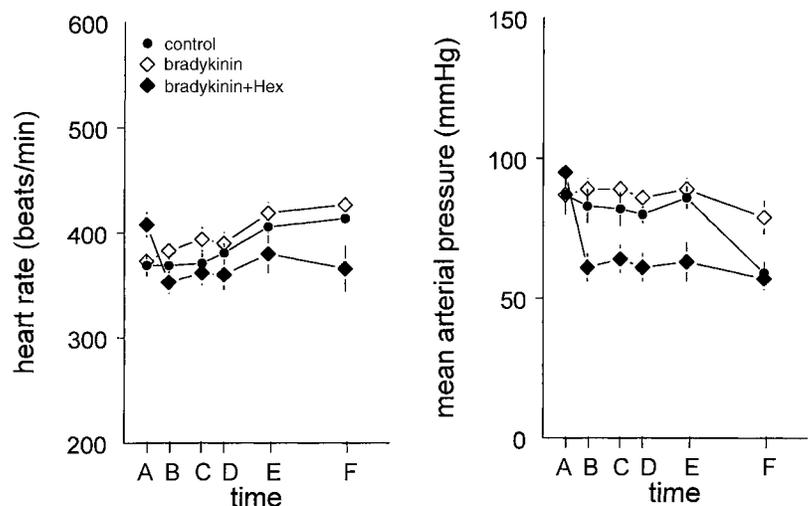
In the present study we investigated the role of BK in the remote cardiac preconditioning by mesenteric artery occlusion-reperfusion. The working hypothesis was that BK that is locally released during ischemia-reperfusion in the mesenteric bed stimulates sensory nerves projecting on efferent nerves to the heart. The hypothesis is supported by our findings that 1) HOE-140, an antagonist for the B₂ receptor involved in sensory nerve stimulation, completely abolished the cardiac protection by mesenteric ischemia-reperfusion; 2) intramesenteric BK, at a dose that stimulates sen-

sory nerves without systemic effects, could mimic cardiac protection by mesenteric ischemia-reperfusion; and 3) the protective effects of intramesenteric BK can be completely blocked by the ganglion blocker hexamethonium.

BK blockade. The B₂ receptor is reported to be the receptor involved in vasodilation as well as sensory nerve stimulation (9) and can be antagonized by HOE-140. In pentobarbital sodium-anesthetized rats, intravenous BK induces a maximal decrease in MAP of ~25 mmHg at a dose of 10 µg/kg (pilot studies). In the present study 4 µg/kg were used as a submaximal dose to examine the time course of BK response inhibition after 300 µg/kg HOE-140 (19). HOE-140 was capable of blocking the response to BK completely for at least 90 min, which meant that the preconditioning protocols were performed under B₂ blockade up until at least the 60-min CAO. The effect of HOE-140 is waning during the 180-min reperfusion period.

Pretreatment with HOE-140 had no effect on infarct size of non-preconditioned rats. This is in general accordance with earlier findings in other studies (2, 4). However, HOE-140 completely abolished the cardiac protection of the remote (by MAO) as well as the classic (by CAO) preconditioning. Attenuation of classic preconditioning by HOE-140 has been reported before but appeared to occur *in vivo* (4, 21) rather than *in vitro* (2, 18). Moreover, cardiac protection could be obtained by intracoronary BK infusion *in vitro* (2, 18) as well as *in vivo* (21). In our previous study (3), in contrast to remote preconditioning, classic preconditioning could not be inhibited by hexamethonium nor had hexamethonium itself cardioprotective effects. Thus although sensory nerves may be present on the ventricle, which could induce efferent nerve stimulation to the heart, results suggest a local effect of BK on the heart rather than the aforementioned neurogenic pathway. This would be in accordance with the study of Goto et al. (4), who suggested that blood-borne kininogens rather than autonomic nerves would be involved in classic preconditioning.

Fig. 5. Heart rate and blood pressure at different time points in protocols described in Fig. 2: A, after 30 min of stabilization; B, 10 min after injection of saline or hexamethonium; C, at end of 5-min intramesenteric saline or BK infusion; D, just before 60-min CAO; E, at end of 60-min CAO; F, at end of 3-h coronary reperfusion.



In contrast, remote preconditioning could well be associated with stimulation of a neurogenic pathway, because cardiac protection can be attenuated through ganglion blockade by hexamethonium (3). However, similar to classic preconditioning, pretreatment with HOE-140 completely abolished the cardiac protection of 15 min of MAO. This result indicates that endogenous BK has an important role in the neurogenic pathway of remote preconditioning.

Locally infused BK in the mesenteric vascular bed could mimic remote cardiac preconditioning by reduction of myocardial infarct size. The calculated local concentration of BK (1 $\mu\text{g}/\text{min}$ BK into ± 15 ml/min mesenteric flow results in 67 $\mu\text{g}/\text{l} = 55$ nM) of 55 nM at intramesenteric infusion closely resembles the cardiac BK concentrations with classic preconditioning in pigs, from 30 nM at baseline to 60 nM during ischemia-reperfusion (17).

The protection could be prevented by ganglion blockade. Because we showed that intravenous injection of BK decreased blood pressure (as we used to determine duration of action of HOE-140) the slight increases of HR and MAP after intramesenteric BK infusion suggest a locally induced rather than a systemic effect. The cardiac protection observed after intramesenteric BK therefore could be attributed to an action of BK in the mesenteric bed rather than the well-known effects of circulating BK on the heart itself (2, 4, 18, 21).

The route of administration and the dose of local BK in the present study have been reported to stimulate afferent mesenteric nerves (7), but we cannot exclude effects of BK leaking into the systemic circulation. However, the observation that the cardioprotective effect of intramesenteric BK infusion could be prevented by ganglion blockade with hexamethonium suggests a neuronal rather than a circulating mechanism. This is supported by the observation that intramesenteric injection of capsaicin, at a dose that stimulates but does not desensitize sensory nerves (22), caused similar cardioprotection as BK (data not shown).

However, we cannot exclude one hypothetical mechanism for the remote preconditioning. Because BK also has been reported to have a role in sensory function in other organs and even up to the level of the spinal cord (11), which may include hexamethonium-sensitive pathways as well, intramesenteric infused BK could have leaked out to other organs, including the heart or the spinal cord, and have exerted its neuronal stimulation there.

In addition to a direct role of BK in sensory function (11), intramesenteric BK infusion caused a substantial reduction of mesenteric blood flow (7). Regarding the slightly increased MAP, this may result in $\pm 40\%$ increase in mesenteric vascular resistance at the dose of BK used (7). This by itself could cause mesenteric ischemia, which, as we have shown, may result in cardiac protection (present study and Ref. 3). Thus whether intramesenteric endogenous or exogenous BK directly stimulates afferent nerves or acts in association with mesenteric ischemia-reperfusion is not known.

The mechanism at the other end of the neurogenic pathway, that is on the heart, is not yet clear. Iwamoto and co-workers (6) indicate that the heart can be preconditioned by stimulation of the left stellate cardiac nerve, if simultaneously the increase in coronary blood flow was restricted. Moreover, cardiac preconditioning can be achieved by increasing endogenous catecholamine release (1). These studies indicate that under certain conditions the sympathetic nervous system may have a role in cardiac preconditioning. Moreover, two recent studies (14, 20) indicate that cardiac adenosine receptors may be involved in remote preconditioning by renal artery occlusion-reperfusion. Adenosine activates a pertussis toxin-sensitive inhibitory G protein, which can facilitate the activation of ATP-sensitive K^+ channels. Stimulation of α -adrenoceptors by endogenous catecholamines can exert preconditioning effects through the activation of protein kinase C via such a G protein (5). However, further investigation is needed for its role in remote preconditioning.

In conclusion, data from the present study showed evidence for an important role for BK in remote preconditioning. Results support the hypothesis that remote preconditioning could act through sensory nerve stimulation in the ischemic organ. The consecutive mechanism within the heart is not yet clear but may involve the endogenous release of catecholamines.

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