Sites of action of adenosine in interorgan preconditioning of the heart

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The mechanism underlying interorgan preconditioning of the heart remains elusive, although a role for adenosine and activation of a neurogenic pathway has been postulated. We tested in rats the hypothesis that adenosine released by the remote ischemic organ stimulates local afferent nerves, which leads to activation of myocardial adenosine receptors. Preconditioning with a 15-min mesenteric artery occlusion (MAO15) reduced infarct size produced by a 60-min coronary artery occlusion (60-min CAO) from 68 ± 2% to 48 ± 4% (P < 0.05). Pretreatment with the ganglion blocker hexamethonium at 5 min of reperfusion following MAO15 had no effect, 8-SPT at 5 min of reperfusion abolished the protection. Permanent reocclusion of the mesenteric artery before the 60-min CAO enhanced the cardioprotection by MAO15 (30 ± 5%), but all protection was abolished when 8-SPT was administered after reocclusion of the mesenteric artery. Together, these findings demonstrate the involvement of myocardial adenosine receptors. We therefore conclude that locally released adenosine during small intestinal ischemia stimulates afferent nerves in the mesenteric bed during early reperfusion, initiating a neurogenic pathway that leads to activation of myocardial adenosine receptors.

Adenosine receptors; myocardial infarction; neurogenic pathway; remote myocardial preconditioning; preconditioning at a distance; small intestine

Ischemic preconditioning is not organ specific because it has not only been demonstrated for the heart (17), but also for the kidneys, liver, brain, skeletal muscle, and lung (6). Przyklenk et al. (19) expanded the concept of ischemic preconditioning from intraregional to interregional myocardial protection by showing that a brief coronary artery occlusion (CAO) not only preconditioned the myocardium nourished by that coronary artery but also protected the adjacent virgin myocardium. Gho et al. (10) subsequently showed that 15 min of small intestinal or renal ischemia preceding a 60-min CAO by 10 min was also capable of limiting myocardial infarct size. This interorgan preconditioning (IOPC, remote myocardial preconditioning; preconditioning at a distance) of the heart by preceding transient ischemia in remote organs has been confirmed for the small intestine (24, 28), kidney (8, 18, 23), and skeletal muscle (1). However, not all remote organs may be able to protect the myocardium because De Zeeuw et al. (5) did not find any infarct size limitation when a 60-min CAO was preceded by global cerebral ischemia. Although not yet extensively studied, IOPC of the heart also appears to involve a delayed phase (24, 28).

The mechanism underlying classical ischemic myocardial preconditioning is still incompletely understood, but there is now consensus that it involves the release of a number of local mediators such as adenosine, norepinephrine, and bradykinin during the preconditioning stimulus, which, most likely via different signal transduction pathways (3), activate the mitochondrial ATP-sensitive K⁺ (KATP) channels (11). The mechanism underlying IOPC is less clear. Gho et al. (10) showed the involvement of a neurogenic pathway in IOPC by preceding small intestinal ischemia as pretreatment with the ganglion blocker hexamethonium abolished the cardioprotection. In that study it was also shown that reperfusion of the occluded mesenteric artery, responsible for small intestinal ischemia, was mandatory [an observation confirmed for the renal bed (23)]. The latter suggests that activation of the neurogenic pathway occurs upon reperfusion (10) or that reperfusion facilitates the transfer of hormonal preconditioning factors from the ischemic organ to the heart (7). Bradykinin (21) and adenosine (18, 23) may also be involved in IOPC of the heart, but the site(s) of action of these mediators, i.e., the ischemic organ or the heart or both, has not been investigated. Takaoka et al. (23) suggested that myocardial adenosine recep-
tors might be involved because the adenosine concentra-
tions in the carotid artery were 10 times higher after renal ischemia than after regional myocardial ischemia. However, based on the hypotension following renal artery reperfusion, Pell et al. (18) proposed that adenosine release from the kidney was insufficient to produce cardioprotection via the circulation. These in-
vestigators suggested, based on the observations by Gho et al. (10), that adenosine released in the ischemic kidney stimulated the afferent renal nerves and thereby protected the myocardium. The latter hypoth-
esis was recently confirmed by Ding et al. (8) in anes-
thetized rabbits.

To further elucidate the mechanism of IOPC, we not only investigated whether activation of adenosine recep-
tors (and which of its subtypes) is involved in the protection by small intestinal ischemia, but we also determined the location(s) (myocardium and/or small intestine) of the adenosine receptors involved. Studies were performed in anesthetized rats. Until recently, it has been assumed that adenosine is not involved in ischemic preconditioning in the rat (9, 14), despite its capability to limit infarct size in this species (27). We have recently shown, however, that adenosine is in-
volved in classical ischemic preconditioning in the rat, but that its role depends critically on the duration of the preconditioning stimulus (15). Capitalizing on our earlier observation that hexamethonium abolished the cardioprotection by small intestinal ischemia (10), we hypothesized that adenosine locally released during small intestinal ischemia stimulates afferent nerves within the mesenteric bed, which via a neurogenic pathway leads to activation of myocardial adeno-
sine receptors before the sustained CAO, thereby pre-
conditioning the heart. This hypothesis also implies that once the myocardial adenosine receptors have been activated, blockade of the neurogenic pathway will not abolish the cardioprotection by small intestinal ischemia.

METHODS

Experiments were performed in 228 ad libitum-fed male Wistar rats (280–360 g) in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Publication 86-23, Revised 1996) and with approval of the Erasmus University Rotterdam Animal Care Committee.

Experimental Procedures

Pentobarbital-anesthetized (60 mg/kg ip) rats were intu-
bated (polyethylene, PE-240) for positive pressure ventila-
tion (Harvard rodent ventilator) with room air (10, 15, 26). The thoracic aorta was cannulated via the carotid artery with a PE-50 catheter for measurement of arterial blood pressure and computation of heart rate. A catheter was positioned in the femoral vein for infusion of Haemaccel (Behringwerke) to maintain fluid balance. After thoracotomy via the left third intercostal space and opening of the pericardium, a silk 6-0 suture was looped under the coronary artery for later occlu-
sion. After laparotomy, the superior mesenteric artery was dissected free and looped by a loose suture to allow later mesenteric artery occlusion (MAO) with an atraumatic clamp. Pentobarbital was infused in the abdominal cavity to maintain anesthesia. Rectal temperature was continuously measured with an electronic thermometer (Electromedics) and was maintained at 36.5–37.5°C (10, 15, 26).

Rats that fibrillated during occlusion or reperfusion were allowed to complete the protocol when conversion to normal sinus rhythm occurred spontaneously within 1 min or when resuscitation by gently thumping on the thorax or defibrilla-
tion with a modified battery of 9 V was successful within 2 min after onset of fibrillation. Occlusion and reperfusion were visually verified by appearance and disappearance of myocardial cyanosis.

Experimental Design

After surgery, a 30-min stabilization period was allowed before the start of the experimental protocol. All animals were subjected to a 60-min CAO followed by 120 min of reperfusion, after which the area at risk (AR) and infarct area (IA) were determined using trypan blue and nitro-blue tetrazolium staining (10, 15, 26). Infarct size (IS) was defined as 100 × IAR/AR (%). Protocol I: Choice of IOPC stimulus. Using the identical protocol of Li and Kloner (14), we recently observed (15) that three cycles of 3-min CAO interspersed by 5 min of reperfu-
sion (3-CAO3) provided a greater degree of myocardial pro-
tection than a 15-min CAO (CAO15, Fig. 1). Therefore, we first investigated the cardioprotection afforded by IOPC elic-
ited by three cycles of 3-min MAO interspersed by 5 min of reperfusion (3-MAO3, group 1) and compared this with the protection afforded by a single 15-min MAO followed by 10 min of reperfusion [MAO15, historic data (10)]. Because 3-MAO3 failed to afford cardioprotection (see RESULTS), we selected MAO15 as the IOPC stimulus.

Protocol II: Involvement of adenosine receptor stimulation in IOPC. To investigate the role of adenosine in IOPC, four groups of rats were studied (Fig. 1). The effect of a single MAO15 on infarct size by 60-min CAO was determined in the absence (Sham, group 2; MAO15, group 3) and presence (50SPT + Sham, group 4; 50SPT + MAO15, group 5) of a nonselective dose (2 × 25 mg/kg iv, 50SPT) of the adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-SPT). Because we established the involvement of adenosine in this model of IOPC (see RESULTS), we further investigated whether the A1- and A2-receptor subtypes are involved. For this pur-
pose, Sham and IOPC animals were pretreated with either an A1-selective dose (2 × 5 mg/kg iv) of 8-SPT (10SPT, groups 6 and 7) or an A2-selective dose (3.3 mg/kg iv) of the adeno-
sine receptor antagonist MRS 1191 (MRS, groups 8 and 9) (15). Finally, IOPC animals were pretreated with a combina-
tion of 10SPT and MRS (group 10).

Protocol III: Effect of small intestinal adenosine receptor stimulation on myocardial infarct size. To investigate whether activation of adenosine receptors in the small intesti-
tine contributes to IOPC, we employed intramesenteric ar-
tery infusions of vehicle (50 al/min, saline MA, group 11) or adenosine (10 μg/min, ADO MA, group 12). This dose of adenosine has been shown to stimulate intestinal afferent nerves (12). The involvement of the neurogenic pathway in the cardioprotection by intramesenteric adenosine was veri-
fied by pretreating animals with hexamethonium (20 mg/kg iv, Hex + ADO MA, group 13). To exclude that the intra-
mesenteric artery infusion of adenosine protected the myo-
cardium by direct stimulation of myocardial adenosine re-
cptors (after recirculation), we also infused this dose of adenosine intravenously (ADO IV, group 14). Finally, to exclude that stimulation of the afferent nerves in the liver contributed to the cardioprotection, we also studied the effect

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of infusion of 10 μg/min adenosine into the portal vein (ADO IPV, group 15).

Protocol IV: Involvement of myocardial adenosine receptor stimulation in IOPC. It is well established that adenosine receptor blockade between the preconditioning stimulus and the sustained CAO abolishes the cardioprotection in ischemic myocardial preconditioning (25). We therefore followed two approaches to study the involvement of myocardial adenosine receptors in IOPC (Fig. 2). First, we established that the neurogenic pathway (required for IOPC of the heart; Hex / MAO15, see Ref. 10) was no longer required for IOPC after 5 min of mesenteric artery reperfusion (MAO15 / MAO-P, group 18), indicating that the neurogenic pathway had already activated cardiac adenosine receptors. Subsequently, we determined the role of myocardial adenosine receptors by administering 50SPT at 5 min of mesenteric artery reperfusion (MAO15 + 50SPT, group 17). The second approach involved reocclusion of the mesenteric artery 5 min before the 60-min CAO to prevent the subsequently administered 8-SPT would reach the small intestine. To exclude that 8-SPT would reach the small intestine via collaterals, we also occluded in a number of animals the collaterals in the mesenteric vascular bed (16). We first confirmed that a permanent MAO (MAO-P, group 18) was not cardioprotective (10) and then studied IOPC produced by MAO15 + MAO-P (group 19). These experiments revealed a potentiation of the cardioprotection by MAO15 + MAO-P (see RESULTS). To establish whether this potentiated cardioprotection also involved the neurogenic pathway, we determined the effects of treatment with hexamethonium administered before MAO15 (Hex + MAO15 + MAO-P, group 20) and more importantly when administered after 5 min of mesenteric artery reperfusion just before
MAO-P (MAO15 + Hex + MAO-P, group 21). We then studied the effect of 8-SPT administered after reocclusion of the mesenteric artery (MAO15 + MAO-P + 50SPT, group 22) to obtain further evidence for the involvement of myocardial adenosine receptor activation in IOPC. Finally, we studied the role of myocardial A1 (MAO15 + MAO-P + MRS, group 24) and A3 (MAO15 + MAO-P + MRS, group 24) adenosine receptors in IOPC.

Small Intestinal Collateral Blood Flow

Megison et al. (16) reported that after MAO in rats, small intestinal collateral blood flow amounted 17 ± 6% of basal flow and after subsequent occlusion of collateral blood vessels amounted 2 ± 1% of basal flow. To determine small intestinal blood flow in our model, radioactive microspheres were injected in four additional rats at baseline, after MAO and after additional occlusion of collateral vessels [i.e., arcades between right colic artery and ileocolic artery and between jejunal branches just proximal and distal to the point of the superior mesenteric artery occlusion (16)].

Data Analysis and Presentation

Infarct size was analyzed by one-way analysis of variance for all groups followed by one-way analysis of variance within each protocol and Student-Newman-Keuls test. Hemodynamic variables were compared by two-way (time and treatment) analysis of variance for repeated measures followed by the paired or unpaired t-test with Bonferroni correction for multiple comparisons. Statistical significance was accepted when $P < 0.05$. Data are means ± SE.

RESULTS

Mortality

Of the 224 rats that entered the IOPC study, 30 animals were excluded because of sustained ventricular fibrillation during the 60-min CAO or cardiac pump failure. Because the excluded rats were equally distributed over the various groups, the exclusion did not cause a bias toward any intervention. In 13 animals the area at risk was <15% of total left ventricular mass and were therefore also excluded. Data are presented for 181 animals.

Area At Risk

There were no differences between the area at risk of the experimental groups (30.5 ± 0.7%, $n = 181$, $P = 0.16$).

Infarct Size

Protocol I: Choice of IOPC stimulus. Figure 3 shows that MAO15 and CAO15 afford similar cardioprotec-
tion. However, in contrast to 3-CAO3, 3-MAO3 failed to protect the myocardium. Consequently, MAO15 was selected as the stimulus for IOPC.

Protocol II: Involvement of adenosine receptor stimulation in IOPC. Pretreatment with a nonselective dose of 8-SPT (50SPT) abolished the cardioprotection by MAO15 (Fig. 4). The A1-selective dose of 8-SPT (10SPT) and the A3-selective dose of MRS-1191 each attenuated the cardioprotection by MAO15 by 65%. Combined pretreatment with the A1- and A3-selective doses of these antagonists (10SPT + MRS) abolished the cardioprotection.

Protocol III: Effect of small intestinal adenosine receptor stimulation on myocardial infarct size. Intramesenteric artery infusion of 10 μg/min adenosine (ADO IMA), but not of its vehicle, limited infarct size (47 ± 4%) to the same extent as MAO15 (Figs. 4 and 5). Pretreatment with hexamethonium abolished the cardioprotection by ADO IMA (Hex + ADO IMA), implying involvement of a neurogenic pathway in the cardioprotection by locally administered adenosine. This is also confirmed by the lack of effect of intravenous adenosine infusion (ADO IV), excluding that recirculation of adenosine during ADO IMA does not contribute to the protection by ADO IMA, because adenosine infusion into the portal vein (ADO IPV) was ineffective. *P < 0.05 vs. Sham; †P < 0.05 vs. MAO15. See text for details and abbreviations.
Protocol IV: Involvement of myocardial adenosine receptor stimulation in IOPC. Figure 6 shows that whereas pretreatment with hexamethonium (MAO15 + Hex) abolished IOPC (infarct size 74 ± 2%), hexamethonium did not abrogate IOPC when administered at 5 min of mesenteric artery reperfusion (MAO15 + Hex, infarct size 54 ± 2%). In contrast, both pretreatment (50SPT + MAO15, infarct size 74 ± 1%) and treatment at 5 min of mesenteric reperfusion with 8-SPT (MAO15 + 50SPT, infarct size 67 ± 3%) abolished IOPC. Figure 6 also confirms that permanent occlusion of the MAO (MAO-P) did not confer significant cardioprotection (infarct size 63 ± 2%). However, MAO-P potentiated the cardioprotection by MAO15 because infarct size was only 30 ± 5% after MAO15 + MAO-P versus 49 ± 3% after MAO15 (P < 0.05). This enhanced protection was also mediated via a neurogenic pathway as 1) pretreatment with hexamethonium (Hex + MAO15 + MAO-P) completely abrogated all cardioprotection and 2) posttreatment selectively abolished the protection leaving the protection by MAO15 unperturbed (MAO15 + MAO-P + Hex). Administration of 8-SPT after the mesenteric artery was reoccluded following MAO15 (MAO15 + MAO-P + 50SPT) entirely abolished the protection. In several of these experiments, the collaterals of the mesenteric vascular bed were also occluded to minimize the adenosine antagonist from entering the mesenteric bed via these collaterals and blocking the adenosine receptors in the small intestine. A1- and A3-receptor blockade blunted the cardioprotection by MAO15 because infarct size was 61 ± 2% after 10SPT and 53 ± 3% after MRS, respectively.

Heart Rate and Blood Pressure

Baseline heart rate and mean arterial blood pressure for all 181 animals were 381 ± 3 beats/min and 93 ± 2 mmHg, respectively, with minimal differences between the experimental groups with the exception of 50SPT + MAO15, in which baseline mean arterial blood pressure was 135 ± 4 mmHg. Similar to earlier reports (26), there was no correlation between the product of heart rate and mean aortic pressure of the individual animals at the onset of the 60-min CAO and their infarct size (linear regression: r² = 0.01; P = 0.88).

Small Intestinal Collateral Blood Flow

After occlusion of the mesenteric artery, small intestinal blood flow was reduced to 5 ± 2% (range 0–10%) of baseline. Subsequent ligation of the collateral ves-
sels further reduced flow to 0.7 ± 0.7% (range 0–2%) of baseline, confirming previous observations (16).

DISCUSSION

The major findings of the present study are the following. First, in contrast to classical ischemic myocardial preconditioning in which 3-CAO3 provided greater cardioprotection than CAO15, 3-MAO3 was unable to limit myocardial infarct size. Second, pretreatment with a nonselective dose of the adenosine receptor antagonist 8-SPT abolished the cardioprotection by MAO15, whereas pretreatment with an A1-selective dose of 8-SPT or the A3-selective antagonist MRS-1191 attenuated cardioprotection by MAO15. Third, intramesenteric artery infusion of a dose of adenosine, which was ineffective when infused into either the portal or inferior caval vein, mimics the IOPC by MAO15, which was abolished by pretreatment with hexamethonium. Fourth, hexamethonium abolished IOPC by MAO15 but only when administered before the MAO and not when administered after 5-min of mesenteric artery reperfusion. In contrast, 8-SPT abolished IOPC also when administered after 5-min of mesenteric artery reperfusion. Fifth, whereas a permanent MAO was not cardioprotective by itself, it potentiated the protection by MAO15; this enhanced protection was also abolished by ganglion blockade. Finally, after the myocardium was preconditioned by MAO15, administration of 8-SPT, at a time point that the mesenteric artery was permanently reoccluded to prevent 8-SPT to reach the small intestine, abolished all cardioprotection by IOPC.

IOPC Stimulus

In our original study (10), MAO15 was as effective as CAO15 in limiting myocardial infarct size produced by a subsequent 60-min CAO. Because 3-CAO3 afforded greater protection than CAO15 (15), we investigated whether 3-MAO3 was also more effective than MAO15 in eliciting cardioprotection. Our data showed that these multiple brief MAOs were unable to precondition the myocardium. In this respect, it is of interest that Tang et al. (24) recently showed that a single 10-min episode of small intestinal ischemia was equally effective in producing early and delayed (24–72 h) IOPC. The latter could also be elicited by six cycles of 4-min small intestinal ischemia and 4 min of reperfusion (28). Although in this latter study (28), only the delayed preconditioning phase was investigated, it cannot be excluded that a larger number and/or longer duration than the 3-min periods of small intestinal ischemia used in the present study might have provided an effective stimulus for IOPC.

That MAO15 does not yet provide optimal protection was shown when we reoccluded the mesenteric artery permanently after the MAO15. Thus, whereas MAO-P alone was not cardioprotective, we observed that MAO-P potentiated the cardioprotection by MAO15. The reason for the activation of the neurogenic pathway during MAO-P by the preceding of MAO15 is not clear. However, it is likely that the MAO15 not only preconditioned the heart but also preconditioned the small intestine itself possibly via adenosine, calcitonin gene-related peptide, or endogenous opioids (2, 4). It may then be postulated that, whereas the virgin small intestine is unable to activate the neurogenic pathway during MAO and requires reperfusion (10), the preconditioned small intestine is capable of activating the neurogenic pathway during occlusion and thereby enhances the protection by the MAO15. Future studies involving measurement of interstitial adenosine concentrations in the small intestine and discharge rate of the small intestinal afferent nerves are needed to test this hypothesis.

Involvement of Adenosine Receptor Stimulation

The cardioprotection by small intestinal ischemia was completely prevented by pretreating rats with a high nonselective dose of 8-SPT (50 mg/kg), implying that adenosine is at least one of the mediators leading to cardioprotection in this model of IOPC. The present study also reveals that, at least with the currently used stimulus, both the A1- and A3-receptor subtypes contribute. A role for adenosine has also been suggested for IOPC by renal ischemia (8, 18, 23), but in none of these studies the site of location or the subtype of the involved adenosine receptors was investigated.

Myocardial adenosine receptors. Because in protocol II the adenosine receptor antagonists were administered intravenously and before both MAO15 and 60-min CAO, the data in Fig. 4 do not reveal the site of action of adenosine. To investigate whether the myocardial adenosine receptors were involved, we administered 8-SPT 1) after 5 min of mesenteric artery reperfusion at a time when the cardioprotective mechanism no longer required continued activation of the neurogenic pathway, and 2) after the mesenteric artery had been reoccluded following MAO15 to prevent 8-SPT from reaching the small intestine. In several animals, we additionally ligated the collaterals in the mesenteric vascular bed (16) to exclude that 8-SPT would still reach the small intestine via these vessels during MAO-P in sufficient amounts to prevent activation of the neurogenic pathway. In this situation (MAO15 + MAO-P + 50SPT), 8-SPT abolished all cardioprotection by IOPC (and not only the potentation by MAO-P), implying that the myocardial adenosine receptors must be involved in the mechanism underlying this phenomenon. The myocardial adenosine receptors involved are of both the A1- and A3-subtypes. This observation is in agreement with earlier studies in which a role for both receptor subtypes was established when CAO15 was used to precondition the myocardium (15).

Small intestinal adenosine receptors. Intramesenteric infusion of 10 μg/min of adenosine limited myocardial infarct size to the same extent as MAO15. Moreover, because infusion of the same dose of adenosine in the portal vein or inferior caval vein did not protect the myocardium, adenosine must have acted in the small intestine. The action of locally administered
Adenosine was abolished when rats were pretreated with hexamethonium, which implies that the neurogenic pathway was also involved in this model of pharmacological IOPC. The intramesenteric artery infusion rate of adenosine was chosen such that only the adenosine receptors in the small intestine were activated. However, we do not know how the adenosine concentrations in the small intestine achieved by this route of administration compare with the adenosine concentrations that are achieved during MAO15. It cannot be excluded that in the portal vein higher concentrations were reached upon reperfusion of the ischemic small intestine than during the direct infusion of adenosine into the portal vein. If true, these higher concentrations might have been sufficient to stimulate afferent nerves in the liver and thereby have contributed to the cardioprotection by small intestine ischemia. That the concentrations during intramesenteric adenosine infusion and MAO15 were similar is suggested by the observation that the protection by the intramesenteric artery infusion of adenosine was very similar to the protection by MAO15.

To definitively demonstrate the involvement of adenosine receptor stimulation in the small intestine in IOPC requires blockade of these receptors, while leaving the myocardial receptors unaffected. Because of the half-life of the antagonists used, which would result in recirculation and concomitant adenosine receptor blockade in the heart, this is technically not feasible. Nevertheless, the present observations are consistent with the hypothesis that in IOPC by small intestinal ischemia, locally released adenosine triggers afferent nerves which in turn lead to stimulation of myocardial adenosine receptors.

The findings in the present study do not exclude that mediators other than adenosine may also contribute to IOPC similar to classical ischemic myocardial preconditioning. For example, Schoemaker and Van Heijningen (21) showed, without determining the site(s) of action, that the cardioprotection by transient small intestinal ischemia could be mimicked by intramesenteric artery infusion of bradykinin and abolished by pretreatment with the bradykinin antagonist HOE-140. Similarly, a role for calcitonin gene-related peptide, of which the release can be modulated by adenosine (22), has been implicated in the cardioprotection in the rat heart. Cardioprotection in pigs by exogenous norepinephrine but not by cerebral ischemia-induced release of endogenous norepinephrine. Stroke 28: 767–774, 1991.


