Intestinal ischemia induces late preconditioning against myocardial infarction: a role for inducible nitric oxide synthase

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Abstract

Objective: We tested the hypothesis that occlusion of the superior mesenteric artery induces late preconditioning against myocardial infarction and examined the effects of pharmacological modifiers of inducible nitric oxide synthase activity on the late preconditioning in anesthetized rats.

Methods: Rats underwent an intestinal ischemia preconditioning protocol (30 min occlusion of the superior mesenteric artery) or were sham-operated. They were subjected to a sustained 30 min of coronary occlusion and 180 min of reperfusion 24 h later.

Results: In rats receiving no pharmacological intervention, the percentage of myocardial infarct within the area at risk and left ventricle was 72 ± 4% and 31 ± 2%, respectively, in sham-operated rats, and these were significantly reduced to 44 ± 4% and 23 ± 2% (P < 0.01) 24 h after intestinal ischemia preconditioning. Myeloperoxidase activity was significantly reduced by intestinal ischemia preconditioning. Administration of aminoguanidine (300 mg/kg, s.c.) or S-methylisothiourea sulfate (3 mg/kg, i.v.), both relative inducible NO synthase inhibitors, 60 or 30 min before sustained myocardial ischemia not only abolished the late preconditioning afforded by intestinal ischemia, but also inhibited the ability of intestinal ischemia preconditioning to significantly reduce neutrophil infiltration. A change in inducible NO synthase activity was not observed in normal myocardium 24 h after intestinal ischemia, but 30 min of coronary occlusion significantly increased the inducible NO synthase activity in the preconditioned group, which was abolished by aminoguanidine or S-methylisothiourea sulfate.

Conclusions: These data provide pharmacological evidence that induction of inducible nitric oxide synthase, following intestinal ischemia, is associated with increased myocardial tolerance to infarction 24 h later. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Infarction; Ischemia; Nitric oxide; Preconditioning; Reperfusion

1. Introduction

The preconditioning phenomenon was first described in 1986 by Murry et al. [1], who showed that myocardial infarct size was paradoxically reduced if a sustained myocardial ischemic period was preceded by a brief myocardial ischemic episode. Since then, numerous studies have shown that ischemic preconditioning elicits an early and a late phase of protection against myocardial ischemic injury [1–3]. Furthermore, McClanahan et al. [4] and Gho et al. [5] have shown that a brief ischemic period of the kidney or intestine followed by reperfusion limits myocardial infarct size. The phenomenon is termed ‘interorgan’ or ‘remote organ preconditioning of the myocardium’.

Recent studies have demonstrated that the protective effects of late preconditioning against myocardial stunning and myocardial infarction are mediated by the inducible nitric oxide synthase (iNOS) [6,7]. It has been well established that a variety of mediators are released in the venous effluent or activated following intestinal ischemia–reperfusion, including reactive oxygen metabolites and cytokine [8,9], which could, in themselves, induce iNOS [10].

We therefore tested the hypothesis that intestinal ischemia results in late preconditioning against myocardial infarction through the enhanced production of nitric oxide synthase...
(NO). To investigate whether iNOS contributes to the protective effects of late preconditioning, we examined the effects of the relatively selective iNOS inhibitor amino-guanidine (AG) or S-methylisothiourea sulfate (SMT) [11,12], given before sustained myocardial ischemia, on the late preconditioning.

2. Methods

This study was conducted in accordance with the committee on animals of Kagawa Medical University and conforms with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health; NIH Publication No. 85-23, revised 1996).

2.1. Surgical preparation and experimental protocol

Adult male Sprague–Dawley rats were fasted 12 h prior to the abdominal surgery, but given free access to water. Rats were anesthetized with 1–2% halothane delivered via a face mask in oxygen-enhanced air. All procedures were performed using aseptic technique with the rats breathing spontaneously. A polyethylene catheter (PE-10 fused to PE-50) was inserted into the abdominal aorta via the femoral artery for measurement of arterial pressure, heart rate (HR), and a second catheter was inserted into the vena cava via the femoral vein for administration of drugs.

Through a midline abdominal incision, the superior mesenteric artery (SMA) was isolated meticulously at its origin. The rats were given 200 I.U. heparin intravenously. The SMA was occluded at its origin for 30 min and reopened. The abdominal incision was closed. Sham rats were treated identically, omitting the SMA occlusion. Catheters filled with heparin were exteriorized at the base of the neck, and the incision was closed. All rats were closely monitored after recovering from anesthesia, and given free access to food and water.

After 24 h, the rats were reanesthetized with pentobarbi- tal (60 mg/kg, i.p.) and the anesthesia was maintained by administration of pentobarbital intravenously when required. A tracheotomy was performed, and the animals were mechanically ventilated with oxygen-supplemented room air. Arterial blood gas parameters were maintained within a normal physiological range. The body core temperature was maintained at 36.5–37.5°C using a heating pad. A thoracotomy was performed in the left fourth intercostal space. A 6-0 silk suture was passed loosely around the left anterior descending coronary artery 2–3 mm distal from its point of origin. Once the hemodynamics had stabilized, heparin (200 I.U., i.v.) was given and a coronary occlusion was performed by tightening the ligature for 30 min. The occlusion was verified by regional cyanosis of the myocardial surface distal to the suture, accompanied by changes in arterial pressure. The ligature was untied after 30 min, and reperfusion was confirmed by visualizing an epicardial hyperemia. The ischemic myocardium was reperfused for 3 h.

For infarct size analysis, the rats were randomly divided into the following groups (Fig. 1): Group I (Sham group) underwent the coronary occlusion–reperfusion with no intestinal ischemia and no drug treatment. Group II (PC group) underwent intestinal ischemia 24 h before the coronary occlusion with no drug treatment. Group III (PC+AG) underwent the same protocol as group II except that the rats received a subcutaneous injection of AG (300

![Fig. 1. Experimental protocol. On day 2, all groups underwent a 30-min coronary occlusion followed by 180-min of reperfusion. Group I, sham period corresponding to intestinal ischemia preconditioning (Sham); group II, intestinal ischemia preconditioning (PC); group III, PC+AG; group IV, Sham+AG; group V, PC+SMT; group VI, Sham+SMT. AG, aminoguanidine; SMT, S-methylisothiourea sulfate.](image-url)
mg/kg, Sigma, St. Louis, MO, USA) 1 h before the coronary occlusion [7]. Group IV (Sham+AG) underwent the same protocol as group III except that the rats did not undergo intestinal ischemia. Group V (PC+SMT) underwent the same protocol as group II except that the rats received an intravenous injection of SMT sulfate (3 mg/kg, Sigma) 30 min before the coronary occlusion [12]. Group VI (Sham+SMT) underwent the same protocol as group V except that the rats did not undergo intestinal ischemia. AG hydrochloride or SMT sulfate was dissolved in normal saline, and the pH of the solution was adjusted to 7.4 with 0.1 N NaOH.

Myocardial tissue myeloperoxidase (MPO) activity was analyzed in Group I (Sham group), Group II (PC group), Group III (PC+AG), and Group IV (PC+SMT).

Myocardial iNOS activity was analyzed in additional Group I (Sham group), Group II (PC group), Group III (PC+AG), Group IV (PC+SMT). To exclude the effect of the difference in infarct size on the iNOS activity, we occluded the coronary artery for 30 min and did not recover perfusion. In the preliminary studies, no differences in infarct size were observed between any groups after only 30 min of coronary occlusion.

2.2. Infarct size analysis

After 180 min of reperfusion, an additional injection of heparin (200 I.U.) was administered. The ligature about the coronary artery was retightened, and 1 ml of 5% Evans blue was given intravenously to distinguish the non-ischemic area from the area at risk (RA). The heart was then excised, and the atria, great vessels, and right ventricle were dissected at 0°C. The left ventricle (LV) was weighed and cut into slices of 2 mm perpendicular to the base–apex axis. Each slice of the LV was divided into the RA and the remaining LV, and the different areas of the LV were weighed separately. The RA was then incubated for 15–20 min at 37°C in 1% triphenyltetrazolium chloride in phosphate-buffered solution (pH 7.4). Noninfarcted myocardium stained brick red, whereas the infarcted area (IA) remained unstained. The slices were fixed in a 10% formaldehyde solution for 24 h to distinguish clearly between stained viable tissue and unstained necrotic tissue. Each slice was photographed, magnified, and projected onto a screen. A planimeter was used for measurement of IA (uncolored) and RA (uncolored plus brick red). The results are expressed as the percentage of the IA to the RA or the total LV, and the percentage of the RA to the total LV.

2.3. Myocardial tissue MPO activity

After 180 min of reperfusion, the RA and the remaining LV were rapidly isolated at 0°C, weighed, and stored (−80°C). The myocardial MPO activity was determined as described previously [13]. Briefly, frozen myocardium of RA was homogenized at 0°C in 3 volumes buffer containing 50 mmol/l potassium phosphate, pH 6.0, 0.5% hexadecyltrimethyl ammonium bromide (Sigma), with three 10-s bursts in a polytron homogenizer at 10 000 rpm. The homogenate was freeze–thawed three times and sonicated twice to disrupt the cells, and centrifuged at 36 000 g for 15 min at 4°C. The MPO activity in the supernatant was assayed by measuring the change in absorbance (460 nm) resulting from decomposition of H₂O₂ in the presence of o-dianisidine dihydrochloride (Sigma).

The protein concentration was determined with Bio-Rad protein assay solution using bovine serum albumin as a standard.

2.4. Myocardial iNOS activity

After 30 min of coronary occlusion, the RA and the non-ischemic area were rapidly isolated at 0°C, weighed, and stored at −80°C for assay of iNOS activity. The enzyme activity was assayed by measuring the conversion of L-[¹⁴C]arginine to L-[¹⁴C]citrulline as described by Salter et al. [14]. A tissue sample was homogenized (8% w/v) in the appropriate Tris–HCl buffer (50 mmol/l, pH 7.4). The supernatant was passed over a column of Dowex AG-50 WX8 (Na+ form) to remove endogenous arginine, and the eluate was used for the assay of the iNOS activity and the protein concentration determined as described above. The eluate was incubated in a reactive mixture of 50 mmol/l (pH 7.4) N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) containing 1 mmol/l nicotinamide adenine dinucleotide phosphate (NADPH), 5 μmol/l tetrahydrobiopterin, 25 μmol/l L-1⁴C]arginine, 10 μmol/l flavin adenine dinucleotide, 10 μmol/l flavin mononucleotide, 5 μmol/l calmodulin, 50 mmol/l L-valine and 2 mmol/l EGTA but without Ca²⁺. After 60 min of incubation at 37°C, the reaction was terminated using a stop buffer (4°C, pH 5.3) containing 0.1 mol/l HEPES and 10 mmol/l EDTA. L-[¹⁴C]citrulline was eluted on a Dowex AG-50 WX8 (Na⁺ form) column and quantified using a liquid scintillation counter. The enzyme activity was expressed as pmol citrulline/min/mg protein. All drugs mentioned above were purchased from Sigma.

2.5. Statistical analysis of data

All values are expressed as mean±S.E. Analysis of variance was used to determine differences among the groups for infarct size, MPO and iNOS activity. Hemodynamic data within the groups as well as among the groups were compared using one-way ANOVA and two-way ANOVA for repeated measurements, respectively. Statistical differences were considered significant if the P value was <0.05.
3. Results

3.1. Exclusions

A total of 121 rats underwent the first operation (Intestinal ischemia=80; Sham=41). After the first procedure, no sham rats died and three intestinal ischemia-treated rats died early following the SMA releasing. No rats died during the recovery period. Of the 118 rats entering the second procedure, 8 of 55 rats used in the infarct size analysis (3 in Sham, 2 in PC, 1 in Sham+AG, 2 in PC+SMT), 3 of 32 rats used in myocardial MPO analysis (1 in Sham, 2 in PC+AG) and 3 of 31 rats used in myocardial iNOS analysis (1 in PC, 2 in PC+SMT) were excluded from data analysis because of ventricular fibrillation or severe hypotension during coronary occlusion and reperfusion. Final numbers in the groups were shown as follows: infarct size analysis group, Sham (n=5), Sham+AG (n=6), Sham+SMT (n=7); myocardial MPO analysis group, Sham (n=7), Sham+AG (n=8), Sham+SMT (n=7); myocardial iNOS analysis group, Sham (n=7), PC (n=8), PC+AG (n=7), PC+SMT (n=6).

3.2. Hemodynamics

In intestinal ischemia preconditioned groups, occlusion of SMA produced an initial rise in mean arterial pressure (MAP) followed by a gradual decline in MAP during the late period of SMA occlusion (Table 1). During reperfusion of the preconditioned groups, MAP significantly decreased initially and then returned to baseline 30 min after release of SMA. In the preconditioned groups, HR did not change significantly during SMA occlusion and reperfusion. MAP and HR did not change significantly with time in groups in which rats did not undergo intestinal ischemia.

Before coronary occlusion, there were no differences in the baseline values of HR and MAP between any groups (Table 2). Blood pressure decreased during coronary occlusion and remained reduced during the reperfusion in all groups compared with the baseline. In all groups HR remained unchanged throughout the experiment. During coronary occlusion and reperfusion, there were no differences in HR and MAP between any groups.

3.3. Myocardial infarct size

Fig. 2A and B shows the effect of intestinal ischemia on myocardial infarct size. In the sham control group, the infarct size/risk area was 72±4% and the infarct size/LV was 31±2%. Pretreatment with intestinal ischemia preconditioning significantly reduced the infarct size/risk area to 44±4% and the infarct size/LV to 23±2% (P<0.01). Neither AG nor SMT alone affected the sham control infarct size/risk area (73±5% and 67±4%, respectively) and infarct size/LV (34±3% and 29±2%, respectively). These treatments, however, abolished the infarct size/risk area and the infarct size/LV limitation afforded by intestinal ischemia preconditioning. The risk area/LV was identical in all experimental groups compared with the sham control group (Fig. 2C).

3.4. Myocardial MPO activity

The myocardial MPO activity is shown in Fig. 3. In the sham group, MPO activity was 76±9 units/μg protein. Preconditioning induced by intestinal ischemia significantly reduced the MPO activity to 38±6 units/μg protein (P<0.05). The reduction in MPO activity was abolished by treatment with AG or SMT.

### Table 1

Changes in systemic hemodynamics during intestinal ischemia–reperfusion

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<th>Baseline</th>
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<th>Ischemia 29 min</th>
<th>Reperfusion 10 min</th>
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* Values are mean±S.E. MAP, mean arterial blood pressure; HR, heart rate; Sham, sham period corresponding to intestinal ischemia preconditioning; PC, intestinal ischemia preconditioning; AG, aminoguanidine; SMT, S-methylisothiourea sulfate. *, P<0.05 vs. baseline value.
suggesting that AG is more selective for iNOS than eNOS Mullane et al. [26] observed a very strong relation
In this study, AG had no effect on arterial blood pressure, dium [25].
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cant increase in iNOS activity compared with the non-
detrimental action of AG or SMT on myocardial infarc-
tion, as both of them exacerbated infarct size only in the tissue exposed to 30 min of ischemia displayed a signi-
fication of iNOS activity was abolished by AG or SMT. No signi-
ficant differences in iNOS activity of non-ischemic area were found between any groups.

3.5. Myocardial iNOS activity

The myocardial iNOS activity after 30 min of coronary occlusion is shown in Fig. 4. In the sham group, the enzyme activity was similar in ischemic and non-ischemic area. In the preconditioned group, coronary occlusion significantly increased the iNOS activity to 0.32±0.05 pmol citrulline/min/mg protein (P<0.05) compared with the ischemic area in the sham group. The increase in iNOS activity was abolished by AG or SMT. No significant differences in iNOS activity of non-ischemic area were found between any groups.

4. Discussion

The major finding in this study is that prolonged intestinal ischemia resulted in late preconditioning against myocardial infarction. Two different selective iNOS inhibitors AG or SMT consistently eliminated the protection afforded by the late preconditioning against myocardial infarction. In addition, the increase in myocardial iNOS activity was observed after ischemic insult in precon-
ditioned animals and was abolished by AG or SMT. The results strongly suggest that the late preconditioning afforded by intestinal ischemia may be mediated by the activity of iNOS. This effect was not due to an inherent detrimental action of AG or SMT on myocardial infarction, as both of them exacerbated infarct size only in the preconditioned myocardium rather than the nonpre-
conditioned myocardium.

AG, a relatively selective inhibitor of iNOS, has been shown to have an IC50 of 160.0 μmol/l for the constitutive isoform of NOS versus 5.4 μmol/l for iNOS [11,15]. In this study, AG had no effect on arterial blood pressure, suggesting that AG is more selective for iNOS than eNOS at the dose we used. SMT, a competitive NOS inhibitor with the highest selectivity for the iNOS among other known NOS inhibitors, significantly inhibits iNOS activity without significant effects on eNOS [12]. AG has been described to have other nonspecific effects, including inhibition of the antioxidant enzyme catalase, histamine metabolism, and other copper- or iron-containing enzymes [16,17]. However, Szabó et al. [18] showed that SMT does not inhibit the activities of other enzymes, including xanthine oxidase, monoamine oxidase, catalase, cyto-
chrome P450, or superoxide dismutase. So in this study, we used two different iNOS inhibitors to exclude the possibility that the effects observed would be due to a nonspecific action of either agent.

Intestinal ischemia–reperfusion is associated with the increase in a variety of inflammatory mediators, such as cytokine, reactive oxygen metabolites, NO, and activated neutrophils themselves [8,9,19,20]. The iNOS enzyme is activated by cytokine [10]. In addition, Cuzzocrea et al. [19] found that reactive oxygen metabolites produced in ischemic intestine can reach the remote aorta. It has been demonstrated that reactive oxygen species can activate nuclear factor-kB and protein kinase C [21,22]. These signaling pathways involve the induction of iNOS by cytokine [10]. Furthermore, recent studies have shown that posttranslational modulation of iNOS protein via tyrosine phosphorylation is critical in activating the iNOS [23,24]. Our iNOS activity data show that preconditioned heart tissue exposed to 30 min of ischemia displayed a signifi-
cant increase in iNOS activity compared with the non-
ischemic area in the same group and the ischemic area in the sham group. So myocardial ischemia may promote iNOS activity of the preconditioned myocardium via protein tyrosine kinase, which is activated in the ischemic myocardium [25].

Mullane et al. [26] observed a very strong relation

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<td>Changes in systemic hemodynamics during myocardial ischemia–reperfusion*</td>
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*Values are mean±S.E. MAP, mean arterial blood pressure; HR, heart rate; Sham, sham period corresponding to intestinal ischemia preconditioning; PC, intestinal ischemia preconditioning; AG, aminoguanidine; SMT, S-methylisothiourea sulfate. There were no significant differences between group s.
Fig. 3. Myocardial myeloperoxidase (MPO) activity. Rats were subjected to 30 min of myocardial ischemia and 3 h of reperfusion 24 h after sham operation (Sham) or intestinal ischemia preconditioning (PC). PC inhibited accumulation of MPO vs. sham group ($P<0.05$). The decrease in MPO was abolished by treatment with aminoguanidine (AG) or S-methylisothiourea sulfate (SMT). Values are mean±S.E. *, $P<0.05$ vs. sham group.

between MPO activity and infarct size. Our MPO data show that intestinal ischemia–reperfusion induced preconditioning inhibited neutrophil accumulation in the ischemic–reperfused myocardium. The reduced infarct size may be attributed in part to inhibition of neutrophil-mediated myocyte damage. The decrease in MPO in preconditioned myocardium was abolished by AG or SMT, suggesting that the generation of NO by iNOS may inhibit neutrophil accumulation.

Several other possible mechanisms may underline the beneficial effects of NO on infarct size. NO has been shown to reduce Ca$^{2+}$ overload in ischemic–reperfused myocardium [27], to reduce catecholamine-induced increase in myocardial contractility [28], to increase ATP generation by stimulating glycolysis [29], and reduce myocardial oxygen consumption [30]. In addition, NO-mediated coronary vasodilation may help reduce myocardial ischemia [31]. However, the NO synthase inhibitor preserved myocardial function after ischemia–reperfusion in the isolated rat heart [32]. The reasons for these discrepant observations is unclear, but may be related to differences in duration of the ischemic period, the degree of production of O$_2^-$, or the change in blood pressure induced by L-NAME.

The iNOS is implicated in many pathophysiological...
states involving tissue damage, such as myocardial infarction [33], graft rejection [34], and splanchic artery occlusion shock [20]. However, our results are consonant with other studies showing that NO induced by iNOS is an important mediator of late preconditioning against myocardial stunning and myocardial infarction [6,7]. The reasons for these discrepant results probably relates to the time course of iNOS activation and the amount of NO induced by iNOS. Although massive NO formation is toxic, modest generation of NO and iNOS activated during the early phase of reperfusion may protect the ischemic myocardium. It is recognized that in this study we only measured the infarct size using tetrazolium staining, we did not evaluate the regional myocardial function. Because regional myocardial function is an additional index of myocardial protection independent of histochemical measurements of cell death, further studies are needed to clarify the effects of the late preconditioning afforded by intestinal ischemia on the regional myocardial function.

Our results may have important clinical implications. Clinically, the gastrointestinal tract is often subjected to Ca"-independent nitric oxide synthase. FEBS Lett 1991;291:145±150.

Our results have shown that prolonged intestinal ischemia followed by reperfusion provides a marked protection against myocardial infarction. Thus, patients suffering from acute prolonged intestinal ischemia followed by reperfusion might have a longer window for therapy (e.g. thrombolytic therapy or coronary angioplasty) to salvage ischemic myocardium.

Acknowledgements

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