Intestinal ischemia preconditions myocardium: role of protein kinase C and mitochondrial $K_{\text{ATP}}$ channel

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1. Introduction

Ischemic preconditioning (PC), a phenomenon in which brief episodes of ischemia and reperfusion before prolonged ischemic insult limit myocardial cellular damage, was first described by Murry et al. [1]. There is growing evidence showing that PC elicits an early and a late phase of protection against myocardial ischemic injury [1–3]. Furthermore, several studies [4,5] have shown that a brief ischemic period of the kidney or intestine limits myocardial infarct size. The phenomenon is termed “interorgan” or “remote organ preconditioning of the myocardium”.

There is strong support for the hypothesis that PC results in protein kinase C (PKC) activation in the heart [6,7]. Downstream targets of PKC include activation of an ATP-sensitive potassium channel $K_{\text{ATP}}$, most likely the mitochondrial $K_{\text{ATP}}$ channel ($\text{Mito}K_{\text{ATP}}$ channel) [8,9]. The mechanism by which PC activates PKC has not been elucidated, however, nitric oxide (NO) [10], reactive oxygen species [11], bradykinin [12], and opioid [13] have been suggested as mediators of PKC activation in the heart. Intestinal ischemia/reperfusion is associated with the increase in a variety of inflammatory mediators such as reactive oxygen metabolites, NO, bradykinin and opioid [14–17]. Therefore, we hypothesized that intestinal ischemia results in an early PC against myocardial infarction and that the mechanism of the early PC involves the activation of PKC signaling pathway.

In the present study, we tested this hypothesis by determining whether PKC inhibitors chelerythrine (CHE)
and staurosporine (Stauro), or a specific MitoK<sub>ATP</sub> channel inhibitor 5-hydroxydecanoate (5-HD) could attenuate the early PC induced by intestinal ischemia. Our results support the involvement of PKC and MitoK<sub>ATP</sub> channel in the mechanism of the early PC induced by intestinal ischemia.

2. Methods

2.1. Surgical preparation and experimental protocol

Male Sprague–Dawley rats (300–420 g) were fed a standard diet and acclimated in a quiet quarantine room for 1 week before experimentation. The animal protocol was approved by the Animal Care and Research Committee, Kagawa Medical University. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health; NIH Publication No. 85–23, revised 1996).

All rats were fasted 12 h prior to the experimental day, but given free access to water. Rats were anesthetized with pentobarbital (60 mg/kg, i.p.), and mechanically ventilated with oxygen-supplemented room air. Catheters were placed in the femoral artery and femoral vein to monitor arterial pressure, heart rate (HR), and inject drugs, respectively. Arterial blood gas parameters were maintained within a normal physiological range.

A rat model of intestinal and myocardial ischemia/reperfusion was used as previously described [18]. Briefly, after laparotomy, the superior mesenteric artery (SMA) was dissected free. The chest was opened via left thoracotomy, and a reversible coronary artery snare occluder was placed around the proximal left anterior descending coronary artery. Once the hemodynamics had stabilized, heparin (200 I.U., i.v.) was given, and the SMA was occluded for 25 min and reopened. Heat and evaporative water loss from the incision were minimized with a cellophane wrap and blanket. After 15 min of reperfusion, a coronary artery was occluded for 30 min followed by 180 min of reperfusion. The body core temperature was maintained at 36.5–37.5 °C using a heating pad throughout the experiment.

For infarct size analysis, the rats were randomly assigned 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 15 min before the coronary occlusion with no drug treatment. Group III (PC+CHE) underwent the same protocol as group II except that the rats received an intravenous injection of CHE (5 mg/kg, Sigma Chemical Co, MO, USA) 5 min before the coronary occlusion. Group IV (Sham+CHE) underwent the same protocol as group III except that the rats did not undergo intestinal ischemia. Group V (PC+Stauro) underwent the same protocol as group II except that the rats received an intravenous injection of Stauro (50 μg/kg, Sigma Chemical Co, MO, USA) 5 min before the coronary occlusion [19]. Group VI (Sham+Stauro) underwent the same protocol as group V except that the rats did not undergo intestinal ischemia. Group VII (PC+5-HD) underwent the same protocol as group II except that the rats received an intravenous injection of 5-HD (5 mg/kg, ICN Pharmaceuticals, Inc.) 5 min before the coronary occlusion. Group VIII (Sham+5-HD) underwent the same protocol as group VII except that the rats did not undergo intestinal ischemia. To determine the role of the neurogenic pathway in the preconditioning induced by intestinal ischemia, group IX (PC+HEX) underwent the same protocol as group II except that the rats were pretreated with the ganglion blocker hexamethonium (HEX, 20 mg/kg, i.v., Sigma Chemical Co, MO, USA) 15 min before the preconditioning. Group X (Sham+HEX) underwent the same protocol as group IX except that the rats did not undergo intestinal ischemia. To investigate the role of intestinal reperfusion in the preconditioning, in group XI myocardial infarct size was studied in the presence of permanent mesenteric artery occlusion (P-MAO). Group XI (P-MAO) underwent the same protocol as group II except for the permanent intestinal ischemia.

CHE and 5-HD were dissolved in normal saline. Stauro was dissolved in dimethyl sulfoxide (DMSO), and this solution was further diluted in normal saline immediately before use. The final concentration of DMSO in the solution was <5%. The concentration of DMSO (~0.15 ml) did not significantly affect infarct size compared with Sham group (n=4, data not shown).

2.2. Infarct size analysis

Infarcted area (IA) and area at risk (RA) were determined as described previously [18]. After 180 min of reperfusion, the coronary artery was reoccluded, and 1 ml of 5% Evans blue was given intravenously to distinguish the non-ischemic area from the RA. The heart was then excised, and the left ventricle (LV) was transversely cut into slices 2 mm thick. Each slice was divided into the RA and the remaining LV, and the RA was incubated for 20 min at 37 °C in triphenyltetrazolium chloride solution (1% in phosphate buffer, pH 7.4). The slices were then kept in a 10% formaldehyde solution for 24 h. At the end of these procedures, the viable tissue was stained red, and the IA remained pale. The RA and IA volumes were calculated by computerized planimetry, and results were expressed as the percentage of IA to RA or total LV, and the percentage of RA to total LV.

2.3. Statistical analysis of data

All values are expressed as mean±S.E. Differences in infarct size among groups were compared by one-way ANOVA followed by an unpaired t-test with modified
Bonferroni correction. Hemodynamic data within and among the groups were compared using two-way ANOVA for repeated measurements followed by the paired or unpaired t-test with modified Bonferroni correction. Hemodynamic responses to various inhibitors within the same group were compared by the paired t-test. Statistical differences were considered significant if the P value was <0.05.

3. Results

3.1. Exclusions

A total of 92 rats were used in this study. No rats died during the period of intestinal ischemia and reperfusion. Nine of the initial 92 rats entering the procedure of infarct size analysis (two in Sham, one in PC, one in Sham+CHE, one in PC+Stauro, one in PC+5-HD, one in Sham+HEX, two in PC+HEX) 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: Sham (n=8), PC (n=8), PC+CHE (n=8), Sham+CHE (n=7), PC+Stauro (n=8), Sham+Stauro (n=7), PC+5-HD (n=8), Sham+5-HD (n=7), PC+HEX (n=8), Sham+HEX (n=7), and P-MAO (n=7).

3.2. Hemodynamics

There were no differences in baseline values of mean arterial blood pressure (MAP) and HR between any groups. In group I–VIII shown in Table 1, occlusion of SMA produced a significant rise in MAP followed by a gradual decline in MAP during the late period of SMA occlusion. MAP was not significantly different from baseline value 25 min after occlusion of SMA. During reperfusion of the ischemic intestine, MAP significantly decreased initially and then recovered to almost the same level as the baseline values 15 min after release of SMA.
HR did not change significantly during SMA occlusion and reperfusion. Administration of Stauro resulted in a significant decrease in MAP, which persisted throughout the study. No effect on HR was seen with Stauro.

In group IX–X shown in Table 2, administration of HEX resulted in a significant decrease in MAP and HR. Occlusion of SMA produced a significant rise in MAP until 25 min of SMA occlusion. During reperfusion of ischemic intestine, MAP significantly decreased initially and then recovered to the values of 15 min of HEX administration. In group XI, MAP and HR did not change significantly during SMA occlusion.

In all groups, MAP decreased significantly during coronary occlusion and remained reduced during the reperfusion, but HR did not change significantly. During coronary occlusion and reperfusion, there were no differences in MAP and HR between any groups.

### 3.3. Myocardial infarct size

Fig. 2A and B show the effect of intestinal ischemia on myocardial infarct size. In the sham control group, the infarct size/RA was 73±4%, and the infarct size/LV was 31±2%. Intestinal ischemia preconditioning significantly reduced the infarct size/RA to 44±4% and the infarct size/LV to 23±1% (P<0.01). CHE, Stauro, 5-HD and HEX alone affected neither the sham control infarct size/RA (66±4%, 65±3%, 73±5% and 67±4%, respectively) nor the infarct size/LV (30±2%, 33±3%, 34±2% and 30±2%, respectively). However, CHE, STAuro and 5-HD,

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**Table 1**

Changes in systemic hemodynamics in rats of group I–VIII

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>MAO (10 min)</th>
<th>MAO (25 min)</th>
<th>Rep (15 min)</th>
<th>CAO (30 min)</th>
<th>Rep (180 min)</th>
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<tbody>
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<td><strong>MAP (mmHg)</strong></td>
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<td></td>
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<tr>
<td>Sham</td>
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<td>96±5</td>
<td>93±6</td>
<td>76±5*</td>
<td>63±6*</td>
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<td>103±6</td>
<td>95±4</td>
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<td>376±11</td>
<td>367±12</td>
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<td>347±13</td>
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</table>

Values are expressed as mean±S.E. MAP, mean arterial blood pressure; HR, heart rate; Sham, sham period corresponding to intestinal ischemia preconditioning; PC, intestinal ischemia preconditioning; MAO, mesenteric artery occlusion; CAO, coronary artery occlusion; Rep, reperfusion; HEX, hexamethonium. *P<0.05 vs. baseline value; †P<0.05 vs. sham group.

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**Table 2**

Changes in systemic hemodynamics in rats of group IX–X

<table>
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<th></th>
<th>Baseline</th>
<th>HEX (15 min)</th>
<th>MAO (25 min)</th>
<th>Rep (15 min)</th>
<th>CAO (30 min)</th>
<th>Rep (180 min)</th>
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<tbody>
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<td><strong>MAP (mmHg)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>PC+HEX</td>
<td>103±7</td>
<td>79±5*</td>
<td>87±5*</td>
<td>76±7*</td>
<td>69±7*</td>
<td>61±5*</td>
</tr>
<tr>
<td>Sham+HEX</td>
<td>98±7</td>
<td>74±6*</td>
<td>70±4*</td>
<td>72±6*</td>
<td>66±5*</td>
<td>58±7*</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>PC+HEX</td>
<td>374±12</td>
<td>312±12*</td>
<td>311±14*</td>
<td>319±12*</td>
<td>323±10*</td>
<td>325±14*</td>
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<tr>
<td>Sham+HEX</td>
<td>370±14</td>
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<td>310±15*</td>
<td>321±12*</td>
<td>316±12*</td>
<td>329±11*</td>
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</table>

Values are expressed as mean±S.E. MAP, mean arterial blood pressure; HR, heart rate; Sham, sham period corresponding to intestinal ischemia preconditioning; PC, intestinal ischemia preconditioning; MAO, mesenteric artery occlusion; CAO, coronary artery occlusion; Rep, reperfusion; HEX, hexamethonium. *P<0.05 vs. baseline value; †P<0.05 vs. HEX 15 min.
Fig. 2. (A and B) Effects of chelerythrine (CHE), staurosporine (Stauro), 5-hydroxydecanoate (5-HD), hexamethonium (HEX) and permanent mesenteric artery occlusion (P-MAO) on myocardial infarct size in sham-operated (Sham) or intestinal ischemia preconditioned (PC) rat hearts. Infarct size is expressed as a percentage of the area at risk (A) and the left ventricle (LV) (B). (C) Myocardial area at risk is expressed as a percentage of the LV in all groups. Values are expressed as mean±S.E. *P<0.05 vs. sham group.
but not HEX, abolished the infarct size/RA and the infarct size/LV limitation afforded by intestinal ischemia preconditioning. Permanent MAO did not reduce the infarct size/RA and the infarct size/LV. The RA/LV was identical in all groups compared with the sham control group (Fig. 2C).

4. Discussion

The present study shows that intestinal ischemia resulted in early PC against myocardial infarction. The relatively selective PKC inhibitors CHE and Stauro, or a specific MitoK<sub>ATP</sub> channel inhibitor 5-HD eliminated the protection afforded by the early PC with infarct size used as the end point. The present study provides evidence for the hypothesis that PKC and MitoK<sub>ATP</sub> channel activation is a pivotal step in the intracellular signaling pathway of intestinal ischemia-induced early PC in the rat. This effect was not due to an inherent detrimental action of CHE, Stauro and 5-HD on myocardial infarction, as all of them exacerbated infarct size only in the preconditioned myocardium rather than non preconditioned myocardium.

The signaling pathways involved in PC have been investigated intensively. There is enough evidence showing a central role of PKC-MitoK<sub>ATP</sub> channel signaling pathway responsible for both early and late PC against ischemia. PKC has been shown to be involved in the protection provided by PC in rabbits, rats, and dogs. Ytrehus et al. [19] and Mitchell et al. [20] have demonstrated that blockade of PKC alone aborts protection from PC in rabbits and rats, indicating that activation of PKC is necessary to mediate the protection. In addition, 4β-phorbol 12-myristate 13-acetate (PMA) and diacylglycerol, openers. Although 5-HD has been shown to inhibit K<sub>ATP</sub> channel, which are the PKC agonists, have been used to stimulate the effect of (or did mimic) PC in the rabbit and rat model [19,20]. Furthermore, several studies [20,21] used Western blotting with subtype-specific peptide antibodies to determine which PKC subtypes are involved in PC. Those studies showed that PKC<sub>ε</sub> and PKC<sub>δ</sub> are translocated to the membrane fraction after PC, and are involved in the development of protection against ischemic injury in the rat.

Wang and Ashraf et al. have recently demonstrated that activation of MitoK<sub>ATP</sub> channel elicits strong protection against Ca<sup>2+</sup> overload [22] and ischemic injury [23]. These conclusions are well supported by several studies [8,24,25] indicating that MitoK<sub>ATP</sub> channel is the end effector in PC-induced cardioprotection against ischemia. Sato and colleagues [9] reported that the activity of the MitoK<sub>ATP</sub> channel could be regulated by PKC in intact heart cells. Exposure to the PKC activator, PMA, potentiated and accelerated the effect of diazoxide, a potent opener of the MitoK<sub>ATP</sub> channel. The effects of PMA were blocked by the MitoK<sub>ATP</sub> channel blocker 5-HD, which was verified with simultaneous recordings of membrane current and flavoprotein fluorescence. In the latter studies, DeWeille and colleagues [26] also demonstrated that stimulation of PKC by the PMA activated the K<sub>ATP</sub> channel. Therefore, the PKC-mediated pathway appears to be important in preconditioning of rabbit and rat heart. It is reasonable to speculate that PKC activation might phosphorylate the MitoK<sub>ATP</sub> channel.

The mechanism by which opening of the MitoK<sub>ATP</sub> channel produces protection against reperfusion injury remains to be determined. The mitochondrial role in regulating Ca<sup>2+</sup> homeostasis may be pivotal in cardioprotection. As reviewed by Gross and Fryer [27], the opening of the MitoK<sub>ATP</sub> channel causes depolarization of mitochondria, thus reducing Ca<sup>2+</sup> overload during reperfusion. In addition, the opening of the MitoK<sub>ATP</sub> channel may lead to increased amounts of ATP during ischemia [23]. Thus, these beneficial effects of MitoK<sub>ATP</sub> channel activation could be operative as a consequence of MitoK<sub>ATP</sub> channel-mediated cardioprotection.

To inhibit PKC, we used two different PKC inhibitors, CHE and Stauro. CHE is a potent inhibitor of PKC (IC<sub>50</sub>, ≈ 0.7 μmol/l) with very high selectivity for PKC compared with protein kinase A or protein tyrosine kinase [28], at a dose (5 mg/kg) that has been shown to block both the early [29] and the late [30] phases of PC after an ischemic stimulus. Stauro blocks the ATP binding site of PKC with lower K<sub>i</sub> compared with other protein kinase inhibitors [31,32]. Stauro also inhibits protein kinase A and phosphorylase kinase [31,32]. So in this study, we used two different PKC inhibitors, CHE and Stauro, to exclude the possibility that the effects observed would be due to a nonspecific action of either agent. 5-HD is widely used to block PC and cardioprotection induced by K<sub>ATP</sub> channel openers. Although 5-HD has been shown to inhibit K<sub>ATP</sub> channels in sarcolemma [33] and isolated mitochondria [34], several studies showed that 5-HD is an effective blocker of MitoK<sub>ATP</sub> channel [8,34]. Recently, Sato et al. [9] demonstrated that 5-HD abolished the pinacidil-induced flavoprotein oxidation, rather than surface membrane K<sub>ATP</sub> current. These results indicate that 5-HD selectively inhibits MitoK<sub>ATP</sub> channel without affecting surface K<sub>ATP</sub> channels (sarcomemal K<sub>ATP</sub> channels).

Gho et al. [5] reported that ganglion blockade abolished the cardioprotection by 15-min intestinal ischemia, suggesting that the neurogenic pathway is involved in the protection by 15-min brief intestinal ischemia. Furthermore, Schoemaker and van Heijningen [35] showed that pretreatment with bradykinin B<sub>2</sub> receptor antagonist completely abolished the cardioprotection by 15-min intestinal ischemia. This result indicates that bradykinin released during ischemia/reperfusion in the intestine has an important role in the neurogenic pathway of remote preconditioning. However, in the present study, we showed that ganglion blockade had no effect on the PC induced by intestinal ischemia, suggesting that during reperfusion of prolonged ischemic intestine, substances released into
blood were sufficient to precondition the myocardium. These findings indicate that the neurogenic pathway is not involved in the cardioprotection induced by prolonged intestinal ischemia. Further investigation is needed for examining the factors involved in activating myocardial PKC at release of the SMA.

The present results may have important clinical implications. Clinically, the gastrointestinal tract is often subjected to ischemia followed by reperfusion in the settings of trauma, hemodynamic instability, and vascular occlusive disease. Our findings have indicated that prolonged intestinal ischemia followed by reperfusion provides 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.

References


