Activation of Mitochondrial K\textsubscript{ATP} Channel Elicits Late Preconditioning Against Myocardial Infarction via Protein Kinase C Signaling Pathway

En Takashi, Yigang Wang, Muhammad Ashraf

Abstract—Activation of mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channel induces acute ischemic preconditioning (PC) against ischemic injury. The ability of this channel to elicit late PC remains unknown. The present study tests the hypothesis that stimulation of mitoK\textsubscript{ATP} channel induces late PC via the protein kinase C (PKC) signaling pathway. Rats were subjected to 30 minutes of regional ischemia and 120 minutes of reperfusion (I/R). In other groups, rats were pretreated with diazoxide, a specific opener of the mitoK\textsubscript{ATP} channel (7 mg/kg, IV), 12, 24, 48, and 72 hours before they were subjected to I/R. A maximum reduction in infarct size was observed after 24 hours (33.3 ± 2.2\% versus I/R group, 62.1 ± 2.4\%). Pretreatment with diazoxide did not reduce the infarct size significantly after 12, 48, and 72 hours (50.2 ± 4.3\%, 50.5 ± 4.6\%, and 58.2 ± 4.9\%) compared with the I/R group. The protection was blocked with 5-hydroxydecanoic acid (5-HD, 5 mg/kg IV), a relatively selective mitoK\textsubscript{ATP} channel blocker (56.5 ± 2.7\%), and chelerythrine (5 mg/kg IV), an effective PKC inhibitor (57.1 ± 3.4\%) administered either on the first day before diazoxide pretreatment or 10 minutes before I/R on the second day. Cell necrosis was decreased by ≈50\% in the diazoxide preconditioned hearts compared with control I/R hearts. Cell death by apoptosis was also significantly decreased in diazoxide pretreated hearts (3.2\%) as compared with I/R (11.3\%). In conclusion, activation of mitoK\textsubscript{ATP} channel with diazoxide produces late PC against reperfusion injury. The effect of mitoK\textsubscript{ATP} channel appears to be dependent on the PKC-mediated signal pathway. (Circ Res. 1999;85:1146-1153.)

Key Words: mitochondrial K\textsubscript{ATP} channel ■ myocardial infarction ■ apoptosis ■ protein kinase C ■ electron microscopy

Ischemic preconditioning (PC), a phenomenon in which brief episodes of ischemia and reperfusion before a prolonged ischemic event limit myocardial cellular damage, was first described by Murry et al.\textsuperscript{1} The protection lasts ≈2 hours\textsuperscript{2} and reappears after 24 hours; this reappearance is referred to as a delayed phase of cardioprotection or a second window of protection.\textsuperscript{3,4} Since these initial observations, several studies have been performed to determine the mechanism(s) responsible for this remarkable cardioprotective effect in the eventual hope of finding a clinically useful PC-like drug. In this regard, administration of the adenosine A1 receptor agonist 2-chloro-N\textsubscript{6}-cyclopentyladenosine\textsuperscript{5} induced cardioprotection through PKC activation\textsuperscript{6} and heat shock proteins\textsuperscript{7} could be responsible for late cardioprotection. The endotoxin derivative monophosphoryl lipid A also induces delayed PC possibly via inducible NO synthase and protein kinase C (PKC) signaling pathway\textsuperscript{8} and through K\textsubscript{ATP} channels.\textsuperscript{9} Fryer et al.\textsuperscript{10} reported that 6-opioid receptor stimulation also produced a delayed cardioprotection perhaps via the mitochondrial K\textsubscript{ATP} (mitoK\textsubscript{ATP}) channel. Wang and Ashraf\textsuperscript{11} have recently demonstrated that an opener of the mitoK\textsubscript{ATP} channel, diazoxide, induced PC through the activation of the PKC and mitoK\textsubscript{ATP} channel against Ca\textsuperscript{2+} overload and ischemic injury in the rat heart. Thus, several studies have reported that mitoK\textsubscript{ATP} channel is the end effector of PC\textsuperscript{11–14} and PKC activity is important in mitoK\textsubscript{ATP} channel-mediated protection.\textsuperscript{11,12} However, there is no evidence yet whether the activation of mitoK\textsubscript{ATP} channel also leads to a second window of protection. Therefore, the present study tested the hypothesis that activation of mitoK\textsubscript{ATP} channel can induce delayed protection of myocardium against lethal ischemic injury via the PKC signaling pathway.

Materials and Methods

General Surgical Procedures and Preparation of Tissue

Adult male Sprague-Dawley rats were anesthetized by peritoneal injection of 30 mg/kg pentobarbital. The chest was opened, and then the left coronary artery (LCA) 2 to 3 mm away from the origin was ligated for 30 minutes and reopened for 120 minutes. In each group, 100 mg/kg of horseradish peroxidase (HRP) was administered...
intravenously. Hearts were removed and retrogradely perfused with Krebs-Henseleit buffer as previously described.15 The present study was performed in accordance with the guidelines of the Animal Care Committee of the Medical Center of the University of Cincinnati, which is accredited by the American Association of Laboratory Animal Care.

Experimental Protocols

The entire experimental protocol is summarized in Figure 1.

Control Groups

Vehicle saline or 5-hydroxydecanoic acid (5-HD) or chelerythrine was injected 24 hours before sham operation.

Ischemia/Reperfusion (I/R) Group

Hearts were subjected to reperfusion for 120 minutes after 30 minutes of LCA occlusion.

Diazoxide Pretreatment Group

Diazoxide was found to be a relatively specific opener of mitoK\textsubscript{ATP} channel at low concentration without any effect on sarcolemmal K\textsubscript{ATP} channels.13,14 The dosage of diazoxide used was based on a previous study.16 Rats were given an intravenous dose of 7 mg/kg diazoxide. These animals were pretreated with diazoxide for 12, 24, 48, and 72 hours before I/R.

Blockade of MitoK\textsubscript{ATP} Channel

5-HD was given to test whether a specific blocker of the mitoK\textsubscript{ATP} channel can abolish the protection. Diazoxide-pretreated rats were given 5-HD (5 mg/kg, IV), a selective K\textsubscript{ATP} channel antagonist,13 10 minutes before I/R on the second day. In another group, 5-HD was given before diazoxide pretreatment on the first day.

PKC and MitoK\textsubscript{ATP} Channel

To determine whether the mitoK\textsubscript{ATP} channel elicits protection via PKC, chelerythrine (5 mg/kg, IV), a selective PKC inhibitor, was administered before diazoxide pretreatment on the first day. In another group, it was given 10 minutes before I/R in diazoxide-pretreated rats on the second day.

Determination of Infarct Size and Assessment of Ischemic Injury With Molecular and Morphological Markers

Modified 2,3,5-Triphenyltetrazolium Chloride (TTC) Staining

Hearts were perfused retrogradely with 2% TTC followed by fixation with 4% paraformaldehyde. The heart was sliced transversely into 3 to 4 slices (2–3 mm), and thinner sections (100 μm) were also cut with a microtome (Vibratome, Oxford Co). Risk and ischemic regions were measured.17

HRP Technique

To investigate sarcolemmal integrity and permeability alterations, the slices after fixation were frozen in liquid nitrogen. Frozen sections (8 μm) were processed as previously described.18 The number of HRP-positive cells was counted. Strongly stained cells were categorized as necrotic cells and lightly stained cells represented the transient stage leading to necrosis as confirmed by electron microscopy.19

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling (TUNEL) Assay

Apoptosis was assessed with the TUNEL method (MEBSTAIN Apoptosis Kit II, MBL Co). The frozen sections were first reacted with 3,3'-diaminobenzidine for HRP staining, and immediately terminal deoxynucleotidyl transferase buffer was applied to the specimens as described in the kit. Sections were then stained with propidium iodide (PI) to visualize nuclei and photographed with a light microscope equipped with fluorescence optics.

Classification of Cell Injury

The cells were classified as normal, reversibly injured, and necrotic cells as previously characterized.15,20

An expanded Materials and Methods section is available online at http://www.circresaha.org.

<table>
<thead>
<tr>
<th>TABLE 1. Hemodynamic Changes</th>
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<td>Groups</td>
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<td>Sham/control</td>
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<tr>
<td>I/R</td>
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<tr>
<td>DE+I/R</td>
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<td>DE/5-HD+I/R</td>
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<td>DE/CHE+I/R</td>
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Baseline data were obtained before ischemia; values are mean±SEM. HR indicates heart rate; MBP, mean blood pressure; DE, diazoxide; and CHE, chelerythrine.

*P<0.05 vs control.
Results

Hemodynamics
Table 1 summarizes hemodynamic changes including heart rate and mean arterial blood pressure in various groups. The data were recorded at baseline before ischemia, after 10 minutes of LCA occlusion, and after 120 minutes of reperfusion. No significant differences existed in the hemodynamics of diazoxide-pretreated group with or without 5-HD or chelerythrine treatment as compared with the I/R group.

Time-Dependent Reduction of Myocardial Infarct by Stimulation of MitoK<sub>ATP</sub> Channel
In the I/R group, 62.1±2.4% of the risk area was infarcted. In the sham control hearts, the left ventricular slices were stained a brick-red color with TTC, and no HRP-positive material was observed within myocytes. After 30 minutes of LCA occlusion and 120 minutes of reperfusion, the ischemic region lost the TTC staining uniformly and a large number of cells were HRP positive as observed in thick sections (Figures 2 and 3). In the diazoxide-pretreated group 24 hours before I/R, the infarct size was significantly decreased compared with the I/R group (33.3±2.2% versus 62.1±2.4%, P<0.001) (Table 2). HRP-positive cells were also decreased accordingly (Figure 3). The cardioprotective effect of pretreatment 12, 48, and 72 hours before I/R almost disappeared, as shown in Figure 4.

Inhibition of the channel with 5-HD 10 minutes before I/R on the second day completely abolished the delayed cardioprotection (Table 2). Similarly, the protection also disappeared in rats that received 5-HD before diazoxide pretreatment on the first day. The control experiments in which 5-HD or chelerythrine was given with diazoxide on the first day or before I/R on the second day exhibited only a 0.24% and 0.19% TTC-negative area, respectively, in the risk area.

Role of PKC in MitoK<sub>ATP</sub> Channel-Mediated Late PC
To test the hypothesis that the activation of PKC is important for the mitoK<sub>ATP</sub> channel-mediated reduction of cellular injury, chelerythrine, which is an inhibitor of PKC, was given either before diazoxide pretreatment on the first day or before I/R on the second day. The protection was totally abolished in both groups and the infarct size was similar to that of the control I/R group (Table 2).

Detection and Assessment of In Situ DNA Fragmentation by the TUNEL Method
In the I/R group, a large number of cells in the infarcted region were necrotic and had numerous contraction bands, especially in the center of the infarct zone. These cells were
strongly HRP positive, whereas only a few scattered cells were TUNEL positive. In the border area adjacent to normal noninfarcted myocardium, many myocytes underwent apoptosis, ie, were TUNEL positive, and these cells were slightly HRP positive (Figure 5A and 5C). There were still many myocytes in the ischemic region, especially in the border zone adjacent to the normal region, which were not stained with TTC, HRP, or TUNEL, which suggests that these cells were perhaps reversibly injured. In the diazoxide-pretreated group, the number of dead cells was significantly decreased and the number of TTC-positive cells was also decreased to 3.2±0.6% as compared with the I/R group (11.3±2.0% vs I/R). The cells were swollen and myofibrils were slightly strongly HRP positive, whereas only a few scattered cells were TUNEL positive. In the border area adjacent to normal noninfarcted myocardium, many myocytes underwent apoptosis, ie, were TUNEL positive, and these cells were slightly HRP positive (Figure 5A and 5C). There were still many myocytes in the ischemic region, especially in the border zone adjacent to the normal region, which were not stained with TTC, HRP, or TUNEL, which suggests that these cells were perhaps reversibly injured. In the diazoxide-pretreated group, the number of dead cells was significantly decreased and the number of TTC-positive cells was also decreased to 3.2±0.6% as compared with the I/R group (11.3±1.0%) (Figure 6 and Table 2). In the animals treated with 5-HD or chelerythrine, the cell necrosis was similar to that of the control I/R group.

**Subcellular Pathology**

The semiquantitative data on the cell injury in various groups are given in Table 2. By electron microscopy, the myocytes that were TTC positive and HRP negative exhibited uniformly dispersed nuclear chromatin and elongated mitochondria and abundant glycogen dispersed between myofibrils. These myocytes were classified as normal (Figure 7A). The myocytes that were TTC and HRP negative were classified as reversibly injured cells. They were swollen and nuclear chromatin was slightly aggregated. HRP reaction material was restricted to T-tubules and to the extracellular space. These cells were commonly observed in the ischemic area adjacent to normal myocardium (Figure 7B and 7C). HRP-positive myocytes were placed into 2 categories. In the first category, the cells were swollen and myofibrils were slightly stained (Figure 5C), but no HRP reaction material was seen in T-tubules and mitochondrial cristae were broken, without the presence of electron-dense deposits. These cells were found to be TUNEL positive (Figure 7D). The second category included myocytes darkly stained with HRP. These cells were highly swollen; nuclear chromatin was clumped and marginated. Electron-dense deposits were commonly observed in mitochondria and no glycogen was present (Figure 7E). Semiquantitative cell injury is given in Table 2.

**Discussion**

This study demonstrates for the first time that opening of mitoKATP channel induces delayed cardioprotection in vivo 24 hours after the channel activation. It is further demonstrated that this cardioprotection is mediated via the PKC signaling pathway and that the mitoKATP channel is the end effector in cardioprotection.

**Late PC via Memory of MitoKATP Channel**

We have previously demonstrated that activation of mitoKATP channel elicits strong protection against Ca²⁺ overload 11 and ischemic injury.12 These conclusions are well supported by several recent studies10,13,14 indicating that mitoKATP channel is the end effector in cardioprotection against ischemia during acute PC.

There is enough evidence that many triggering agents of acute PC are also capable of inducing delayed PC. The PC molecules via Gi- or Gq-coupled receptors activate phospholipase C or D and PKC,21 ultimately resulting in opening of ATP-sensitive potassium channels.14,22 Since the discovery of the effect of the mitoKATP channel on ischemia, it has become clear that the mitoKATP channel may play a greater role in late PC. The significance of this channel is further increased by the fact that the mitochondrion is a complex organelle with multiple functions and occupies approximately one third of the myocyte volume.23 It has been reported that synthesis of mitochondrial superoxide dismutase,5,7 catalase,24 nitric oxide

**Table 2. Infarct Size (Expressed as Percentage of Risk Area) and Semiquantitative Estimate of Cell Injury in Rats Treated With Diazoxide 24 Hours Before I/R**

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Risk Area</th>
<th>Infarct Size</th>
<th>Degree of Myocyte Injury in Risk Area (% of Cells)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
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<tr>
<td>Sham/control (n=6)</td>
<td>46.8±2.8</td>
<td>0.05±0.03</td>
<td>99.9±0.07</td>
</tr>
<tr>
<td>I/R (n=8)</td>
<td>44.4±2.3</td>
<td>62.1±2.4</td>
<td>35.0±3.2</td>
</tr>
<tr>
<td>DE+/I/R (n=8)</td>
<td>48.4±2.0</td>
<td>33.3±2.2*</td>
<td>63.4±1.8</td>
</tr>
<tr>
<td>DE/5-HD‡+I/R (n=7)</td>
<td>46.3±2.0</td>
<td>56.5±2.7</td>
<td>39.4±3.0</td>
</tr>
<tr>
<td>DE/CHE‡+I/R (n=7)</td>
<td>46.1±2.7</td>
<td>57.1±3.4</td>
<td>38.7±2.3</td>
</tr>
</tbody>
</table>

DE indicates diazoxide; CHE, chelerythrine; and –, negative.

*P<0.001, †P<0.05 vs I/R.‡ Given before I/R on the second day.
synthase, and heat shock proteins may be important in the development of late PC. Heat shock proteins may allow opening of ATP-sensitive potassium channels during delayed cardiac protection. The opening of the mitoK<sub>ATP</sub> channel may lead to increased amounts of ATP during ischemia and increased antioxidant, manganese superoxide dismutase, after 24 hours of ischemic insult. These 2 factors could be operative as a consequence of mitoK<sub>ATP</sub> channel activities. The mitoK<sub>ATP</sub> channel also regulates Ca<sup>2+</sup> homeostasis in mitochondria.

How the protection by the opening of mitoK<sub>ATP</sub> channel in acute PC disappears within hours and reappears after 24 hours remains to be elucidated. This problem is further hampered by the lack of molecular characterization of this channel. The participation of PKC in the opening of these channels is, however, highly attractive. Activation of K<sub>ATP</sub> channels via PKC could be an inducer of PC. The administration of PKC inhibitor, chelerythrine (before activation of the channel or 10 minutes before I/R after 24 hours of diazoxide-induced activation of mitoK<sub>ATP</sub> channel) resulted in loss of protection against infarct, suggesting that PKC activity is important for the mitoK<sub>ATP</sub> channel–mediated effects. Recently, Fryer et al demonstrated that δ-opioid receptor stimulation produced a delayed protection that was lost by administering a relatively specific blocker of mitoK<sub>ATP</sub> channel, 5-HD, 5 minutes before I/R in 48 hours δ<sub>1</sub> receptor agonist–pretreated rats. We recently demonstrated that the activity of PKC is important for mitoK<sub>ATP</sub> channel–mediated effects on the Ca<sup>2+</sup> paradox and ischemic injury. PKC isoforms δ and β1 are translocated to the myocyte nuclei and PKCδ to mitochondria after diazoxide pretreatment, and PKC activates the mitoK<sub>ATP</sub> channel simultaneously. This finding may have implications for the late PC induced by diazoxide treatment. It has also been reported that 2 major

Figure 5. Photomicrographs from a section from border zone after 30-minute LCA occlusion and 120-minute reperfusion, stained simultaneously for TUNEL (A), PI (B), and HRP (C). Many TUNEL-positive myocytes (A, green) are observed in the slightly HRP-positive area (C, upper area). Necrotic cells (strongly HRP positive, arrowhead) and reversibly injured cells (TUNEL and HRP negative) were also observed (arrow). Bars=10 μm.

Figure 6. Photomicrographs showing TUNEL-positive cells in the border zone of infarcted myocardium. TUNEL-positive cells (green) were significantly decreased in the diazoxide-pretreated heart (B) as compared with the I/R group (A). Bars=10 μm.
isoforms in rat heart, PKCδ and ε, are translocated during brief episodes of transient ischemia from the cytosol to the membrane and nucleus.31 As suggested by Yellon and Baxter,32 the nuclear translocation of PKC isoforms may be important in the modification of gene-regulatory processes leading to synthesis of effector proteins responsible for delayed PC. To support this rationale, administration of chelerythrine before activation of mitoKATP channel on the first day or before I/R on the second day abolished the late PC. The present study supports a central role of PKC-mitoKATP channel signaling pathway responsible for both acute12 and late PC against ischemia. Therefore, in light of existing knowledge, it is likely that PKC could modulate the increased mitoKATP channel activity in the late PC.

The mitoKATP channel was activated with diazoxide, which is quite a potent antihypertensive agent that acts as a result of relaxation of arteriolar smooth muscle while having no direct effect on cardiac function.33 Diazoxide is also highly protective against the ischemic injury in low concentrations because of the opening of the mitoKATP channel, but it is without any effect on action potential duration.14 The acute effect of diazoxide on ischemic injury has been attributed to the direct activation of mitoKATP channel.12–14 However, this study demonstrates that opening of the mitoKATP channel also produces delayed cardioprotection 24 hours after initial treatment. Because of the prolonged half-life of diazoxide in human (72 hours),34 it could also be argued that the drug is trapped in mitochondrial membranes for a longer duration and thus induces the PC effect. The half-life of diazoxide is even smaller in rats after ~1–2 hours.35 However, the lack of effect after 12 hours (Figure 4) supports this argument.

It is well established that diazoxide is a relatively selective opener of the mitoKATP channel.13,14 It is possible that it also works on the sarcolemmal KATP channel. However, at a low dose, it rather opens the mitoKATP channel14 and also had very little effect on the plasma glucose in the rat.36

The mild and brief hypotension associated with diazoxide treatment could possibly trigger PC responses regardless of
mitoK$_{ATP}$ channel activation. It is unlikely that brief hemodynamic responses caused by diazoxide treatment are sufficient to induce myocardial ischemia to elicit adaptive responses. Thornton et al$^{13}$ recently reported that PC induced by adenine A1 receptor activation with R-N$_2$-(phenyl-2R-isopropyl)-adenosine was not caused by bradycardia, because cardiac pacing could not prevent the protection. Thus, the use of relatively specific inhibitors of both mitoK$_{ATP}$ channel and PKC preclude the involvement of hemodynamic factors in the delayed PC reported in this study.

A major effect of diazoxide pretreatment was the reduction in infarct size. The mechanism by which opening of the mitoK$_{ATP}$ channel produces protection against reperfusion injury remains to be determined. The mitochondrial role in regulating Ca$^{2+}$ homeostasis may be pivotal in cardioprotection. As reviewed by Gross and Fryer,$^{38}$ the opening of the mitoK$_{ATP}$ channel causes depolarization of mitochondria, thus reducing Ca$^{2+}$ overload during reperfusion. The electron microscopic examination of lightly HRP-stained areas within the infarct zone revealed that mitochondria were swollen but were devoid of calcium containing electron-dense deposits known to be present in the dead cells.$^{39,40}$ The study by Holmuhamedov et al$^{27}$ in which isolated preconditioned hearts released their Ca$^{2+}$ contents on opening of the mitoK$_{ATP}$ channel with diazoxide supports our findings on reduced Ca$^{2+}$ accumulation by mitochondria in the diazoxide-pretreated hearts. Thus, one of the beneficial effects of mitoK$_{ATP}$ channel activation could be reduced Ca$^{2+}$ overload in mitochondria and an increased amount of ATP contents,$^{12}$ both being the major parameters of cell viability.

Finally, the cell death by both necrosis (oncosis) and apoptosis was drastically reduced in the preconditioned myocardium. Although the occurrence of apoptosis in the ischemic myocardium is controversial,$^{41}$ reperfusion is known to accelerate its presence in the ischemic myocardium.$^{42}$ In this study, TUNEL-positive cells were significantly reduced after PC in the border areas compared with the center of the infarcted zone where only a few TUNEL-positive cells were present. By electron microscopy, these cells in the preconditioned myocardium were nearly normal, with intact sarcolemma exhibiting no altered permeability. However, a limited number of cells lost their cell membrane permeability allowing the entry of extracellular tracer (HRP) into the cytoplasm; mitochondria were swollen but without the presence of electron-dense deposits. Although these ischemic cells do not meet the typical criteria for an apoptotic cell, it appears that TUNEL positivity in the ischemic myocardium indicates a transient stage of cell necrosis.$^{43,44}$ This study is in agreement with previous studies$^{45,46}$ that found that PC reduces apoptosis.

In summary, the data support our hypothesis that opening of the mitoK$_{ATP}$ channel leads to late PC via the PKC signaling pathway. Translocation of PKC to nuclei and mitochondria may be essential in the signal transduction for late PC.

**Acknowledgment**

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**References**


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