Opening of Mitochondrial K\textsubscript{ATP} Channels Triggers the Preconditioned State by Generating Free Radicals

Tilley Pain, Xi-Ming Yang, Stuart D. Critz, Yankun Yue, Atsushi Nakano, Guang S. Liu, Gerd Heusch, Michael V. Cohen, James M. Downey

Abstract—The critical time for opening mitochondrial (mito) K\textsubscript{ATP} channels, putative end effectors of ischemic preconditioning (PC), was examined. In isolated rabbit hearts 29±3\% of risk zone infarcted after 30 minutes of regional ischemia. Ischemic PC or 5-minute exposure to 10 \textmu mol/L diazoxide, a mito K\textsubscript{ATP} channel opener, reduced infarction to 3±1\% and 8±1\%, respectively. The mito K\textsubscript{ATP} channel closer 5-hydroxydecanoate (200 \textmu mol/L), bracketing either 5-minute PC ischemia or diazoxide infusion, blocked protection (24±3 and 28±6\% infarction, respectively). However, 5-hydroxydecanoate starting 5 minutes before long ischemia did not affect protection. Glibenclamide (5 \textmu mol/L), another K\textsubscript{ATP} channel closer, blocked the protection by PC only when administered early. These data suggest that K\textsubscript{ATP} channel opening triggers protection but is not the final step. Five minutes of diazoxide followed by a 30-minute washout still reduced infarct size (8±3\%), implying memory as seen with other PC triggers. The protection by diazoxide was not blocked by 5 \textmu mol/L chelerythrine, a protein kinase C antagonist, given either to bracket diazoxide infusion or just before the index ischemia. Bracketing preischemic exposure to diazoxide with 50 \textmu mol/L genistein, a tyrosine kinase antagonist, did not affect infarction, but genistein blocked the protection by diazoxide when administered shortly before the index ischemia. Thus, although it is not protein kinase C-dependent, the protection by diazoxide involves tyrosine kinase. Bracketing diazoxide perfusion with N-(2-mercaptobutyl) glycine (300 \textmu mol/L) or Mn(III)tetrakis(4-benzoic acid) porphyrin chloride (7 \textmu mol/L), each of which is a free radical scavenger, blocked protection, indicating that diazoxide triggers protection through free radicals. Therefore, mito K\textsubscript{ATP} channels are not the end effectors of protection, but rather their opening before ischemia generates free radicals that trigger entrance into a preconditioned state and activation of kinases. (Circ Res. 2000;87:460-466.)

Key Words: diazoxide ■ 5-hydroxydecanoate ■ ischemic preconditioning ■ K\textsubscript{ATP} channels ■ myocardial infarction

Ischemic preconditioning (PC) confers protection to the myocardium through a signal-transduction pathway that may conceptually be divided into 2 phases. The first is the trigger phase, which occurs before the index ischemia, followed by the mediator/effector phase during the prolonged ischemia. There is consensus that the triggering of PC involves agonist binding to membrane receptors such as adenosine, bradykinin, and opioid receptors. Ligand-receptor binding is thought to initiate the intracellular mediator phase by activation of protein kinase C (PKC). The kinase cascade that follows PKC activation has not been clearly defined but appears to involve at least 1 tyrosine kinase and perhaps p38 mitogen-activated protein (MAP) kinase. The trigger role of adenosine receptors can be demonstrated by showing that bracketing the preconditioning ischemia with an adenosine receptor blocker eliminates protection. Similarly, PKC can be shown to be a mediator, given that PKC blockers eliminate protection only if the blockers are present during the prolonged index ischemia. It has been assumed that the end effector of this signal-transduction cascade is the mitochondrial (mito) K\textsubscript{ATP} channel. No previous study has defined the critical timing for the opening of mito K\textsubscript{ATP} channels in the PC signal-transduction pathway. If mito K\textsubscript{ATP} channels are the end effectors of protection, then they must be downstream of the kinase cascade, and the critical time for their blockade would be during the index ischemia. Administration of 5-hydroxydecanoate (5HD), a selective blocker of mito K\textsubscript{ATP} channels, during either the trigger or the mediator/effector phase should allow identification of the critical timing of channel opening. Furthermore, if mito K\textsubscript{ATP} channels are the end effectors, then we would not expect a channel opener to show a memory (ie, protection to be still evident after the agent has been washed out), because the memory can be shown to reside upstream of PKC.
Infarct Size Studies

New Zealand White rabbits (1.5–2.5 kg) were anesthetized with sodium pentobarbital (30 mg/kg IV). As previously described, the heart was exposed and a 2-0 silk suture was passed around a branch of the left coronary artery to form a snare. The heart was rapidly excised, mounted on a Langendorff apparatus, and retrogradely perfused via the aorta with warmed Krebs-Henseleit buffer gassed with 95% O2-5% CO2. Perfusion pressure was set at 75 mm Hg by adjusting the height of the reservoir. A fluid-filled latex balloon was inserted into the left ventricle and inflated to set an end-diastolic pressure of 5 mm Hg at baseline. Atrial pacing at 200 bpm was performed if the spontaneous rate was slower.

Figure 1 summarizes the protocol for each of the 18 experimental groups. All hearts experienced 30 minutes of coronary branch occlusion and 2 hours of reperfusion. The following drugs were added to the perfusate as detailed in Figure 1 (in μmol/L): diazoxide 10, pinacidil 100, 5HD 200, glibenclamide 5, chelerythrine 5, genistein 50, N-(2-mercaptopyridinyl)-glycine (MPG) 300, and Mn(II)tetrakis(4-benzoic acid) porphyrin chloride (TBAP) 7. The drugs were added in either an early (E) protocol to bracket some other intervention or a late (L) protocol just before and during all or part of the 30-minute ischemic period. Chelerythrine and diazoxide were dissolved in DMSO. Final concentrations of DMSO in the solution were ≤0.05%.

At the end of the study the risk zone was marked with 1- to 10-μm zinc/cadmium sulfide particles. The heart was weighed, frozen, cut into 2-mm-thick slices, and incubated in 1% triphenyltetrazolium chloride in sodium phosphate buffer to visualize the infarcts. Infarct size was expressed as a percentage of the risk zone.

Western Blotting for p38 MAP Kinase

We tested for activation of p38 MAP kinase with a phosphospecific antibody (New England Biolabs) recognizing phosphorylation of the 2 activation sites of p38 MAP kinase. Biopsies were obtained from each isolated heart, as follows: before a 5-minute infusion of diazoxide; after 10 minutes of washout; and after 5, 10, 20, and 30 minutes of global ischemia. Biopsies were homogenized and subjected to SDS-PAGE and standard Western blotting. Membranes were probed with the phosphospecific antibody and then stripped and reprobed with the nonphosphospecific antibody. Lane densities were normalized to the baseline value. The normalized phospho-p38 MAP kinase density in each lane was divided by the total p38 MAP kinase density in that lane. This yielded an activation value independent of any variations in protein loading.

Statistics

All data are presented as mean±SEM. One-way ANOVA with the Tukey post hoc test was performed on baseline hemodynamics and

### Infarct Size Data

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, kg</th>
<th>Heart Weight, g</th>
<th>Risk Zone, cm³</th>
<th>Infarct Size, cm³</th>
<th>I/R, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>2.3±0.0</td>
<td>8.9±0.1</td>
<td>0.89±0.14</td>
<td>0.28±0.07</td>
<td>29.0±3.1</td>
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<td>PC</td>
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<td>2.0±0.1</td>
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<td>1.17±0.23</td>
<td>0.04±0.01</td>
<td>3.2±1.0</td>
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<tr>
<td>PC/5HD(E)</td>
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<td>8.8±0.1</td>
<td>0.76±0.06</td>
<td>0.19±0.03</td>
<td>23.9±2.9</td>
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<tr>
<td>PC/5HD(L)</td>
<td>6</td>
<td>2.2±0.1</td>
<td>8.5±0.3</td>
<td>0.72±0.08</td>
<td>0.05±0.02</td>
<td>5.4±2.2</td>
</tr>
<tr>
<td>PC Shift/5HD(E)</td>
<td>6</td>
<td>2.1±0.1</td>
<td>7.9±0.2</td>
<td>0.80±0.11</td>
<td>0.06±0.05</td>
<td>5.3±3.4</td>
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<td>Diaz 15</td>
<td>6</td>
<td>2.1±0.1</td>
<td>6.9±0.5</td>
<td>1.29±0.13</td>
<td>0.11±0.03</td>
<td>8.1±1.3</td>
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<td>Diaz 30</td>
<td>6</td>
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<td>6.1±0.3</td>
<td>1.16±0.15</td>
<td>0.09±0.02</td>
<td>7.5±2.6</td>
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<td>Pinacidil</td>
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<td>5.7±0.2</td>
<td>1.25±0.14</td>
<td>0.12±0.04</td>
<td>9.6±3.5</td>
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<tr>
<td>Diaz 15/5HD(E)</td>
<td>7</td>
<td>2.1±0.0</td>
<td>6.1±0.5</td>
<td>1.11±0.10</td>
<td>0.30±0.06</td>
<td>27.9±5.5</td>
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<tr>
<td>Diaz 15/5HD(L)</td>
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<td>2.2±0.0</td>
<td>6.8±0.2</td>
<td>1.17±0.07</td>
<td>0.09±0.03</td>
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<tr>
<td>PC/Glib(E)</td>
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<td>0.08±0.03</td>
<td>6.0±2.5</td>
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<td>Diaz 15/Gen(E)</td>
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<td>7.8±0.2</td>
<td>1.03±0.08</td>
<td>0.06±0.02</td>
<td>6.5±2.6</td>
</tr>
<tr>
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<td>0.91±0.09</td>
<td>0.32±0.04</td>
<td>35.1±3.2</td>
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<td>TBAP</td>
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<td>0.96±0.08</td>
<td>0.25±0.04</td>
<td>25.8±3.0</td>
</tr>
<tr>
<td>Diaz/TBAP</td>
<td>5</td>
<td>1.9±0.0</td>
<td>7.0±0.1</td>
<td>1.03±0.11</td>
<td>0.23±0.03</td>
<td>23.0±3.3</td>
</tr>
<tr>
<td>Diaz/MPG</td>
<td>6</td>
<td>1.9±0.0</td>
<td>7.9±0.2</td>
<td>1.00±0.08</td>
<td>0.30±0.05</td>
<td>28.8±2.4</td>
</tr>
</tbody>
</table>

Data are mean±SEM. I/R indicates infarct size/risk zone; E, early; L, late; Diaz, diazoxide; Glib, glibenclamide; Chel, chelerythrine; and Gen, genistein. 15 and 30 refer to minutes of washout after treatment.

*P<0.001, †P<0.01 vs control.

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The text contains a detailed description of the experimental protocol, materials and methods, and the results of the infarct size studies. It also includes a table summarizing the results and statistical analyses. The study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the NIH. All data are presented as mean±SEM.
infarct measurements. ANOVA for repeated measures was used to test for differences in hemodynamics within any given group and for differences in the Western blots. A value of $P<0.05$ was considered significant.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

**Results**

**Hemodynamics**

No differences were noted between the experimental groups for basal heart rate, developed pressure, or coronary flow. Diazoxide did not affect heart rate, developed pressure, or coronary flow in any of the groups. None of the inhibitors, 5HD, chelerythrine, or genistein, had any effect on hemodynamic parameters in any of the groups. Pinacidil caused a significant reduction in developed pressure to 22 ± 4 mm Hg ($P<0.05$), which quickly returned to baseline levels after discontinuation of the infusion. Heart rate and coronary flow were not altered by pinacidil.

**Infarct Size**

There was no significant difference in body weight, heart weight, or risk zone size between the groups (Table). In control hearts, infarct size was 29.0 ± 3.1% of the risk zone (Figure 2). In the first series of experiments, we tested whether there was a memory component to the protection associated with KATP channel opening, as there is in ischemic PC. Diazoxide conferred protection for at least 30 minutes after its washout, resulting in infarct sizes of 8.1 ± 1.3% and 7.5 ± 2.6% with 15 and 30 minutes of washout, respectively. Pinacidil followed by a 15-minute washout was equally as cardioprotective as diazoxide (9.5 ± 3.5% infarction), indicating that the memory feature of diazoxide could be duplicated by a nonselective KATP channel opener.

We determined the critical time for mito K$_{ATP}$ channel opening to protect the heart. Figure 3A reveals that early administration of 5HD, a selective mito K$_{ATP}$ channel closer, to diazoxide-treated hearts abolished protection, resulting in 27.9 ± 5.5% infarction of the risk zone. When 5HD was administered shortly before and during the long ischemic period, the myocardium remained in a protected state with an infarct size of 7.8 ± 2.1%. Figure 3B shows that PC reduced infarct size to 3.2 ± 1.0% of the risk zone. Early 5HD blocked protection from PC with 23.9 ± 2.9% infarction, whereas late 5HD had no effect on the protection by PC (5.5 ± 2.2% infarction). We also included the PC-shifted group (Figure 1), in which the PC ischemia was shifted earlier out of the 5HD-infusion window. The timing of 5HD infusion was identical to that in the PC/5HD(E) protocol in which protection was blocked. 5HD failed to block in this group (Figure 3B), which argues against incomplete washout as a possible explanation for the blockade of protection in PC/5HD(E). In addition, we repeated the PC protocols using 5 μmol/L glibenclamide, a potent but nonselective K$_{ATP}$ blocker. Glibenclamide blocked the protection by PC only in the early protocol (Figure 3B).

Inhibition of PKC by chelerythrine, either early or late, did not block the protection by diazoxide (Figure 4). Nor did tyrosine kinase inhibition with genistein infused early block protection from diazoxide. However, genistein late completely abolished the protection by diazoxide, resulting in an infarct size of 35.1 ± 3.2% (Figure 4). We have previously shown that genistein alone at this dose has no effect on infarction.3

Bracketing the diazoxide infusion with the free radical scavenger MPG completely blocked the protection with an
Because MPG is a sulfhydryl-reducing agent, we tested TBAP in the same protocol. TBAP catalyzes the dismutation of superoxide radical and breakdown of $H_2O_2$ to water. TBAP in untreated hearts had no effect on infarct size ($25.8 \pm 3.0\%$ infarction), but it completely blocked protection in diazoxide-treated hearts ($23.0 \pm 3.3\%$ infarction). Thus, the protection by diazoxide was found to be dependent on free radicals.

### Western Blotting

We previously found that the activation site for p38 MAP kinase becomes phosphorylated during ischemia only if the heart is in a preconditioned state. In this study we tested whether diazoxide would cause a similar pattern of activation. We studied 5 hearts exposed to 10 $\mu$mol/L diazoxide for 5 minutes followed by 10 minutes of washout and 30 minutes of global ischemia. Figure 6 shows the ratio of normalized phospho–p38 MAP kinase to total p38 MAP kinase, an index of the activation of p38 MAP kinase, at various times before and during ischemia. By 30 minutes of ischemia the ratio had increased 3-fold in the diazoxide-treated hearts ($P, 0.01$). In contrast, activity did not significantly change in the 7 untreated hearts. These measurements reveal that, like PC, diazoxide causes a marked activation of p38 MAP kinase during an ischemic insult, which suggests that the mechanisms of protection are similar.

### Discussion

The present study clearly demonstrates that the critical time for mito $K_{ATP}$ channel opening to be protective is before the sustained ischemic insult. This was true for channel opening induced by both diazoxide infusion and PC. Thus, the timing of mito $K_{ATP}$ channel opening is more compatible with the...
channels performing a trigger rather than an end-effector role. Like PC, transient mito K<sub>ATP</sub> channel opening puts the heart into a protected state that lasts for at least 30 minutes after drug washout, again suggesting a trigger effect. Further evidence that mito K<sub>ATP</sub> channels reside upstream of kinases in the signal-transduction pathway is the observation that blockade of tyrosine kinases during the index ischemia blocked the protection by diazoxide. Finally, it was found that diazoxide confers its protection through free radicals, a known trigger of the preconditioned state. We propose that opening of these channels, during either preconditioning ischemia or diazoxide infusion, generates free radicals, which then trigger the PC memory. Then, during the subsequent ischemic insult, a kinase cascade modulates some unidentified end effector that actually protects the heart.

Previous studies have largely ignored any possible effect of timing of 5HD administration on the ability of this K<sub>ATP</sub> channel to block protection from diazoxide or ischemic PC. Previous studies have revealed that the critical time for kinase activation in the preconditioned heart is during the index ischemia. If the mito K<sub>ATP</sub> channels reside downstream of the kinases in the PC signal-transduction pathway, then the critical time for their opening must also be during the index ischemia. Our present findings, however, do not support this model. The protection by diazoxide was blocked by 5HD only when the latter was present during the diazoxide exposure before the index ischemia. More revealing was the observation that an identical pattern existed for PC.

Close examination of the protocols of previous studies reveals that this trigger behavior has actually been seen before. Bracketing diazoxide infusion with 5HD followed by a 5-minute washout before ischemia blocked the protection by diazoxide measured by LDH release in isolated rat hearts. Preincubation of isolated canine myocytes with morphine followed by a 10-minute drug-free incubation was protective against a 90-minute ischemic episode, whereas morphine followed by a 10-minute drug-free incubation was free radicals, which then trigger the PC memory. Then, during the subsequent ischemic insult, a kinase cascade modulates some unidentified end effector that actually protects the heart.

Performance of PKC and tyrosine kinase in the PC signal-transduction pathway differs with species. Although blockade of PKC alone abets protection from ischemic PC in rats and rabbits, combined inhibition of both PKC and tyrosine kinase is required in pigs, which suggests that these kinases act in parallel pathways. PKC and an unidentified tyrosine kinase may also act via parallel pathways in the rat heart, given that both inhibitors must be present to completely abolish protection from multiple cycles of ischemic PC, whereas either is sufficient to block protection from a single cycle of PC. With a near-threshold stimulus, activation of both PKC and protein tyrosine kinase may be necessary to mediate protection. With a stronger stimulus, both protein kinases may be
activated to such an extent that either is sufficient to mediate protection. Also, the activation of either protein kinase pathway may depend not only on the strength of the stimulus but on the sensitivity of the pathway to the nature of the stimulus as well. This may explain why the protection by diazoxide was aborted by genistein but not by chelerythrine.

We were able to block the protection by diazoxide with the free radical scavengers MPG and TBAP. Free radicals have been previously proposed to be an important part of the mechanism of PC. The source of free radicals in PC has never been identified, but their production is thought to accompany reperturbation after the brief ischemia. The present study suggests, but does not prove, that diazoxide also protects by a free radical–dependent mechanism. Because 5HD blocked that protection, the radical generation could very likely be related to the opening of mito K_ATP channels. It has never been understood why opening of mito K_ATP channels should be protective. Mito K_ATP channel opening should slightly uncouple the mitochondria and cause them to swell. Neither action would be expected to lead to protection. The present data suggest that generation of free radicals may be an explanation of the protective effect of the mito K_ATP channel.

We are not the first to propose that diazoxide protects by a free radical mechanism. Forbes et al. found that the free radical scavenger N-acetylcysteine could block the protective effect of diazoxide in isolated rat hearts. Yao et al. recently showed that preconditioning chick myocytes with acetylcholine was accompanied by a burst of free radicals concurrent with drug administration and that this burst could be eliminated by 5HD. The observation that myxothiazol could also block the burst suggested that the radicals came from site III electron transport within the mitochondria. Finally, Becker et al. found that simulated ischemia in chick myocytes was accompanied by myxothiazol–dependent free radical generation and that these radicals were not from xanthine oxidase or NO. Although the effect of opening of mito K_ATP channels was not tested in that study, the data are compatible with the opening of these channels acting to trigger generation of free radicals. The coupling between adenosine receptors and PKC has never been clearly understood. The study of Becker et al. suggests that G1-coupled receptors, eg, adenosine A1 or muscarinic M2, may first open mito K_ATP channels, which will then activate PKC through the generation of free radicals. Arguing against this, however, is the failure of adenosine to directly open mito K_ATP channels as measured by flavoprotein fluorescence. We have previously reported that 5HD blocks protection from anisomycin, an activator of p38 MAP kinase. At the same time, we have proposed that p38 MAP kinase is downstream of PKC, which would obviously put the mito K_ATP channel very distal in the pathway. The mechanism by which anisomycin activates p38 MAP kinase is unknown, however, and could involve opening of mito K_ATP channels to generate free radicals, which are classical activators of the p38 MAP kinase pathway.33

In Figure 7, we propose a new paradigm of how mito K_ATP channels might function in PC. It is suggested that the preconditioning ischemia via G1-coupled receptors acts to open mito K_ATP channels, resulting in free radical generation.


Opening of Mitochondrial K$_{ATP}$ Channels Triggers the Preconditioned State by Generating Free Radicals

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