Ischemic preconditioning depends on interaction between mitochondrial K\textsubscript{ATP} channels and actin cytoskeleton

CHRISTOPHER P. BAINES, GUANG S. LIU, MUSTAFA BIRINCIIOGLU, STUART D. CRITZ, MICHAEL V. COHEN, AND JAMES M. DOWNEY

Departments of Physiology, Structural and Cellular Biology, and Medicine,
University of South Alabama, Mobile, Alabama 36688-0002

Baines, Christopher P., Guang S. Liu, Mustafa Birincioglu, Stuart D. Critz, Michael V. Cohen, and James M. Downey. Ischemic preconditioning depends on interaction between mitochondrial K\textsubscript{ATP} channels and actin cytoskeleton. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1361–H1368, 1999.—Both mitochondrial ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels and the actin cytoskeleton have been proposed to be end-effectors in ischemic preconditioning (PC). For evaluation of the participation of these proposed end-effectors, rabbits underwent 30 min of regional ischemia and 3 h of reperfusion. PC by 5-min ischemia + 10-min reperfusion reduced infarct size by 60%. Diazoxide, a mitochondrial K\textsubscript{ATP}-channel opener, administered before ischemia was protective. Protection was lost when diazoxide was given after onset of ischemia. Anisomycin, a p38\textsuperscript{JNK} activator, reduced infarct size, but protection from both diazoxide and anisomycin was abolished by 5-hydroxydecanoate (5-HD), an inhibitor of mitochondrial K\textsubscript{ATP} channels. Isolated adult rabbit cardiomyocytes were subjected to simulated ischemia by centrifuging the cells into an oxygen-free pellet for 3 h. PC was induced by prior pelleting for 10 min followed by resuspension for 15 min. Osmotic fragility was assessed by adding cells to hypotonic (85 mosmol) Trypan blue. PC delayed the progressive increase in fragility seen in non-PC cells. Incubation with diazoxide or pinacidil was as protective as PC. Anisomycin reduced osmotic fragility, and this was reversed by 5-HD. Interestingly, protection by PC, diazoxide, and pinacidil could be abolished by disruption of the cytoskeleton by cytochalasin D. These data support a role for both mitochondrial K\textsubscript{ATP} channels and cytoskeletal actin in protection by PC.

The nature of the end effector(s) responsible for ischemic preconditioning's protection has so far proved elusive. However, opening of ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels or changes in the myocyte's actin cytoskeleton have been proposed as two possible candidates. The K\textsubscript{ATP}-channel blockers glibenclamide and 5-hydroxydecanoate (5-HD) have been reported to block ischemic preconditioning's protection in a variety of species, including rabbits (17, 40, 42), dogs (2, 12), pigs (34), and rats (33). Preconditioning in human tissue also appears to involve K\textsubscript{ATP} channels because glibenclamide could block the effects of ischemic preconditioning and protein kinase C activation in human atrial trabeculae (35). Conversely, K\textsubscript{ATP}-channel openers are cardioprotective. Nicorandil (28), bimakalim (44), and cromakalim (15) reduce infarct size in dogs. Pinacidil can protect rabbit myocytes against simulated ischemia (4). In guinea pig hearts postischemic recovery of function was greatly improved by the K\textsubscript{ATP}-channel openers BMS-180448 and cromakalim (14).

Several discrepancies in the literature have caused investigators to be cautious about accepting the K\textsubscript{ATP} hypothesis, however. It was thought that by opening of sarcolemmal K\textsubscript{ATP} channels, action potential duration (APD) would be shortened, which would reduce calcium loading of the myocyte and hence exert a cardioprotective action (13). However, bimakalim (44), BMS-180448 (14), and cromakalim (15) are protective independent of APD shortening. Furthermore, 5-HD did not block the cromakalim-induced sarcolemmal K\textsubscript{ATP} currents (26), yet it was still able to block the anti-ischemia action of the opener.

Instead, mitochondrial K\textsubscript{ATP} channels may mediate the observed cardiac protection. Garlid et al. (9, 10) have demonstrated that whereas both cromakalim and diazoxide stimulated K\textsubscript{ATP} activity in purified mitochondria, sarcolemmal K\textsubscript{ATP} channels were insensitive to diazoxide. Diazoxide was just as protective in preserving postischemic function in isolated rat hearts as cromakalim (9). In addition, diazoxide-induced protection and cardiac mitochondrial K\textsuperscript{+} flux could be reversed by 5-HD. Similar results have been obtained in isolated rabbit myocytes (23, 32). Therefore, diazoxide appears to be a selective opener and 5-HD a selective blocker of mitochondrial K\textsubscript{ATP} channels.

There is also evidence to suggest that preconditioning may involve changes in the actin cytoskeleton. Activation of stress-activated protein kinase pathways, p38 mitogen-activated protein (MAP) kinase and JNK, has been documented in preconditioned rabbit (41) and rat (25) hearts. Among other things, this pathway activates MAP kinase-activated protein kinase-2 (MAPKAPK-2). This enzyme can, in turn, phosphorylate the small heat shock protein 27 (HSP27) (5, 36), an important regulator of actin dynamics that promotes polymerization of actin filaments, thus increasing the stability of the cytoskeleton (16, 21). Activation of p38 MAP kinase has been shown to prevent cytochalasin D-induced fragmentation of actin filaments, thus preserving cell viability (16, 18). Brief exposure to adenosine increases p38 MAP kinase activity and HSP27 phosphorylation in cardiomyocytes (20). Furthermore, overexpression of HSP27 in isolated rat ventricular myocytes confers protection against simulated ischemia (24). Because prolonged ischemia is known to cause cytoskeletal disruption (7), activation of the MAPKAPK-2/HSP27 pathway and preservation of the actin fila-

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ments could well explain the beneficial action of ischemic preconditioning. Thus there are suggestive data to support the role of both the \( K_{\text{ATP}} \) channel and the actin cytoskeleton as the end effector in cardioprotection. However, there has not been a systematic analysis of the importance of the mitochondrial \( K_{\text{ATP}} \) channel in the signaling pathway of preconditioning, and no attempt has been made to investigate any possible interaction between these two putative end effectors. Accordingly, the present study tested whether the mitochondrial \( K_{\text{ATP}} \) channel opener diazoxide could mimic preconditioning in both an anesthetized rabbit infarct size model and isolated cardiomyocytes. Whether protection by anisomycin, a direct activator of the p38 MAP kinase/MAPKAPK-2 pathway, could be prevented by blockade of mitochondrial \( K_{\text{ATP}} \) channels with 5-HD was also examined. Finally, activator of the p38 MAP kinase/MAPKAPK-2 pathway, whether disruption of actin filaments with cytochalasin D would affect protection mediated by either ischemic preconditioning, pinacidil, or diazoxide.

**MATERIALS AND METHODS**

All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with recommendations published in the Guide for the Care and Use of Laboratory Animals.

**In Situ Rabbit Heart Model**

New Zealand White rabbits of either sex weighing between 1.5 and 2.5 kg were anesthetized with pentobarbital sodium (30 mg/kg iv) via a marginal ear vein. A tracheotomy was performed, and the rabbits were ventilated with 100% oxygen using a positive-pressure ventilator at a rate of 30–35 breaths/min and a tidal volume of ~15 ml. The respiratory rate was adjusted to maintain blood pH in the physiological range (pH 7.35–7.45), and a heating pad was placed under the rabbit to maintain rectal temperature between 38 and 39°C. The left carotid artery and jugular vein were cannulated with PE-90 catheters filled with heparinized (10 U/ml) saline for measurement of arterial blood pressure and infusion of anesthetics and drugs, respectively. A left thoracotomy was performed in the fourth intercostal space to expose the heart, and a 2-0 silk suture was passed around a main branch of the left coronary artery to form a snare by passing the ends of the thread through a small vinyl tube. Coronary occlusion was implemented by pulling the snare tight and clamping the tube with a hemostat. Ischemia was confirmed by regional cyanosis. Reperfusion was achieved by releasing the snare and confirmed by visible hyperemia. The animals were allowed to stabilize for 20 min before the start of the experimental protocol.

**Infarct Size Measurement**

At the end of the experiment, the hearts were quickly excised and mounted on a Langendorff apparatus, where they were perfused for 1 min with saline to wash out blood. The snare around the coronary artery was again pulled to reocclude the coronary artery, and 1- to 10-µm zinc cadmium fluorescent particles were infused into the perfusate to demarcate the ischemic (tissue at risk) zone as the tissue without fluorescence under ultraviolet light. The hearts were then weighed and frozen. The frozen hearts were cut into slices 2 mm in thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) in sodium phosphate buffer (pH 7.4) for 20 min at 37°C. TTC stains viable tissue a deep red, whereas necrotic tissue remains unstained and appears tan to brown. The slices were then immersed in 10% Formalin to enhance the contrast between stained and unstained tissue. The areas of infarct and risk zone in each slice were determined by planimetry. The volumes of both regions of each slice were calculated and then summed for the entire heart. The amount of infarction for each heart was expressed as a percentage of the risk (ischemic) zone. Only hearts with a risk zone >0.5 cm³ were included because smaller ischemic zones are associated with small infarcts even in nonpreconditioned hearts (46).

**Isolated Rabbit Myocyte Model**

New Zealand White rabbits of either sex weighing between 1.5 and 2.5 kg were used in these studies. Rabbits were anesthetized and surgically prepared as described above. After exposure of the myocardium, the heart was rapidly excised and mounted on a Langendorff apparatus. The heart was perfused for 5 min with calcium-free buffer containing 90 mM NaCl, 30 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, 20 mM creatine, 60 mM taurine, and 1% BSA and supplemented with basal medium Eagle (BME) vitamin and amino acid mixture. The buffer was then recirculated with 1 mg/ml collagenase and 20 μM CaCl₂ for 15 min. The hearts were removed, and the left ventricle was macerated and dispersed in buffer containing 115 mM NaCl, 5 mM KCl, 1.2 mM MgCl₂, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 10 mM glucose, 20 mM creatine, 60 mM taurine, 10 mM HEPES, and 4% BSA and supplemented with BME vitamin and amino acid mixture. After the cells were filtered through nylon mesh, they were washed two to three times and then made calcium tolerant by slowly restoring calcium in the medium to 1.25 mM. All buffers were gassed with 95% O₂-5% CO₂ and maintained at 37°C. Ischemia was simulated by pelleting the cells (~0.5 ml packed volume) at 200 rpm, and the supernatant was replaced with microballoons to exclude oxygen. Every 30 min for 3 h, a 25-µl aliquot of cells was removed for determination of osmotic fragility by observing whether the cells could exclude trypan blue dye in a hypotonic (85 mosmol) solution. Those cells unable to exclude dye are considered to have undergone membrane failure and are therefore deemed nonviable. Three hundred cells were counted in each sample, and the percentage of stained cells was determined. An index of fragility was calculated as the area under the curve of percent stained cells vs. time.

**Materials**

Anisomycin was obtained from Sigma (St. Louis, MO). Cytochalasin D was purchased from Fluka (Ronkonkoma, NY). 5-HD and diazoxide were purchased from Research Biochemicals International (Natick, MA). Anisomycin was dissolved in DMSO and then diluted in either 0.9% saline or buffer. Cytochalasin D was dissolved in DMSO and diluted in buffer. Diazoxide was dissolved in a vehicle consisting of 1 N sodium hydroxide and 0.9% saline mixed in a 1:3 proportion. 5-HD was dissolved directly in 0.9% saline or buffer.

**Experimental Protocols**

In situ rabbit hearts. Nine groups of in situ hearts were studied, and the timing of interventions is illustrated in Fig. 1. In the control (Con) group, hearts experienced only 30 min of regional ischemia and 3 h of reperfusion. The PC group was ischemically preconditioned by means of 5 min of regional...
ANOVA with repeated measures was used to test for differences in hemodynamics within any given group. A P value < 0.05 was considered to be significant.

RESULTS

In Situ Rabbit Hearts

Fifty-four in situ rabbit hearts were prepared for this investigation, with six in each group. Table 1 shows that baseline hemodynamics were not statistically different between groups. Diazoxide (10 mg/kg) injected 10 min before the ischemic period (Diaz-Pre) induced a marked reduction in mean arterial pressure (P < 0.05) without a significant change in heart rate. The peak effect was observed 1 min after injection, with the change in arterial pressure still present (48 ± 5 mmHg, P < 0.05) at the onset of the sustained ischemia. Similar hemodynamic changes were seen when diazoxide was given 5 min into the ischemic period (Diaz-Post). Administration of 5 mg/kg 5-HD 15 min before the ischemic period elicited a small but significant increase in arterial pressure (P < 0.05) but did not affect heart rate. 5-HD had no appreciable effect on the diazoxide-induced hypotension at either 1 or 10 min after the latter's injection. However, combination of the two drugs led to a significant elevation in heart rate by the onset of ischemia (256 ± 15 beats/min, P < 0.05), which was sustained for the remainder of the experiment. Neither dose of anisomycin (50 or 100 µg/kg) had any appreciable hemodynamic effect.

Table 2 shows the heart weight and infarct size data with no apparent differences in body weight, heart weight, or risk zone size among the various groups.
Figure 2 shows that pretreatment with diazoxide 10 min before occlusion was just as protective as ischemic preconditioning (19.1 ± 4.0 and 16.0 ± 2.1%, respectively; \( P < 0.05 \) vs. control). However, when administration of diazoxide was delayed until 5 min after the onset of ischemia, the resulting infarct size (30.7 ± 3.9%; \( P = \text{NS} \) vs. control) was similar to that in the control group. As expected, prior blockade of mitochondrial K\(_{\text{ATP}}\) channels with 5-HD, which alone did not change infarct size from control levels (38.9 ± 1.5%), completely abolished the protective effect of diazoxide given 10 min before ischemia (37.3 ± 3.8%). A plot of infarct size vs. risk zone size for these groups is shown in Fig. 3. Anisomycin at 50 µg/kg also reduced infarct size (21.0 ± 3.9%; \( P < 0.05 \)), as shown in Fig. 4. Increasing the dose to 100 µg/kg afforded no additional protection (21.6 ± 4.1%; \( P < 0.05 \)). However, the protective effect of the higher dose of anisomycin was blocked by 5-HD (40.8 ± 3.9%).

### Table 2. Infarct size data for in situ rabbit hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Wt, kg</th>
<th>Heart Wt, g</th>
<th>Risk Zone Volume, cm(^3)</th>
<th>Infarct Volume, cm(^3)</th>
<th>I/R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2 ± 0.1</td>
<td>7.2 ± 0.2</td>
<td>0.97 ± 0.08</td>
<td>0.39 ± 0.05</td>
<td>39.4 ± 2.2</td>
</tr>
<tr>
<td>PC</td>
<td>2.3 ± 0.0</td>
<td>7.9 ± 0.3</td>
<td>1.00 ± 0.10</td>
<td>0.17 ± 0.03</td>
<td>16.0 ± 2.1</td>
</tr>
<tr>
<td>Diaz-Pre</td>
<td>2.3 ± 0.1</td>
<td>6.6 ± 0.4</td>
<td>0.93 ± 0.08</td>
<td>0.19 ± 0.05</td>
<td>19.1 ± 4.0</td>
</tr>
<tr>
<td>Diaz-Post</td>
<td>2.6 ± 0.1</td>
<td>7.2 ± 0.3</td>
<td>0.98 ± 0.06</td>
<td>0.30 ± 0.04</td>
<td>30.7 ± 3.9</td>
</tr>
<tr>
<td>5-HD</td>
<td>2.1 ± 0.1</td>
<td>7.3 ± 0.3</td>
<td>1.19 ± 0.10</td>
<td>0.46 ± 0.05</td>
<td>38.8 ± 1.5</td>
</tr>
<tr>
<td>Diaz + 5-HD</td>
<td>2.2 ± 0.1</td>
<td>7.5 ± 0.3</td>
<td>1.09 ± 0.10</td>
<td>0.42 ± 0.06</td>
<td>37.3 ± 3.8</td>
</tr>
<tr>
<td>Aniso(50)</td>
<td>2.0 ± 0.1</td>
<td>6.7 ± 0.2</td>
<td>0.85 ± 0.10</td>
<td>0.20 ± 0.06</td>
<td>21.0 ± 3.9</td>
</tr>
<tr>
<td>Aniso(100)</td>
<td>2.3 ± 0.1</td>
<td>6.8 ± 0.3</td>
<td>0.94 ± 0.07</td>
<td>0.22 ± 0.05</td>
<td>21.6 ± 4.1</td>
</tr>
<tr>
<td>Aniso + 5-HD</td>
<td>2.3 ± 0.1</td>
<td>7.5 ± 0.3</td>
<td>1.05 ± 0.12</td>
<td>0.46 ± 0.09</td>
<td>40.8 ± 3.8</td>
</tr>
</tbody>
</table>

Values are means ± SE. Wt, weight; I/R, ratio of infarct volume to risk zone volume. *\( P < 0.05 \) vs. control.
Figure 7 shows that anisomycin reduced the number of stained cells (62.0 ± 2.8%·h) compared with controls. However, 5-HD completely reversed this protective effect (72.6 ± 3.4%·h). The survival rate of oxygenated cells treated with either cytochalasin D or 5-HD was no different from oxygenated controls over 3 h.

**DISCUSSION**

Previous studies have shown that adenosine and other receptors trigger ischemic preconditioning in the rabbit heart (11, 22, 27). Subsequent activation and translocation of protein kinase C are then required (29, 45). Downstream signaling components include tyrosine kinase (3) and MAP kinases (43). However, the final effector protein(s) activated during preconditioning have remained controversial.

The observation that the benzopyran derivatives such as cromakalim and bimakalim were able to induce cardioprotection independently of APD shortening suggested that sarcolemmal KATP channels were not the site of action (14, 15, 44). Demonstration that the reportedly selective KATP-channel blocker 5-HD could inhibit protection without affecting activation of sarcolemmal KATP current induced by cromakalim (26) further eroded support of the sarcolemmal channels as the
end effector. Garlid et al. (10) reported that diazoxide and cromakalim were able to restore ATP-inhibited K⁺ flux in bovine cardiac mitochondria. Therefore, cromakalim was also opening the mitochondrial K⁺ channel within the same concentration range as its protective effect. Hence it was suggested that the protection of openers such as cromakalim was more likely to be related to the drug’s action on mitochondrial channels rather than those in the sarcolemma. However, although cromakalim was also a potent activator of cardiac sarcolemmal K⁺ channels, very high (near millimolar) concentrations of diazoxide were required to open those channels on the surface membrane (9). Therefore, diazoxide is ~2,000-fold more selective for mitochondrial versus sarcolemmal K⁺ channels.

The present study demonstrates that intravenous injection of diazoxide 10 min before ischemia greatly reduces infarct size in the rabbit heart. These are the first data indicating that direct opening of mitochondrial K⁺ channels by diazoxide can mimic the anti-infarct effect of ischemic preconditioning. Garlid et al. (9) utilized 30 µM diazoxide to protect isolated rat hearts. Consequently, the present study used a dose of 10 mg/kg. If diazoxide distributes in total body water, then a dose of 10 mg/kg would yield an approximate plasma concentration of 40 µM, which should, therefore, be protective. We did not test whether lower doses would still provide a similar degree of protection. Protection was also observed in isolated rabbit ventricular myocytes exposed to diazoxide. These data confirm the recent observations of Liu et al. (23), who also showed that diazoxide reduced osmotic fragility in rabbit myocytes subjected to 2 h of simulated ischemia. The link between mitochondrial K⁺ channels and osmotic fragility is enigmatic. An improved energetic state seems unlikely because preserved cytosolic ATP is not a feature of the preconditioned rabbit cardiomyocyte model (1).

If opening of these K⁺ channels is indeed the end step in the mechanism of preconditioning, then it could be reasoned that diazoxide should still exert a protective effect if given after the onset of ischemia because the delay associated with the upstream signal transduction pathway would be bypassed. However, when diazoxide was administered 5 min into the 30-min ischemia, a decrease in infarct size was no longer observed. This is probably because collateral flow is very low (~5 ml·min⁻¹·100 g tissue⁻¹) in the rabbit. For example, if we apply a simple first-order wash-in model (half-time = time to achieve 50% concentration = (100 × 0.69)/flow rate), it can be calculated that diazoxide would enter the ischemic zone with a half-time of 14 min, i.e., very slowly. At the end of the 30-min ischemic insult, tissue concentration would have reached only ~78% of that in plasma. Obviously, the drug must have arrived too late and in too small an amount to produce any beneficial effect in the posttreatment group. Whether this schedule might be beneficial in a model with a higher collateral flow such as a dog is unknown. In a dog in which collateral flow averages 20 ml·min⁻¹·100 g tissue⁻¹, drug would reach the heart with a half-time of only 3.5 min.

To confirm that diazoxide was indeed acting through mitochondrial K⁺ channels, we tested whether 5-HD could inhibit the reduction in infarct size. 5-HD has been shown to reverse diazoxide-induced mitochondrial K⁺ flux (9). This inhibition is dependent on the presence of ATP, Mg²⁺, and a physiological or pharmacological opener such as diazoxide (19). Increases in mitochondrial redox state in response to diazoxide can also be prevented with this blocker (23). However, 5-HD does not affect sarcolemmal K⁺ channels (26, 32; unpublished results with rabbit cardiomyocytes). Therefore, 5-HD appears to selectively block mitochondrial K⁺ channels, and, as expected, pretreatment with 5 mg/kg 5-HD completely abolished the protective effect of diazoxide in the intact rabbit heart.

Diazoxide is a potent vasodilator (9, 30) and is used clinically as an antihypertensive agent. In the anesthetized rabbits, diazoxide elicited a 25-mmHg reduction in mean arterial blood pressure. Such a modest reduction in driving pressure should not have preconditioned the heart because in a previous study we lowered the rabbit’s systemic pressure to 41 mmHg with the A₁-selective adenosine analog CGS-21680 with no sign of protection (39). In addition, as noted above, diazoxide also protected isolated myocytes as well as isolated rat hearts (9) in which hypotension was not a complicating factor, suggesting that the infarct-reducing effect of the K⁺ opener is not dependent on any hemodynamic alteration.

It might also be argued that in lieu of a specific effect of 5-HD on mitochondrial channels the tachycardia induced by the combination of diazoxide and 5-HD could have contributed to the increase in infarction in this group. However, in a previous study from our laboratory, injection of the norepinephrine precursor tyramine caused an elevation in heart rate similar to that seen in the present study, but in fact reduced infarct size (38). This protective effect was mediated by α₁-receptors. Therefore, increased heart rate per se does not exacerbate ischemic injury in the anesthetized rabbit.

Previous studies have strongly implicated a role for p38 MAP kinase in ischemic preconditioning in the rabbit (41, 43). Furthermore, anisomycin, which activates MAP kinase kinases 3, 4, 6, and 7 and hence the p38 MAP kinase and JNK pathways, mimics preconditioning in isolated rabbit hearts and myocytes (3, 43). Intravenous injection of 50 µg/kg anisomycin, which would approximate the concentration used in vitro (3), 10 min before ischemia was able to reduce infarct size in the in situ rabbit model. Doubling the dose offered no additional protection. Pretreatment with anisomycin before simulated ischemia was just as protective as ischemic preconditioning in the isolated ventricular myocytes. If stress-activated MAP kinases truly are involved in the mechanism of ischemic preconditioning, then protection elicited by direct activation of p38 MAP kinase/JNK should also be dependent on mitochondrial K⁺ channels, which seem to be so important in
preconditioning. In the present study, 5-HD completely blocked the protective effects of anisomycin in both the anesthetized rabbits and the isolated myocytes. Therefore, like the protection mediated by both diazoxide and ischemic preconditioning, that induced by anisomycin is also sensitive to 5-HD. This confirms that anisomycin is stimulating the same signaling pathway used in ischemic preconditioning and supports the hypothesis that p38 MAP kinase and/or JNK plays an important role in ischemic preconditioning in the rabbit heart.

The involvement of p38 MAP kinase would suggest that changes in the cytoskeleton may also be involved in cardioprotection because activation of the p38 MAP kinase pathway ultimately leads to the serine phosphorylation of HSP27 via the action of MAPKAPK2 (5, 31, 36). The phosphorylated form of HSP27 can act as an end cap, thus stabilizing actin filaments, and can even promote actin polymerization (21). Guay et al. (16) have shown that activation of the p38 MAP kinase pathway greatly increased phosphorylation of HSP27 in CCL39 cells and prevented the disruption of actin filaments by cytochalasin D. Overexpression of HSP27 in these cells also conferred cytoskeletal stability against either cytochalasin or hydrogen peroxide (16, 18). Importantly, increased expression of HSP27 in rat myocytes rendered them resistant to simulated ischemia (24).

In the present investigation, cytochalasin D, which would oppose actin filament polymerization, abolished the reduction in osmotic fragility seen with preconditioning in rabbit myocytes but was without effect in control cells. Therefore, modulation of the actin cytoskeleton appears to be a necessary step in this mechanism. Unexpectedly, cytochalasin D was also able to prevent protection by the direct K\textsubscript{ATP} openers pinacidil and diazoxide. These data suggest that changes in actin filaments and opening of mitochondrial K\textsubscript{ATP} channels may somehow be linked in ischemic preconditioning. Previously, Terzic and Kurachi (37) reported that disruption of actin filaments could reverse ATP-induced inhibition of sarcolemmal K\textsubscript{ATP} channels. This would imply that disruption of actin opens these channels, thus contrasting with our conclusion. However, the opposite was seen in guinea pig myocyte patches in which cytochalasin D induced K\textsubscript{ATP}-channel rundown, which could be reversed with application of purified F-actin and MgATP (6). Therefore, the integrity of the cytoskeleton can markedly influence the activity of at least sarcolemmal K\textsubscript{ATP} channels, but whether cytoskeletal disruption results in opening or closing of the channel is still controversial. There have been no studies examining effects of cytochalasin directly on mitochondrial channels. It is conceivable that ischemic preconditioning preserves actin filaments during ischemia by activation of the p38 MAP kinase/HSP27 pathway. This in turn may initiate and maintain opening of mitochondrial K\textsubscript{ATP} channels with ultimate salvage of cell integrity.

It still is not clear why opening of mitochondrial K\textsubscript{ATP} channels is cardioprotective. Opening would cause a net influx of K\textsuperscript{+}, which should increase matrix volume (8), dissipate energy, and uncouple oxidative phosphorylation. This may be beneficial in ischemia by preventing wasteful ATP hydrolysis (9) and reducing the electrical gradient favoring Ca\textsuperscript{2+} influx into mitochondria (23).

In conclusion, activation of mitochondrial K\textsubscript{ATP} channels by diazoxide reduces infarct size and protects isolated rabbit myocytes. Disruption of the actin cytoskeleton blocks the protection mediated by both ischemic preconditioning and diazoxide, suggesting that cardioprotection in the rabbit heart requires a coupling between mitochondrial K\textsubscript{ATP} channels and actin filaments.

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