Bradykinin induces mitochondrial ROS generation via NO, cGMP, PKG, and mitoK\textsubscript{ATP} channel opening and leads to cardioprotection

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Bradykinin (BK) mimics ischemic preconditioning by generating reactive oxygen species (ROS). To identify intermediate steps that lead to ROS generation, rabbit cardiomyocytes were incubated in reduced MitoTracker Red stain, which becomes fluorescent after exposure to ROS. Fluorescence intensity in treated cells was expressed as a percentage of that in paired, untreated cells. BK (500 nM) caused a 51.1% increase in ROS generation (P < 0.001). Coincubation with either the BK B\textsubscript{2}-receptor blocker HOE-140 (5 μM) or the free radical scavenger N-(2-mercaptopropionylylglycine (1 mM) prevented this increase, which confirms that the response was receptor mediated and ROS were actually being measured. Closing mitochondrial ATP-sensitive K\textsuperscript{+} (mitoK\textsubscript{ATP}) channels with 5-hydroxydecanoate (5-HD, 1 mM) prevented increased ROS generation. BK-induced ROS generation was blocked by N\textsuperscript{ω}-nitro-L-arginine methyl ester (L-NAME, 200 μM) or the nitric oxide donor S-nitroso-N-acetylpenicillamine (1 μM) increased ROS by 34.3% (P < 0.001). This protection was abolished by 5-HD. Finally, the nitric oxide donor S-nitroso-N-acetylpenicillamine (1 μM) increased ROS by 34.6%. This increase was also blocked by 5-HD. In intact rabbit hearts, BK (400 nM) decreased infarction from 30.5 ± 3.0% of the risk zone in control hearts to 11.9 ± 1.4% (P < 0.001). This protection was abolished by either 200 μM L-NAME or 2 μM ODQ (35.4 ± 5.7 and 30.4 ± 3.0% infarction, respectively; P = not significant vs. control). Hence, BK preconditions through receptor-mediated production of nitric oxide, which activates guanylyl cyclase. The resulting cGMP activates PKG, which opens mitoK\textsubscript{ATP}. Subsequent release of ROS triggers cardioprotection.

mitochondrial ATP-sensitive potassium channel; nitric oxide; preconditioning; reactive oxygen species; protein kinase G

BRIEF ISCHEMIA CAN PROTECT the heart against lethal injury following a sustained ischemic period (17), a phenomenon known as ischemic preconditioning. In the last decade, several endogenous and exogenous substances have been identified that are able to mimic this protective effect of ischemic preconditioning (32).

Whereas the complete mechanism by which brief ischemia actually protects the heart is still unknown, the opening of mitochondrial ATP-sensitive K\textsuperscript{+} (mitoK\textsubscript{ATP}) channels and the generation of reactive oxygen species (ROS) play crucial roles in the signaling pathway of ischemic and drug-induced preconditioning (21). We previously reported that opening mitoK\textsubscript{ATP} channels triggers the onset of the preconditioned state by causing the generation of ROS (24). Several receptor agonists able to mimic ischemic preconditioning do so by opening mitoK\textsubscript{ATP} channels and inducing ROS generation and thus share elements of the signaling cascade of ischemic preconditioning (6).

In our recent studies of the preconditioning signaling pathway, we principally examined the functional and biochemical effects of ACh, which is a known exogenous trigger of cardioprotection in intact hearts (6, 37). We found that ACh induces ROS production in both vascular smooth muscle cells (13, 23) and isolated rabbit ventricular myocytes (22). We also demonstrated that muscarinic surface receptors, G\textsubscript{i} proteins, phosphatidylinositol 3-kinase, and Src are all signaling elements in ACh-mediated mitoK\textsubscript{ATP} channel opening and ROS generation (22). Moreover, the critical time for activation of all of the above is the trigger phase of preconditioning, i.e., before the index ischemia (28). However, ACh is not an endogenous cardioprotective agent, as there is no mechanism for its release during the preconditioning ischemia.

It has been shown that bradykinin (BK) along with adenosine and opioids is important in the mechanism of ischemic preconditioning (9, 35) and may even mediate remote preconditioning (31). It is a preconditioning mimic, and its interstitial concentration increases during ischemic preconditioning (25). Moreover, in patients undergoing coronary angioplasty, BK was able to induce preconditioning (14). As was the case with ACh, opening of mitoK\textsubscript{ATP} channels (6, 12) and generation of ROS (6) were also found to be key signaling elements in the BK signaling cascade.

The aim of the present study was to examine the BK signaling pathway leading to ROS generation in cardiomyocytes. We wanted to see whether BK, like ACh, could trigger ROS generation; if so, we sought to identify the pathway involved. These studies were conducted on isolated adult rabbit cardiomyocytes, which is a convenient model for scrutiny of this intricate signaling pathway. The use of cardiomyocytes
rather than other available cell types increased the likelihood that the data could be extrapolated to intact heart. Parts of the pathway were indeed confirmed in intact rabbit heart.

METHODS

This study was performed in accordance with The Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996).

Adult Rabbit Cardiomyocytes

Rabbit ventricular myocytes were isolated by collagenase perfusion (type 2, Worthington; Lakewood, NJ) as described previously in detail (1, 22). Briefly, New Zealand White rabbits were anesthetized with pentobarbital sodium (30 mg/kg iv), anticoagulated with heparin (1,000 UI/kg iv), and ventilated with 100% oxygen. The heart was excised and quickly mounted on a Langendorff apparatus where it was retrogradely perfused with calcium-free Krebs-Henseleit-HEPES buffer that contained collagenase (200 U/ml) at 37°C. After the heart became soft, it was minced and passed through a nylon mesh (diameter, 200–350 μm). Viable myocytes were separated by repetitive slow-speed centrifugation in buffer that contained 2% bovine serum albumin. Cells were made calcium tolerant by stepwise restoration of calcium in the medium to 1.25 mM. Usually, 30–35 million viable, calcium-tolerant cells were extracted per heart. Preparations were considered satisfactory if rod-shaped cells accounted for >65% of the cells.

Immediately after the isolation and separation procedure, cells were plated on no. 1.0 borosilicate glass coverslips in four-well chambers (Lab-Tek II, Nunc; Naperville, IL) using creatine (5 mM), L-carnitine (2 mM), and taurine (5 mM)-supplemented medium 199 (CCT-medium 199) as described by Piper et al. (26, 27) and Mitcheson et al. (16). Penicillin (10 U/ml) and streptomycin (10 μg/ml) were added as antibiotics. Cells were stored in incubators at 37°C in air enriched with 5% CO2. A first medium change was performed after 3–4 h; afterward, cells were allowed to adjust for at least 24 h. Experiments were performed 2–5 days after myocyte isolation.

Experimental Design

Each experiment started with a change of medium in the wells for 10 min. The medium was then removed and replaced with one that contained the drug or the drug with blocker (if required) and reduced MitoTracker Red as a dye (500 nM, Molecular Probes; Eugene, OR). This reduced form of the probe is nonfluorescent, but it becomes fluorescent when oxidized by ROS. Some of the oxidized product becomes bound to thiol groups and proteins within the cell. After a 15-min incubation with reduced MitoTracker Red, cells were washed twice with fresh MitoTracker Red-free CCT-medium 199. The wash serves two purposes. First, it removes any unbound and thus voltage-dependent pool of oxidized MitoTracker Red held in the mitochondria (13). Second, it removes any unreacted reduced MitoTracker Red to stop the reaction. All that should remain after washing is the protein-bound fluorescent product. Indeed, we found the fluorescence to be stable after the wash for at least 30 min.

In experiments in which the effects of the blockers HOE-140 (5 μM), N-(2-mercaptopropionyl)glycine (MPG, 1 mM), glibenclamide (Gli, 50 μM), 5-hydroxydecanoate (5-HD, 1 mM), myxothiazol (Myxo, 200 nM), N-nitro-l-arginine methyl ester (l-NNAME, 200 μM), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), or 8-bromoguanosine-3’-5’-cyclic monophosphorothioate, Rp-isomser (Rp-8-Br-cGMP, 25 μM) on ROS production by either BK (500 nM), diazoxide (Diaz, 50 μM), or 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP, 100 μM) were to be examined, each blocker was present in the medium during the 10-min period before the additions of reduced MitoTracker Red and agonist.

To further evaluate the role of nitric oxide (NO) in this signaling pathway, we exposed cells to the NO donor S-nitroso-N-acetylpenicillamine (SNAP, 1 μM) in lieu of BK both in the presence and absence of 5-HD (200 μM).

Measurement of ROS Production

Experiments were designed such that four different conditions were always simultaneously evaluated. ROS generation was analyzed by measuring the fluorescence of 15–30 individual rod-shaped cells that were randomly selected within each well of a four-well chamber. In each field, cells were either obviously rod shaped and considered to be viable or clearly round and considered to be dead. There were no intermediate shapes. Rods comprised at least 50% of the cells in each field. The average fluorescence for the selected cells in each well was computed and compared with the average single-cell fluorescence in the respective control well that was always included in the same four-well chamber. Thus the treated cells were only compared with untreated cells of the same age and isolation and were stained with the same reduced MitoTracker Red lot. Single-cell fluorescence was quantified as described previously (13, 23) using a Nikon TMS-F microscope with a ×20 objective, an XF filter set (Omega Optical; Brattleboro, VT), a xenon light source with a Lambda 10-2 optical filter changer (excitation, 560 nm; emission, 610 nm; light exposure, 0.1 s; Sutter Instruments; Novato, CA), and a COHU 6600 scan charge-coupled device camera (COHU; San Diego, CA) using software from Intracelllar Imaging (Cincinnati, OH). Each set of experiments was repeated 8–10 times on different days with cells of different ages. Approximately 200–250 typical rod-shaped cells contributed data for each experiment.

Isolated Heart Model

New Zealand White rabbits of either sex were used. As previously described (9), hearts from animals anesthetized with pentobarbital sodium were exposed through a left thoracotomy, and a suture was passed around a prominent coronary artery branch visible on the epicardial surface. The heart was then removed, mounted on a Langendorff apparatus, and perfused with modified Krebs-Henseleit bicarbonate buffer that contained (in mM) 118.5 NaCl, 24.7 NaHCO3, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 2.5 CaCl2, and 10 glucose. The buffer was gassed with 95% O2–5% CO2, which resulted in a pH value of 7.4–7.5. The temperature of the perfusate was maintained at 37°C. A fluid-filled latex balloon connected to a transducer with polyethylene-240 tubing was inserted into the left ventricle. Balloon volume was adjusted to set the left ventricular end-diastolic pressure equal to 5–10 mmHg at the beginning of the experiment. Total coronary flow was measured by timed collection of perfusate that dripped from the right heart into a graduated cylinder.

Six groups of hearts were studied. All hearts were subjected to 30 min of global ischemia by arrest of retrograde aortic perfusion and were then reperfused for 2 h. Control hearts were subjected to only 30 min of global ischemia and then reperfusion. One group of hearts was treated with BK (400 nM) for 5 min, followed by 10 min of washout before the long ischemia. A third group was also treated with BK. In addition, l-NNAME (200 μM) was added to the perfusate for 15 min starting 5 min before and ending 5 min after BK treatment. Therefore, there was 5 min of washout before coronary occlusion. The fourth group of hearts was treated with only l-NNAME as noted above before commencement of the ischemic period. In the final two groups, ODQ (2 μM) was infused either alone for 15 min as was done with l-NNAME or to bracket a 5-min infusion of BK.

Measurement of Infarct Size

At the end of the experiment, the coronary artery was reoccluded, and 2- to 9-μm fluorescent polymer microspheres (Duke Scientific; Palo Alto, CA) were infused into the perfusate to demarcate the ischemic zone (region at risk) as the area of tissue without fluorescence. The heart was weighed and frozen and then cut into 2-mm-thick slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) in sodium phos-
phate buffer (pH 7.4) at 37°C for 20 min. TTC stains noninfarcted myocardium brick red. The slices were then immersed in 10% formalin to preserve the stained (viable) and unstained (necrotic) tissue. The risk zone was identified by illuminating the slices with ultraviolet light. The areas of infarct and risk zone were determined by planimetry of each slice, and the volumes were calculated by multiplying each area by the slice thickness and summing them for each heart. Infarct size is expressed as a percentage of the risk zone.

Chemicals

All drugs required for cell isolation and culture were purchased from Sigma Chemical (St. Louis, MO). Reduced MitoTracker Red was purchased from Molecular Probes. BK, HOE-140, Gli, 5-HD, Myxo, and SNAP were obtained from Sigma; L-NAME, ODQ, and Rp-8-Br-cGMPS were from Alexis Biochemicals (Qbiogene; Carlsbad, CA), and 8-pCPT-cGMP was from Biolog Life Science (Bremen, Germany). Either distilled water or DMSO was used to dissolve the drugs and to prepare stock solutions. The final DMSO concentration was kept <1%.

Data Analysis

Fluorescence measurements present the data in arbitrary units (au). To remove the variability introduced by different lots of reduced MitoTracker Red, cell age, and environmental conditions, the average cell fluorescence was calculated and compared to that of simultaneously studied control cells as described. Therefore, fluorescence data are provided as a percentage of the respective controls (means ± SE). To further minimize the possible influence of these variables on the data, ANOVA for repeated measures with Bonferroni’s correction was used to test for differences in mean fluorescence (in au) of the groups within the same four-well chamber. For the isolated heart studies, baseline hemodynamic variables in experimental groups were compared with those in the control group by one-way ANOVA, and changes with time in any given group were compared to baseline by ANOVA with repeated measures. Infarct size data were compared by one-way ANOVA. Differences were considered to be significant if the P value was <0.05.

RESULTS

Isolated Cardiomyocytes

BK B2-receptor activation. Exposure of cardiomyocytes to BK led to a robust increase in ROS production (51 ± 16%; P < 0.01; Fig. 1). Coincubation with the highly selective B2-receptor blocker HOE-140 prevented the BK effect on ROS generation [−19 ± 8%; P = not significant (NS)]. HOE-140 itself had no effect on ROS production (−5 ± 8%; P = NS).

Scavenging of free radicals. To confirm that the increase in MitoTracker Red fluorescence induced by BK was caused by free radical production, experiments were performed with the free radical scavenger MPG (Fig. 2). In these experiments, BK led to the expected increase in fluorescence (56 ± 11%; P < 0.001). Coincubation with MPG abolished the increase in fluorescence (−6 ± 6%; P = NS). As in previous experiments, MPG itself had no impact on cell fluorescence (8 ± 12%; P = NS).

KATP channel blockade. To test whether BK-induced ROS formation is dependent on KATP channel opening, two different KATP channel antagonists were used. In these experiments, BK led to the expected increase in ROS production (49 ± 7%; P < 0.001), and Gli, which is a nonselective closer of KATP channels, was able to block that increase (−4 ± 10%; P = NS; Fig. 3). Gli itself had no impact on ROS generation (5 ± 9%; P = NS). Selective blockade of mitoKATP channels with 5-HD produced similar results (Fig. 4). The increase in ROS formation triggered by BK (54 ± 10%; P < 0.001) was blocked by 5-HD (5 ± 10%; P = NS). As before, 5-HD had no independent influence on ROS generation (7 ± 10%; P = NS).
Source of ROS. Blockade of the mitochondrial respiratory chain at site III with the selective blocker Myxo aborted the BK-induced ROS generation, which suggests that the mitochondrial respiratory chain is the source of the free radicals (Fig. 5). The increase in ROS following BK exposure (42 ± 11%; *P* < 0.03) was abolished by Myxo (2 ± 13%; *P* = NS). Myxo itself had no impact on ROS formation (7 ± 5%; *P* = NS).

NO dependency. Coincubation of cells with l-NAME, which is an inhibitor of NO synthase (NOS), and BK completely abolished (−6 ± 7%; *P* = NS) the increase in ROS production that was seen with BK alone (50 ± 9%; *P* < 0.001; Fig. 6). l-NAME exposure itself had no impact on ROS generation (−4 ± 12%; *P* = NS). To determine whether NO signaling is located upstream of the mitoKATP channel, diazoxide, which is a direct opener of mitoKATP channels, was used to trigger ROS production. Diazoxide was previously reported to increase ROS generation in this model by 50% (22). l-NAME was unable to block the increase in ROS production from diazoxide (54 ± 19%; *P* < 0.02; Fig. 6).

NO-sensitive guanylyl cyclase. Inhibition of NO-sensitive guanylyl cyclase with ODQ completely abolished BK-induced ROS production (−12 ± 7%; *P* = NS vs. 47 ± 9% for BK alone; *P* < 0.02; Fig. 7). ODQ itself had no impact on ROS generation (10 ± 8%; *P* = NS). The increased ROS production from the mitoKATP opener diazoxide could not be blocked by ODQ (32 ± 10%; *P* < 0.02; Fig. 7).

cGMP-dependent protein kinase G. Inhibition of protein kinase G (PKG) by the cell-permeant PKG inhibitor Rp-8-Br-cGMPS blocked BK-induced ROS generation in cardiomyocytes (Fig. 8). Whereas BK alone caused a significant increase in ROS production (45 ± 8%; *P* = 0.003), this increase was aborted by coincubation with Rp-8-Br-cGMPS (6 ± 11%; *P* = NS). The latter by itself had no influence on fluorescence (9 ± 9%; *P* = NS). On the other hand, direct activation of PKG with 8-pCPT-cGMP (Fig. 9) led to an increase in ROS production (39 ± 15%; *P* = 0.004) comparable to that seen previously with BK. This PKG-dependent ROS generation was blocked by 5-HD (0 ± 6%; *P* = NS), which suggests that PKG is upstream of mitoKATP channels. Again, 5-HD alone had no influence on ROS generation (−7 ± 4%; *P* = NS).

Intact Rabbit Heart

Hemodynamics are presented in Table 1. At baseline, there were no differences between groups except for the BK hearts,
which had developed pressure that was modestly higher than control hearts, and the mildly elevated heart rate in hearts destined to be exposed to the combination of BK and ODQ. The vasodilator BK tended to decrease left ventricular developed pressure and coronary flow, whereas L-NAME significantly decreased coronary flow and developed pressure. ODQ had only a mild depressant effect on developed pressure and coronary flow. In all groups, developed pressure and coronary flow measurements were significantly lower than baseline values during coronary occlusion with partial recovery during reperfusion.

Table 2 contains the infarct size data. There were no differences in risk zone volume in the six groups. Infarction in control hearts averaged 30.5 ± 3.0% of the risk zone (Fig. 11). BK salvaged ischemic tissue and reduced infarct size to 11.9 ± 1.4% of the risk zone (P < 0.01 vs. control). However, this protection was aborted when the BK infusion was bracketed with a simultaneous infusion of L-NAME or ODQ, there continued to be a modest decline in developed pressure and coronary flow. In all groups, developed pressure and coronary flow measurements were signifi cantly lower than baseline values during coronary occlusion with partial recovery during reperfusion.

**DISCUSSION**

The mechanism of the protective effect of ischemic preconditioning remains elusive after nearly 15 years of intensive effort. The end effector is not known, nor is the complete signaling pathway. Thorough identification of either should provide enough insight to make extrapolation to clinical areas possible. Examination of the steps of the signaling pathway is greatly facilitated by using an isolated cardiomyocyte model. Multiple agonists and blockers can be studied without concern for hemodynamic effects, confusing independent endothelial interactions, and drug costs. Using this model, we have already confirmed that ACh leads to ROS production in the mitochondria of the cardiomyocyte as a result of the opening of mitochondrial K<sub>ATP</sub> channels (22). The present data confirm the same step for BK, which suggests a possible common pathway for all Gi-coupled receptor agonists. The present study provides important new insights into this pathway. Importantly, NO, guanylyl cyclase, cGMP, and PKG are also key players in opening mitochondrial K<sub>ATP</sub> channels and in production of ROS in rabbit cardiomyocytes.

By activating the B<sub>2</sub> receptor, BK becomes a physiological trigger of ischemic preconditioning (9, 35). HOE-140, a selective antagonist of this receptor subtype, blocks BK-induced cardioprotection in intact hearts (9, 34). Recently it was shown that ACh leads to ROS production in the mitochondria of the cardiomyocyte as a result of the opening of mitochondrial K<sub>ATP</sub> channels (22). The present data confirm the same step for BK, which suggests a possible common pathway for all G<sub>i</sub>-coupled receptor agonists. The present study provides important new insights into this pathway. Importantly, NO, guanylyl cyclase, cGMP, and PKG are also key players in opening mitochondrial K<sub>ATP</sub> channels and in production of ROS in rabbit cardiomyocytes.

By activating the B<sub>2</sub> receptor, BK becomes a physiological trigger of ischemic preconditioning (9, 35). HOE-140, a selective antagonist of this receptor subtype, blocks BK-induced cardioprotection in intact hearts (9, 34). Recently it was shown that protection from BK, just like that from ACh, could be blocked by the ROS scavenger MPG (6). Indeed, we now show that exposure of myocytes to BK causes a robust increase in cell fluorescence that is completely blocked by coincubation with the B<sub>2</sub>-receptor blocker HOE-140. MPG, a free radical scavenger, also eliminates this increase in fluorescence, which confirms that ROS generation was being monitored. Myxo, a blocker of site III of the mitochondrial respiratory chain, interferes with ROS generation as well, thereby confirming mitochondrial electron transport as the source of free radical generation. These results agree closely with previous reports of other receptor agonists by us (22, 23) and others (4, 38).

NO was previously proposed to be part of the protective mechanism of BK. BK was shown to improve recovery of
ventricular function after cardioplegic ischemia in isolated rabbit hearts, and L-NAME, a NOS inhibitor, was able to block this beneficial effect (8). Of course, these experiments in intact heart could not distinguish between signal elements located in endothelial cells and myocytes. Classically, BK is assumed to stimulate NO production in endothelial cells. Activation of BK receptors on the surface of endothelial cells leads to calcium-mediated NO production via NOS. Feng et al. (8) speculated that NO produced in endothelial cells acted as a second messenger between the vascular endothelium and the myocytes. However, our cell culture has very few, if any, endothelial cells present, and fluorescence was measured only in rod-shaped cardiomyocytes. Our results indicate that BK causes NO production in ventricular myocytes because BK-induced ROS generation in these cells could be blocked by L-NAME.

It has been known for some time that NO can mimic ischemic preconditioning. Nakano et al. (18) noted that the NO donor SNAP could precondition the rabbit heart against infarction. Because they found that a free radical scavenger could block the protection, they speculated that NO could be forming peroxynitrite, which may directly activate protective kinases. Nakano et al. could not block ischemic preconditioning with 100 μM L-NAME. Rakhit et al. (29) found that neonatal rat

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### Table 1. Hemodynamics of isolated hearts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>HR (beats/min)</th>
<th>BP (mmHg)</th>
<th>CF (ml/min g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>305 ± 15</td>
<td>112 ± 12</td>
<td>192 ± 6</td>
</tr>
<tr>
<td>Bradykinin + L-NAME</td>
<td>205 ± 8</td>
<td>121 ± 7</td>
<td>192 ± 2.2</td>
</tr>
<tr>
<td>ODQ + Bradykinin</td>
<td>230 ± 8</td>
<td>110 ± 3</td>
<td>197 ± 2.2</td>
</tr>
<tr>
<td>Values are mean ± SE; n = 5 hearts for ODQ group; n = 6 hearts for all other groups.</td>
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</tbody>
</table>

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### Table 2. Infarct data for isolated hearts

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Wt, kg</th>
<th>Heart Wt, g</th>
<th>Risk Zone, cm³</th>
<th>Infarct Size, cm³</th>
<th>I/R, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2 ± 0.1</td>
<td>5.6 ± 0.1</td>
<td>1.20 ± 0.08</td>
<td>0.37 ± 0.05</td>
<td>30.5 ± 3.0</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>2.0 ± 0.1</td>
<td>5.3 ± 0.2</td>
<td>1.17 ± 0.16</td>
<td>0.15 ± 0.04*</td>
<td>11.9 ± 1.4†</td>
</tr>
<tr>
<td>L-NAME</td>
<td>2.1 ± 0.1</td>
<td>7.9 ± 0.3†</td>
<td>1.55 ± 0.11</td>
<td>0.47 ± 0.03</td>
<td>30.8 ± 2.4</td>
</tr>
<tr>
<td>Bradykinin + L-NAME</td>
<td>2.2 ± 0.2</td>
<td>5.9 ± 0.5</td>
<td>1.27 ± 0.14</td>
<td>0.45 ± 0.07</td>
<td>35.4 ± 5.7</td>
</tr>
<tr>
<td>ODQ + Bradykinin</td>
<td>2.3 ± 0.1</td>
<td>5.8 ± 0.4</td>
<td>1.30 ± 0.08</td>
<td>0.36 ± 0.05</td>
<td>27.2 ± 2.3</td>
</tr>
<tr>
<td>ODQ + Bradykinin</td>
<td>1.9 ± 0.1</td>
<td>6.0 ± 0.5</td>
<td>1.22 ± 0.15</td>
<td>0.39 ± 0.08</td>
<td>30.4 ± 3.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 hearts for ODQ group; n = 6 hearts for all other groups. I/R, ratio of infarct volume to risk zone volume. *P < 0.05, †P < 0.01, statistical significance of difference between experimental group and control.

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Fig. 11. Infarct size as a percentage of risk zone. Individual hearts (○) and group means with superimposed standard errors (●) are shown. At reperfusion, bradykinin decreased infarct size, and this salutary effect was blocked by both the NO synthase inhibitor L-NAME and the guaneryl cyclase antagonist ODQ; *P < 0.01.
ventricular myocytes could be pharmacologically preconditioned by application of SNAP. In addition, cardioprotection from simulated ischemia was blocked in these cells by the nonspecific NOS inhibitor \( \text{L-NAME} \). However, in the experiments of Rakhit et al. (29), ODQ, the guanylyl cyclase inhibitor, was also able to block SNAP-induced preconditioning, which indicates the dependency of NO-triggered cardioprotection on cGMP. These results agree closely with our observation that ODQ blocked BK-induced ROS generation. In the present study, we too observed that SNAP increased ROS generation. Therefore, these data are consistent with the role of NO as an important second messenger in the signaling that leads to ROS production and ultimately cardioprotection.

Despite these similarities between the signaling in neonatal rat ventricular myocytes (29) and our present data in adult rabbit cardiomyocytes, one obvious difference was the failure of Rakhit et al. to abort SNAP-induced cardioprotection by blockade of KATP channels with either Gli or 5-HD. This led the authors to conclude that SNAP-induced protection was independent of sarcoclemmal/mitochondrial ATP-sensitive K\(^+\) channel opening. However, our prior study clearly showed a role for both mitoK\(_{\text{ATP}}\) channels and ROS in the anti-infarct mechanism of BK in rabbit hearts (6). And our present data demonstrate that 5-HD completely blocks the ability of SNAP to increase ROS generation. The source of the discrepancy is not clear. Species difference certainly could be a factor; also, the end points were quite different. There is also evidence that neonatal ventricular myocardium lacks functional mitoK\(_{\text{ATP}}\) channels. Preliminary studies have demonstrated that, in contrast with adult rat hearts, neonatal hearts cannot be protected by preischemic exposure to the K\(_{\text{ATP}}\) channel openers diazoxide and cromakalim (2). And as demonstrated in at least human neonatal cardiomyocytes, there is a paucity of mitoK\(_{\text{ATP}}\) channels (7).

Han and coworkers (11) investigated surface K\(_{\text{ATP}}\) channel activity in adult rabbit ventricular myocytes using the patch-clamp technique. Their study showed that K\(_{\text{ATP}}\) channel activation in these cells can occur through a signal transduction pathway that involves guanylyl cyclase activation, increased production and accumulation of cGMP, and activation of PKG, and they proposed that K\(_{\text{ATP}}\) channel phosphorylation and activation resulted. This signaling pathway is quite similar to ours, although it should be noted that Han et al. were studying the sarcoclemmal as opposed to the mitochondrial K\(_{\text{ATP}}\) channel. In our experiments, Gli, a nonselective blocker of K\(_{\text{ATP}}\) channels, as well as the highly selective mitoK\(_{\text{ATP}}\) channel blocker 5-HD were able to completely abolish the increase in BK-dependent ROS production. Also, in rabbit ventricular myocytes, Sasaki et al. (30) used the mitochondrial redox potential as an index of mitoK\(_{\text{ATP}}\) channel opening to investigate activation of these channels by NO. The NO donor SNAP opened mitoK\(_{\text{ATP}}\) channels, whereas coadministration of either the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide or 5-HD was able to block this effect. These observations confirm the involvement of the mitoK\(_{\text{ATP}}\) channel and are in agreement with our present data and the studies of others (12).

Lochner et al. (15) noted that 50 \( \mu \text{M L-NAME} \) could block the improvement in postischemic left ventricular function that is observed after ischemic preconditioning in rat hearts, but we were unable to block the anti-infarct effects of either ischemic preconditioning (18) or BK (9) with 100 \( \mu \text{M L-NAME} \). Because of those studies, NO has received little attention in classical preconditioning, although its involvement in delayed preconditioning is undeniable (5). In an attempt to resolve the discrepancy between our present cell data in which 200 \( \mu \text{M L-NAME} \) was used and our previous infarct data in which a lower dose of L-NAME was employed, we repeated the intact, isolated heart studies with the higher dose of L-NAME. As seen in Fig. 11, the infarct-sparing effect of BK was blocked by 200 \( \mu \text{M L-NAME} \). Therefore, cardiomyocyte and whole heart data are in agreement. It is interesting to note that the higher dose of L-NAME still failed to abort the protection of ischemic preconditioning (data not shown), presumably because in the isolated heart, enough adenosine is released to trigger protection by a pathway not thought to be dependent on NO. We further explored BK signaling in the intact heart by coadministering ODQ. As noted in Fig. 11, this guanylyl cyclase antagonist blocked the salvage of ischemic myocardium by BK. These data confirming the central role of NO in BK-triggered cardioprotection support the isolated cardiomyocyte data.

NO activates guanylyl cyclase to produce cGMP, which in turn stimulates PKG. Our data confirm the involvement of all of these intermediates in the pathway leading to mitoK\(_{\text{ATP}}\) opening and ROS generation. Recently, Ockaili et al. (19) reported that sildenafil (Viagra), a selective inhibitor of cGMP-specific phosphodiesterase type 5 that leads to an accumulation of cGMP, can produce acute and delayed preconditioning in rabbit hearts. Intravenous or oral administration of sildenafil markedly reduced infarct size after coronary occlusion. This cardioprotective effect was dependent on opening of mitoK\(_{\text{ATP}}\) channels during the preischemic trigger phase. Our results are certainly consistent with this report. Furthermore, we extended the observations to include PKG activation as an important step. Direct activation of PKG leads to an increase in ROS generation, and this effect is dependent on mitoK\(_{\text{ATP}}\) channel opening. Moreover, BK-induced ROS generation could be prevented by blockade of PKG, thus providing the link between the cardioprotective effects of sildenafil and BK.

We have previously documented that the mitoK\(_{\text{ATP}}\) channel serves as a trigger of preconditioning (20), and the present data support this conclusion. Opening of the channel before the long ischemia is required to “prime” the myocardium. Controversy concerning the possible role of the channel as a mediator during the ischemic phase has not yet been completely resolved. Gross and Auchampach (10) administered the K\(_{\text{ATP}}\) channel closer Gli to dogs from the time of completion of the preconditioning ischemia to the onset of the index ischemia and blocked protection. Because the drug had an effect after completion of the preconditioning phase, it was concluded that the K\(_{\text{ATP}}\) channel must be a mediator. However, Schulz et al. (33) noted that if Gli were administered before the index ischemia but delayed for just 5 min after the end of the preconditioning ischemia, then it could no longer block protection, which again establishes K\(_{\text{ATP}}\) as a trigger rather than a mediator. Indeed, it makes sense that a short period of reperfusion following the preconditioning ischemia would be required to allow generation of the signaling ROS by the mitochondria. Nonetheless, Wang et al. (36) could block protection from diazoxide when 5-HD was confined to the index ische-
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mia, although a fourfold higher 5-HD concentration was needed to block protection than when it was infused during the trigger phase.

We can speculate as to how opening of the mitochondrial $K_{ATP}$ channels might lead to production of ROS. On the basis of prior studies in isolated mitochondria, it is known that ROS production increases with increasing matrix pH (3). Matrix alkalization will normally accompany $K^+$ uptake because there is an imbalance between uptake of $K^+$ and anionic equivalents. $K^+$ uptake is driven by proton ejection by electron transport leading to profound alkalization. Although there will also be uptake of phosphate and other substrate anions, the cytosolic concentrations of these anions are far lower than that of $K^+$, and the imbalance results in a higher matrix pH value at the new steady state.

This state has examined the effects of BK in an isolated cell model and in the intact isolated heart. We were unable to study the cardioprotective properties of this agonist in an in situ preparation because of the profound hypotensive effects of intravenous drug administration. In an unpublished study in rabbits, we advanced a catheter under fluoroscopy in the laboratory, however, such studies are not feasible and we must rely on the isolated heart model.

BK causes mito$K_{ATP}$ channels to open with subsequent release of ROS. The signaling pathway between surface receptor and mito$K_{ATP}$ channel includes activation of NOS with production of NO within the myocardial cell. NO stimulates guanylyl cyclase, which leads to an increased level of cGMP that in turn activates PKG. At this point, it is unclear whether PKG directly phosphorylates mito$K_{ATP}$ channels to open them or instead activates some intermediate. The released ROS then are assumed to stimulate a protective kinase cascade that results in cardioprotection.

GRANTS

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REFERENCES


