Reactive Oxygen Species and Mitochondrial Adenosine Triphosphate–Regulated Potassium Channels Mediate Helium-Induced Preconditioning Against Myocardial Infarction In Vivo

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Objectives: Helium produces preconditioning by activating prosurvival kinases, but the roles of reactive oxygen species (ROS) or mitochondrial adenosine triphosphate–regulated potassium (K_{ATP}) channels in this process are unknown. The authors tested the hypothesis that ROS and mitochondrial K_{ATP} channels mediate helium-induced preconditioning in vivo.

Design: A randomized, prospective study.

Setting: A university research laboratory.

Participants: Male New Zealand white rabbits.

Interventions: Rabbits (n = 64) were instrumented for the measurement of systemic hemodynamics and subjected to a 30-minute left anterior descending coronary artery (LAD) occlusion and 3 hours of reperfusion. In separate experimental groups, rabbits (n = 7 or 8 per group) were randomly assigned to receive 0.9% saline (control) or 3 cycles of 70% helium–30% oxygen administered for 5 minutes interspersed with 5 minutes of an air-oxygen mixture before LAD occlusion with or without the ROS scavengers N-acetylcysteine (NAC; 150 mg/kg) or N-2 mercaptopropionyl glyoxine (2-MPG; 75 mg/kg), or the mitochondrial K_{ATP} antagonist 5-hydroxydecanoate (5-HD; 5 mg/kg). Statistical analysis of data was performed with analysis of variance for repeated measures followed by Bonferroni’s modification of a Student t test.

Measurements and Main Results: The myocardial infarct size was determined by using triphenyltetrazolium chloride staining and presented as a percentage of the left ventricular area at risk. Helium significantly (p < 0.05) reduced infarct size (23 ± 4% of the area at risk; mean ± standard deviation) compared with control (46 ± 3%). NAC, 2-MPG, and 5-HD did not affect irreversible ischemic injury when administered alone (49 ± 5%, 45 ± 6%, and 45 ± 3%), but these drugs blocked reductions in infarct size produced by helium (45 ± 4%, 45 ± 2%, and 44 ± 3%).

Conclusions: The results suggest that ROS and mitochondrial K_{ATP} channels mediate helium-induced preconditioning in vivo.

KEY WORDS: myocardial ischemia, preconditioning, helium, reactive oxygen species, mitochondrial adenosine triphosphate–regulated potassium channels

By this form of pharmacologic preconditioning mediated reductions in myocardial infarct size produced by the volatile agent.5-7,10 Brief, intermittent administration of helium before prolonged coronary artery occlusion and reperfusion was recently shown to protect myocardium against infarction by activating prosurvival signaling kinases (eg, phosphotyrosylinositol-3-kinase, extracellular signal-regulated kinases, and endothelial nitric oxide synthase), attenuating the detrimental actions of glycogen synthase kinase, and inhibiting mitochondrial transition in vivo.11-13 Whether oxygen-derived free radical intermediates play a role in helium-induced preconditioning is unknown. Brief exposure to the anesthetic noble gas xenon produced cardioprotection by activating mitochondrial K_{ATP} channels,14 but the role of mitochondrial K_{ATP} channels in cardioprotection by anesthetic noble gases remains undefined. Thus, the current investigation also tested the hypothesis that the activation of mitochondrial K_{ATP} channels mediates helium-induced preconditioning in rabbits.

METHODS

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. Furthermore, all conformed to the Guiding Principles in the Care and Use of Animals of the American Physiologic Society and were in accordance with the Guide for the Care and Use of Laboratory Animals.

Male New Zealand white rabbits weighing between 2.5 and 3.0 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg) as previously described.11 Additional doses of pentobarbital were titrated as required to ensure that pedal and palpebral reflexes were absent throughout the experiment. Briefly, a tracheostomy was performed through a midline incision, and each rabbit was ventilated with positive

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pressure using an air-oxygen mixture (fractional inspired oxygen concentration = 0.30). Arterial blood gas tensions and acid-base status were maintained within a normal physiologic range by adjusting the respiratory rate or tidal volume throughout the experiment. A pulse oximeter was placed on the right hind paw of each rabbit for the measurement of continuous arterial oxygen saturation. Heparin-filled catheters were positioned in the right carotid artery and the left jugular vein for measurement of arterial blood pressure and fluid or drug administration, respectively. Maintenance fluids (0.9% saline; 15 mL/kg/min) were continued for the duration of each experiment. A thoracotomy was performed at the left fourth intercostal space, and the heart was suspended in a pericardial cradle. A prominent branch of the left anterior descending coronary artery (LAD) was identified, and a silk ligature was placed around this vessel approximately halfway between the base and the apex for the production of coronary artery occlusion and reperfusion. Intravenous heparin (500 U) was administered immediately before LAD occlusion. Coronary artery occlusion was verified by the presence of epicardial cyanosis and regional dyskinesia in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response. Hemodynamics were continuously recorded on a polygraph throughout each experiment.

The experimental design is shown in Figure 1. Baseline hemodynamics, arterial blood gas tensions, and arterial oxygen saturation were recorded 30 minutes after instrumentation was completed. All rabbits underwent a 30-minute LAD occlusion followed by 3 hours of reperfusion. In 8 separate groups, rabbits (n = 7 to 8 per group) were randomly assigned (Latin square design) to receive 0.9% saline (control) or 3 cycles of 70% helium–30% oxygen administered for 5 minutes interspersed with 5 minutes of 70% nitrogen–30% oxygen before coronary artery occlusion in the presence or absence of the ROS scavengers N-acetylcysteine (NAC, 150 mg/kg) or N-2 mercaptopropionyl glycine (2-MPG, 75 mg/kg) or the selective mitochondrial K<sub>AChP</sub> channel antagonist 5-hydroxydeconate (5-HD, 5 mg/kg). NAC and 2-MPG were dissolved in 0.9% saline and administered as intravenous infusions over 30 and 75 minutes, respectively. 5-HD was dissolved in 0.9% saline and administered intravenously 30 minutes before LAD occlusion. The doses of NAC, 2-MPG, and 5-HD used in the current investigation did not produce hemodynamic effects nor affect infarct size when administered alone in an identical rabbit model. The doses of NAC, 2-MPG, and 5-HD also abolished isoflurane-induced production of ROS as detected by dihydroethidium staining independent of prolonged coronary artery occlusion and reperfusion in rabbits. Taken together, these previous findings suggest that these particular antagonists are effective and selective in the doses that were used in the current investigation.

Myocardial infarct size was measured as previously described. Briefly, the LAD was reoccluded at the completion of each experiment, and 3 mL of patent blue dye were injected intravenously. The left ventricular area at risk for infarction was separated from the surrounding normal areas (stained blue), and the 2 regions were incubated at

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**Fig 1.** Schematic illustration depicting the experimental protocol used in the current investigation.
37°C for 20 minutes in 1% 2,3,5 triphenyltetrazolium chloride in 0.1 mol/L of phosphate buffer adjusted to pH 7.4. Infarcted and noninfarcted myocardium within the area at risk were carefully separated and weighed after storage overnight in 10% formaldehyde. Myocardial infarct size was expressed as a percentage of the area at risk. Rabbits that developed intractable ventricular fibrillation and those with an area at risk less than 15% of the total left ventricular mass were excluded from subsequent analysis.

Statistical analysis of data within and between groups was performed with multiple analysis of variance for repeated measures followed by Bonferroni’s modification of a Student t test. Changes were considered statistically significant when p < 0.05. All data are expressed as mean ± standard deviation.

RESULTS

Sixty-four rabbits were instrumented to obtain 59 successful infarct size experiments. Two rabbits were excluded because the left ventricular area at risk was less than 15% of the total left ventricular mass. Three rabbits were excluded because intractable ventricular fibrillation occurred during coronary artery occlusion. Arterial blood gas tensions were maintained within the physiologic range during the administration of helium in all groups (data not shown). Arterial oxygen saturation remained at 100% during and after the administration of helium with or without other drug interventions (data not shown). Baseline systemic hemodynamics were similar between groups (Table 1), but the mean arterial pressure was greater in rabbits randomized to receive 2-MPG compared with 0.9% saline. Helium did not affect hemodynamics. The mean arterial pressure and rate-pressure product were greater in rabbits receiving NAC or 2-MPG alone before LAD occlusion compared with those treated with 0.9% saline. Brief coronary artery occlusion and reperfusion significantly (p < 0.05) reduced rate-pressure product in all experimental groups. There were no differences in hemodynamics between groups during LAD occlusion and reperfusion. Body weight, left ventricular mass, area at risk weight, and the ratio of area at risk to left ventricular mass were similar between groups (Table 2). Left ventricular weight and area at risk weight were less in rabbits randomized to receive NAC compared with 0.9% saline, but the ratio of area at risk to the left ventricular mass was similar between these groups. Brief, intermittent exposure to 70% helium before LAD occlusion reduced myocardial infarct size (23 ± 4% of the left ventricular area at risk) as compared with control rabbits (46 ± 3%, Fig 2). The administration of NAC, 2-MPG, or 5-HD alone did not affect infarct size (49 ± 5%, 45 ± 6%, and 45 ± 3%.

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<thead>
<tr>
<th>Table 1. Hemodynamics</th>
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<td>HR (beats/min)</td>
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<td>CON</td>
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<tr>
<td>He</td>
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<tr>
<td>5-HD</td>
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<tr>
<td>5-HD + He</td>
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<tr>
<td>NAC</td>
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<tr>
<td>NAC + He</td>
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<tr>
<td>2-MPG</td>
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<tr>
<td>2-MPG + He</td>
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<tr>
<td>MAP (mmHg)</td>
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<tr>
<td>CON</td>
</tr>
<tr>
<td>He</td>
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<tr>
<td>5-HD</td>
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<tr>
<td>5-HD + He</td>
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<tr>
<td>NAC</td>
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<tr>
<td>NAC + He</td>
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<tr>
<td>2-MPG</td>
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<tr>
<td>2-MPG + He</td>
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<tr>
<td>RPP (min/mmHg/10³)</td>
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<tr>
<td>CON</td>
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<tr>
<td>He</td>
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<tr>
<td>5-HD</td>
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<tr>
<td>5-HD + He</td>
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<tr>
<td>NAC</td>
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<tr>
<td>NAC + He</td>
</tr>
<tr>
<td>2-MPG</td>
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<tr>
<td>2-MPG + He</td>
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</tbody>
</table>

NOTE. Data are mean ± standard deviation.
Abbreviations: HR, heart rate; MAP, mean arterial pressure; RPP, rate-pressure product; CON, control; He, helium.
*Significantly (p < 0.05) different from baseline.
†Significantly (p < 0.05) different from the corresponding control.
respectively), but these drugs abolished helium-induced cardioprotection (45 ± 4%, 44 ± 2%, and 44 ± 3%, respectively).

**DISCUSSION**

The current results confirm the previous findings, indicating that 3 cycles of 5 minutes of 70% helium–30% oxygen preconditioning interspersed with 5-minute washout periods of an air-oxygen mixture reduce myocardial necrosis after prolonged coronary artery occlusion and reperfusion. The results show for the first time that pretreatment with NAC or 2-MPG abolishes this helium-induced cardioprotection, suggesting that ROS mediate preconditioning by the nonanesthetic noble gas in vivo. The results further indicate that 5-HD pretreatment blocks reductions in myocardial infarct size produced by brief, intermittent administration of helium, implicating mitochondrial KATP channels in this process as well. Weber et al. showed that mitochondrial KATP channels mediated cardioprotection produced by the anesthetic inert gas xenon, and the current results extend this observation to another noble gas that is devoid of anesthetic properties, even under extreme hyperbaric conditions (>200 atm). Collectively, the current and previous data with noble gases also lend support to the hypothesis that ROS are a ubiquitous feature in preconditioning phenomena in conjunction with mitochondrial KATP channels. Treatment with low concentrations of ROS mimicked the beneficial effects of ischemic preconditioning, and small quantities of ROS generated by mitochondria during brief periods of ischemia or after exposure to the mitochondrial KATP channel agonist diazoxide have been shown to play a central role in ischemic and pharmacologic preconditioning, respectively. Evidence that mitochondrial KATP activation mediates this ROS-induced cardioprotection also was provided by the observations that diazoxide enhances oxidation of the ROS probe MitoTracker orange (Molecular Probes, Eugene, OR) and increases ROS production as measured using 2',7'-dichlorofluorescein diacetate in rat ventricular myocytes or isolated hearts. Pretreatment with 5-HD abolished ROS generation produced by the mitochondrial KATP channel openers nicorandil and cromakalim in isolated rat hearts. Similarly, the ROS scavengers NAC, 2-MPG, and Mn(III)tetrakis (4-benzoic acid) porphyrin chloride inhibited the cardioprotective effects of isoflurane in isolated and intact rabbit hearts. Scavengers of ROS also

### Table 2. Left Ventricular Area at Risk

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Body Weight (g)</th>
<th>LV (g)</th>
<th>AAR (g)</th>
<th>AAR/LV (%)</th>
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<tbody>
<tr>
<td>CON</td>
<td>8</td>
<td>2,703 ± 298</td>
<td>3.90 ± 0.46</td>
<td>1.63 ± 0.20</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>He</td>
<td>8</td>
<td>2,803 ± 243</td>
<td>3.84 ± 0.21</td>
<td>1.26 ± 0.08</td>
<td>33 ± 2</td>
</tr>
<tr>
<td>5-HD</td>
<td>7</td>
<td>2,789 ± 231</td>
<td>3.49 ± 0.39</td>
<td>1.36 ± 0.34</td>
<td>39 ± 6</td>
</tr>
<tr>
<td>5-HD + He</td>
<td>7</td>
<td>2,487 ± 72</td>
<td>3.58 ± 0.25</td>
<td>1.34 ± 0.17</td>
<td>37 ± 4</td>
</tr>
<tr>
<td>NAC</td>
<td>8</td>
<td>2,709 ± 280</td>
<td>3.08 ± 0.48*</td>
<td>1.02 ± 0.33*</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>NAC + He</td>
<td>7</td>
<td>2,594 ± 82</td>
<td>3.74 ± 0.45</td>
<td>1.35 ± 0.37</td>
<td>36 ± 7</td>
</tr>
<tr>
<td>2-MPG</td>
<td>7</td>
<td>2,847 ± 170</td>
<td>3.32 ± 0.63</td>
<td>1.18 ± 0.48</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>2-MPG + He</td>
<td>7</td>
<td>2,726 ± 112</td>
<td>3.58 ± 0.44</td>
<td>1.47 ± 0.25</td>
<td>41 ± 4</td>
</tr>
</tbody>
</table>

*Significantly (p < 0.05) different from the corresponding CON value.

Note: Data are mean ± standard deviation.

Abbreviations: LV, left ventricle; AAR, area at risk; CON, control; He, helium.
abolished the salutary actions of sevoflurane against ischemic damage in isolated guinea pig hearts.\textsuperscript{23,24} Preconditioning by isoflurane directly increased the production of superoxide anion (O$_2^-$) as determined using dihydroethidium staining through a mitochondrial K$_{\text{ATP}}$ channel-mediated mechanism.\textsuperscript{5,10} Thus, it has become abundantly clear that ROS and mitochondrial K$_{\text{ATP}}$ channels play complementary roles during ischemic, pharmacologic, and anesthetic preconditioning, and the current results with helium suggest that ROS and mitochondrial K$_{\text{ATP}}$ channels are also essential in preconditioning by the nonanesthetic noble gas.

The current results must be interpreted within the constraints of several potential limitations. Based on previous experiments conducted with volatile anesthetics,\textsuperscript{7,8,10} it appears highly likely that O$_2^-$ generated from the electron transport chain though mitochondrial K$_{\text{ATP}}$ channel opening may also be responsible for the observed results with helium. The sulfhydryl-containing glutathione precursor NAC produces antioxidant effects by enhancing glutathione synthesis, acting as a substrate for glutathione peroxidase, and facilitating metabolism of hydrogen peroxide (H$_2$O$_2$) by univalent reduction of O$_2^-$ via preservation of intracellular-reduced glutathione concentration.\textsuperscript{25} Large amounts of superoxide dismutase are contained within mitochondria, and this enzyme is primarily responsible for the chemical conversion of O$_2^-$ to H$_2$O$_2$ and water. The subsequent reduction of H$_2$O$_2$ is catalyzed by glutathione peroxidase to which reduced glutathione serves as an electron donor during the reaction. Thus, the current observation that NAC abolishes helium-induced preconditioning indirectly infers that O$_2^-$ or one of its immediate derivatives is responsible for this cardioprotective effect; 2-MPG also donates sulfhydryl groups to glutathione peroxidase and may be more mitochondria-specific than NAC.\textsuperscript{26-28} These observations also indirectly suggest that O$_2^-$ derived from mitochondria or another oxygen-derived free radical intermediate produced by O$_2^-$ metabolism is involved in cardioprotection by helium. Nevertheless, such conclusions must be qualified because the authors did not specifically determine the identity or define the source of the ROS involved in helium-induced preconditioning in the current investigation. It is also unclear based on the current results whether mitochondrial K$_{\text{ATP}}$ opening acts as a trigger or end-effector for preconditioning by helium through ROS generation. The authors’ laboratory is currently examining this hypothesis.

In addition to previously described limitations, the current results must be interpreted within the constraints of several other potential shortcomings. The duration of administration of NAC, 2-MPG, and 5-HD were heterogenous, and these pharmacokinetic factors may have influenced the results. Plasma concentrations of NAC, 2-MPG, and 5-HD also were not determined nor were dose-response relationships to these drugs performed. Myocardial infarct size is determined primarily by the size of the area at risk and the extent of coronary collateral perfusion. The area at risk expressed as a percentage of total left ventricular mass was similar between groups in the current investigation, and coronary collateral blood flow has been shown to be minimal in rabbits.\textsuperscript{29} Thus, differences in collateral perfusion between groups probably did not account for the observed results, but coronary collateral blood flow was not specifically quantified. The reductions in myocardial necrosis produced by helium in the absence or presence of other drug interventions occurred independent of changes in major determinants of myocardial oxygen consumption. Nevertheless, coronary venous oxygen tension was not directly measured nor was myocardial oxygen consumption calculated. Notably, no significant differences in hemodynamics were observed between groups before and during coronary artery occlusion that may account for differences in infarct size observed between groups. Finally, the current results implicating a role for ROS and mitochondrial K$_{\text{ATP}}$ channels in helium-induced cardioprotection were obtained in barbiturate-anesthetized, acutely instrumented rabbits. Whether similar results occur in other animal species or humans is unknown. Helium preconditioning has yet to be established in humans, but the administration of this noble gas before a defined period of myocardial ischemia may be beneficial in a clinical setting in which an anesthetic is not required (eg, inflation of an angioplasty balloon during cardiac catheterization). However, additional investigation will be required to test this intriguing hypothesis.

In summary, the current results confirm that brief, intermittent administration of helium before prolonged coronary artery occlusion and reperfusion protects myocardium against infarction. The findings further suggest that ROS and mitochondrial K$_{\text{ATP}}$ channels mediate this helium-induced preconditioning in vivo.

REFERENCES


