Decreased tetrahydrobiopterin and disrupted association of Hsp90 with eNOS by hyperglycemia impair myocardial ischemic preconditioning

Nikolina Vladic, Zhi-Dong Ge, Thorsten Leucker, Anna K. Brzezinska, Jian-Hai Du, Yang Shi, David C. Warltier, Phillip F. Pratt Jr., and Judy R. Kersten

Departments of Anesthesiology, Pediatric Surgery, and Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin

Submitted 26 October 2010; accepted in final form 19 August 2011

Vladic N, Ge ZD, Leucker T, Brzezinska AK, Du J, Shi Y, Warltier DC, Pratt Jr PF, Kersten JR. Decreased tetrahydrobiopterin and disrupted association of Hsp90 with eNOS by hyperglycemia impair myocardial ischemic preconditioning. Am J Physiol Heart Circ Physiol 301: H2130–H2139, 2011. First published September 9, 2011; doi:10.1152/ajpheart.01078.2010.—Cardioprotection by ischemic preconditioning (IPC) is impaired during hyperglycemia, but the mechanisms underlying this phenomenon are poorly understood. This study investigated the role of hyperglycemia to adversely modulate tetrahydrobiopterin (BH4) and heat shock protein 90 (Hsp90) during cardioprotection by IPC. Rabbits or mice underwent 30 min of coronary occlusion followed by reperfusion with or without IPC in the presence or absence of hyperglycemia. IPC significantly (P < 0.05) decreased myocardial infarct size (46 ± 1 to 19 ± 2% of the area at risk in control and IPC rabbits, respectively) and increased BH4 concentrations (HPLC; 7.6 ± 0.2 to 10.2 ± 0.3 pmol/mg protein, respectively), Hsp90-endothelial nitric oxide synthase (eNOS) association (coimmunoprecipitation and Western blotting in mice; 4.0 ± 0.3 to 5.4 ± 0.1, respectively), and the ratio of phosphorylated eNOS total eNOS. These beneficial actions of IPC on infarct size, BH4, Hsp90/eNOS, and phosphorylated eNOS were eliminated by hyperglycemia. Pretreatment of animals with the Hsp90 inhibitor geldanamycin (0.6 mg/kg) or the BH4 synthesis inhibitor diaminooxyproimidine (1.0 g/kg) also eliminated cardioprotection produced by IPC. In contrast, the BH4 precursor sepiapterin (2 mg/kg iv) restored the beneficial effects of IPC on myocardial BH4 concentrations, eNOS dimerization, and infarct size during hyperglycemia. A-23871 increased Hsp90-eNOS association (0.33 ± 0.06 to 0.59 ± 0.3) and nitric oxide production (184 ± 17%) in human coronary artery endothelial cells cultured in normal (5.5 mM) but not high (20 mM) glucose media. These data indicate that hyperglycemia eliminates protection by IPC via decreases in myocardial BH4 concentration and disruption of the association of Hsp90 with eNOS. The results suggest that eNOS dysregulation may be a central mechanism of impaired cardioprotection during hyperglycemia.

hyperglycemia; ischemia reperfusion; tetrahydrobiopterin; heat shock protein; nitric oxide; endothelial nitric oxide synthase

HYPERGLYCEMIA (HG) is an independent risk factor for increased cardiovascular morbidity and mortality (9, 12, 13). Previous studies have suggested that HG-associated risk of death is greater in patients with acute myocardial infarction who do not have antecedent diabetes than in those with diabetes, although the responsible mechanisms are not entirely clear (9, 32, 52). Available evidence indicates that endogenous cardioprotective mechanisms such as ischemic preconditioning (IPC) and pharmacological activation of cardioprotective signal transduction pathways are eliminated by HG in animals and in humans (2, 16, 26, 29, 31). We tested the hypothesis that regulation of endothelial nitric oxide synthase (eNOS) by its cofactors and protein chaperones is a key mechanism by which HG impairs cardioprotection.

Nitric oxide (NO) has been demonstrated to play a critical role in cardioprotective signaling (2, 19, 28, 40, 43, 46). Three isoforms of NO synthase (NOS), neuronal, inducible, and endothelial, are expressed in cardiomyocytes and in coronary artery endothelial cells (35, 36). NOS catalyzes the oxidation of L-arginine to produce L-citrulline and NO in the presence of its essential NOS cofactor tetrahydrobiopterin (BH4), physiological binding partner heat shock protein 90 (Hsp90), and cofactors NADPH, FAD, and flavin mononucleotide (11, 35). Biochemical and pharmacological evidence indicates that BH4 deficiency or disruption of the association of Hsp90 with eNOS results in “uncoupling” of eNOS with L-arginine, leading to the production of superoxide anion rather than NO by this enzyme (4, 21, 41, 44, 51). Interestingly, diabetes and HG have been shown to modulate these regulators of eNOS activity (1). For example, overproduction of reactive oxygen species by mitochondria during diabetes and HG results in oxidation of intracellular BH4 to the catalytically incompetent species dihydrobiopterin. Similarly, Hsp90-eNOS interactions and NO production are reduced in endothelial cells cultured in media containing excess glucose (7, 10, 27, 39, 50). However, the roles of BH4 and Hsp90 to mediate IPC during HG have not been investigated. The purpose of the current study was to examine the mechanisms underlying eNOS dysfunction caused by HG, with special emphasis on the importance of BH4 and Hsp90 in the regulation of eNOS function during IPC and HG.

METHODS

Animals

Male New Zealand White rabbits (wt: 2.5–3.0 kg; age: 8–10 wk) and C57BL/6 mice (wt: 25.3 ± 0.5 g; age: 9–12 wk) were purchased from Kuiper Rabbit Ranch (Gary, IN) and the Jackson Laboratory (Bar Harbor, ME), respectively. The animals were kept on a 12:12-h light-dark cycle in a temperature-controlled room. All experimental procedures used in this study were approved by the Animal Care and Use Committee of the Medical College of Wisconsin and conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996).

Rabbit Myocardial Ischemia and Reperfusion Injury in Vivo

Surgical preparation. We have previously described the rabbit in vivo model of myocardial ischemia and reperfusion injury (2, 48). Briefly, pentobarbital (30 mg/kg iv)-anesthetized rabbits were venti-
ltered 90 min before IPC. In HG, IPC alent amount of vehicle [dimethyl sulfoxide (DMSO)] was adminis-
coronary occlusion followed by 15 min of reperfusion immediately
followed by 3 ho freperfusion. IPC was induced by a single 5-min
for 30 min and subjected to 30 min of coronary artery occlusion
ments, myocardial BH4 concentrations were measured to examine the
effects of HG, IPC, and IPC as described above but not subjected to prolonged coronary occlusion. Rabbits in
CON, HG, SEP, and SEP + HG groups underwent no coronary occlusion. Myocardial BH4 concentrations were determined using
protocol 4
The effects of diamo-no-6-hydroxyprymidine (DAHP, an inhibitor of BH4
synthesis) on IPC-induced cardioprotection against infarction were
investigated in four experimental groups (8 rabbits/group): CON, DAHP, IPC, and DAHP (protocol 2). Rabbits were injected
with a bolus of DAHP (1 g/kg ip) 140 min before the 30-min coronary
occlusion in DAHP and IPC + DAHP groups. In separate experi-
ments, myocardial BH4 concentrations were measured to examine the
inhibitory effect of DAHP on BH4 synthesis. The animals were treated
with DAHP and IPC as described above but not subjected to 30 min of coronary occlusion and 180 min of reperfusion.
Measurement of BH4. Myocardial BH4 was quantified by HPLC
with an electrochemical detector (ESA Biosciences CoulArray Sys-
I. Experimental protocol in rabbits

A Protocol 1: Effects of HG and SEP on myocardial BH4 and infarct size in rabbits subjected to ischemia/reperfusion injury alone or with IPC

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>HG</th>
<th>IPC</th>
<th>IPC+HG</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>BASELINE</td>
<td>HYPERG/LCARBA 300 mg/l</td>
<td>BASELINE</td>
<td>BASELINE</td>
</tr>
<tr>
<td>DMSO, i.v.</td>
<td>BASELINE</td>
<td>CORONARY OCCLUSION</td>
<td>IPC</td>
<td>IPC+HG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B Protocol 2: Effects of DAHP on BH4 and infarct size in rabbits subjected to ischemia/reperfusion injury alone or with IPC

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>DAHP</th>
<th>IPC</th>
<th>IPC+DAHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>GH</td>
<td>BASELINE</td>
<td>DAHP 1 g/kg, ip</td>
<td>BASELINE</td>
<td>BASELINE</td>
</tr>
<tr>
<td>DMSO, i.v.</td>
<td>BASELINE</td>
<td>CORONARY OCCLUSION</td>
<td>IPC</td>
<td>IPC+DAHP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Schematic diagram depicting the experimental protocols. The effects of hyperglycemia (HG) with or without sepiapterin (SEP, protocol 1; A) or diamo-no-6-hydroxyprymidine (DAHP, protocol 2; B) on myocardial tetrabhydrobiopterin (BH4) and infarct size were determined in rabbits subjected to ischemia and reperfusion injury in the absence [control (CON)] or presence of ischemic preconditioning (IPC). The effects of HG (protocol 3; C) or geldanamycin (GEL, protocol 4; D) on heat shock protein 90 (Hsp90)-endothelial nitric oxide synthase (eNOS) association and infarct size in mice subjected to ischemia and reperfusion injury in the absence or presence of IPC. DMSO, dimethyl sulfoxide.

AJP-Heart Circ Physiol • VOL. 301 • NOVEMBER 2011 • www.ajpheart.org
tem, Chelmsford, MA), as previously described (1). Briefly, rabbit myocardium from the risk region was homogenized in 50 mM phosphate buffer (pH 2.6) containing 0.2 mM diethylenetriaminepentaaetic acid and 1 mM dithioerythritol. Samples were centrifuged (12,000 g, 10 min, two times) and supernatants were filtered through a 10-kDa cutoff column (Millipore, Billerica, MA). Multichannel coulometric detection was set between 0 and 600 mV. One channel was set at −250 mV to verify the reversibility of BH₄ oxidative peak detection. Calibration curves using authentic BH₄ were constructed by summation of the peak areas collected at 0 and 150 mV for BH₄. Myocardial BH₄ was calculated from the calibration curves and normalized to protein concentration.

**Mouse Myocardial Ischemia and Reperfusion Injury In Vivo**

Experiments were completed in mice to complement those conducted in rabbits in vivo. Because of cross-reactivity of antibodies in rabbit myocardium, we were unable to complete Hsp90/eNOS immunoprecipitation/immunoblotting experiments in rabbit myocardium.

**Surgical preparation.** Mice were anesthetized with pentobarbital sodium (100 mg/kg ip) and ventilated with room air supplemented with 100% oxygen at a rate of ~104 breaths/min with a tidal volume of ~225 μl using a mouse ventilator (Hugo Sachs Elektronic, Hugsteten, Germany), as previously described (5, 22–25). The heart was exposed via a thoracotomy at the fourth intercostal space. Myocardial ischemia was produced by occluding the left anterior descending coronary artery, and reperfusion was initiated by loosening the suture. The infarct area was delineated by perfusing the coronary arteries with 2,3,5-triphenyltetrazolium chloride via the aortic root, and the AAR was delineated by perfusing phthalo blue dye (Heucotech, Fairless Hill, PA) in the aortic root after ligating the coronary artery. As a result, the nonischemic portion of the LV was stained dark blue. Viable myocardium within the AAR was stained bright red, and infarcted tissue was unstained. Throughout the experiment, body temperature was maintained between 36.8 and 37.5°C by using a heating pad (CWE, Ardmore, PA).

**Experimental protocol.** Mouse protocols are depicted in Fig. 1. The effect of HG on IPC-elicited changes in infarct size was determined in C57BL/6 mice assigned to one of four experimental groups (7 mice/group): CON, HG, IPC, and IPC + HG (protocol 3). After instrumentation was completed, all mice were stabilized for 30 min and subjected to 30 min of coronary artery occlusion followed by 2 h of reperfusion. IPC was produced via four cycles of 5 min of coronary occlusion, each followed by 5 min of reperfusion. D-Glucose (2 g/kg ip) was administered to produce HG 10 min before IPC. A few drops of tail blood were sampled at baseline, 10 min after D-glucose injection, 30 min after coronary occlusion, and 30, 60, and 120 min after reperfusion for measurement of blood glucose concentrations (Glucometer). Heart rate was monitored from the electrocardiogram.
and AAR and infarct size were determined following each experiment. In separate animals, the effects of IPC and HG on Hsp90-eNOS association were investigated in mice subjected to sham surgery (CON) and IPC with and without HG. The effects of geldanamycin (GEL, an inhibitor of Hsp90 synthesis) on IPC-elicted changes in Hsp90-eNOS association and infarct size were investigated in four groups of mice (7 mice/group): CON, GEL, IPC, and IPC + GEL (protocol 4). GEL (0.6 mg/kg iv) or equivalent amount of vehicle (DMSO) was administered 70 min before the prolonged occlusion of the coronary artery.

Culture of human coronary artery endothelial cells. Human coronary artery endothelial cells (HCAECs) isolated from healthy human donors were purchased from Cell Applications (San Diego, CA). HCAEC were cultured in normoglycemic media containing 5.5 mM d-glucose, 14.5 mM mannitol, 81 mM NaCl, 4.0 mM KCl, 1.6 mM CaCl₂ (pH 7.40), or hyperglycemic media containing 20.0 mM d-glucose, 81 mM NaCl, 4.0 mM KCl, and 1.6 mM CaCl₂, as previously described (2). Osmolarity was maintained at 290 mosmol/l in normoglycemic and hyperglycemic media.

Measurement of Hsp90-eNOS association. Myocardium from the AAR of mouse hearts or HCAECs were homogenized in a buffer containing 20.0 mM MOPS, 2.0 mM EGTA, 5.0 mM EDTA, protease inhibitor cocktail (1:100; Calbiochem, San Diego, CA), phosphatase inhibitor cocktail (1:100; Calbiochem), and 0.5% detergent (Nonidet P-40 detergent, pH 7.4; Sigma-Aldrich, St. Louis, MO) and processed for immunoprecipitation or immunoblotting. Immunoprecipitation and

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>Coronary Occlusion</th>
<th>Reperfusion, min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart rate, min⁻¹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>246 ± 9</td>
<td>233 ± 6</td>
<td>221 ± 6*</td>
</tr>
<tr>
<td>HG</td>
<td>257 ± 8</td>
<td>246 ± 7</td>
<td>221 ± 8*</td>
</tr>
<tr>
<td>IPC</td>
<td>246 ± 10</td>
<td>230 ± 7</td>
<td>229 ± 8</td>
</tr>
<tr>
<td>IPC + HG</td>
<td>260 ± 11</td>
<td>233 ± 5</td>
<td>234 ± 6</td>
</tr>
<tr>
<td>SEP</td>
<td>258 ± 6</td>
<td>237 ± 8</td>
<td>229 ± 9*</td>
</tr>
<tr>
<td>SEP + HG</td>
<td>255 ± 14</td>
<td>233 ± 10</td>
<td>215 ± 8*</td>
</tr>
<tr>
<td>IPC + SEP</td>
<td>263 ± 8</td>
<td>248 ± 8</td>
<td>236 ± 8*</td>
</tr>
<tr>
<td>IPC + HG + SEP</td>
<td>246 ± 7</td>
<td>220 ± 7*</td>
<td>208 ± 9*</td>
</tr>
<tr>
<td>Mean arterial pressure, mmHg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>78 ± 2</td>
<td>69 ± 3</td>
<td>71 ± 2</td>
</tr>
<tr>
<td>HG</td>
<td>76 ± 4</td>
<td>67 ± 3</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>IPC</td>
<td>70 ± 4</td>
<td>61 ± 6</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>IPC + HG</td>
<td>75 ± 4</td>
<td>55 ± 4</td>
<td>64 ± 6</td>
</tr>
<tr>
<td>SEP</td>
<td>81 ± 4</td>
<td>67 ± 6*</td>
<td>69 ± 3*</td>
</tr>
<tr>
<td>SEP + HG</td>
<td>83 ± 4</td>
<td>66 ± 4*</td>
<td>64 ± 3*</td>
</tr>
<tr>
<td>IPC + SEP</td>
<td>80 ± 4</td>
<td>66 ± 3*</td>
<td>69 ± 3*</td>
</tr>
<tr>
<td>IPC + HG + SEP</td>
<td>76 ± 4</td>
<td>57 ± 4*</td>
<td>57 ± 4*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 8 rabbits/group. CON, control; DAHP, diamino-6-hydroxypyrimidine; HG, hyperglycemia; IPC, ischemic preconditioning; SEP, sepiapterin. *P < 0.05 vs. baseline.

Fig. 3. Effects of DAHP on cardiac BH₄ concentrations (A), area at risk for infarction (B), and myocardial infarct size (C) in rabbits subjected to ischemia and reperfusion injury in the absence (CON) or presence of IPC. *P < 0.05 vs. CON; †P < 0.05 vs. IPC (n = 8 rabbits/group).
was performed by incubating cell lysates and homogenized tissue (200 μg) with eNOS monoclonal antibody (2 μg/mg of total cell protein; Biomol) for 16 h at 4°C, followed by 2 h incubation with a 1:1 protein A-to-protein G ratio of Sepharose beads, as previously described (2). After being boiled for 5 min, samples were resolved using precast 7.5% Tris·HCl gels (Criterion; Bio-Rad, Hercules, CA), and protein was transferred to a polyvinylidene fluoride membrane. Immunoblots were performed with rabbit polyclonal anti-eNOS (1:5,000 for cells and 1:750 for tissue; Santa Cruz Biotechnologies, Delaware, CA) and mouse monoclonal anti-Hsp90 (1:1,000 for cells and 1:750 for tissue; Santa Cruz Biotechnologies) and were incubated overnight at 4°C. Membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG for eNOS (1:10,000 for the cells and 1:5,000 for the tissue; Santa Cruz Biotechnologies) and goat anti-mouse for Hsp90 (1:8,000 for cells and 1:5,000 for the tissue; Bio-Rad) and developed using ECL plus Western blot chemiluminescence detection reagent (GE Healthcare). Densitometric analysis was performed using image acquisition and analysis software (Image J, Bethesda, MD). Results were expressed as the fraction of Hsp90 coimmunoprecipitation with eNOS.

Measurement of NO. Nitrite concentration corresponding to the stable byproduct of NO released by HCAECs in aqueous solution was quantified by ozone chemiluminescence (1). Samples (20 μl) were refluxed in glacial acetic acid containing potassium iodide and nitrite quantified in a NO chemiluminescence analyzer (Sievers Instruments, Boulder, CO). Nitrite concentrations were calculated after subtraction of background levels and normalized to protein content (Bradford method).

Table 2. Heart rate during in vivo mouse experiments

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline (min)</th>
<th>Coronary Occlusion (min)</th>
<th>30</th>
<th>60</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>407 ± 16</td>
<td>418 ± 21</td>
<td>422 ± 22</td>
<td>404 ± 28</td>
<td>384 ± 19</td>
</tr>
<tr>
<td>HG</td>
<td>392 ± 22</td>
<td>419 ± 13</td>
<td>421 ± 11</td>
<td>420 ± 14</td>
<td>399 ± 14</td>
</tr>
<tr>
<td>IPC</td>
<td>427 ± 19</td>
<td>400 ± 14</td>
<td>430 ± 14</td>
<td>410 ± 17</td>
<td>399 ± 12</td>
</tr>
<tr>
<td>IPC + HG</td>
<td>394 ± 29</td>
<td>410 ± 18</td>
<td>406 ± 13</td>
<td>392 ± 16</td>
<td>403 ± 19</td>
</tr>
<tr>
<td>Protocol 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>403 ± 11</td>
<td>409 ± 16</td>
<td>405 ± 11</td>
<td>389 ± 12</td>
<td>382 ± 12</td>
</tr>
<tr>
<td>GEL</td>
<td>410 ± 18</td>
<td>399 ± 16</td>
<td>410 ± 10</td>
<td>412 ± 7</td>
<td>421 ± 13</td>
</tr>
<tr>
<td>IPC</td>
<td>409 ± 17</td>
<td>406 ± 18</td>
<td>439 ± 16</td>
<td>421 ± 20</td>
<td>397 ± 10</td>
</tr>
<tr>
<td>IPC + GEL</td>
<td>403 ± 9</td>
<td>439 ± 27</td>
<td>432 ± 12</td>
<td>419 ± 27</td>
<td>433 ± 28</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6–7 mice/group. Units are min⁻¹. GEL, geldanamycin. There were no significant differences among groups.
Western blot analysis of phosphorylated eNOS. HCAECs were randomized to the following four groups (n = 4 cell dishes/group): CON, HG, hypoxic preconditioning (HP), and HP + HG. HP was induced by three cycles of 3 h of hypoxia/1 h of reoxygenation in three consecutive days, as previously described (54). Briefly, the first exposure to hypoxia lasted 3 h, followed by a 1-h period of normoxia (21% O2-5% CO2-74% N2), after which the cells received fresh hypoxia media overnight for a second 3-h hypoxia exposure. With each return to normoxia, oxygenated complete M199 containing 20% serum was added. Three consecutive days of these same paired 3 h of hypoxia exposures constituted the HP stimulus. Hypoxia was produced by exposure to 5% CO2 and 95% N2 in an airtight chamber in hypoxia exposures constituted the HP stimulus. Hypoxia was produced by exposure to 5% CO2 and 95% N2 in an airtight chamber in the presence of serum- and glucose-free DMEM containing 10 mM deoxyglucose to inhibit glycolysis. HCAECs were cultured in media containing 5 mM (CON and HP groups) or 20 mM (HG and HP groups) of glucose for 12 h before the HP protocol and for 1 h between hypoxia intervals. The cells were lysed in 500 μl of lysis buffer [20.0 mM MOPS, 2.0 mM EGTA, 5.0 mM EDTA, and protease inhibitor cocktail (1:100; Sigma-Aldrich)], phosphatase inhibitor cocktail (1:100; Calbiochem), or 0.5% detergent (Nonidet P-40 detergent, pH 7.4; Sigma-Aldrich). Fifteen to 25 μg of protein were loaded on precast 10% Tris·HCl gels (Criterion; Bio-Rad) and trans- ferred to polyvinylidene fluoride membranes. After the membranes were incubated in 5% milk in Tris-buffered saline, immunoblots were transferred to polyvinylidene fluoride membranes. After the membranes were blocked in 5% milk in Tris-buffered saline, immunoblots were transferred with secondary antibodies horseradish peroxidase-conjugated donkey anti-rabbit IgG for eNOS (1:5,000; Santa Cruz Biotechnologies) and goat anti-rabbit for phospho-eNOS (1:2,000; Bio-Rad). Bands were visualized using chemiluminescence and quantified using NIH image J software.

Measurements of eNOS expression and dimerization. HCAECs were randomly assigned to the following four groups (n = 3 cell dishes/group): CON, SEP, HP, and HP + SEP. HP was produced as described above. In the HP + SEP group, the HCAECs were cultured in media containing 10 μM SEP for 1 h before the HP protocol and for 1 h between hypoxia intervals. To investigate eNOS homodimer formation in HCAECs, nonboiled cellular lysate was resolved with 5% SDS-PAGE at 4°C overnight, as previously described (8). Membranes were incubated with a 1:2,000 dilution of mouse anti-eNOS monoclonal antibody (BD Transduction Laboratories). Densitometric analysis was conducted using NIH image J software.

Statistical Analysis

All data are expressed as means ± SE. Statistical analysis was performed with one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons of multiple group means. A value of P < 0.05 was considered as statistically significant.

RESULTS

HG Eliminated Increases in BH4 and Decreases in Infarct Size Produced by IPC in Rabbits

Baseline blood glucose, AAR, heart rate, and mean arterial blood pressure were comparable among the eight experimental groups (Fig. 2 and Table 1). IPC significantly increased myocardial BH4 concentrations (10.2 ± 0.3 pmol/mg protein in IPC vs. 7.6 ± 0.2 pmol/mg protein in CON, n = 6–7 hearts/group, P < 0.05) and decreased myocardial infarct size (19 ± 2% of AAR in IPC vs. 46 ± 1% in CON, n = 8, P < 0.05) compared with CON. HG alone did not change BH4 concentrations or infarct size but blocked IPC-induced increases in
BH₄ concentrations (7.4 ± 0.6 pmol/mg protein in IPC + HG) and decreases in infarct size (42 ± 2% in IPC + HG) (Fig. 2). Pretreatment of rabbits with SEP significantly elevated BH₄ concentrations (11.5 ± 0.7 pmol/mg protein in IPC + HG + SEP) during IPC in the presence of HG and decreased infarct size to 24 ± 2%, suggesting that supplementation of BH₄ restores IPC-induced cardioprotection against infarction.

**Inhibition of BH₄ Blocked Decreases in Infarct Size by IPC in Rabbits**

DAHP did not significantly alter myocardial BH₄ concentrations (6.1 ± 0.4 pmol/mg protein in DAHP vs. 7.7 ± 0.4 pmol/mg protein in CON, n = 6 rabbits/group) and infarct size compared with control (44 ± 1% of AAR in DAHP vs. 48 ± 0.8% in CON, n = 8 rabbits/group); however, DAHP blocked the increase in BH₄ concentrations (10.9 ± 0.6 pmol/mg protein in IPC vs. 7.9 ± 0.7 pmol/mg protein in IPC + DAHP, n = 6–7 rabbits/group) and the reduction in infarct size produced by IPC (19 ± 2% of AAR in IPC vs. 41 ± 3% of AAR in IPC + DAHP, n = 8 rabbits/group, P < 0.05) (Fig. 3), suggesting that the synthesis of BH₄ is necessary for the cardioprotection produced by IPC. There were no significant differences in heart rate and mean arterial blood pressure between groups at baseline and during coronary occlusion and reperfusion (Table 1).

**HG Eliminated Increases in Hsp90-eNOS Association by IPC in Mice**

Baseline blood glucose was 209 ± 15 mg/dl (n = 21 mice) in unfasted mice. Coronary occlusion for 30 min and subsequent reperfusion for 2 h did not significantly alter blood glucose concentration (Fig. 4A). No significant differences existed between CON and IPC groups. A bolus injection of d-glucose (2 g/kg ip) significantly increased blood glucose concentration compared with control and IPC groups.

AAR and heart rate were comparable among four experimental groups (Fig. 4B and Table 2). IPC increased the ratio of Hsp90/eNOS from 4.0 ± 0.3 in CON to 5.4 ± 0.1 (n = 5, P < 0.5) (Fig. 4C) and decreased infarct size from 53 ± 4% of AAR in control to 29 ± 4% (n = 7 mice, P < 0.05) (Fig. 4D). HG eliminated IPC-induced increases in the ratio of Hsp90/eNOS (3.2 ± 0.1) and decreases in infarct size (46 ± 3%).

**Inhibition of Hsp90 and eNOS Association Blocked the Cardioprotective Effect of IPC in Mice**

GEL did not significantly affect the ratio of Hsp90/eNOS (2.8 ± 0.5 in GEL vs. 4.0 ± 0.4 in CON, n = 5, P < 0.05) but blocked the IPC-induced increase in the ratio of Hsp90/eNOS (5.7 ± 0.4 in IPC vs. 3.4 ± 0.3 inIPC + GEL, n = 5, P < 0.5) (Fig. 5). Infarct size was 30 ± 4% of AAR (n = 7 mice) in the IPC group. GEL did not significantly change AAR or heart rate (Table 2); however, GEL blocked the decrease in infarct size produced by IPC (49 ± 3% of AAR, n = 7, P < 0.05 vs. IPC).

**HG Disrupted Hsp90 and eNOS Association and Decreased NO Production by Endothelial Cells**

There were no significant differences in the ratio of Hsp90/eNOS and NO production by HCAECs cultured in media containing 5.5 or 20 mM glucose. Treatment of HCAECs with the calcium ionophore A-23187 increased the ratio of Hsp90/eNOS (5.7 ± 0.4 in IPC vs. 3.4 ± 0.3 in IPC + GEL, n = 5, P < 0.5) (Fig. 5). Infarct size was 30 ± 4% of AAR (n = 7 mice) in the IPC group. GEL did not significantly change AAR or heart rate (Table 2); however, GEL blocked the decrease in infarct size produced by IPC (49 ± 3% of AAR, n = 7, P < 0.05 vs. IPC).
HG Abolished the HP-Induced Increase in the Ratio of Phosphorylated eNOS/eNOS in Endothelial Cells

The ratio of phosphorylated eNOS/total eNOS was decreased significantly in HCAECs cultured in media containing 20 mM glucose compared with 5.5 mM glucose. Three cycles of HP enhanced the ratio of phosphorylated eNOS/total eNOS in HCAECs cultured in media containing 5.5 mM but not 20 mM glucose (Fig. 7).

HP and SEP Increase eNOS Dimerization in Endothelial Cells

In cultured HCAEC extract, the homodimer and monomer of eNOS were detected by Western blot. Pretreatment of HCAECs with 10 μM SEP significantly increased the eNOS dimer but not monomer. HP alone or combined with SEP significantly increased the eNOS dimer and decreased monomer (Fig. 8).

DISCUSSION

The results of this investigation demonstrate that IPC increases myocardial BH4 concentrations; enhances the association of Hsp90 with eNOS, phosphorylated eNOS, and eNOS dimerization; and protects against myocardial infarction in rabbits and mice. These beneficial actions of IPC are eliminated by acute HG and by inhibition of BH4 synthesis or Hsp90-eNOS interactions using pharmacological inhibitors. In contrast, exogenous SEP restores myocardial BH4 concentrations, eNOS dimerization, and the cardioprotective effect produced by IPC during HG. The results confirm and extend previous findings and indicate that HG adversely modulates eNOS cofactor and chaperone function, impairing cardioprotective signaling.

BH4 is an endogenous molecule synthesized from GTP in the cytoplasm (49). Intracellular BH4 concentration is dependent on de novo and salvage pathways and on its degradation via catalytic processes. The de novo pathway converts GTP to BH4 through the sequential action of three enzymes: GTP cyclohydrolase 1, 6-pyruvyl-tetrahydropterin synthase, and sepiapterin reductase (49). The salvage pathway synthesizes BH4 from BH2 via NADPH-dependent dihydrofolate reductase. Although SEP is not an endogenous precursor of BH4, it is metabolized to BH2 by intracellular conversion of BH2 to BH4 by dihydrofolate reductase. Therefore, SEP can serve as an effective substrate for BH4 synthesis through the salvage pathway (49).

BH4 has profound effects on eNOS function, acting to increase the affinity of eNOS for L-arginine and to stabilize and increase eNOS dimers, an important mechanism in the regulation of eNOS function (6, 11, 38). In the presence of adequate substrate, L-arginine, and BH4, the heme-containing oxygenase domain of eNOS binds molecular oxygen and L-arginine, and the transfer of electrons from reduced NADPH to FAD and FMN in the reductase domain is coupled to the synthesis of NO. However, electron transfer within the active site of eNOS can become uncoupled from L-arginine oxidation during conditions of low intracellular BH4 and results in increased production of superoxide anion. During HG and myocardial ischemia, reactive oxygen species are also produced in excessive amounts by mitochondria (10, 14, 15). An increased production of superoxide anion and subsequent reaction with NO increases the formation of peroxynitrite and oxidation of BH4 to BH2, thus resulting in BH4 deficiency and eNOS uncoupling (17, 33, 37, 45, 53).

The current findings support previous findings in the vasculature and further indicate that adequate concentrations of BH4 are required to facilitate cardioprotective signaling in the myocardium. This process may be eNOS dependent, although an obligatory role for eNOS during IPC is controversial. We, and others, have shown that the nonselective NOS inhibitor N\(^G\)-nitro-L-arginine methyl ester does not block IPC in normal myocardium but abolishes enhanced protection elicited with IPC and statin drugs during HG (26). Findings that inhibition of GTP cyclohydrolase-I with DAHP abolished IPC, whereas N\(^G\)-nitro-L-arginine methyl ester does not, suggests that decreases in NO production alone may not be responsible for impaired cardioprotection. Inhibition of BH4 synthesis with pharmacological antagonists (DAHP) or HG may reduce Hsp90-eNOS association and uncouple eNOS and result in increases in superoxide anion generated from eNOS. Interestingly, reactive oxygen species scavengers such as N-acetylcysteine have been shown to restore pharmacological preconditioning during HG, although an effect related specifically to eNOS function was not determined (30). In the present investigation, augmenting BH4 bioavailability with SEP rescued eNOS dimerization and cardioprotection by IPC during HG. These results extend previous evidence that supplementation of BH4 in diabetes increases NO production and decreases superoxide anion production (27). It is possible that eNOS uncoupling during HG and not merely decreases in NO may impair IPC, although, this hypothesis remains to be completely tested.

Hsp90 is an essential, highly expressed cytosolic protein that mediates conformational regulation of signal transduction molecules, including members of the Src-kinase family of non-receptor tyrosine kinases, Raf and other serine/threonine kinases, transcription factors such as steroid hormone receptors and p53, and eNOS (3). In the resting state, Hsp90 is associated with eNOS, and upon stimulation such as by shear stress, statins, certain pharmacological agents, or vascular endothelial growth factor, the association between the two proteins is increased (2, 21). The association of Hsp90 with eNOS enhances phosphorylation of the enzyme by serine/threonine kinase Akt (47) and stimulates eNOS-dependent NO production (18, 21). Pharmacological inhibition of Hsp90 alters the conformational state of the protein by binding to its unique adenosine triphosphate binding site (20). This action prevents eNOS phosphorylation (47), and results in eNOS uncoupling (41). The present results indicate that HG decreases the association between eNOS and Hsp90, and increases in glucose concentration or inhibition of Hsp90 with GEL eliminated the cardioprotective effects of IPC. In addition, pharmacological inhibition of Hsp90 may impair the cardioprotective effect of IPC through attenuating mitochondrial import of connexin 43 (42).

Hsp90 may have additional binding partners that could play a role in cardioprotective signaling. For example, caveolin-1, eNOS and Hsp90 can be immunoprecipitated from endothelial cells, and Hsp90 decreases the inhibitory effect of caveolin-1 on eNOS activity (34). Taken together, the results support the contention that eNOS uncoupling by HG, or with GTP cyclohydrolase 1 and Hsp90 inhibition impairs cardioprotective signaling by NO. In
contrast, the adverse effects of HG may be mitigated by strategies that increase BH4 bioavailability.

The present results should be interpreted within the constraints of several potential limitations. The actions of IPC and HG to alter BH4 and Hsp90-eNOS interactions were measured in endothelial cells and myocardium, however, the differential contributions of NO derived from endothelial cells versus cardiomyocytes was not determined. For example, it is possible that HG impaired paracrine interactions between endothelial cells and cardiomyocytes and this possibility is the focus of ongoing experiments in the laboratory. Moreover, NO isoforms are present in cardiomyocytes, and it has been recently suggested that uncoupled myocardial NOS might be a mechanism involved during the pathogenesis of cardiovascular disease (35). Whether HG alters eNOS coupling in cardiomyocytes is an important focus for future investigations.

In conclusion, the present study indicates that HG abolishes the cardioprotective effects of IPC by decreasing myocardial BH4 concentration and by disrupting the association of Hsp90 with eNOS. Maintenance of adequate amounts of BH4 in myocardium and association of Hsp90 and eNOS may enhance eNOS coupling and preserve cardioprotective signal transduction during HG.

ACKNOWLEDGMENTS

We are grateful to John G. Krolikowski, Tongju Guan, Shelley L. Baumgardt, and David Schwabe for excellent technical assistance.

GRANTS

This work was supported, in part, by National Institutes of Health Research Grants HL-063705 (to J. R. Kersten) and GM-066730 (to J. R. Kersten and D. C. Warltier).

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


