Volatile Anesthetic Preconditioning Attenuates Myocardial Apoptosis in Rabbits after Regional Ischemia and Reperfusion via Akt Signaling and Modulation of Bcl-2 Family Proteins

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ABSTRACT

We tested whether isoflurane preconditioning inhibits cardiomyocyte apoptosis and evaluated the role of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway in anesthetic preconditioning and determined whether PI3K/Akt signaling modulates the expression of pro- and antiapoptotic proteins in anesthetic preconditioning. Six-month-old New Zealand rabbits subjected to 40 min of myocardial ischemia followed by 180 min of reperfusion were assigned to the following groups: ischemia-reperfusion (I/R), isoflurane preconditioning and isoflurane plus PI3K inhibitors, wortmannin and 2-(4-morpholinyl)-8-phenyl-4H-L-benzopyran-4-one (LY294002) (0.6 and 0.3 mg/kg i.v., respectively). Sham-operated, wortmannin- and LY294002-treated rabbits were also included. Infarct size was assessed by triphenyltetrazolium chloride staining. Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling and activated caspase-3 assays. Akt phosphorylation, Bax, Bcl-2, Bad, and phosphorylated Bad (phospho-Bad) expression was assessed by immunoblotting. Isoflurane preconditioning reduced infarct size compared with the I/R group: 22 ± 4 versus 41 ± 5% (p < 0.05). The percentage of apoptotic cells decreased in the isoflurane group (3.8 ± 1.2%) compared with the I/R group (12.4 ± 1.6%; p < 0.05). These results were also confirmed by the activated caspase-3 assay. Wortmannin and LY294002 inhibited the effects of isoflurane. Myocardial infarction increased to 44 ± 3 and 45 ± 2% and the percentage of apoptotic cells was 11.9 ± 2.1 and 11.7 ± 3.3%, respectively. Akt phosphorylation and Bcl-2 and phospho-Bad expression increased after isoflurane preconditioning, whereas Bax expression decreased. These effects were inhibited by wortmannin and LY294002. The data indicate that isoflurane preconditioning reduces infarct size and myocardial apoptosis after I/R. Activation of PI3K and modulation of the expression of pro- and antiapoptotic proteins may play a role in isoflurane-induced myocardial protection.

Myocardial cell death via necrosis and apoptosis is the main feature of pathological conditions associated with ischemia and reperfusion. Reducing myocyte loss through suppression of cell death pathways represents a logical strategy to protect cardiac function. It has been shown that volatile anesthetics produce pharmacological preconditioning and protect the heart against myocardial infarction in a variety of experimental animal models as well as in humans (Cason et al., 1997; Cope et al., 1997; Tanaka et al., 2004a; Zaugg et al., 2004). The mechanisms by which volatile anesthetics protect the heart have been extensively investigated and are believed to involve activation of adenosine receptors (Kersten et al., 1997) and protein kinase C (Novalija et al., 2003b), release of reactive oxygen species (Mullenheim et al., 2002), and opening of ATP-regulated potassium channels (Pain et al., 2000).

Ischemic preconditioning, a phenomenon whereby exposure of the myocardium to a brief ischemic episode protects it from infarction during a subsequent sustained ischemic insult, was found to decrease myocardial infarction by reducing cell necrosis as well as apoptosis (Piot et al., 1997; Zhao and Vinten-Johansen, 2002). In brain models of ischemia and reperfusion, volatile anesthetics were found to have anti-
apoptotic effects in addition to antinecrotic properties (Wise-Faberowski et al., 2001; Kawaguchi et al., 2004). However, whether volatile anesthetics are also capable in decreasing myocardial apoptosis after ischemia-reperfusion injury in the heart is unknown.

Isoflurane is one of the most frequently used volatile anesthetic agents as part of a balanced anesthetic regimen during surgery. Identifying the mechanisms by which isoflurane and other volatile anesthetic agents mediate their anti-ischemic actions may be of special clinical significance to protect the myocardium against ischemia that frequently occurs in patients with coronary artery disease in the peri-operative period.

Activation of the phosphatidylinositol-3-kinase (PI3K)/Akt pathway has been shown to be essential for the antiapoptotic effects of hypoxic preconditioning in cardiomyocytes (Uchiyama et al., 2004; Hausenloy et al., 2005) as well as for infarct size reduction (Murphy, 2004). Recently, we have demonstrated that isoflurane-induced myocardial protection in rabbits is dependent on activation of the PI3K/Akt survival pathway (Raphael et al., 2005); however, whether PI3K/Akt signaling is also essential for anesthetic preconditioning-induced attenuation of apoptosis is unknown.

The signal transduction pathways that are involved in anesthetic-induced preconditioning share many common steps with the pathways that are activated by ischemic preconditioning. Therefore, the primary aim of the current investigation was to evaluate whether isoflurane administration before regional myocardial ischemia and reperfusion would inhibit myocardial apoptosis and whether this effect is mediated by the PI3K/Akt pathway. A main pathway of apoptosis in myocytes is by ischemia-reperfusion-induced expression of the Bcl-2 family proteins (Gross et al., 1999). Bad and Bax are proapoptotic, whereas phosphorylation of Bad inhibits its binding to, and inactivation of, the antiapoptotic protein, Bcl-2. Thus, increases in the expression of phospho-Bad and/or Bcl-2 would be antiapoptotic (Steenbergen et al., 2003). Therefore, an additional aim of the present study was to evaluate whether isoflurane-induced cardioprotection involves modulation of the expression of the Bcl-2 family proteins, specifically, Bcl-2, Bax, Bad, and phospho-Bad.

Materials and Methods

All experiments were conducted following the approval of the institutional Committee for Animal Care and Laboratory Use and in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication 85-23, revised 1996).

General Preparation. The rabbit model of regional myocardial ischemia and reperfusion has been described previously in detail (Raphael et al., 2005). In brief, 6-month-old New Zealand white male rabbits, weighing 2.8 to 3.2 kg, were anesthetized with i.v. pentobarbital (30 mg/kg) via a 20-gauge catheter in a marginal ear vein, followed by a 5 mg/kg/h infusion. Isoflurane was used according to the study protocol. Neuromuscular blocking agents were not administered, and anesthetic depth was assessed according to the eyelash reflex. A tracheostomy was performed using a ventral midline incision, and the rabbits were mechanically ventilated with positive pressure ventilation using 30 to 40% oxygen/air mixture to maintain an arterial oxygen partial pressure of 100 to 150 mm Hg. Ventilation rate was 30 to 35 breaths/min, and tidal volume was set at 12 ml/kg. The respiratory rate was adjusted to keep the blood pH in the range of 7.35 to 7.45. End expiratory carbon dioxide tension was monitored continuously. Catheters filled with heparinized saline (10 U/ml) were inserted in a carotid artery for arterial pressure monitoring and blood sampling and in an internal jugular vein for i.v. drug administration. Maintenance fluids (0.9% NaCl) were administered at 15 ml/kg/h during the experiment. Core body temperature was measured via a rectal temperature probe and maintained at 38.5 ± 0.2°C (normothermia for rabbits) with radiant heat and a warming blanket. A three-lead electrocardiogram was continuously recorded. A left thoracotomy was performed in the fourth intercostal space, the pericardium was opened, and the heart was suspended in a pericardial cradle. A 4-0 silk suture was passed around the left anterior descending coronary artery just distal to the first diagonal branch with a tapered needle, and the ends of the suture were threaded through a small vinyl tube to form a snare. Coronary artery occlusion was performed by tightening the snare around the coronary artery. Myocardial ischemia was confirmed by regional epicardial cyanosis and ST segment elevation in the electrocardiogram. Reperfusion was achieved by releasing the snare and confirmed by visual observation of reactive hyperemia.

Experimental Design. The experimental design is illustrated in Fig. 1. After a 30-min stabilization period, heparin (500 units) was administered i.v. to all the rabbits. All animals were subjected to 40 min of regional myocardial ischemia followed by 180 min of reperfusion. Rabbits were randomly assigned to one of the following groups: group 1, a nonischemic control group of sham-operated rabbits (n = 8); group 2, an ischemia-reperfusion group (40 min of myocardial ischemia and 180 min of reperfusion; I/R, n = 10); and group 3, isoflurane preconditioning group (Iso, n = 10). One minimum alveolar concentration of isoflurane (2.1%) (Drummond, 1985) was started at the end of the stabilization period and administered for 30 min, followed by 30 min of washout before coronary occlusion. End-tidal concentrations of isoflurane were measured at the tip of the tracheostomy tube using an infrared anesthetic analyzer (Dräger Medical, Lübeck, Germany) that was calibrated with known standards.

To evaluate the role of the PI3K/Akt pathway, the PI3K inhibitors, wortmannin, and LY294002 (Sigma, St. Louis, MO), were administered i.v. to the next six experimental groups. To rule out any direct effects of wortmannin or LY294002 on the heart during baseline conditions or during ischemia and reperfusion, additional control groups were evaluated. In group 4 (Wort + Sham, n = 8), rabbits were treated with wortmannin (0.6 mg/kg) (Chiari et al., 2005a) but did not undergo any coronary intervention. In group 5, wortmannin (0.6 mg/kg) was administered 70 min before coronary ischemia and reperfusion (Wort + I/R, n = 10). In group 6, wortmannin was administered i.v. (0.6 mg/kg) 10 min before isoflurane preconditioning (Wort + Iso, n = 10), and then the rabbits were subjected to I/R. Likewise, in group 7 (LY + Sham, n = 8), rabbits were treated with LY294002 (0.3 mg/kg) (Gross et al., 2004) but did not undergo any coronary intervention. In group 8, LY294002 was administered 70 min before coronary ischemia and reperfusion (LY + I/R, n = 10). In group 9, LY294002 was administered i.v. (0.3 mg/kg) 10 min before isoflurane preconditioning (LY + Iso, n = 10), and then the rabbits were subjected to I/R.

Measurements of Hemodynamics. Hemodynamic measurements included heart rate and mean arterial pressure. The rate-pressure product was calculated as the product of the heart rate and the peak mean arterial pressure. These parameters were continuously measured during the experimental protocol (Table 1).

Determination of the Area at Risk and Infarct Size. Determination of infarct size was done as described previously (Raphael et al., 2005). In brief, at the end of the experimental protocol, hearts were excised, mounted on a Langendorff apparatus, and perfused with phosphate-buffered saline at 100 cm of H₂O for 1 min to wash out intravascular blood. The coronary artery was reocluded, and 10 ml of 0.1% methylene blue was infused into the aortic root to label the normally perfused zone with deep blue color, thereby delineating the risk zone as a nonstained area. The hearts were then removed
from the Langendorff apparatus, trimmed of atria and great vessels, weighed, and frozen (in a cold chamber with a temperature of −18°C). Hearts were then cut into 2-mm transverse slices. The slices were incubated in 1% 2,3,5-triphenyltetrazolium chloride in pH 7.4 buffer for 20 min at 37°C. The slices were then placed in 10% neutral buffered formalin for 10 min to increase the contrast between stained and nonstained tissue. Because triphenyltetrazolium chloride stains viable tissue a deep red color, nonstained tissue was presumed to be infarcted. Slices were then photographed, the risk and infarct areas in each slice were measured by computer morphometry using the Bioquant imaging software, and the percentages of the at-risk and infarcted areas were calculated.

In the second set of experiments, nine similar experimental groups of rabbits (n = 5 in each group) were subjected to the same experimental procedures. At the end of reperfusion, the animals were sacrificed by an i.v. injection of 10% KCl solution, and myocardial samples were collected from ischemic left ventricle regions for evaluation of myocardial apoptosis and protein analysis. Samples were frozen in liquid nitrogen and kept in −80°C until further processed (for Western blotting) or were fixed in paraformaldehyde (for in situ identification of nuclear DNA fragmentation). All assays were done in triplicate.

Evaluation of Apoptosis. Detection of apoptotic cells was carried out using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. Terminal deoxynucleotidyl transferase binds to the exposed 3'-OH fragmented DNA ends and catalyzes the addition of conjugated deoxynucleotides for identification of apoptotic nuclei. LV tissues from the area at risk were

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**TABLE 1**
Systemic hemodynamics

<table>
<thead>
<tr>
<th>No.</th>
<th>Baseline</th>
<th>Coronary Occlusion (min)</th>
<th>Reperfusion (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>I/R</td>
<td>8</td>
<td>253 ± 6</td>
<td>257 ± 4</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>247 ± 4</td>
<td>261 ± 11</td>
</tr>
<tr>
<td>Iso</td>
<td>9</td>
<td>259 ± 7</td>
<td>254 ± 8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>255 ± 8</td>
<td>263 ± 13</td>
</tr>
<tr>
<td>Wort + Sham</td>
<td>8</td>
<td>252 ± 5</td>
<td>258 ± 7</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>248 ± 8</td>
<td>253 ± 6</td>
</tr>
<tr>
<td>MAP (mm Hg)</td>
<td>8</td>
<td>84 ± 3</td>
<td>66 ± 5*</td>
</tr>
<tr>
<td>I/R</td>
<td>9</td>
<td>87 ± 6</td>
<td>82 ± 7</td>
</tr>
<tr>
<td>Iso</td>
<td>9</td>
<td>83 ± 5</td>
<td>75 ± 2*</td>
</tr>
<tr>
<td>Wort + I/R</td>
<td>9</td>
<td>86 ± 8</td>
<td>71 ± 6*</td>
</tr>
<tr>
<td>Wort + Iso</td>
<td>8</td>
<td>84 ± 7</td>
<td>70 ± 4*</td>
</tr>
<tr>
<td>LY + I/R</td>
<td>9</td>
<td>88 ± 5</td>
<td>72 ± 6*</td>
</tr>
<tr>
<td>LY + Iso</td>
<td>8</td>
<td>216 ± 0.8</td>
<td>17 ± 1*</td>
</tr>
<tr>
<td>I/R</td>
<td>9</td>
<td>22 ± 1.1</td>
<td>20.6 ± 0.7*</td>
</tr>
<tr>
<td>Iso</td>
<td>9</td>
<td>21.5 ± 1.1</td>
<td>19.1 ± 1.2*</td>
</tr>
<tr>
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<td>21.9 ± 0.6</td>
<td>18.7 ± 0.6*</td>
</tr>
<tr>
<td>Wort + Iso</td>
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<td>21.2 ± 0.4</td>
<td>18.2 ± 0.5*</td>
</tr>
<tr>
<td>LY + I/R</td>
<td>9</td>
<td>21.8 ± 0.6</td>
<td>18.2 ± 0.8*</td>
</tr>
</tbody>
</table>

HR, heart rate; MAP, mean arterial pressure; RPP, rate-pressure product.

*, p < 0.05 compared with baseline values.

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**Fig. 1.** Diagram of the experimental protocol. Animals were subjected to 40 min of regional myocardial ischemia and 180 min of reperfusion. One MAC of isoflurane was administered for 30 min followed by 30 min of washout before coronary occlusion. Wortmannin or LY294002 (0.6 mg/kg and 0.3 mg/kg i.v., respectively) was administered 10 min before isoflurane preconditioning. Iso, isoflurane; Wort + sham, wortmannin + sham; Wort + I/R, wortmannin + ischemia reperfusion; Wort + Iso, wortmannin + isoflurane; LY + sham, LY294002 + sham; LY + I/R, LY294002 + isoflurane (here and in subsequent figures).
fixed in formalin for 24 h, embedded in paraffin, and 4-μm sections were obtained. The sections were then deparaffinized and rehydrated with xylene and graded alcohol series. The sections were stained using the Apoptosis Detection System (Promega, Madison, WI) according to the manufacturer’s instructions. Fluorescence staining was viewed with a confocal laser microscope (Olympus, Tokyo, Japan). Apoptotic cells exhibited strong nuclear green fluorescence with a standard fluorescence filter set (520 ± 20 nm). All cells stained with propidium iodide exhibited strong red cytoplasmic fluorescence when viewed at >620 nm. The percentage of TUNEL-positive cardiomyocyte nuclei was determined using ×200 magnification. Three sections from each myocardial sample were randomly selected, and 10 microscopic fields per section were evaluated by two independent observers. In each field, cells were counted, and the percentage of apoptotic cardiomyocytes was calculated.

Western Blotting for Total Akt, Phosphorylated Akt, Activated Caspase-3, Bcl-2, Bax, Bad, and Phospho-Bad. Myocardial samples were homogenized in ice-cold lysis buffer containing 20 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM Na₃VO₄, and complete protease inhibitor cocktail (one tablet per 10 ml; Roche and mouse monoclonal antiphospho-Bad (at Ser112) (Cell Signaling Technology) 1:1000 (v/v) dilution in 5% nonfat dry milk; Roche Diagnostics Corporation, Indianapolis, IN). The homogenate was centrifuged at 10,000g for 15 min at 4°C to remove cellular debris and isolate total protein. Protein concentrations were determined using a modified Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard. Equivalent amounts (50 μg) of protein samples were loaded and separated on 10% SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 (TBST), membranes were incubated for 1 h in room temperature with the following antibodies: mouse monoclonal antiphospho-Akt (at Ser473) (Cell Signaling Technology, Beverly, MA) 1:1000 (v/v) dilution in 5% nonfat dry milk; rabbit polyclonal anti-Bcl-2, Bad, and Bax (Cell Signaling Technology) 1:1000 (v/v) dilution in 5% nonfat dry milk; and mouse monoclonal antiphospho-Bad (at Ser112) (Cell Signaling Technology) 1:2000 (v/v) dilution with 5% bovine serum albumin, rabbit polyclonal anticaspase-3, and mouse polyclonal anti-Akt (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) 1:1000 (v/v) dilution in 5% nonfat dry milk. The membranes were then washed three times with TBST for 10 min and subsequently incubated for 1 h in 5% nonfat dry milk in TBST containing the appropriate secondary antibody of either a sheep anti-rabbit or sheep anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Jackson Immunolabs, West Grove, PA) at 1:3000 dilution. Peroxidase activity was visualized by means of an enhanced chemiluminescence substrate system (Amersham Pharmacia Biotech, Piscataway, NJ), followed by exposure to hyperfilm (Amersham Pharmacia Biotech). B-Actin (1:2000 dilution; Santa Cruz Biotechnology, Inc.) was detected on immunoblots as a loading control for protein quantity. Optical density for each band was determined using the NIH Image 1.6 (National Institutes of Health, Bethesda, MD) and normalized against background density for each gel.

Statistical Analysis. Statistical analysis was performed using SPSS 10.0 for Windows software (SPSS Inc., Chicago, IL). Values are expressed as means ± S.E.M. The statistical significance was determined by analysis of variance with repeated measurements followed by the Bonferroni correction for post hoc Student’s t tests, p < 0.05 was considered to be statistically significant.

Results

One hundred twenty-nine rabbits were studied to successfully complete 121 experiments; eight animals were excluded because of refractory ventricular fibrillation. The incidence of refractory ventricular fibrillation was not significantly different among the groups (2/10 I/R, 1/10 Iso, 1/10 Wort + Iso, 1/10 Wort + I/R, 2/10 LY + I/R, and 1/10 LY + Iso). There was no mortality in the sham-operated animals.

Systemic Hemodynamics. Hemodynamic data are presented in Table 1. There were no differences in baseline hemodynamic parameters among groups, nor were there any differences during the experiment in the two sham-operated animal groups. Mean arterial pressure decreased in the isoflurane group during administration of the agent. After the washout period, the pressure returned to baseline values. A transient increase in heart rate (p = N.S.) was noted during isoflurane administration. Left anterior descending occlusion significantly decreased (p < 0.05) mean arterial pressure and rate-pressure product in the I/R, Wort + I/R, Wort + Iso, LY + I/R, and LY + Iso groups. Heart rate, mean arterial pressure, and rate-pressure product decreased over time during reperfusion in all experimental groups. This reduction, which can be explained, at least partially, by a decrease in the surgical stimulus, was significant compared with baseline values (p < 0.05). However, there were no significant differences in the hemodynamic parameters among groups during reperfusion.

Isoflurane Preconditioning Reduces Infarct Size. The ratio of area at risk to left ventricular mass (AR/LV) did not differ significantly among the groups (51 ± 2% in the I/R group, 54 ± 3% in the Iso group, 47 ± 4% in the Wort + I/R group, 48 ± 3% in the Wort + Iso group, 48 ± 4.2% in the LY + I/R group, and 52 ± 1.7% in the LY + Iso group, p = N.S.) (Fig. 2A). These data suggest that changes in the infarct sizes observed in the various experimental groups cannot be related to the percentage of the left ventricular myocardium that was occluded.

In the I/R group, the infarct size was 41 ± 5% of the area at risk. Pretreatment with isoflurane reduced the infarct size to 22 ± 4% (p < 0.05 compared with the I/R group). Administration of the PI3K inhibitors wortmannin or LY294002 was considered to be statistically significant.
before myocardial ischemia and reperfusion did not affect infarct size (38 ± 3 and 42 ± 3.2%, respectively, \( p = \text{N.S.} \) versus I/R group). Both inhibitors eliminated the cardioprotection produced by 1 minimum alveolar concentration (MAC) of isoflurane (infarct size was 44 ± 3 and 45 ± 2.6%, respectively, \( p < 0.05 \) compared with the Iso group). No myocardial infarctions were measured in the various groups of sham-operated animals (Fig. 2B).

**Isoflurane Preconditioning Decreases Myocardial Apoptosis.** Analysis of DNA fragmentation was performed using the TUNEL method at single-cell level. The mean percentage of TUNEL-positive cardiomyocyte nuclei was 12.4 ± 1.6% in the I/R group. Isoflurane preconditioning significantly reduced the percentage of apoptotic myocytes to 3.8 ± 1.2% \( (p < 0.05) \). This effect was abolished by PI3K inhibition, and the percentage of TUNEL-positive nuclei increased to 11.9 ± 1.7% in the Wort + Iso and 11.7 ± 2.2% in the LY + Iso groups. Wortmannin and LY294002 did not have any direct effect on the percentage of apoptotic nuclei as observed in the Wort + I/R and LY + I/R groups (11.4 ± 2.1% and 12.6 ± 2.2%, respectively, \( p = \text{N.S.} \) versus the I/R group). TUNEL-positive nuclei were not detected in the sham-operated animals or Wort + sham or LY + sham groups (Fig. 3).

To determine whether caspase-3 was activated, we detected the cleaved caspase-3 protein by Western blotting. Caspase-3 normally exists as a 32-kDa inactive precursor that is cleaved proteolytically to an active p17 subunit when cells are induced to undergo apoptosis. As shown in Fig. 4, caspase-3 expression was comparable in all experimental groups. The tissue abundance of the cleaved caspase-3 significantly increased in the I/R group after 40 min of myocardial ischemia and 180 min of reperfusion. Isoflurane inhibited the increase in cleaved caspase-3 to a degree comparable with the sham animals. Pretreatment with wortmannin or LY294002 abolished the effect of isoflurane on cleaved caspase-3 expression.

**Isoflurane Preconditioning Increases the Expression of Phosphorylated Akt.** PI3K and its downstream-regulated protein Akt are known to play an important role in survival against ischemia/reperfusion-induced myocardial damage. To investigate whether Akt is involved in isoflurane-induced cardioprotection, we evaluated the expression of Akt and its activated, phosphorylated form (phospho-Akt) at Ser473 during myocardial ischemia and reperfusion in the various experimental conditions (Fig. 5). Total Akt expression was comparable in all experimental groups. Phospho-Akt expression was significantly increased in the I/R and preconditioning groups compared with sham-operated controls. Pretreatment by wortmannin and LY294002 inhibited the phosphorylation of Akt in isoflurane-treated and nontreated rabbits to a degree comparable with sham-operated animals.

**Bcl-2 Family Protein Expression and Bad Phosphorylation.** The changes in expression of Bcl-2, Bax, and Bad proteins were analyzed by Western blotting (Figs. 6 and 7).
In the I/R group, the tissue abundance of Bcl-2 was significantly reduced compared with the sham-operated animals. Isoflurane preconditioning restored Bcl-2 expression to a level similar to that of the sham group ($p < 0.05$ versus I/R). Tissue expression of the proapoptotic protein Bax was significantly increased in the I/R group. Isoflurane preconditioning inhibited the increase in Bax ($p < 0.05$ versus I/R) and maintained it at a level comparable with the sham groups. PI3K inhibition with wortmannin or LY294002 abolished the effects of isoflurane: Bcl-2 tissue abundance was decreased, and Bax expression was increased to a degree similar to that of the I/R group (Fig. 7).

Discussion

The major findings of the current study are as follows. First, we demonstrated that isoflurane preconditioning attenuated myocardial apoptosis and reduced infarct size after I/R injury. Second, the cardioprotective effects of anesthetic preconditioning are dependent upon activation of the PI3K...
survival pathway and phosphorylation of Akt. Third, inhibition of the myocardial apoptotic processes by volatile anesthetics may involve modulation of the expression of pro- and antiapoptotic proteins, specifically Bax, phospho-Bad, and Bcl-2.

Apoptosis is a fundamental process of cell death that occurs via activation of distinct signaling pathways involving mitochondria, mitochondrial regulatory proteins, and activation of caspases. Ultimately, cells undergo nuclear chromatin condensation, DNA fragmentation, and formation of apoptotic bodies (van Heerde et al., 2000). Ischemia and reperfusion, but not ischemia alone, have been shown to initiate this apoptotic cascade in myocardial cells (Freude et al., 2000). A study by Kajstura et al. (1996) demonstrated that apoptosis is the predominant form of ischemia and reperfusion-related cell death in the heart. Both DNA fragmentation and infarct size were significantly decreased after prolonged ischemia and reperfusion in the preconditioned rat heart in vivo (Piot et al., 1997). Moreover, a direct correlation between myocardial infarct size and internucleosomal DNA fragmentation was reported (Piot et al., 1997).

Using an in vivo model of regional myocardial ischemia and reperfusion, our data demonstrate significant reduction of apoptosis after isoflurane preconditioning, which correlated with reduction of infarct size in the heart. Furthermore, the findings of the current investigation indicate that these effects of isoflurane are mediated by the PI3K/Akt survival pathway.

The Bcl-2 family of proteins regulates apoptosis by modulating mitochondrial permeability and the release of cytochrome c (Zhao and Vinten-Johansen, 2002; Valen, 2003). The antiapoptotic protein Bcl-2 resides in the outer mitochondrial wall and inhibits cytochrome c release. The proapoptotic proteins, such as Bad and Bax, are located in the cytosol but translocate to the mitochondria and form a proapoptotic complex with Bcl-2. This translocation is inhibited by phosphorylation of Bad, leading to its cytosolic sequestration. Thus, phosphorylation of Bad may promote cell survival (Steenbergen et al., 2003). Moreover, Steenbergen et al. (2003) recently found a significant decrease in phospho-Bad (Ser112) in failing hearts, and this was also consistent with a proapoptotic shift in heart failure. Baines et al. (2002) reported that activation of protein kinase C/ extracellular regulated proteins was associated with phosphorylation of Bad at Ser112. Bad can be phosphorylated on Ser 112, Ser136, Ser155, and Ser170, but the relative importance of phosphorylation at different sites is still unclear (Steenbergen et al., 2003).

The ratio of Bcl-2/Bax protein has also been suggested to determine survival or death after ischemia and reperfusion (Zhao et al., 2000a,b). Furthermore, it has also been demonstrated that ischemia and reperfusion significantly decreased the expression of Bcl-2 and increased the expression of Bax in the ischemic-reperfused myocardium (Zhao et al., 2000a,b).

Consistent with these studies, our results indicate that 40 min of regional myocardial ischemia and 180 min of reperfusion caused a decrease in myocardial expression of the antiapoptotic protein Bel-2 and an increase in the tissue abundance of the proapoptotic protein Bax. Isoflurane preconditioning prevented these changes, thus inhibiting the decrease in the expression of Bel-2 and the increase in the expression of Bax, leading to an increased Bel-2/Bax ratio. Pretreatment with the PI3K inhibitors wortmannin or LY294002 before isoflurane preconditioning inhibited the protective effects of isoflurane on Bel-2 and Bax expression, suggesting that the effect of isoflurane is mediated through PI3K/Akt signaling. There was no significant difference in the tissue abundance of total Bad among the various exper-
imental groups. However, Bad phosphorylation was reduced in the I/R group. Isoflurane preconditioning prevented the decrease in Bad phosphorylation, thus maintaining it in its nonactivated form. Again, pretreatment with wortmannin abolished the effect of isoflurane, leading to a reduction in Bad phosphorylation.

Anesthetic preconditioning confers protection against ischemia and reperfusion in the heart (Tanaka et al., 2004a). Isoflurane was reported to decrease norepinephrine-induced apoptosis in isolated cardiomyocytes (Zaugg et al., 2000) as well as protect against cytokine-induced injury in endothelial and vascular smooth muscle cells (de Klaver et al., 2002). The current investigation, however, demonstrates, for the first time, attenuation of myocardial apoptosis and reduction in infarct size in the heart, using an in vivo model of regional myocardial ischemia and reperfusion. Furthermore, our data indicate that the cardioprotective effect of isoflurane preconditioning may involve activation of the PI3K/Akt survival pathway. Activation of the PI3K/Akt pathway has been demonstrated to play a key role in both early and delayed myocardial preconditioning (Hausenloy and Yellon, 2004; Murphy, 2004; Hausenloy et al., 2005). PI3K converts phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate (Hausenloy and Yellon, 2004). Phosphatidylinositol-3,4,5-trisphosphate-stimulated phosphorylation of the serine/threonine kinase Akt by phosphoinositide-dependent kinase 1 subsequently inhibits formation of the pro-apoptotic proteins Bad, Bax, and caspase-9 (Cantley, 2002), as well as maintaining the levels of the antiapoptotic protein Bcl-2 (Uchiyama et al., 2004). Moreover, Akt has been shown to increase the formation of nitric oxide (Dimmel et al., 1999). The protective actions of nitric oxide during ischemic and anesthetic preconditioning have been already demonstrated (Novalija et al., 2003a; Chiari et al., 2005b). In addition, phosphoinositide-dependent kinase 1 is a potent activator of other protein kinases, including protein kinase C, which has been implicated in the protection of myocardium against ischemia and reperfusion injury by reducing apoptosis as well as decreasing myocardial infarct size (Liu et al., 2002). Therefore, it seems that the PI3K signaling cascade may contribute to the recruitment of multiple endogenous cardioprotective pathways to reduce myocardial damage after ischemia and reperfusion.

Our results are in agreement with a recent investigation conducted by Chiari et al. (2005a) who demonstrated a role for the PI3K/Akt survival pathway in anesthetic postconditioning. However, the current study extends these findings by demonstrating that preconditioning by isoflurane attenuates myocardial apoptosis and reduces infarct size. This effect is mediated by activation of PI3K signaling and phosphorylation of the downstream kinase Akt.

In the present study, we assessed apoptosis after a relatively short period of reperfusion (i.e., 3 h) by TUNEL staining and measurement of activated caspase-3 tissue abundance, which are known to measure the later stages of cell death. It has been reported that the extent of necrosis peaks at 24 h of reperfusion and remains constant thereafter (Zhao et al., 2000a). On the other hand, the appearance of apoptotic cells in the perinecrotic area progressively increases up to 72 h, suggesting that necrosis and apoptosis occur simultaneously during reperfusion, with a rapidly developing necrotic cell death during the early phase of reperfusion, followed by a slower appearance of apoptosis during the late phase of reperfusion. The number of apoptotic cells in the perinecrotic myocardium progressively increases during the extended period of reperfusion, which suggests the role of apoptosis in the development of infarction (Zhao et al., 2001b). Considering the reported delayed preconditioning effects of isoflurane (Tonkovic-Capin et al., 2002; Tanaka et al., 2004b), it would not be surprising if isoflurane also inhibits the extent of apoptosis in models of delayed preconditioning. This is an interesting question, which warrants future investigation.

Our results demonstrate that I/R itself caused a significant increase in Akt phosphorylation; however, expression of the proapoptotic proteins Bax increased after I/R and the level of Bcl-2 and phospho-Bad decreased. Other factors like extracellular signal-regulated kinase (ERK) 1 and 2 were found to be involved in targeting the apoptotic component of reperfusion-induced cell death through modulation of pro- and anti-apoptotic protein levels (Hausenloy and Yellon, 2004). Although there are no reports regarding the effect of isoflurane on ERK1/2 levels in myocardial ischemia and reperfusion, the anesthetic agent desflurane was found to provide myocardial protection through activation of ERK1/2 (Toma et al., 2004).

The current results must be interpreted within the constraints of several potential limitations. Myocardial infarct size is determined primarily by the size of the area at risk and extent of coronary collateral perfusion. The area at risk, expressed as a percentage of total LV mass, was similar among groups in the current investigation. Rabbits have also been shown to possess little if any coronary collateral blood flow (Maxwell et al., 1987). Thus, it seems unlikely that differences in collateral perfusion among groups account for the observed results. However, coronary collateral blood flow was not specifically quantified in the current investigation. Phosphorylation of Akt by isoflurane and its inhibition by the PI3K antagonist wortmannin provides strong supportive evidence for the involvement of PI3K in isoflurane-induced myocardial protection. Nevertheless, the possibility that other unrelated protein kinases were responsible for phosphorylation of Akt cannot be entirely excluded based on the current results.

In summary, the current investigation suggests that preconditioning by administration of 1 MAC of isoflurane before myocardial ischemia and reperfusion protects the heart by attenuating myocardial apoptosis that also correlates with reduction in infarction size. These effects of isoflurane preconditioning seem to be mediated by activation of the PI3K pathway and phosphorylation of Akt. Additional research is required to identify other signaling elements involved in anesthetic preconditioning and clarify the mechanisms responsible for this phenomenon.

References


Chiari PC, Bienengraeber MW, Pagel FS, Krokowski JG, Kersten JR, and Warrick DC (2005a) Isoflurane protects against myocardial infarction during early reper-

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