Activation of Adenosine Triphosphate–regulated Potassium Channels during Reperfusion Restores Isoflurane Postconditioning-induced Cardiac Protection in Acutely Hyperglycemic Rabbits

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ABSTRACT

Background: Hyperglycemia is known to inhibit myocardial anesthetic postconditioning. The authors tested whether activation of adenosine triphosphate–regulated potassium (K_{ATP}) channels would restore anesthetic postconditioning during acute hyperglycemia.

Methods: Rabbits subjected to 40-min myocardial ischemia and 3-h reperfusion (ischemia–reperfusion [I/R]) were assigned to groups (n = 10 in each group) with or without isoflurane postconditioning (2.1% for 5 min) in the presence or absence of hyperglycemia and/or the K_{ATP} channel agonist diazoxide. Creatine kinase MB fraction and infarct size were measured. Phosphorylated protein kinase B (Akt) and endothelial nitric oxide synthase (eNOS) were assessed. Oxidative stress was evaluated by measuring malondialdehyde, and apoptosis was assessed by dUTP nick-end labeling and activated caspase-3.

Results: Postconditioning significantly reduced myocardial infarct size (26 ± 4% in the isoflurane [ISO] group *vs.* 53 ± 2% in the I/R group; $P = 0.007$); whereas, hyperglycemia inhibited this effect (infarct size: 47 ± 2%, $P = 0.02$ *vs.* the ISO group). Phosphorylated and eNOS levels increased, whereas malondialdehyde and myocardial apoptosis were significantly lower after isoflurane postconditioning compared with I/R. These effects were inhibited by acute hyperglycemia. Diazoxide restored the protective effect of isoflurane in the hyperglycemic animals (infarct size: 29 ± 2%; $P = 0.01$ *vs.* the I/R group), reduced malondialdehyde levels and myocardial apoptosis, but did not affect the expression of phosphorylated Akt or eNOS.

Conclusions: K_{ATP} channel activation restored anesthetic postconditioning-induced myocardial protection under acute hyperglycemia. This effect occurred without increasing Akt or eNOS phosphorylation, suggesting that K_{ATP} channels are located downstream to Akt and eNOS in the pathway of isoflurane-induced myocardial postconditioning. **(ANESTHESIOLOGY 2015; 122:1299-311)**

ACUTE hyperglycemia is a common perioperative finding, especially after cardiac surgery[1] and is associated with increased morbidity and mortality in diabetics as well as in nondiabetic patients.[2] Hyperglycemia is also associated with a larger myocardial infarct size and decreased postischemic ventricular function.[3] Furthermore, acute hyperglycemia is a common finding on admission in patients with myocardial infarction (MI) and is associated with worse outcome.[4]

It has been demonstrated that volatile anesthetics administered during the initiation phase of reperfusion provide cardiac protection and reduce infarct size to an extent similar to that caused by ischemic and pharmacological preconditioning.[5,6] We and others have reported that this phenomenon, termed anesthetic postconditioning, is associated with activation of the phosphatidylinositol-3-kinase (PI3K)/Akt survival pathway and up-regulation in nitric oxide availability.[7,8] In addition, similar to preconditioning, ATP-regulated potassium (K_{ATP}) channels were found to have an important role in volatile anesthetic-induced postconditioning.[9]

What We Already Know about This Topic

- Acute hyperglycemia has been shown to inhibit the cardioprotective effects of ischemic- and anesthetic-induced preconditioning. This loss of protection is associated with increased oxidative stress and decreased production of nitric oxide.
- This study determined whether activation of adenosine triphosphate–regulated potassium (K_{ATP}) channels would restore anesthetic postconditioning during acute hyperglycemia.

What This Article Tells Us That Is New

- Adenosine triphosphate–regulated potassium (K_{ATP}) channel activation restored anesthetic postconditioning-induced myocardial protection under acute hyperglycemia. This effect occurred without increasing protein kinase B (Akt) or endothelial nitric oxide synthase (eNOS) phosphorylation, suggesting that K_{ATP} channels are located downstream to Akt and eNOS in the pathway of isoflurane-induced myocardial postconditioning.

Acute hyperglycemia has been shown to inhibit the cardioprotective effects of ischemic and anesthetic-induced preconditioning.[10,11] This loss of protection is associated

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with increased oxidative stress[11] and decreased production of nitric oxide.[12] We have recently demonstrated that acute hyperglycemia inhibited anesthetic-induced myocardial postconditioning in rabbits *via* inhibition of the phosphorylation of Akt and endothelial nitric oxide synthase (eNOS) as well as decrease in myocardial nitric oxide content.[7] In the current investigation, we sought to examine whether activation of the mitochondrial ATP-mediated potassium (K_{ATP}) channels would restore the cardioprotective effects of anesthetic postconditioning in acutely hyperglycemic rabbits, and whether this effect was also mediated *via* PI3K/Akt and nitric oxide signaling. Furthermore, because hyperglycemia is associated with production of large amounts of reactive oxygen species (ROS) and increased apoptotic cell death,[13–15] both of which may contribute to the pathogenesis of myocardial injury and infarction, we also evaluated the effects of anesthetic postconditioning with or without K_{ATP} channel activation under normoglycemia and acute hyperglycemia on lipid peroxidation and apoptosis.

Materials and Methods

All experiments were conducted after the approval of the institutional board for Animal Care and Laboratory Use of the Hebrew University-Hadassah Medical School, Jerusalem, Israel. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, National Academy of Sciences, 8th edition, 2011).

General Preparation

The rabbit model of regional myocardial ischemia and reperfusion has been previously described in the Method section of our previous publication.[7] In brief, 6-month-old New Zealand white male rabbits weighing 2.5 to 3.0 kg were anesthetized with intravenous sodium thiopental (30 mg/kg), followed by a 5 mg kg^{-1} h^{-1} infusion. Neuromuscular-blocking agents were not used. Anesthetic depth was assessed repeatedly using the corneal and pedal reflexes. A tracheostomy was performed, and the rabbits were mechanically ventilated using positive pressure ventilation with 33% oxygen–air mixture to maintain an arterial oxygen partial pressure of 100 to 150 mmHg. Ventilation rate and tidal volumes were set to maintain the blood pH in the range of 7.35 to 7.45. End-expiratory carbon dioxide tension was monitored continuously and maintained at 35 to 45 mmHg. Heparinized saline-filled catheters were inserted in the right carotid artery and right internal jugular vein for arterial pressure monitoring, blood sampling, and intravenous drug administration, respectively. Intravenous 0.9% NaCl was administered at 5 to 7 ml kg^{-1} h^{-1} throughout the experiment. The rabbits' body temperature was monitored using a rectal temperature probe and maintained at 38.5° ± 0.2°C with an underlying warming blanket. A 3-lead electrocardiogram was continuously recorded to monitor for myocardial ischemia. The myocardium was accessed *via* a left thoracotomy in the fourth intercostal space. A 4-0 silk suture was passed around the left anterior descending (LAD) coronary artery immediately distal to the first diagonal branch, and the suture's ends threaded through a small vinyl tube to form a snare. Coronary artery occlusion was achieved by tightening the snare, and myocardial ischemia was confirmed by epicardial cyanosis and ST-segment elevation in the electrocardiogram. Reperfusion was achieved by releasing the snare and confirmed by visualization of reactive hyperemia.

Experimental Design

The experimental design is illustrated in figure 1. Baseline hemodynamics were recorded at the end of a 30-min stabilization period, and heparin (300 units/kg) was administered intravenously. All animals were subjected to 40 min of myocardial ischemia and 3 h of reperfusion. After computer-based randomization, the animals were assigned to one of the following six groups (n = 10 in each group): group 1 ischemia–reperfusion (I/R): (40-min ischemia and 3-h reperfusion) control group; group 2 isoflurane (ISO): postconditioning with 1.0 minimum alveolar concentration of isoflurane (1.0 minimum alveolar concentration of isoflurane = 2.1% in rabbits[16]) administered for 5 min: 2 min before and 3 min after the initiation of reperfusion; group 3 (HG + I/R): hyperglycemia was caused by intravenous infusion of 15% dextrose in water for 60 min starting 10 min before the ischemic phase and continued until 10 min after the initiation of reperfusion to increase blood glucose concentration to 300 to 350 mg/dl; group 4 (HG + ISO): postconditioning with isoflurane in the presence of hyperglycemia; group 5 (HG + Diaz): administration of diazoxide (a mitochondrial K_{ATP} channel agonist; Sigma, USA) before reperfusion in the presence of hyperglycemia. Diazoxide was dissolved in 0.9% NaCl and administered intravenously at a dose of 5 mg/kg over the last 5 min of coronary occlusion before reperfusion to achieve a maximal effect at the onset of reperfusion.[17] Group 6 (HG + ISO + Diaz): postconditioning with isoflurane in the presence of hyperglycemia in diazoxide-treated rabbits.

To evaluate a potential effect of diazoxide on PI3K/Akt and eNOS signaling and on myocardial apoptosis in anesthetic postconditioning in the presence of hyperglycemia, a second set of experiments was performed: rabbits (n = 5 in each group) were subjected to the same experimental conditions as above. At the end of the reperfusion period, the animals were euthanized by an intravenous injection of sodium thiopental (100 mg/Kg), and myocardial samples were collected from the ischemic and transition zone of the left ventricle 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 paraffin (for identification of apoptotic cells). All assays were repeated in duplicates.

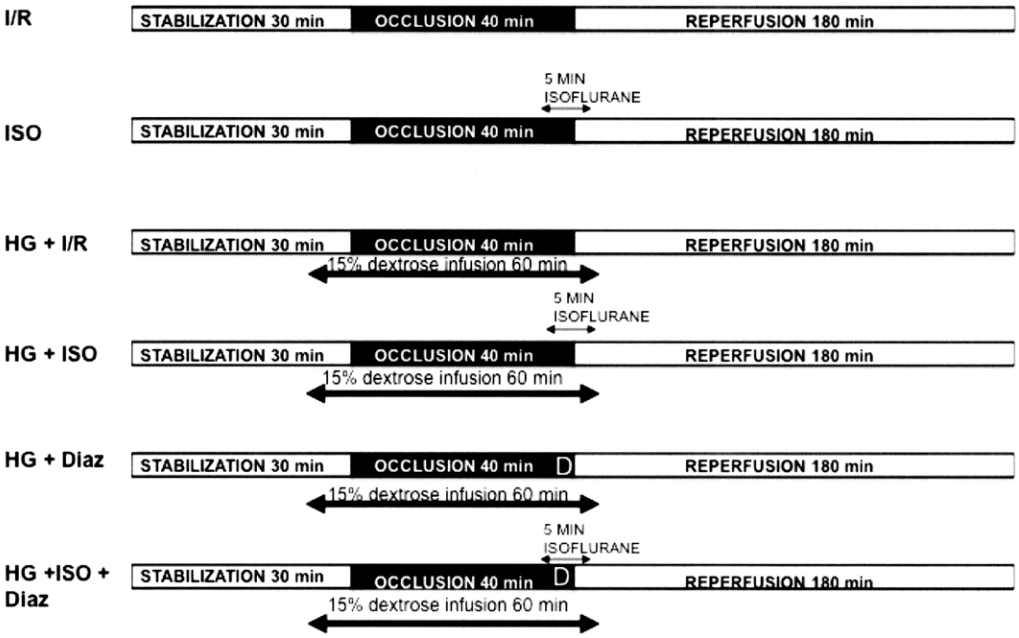

Fig. 1. Diagram of the experimental protocol. Animals were subjected to 40 min of regional myocardial ischemia and 180 min of reperfusion. One minimal alveolar concentration of isoflurane was administered for 3 min before and 2 min after reperfusion. Dextrose 15% in water was administered intravenously for 60 min starting 10 min before the ischemia and continued until 10 min of reperfusion. D = diazoxide administration; HG + Diaz = hyperglycemia + diazoxide; HG + I/R = hyperglycemia + ischemia–reperfusion; HG + ISO = hyperglycemia + isoflurane postconditioning; HG + ISO + Diaz = diazoxide administration to isoflurane-postconditioned rabbits in the presence of hyperglycemia; I/R = ischemia–reperfusion; ISO = isoflurane postconditioning.

Hemodynamic Measurements

Hemodynamic measurements were arterial blood pressure and heart rate. The data were digitalized using an analog to digital converter (PowerLab/8SP; AD Instruments, Australia) using a sampling rate of 500 Hz and constantly recorded on a computer using Chart for Windows v5.0 software (AD Instruments). The rate–pressure product was calculated as the product of the heart rate and the peak mean arterial pressure (table 1).

Determination of the Area at Risk and Infarct Size

Myocardial infarction was measured as previously described.[7] In brief, at the end of reperfusion, hearts were excised, mounted on a Langendorff apparatus (AD Instruments, Australia), and perfused with phosphate-buffered saline. The LAD coronary artery was reoccluded, and 10 ml of 0.1% Evans blue dye (Sigma-Aldrich, USA) were infused into the aortic root to label the perfused zone with deep blue color, hence delineating the risk zone as a nonstained area. The hearts were then removed from the Langendorff device. The atria and great vessels removed, and the remainder tissue weighed and frozen. The hearts were then cut into 2-mm transverse slices and incubated in 1% 2,3,5-triphenyltetrazolium chloride (TTC) in pH 7.4 buffer for 20 min at 37°C. The slices were then incubated in 10% neutral buffered formaldehyde overnight to increase the contrast between stained and nonstained tissue. Because TTC stains viable tissue to a deep red color, nonstained tissue was presumed to be infarcted. Slices were then photographed, and the risk and infarct areas in each slice were measured by computer morphometry using the Bioquant imaging software (Bioquant Imaging Corporation, USA), and the percentages of the at-risk and infracted areas were calculated.

Measurements of Serum Creatine Kinase-MB, Blood Glucose, and Plasma Insulin Levels

Blood samples were obtained and analyzed for serum concentrations of the MB fraction of creatine kinase (CK-MB) using a CK assay kit (Sigma Diagnostics) per the manufacturer's specifications. Samples were collected at the following time points: at baseline (just before coronary occlusion), 20 and 40 min after LAD occlusion, and 30, 60, 120, and 180 min during reperfusion. Blood samples for glucose measurement in the various experimental groups (using the Free-Style Freedom Blood Glucose Meter; Abbott, United Kingdom) were collected at baseline, 10, 30, and 60 min after initiation of the dextrose infusion and 30, 60, and 120 min after the dextrose infusion was discontinued.

Insulin plasma levels were measured using a Rabbit/Dog Insulin Elisa Kit (Cosmo Bio USA, USA) to determine an appropriate physiological endocrine response to hyperglycemia. Samples were collected at baseline, 20 min after initiation of ischemia, and after 30 min of reperfusion. During ischemia, plasma insulin level was two-three times higher

Table 1. Systemic Hemodynamics

			Coronary Occlusion (min)		Reperfusion (min)				
	No.	Baseline	20	40	10	30	60	120	180
HR(beats/min)									
I/R	8	266±8	251±4	260±8	239±14*	228±5*	227±6*	220±9*	219±11*
ISO	8	254±7	264±11	266±7	239±8*	236±3*	233±7*	215±6*	222±9*
HG + I/R	9	262±5	255±6	250±3	251±9	232±7*	229±4*	221±11*	217±12*
HG + ISO	8	261±6	266±11	262±10	238±7	231±7*	234±8*	216±5*	223±6*
HG + Diaz	10	270±6	273±9	259±7	242±13	241±5*	228±8*	217±7*	221±11*
HG + ISO + Diaz	8	260±10	269±7	263±11	252±7	247±6	231±11*	226±5*	222±9*
MAP (mmHg)									
I/R	8	83±7	66±5*	62±9*	66±7*	58±4*	55±4*	54±4*	52±4*
ISO	8	85±4	82±7	77±7	61±5*	61±6*	57±7*	55±6*	55±5*
HG + I/R	9	86±5	76±2*	77±9	72±5*	59±5*	60±5*	61±9*	63±7*
HG + ISO	8	81±7	71±8*	72±12*	66±6*	57±3*	56±7*	53±8*	55±6*
HG + Diaz	10	79±8	69±6	66±7*	66±4*	61±3*	55±4*	52±6*	55±8*
HG + ISO + Diaz	8	82±6	77±4	72±6	68±9*	57±5*	60±7*	53±4*	53±7*
RPP (min^{-1} mmHg 10^3)									
I/R	8	21.4±0.8	17±1	16.1±1.4*	14.6±0.7*	13.2±0.6*	12.7±0.5*	11.8±0.7*	11.5±0.3*
ISO	8	23.2±1.1	20.6±0.7	19.9±0.6*	14.4±1.4*	14.2±0.5*	13.7±1.2*	11.6±0.9*	11.8±0.8*
HG + I/R	9	21.5±1.2	19.1±1.2	19.2±0.9*	17.1±1.1*	14±1.4*	13.7±0.4*	13.3±1.1*	12.8±1.4*
HG + ISO	8	21.7±0.5	18.7±0.6*	19.8±1.5*	16.0±0.9*	13.6±1.1*	12.3±1.4*	11.6±1.2*	11.6±1.4*
HG + Diaz	10	21.3±0.8	18.8±1.1	17.1±1.1*	16.0±1.3*	12.5±1.2*	11.3±1.2*	11.2±0.5*	12.1±0.4*
HG + ISO + Diaz	8	21.3±0.6	20.5±0.4	18.9±1.3*	17.1±0.7*	13.8±0.6*	13.6±0.9*	12.1±0.8*	11.7±1.3*

Nine animals were excluded because of refractory ventricular fibrillation (seven rabbits) or area at risk <20% of the left ventricle (two rabbits). The sizes of the groups presented in the table are after exclusion of the rabbits that did not complete the experiments. Data are presented as mean ± SD.

*$P < 0.05$ compared with baseline values.

HG + Diaz = hyperglycemia + diazoxide; HG + I/R = hyperglycemia + ischemia–reperfusion; HG + ISO = hyperglycemia + isoflurane postconditioning; HG + ISO + Diaz = diazoxide administration to isoflurane-postconditioned rabbits in the presence of hyperglycemia; HR = heart rate; I/R = ischemia–reperfusion; ISO = isoflurane postconditioning; MAP = mean arterial pressure; RPP = rate–pressure product.

in the hyperglycemic animals compared with nonhyperglycemic animals. At 30 min of reperfusion, insulin was still three-fold higher in the hyperglycemic groups compared with nonhyperglycemic animals.

Quantification of Plasma Malondialdehyde

Plasma malondialdehyde, a marker of lipid peroxidation, was measured to estimate the extent of lipid peroxidation in the myocardium. Malondialdehyde levels were measured at baseline and 30, 60, 120, and 180 min after reperfusion. At each time point, 1 ml of venous blood was collected and centrifuged (5,000*g* at 4°C) for 10 min, and the plasma stored at −80°C until further analyzed. Malondialdehyde activity was measured using a commercial colorimetric assay kit (Cayman Chemical Company, USA) according to the manufacturer's instructions and expressed as micromoles per liter.

Terminal Deoxynucleotidyl dUTP Nick-end Labeling Staining

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining was performed as previously reported.[18] In brief, left ventricular samples from the area at risk were 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. Apoptotic cells were identified by TUNEL using an apoptosis detection kit (Promega, USA) according to the manufacturer's instructions.

The percentage of TUNEL-positive nuclei was determined using ×200 magnification. Three sections from each sample were randomly selected. Ten microscopic fields per section were evaluated by a blinded independent observer. In each field, cells were counted, and the percentage of apoptotic-positive nuclei was calculated.

Caspase-3 Activity

Caspase-3 activity was determined as previously described by Kin *et al.*,[19] using a colorimetric activity assay kit (Chemicon International Molecular, USA). Left ventricular samples from the area at risk were homogenized in lysis buffer (1% Triton X-100, 0.32 sucrose, NaCl, 1 mmol/l phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mmol/l Tris-HCl, and at pH 8.0) and then centrifuged (16,000*g* in 4°C) for 15 min. After protein content determination, equal amounts of protein (100 μg) were incubated with the reaction buffer (containing 10 mmol/l dithiothreitol) and 50 μmol/l of the caspase-3 substrate DEVD-7-amino-4-trifluromethyl coumarin (AFC) at 37°C for 60 min in a 96-well

plate. Caspase-3 activity was detected using a luminescence spectrometer (Molecular Devices, USA) by measuring the proteolytic cleavage of the DEVD-AFC substrate and AFC as standard at an excitation and emission wavelengths of 405 and 505 nm, respectively. Fold change in caspase-3 activity was calculated by normalizing the results from groups two through six to that of the I/R group.

Western Blotting for Total and Phosphorylated Akt and eNOS

Phosphatidylinositol-3-kinase/Akt and eNOS are known to play important roles in anesthetic-induced survival against ischemia–reperfusion injury.[5] We evaluated the expression of Akt and its activated/phosphorylated form (phospho-Akt) at Ser^{473} as well as total and phosphorylated eNOS (at Ser^{1177}) expression at the end of the reperfusion period in ischemic left ventricular myocardial samples from the various experimental groups. Western Blot analysis was performed as previously reported.[7] Protein concentrations were determined by the modified Bradford assay (Bio-Rad, USA) using bovine serum albumin as a standard. Equivalent amounts (100 μg) of protein samples were loaded and electrophoretically separated using 10% sodium dodecyl sulfate polyacrilamide gel and 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 anti-phospho-Akt (at Ser^{473}) (Cell Signaling Technology, USA) 1:1,000 (v/v) dilution in 5% nonfat dry milk, rabbit monoclonal anti-phospho-eNOS (at Ser^{1177}) (Cell Signaling Technology) 1:1,000 (v/v) dilution in 5% nonfat dry milk, mouse monoclonal anti-eNOS (Biomol, USA) 1:2,000 (v/v) dilution in 5% nonfat dry milk, and mouse polyclonal anti-Akt (Santa Cruz Biotechnology, USA) 1:1,000 (v/v) dilution in 5% nonfat dry milk. The membranes were washed three times with TBST and then incubated for 1 h in 5% nonfat dry milk in TBST with diluted horseradish peroxidase–conjugated appropriate secondary antibodies (Bio-Rad). Immune complexes were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, USA), followed by exposure to hyperfilms (Amersham Pharmacia Biotech). β-Actin (Santa Cruz Biotechnology) was detected on immunoblots as a loading control for protein quantity. Optical density for each band was determined using the National Institute of Health image program (NIH Image 1.6, USA) and normalized against background density for each gel.

Sample Size Estimate

Based on the results from our previous experience,[18,20] we assumed that the minimum detectable difference in infarct size among the six experimental groups is 50% with an expected SD of residuals of 20%. This assumption led to eight trials (repetitions) in each group to have a desired power of 0.8 at an α level of 0.05 using one-way ANOVA. Furthermore, because animal mortality could be as high as 20%, we added two rabbits to each group to ensure each group has results of at least eight animals. This has resulted in n = 10 in each experimental group.

Statistical Analysis

Data are expressed as mean ± S.D. Statistical analysis was performed using SPSS 12.0.1 for Windows software (SPSS Inc., USA). Hemodynamic data were analyzed by repeated-measures ANOVA followed by *post hoc* Bonferroni correction. Differences in glucose levels and CK-MB concentrations between groups were analyzed by two-way repeated-measures ANOVA (group × time). A high-order analysis was performed and revealed a significant interaction between groups and time. Therefore, a stepdown approach was taken to consider comparisons between two groups at each time. The comparison of glucose levels and CK-MB concentrations within each group was analyzed by repeated-measures ANOVA followed by Bonferroni *post hoc* analysis. All other data were compared by one-way ANOVA followed by Bonferroni *post hoc* correction. The nature of all tests was two tailed. *P* value less than 0.05 was considered to be statistically significant.

Results

Ninety rabbits were used to successfully complete 81 experiments. Nine animals were excluded because of refractory ventricular fibrillation (seven rabbits) or area at risk less than 20% of the left ventricle (two rabbits).

Systemic Hemodynamics

Hemodynamic data are reported in table 1. There were no differences in baseline parameters among the various experimental groups. After isoflurane administration, there was a transient, nonsignificant, increase in heart rate. LAD occlusion resulted in a significant decrease ($P = 0.04$) in mean arterial blood pressure and thus the rate–pressure product in the I/R, HG + I/R, HG + ISO, and HG + Diaz groups. Heart rate, mean arterial blood pressure, and rate–pressure product decreased over time during reperfusion in all the experimental groups. This reduction, which can be explained, at least partially, by a decrease in the surgical stimulus, was significant compared with baseline values ($P = 0.04$); however, there were no significant differences in the hemodynamic parameters among groups during reperfusion.

Blood Glucose Concentrations

Blood glucose concentrations are shown in figure 2A. Mean blood glucose concentration at baseline was 136 ± 14 mg/dl and did not differ among the various groups. Blood glucose concentrations did not change significantly over time in the I/R and the ISO groups. Dextrose infusion caused a very large increase in blood glucose levels at the time points tested in the HG + I/R, HG + ISO, HG + DIAZ, and HG + ISO + DIAZ groups ($F(1,15) = 340.26$, $P < 0.001$ for

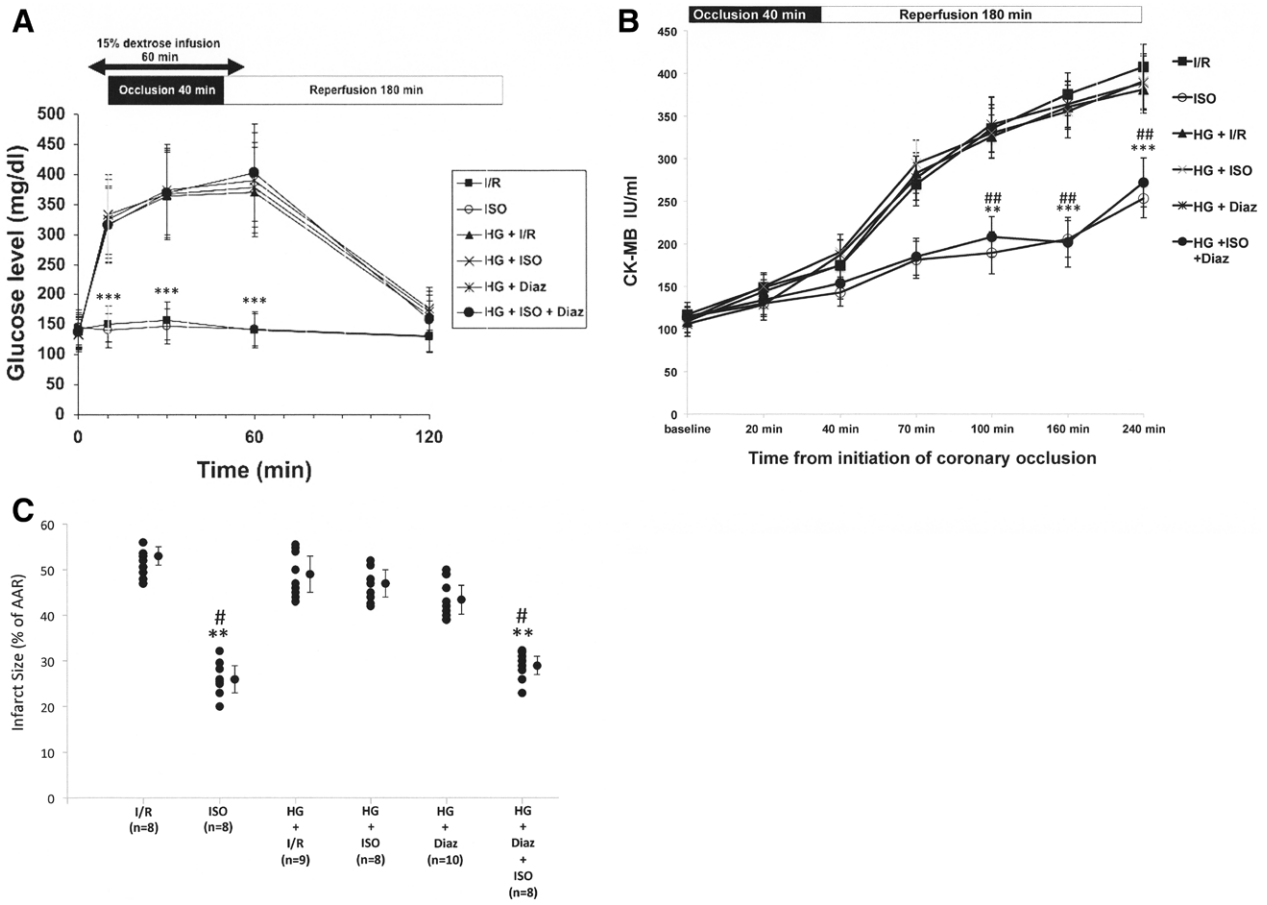

Fig. 2. Blood glucose concentrations over time in the various experimental groups (*A*). MB fraction of creatine kinase (CK-MB) concentrations (*B*) and infarct size as percentage of the area at risk (*C*). Data are presented as mean ± SD. AAR = area at risk; HG + Diaz = hyperglycemia + diazoxide; HG + I/R = hyperglycemia + ischemia–reperfusion; HG + ISO = hyperglycemia + isoflurane postconditioning; HG + ISO + Diaz = diazoxide administration to isoflurane-postconditioned rabbits in the presence of hyperglycemia; I/R = ischemia–reperfusion; ISO = isoflurane postconditioning. $^{**}P \leq 0.01$ compared with the I/R group; $^{***}P \leq 0.001$ compared with the I/R group; $\#P \leq 0.05$ compared with the HG + ISO group; $\#\#P \leq 0.01$ compared with the HG + ISO group.

the HG + I/R group *vs.* the I/R group); ($F(1,14) = 260.65$, $P < 0.001$ for the HG + ISO group *vs.* the I/R group); ($F(1,16) = 342.38$, $P < 0.001$ for the HG + Diaz group *vs.* the I/R group); ($F(1,14) = 228.83$, $P < 0.001$ for the HG + ISO + Diaz group *vs.* the I/R group), respectively. There was a significant interaction between dextrose infusion and time ($F(3,45) = 153.11$, $P < 0.001$), HG + ISO and time ($F(3,42) = 150.78$, $P < 0.001$), HG + DIAZ and time ($F(3,48) = 106.99$, $P < 0.001$), and HG + ISO + DIAZ and time ($F(3,42) = 136.99$, $P < 0.001$). Blood glucose concentrations returned to baseline values in the hyperglycemic rabbits after discontinuation of the dextrose infusion during reperfusion.

CK-MB Concentrations and Myocardial Infarct Sizes

In the I/R group, the CK-MB concentration increased from 119.2 ± 14 IU/ml at baseline to 407.8 ± 26.6 IU/ML after 180 min of reperfusion. Isoflurane postconditioning significantly attenuated this response, and the CK-MB concentration increased from 114 ± 11.6 IU/ml to 253.5 ± 23.1 IU/ml after 180 min of reperfusion ($F(1,14) = 119.65$, $P < 0.001$; interaction between ISO and time: $F(6,84) = 95.49$, $P < 0.001$) (fig. 2B). Administration of dextrose to the HG + I/R group resulted in a CK-MB increase that was similar to the I/R group: from 109 ± 13 IU/ml at baseline to 381.3 ± 27.8 IU/ml after 180 min of reperfusion (I/R *vs.* HG + I/R: $F(1,15) = 0.88$, $P = 0.36$; interaction between HG and time: $F(6,90) = 2.67$, $P = 0.02$). Dextrose administration to the HG + ISO group, however, significantly inhibited the cardioprotective effect of isoflurane (CK-MB increased from 111 ± 15.3 IU/ml at baseline to 391 ± 32.4 IU/ml after 180 min of reperfusion; ISO *vs.* HG + ISO: $F(1,14) = 101.59$, $P < 0.001$; interaction between HG and time: $F(6,84) = 62.67$, $P < 0.001$) (fig. 2B). In the HG + Diaz group, the CK-MB level increased from 106 ± 14.6 IU/ml at baseline to 389.1 ± 31.6 IU/ml at the end of reperfusion (I/R *vs.* HG + Diaz: $F(1,16) = 0.43$, $P = 0.52$; interaction between Diaz and time: $F(6,96) = 2.77$, $P = 0.016$). In contrast, administration of diazoxide to isoflurane-treated acutely hyperglycemic animals (HG + ISO + Diaz group) resulted in

attenuation of CK-MB release to a similar level of that measured in the ISO group (from 114.6 ± 12.1 IU/ml at baseline to 272.1 ± 28.8 IU/ml at the end of reperfusion; I/R *vs.* HG + ISO + Diaz: $F(1,14) = 77.47$, $P < 0.001$; interaction between ISO + Diaz and time: $F(6,84) = 76.38$, $P < 0.001$) (fig. 2B).

The ratio of area at risk to left ventricular mass did not differ significantly among groups: 49 ± 3% in the I/R group, 44 ± 2% in the ISO group, 45 ± 5% in the HG + I/R group, 49 ± 2 in the HG + ISO group, 46 ± 6% in the HG + Diaz group, and 51 ± 4% in the HG + ISO + Diaz group. These data suggest that changes in myocardial injury and infarction size observed in the various experimental groups cannot be attributed to the percentage of the left ventricular myocardium whose blood supply was occluded.

There was a significant difference in infarct size among the experimental groups ($F(5,45) = 78.75$, $P < 0.001$). In the I/R group, myocardial infarct size was 53 ± 2% of the area at risk. Postconditioning with isoflurane decreased the infarct size to 26 ± 4% ($P < 0.001$ for ISO *vs.* I/R). Dextrose administration before I/R did not affect infarct size (49 ± 5%; $P = 0.48$ for HG + I/R *vs.* I/R); however, hyperglycemia significantly inhibited the cardiac protection produced by isoflurane (47 ± 2%; $P = 0.02$ for HG + ISO *vs.* ISO) (fig. 2C). Administration of diazoxide to hyperglycemic rabbits (HG + Diaz group) resulted in an infarct size of 43 ± 4% ($P = 0.21$ for HG + Diaz *vs.* I/R). In contrast, administration of diazoxide has restored cardiac protection in isoflurane-postconditioned rabbits in the presence of hyperglycemia, and the infarct size decreased to 29 ± 2% (fig. 2C; $P = 0.001$ for HG + ISO + Diaz *vs.* I/R).

Plasma Malondialdehyde Levels

Plasma malondialdehyde levels are shown in figure 3. Malondialdehyde levels at baseline did not differ among the various experimental groups. During reperfusion, there was a significant difference in the plasma malondialdehyde levels among the groups ($F(5,90) = 18.55$, $P < 0.001$). Plasma malondialdehyde levels in the ISO group were significantly lower at all time points compared with the I/R group. Hyperglycemia blocked the effects of isoflurane postconditioning on lipid peroxidation (HG + ISO group), resulting in a significant increase in malondialdehyde levels ($P = 0.002$ for HG + ISO *vs.* ISO). Administration of diazoxide to hyperglycemic rabbits (HG + Diaz group) did not change plasma malondialdehyde levels compared with the HG + ISO or I/R groups. In contrast, administration of diazoxide before reperfusion in the HG + ISO + Diaz group restored the effects of isoflurane postconditioning, resulting in a significant decrease in plasma malondialdehyde levels ($P = 0.01$ for HG + ISO + Diaz *vs.* HG + ISO).

TUNEL Staining for Apoptosis and Caspase-3 Activity

Apoptosis as assessed by TUNEL staining is reported in figure 4A. There was a significant difference in the number of TUNEL-positive stained cells among the various experimental groups ($F(5,145) = 21.01$, $P < 0.001$). The percentage of TUNEL-positive nuclei was significantly lower in the ISO group compared with the I/R group (3.4 *vs.* 9.1%, $P = 0.02$). Hyperglycemia blocked the effects of isoflurane, resulting in a significant increase in the percentage of TUNEL-positive nuclei to 11.1% ($P = 0.01$ for HG + ISO *vs.* ISO). Administration of diazoxide alone to hyperglycemic rabbits (HG + Diaz group) slightly decreased the percent of TUNEL-positive cells (8.6%) compared with the HG + ISO or I/R groups; however, this was not statistically significant ($P = 0.42$ for HG + Diaz *vs.* HG + ISO and $P = 0.27$ for HG + Diaz *vs.* I/R). In contrast, the administration of diazoxide

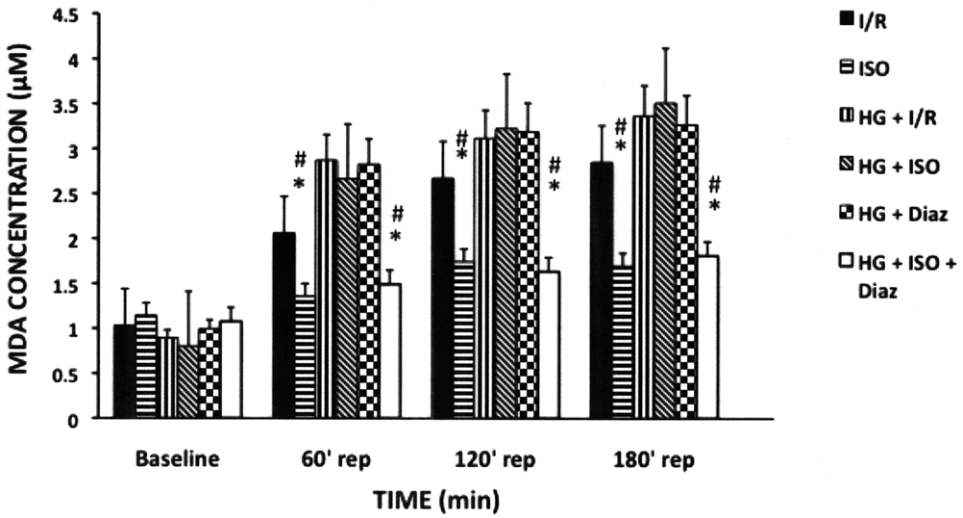

Fig. 3. Plasma malondialdehyde (MDA) levels during reperfusion in the various experimental groups. Data are presented mean ± SD. HG + Diaz = hyperglycemia + diazoxide; HG + I/R = hyperglycemia + ischemia–reperfusion; HG + ISO = hyperglycemia + isoflurane postconditioning; HG + ISO + Diaz = diazoxide administration to isoflurane-postconditioned rabbits in the presence of hyperglycemia; I/R = ischemia–reperfusion; ISO = isoflurane postconditioning. *$P < 0.05$ compared with I/R group; #$P < 0.05$ compared with the HG + ISO group.

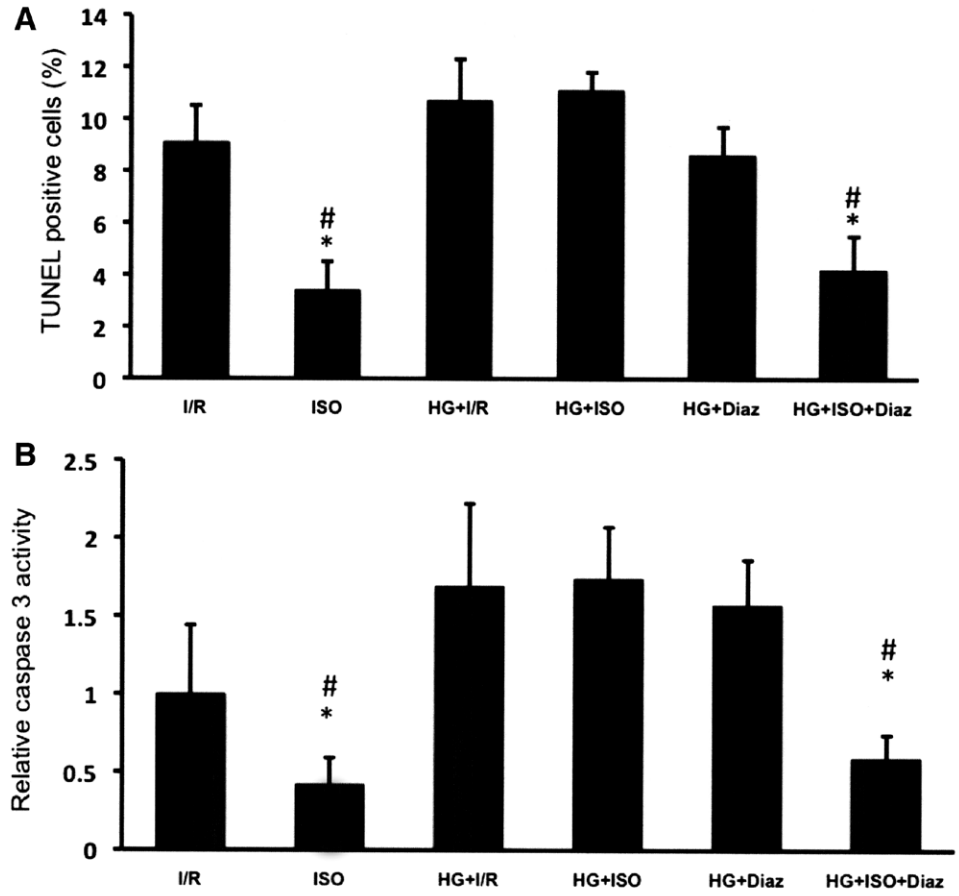

Fig. 4. Percent of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive cells (*A*) and caspase-3 activity (*B*) in the various experimental groups. Data are presented as mean ± SD. HG + Diaz = hyperglycemia + diazoxide; HG + I/R = hyperglycemia + ischemia–reperfusion; HG + ISO = hyperglycemia + isoflurane postconditioning; HG + ISO + Diaz = diazoxide administration to isoflurane-postconditioned rabbits in the presence of hyperglycemia; I/R = ischemia–reperfusion; ISO = isoflurane postconditioning. *$P < 0.05$ compared with I/R group; #$P < 0.05$ compared with the HG + ISO group.

before reperfusion in the HG + ISO + Diaz group restored the effects of isoflurane postconditioning, resulting in a significant decrease in TUNEL-positive cells (4.2 *vs.* 11.1%, $P = 0.02$ compared with HG + ISO).

Similarly, there was a significant difference in the caspase-3 activity among the various groups ($F(5,45) = 39.62$, $P < 0.001$). Caspase-3 activity was significantly lower in the ISO group compared with the I/R group ($P = 0.018$). This effect, however, was completely reversed by hyperglycemia, resulting in a caspase activity level similar to that measured in the I/R group. Diazoxide alone did not change caspase-3 activity in the HG + Diaz group; however, administration of diazoxide before reperfusion in the HG + ISO + Diaz group restored the cardioprotective effect of isoflurane and significantly reduced caspase-3 activity ($P = 0.035$ for HG + ISO + Diaz *vs.* HG + ISO; fig. 4B).

Western Immunoblotting for Total and Phosphorylated Akt and eNOS

Total Akt and eNOS (fig. 5) expression was comparable in all groups. There was a significant difference in the expression of phosphorylated Akt and eNOS among the groups ($F(5,18) = 5.03$, $P = 0.012$ and $F(5,18) = 9.38$, $P = 0.003$, respectively). Phospho-Akt and phospho-eNOS expression was significantly higher after postconditioning with isoflurane in the ISO group compared with the I/R control group. Hyperglycemia completely inhibited the increase in Akt and eNOS phosphorylation in isoflurane-treated and nontreated rabbits (groups HG + I/R and HG + ISO; fig. 5, A and C, respectively). Administration of diazoxide to hyperglycemic rabbits with and without isoflurane postconditioning did not change the expression of phospho-Akt or phospho-eNOS.

Discussion

The findings of the current investigation confirm our previous work in which isoflurane postconditioning protected against MI, whereas acute hyperglycemia abolished this protection.[7] In our current study, we report that the cardioprotective effects of isoflurane are associated with a reduction in myocardial apoptosis and decrease in lipid peroxidation. Under acute hyperglycemia, however, the cardioprotective

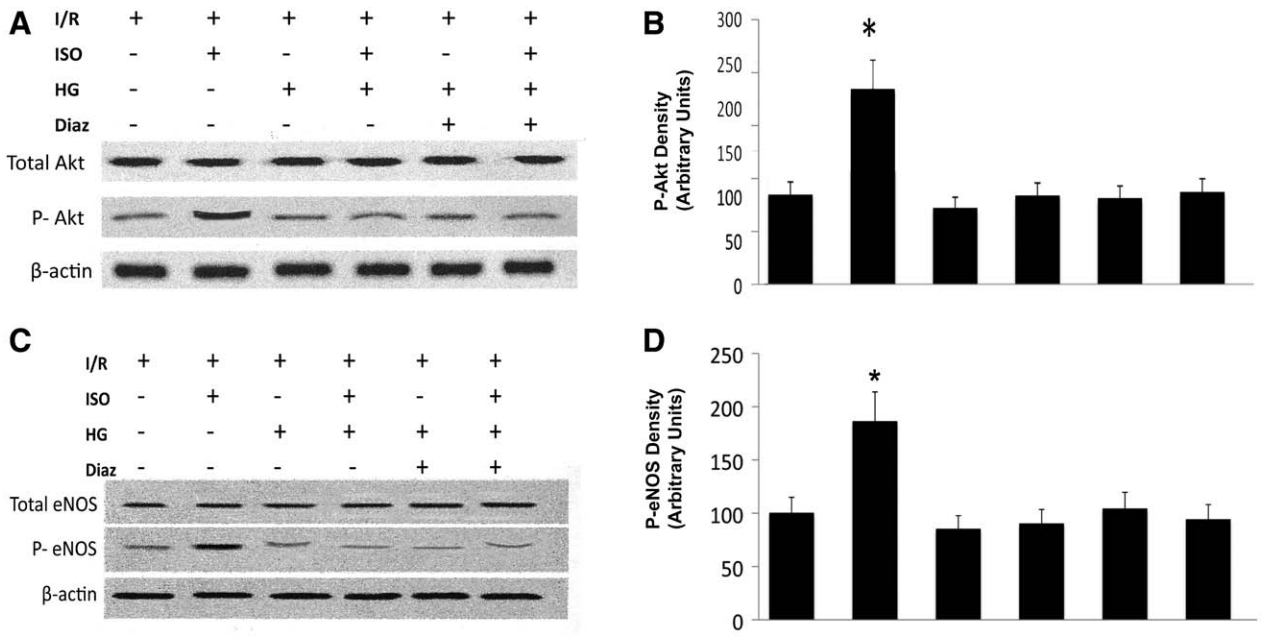

Fig. 5. (*A*) Representative Western blot analysis of total protein kinase B (Akt) and phosphorylated Akt (P-Akt) at Ser[473] in left ventricular myocardial samples after anesthetic postconditioning with or without hyperglycemia. In each lane, the protein content was 100 μg. β-Actin was used to demonstrate equal protein loading. (*B*) Graphic presentation of P-Akt quantified by integrating the volume of autoradiograms from three separate experiments. (*C*) Representative Western blot analysis of total and phosphorylated eNOS (P-eNOS) at Ser[1177] in left ventricular myocardial samples after anesthetic postconditioning with or without hyperglycemia. In each lane, the protein content was 100 μg. β-Actin was used to demonstrate equal protein loading. (*D*) Graphic presentation of P-eNOS expression from the various experimental groups quantified by integrating the volume of autoradiograms from three separate experiments. Values in the graphs are presented as mean ± SD. Diaz = diazoxide; eNOS = endothelial nitric oxide synthase; HG = hyperglycemia; I/R = ischemia–reperfusion; ISO = isoflurane postconditioning. *$P < 0.05$ compared with the I/R group.

effects of isoflurane were lost. This was associated with inhibition of Akt and eNOS phosphorylation/activation and increased apoptosis as well as production of ROS during reperfusion. Administration of the mitochondrial K_{ATP} channel agonist, diazoxide, restored the cardioprotective effects of isoflurane postconditioning in the presence of acute hyperglycemia and attenuated the level of myocardial apoptosis and lipid peroxidation. This effect, however, was not associated with a corresponding increase in Akt or eNOS phosphorylation. Taken together, these data may suggest that acute hyperglycemia antagonizes anesthetic postconditioning by attenuating the activation of PI3K/Akt and eNOS and that the mitochondrial K_{ATP} channels are probably located downstream of these signaling molecules in the postconditioning pathway (fig. 6).

Myocardial infarction is a serious perioperative complication that carries significant morbidity and mortality.[21] Acute hyperglycemia is a common metabolic abnormality that can be found in diabetic as well as in nondiabetic patients in response to surgical stress[22] and may be associated with poor outcome after cardiac[2] or noncardiac surgery.[23] Moreover, acute hyperglycemia on hospital admission was reported as an independent risk factor predicting mortality in patients with acute MI.[24]

The mechanisms responsible for the increased morbidity and mortality in patients with perioperative hyperglycemia are not completely known. Potential mechanisms may involve inhibition of endogenous protective pathways which could result in increased oxidative stress and endothelial dysfunction.[25] Kersten *et al.*[26] found that hyperglycemia blocked ischemic and anesthetic preconditioning and that infarct size was directly related to the degree of hyperglycemia.[27]

Our results are in agreement with the results published by Huhn *et al.*,[28] who demonstrated that anesthetic postconditioning with sevoflurane was inhibited by hyperglycemia and restored by using cyclosporin A. Our current findings, however, extend these observations. We provide data on the effects of acute hyperglycemia and K_{ATP} channel activation on PI3K/Akt and eNOS phosphorylation, as well as on oxidative stress and myocardial apoptosis. In contrast to Huhn *et al.*, we have chosen a lower blood glucose target for our hyperglycemic animals. This glucose concentration has already been reported to block ischemic preconditioning[10] and, in our opinion, better reflects the level of hyperglycemia that patients are likely to experience.

Several other groups have recently reported their results regarding anesthetic-induced protection in the context of diabetes mellitus.[29,30] Although there are similarities with the results presented here, our work focused on the effects of acute hyperglycemia on the myocardium after I/R in nondiabetic animals. Animals with diabetes and chronic hyperglycemia present experimental models that are different from

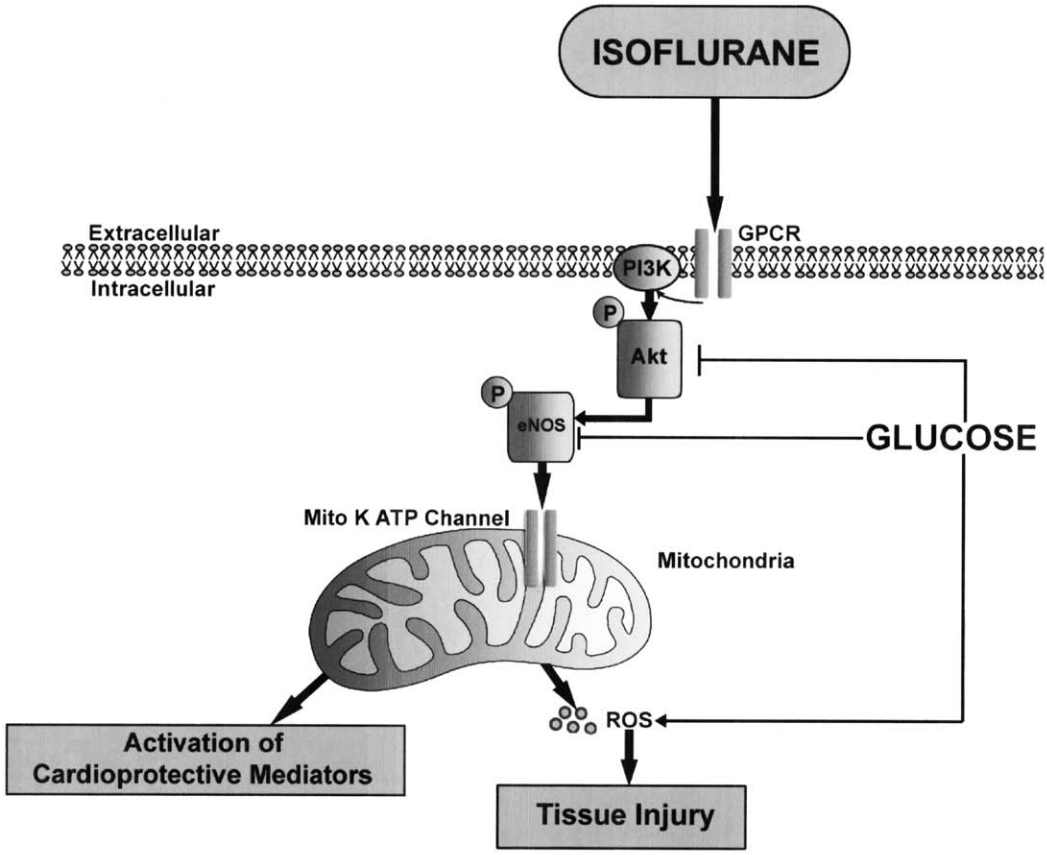

Fig. 6. A hypothetical illustration that explains the potential interaction between phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt), endothelial nitric oxide synthase (eNOS), and the mitochondrial (Mito) K_{ATP} channels during the process of isoflurane-induced myocardial postconditioning. Isoflurane activates G-protein–coupled receptors that are located in the cell membrane. This leads to activation of PI3K. PI3K phosphorylates its downstream target, Akt. Akt mediates the phosphorylation (activation) of eNOS, which acts on the mitochondria (*via* the mitochondrial K_{ATP} channels) to decrease oxidative stress and activate other downstream cardioprotective mediators. Acute hyperglycemia blocks the phosphorylation (P) of both Akt and eNOS, thus leading to inhibition of cardioprotective mechanisms. In addition, hyperglycemia also directly stimulates the release of large quantities of ROS from the mitochondria and this further exacerbates myocardial tissue damage. GPCR = G-protein–coupled receptors; K_{ATP} channel = potassium-regulated adenosine triphosphate channel; ROS = reactive oxygen species. An *arrow* symbolizes positive feedback; *blocked line* symbolizes negative feedback (inhibition).

ours, with different clinical implications; therefore, results derived from the use of diabetic subjects cannot be extrapolated to healthy subjects with acute hyperglycemia.

The PI3K/Akt pathway is known as a key mediator of ischemic and anesthetic postconditioning.[5,31] Activation of PI3K results in phosphorylation of the its downstream target, Akt (also known as protein kinase B).[31] This activated (phosphorylated) kinase inhibits proapoptotic proteins, such as Bad, Bax, and caspase-9,[32] and maintains high levels of the antiapoptotic protein Bcl-2.[18] In addition, Akt has been shown to increase the formation of nitric oxide, specifically *via* activation of eNOS.[31] Nitric oxide is known to have an important role in ischemic and pharmacologic preconditioning as well as postconditioning of the heart.[5,8,33,34] Acute hyperglycemia was found to reduce the availability of nitric oxide in humans by enhancing ROS production[12] and this could exacerbate myocardial infarct size. Gross *et al.*[25] reported that hyperglycemia-induced ROS production enhanced endothelial dysfunction and decreased coronary wall shear stress in dog hearts. Moreover, nitric oxide directly activates mitochondrial K_{ATP} channels and potentiates agonist-induced increases in channel activity.[35]

The ability of hyperglycemia to generate large quantities of ROS has been well established.[13] Oxygen-derived free radicals are released during I/R and cause direct lipid peroxidation and mitochondrial damage.[36] It has been reported that this may lead to significant intracellular calcium overload in cardiomyocytes and coronary endothelial cells, thus resulting in decreased myocardial contractility and increased coronary vascular resistance.[37] Hyperglycemia is also associated with increased apoptosis, which could further contribute to myocardial cell death.[38–41] Taken together, it seems that hyperglycemia may increase oxidative stress, induce apoptosis, and further exacerbate I/R injury and myocardial cell damage. This is mediated, at least in part, by increased formation and release of ROS and decreased nitric oxide activity.

The K_{ATP} channels were first described in the myocardium in 1983.[42] These channels were found to have a key role in ischemic and pharmacologic (anesthetic) preconditioning.[43,44] More recently, these channels were also found to mediate the protective effects of anesthetic postconditioning.[9] We have demonstrated that glibenclamide, a nonspecific K_{ATP} channel blocker, inhibited ischemic preconditioning in rabbits, and this was associated with increased ROS production during reperfusion.[45] It was also reported that diazoxide markedly reduced ROS formation in isolated perfused hearts[46] and in cardiac mitochondria after hypoxia and reoxygenation.[47] In addition, activation of K_{ATP} channels also inhibited myocardial apoptosis after I/R.[48,49] Kersten *et al.*[17] reported that profound hyperglycemia (target glucose of 600 mg/dl) blocked cardiac protection *via* inhibition of K_{ATP} channels. Similar to our results, they also demonstrated that the loss of protection caused by moderate and more clinically relevant hyperglycemia (target blood glucose of 300 mg/dl) can be successfully restored by diazoxide. Our results confirm these findings and extend these observations to myocardial postconditioning—an approach that could be clinically more relevant and feasible because the protective intervention is performed just before or at the reperfusion phase and not before the index ischemia (which may be unpredictable in many occasions) as in preconditioning. Taken together, our data seem to indicate that the cardioprotective effects of anesthetic postconditioning are mediated *via* PI3K/Akt and eNOS signaling as well as the activation of mitochondrial K_{ATP} channels that seem to be located downstream of PI3K/Akt and eNOS in the postconditioning pathway. Alternatively, it may be possible that the K_{ATP} channels are located within a protective pathway that does not involve PI3K/Akt or eNOS signaling. Although this hypothesis has not been investigated in the current study, it has been shown by others[50,51] that PI3K/Akt, eNOS, and K_{ATP} channels are all related in protection against I/R injury.

In this study, we evaluated PI3K/Akt and eNOS phosphorylation. In addition to eNOS, inducible nitric oxide synthase and neuronal nitric oxide synthase are known to have a role in cardiac protection by ischemic preconditioning[33]; however, recent data from our laboratory[7] as well as another report[8] do not support the involvement of these isoenzymes in anesthetic-induced postconditioning. Therefore, the current investigation has only evaluated changes in eNOS phosphorylation.

The current results must be interpreted within the constraints of several potential limitations: administration of 15% dextrose increases osmolality. Although the current study did not address the effect of changes in plasma osmolality on infarct size, Kersten *et al.*[26] demonstrated that neither osmolality nor insulin concentration had an effect on infarct size in dogs. Therefore, it seems unlikely that increased osmolality has an effect on isoflurane-induced postconditioning.

Evaluation of apoptosis was performed on left ventricular samples that were harvested from the area at risk. The assays require fresh tissue that cannot be stained with TTC to further distinguish between infarcted or at-risk zones. Hence, one could suggest that our samples contained only infarcted myocardium. Nevertheless, necrotic tissues rarely contain apoptotic cells, and because we have detected a considerable degree of apoptosis in our samples, it seems unlikely that they were taken from infarct-only zones.

Myocardial infarct size is determined primarily by the area at risk and the extent of coronary collateral perfusion. The area at risk, expressed as a percentage of total left ventricle mass, was similar between groups. Coronary collateral blood flow, however, was not measured in the present investigation. However, rabbits have been shown to possess little if any coronary collateral blood flow.[52] Thus, it appears unlikely that differences in collateral perfusion between groups account for the observed results.

In conclusion, the results of the present investigation confirm that administration of the inhaled anesthetic isoflurane at the early stages of reperfusion (postconditioning) protects the myocardium against infarction. These beneficial effects seem to be associated with activation of PI3K/Akt and eNOS signaling as well as inhibition of apoptosis and lipid peroxidation. Acute hyperglycemia inhibits the cardioprotective effects of isoflurane-induced postconditioning. This is associated with decrease in Akt and eNOS activation and increased apoptosis and oxidative stress, which may further exacerbate myocardial injury. Activation of K_{ATP} channels with diazoxide restored isoflurane-induced cardiac protection in the presence of hyperglycemia, without affecting the protein expression of phospho-Akt or phospho-eNOS, suggesting that K_{ATP} channels are probably located downstream to Akt and eNOS in the pathway of anesthetic postconditioning.

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Competing Interests

The authors declare no competing interests.

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