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# *Isoflurane Preconditioning Decreases Myocardial Infarction in Rabbits* via *Up-regulation of Hypoxia Inducible Factor 1 That Is Mediated by Mammalian Target of Rapamycin*

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***Background:*** **Volatile anesthetics are known to protect the heart against ischemia–reperfusion injury. The authors tested whether anesthetic preconditioning with isoflurane is mediated *via* activation of the transcription factor hypoxia inducible factor 1 (HIF-1) and evaluated the role of mammalian target of rapamycin signaling in this process.**

***Methods:*** **New Zealand White rabbits subjected to 40 min of regional myocardial ischemia, followed by 180 min of reperfusion, were assigned to the following groups: ischemia and reperfusion (I/R) only, isoflurane (1 minimal alveolar concentration) preconditioning, and isoflurane preconditioning in the presence of the mammalian target of rapamycin inhibitor rapamycin (0.25 mg/kg). Sham-operated, isoflurane + sham, rapamycin + sham, rapamycin + I/R, and dimethyl sulfoxide + I/R groups were also included. Creatine kinase-MB levels were assessed as an indicator of myocardial damage, and infarct size was evaluated by triphenyl tetrazolium chloride staining. HIF-1$\alpha$ expression and DNA binding were assessed by Western blotting and electrophoretic mobility shift analysis, respectively.**

***Results:*** **Isoflurane preconditioning reduced infarct size compared with the I/R group: 26 ± 4% *versus* 44 ± 6% ($P < 0.05$). Creatine kinase-MB concentrations in the preconditioned animals (103 ± 8% above baseline) were lower than in the I/R group (243 ± 12% above baseline; $P < 0.05$). Rapamycin inhibited the cardioprotective effect of isoflurane: myocardial infarction increased to 44 ± 4% and creatine kinase-MB level increased to 254 ± 9% above baseline. HIF-1$\alpha$ protein expression and DNA binding activity increased after isoflurane preconditioning compared with the ischemia group. These effects were also inhibited by rapamycin.**

***Conclusions:*** **The current results indicate that isoflurane-induced myocardial protection involves activation of the HIF-1 pathway that is mediated by the mammalian target of rapamycin.**

REPEATED brief episodes of ischemia protect the myocardium against subsequent prolonged ischemic injuries. This phenomenon, which has been termed *ischemic preconditioning*, has been described in various animal models, including humans.[1,2] In addition to ischemia, myocardial preconditioning can be achieved by several pharmacologic agents, including volatile anesthetics.[3,4] The mechanisms by which volatile anesthetics protect the heart have been investigated extensively and are believed to involve activation of adenosine receptors[5] and protein kinase C,[6] release of reactive oxygen species,[7] opening of adenosine triphosphate–regulated potassium channels,[8] activation of the phosphatidylinositol-3-kinase/Akt pathway[9] and other survival kinases,[10] and eventually activation of downstream targets, such as endothelial and inducible nitric oxide synthase.[11] However, despite extensive research, the precise mechanisms responsible for anesthetic-induced protection against myocardial ischemia and reperfusion are still incompletely understood. Hypoxia inducible factor 1 (HIF-1) is a transcription factor that promotes the expression of several genes that confer protection against hypoxia through angiogenesis, erythropoiesis, vasodilation, and altered glucose metabolism.[12,13] HIF-1 is a heterodimeric protein consisting of two subunits: HIF-1$\alpha$ and HIF-1$\beta$. HIF-1$\beta$ is constitutively expressed. In contrast, HIF-1$\alpha$ is rapidly degraded during normoxia. Upon hypoxia, however, HIF-1$\alpha$ is stabilized through decreased activity of prolyl hydroxylases that target the protein for degradation, therefore allowing it to accumulate within the cell. HIF-1$\alpha$ then translocates to the nucleus, where it forms a heterodimer with HIF-1$\beta$ and becomes an active transcription factor for end products that are important for anaerobic metabolism and cell survival, including erythropoietin, vascular endothelial growth factor (VEGF), glucose transport enzymes, and endothelial and inducible nitric oxide synthase.[12,13]

Several studies have focused lately on activation of HIF-1 to reduce injury after ischemia and reperfusion.[14,15] Isoflurane can induce HIF-1–responsive genes such as inducible nitric oxide synthase,[16] heme oxygenase 1,[17] and VEGF.[18] However, it is not known whether anesthetic-induced cardioprotection is mediated through increased expression and/or activation of HIF-1, which in turn activates downstream targets that provide protection to the myocardium against ischemia–reperfusion injury.[19–21] Identifying the mechanisms by which anesthetic preconditioning mediates its anti-ischemic actions may be of special clinical significance in protection against ischemic events in patients with coronary artery disease in the perioperative period. Therefore, using an *in vivo* model of regional myocardial ischemia and reperfusion in rabbits, we evaluated the effects of anes-

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thetic preconditioning, using isoflurane, on HIF-1 both in the ischemic myocardium and in remote nonischemic myocardial regions.

## Materials and Methods

All experiments were conducted after the approval of the institutional Committee for Animal Care and Laboratory Use of the Hebrew University-Hadassah Medical Center, Jerusalem, Israel. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health publication 85-23, revised 1996).

*General Preparation*

The rabbit model of regional myocardial ischemia and reperfusion has been previously described in detail.[9] Briefly, 6-month-old New Zealand White male rabbits, weighing 2.8–3.2 kg, were anesthetized with intravenous sodium thiopental (30 mg/kg) *via* a 20-gauge catheter in a marginal ear vein, followed by a 5-mg $\cdot$ kg$^{-1}$ $\cdot$ h$^{-1}$ infusion. Neuromuscular blocking agents were not administered, and anesthetic depth was assessed according to the eyelash and pedal reflexes. A tracheostomy was performed using a ventral midline incision, and the rabbits were mechanically ventilated with positive-pressure ventilation using a 21–24% oxygen–air mixture to maintain an arterial oxygen partial pressure of 75–90 mmHg. The ventilation rate was 30–35 breaths/min, and the tidal volume was set at 10 ml/kg. The respiratory rate was adjusted to keep the blood pH in the range of 7.35–7.45. 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 intravenous drug administration. Maintenance fluids (0.9% NaCl) were administered at 15 ml $\cdot$ kg$^{-1}$ $\cdot$ h$^{-1}$ during the experiment. Core body temperature was measured *via* a rectal temperature probe and maintained at 38.5° $\pm$ 0.2°C with radiant heat and a warming blanket. A three-lead electrocardiogram was continuously recorded. To access the heart, 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 epicardial cyanosis and ST-segment elevation in the electrocardiogram. Reperfusion was achieved by releasing the snare and was confirmed by visual observation of reactive hyperemia.

*Experimental Design*

The experimental design is illustrated in figure 1. After a 30-min stabilization period, heparin (300 U/kg) was administered intravenously to all rabbits. All animals (except the sham groups) were subjected to 40 min of regional myocardial ischemia followed by 180 min of reperfusion. These animals were used to determine the hemodynamics, serum MB fraction of creatine kinase (CK-MB) concentrations, and cardiac infarct sizes. Rabbits were randomly assigned to one of the following groups: group 1, a nonischemic control group of sham-operated rabbits (sham, n = 5); group 2, an ischemia–reperfusion group (40 min of myocardial ischemia and 180 min of reperfusion, I/R, n = 8); group 3, isoflurane + sham (Iso + sham, n = 5); or group 4, anesthetic preconditioning group (APC, n = 8). One minimal alveolar concentration of isoflurane (2.1%)[22] 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. Before coronary occlusion, a zero end-tidal isoflurane concentration was confirmed.

To evaluate the potential role of HIF-1 in anesthetic preconditioning, the mammalian target of rapamycin (mTOR) inhibitor rapamycin (0.25 mg/kg intravenous[23]; Sigma, St. Louis, MO) was used in the next experimental groups. This agent has been found to control the expression and reduce cellular levels of HIF-1$\alpha$.[24,25] Rapamycin was dissolved in dimethyl sulfoxide (DMSO) and administered intravenously 10 min before isoflurane administration. To rule out any direct effect of rapamycin on the heart during baseline conditions or during ischemia and reperfusion, rabbits were treated with rapamycin but did not undergo any coronary intervention (group 5, Rap + sham, n = 5). In group 6, rapamycin was administered 70 min before coronary ischemia and reperfusion (Rap + I/R, n = 8), and in group 7, rapamycin was administered intravenously before anesthetic preconditioning (Rap + APC, n = 8) followed by coronary occlusion and reperfusion. An additional group of rabbits (group 8, DMSO, n = 8) was treated with DMSO alone before coronary occlusion and reperfusion to rule out any effect of the vehicle itself on infarct size.

In the second set of experiments, rabbits (n = 5 in each group) were subjected to the various experimental conditions as described above, except they were not subjected to myocardial reperfusion. The hearts were not reperfused because HIF-1$\alpha$ is rapidly metabolized upon reoxygenation.[26] At the end of the 40 min of coronary occlusion, the animals were killed by an intravenous injection of sodium thiopental, and myocardial samples were collected from ischemic left ventricular regions and nonischemic right ventricular regions for evaluation of HIF-1 protein expression and DNA binding

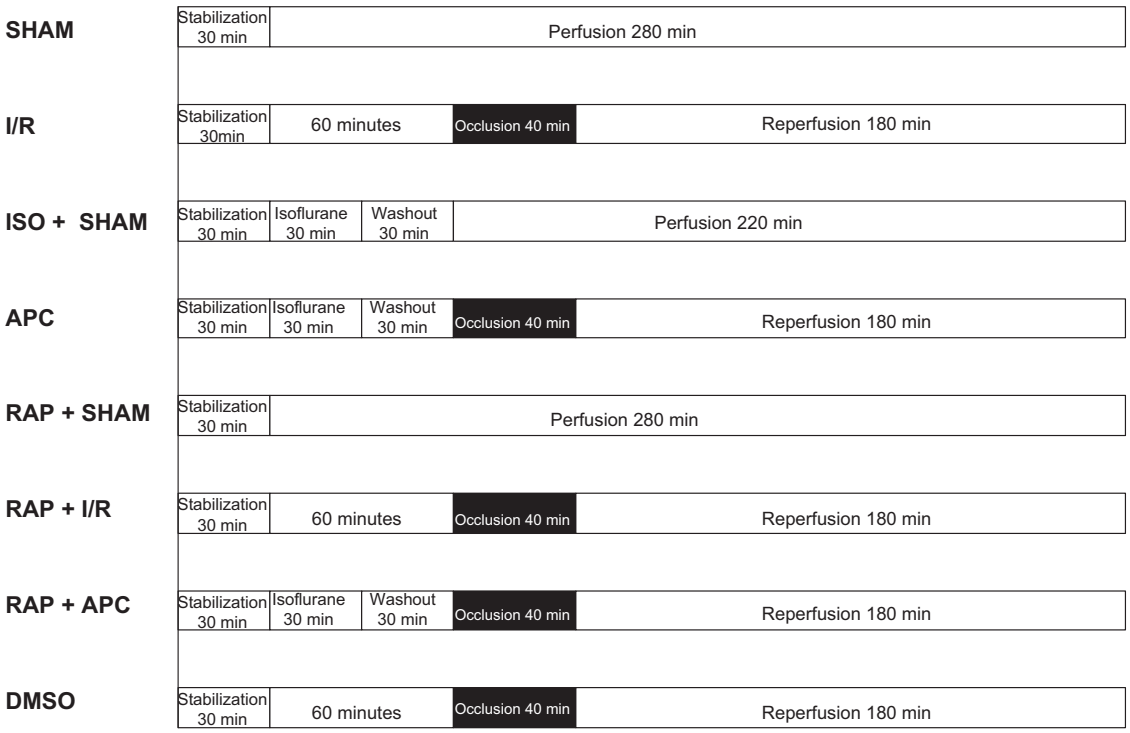


**Fig. 1. Diagram of the experimental protocol. Animals were subjected to 40 min of regional myocardial ischemia and 180 min of reperfusion. One minimum alveolar concentration of isoflurane was administered for 30 min followed by 30 min of washout before coronary occlusion. Rapamycin (0.25 mg/kg intravenous) was administered 10 min before isoflurane preconditioning. APC = anesthetic preconditioning; DMSO = dimethyl sulfoxide; Iso + sham = isoflurane + sham; I/R = ischemia–reperfusion; RAP + APC = rapamycin + anesthetic preconditioning; RAP + I/R = rapamycin + ischemia–reperfusion; RAP + sham = rapamycin + sham.**

activity. Samples were frozen in liquid nitrogen and kept at −80°C until further processed. All assays were repeated in triplicate.

*Measurements of Hemodynamics and Serum CK-MB Concentrations*

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 (table 1). Blood samples were obtained and analyzed for serum concentrations of CK-MB by an enzymatic assay method using a CK assay kit (Sigma Diagnostics, St. Louis, MO). Samples were collected at the following time points: immediately after anesthetizing the animals (baseline sample); 20 and 40 min after the onset of coronary occlusion; and 30, 60, 120, and 180 min after the onset of reperfusion.

*Determination of the Area at Risk and Infarct Size*

Infarct size measurement was performed as previously described.[9] Briefly, at the end of reperfusion, hearts were excised, mounted on a Langendorff apparatus, and perfused with phosphate-buffered saline at 100 cm $H_2O$ for 1 min to wash out intravascular blood. The coronary artery was reoccluded, and 10 ml methylene blue, 0.1%, 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. The hearts were then cut into 2-mm transverse slices and 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 formaldehyde overnight to increase the contrast between stained and nonstained tissue. Because 2,3,5-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 (Bioquant Imaging Corporation, Nashville, TN), and the percentages of the at-risk and infarcted areas were calculated.

*Nuclear Extract Preparation*

For evaluation of HIF-1$\alpha$ expression, nuclear extracts were prepared from ischemic and nonischemic myocardial samples as previously described.[27] Briefly, tissue samples (approximately 200 mg each) were ground in liquid nitrogen and homogenized in 1 ml ice-cold lysis buffer containing 20 mM Tris (pH 7.4), 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 0.5% NP-40, 0.5 mM $Na_3VO_4$, and a cocktail of protease inhibitors (aprotinin, leupeptin, PMSF) using the IKA-Ultra-Turrax homogenizer (Staufen, Germany). The homogenate was centrifuged at 13,000*g* for 15 min at 4°C.

**Table 1. Systemic Hemodynamics**

| | | | Coronary Occlusion, min | | Reperfusion, min | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | n | Baseline | 20 | 40 | 5 | 30 | 60 | 120 | 180 |
| HR, beats/min | | | | | | | | | |
| I/R | 6 | 256 ± 6 | 249 ± 4 | 260 ± 8 | 239 ± 12* | 228 ± 5* | 227 ± 6* | 220 ± 9* | 218 ± 8* |
| APC | 6 | 247 ± 4 | 261 ± 11 | 266 ± 7 | 241 ± 8* | 236 ± 3* | 233 ± 7* | 215 ± 6* | 221 ± 6* |
| Rap + I/R | 7 | 259 ± 7 | 254 ± 8 | 250 ± 3 | 251 ± 12* | 232 ± 5* | 226 ± 4* | 221 ± 9* | 221 ± 8* |
| Rap + APC | 6 | 255 ± 8 | 263 ± 13 | 269 ± 10 | 239 ± 10 | 231 ± 7* | 230 ± 5* | 216 ± 4* | 219 ± 6* |
| DMSO | 7 | 255 ± 5 | 258 ± 7 | 263 ± 11 | 242 ± 11* | 235 ± 3* | 236 ± 3* | 210 ± 9* | 220 ± 6* |
| MAP, mmHg | | | | | | | | | |
| I/R | 6 | 82 ± 5 | 66 ± 5* | 62 ± 9* | 64 ± 7* | 58 ± 4* | 55 ± 2* | 54 ± 4* | 54 ± 4* |
| APC | 6 | 87 ± 6 | 82 ± 7 | 79 ± 5 | 61 ± 3* | 61 ± 6* | 58 ± 7* | 55 ± 6* | 55 ± 8* |
| Rap + I/R | 7 | 83 ± 5 | 75 ± 2* | 77 ± 11 | 70 ± 2* | 61 ± 8* | 60 ± 5* | 60 ± 9* | 60 ± 9* |
| Rap + APC | 6 | 83 ± 8 | 71 ± 8* | 74 ± 10* | 66 ± 6* | 59 ± 3* | 54 ± 5* | 53 ± 8* | 55 ± 6* |
| DMSO | 7 | 84 ± 4 | 70 ± 4* | 72 ± 8* | 70 ± 5* | 62 ± 8* | 55 ± 7* | 55 ± 4* | 51 ± 3* |
| RPP, $min^{-1} \cdot mmHg \cdot 10^3$ | | | | | | | | | |
| I/R | 6 | 21.6 ± 0.8 | 17 ± 1* | 16.1 ± 1.3* | 14.9 ± 0.7* | 13.2 ± 0.6* | 12.5 ± 0.5* | 11.8 ± 0.7* | 11.6 ± 0.7* |
| APC | 6 | 22 ± 1.1 | 20.6 ± 0.7 | 19.9 ± 0.4* | 14.7 ± 1.4* | 14.2 ± 0.4* | 13.5 ± 1.1* | 11.8 ± 0.9* | 11.8 ± 0.6* |
| Rap + I/R | 7 | 21.5 ± 1.1 | 19.1 ± 1.2* | 18.9 ± 0.9* | 17.1 ± 1.1* | 14 ± 1.2* | 13.5 ± 0.4* | 13.3 ± 1.1* | 12.9 ± 1.1* |
| Rap + APC | 6 | 21.9 ± 0.6 | 18.7 ± 0.6* | 19.3 ± 1.5* | 15.8 ± 0.9* | 13.6 ± 1.5* | 12 ± 1.3* | 11.6 ± 1.4* | 11.6 ± 1.4* |
| DMSO | 7 | 21.2 ± 0.4 | 18.2 ± 0.5* | 18.6 ± 1.2* | 16.9 ± 0.8* | 14.6 ± 1.1* | 13 ± 0.8* | 11.7 ± 1.1* | 11.2 ± 0.6* |

Eight animals were excluded from the study because of refractory ventricular fibrillation (2/8 I/R, 2/8 APC, 1/8 Rap + I/R, 2/8 Rap + APC, 1/8 DMSO). The sizes of the groups presented in the table are after exclusion of the eight rabbits that did not complete the experiments. Data are presented as mean ± SEM.

* $P < 0.05$ compared with baseline values.

APC = anesthetic preconditioning; DMSO = dimethyl sulfoxide + ischemia–reperfusion; HR = heart rate; I/R = ischemia–reperfusion; MAP = mean arterial pressure; Rap + APC = rapamycin + anesthetic preconditioning; Rap + I/R = rapamycin + ischemia–reperfusion; RPP = rate–pressure product.

The cell pellets were washed twice in cold phosphate-buffered saline and resuspended in 4 packed cell volumes of buffer A containing 10 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 2 mM dithiothreitol, 0.4 mM PMSF, 1 mM $Na_3VO_4$, 2 g/l leupeptin, 2 g/l pepstatin, and 2 g/l aprotinin. The pellets were then incubated on ice for 10 min and homogenized with 50 strokes of a dounce homogenizer. The nuclei were pelleted and then resuspended in 3 packed volumes of buffer C containing 0.42 mM KCl, 20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 20% glycerol, 2 mM DTT, 0.4 mM PMSF, 1 mM $Na_3VO_4$, 2 g/l leupeptin, 2 g/l pepstatin, and 2 g/l aprotinin. The resulting mixture was then mixed on a rotator for 30 min, centrifuged to remove nuclear debris, and then dialyzed with one change of buffer for 4 h at 4°C in buffer D containing 20 mM Tris-HCl (pH 7.5), 0.1 mM KCl, 0.2 mM EDTA, and 20% glycerol. The extracts were aliquoted and stored at −80°C.

*Western Blotting for HIF-1α*

After determination of protein concentrations by the modified Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard, equivalent amounts (50 μg) of nuclear protein samples were loaded and separated on 10% SDS-PAGE gel 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 3 h in room temperature with anti-rabbit HIF-1α antibodies (Novus Biologicals Inc., Littleton, CO) at 1:1,000 (vol/vol) dilution in 5% nonfat dry milk. 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 goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Jackson Immunolabs, West Grove, PA) at 1:5,000 dilution. Peroxidase activity was visualized by means of enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ), followed by exposure to hyperfilms (Amersham Pharmacia Biotech). HIF-1β expression remained unchanged by ischemia–reperfusion, isoflurane, or any other experimental treatment applied in the study; therefore, HIF-1β (1:2,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA) was detected on immunoblots as a loading control for protein quantity. Optical density for each band was determined using the National Institutes of Health image program (NIH Image 1.6; Bethesda, MD) and normalized against background density for each gel.

*Electrophoretic Mobility Shift Analysis*

Electrophoretic mobility shift analysis was performed to evaluate the effect of isoflurane on HIF-1 DNA binding activity. Nuclear protein extracts (3 μg) from ischemic myocardium were preincubated in binding buffer (10 mM Tris, 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol, and 5% glycerol) for 5 min at 4°C. The oligonucleotides probes for HIF-1 were sense strand 5′-GCCCTACGTGCTGTCTCA-3′ and antisense 5′-GCCCTAAAAGCTGTCTCA-3′.[28] The sense strand was labeled using $T_4$ polynucleotide kinase (Promega, Madison, WI) and $\gamma$-$^{32}$P[ATP] and added (1.5 fmol) to the

nuclear extracts and incubated for 15 min. The mixture was loaded on a 4% nondenaturing polyacrylamide gel, and electrophoresis was performed in 0.3 × TBE (1 × TBE is 89 mM Tris-borate and 20 mM EDTA [pH 8.0]) at 4°C. The gel was dried and autoradiographed overnight. Supershift assay using HIF-1$\alpha$ antibodies and cold competition with unlabeled oligonucleotides (in a concentration 50 times higher than of the labeled oligonucleotides) were performed for specificity.

*Statistical Analysis*

Statistical analysis was performed using SPSS 10.0 for Windows software (SPSS Inc., Chicago, IL). Data within groups at different times were analyzed by one-way analysis of variance for repeated measures followed by Student-Newman-Keuls test. Data between groups at the same time points were analyzed by one-way analysis of variance followed by Student-Newman-Keuls test. All data were confirmed to be normally distributed. Values are expressed as mean ± SEM. $P < 0.05$ was considered to be statistically significant.

## Results

Ninety-five rabbits were used to successfully complete 87 experiments. Eight animals were excluded because of refractory ventricular fibrillation. The incidence of refractory ventricular fibrillation was not significantly different among the groups (2/8 I/R, 2/8 APC, 1/8 Rap + I/R, 2/8 Rap + APC, 1/8 DMSO). 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 between groups, nor were there any differences recorded during the time course of the experiment in the sham-operated animal groups. Mean arterial pressure decreased in the Iso + sham and APC groups during isoflurane administration; however, after the washout period, the pressure returned to baseline values. A transient increase in heart rate (not significant) was also noted in the isoflurane-treated animals. Left anterior descending coronary artery occlusion significantly decreased ($P < 0.05$) mean arterial pressure and the rate–pressure product in the I/R, Rap + I/R, Rap + APC, and DMSO 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 between groups during reperfusion.

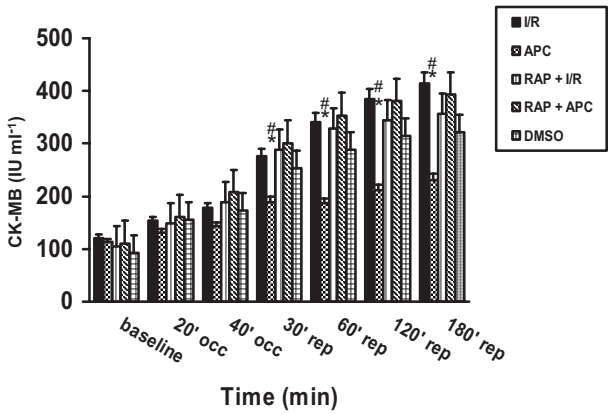


**Fig. 2. MB fraction of creatine kinase (CK-MB) concentrations. Serum CK-MB concentrations were analyzed by an enzymatic assay using a CK assay kit. The increase in CK-MB concentrations was lower in rabbits pretreated with isoflurane than in controls (I/R group). Rapamycin abolished this protective effect, and the increase in CK-MB was similar to that seen in the control group. Data are presented as mean ± SEM. APC = anesthetic preconditioning; DMSO = dimethyl sulfoxide; I/R = ischemia–reperfusion; occ = occlusion; RAP + APC = rapamycin + anesthetic preconditioning; RAP + I/R = rapamycin + ischemia–reperfusion; rep = reperfusion. * $P < 0.05$ *versus* I/R. # $P < 0.05$ *versus* RAP + APC.**

*CK-MB Concentrations and Myocardial Infarct Sizes*

In the I/R group, the CK-MB concentration increased by 243 ± 12% after 180 min of reperfusion, compared with baseline levels,whereas anesthetic preconditioning with isoflurane attenuated this increase to only 103 ± 8% at the same time point (fig. 2; $P < 0.05$). Administration of rapamycin inhibited the cardioprotective effect of isoflurane, and CK-MB concentrations increased maximally by 254 ± 9% (fig. 2; $P < 0.05$ compared with the APC group). In the Rap + I/R group, CK-MB increased by 239 ± 11% above baseline levels after 180 min of reperfusion (not significant compared with the I/R group). Similarly, administration of DMSO before ischemia and reperfusion resulted in a maximal increase of 247 ± 7% in CK-MB after 180 min of reperfusion (fig. 2; not significant compared with the I/R group).

The ratio of area at risk to left ventricular mass did not differ significantly among groups: 51 ± 2% in the I/R group, 54 ± 3% in the APC group, 48 ± 3% in the Rap + I/R group, 49 ± 6% in the Rap + APC group, and 53 ± 6% in the DMSO group. 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, whose blood supply was occluded.

In the I/R group, the infarct size was 44 ± 6% of the area at risk. Pretreatment with isoflurane decreased the infarct size to 26 ± 4% ($P < 0.05$ compared with the I/R group). Rapamycin administration before myocardial ischemia and reperfusion did not affect infarct size (50 ± 3%; not significant compared with the I/R group); how-

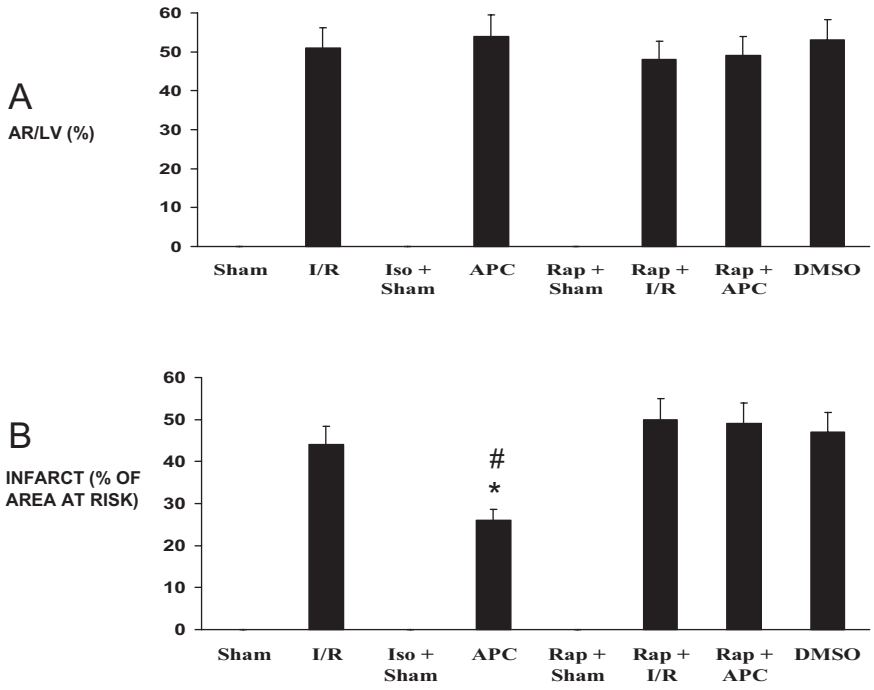


**Fig. 3. Bar graphs showing ratio of area at risk to left ventricular mass (AR/LV; *A*) and infarct size as percentage of the area at risk (*B*). Isoflurane significantly decreased infarct size compared with control (I/R) animals, whereas the administration of rapamycin abolished this protective effect, and infarct size is similar to that of the controls. Data are presented as mean ± SEM. APC = anesthetic preconditioning; DMSO = dimethyl sulfoxide; I/R = ischemia–reperfusion; Iso + sham = isoflurane + sham; RAP + APC = rapamycin + anesthetic preconditioning; RAP + I/R = rapamycin + ischemia–reperfusion; RAP + sham = rapamycin + sham. * $P <$ 0.05 compared with I/R group. # $P <$ 0.05 compared with RAP + APC group.**

ever, rapamycin significantly inhibited the cardioprotective effect produced by 1 minimum alveolar concentration (MAC) of isoflurane (49 ± 4%; $P < 0.05$ compared with the APC group; fig. 3). An infarct size of 47 ± 3% was observed in the DMSO group (not significantly different compared with the I/R group), indicating that administration of DMSO before myocardial ischemia and reperfusion did not affect infarct size. Finally, there was no increase in CK-MB concentration above baseline in the sham-operated animals, nor were any myocardial infarctions observed (data not shown).

*Isoflurane Induces an Increase in HIF-1α Myocardial Expression*

Hypoxia inducible factor-1α expression significantly increased in the ischemia and Iso + sham groups compared with nonischemic sham-operated controls. Isoflurane administration before coronary occlusion in the APC group resulted in a greater increase in HIF-1α protein levels than that seen in the ischemia or Iso + sham groups (fig. 4). Furthermore, this increase in HIF-1α protein expression was found both in the ischemic myocardium and in the remote nonischemic myocardial samples (fig. 5). Rapamycin pretreatment inhibited the increase in HIF-1 caused by ischemia and isoflurane (figs. 4 and 5).

*Isoflurane Increases HIF-1 DNA Binding Activity*

To evaluate the effect of isoflurane on HIF-1 DNA binding activity, electrophoretic mobility shift analysis was performed on nuclear protein samples that were obtained from ischemic myocardium. HIF-1 DNA binding was significantly increased in the coronary ischemia and Iso groups compared with the nonischemic operated sham group (fig. 6). Isoflurane preconditioning resulted in an even greater increase in HIF-1 DNA binding than that seen in the coronary ischemia and Iso groups. Rapamycin pretreatment, however, inhibited the effects of ischemia and isoflurane on HIF-1 DNA binding, returning it to a degree comparable with nonischemic sham-operated animals. Cold competition with excessive unlabeled oligonucleotides and supershift assays confirmed HIF-1 band specificity.

## Discussion

Using an *in vivo* model of regional myocardial ischemia and reperfusion in rabbits, we confirmed findings of previous studies[5,29] demonstrating that anesthetic preconditioning by isoflurane before myocardial ischemia and reperfusion is cardioprotective. These cardioprotective effects of anesthetic preconditioning may be mediated by activation of the transcription factor HIF-1. Administration of the mTOR inhibitor rapamycin, an upstream regulator of HIF-1α expression and activity,[24,25,30] abolished the cardioprotective effect of isoflurane while concomitantly inhibiting HIF-1α protein expression and HIF-1 DNA binding, providing evidence that isoflurane-induced HIF-1α upregulation is mTOR mediated. Our findings are important because it has been known for several years that volatile anesthetics provide protection against ischemia and reperfusion in various organs, including the heart,[3,4,31] kidneys,[32] and brain.[16] However, the mechanisms involved in this protection are not completely understood yet. The results of the current investigation are in agreement with a

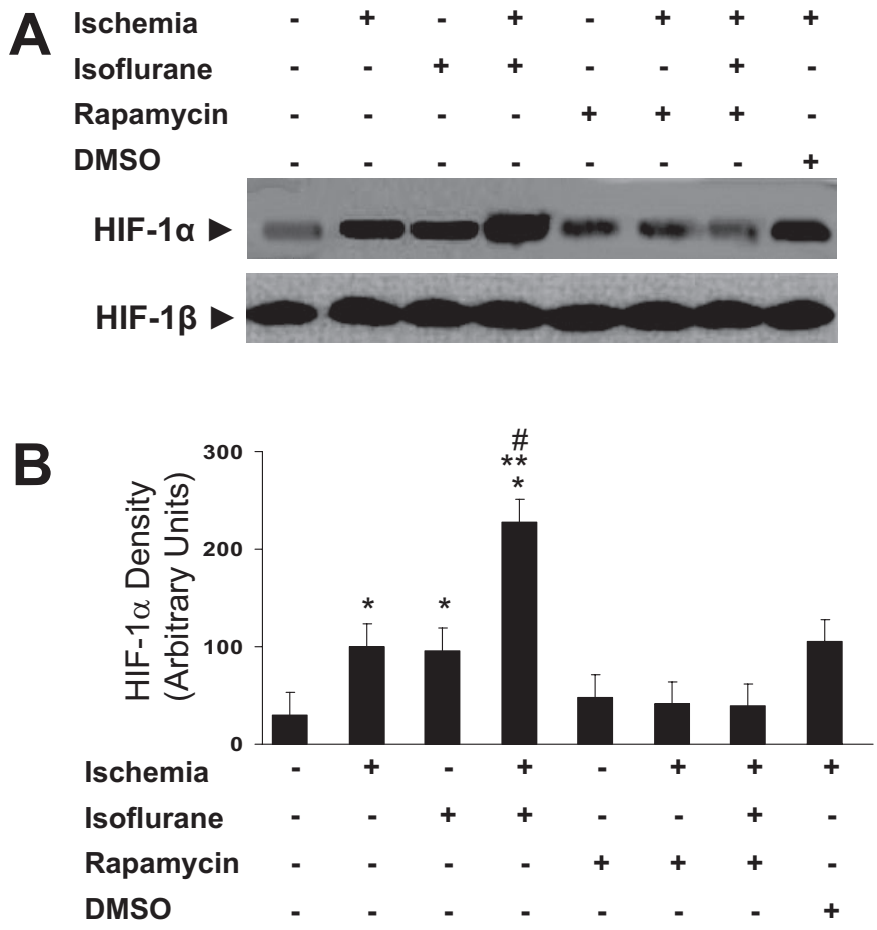


**Fig. 4. (*A*) Representative Western blot analysis of hypoxia inducible factor 1α (HIF-1α) in ischemic myocardium after anesthetic preconditioning with or without the presence of rapamycin (*top*). In each lane, the protein content was 50 μg. HIF-1β was used to demonstrate equal protein loading. (*B*) Graphic presentation of HIF-1α expression in samples of ischemic myocardium from the various experimental groups quantified by integrating the volume of autoradiograms from three separate experiments (*bottom*). Values in the graphs are presented as mean ± SEM. DMSO = dimethyl sulfoxide. * $P < 0.05$ compared with the sham group. ** $P < 0.05$ compared with the ischemia group. # $P < 0.05$ compared with the RAP + APC (rapamycin + anesthetic preconditioning) group.**

recent investigation[33] showing that isoflurane preconditioning is associated with up-regulation of HIF-1α. Our current investigation, however, extends these results. We found that isoflurane exposure resulted in up-regulation of HIF-1α even without ischemia, suggesting that isoflurane may have an ischemia-hypoxia–mimicking effect, to activate the HIF-1 cascade. Furthermore, Li *et al.*[33] tested the response of HIF-1 to isoflurane in a cell culture model without hypoxia and reoxygenation interventions, whereas we have used a more clinically relevant *in vivo* model of coronary ischemia and reperfusion. Our results are also in agreement with a recent study by Wang *et al.*[18] These investigators showed that isoflurane preconditioning increased HIF-1 and VEGF expression in the rat heart and that an extracellular signal–regulated protein kinase inhibitor blocked this protection and concomitantly inhibited the up-regulation in HIF-1 expression. Taken together, our results and the results of other investigations provide strong evidence to support the hypothesis that anesthetic preconditioning is mediated by activation of the HIF-1 cascade.

Our results also showed that HIF-1α protein expression was increased in nonischemic myocardial regions of the heart in response to remote ischemia or isoflurane preconditioning. Similarly, Kim *et al.*[34] demonstrated increased expression of the HIF-1 target VEGF in nonischemic regions of the right ventricle in response to myocardial ischemia. Because these remote areas are neither ischemic nor hypoxic, stimuli other than ischemia seem to be responsible for the accumulation of HIF-1α. One of the possible stimuli is the increased right ventricular diastolic wall stress caused by increased left ventricular end-diastolic pressure typically observed in hearts with severe ischemia or myocardial infarction. Moreover, systemic administration of isoflurane has the same effects on both ventricles, allowing us to assume that in response to isoflurane exposure, the same mechanisms that cause increased HIF-1 expression in the left ventricle are also responsible for the increased expression of HIF-1 in the right ventricle. However, the exact mechanisms involved in this phenomenon are still under investigation by our laboratory.

Intracellular hypoxia is a major stimulus for the production and enhanced activity of HIF-1α, but several other mediators, such as reactive oxygen species, have also been shown to be potent activators of HIF-1α under normoxic conditions.[35] Furthermore, these reactive oxygen intermediates play a key role in triggering isoflurane-induced preconditioning *in vivo*.[7] Isoflurane was also recently shown to increase endothelial nitric oxide synthase transcription and translation before prolonged coronary occlusion and reperfusion.[20] Hence, isoflurane may enhance HIF-1α expression and activity *via* gener-

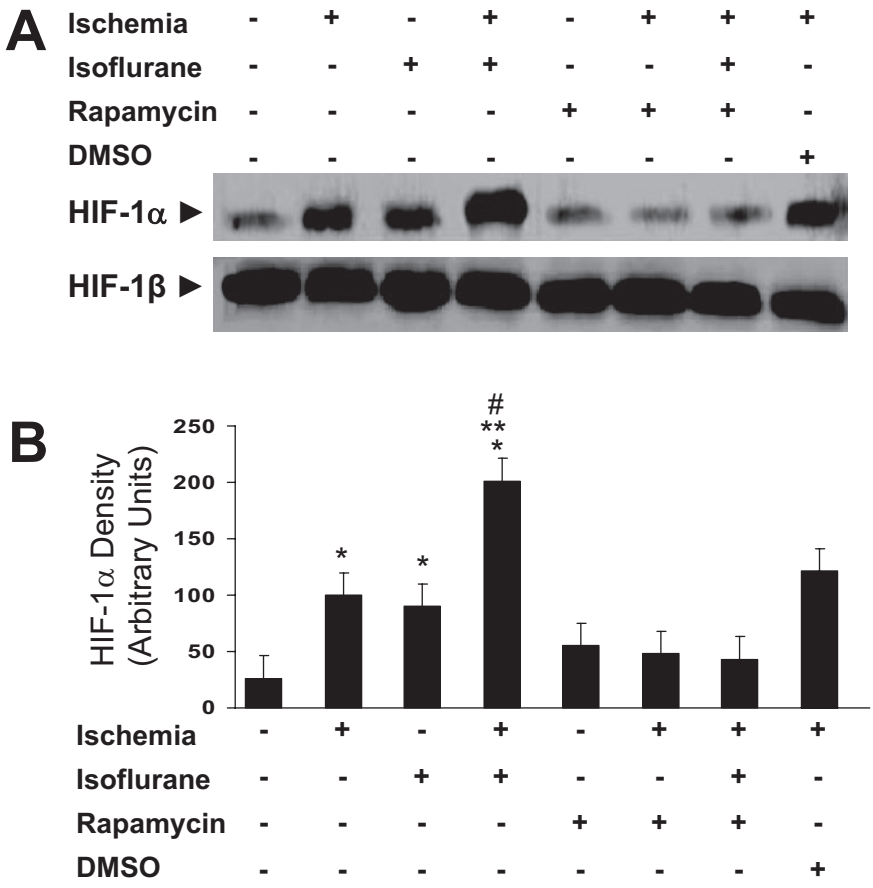


**Fig. 5. (*A*) Representative Western blot analysis of hypoxia inducible factor 1α (HIF-1α) in nonischemic myocardium after anesthetic preconditioning with or without the presence of rapamycin (*top*). In each lane, the protein content was 50 μg. HIF-1β was used to demonstrate equal protein loading. (*B*) Graphic presentation of HIF-1α expression in samples of nonischemic myocardium from the various experimental groups quantified by integrating the volume of autoradiograms from three separate experiments (*bottom*). Values in the graphs are presented as mean ± SEM. DMSO = dimethyl sulfoxide. * $P < 0.05$ compared with the sham group. ** $P < 0.05$ compared with the ischemia group. # $P < 0.05$ compared with the RAP + APC (rapamycin + anesthetic preconditioning) group.**

ation of reactive oxygen species or production of nitric oxide. Furthermore, we have recently shown[9] that isoflurane preconditioning is mediated by activation of the PI3K/Akt survival pathway. This pathway has been demonstrated to control HIF-1α protein expression and activity.[34,36] Therefore, an alternative explanation for HIF-1α up-regulation by isoflurane may be *via* activation of the PI3K/Akt survival pathway.

Several studies have focused lately on activation of the HIF-1 signaling system as a potential therapy to reduce injury after ischemia and reperfusion.[14,15] In this regard, it is important to note that hypoxic or pharmacologic preconditioning–induced neuroprotection in neonatal rats after hypoxic–ischemic brain injury is mediated by activation of HIF-1.[37] However, to our knowledge, our study is the first study to report that anesthetic preconditioning, using an *in vivo* model of regional myocardial ischemia and reperfusion, is related to activation of HIF-1α and that this activation is mediated through mTOR signaling. Furthermore, the increase in HIF-1α expression was demonstrated in both ischemic and remote nonischemic myocardium.

Hypoxia inducible factor 1 is a transcription factor that mediates adaptive mechanisms such as glycolysis, erythropoiesis, and angiogenesis during hypoxic–ischemic injury.[12,13,38] As previously mentioned, during hypoxic conditions, the degradation of HIF-1α is blocked, allowing it to accumulate within the cell and then to translocate to the nucleus, where it forms a heterodimer with HIF-1β, to become the active transcription factor for several key end products that are important for anaerobic metabolism and cell survival, including erythropoietin, VEGF, glucose transport enzymes, endothelial nitric oxide synthase, inducible nitric oxide synthase, and glycolytic enzymes.[38] Although HIF-1 is rapidly degraded upon reperfusion/reoxygenation of tissues or cells, the process to increase the expression of its downstream targets, such as inducible nitric oxide synthase, VEGF, and erythropoietin, should already be activated to provide protection against ischemia–reperfusion. The protective actions of erythropoietin and nitric oxide during ischemic and anesthetic preconditioning have already been demonstrated.[20,39]

Increased HIF-1α protein expression and DNA binding by isoflurane and its inhibition by the mTOR antagonist rapamycin provide strong evidence for the involvement of HIF-1 in isoflurane-induced cardioprotection.

Rapamycin is an immunosuppressive agent that is known to be a specific inhibitor of the high-molecular-mass protein kinase mTOR. This protein kinase has been shown to coordinate growth factor and nutrient availability with cell growth and proliferation.[40] Through its effect on mTOR, rapamycin has been demonstrated as an upstream inhibitor of HIF-1α expression and activity in

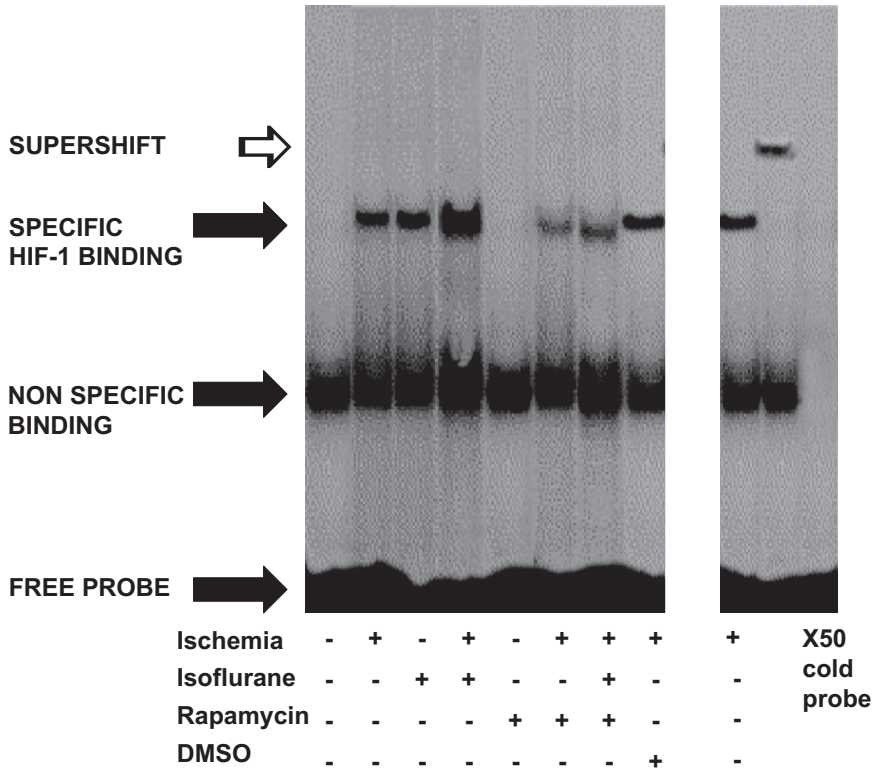


**Fig. 6. Hypoxia inducible factor 1 (HIF-1) DNA binding activity after anesthetic preconditioning. Electrophoretic mobility shift analysis was performed to determine HIF-1 DNA binding activity after anesthetic preconditioning with or without the presence of rapamycin. Specificity of the HIF-1-DNA complex was confirmed by supershift assay and cold competition in the presence of an unlabeled probe in a concentration 50 times higher than the labeled probe. DMSO = dimethyl sulfoxide.**

various experimental models.[25,41] Furthermore, it was recently approved as an antitumor therapy in certain neoplasms that have increased expression of HIF-1.[24,42] For these reasons, we chose to use rapamycin to determine the role of mTOR in the isoflurane-induced up-regulation of HIF-1 expression and activity. Nevertheless, rapamycin has also been used as an antagonist of the p70s6 kinase that is known to play a major role in preconditioning.[43] Therefore, the possibility that rapamycin abolished isoflurane's cardioprotective effect *via* inhibition of the p70s6 kinase or another unrelated protein kinase cannot be entirely excluded.

Recently, Khan *et al.*[44] used rapamycin to induce cardioprotection in an isolated mouse heart model and mouse cardiomyocytes. These findings are not consistent with our results or with results from many other investigators who showed that rapamycin did not induce protection and, instead, blocked the effects of preconditioning. The reasons for this discrepancy are not clear. Species differences may contribute to it.

In contrast to the reported effects of isoflurane on HIF-1, it has been demonstrated, however, that halothane reversibly blocks hypoxia-induced HIF-1$\alpha$ protein accumulation and transcriptional activity at clinically relevant doses in a human hepatoma–derived cell line.[45] Differential physiologic effects of volatile anesthetics are well known. For example, halothane and isoflurane produce different inotropic and vasodilatory effects.[46] In addition, isoflurane induces protection of human myocardium against anoxic injury; however, halothane does not.[47] Therefore, these previous studies may support the hypothesis that isoflurane and halothane differ in their actions on HIF-1 activation.

Cai *et al.*[48] have showed that hypoxic preconditioning before myocardial ischemia and reperfusion attenuated apoptosis and was associated with up-regulation of HIF-1$\alpha$. We have recently demonstrated that isoflurane preconditioning reduced myocardial apoptosis using the same model that was used in the current investigation.[9] These data may support the hypothesis that isoflurane preconditioning inhibits myocardial apoptosis *via* an HIF-1–regulated mechanism; however, this is yet to be demonstrated.

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 the extent of coronary collateral perfusion. The area at risk, expressed as a percentage of total left ventricle mass, was similar between groups in the current investigation. Coronary collateral blood flow was not specifically quantified in the current investigation. However, rabbits have been shown to possess little if any coronary collateral blood flow.[49] Therefore, it seems unlikely that differences in collateral perfusion between groups account for the observed results.

In the current investigation, we used CK-MB to evaluate the extent of myocardial injury. Although troponins are more specific to the myocardium, CK-MB is still in wide use for evaluation of myocardial injury.[50] One may comment that a part of our measured CK-MB

concentrations may originate from muscle injury during the surgical procedure. Indeed, striated muscle damage can result in elevated CK-MB concentrations. However, considering the fact that all animals went through the same surgical procedure, we assume that the extent of muscle injury was similar in all of the experimental groups. Therefore, the differences in CK-MB concentrations probably correlate with differences in the extent of myocardial injury. Furthermore, the increase in CK-MB concentrations occurred mainly during the reperfusion phase, which is more compatible with myocardial injury than with striated muscle damage.

In summary, the current investigation indicates that preconditioning by administration of 1.0 MAC isoflurane before myocardial ischemia and reperfusion salvages myocardium from infarction. These beneficial effects of anesthetic preconditioning may be mediated by increased protein expression and activation of HIF-1$\alpha$, which may be mTOR dependent. Ischemic and anesthetic preconditioning have been shown to be mediated by important downstream targets of HIF-1, such as endothelial nitric oxide synthase and erythropoietin. These data suggest that isoflurane may affect gene expression of cardioprotective elements before coronary occlusion. Additional research will be required to elucidate other signaling elements involved in preconditioning by anesthetics and clarify the role of HIF-1 in anesthetic-induced cardioprotection.

## References


1. Murry CE, Jennings RB, Reimer KA: Preconditioning with ischemia: A delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74:1124–36
2. Alkhulaifi AM: Preconditioning the human heart. Ann R Coll Surg Engl 1997; 79:49–54
3. Cason BA, Gamperl AK, Slocum RE, Hickey RF: Anesthetic-induced preconditioning: Previous administration of isoflurane decreases myocardial infarct size in rabbits. Anesthesiology 1997; 87:1182–90
4. Kersten JR, Schmeling TJ, Pagel PS, Gross GJ, Warltier DC: Isoflurane mimics ischemic preconditioning *via* activation of $K_{ATP}$ channels: Reduction of myocardial infarct size with an acute memory phase. Anesthesiology 1997; 87: 361–70
5. Kersten JR, Orth KG, Pagel PS, Mei DA, Gross GJ, Warltier DC: Role of adenosine in isoflurane-induced cardioprotection. Anesthesiology 1997; 86:1128–39
6. Novalija E, Kevin LG, Camara AK, Bosnjak ZJ, Kampine JP, Stowe DF: Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. Anesthesiology 2003; 99:421–8
7. Mullenheim J, Ebel D, Frassdorf J, Preckel B, Thamer V, Schlack W: Isoflurane preconditions myocardium against infarction *via* release of free radicals. Anesthesiology 2002; 96:934–40
8. Pain T, Yang XM, Critz SD, Yue Y, Nakano A, Liu GS, Heusch G, Cohen MV, Downey JM: Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. Circ Res 2000; 87:460–6
9. Raphael J, Abedat S, Rivo J, Meir K, Beeri R, Pugatsch T, Zuo Z, Gozal Y: Volatile anesthetic preconditioning attenuates myocardial apoptosis in rabbits after regional ischemia and reperfusion *via* Akt signaling and modulation of Bcl-2 family proteins. J Pharmacol Exp Ther 2006; 318:186–94
10. Hausenloy DJ, Tsang A, Yellon DM: The reperfusion injury salvage kinase pathway: A common target for both ischemic preconditioning and postconditioning. Trends Cardiovasc Med 2005; 15:69–75
11. Dawn B, Bolli R: Role of nitric oxide in myocardial preconditioning. Ann N Y Acad Sci 2002; 962:18–41
12. Semenza GL, Roth PH, Fang HM, Wang GL: Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. J Biol Chem 1994; 269:23757–63
13. Semenza GL: Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis. J Lab Clin Med 1998; 131:207–14
14. Xi L, Taher M, Yin C, Salloum F, Kukreja RC: Cobalt chloride induces delayed cardiac preconditioning in mice through selective activation of HIF-1$\alpha$ and AP-1 and iNOS signaling. Am J Physiol Heart Circ Physiol 2004; 287: H2369–75
15. Siddiq A, Ayoub IA, Chavez JC, Aminova L, Shah S, LaManna JC, Patton SM, Connor JR, Cherny RA, Volitakis I, Bush AI, Langsetmo I, Seeley T, Gunzler V, Ratan RR: Hypoxia-inducible factor prolyl 4-hydroxylase inhibition: A target for neuroprotection in the central nervous system. J Biol Chem 2005; 280:41732–43
16. Zhao P, Zuo Z: Isoflurane preconditioning induces neuroprotection that is inducible nitric oxide synthase–dependent in neonatal rats. Anesthesiology 2004; 101:695–703
17. Hoetzel A, Leitz D, Schmidt R, Tritschler E, Bauer I, Loop T, Humar M, Geiger KK, Pannen BH: Mechanism of hepatic heme oxygenase-1 induction by isoflurane. Anesthesiology 2006; 104:101–9
18. Wang C, Weihrauch D, Schwabe DA, Bienengraeber M, Warltier DC, Kersten JR, Pratt PF Jr, Pagel PS: Extracellular signal-regulated kinases trigger isoflurane preconditioning concomitant with upregulation of hypoxia-inducible factor-1$\alpha$ and vascular endothelial growth factor expression in rats. Anesth Analg 2006; 103:281–8
19. Cho S, Park EM, Zhou P, Frys K, Ross ME, Iadecola C: Obligatory role of inducible nitric oxide synthase in ischemic preconditioning. J Cereb Blood Flow Metab 2005; 25:493–501
20. Chiari PC, Bienengraeber MW, Weihrauch D, Krolikowski JG, Kersten JR, Warltier DC, Pagel PS: Role of endothelial nitric oxide synthase as a trigger and mediator of isoflurane-induced delayed preconditioning in rabbit myocardium. Anesthesiology 2005; 103:74–83
21. Joyeux-Faure M, Ramond A, Beguin PC, Belaidi E, Godin-Ribuot D, Ribuot C: Early pharmacological preconditioning by erythropoietin mediated by inducible NOS and mitochondrial ATP-dependent potassium channels in the rat heart. Fundam Clin Pharmacol 2006; 20:51–6
22. Drummond JC: MAC for halothane, enflurane, and isoflurane in the New Zealand White rabbit: And a test for the validity of MAC determinations. Anesthesiology 1985; 62:336–8
23. Kis A, Yellon DM, Baxter GF: Second window of protection following myocardial preconditioning: An essential role for PI3 kinase and p70S6 kinase. J Mol Cell Cardiol 2003; 35:1063–71
24. Abraham RT: mTOR as a positive regulator of tumor cell responses to hypoxia. Curr Top Microbiol Immunol 2004; 279:299–319
25. Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, Giaccia AJ, Abraham RT: Regulation of hypoxia-inducible factor 1$\alpha$ expression and function by the mammalian target of rapamycin. Mol Cell Biol 2002; 22: 7004–14
26. Semenza GL: HIF-1: Mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol 2000; 88:1474–80
27. Jung F, Palmer LA, Zhou N, Johns RA: Hypoxic regulation of inducible nitric oxide synthase *via* hypoxia inducible factor-1 in cardiac myocytes. Circ Res 2000; 86:319–25
28. Jiang BH, Rue E, Wang GL, Roe R, Semenza GL: Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem 1996; 271:17771–8
29. Bienengraeber MW, Weihrauch D, Kersten JR, Pagel PS, Warltier DC: Cardioprotection by volatile anesthetics. Vascul Pharmacol 2005; 42:243–52
30. Abraham RT, Wiederrecht GJ: Immunopharmacology of rapamycin. Annu Rev Immunol 1996; 14:483–510
31. Zaugg M, Lucchinetti E, Garcia C, Pasch T, Spahn DR, Schaub MC: Anaesthetics and cardiac preconditioning: II. Clinical implications. Br J Anaesth 2003; 91:566–76
32. Hashiguchi H, Morooka H, Miyoshi H, Matsumoto M, Koji T, Sumikawa K: Isoflurane protects renal function against ischemia and reperfusion through inhibition of protein kinases, JNK and ERK. Anesth Analg 2005; 101:1584–9
33. Li QF, Wang XR, Yang YW, Su DS: Up-regulation of hypoxia inducible factor 1$\alpha$ by isoflurane in Hep3B cells. Anesthesiology 2006; 105:1211–9
34. Kim CH, Cho YS, Chun YS, Park JW, Kim MS: Early expression of myocardial HIF-1$\alpha$ in response to mechanical stresses: Regulation by stretch-activated channels and the phosphatidylinositol 3-kinase signaling pathway. Circ Res 2002; 90:E25–33
35. Bardos JI, Ashcroft M: Hypoxia-inducible factor-1 and oncogenic signalling. Bioessays 2004; 26:262–9
36. Chachami G, Simos G, Hatziefthimiou A, Bonanou S, Molyvdas PA, Paraskeva E: Cobalt induces hypoxia-inducible factor-1$\alpha$ expression in airway smooth muscle cells by a reactive oxygen species- and PI3K-dependent mechanism. Am J Respir Cell Mol Biol 2004; 31:544–51
37. Bergeron M, Gidday JM, Yu AY, Semenza GL, Ferriero DM, Sharp FR: Role of hypoxia-inducible factor-1 in hypoxia-induced ischemic tolerance in neonatal rat brain. Ann Neurol 2000; 48:285–96
38. Semenza GL: Surviving ischemia: Adaptive responses mediated by hypoxia-inducible factor 1. J Clin Invest 2000; 106:809–12
39. Bullard AJ, Govewalla P, Yellon DM: Erythropoietin protects the myocardium against reperfusion injury *in vitro* and *in vivo*. Basic Res Cardiol 2005; 100:397–403

40. Chiang GG, Abraham RT: Targeting the mTOR signaling network in cancer. Trends Mol Med 2007; 13:433–42

41. Majumder PK, Febbo PG, Bikoff R, Berger R, Xue Q, McMahon LM, Manola J, Brugarolas J, McDonnell TJ, Golub TR, Loda M, Lane HA, Sellers WR: mTOR inhibition reverses Akt-dependent prostate intraepithelial neoplasia through regulation of apoptotic and HIF-1-dependent pathways. Nat Med 2004; 10:594–601

42. Belozerov VE, Van Meir EG: Hypoxia inducible factor-1: A novel target for cancer therapy. Anticancer Drugs 2005; 16:901–9

43. Hausenloy DJ, Tsang A, Mocanu MM, Yellon DM: Ischemic preconditioning protects by activating prosurvival kinases at reperfusion. Am J Physiol Heart Circ Physiol 2005; 288:H971–6

44. Khan S, Salloum F, Das A, Xi L, Vetrovec GW, Kukreja RC: Rapamycin confers preconditioning-like protection against ischemia-reperfusion injury in isolated mouse heart and cardiomyocytes. J Mol Cell Cardiol 2006; 41:256–64

45. Itoh T, Namba T, Fukuda K, Semenza GL, Hirota K: Reversible inhibition of hypoxia-inducible factor 1 activation by exposure of hypoxic cells to the volatile anesthetic halothane. FEBS Lett 2001; 509:225–9

46. Lee HT, Ota-Setlik A, Fu Y, Nasr SH, Emala CW: Differential protective effects of volatile anesthetics against renal ischemia-reperfusion injury *in vivo*. ANESTHESIOLOGY 2004; 101:1313–24

47. Roscoe AK, Christensen JD, Lynch C III: Isoflurane, but not halothane, induces protection of human myocardium *via* adenosine A1 receptors and adenosine triphosphate-sensitive potassium channels. ANESTHESIOLOGY 2000; 92: 1692–701.

48. Cai Z, Manalo DJ, Wei G, Rodriguez ER, Fox-Talbot K, Lu H, Zweier JL, Semenza GL: Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. Circulation 2003; 108: 79–85

49. Maxwell MP, Hearse DJ, Yellon DM: Species variation in the coronary collateral circulation during regional myocardial ischaemia: A critical determinant of the rate of evolution and extent of myocardial infarction. Cardiovasc Res 1987; 21:737–46

50. Korbmacher B, Klein KK, Sunderdiek U, Gams E, Schipke JD: Does adenosine pharmacologically precondition human myocardium during coronary bypass surgery? J Cardiovasc Surg (Torino) 2005; 46:285–90