

# Isoflurane Produces Sustained Cardiac Protection after Ischemia-Reperfusion Injury in Mice

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**Background:** Isoflurane reduces myocardial ischemia-reperfusion injury within hours to days of reperfusion. Whether isoflurane produces sustained cardiac protection has never been examined. The authors studied isoflurane-induced cardiac protection in the intact mouse after 2 h and 2 weeks of reperfusion and determined the dependence of this protection on adenosine triphosphate-dependent potassium channels and the relevance of this protection to myocardial function and apoptosis.

**Methods:** Mice were randomly assigned to receive oxygen or isoflurane for 30 min with 15 min of washout. Some mice received mitochondrial (5-hydroxydecanoic acid) or sarcolemmal (HMR-1098) adenosine triphosphate-dependent potassium channel blockers with or without isoflurane. Mice were then subjected to a 30-min coronary artery occlusion followed by 2 h or 2 weeks of reperfusion. Infarct size was determined at 2 h and 2 weeks of reperfusion. Cardiac function and apoptosis were determined 2 weeks after reperfusion.

**Results:** Isoflurane did not change hemodynamics. Isoflurane reduced infarct size after reperfusion when compared with the control groups (27.7 ± 6.3 vs. 41.7 ± 6.4% at 2 h and 19.6 ± 5.9 vs. 28.8 ± 9.0% at 2 weeks). Previous administration of 5-hydroxydecanoic acid, but not HMR-1098, abolished isoflurane-induced cardiac protection. At 2 weeks, left ventricular end-diastolic diameter was decreased significantly and end-systolic pressure and maximum and minimum  $dp/dt$  were improved by isoflurane. Isoflurane-treated mice subjected to ischemia and 2 weeks of reperfusion showed less expression of proapoptotic genes, significantly decreased expression of cleaved caspase-3, and significantly decreased deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling-positive nuclei compared with the control group.

**Conclusions:** Cardiac protection induced by isoflurane against necrotic and apoptotic cell death is associated with an acute memory period that is sustained and functionally relevant 2 weeks after ischemia-reperfusion injury in mice *in vivo*.

**ANESTHETIC preconditioning** (APC) is a phenomenon by which brief exposure to a volatile anesthetic leads to a subsequent protected state whereby the myocardium is made resistant to ischemia-reperfusion injury.<sup>1,2</sup> Although several studies have evaluated APC induced by

isoflurane in many species,<sup>1-5</sup> it is unknown whether APC occurs in the mouse, a species amenable to genetic manipulation using transgenic mice for molecular dissection of triggers, mediators, and end effectors of APC.

Many characteristics of APC<sup>1,2,6-9</sup> are similar to those of ischemic preconditioning,<sup>10-15</sup> where brief ischemic episodes protect the myocardium from subsequent injury.<sup>16</sup> Most APC and ischemic preconditioning studies focus on the short-term consequences of reperfusion, limiting the endpoint determination of infarct size to a few hours or a day after reperfusion. The long-term consequences of APC on myocardial injury and remodeling have not been examined, although understanding whether APC results in sustained cardiac protection is relevant for extrapolation to the clinic. Experiments in brain suggest that the benefit of APC observed acutely wanes after 2 weeks of reperfusion, suggesting that APC in the brain produces a transient protected state.<sup>17</sup>

The goal of the current study was to determine whether APC induced by isoflurane is sustained in the heart. Isoflurane-induced cardiac protection in the mouse heart was characterized further by examining the role of adenosine triphosphate-dependent potassium ( $K_{ATP}$ ) channels, apoptosis, and assessing cardiac function.

## Materials and Methods

### Animals

All animals were treated in compliance with the *Guide for the Care and Use of Laboratory Animals*,<sup>18</sup> and animal use protocols were approved by the VA San Diego Healthcare System Institutional Animal Care and Use Committee (San Diego, California). C57BL/6 male mice (aged 8-10 weeks, 24-26 g body weight) were purchased from Jackson Laboratory (Bar Harbor, Maine). The animals were kept on a 12-h light-dark cycle in a temperature-controlled room. Mice were placed postoperatively in an animal care unit under daily supervision.

### Experimental Preparation

Mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal), and anesthesia was maintained *via* supplemental doses (20 mg/kg, intraperitoneal) as needed. Twenty-gauge catheters were then inserted into the tracheae, and the mice mechanically ventilated using a pressure-controlled ventilator (TOPO Ventilator; Kent Scientific Co., Torrington, CT; peak inspiratory pressure: 15 cm H<sub>2</sub>O, respiratory rate: 100 breaths/min, inspired oxygen: 100%). A thoracotomy

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was performed to expose the heart. Core temperature was maintained at 36°C with a heating pad, and electrocardiographic leads were placed to record heart rate.

#### Hemodynamics and Blood Gas Analysis

The hemodynamic effects of isoflurane during the ischemia-reperfusion protocol were studied in a separate group of 16 mice. Mice were anesthetized with sodium pentobarbital as above and instrumented with a heparin-filled catheter (Micro-Cannula; Harvard Apparatus, Holliston, MA) inserted into the right carotid artery and connected to a Tru-Wave pressure transducer (Model PX600I; Baxter Healthcare, Deerfield, IL) for determination of heart rate, arterial blood pressure, and rate-pressure product. Pressure signals were amplified and digitally converted (DI-220; DATAQ Instruments, Inc., Akron, OH) and stored on a computer. After stabilization, mice were randomly assigned to receive 100% oxygen or 1.4% isoflurane (1.0 minimum alveolar concentration [MAC] for mice)<sup>19</sup> for 30 min followed by 15 min of washout. Arterial blood gases were analyzed at the preocclusion time point using i-STAT PCA (i-STAT Co., East Windsor, NJ).

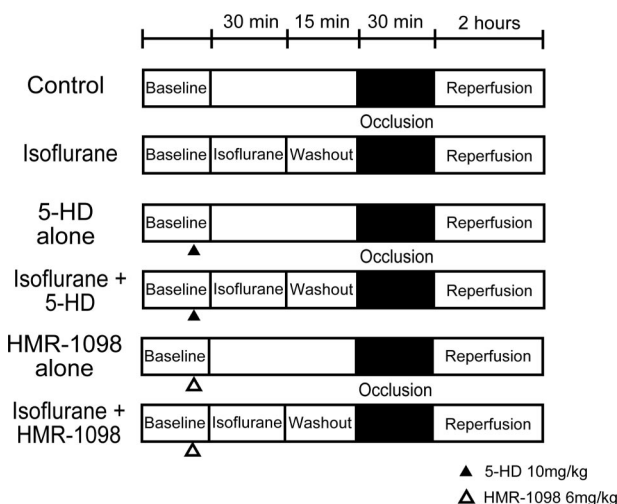
#### Ischemia Reperfusion Protocol and Experimental Groups

After thoracotomy, baseline was established, and mice were randomly assigned to one of six experimental protocols as described in figure 1. Mice received 100% oxygen (45 min) in the control group or 1.0 MAC isoflurane (30 min with 15 min of washout). Other mice received selective mitochondrial  $K_{ATP}$  channel antagonist, 5-hydroxydecanoic acid (5-HD; 10 mg/kg, Sigma Chemical, St. Louis, MO), or selective sarcolemmal  $K_{ATP}$  channel antagonist, HMR-1098 (6 mg/kg), at 10 min before APC or time equivalent for control. Ischemia was produced by occluding the left coronary artery with a 7-0 silk suture on a taper BV-1 needle (Ethicon, Inc., Somerville, NJ). A small piece of polyethylene tubing was used to secure the silk ligature without damaging the artery. After 30 min of occlusion, the ligature was released, and the heart was reperfused for 2 h or 2 weeks. Reperfusion was confirmed by observing return of blood flow in the epicardial coronary arteries. In the 2-week reperfusion groups, the chest was closed, mice were weaned from the ventilator, and the endotracheal tube was removed when spontaneous breathing was evident.

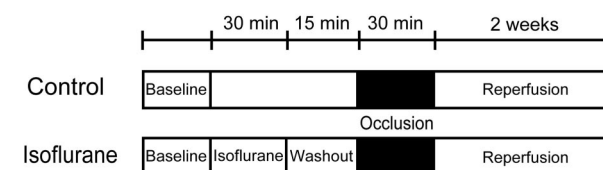
#### Determination of Infarct Size

After 2 h or 2 weeks of reperfusion, mice were heparinized, and the coronary artery was again occluded. The area at risk (AAR) was determined by staining with 1% Evans blue (1.0 ml; Sigma Chemical). The heart was immediately excised and placed into 1% agarose and allowed to harden. Once hardened, the heart was cut

#### A. 2 hours reperfusion protocol



#### B. 2 weeks reperfusion protocol



**Fig. 1. Experimental protocols.** (A) Two-hour reperfusion protocol. Mice were subjected to a 30-min coronary occlusion and 2 h of reperfusion. Isoflurane-treated mice received 1.0 minimum alveolar concentration of isoflurane followed by a memory period of 15 min before coronary occlusion. 5-Hydroxydecanoic acid (5-HD) or HMR-1098 was administered 55 min before occlusion. (B) Two-week reperfusion protocol. The mice were exposed to oxygen or isoflurane as described in the ischemia-reperfusion protocol and allowed to recover for 2 weeks.

into 1.0-mm slices (McIlwain tissue chopper; Brinkmann Instruments, Inc., Westbury, NY). Each slice of left ventricle (LV) was then counterstained with 3.0 ml 2,3,5-triphenyltetrazolium chloride, 1% (Sigma Chemical), for 5 min at 37°C. After overnight storage in 10% formaldehyde, slices were weighed and visualized under a microscope (Leica Microsystems Inc., Bannockburn, IL) equipped with a charge-coupled device camera (Cool SNAP-Pro; Media Cybernetics, Inc., Silver Spring, MD). The images were analyzed (Image-Pro Plus Version 4.5; Media Cybernetics, Inc.), and infarct size was determined by planimetry as previously described.<sup>20</sup> The AAR was expressed as a percentage of the LV (AAR/LV), and infarct size (IS) was expressed as a percentage of the AAR (IS/AAR).

#### Echocardiography and Cardiac Catheterization

Echocardiography and cardiac catheterization was performed after 2 weeks of reperfusion in mice with or without isoflurane. In addition, a group of sham-operated mice with or without isoflurane were studied. Sham mice were anesthetized with sodium pentobarbital as above and underwent thoracotomy. The mice were then exposed to oxygen or isoflurane as described in the

ischemia-reperfusion protocol and allowed to recover for 2 weeks.

Echocardiography was performed 2 weeks after reperfusion or sham operation in mice anesthetized with isoflurane (1.4%) using an echocardiograph and L15/6-MHz transducer (Sonos 5500; Philips Medical Systems, Andover, MA). With mice in the left lateral decubitus position, a parasternal short-axis view was obtained for left ventricular M-mode imaging at the papillary muscle level as described previously.<sup>21</sup> Left ventricular wall thickness and left ventricular internal chamber diameters were determined in diastole and systole, and left ventricular fractional shortening was observed. Echocardiographic measurements were made by one blinded observer. Intraobserver variability for M-mode measurements was  $1.8 \pm 1.5\%$ .

Cardiac catheterization was performed 2 weeks after reperfusion or sham operation in mice anesthetized with sodium pentobarbital using a high-fidelity pressure transducer (1.4-French, SPR-719; Millar Instruments, Houston, TX). Respiration was controlled in mice intubated and ventilated as described in the experimental preparation above. Under microscopic guidance, the right carotid artery was exposed, and the catheter was advanced into the LV. End-diastolic pressure and end-systolic pressure were obtained from the left ventricular waveform and determined by an algorithm from EMKA Technologies (Falls Church, VA). The continuous pressure signals were digitally sampled at 1,000 points/s. The data were displayed in real time using IOX version 1.8.5.11 software (EMKA Technologies) and analyzed by an algorithm from EMKA Technologies. The parameters were averaged over more than 20 successive heartbeats for end-systolic pressure, end-diastolic pressure, maximum  $dP/dt$ , and minimum  $dP/dt$ .

### *Apoptosis*

Real-time polymerase chain reaction analysis of gene expression of proapoptotic and antiapoptotic genes<sup>22</sup> was performed on total RNA isolated from myocardium at risk of ischemia at 2 weeks of reperfusion using an RNeasy Mini Kit (Qiagen Inc., Valencia, CA). First-strand complementary DNA (cDNA) synthesis (Superscript First Strand Synthesis System for real-time polymerase chain reaction; Invitrogen Co., Carlsbad, CA) was performed using random hexamers on 1–2  $\mu\text{g}$  total RNA. The concentration of cDNA was determined and adjusted to 50 ng/ $\mu\text{l}$  for real-time polymerase chain reaction analysis, which was performed on an MJ Research Opticon 2 (BioRad Laboratories, Hercules, CA) in triplicate using the QPCR Mastermix Plus for SYBR Green Kit (Eurogentec North America, Inc., San Diego, CA) with 100 ng cDNA and 0.5  $\mu\text{M}$  forward/reverse primer mix (primers were generated using Primer3 Web-based software; Whitehead Institute for Biomedical Research, Cambridge, MA) in 20  $\mu\text{l}$  final reaction volume. Thermal

cycle conditions were as follows: 94°C–10 min (1 cycle); 94°C–20 s, 55°C–20 s, 72°C–30 s (40 cycles). Resulting polymerase chain reaction products were confirmed by melt curve analysis. Analysis of cycle threshold ( $C_t$ ) was performed using Opticon 2 Analysis Software (BioRad Laboratories); normalized values were obtained for each group by subtracting matched glyceraldehyde-3-phosphate dehydrogenase  $C_t$  values. Fold increase or decrease was determined relative to sham animals that underwent similar handling with the exception of the ischemia-reperfusion protocol.

For immunoblot analysis, myocardium at risk of ischemia after 2 weeks of reperfusion was homogenized in lysis buffer. Protein (5  $\mu\text{g}$ ) from five isoflurane-treated and five control animals was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% polyacrylamide precast gels (Invitrogen Co.) and transferred to a polyvinylidene difluoride membrane by electroelution. Membranes were blocked in phosphate-buffered saline containing 1.5% nonfat dry milk and incubated with primary antibody overnight at 4°C (total caspase-3 and cleaved caspase-3 [1:1,000], Stressgen Biotechnologies Corporation, Victoria, Canada; glyceraldehyde-3-phosphate dehydrogenase [1:1,000], Imgenex Corporation, San Diego, CA). Bound primary antibodies were visualized using secondary antibodies conjugated with horseradish peroxidase from Santa Cruz Biotech (Santa Cruz, CA) and enhanced chemiluminescent reagent from Amersham Pharmacia Biotech (Piscataway, NJ). All displayed bands migrated at the appropriate size, as determined by comparison with molecular weight standards (Santa Cruz Biotech). Densitometry for total caspase-3 and cleaved caspase-3 was normalized to glyceraldehyde-3-phosphate dehydrogenase values.

For terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assays, hearts were removed from the mice after 2 weeks of reperfusion, and the atrial tissue was dissected away. The LV was cut in short axis, 1-mm section of LV at a level distal to the coronary occlusion was fixed in 3.7% formalin and later embedded in paraffin, and sections (5  $\mu\text{m}$ ) were obtained to perform TUNEL assays using the Apoptosis Detection Kit (R&D Systems, Inc., Minneapolis, MN) according to the manufacturer's instructions. Briefly, the deparaffinized sections were pretreated with proteinase K for 15 min at room temperature. The sections were incubated with terminal deoxynucleotidyl transferase for 1 h at 37°C. TUNEL-positive myocytes were determined by randomly counting 10 fields from the region of myocardium at risk by a blinded observer and were expressed as a percentage of the total cardiac myocyte population.

### *Statistical Analysis*

Determination of all data was performed blinded to experimental groups for the observers. Group size to



**Table 1. Hemodynamics**

	Baseline Treatment	30 min	Preocclusion	Ischemia 30 min	Reperfusion 120 min
<b>A. Heart rate, beats/min</b>					
Control (n = 8)	457 ± 29	440 ± 22	441 ± 31	—	—
Isoflurane (n = 8)	452 ± 27	467 ± 44	453 ± 43	—	—
Mean arterial pressure, mmHg					
Control (n = 8)	60 ± 8	61 ± 11	65 ± 12	—	—
Isoflurane (n = 8)	59 ± 8	59 ± 6	64 ± 9	—	—
Rate-pressure product, beats · min <sup>-1</sup> · mmHg · 10 <sup>3</sup>					
Control (n = 8)	27.4 ± 4.8	26.7 ± 5.0	28.5 ± 5.0	—	—
Isoflurane (n = 8)	26.5 ± 4.2	27.5 ± 3.5	29.2 ± 5.8	—	—
<b>B. Heart rate, beats/min, 2-h reperfusion group</b>					
Control (n = 8)	486 ± 37	479 ± 40	470 ± 43	472 ± 23	466 ± 28
Isoflurane (n = 8)	448 ± 37	486 ± 50	477 ± 60	445 ± 47	445 ± 35
5-HD alone (n = 9)	462 ± 56	482 ± 35	481 ± 38	466 ± 58	476 ± 32
ISO + 5-HD (n = 9)	460 ± 38	459 ± 56	472 ± 45	466 ± 52	466 ± 24
Heart rate, beats/min, 2-wk reperfusion group					
Control (n = 11)	459 ± 40	455 ± 31	460 ± 34	443 ± 19	—
Isoflurane (n = 11)	453 ± 32	480 ± 48	482 ± 44	482 ± 52	—

Data are presented as mean ± SD. Thirty-minute treatment values were obtained after 30 min of oxygen or isoflurane. Preocclusion measurements were obtained after 15 min of washout.

5-HD = 5-hydroxydecanoic acid; ISO + 5-HD = isoflurane and 5-hydroxydecanoic acid.

determine the primary outcome variable of infarct size at 2 h and 2 weeks after reperfusion was determined by power analysis. The SD of infarct size was determined from historic control mice of similar strain undergoing a similar ischemia-reperfusion protocol. A sample size of eight mice per experimental group was determined assuming an  $\alpha$  of 0.05, two-tailed at 90% power with a hypothetical mean difference of 10% (StatMate; Graph-Pad Software Inc., San Diego, CA).

Statistical analyses of infarct size at 2 h and 2 weeks of reperfusion were performed by two-way analysis of variance (with time and infarct size being the two factors) followed by Bonferroni *post hoc* test. Statistical analyses of all other data were performed by one-way analysis of variance for repeated measures, followed by Bonferroni *post hoc* test or unpaired Student *t* test. All data are expressed as mean ± SD. Statistical significance was defined as  $P < 0.05$ .

## Results

### Experimental Animals

Experiments were performed in 160 mice. Thirteen mice died during or shortly after ischemia-reperfusion because of arrhythmia (control, 4; isoflurane, 1; 5-HD administration, 5; HMR-1098 administration, 3). Three animals died between 1 and 7 days after operation (control, 1; isoflurane, 2).

### Hemodynamics, Blood Gas Analyses, and Left Ventricular Weight

Table 1A shows hemodynamics (heart rate and rate-pressure product) in mice after carotid artery cannulation and exposure to oxygen or isoflurane followed by

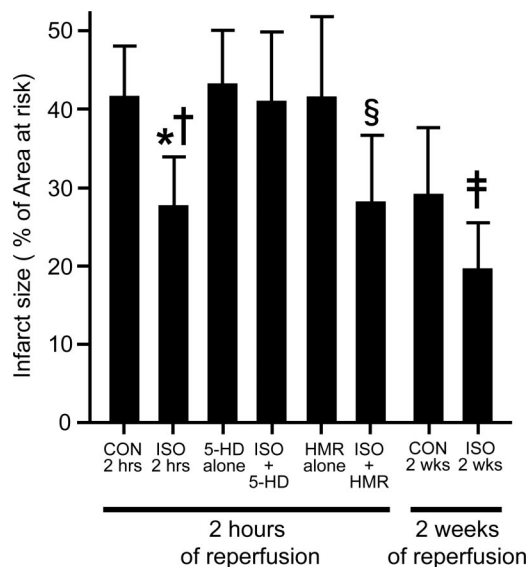
15 min of washout. No significant differences in hemodynamics were observed at any time point. Arterial pH, arterial carbon dioxide tension, and arterial oxygen tension levels were similar after exposure to oxygen or isoflurane followed by 15 min of washout ( $7.39 \pm 0.09$  vs.  $7.36 \pm 0.09$ ,  $37 \pm 8$  vs.  $35 \pm 12$  mmHg, and  $515 \pm 50$  vs.  $498 \pm 90$  mmHg, respectively).

Heart rate was determined in all mice in the ischemia-reperfusion protocols (table 1B). No significant differences in heart rate were found between groups at the preocclusion time point, during coronary artery occlusion, or at reperfusion.

Left ventricular weight was determined after ischemia-reperfusion. Left ventricular weight was significantly increased after 2 weeks of reperfusion; there were no differences in left ventricular weights between control and isoflurane groups at either 2 h ( $72 \pm 6$  and  $72 \pm 6$  mg, respectively) or 2 weeks ( $87 \pm 15$  and  $84 \pm 8$  mg, respectively) of reperfusion.

### Myocardial Area at Risk and Infarct Size

The AAR as a percent of the LV was similar among groups. Two-hour reperfusion in control mice resulted in myocardial infarction of  $41.7 \pm 6.4\%$  (n = 8) of the AAR and  $28.8 \pm 9.0\%$  (n = 11) of the AAR at 2 weeks after reperfusion. Isoflurane (1.0 MAC) reduced infarct size after both 2 h and 2 weeks of reperfusion ( $27.7 \pm 6.3\%$  [n = 8] and  $19.6 \pm 5.9\%$  [n = 11] of the AAR, respectively) as compared with control groups (fig. 2). 5-HD eliminated the protection produced by isoflurane ( $41.0 \pm 8.8\%$  [n = 9] of the AAR; fig. 2), but HMR-1098 did not abrogate APC-induced infarct size reduction ( $28.0 \pm 8.9\%$  [n = 8]; fig. 2).



**Fig. 2.** Infarct size expressed as a percentage of the area at risk. Mice underwent coronary artery occlusion receiving oxygen (CON 2 hrs), isoflurane (ISO 2 hrs), 5-hydroxydecanoic acid (5-HD alone), 5-hydroxydecanoic acid and isoflurane (ISO + 5-HD), HMR-1098 (HMR alone), and HMR-1098 and isoflurane (ISO + HMR) at 2 h of reperfusion and with and without isoflurane (CON 2 wks and ISO 2 wks, respectively) at 2 weeks of reperfusion groups. \* Significant ( $P < 0.05$ ) difference from CON 2 hrs. † Significant ( $P < 0.05$ ) difference from ISO + 5-HD. § Significant ( $P < 0.05$ ) difference from HMR alone. ‡ Significant ( $P < 0.05$ ) difference from CON 2 wks.

### Echocardiography

The results of echocardiography 2 weeks after reperfusion are given in table 2. Compared with the other groups, left ventricular internal cavity diameter in diastole and left ventricular internal cavity diameter in systole were increased significantly in the control group. However, ventricular wall thickness (anterior wall and posterior wall) and fractional shortening were similar in control and isoflurane groups.

**Table 2. Echocardiography**

	Sham-Control	Sham-Isoflurane	Control	Isoflurane
n	8	8	8	8
Awd, mm	0.65 ± 0.04	0.67 ± 0.05	0.65 ± 0.04	0.65 ± 0.04
LVIDd, mm	3.80 ± 0.32	3.77 ± 0.38	4.37 ± 0.36*	3.80 ± 0.27†
PWd, mm	0.67 ± 0.04	0.67 ± 0.05	0.68 ± 0.06	0.63 ± 0.06
AWs, mm	1.06 ± 0.06	1.07 ± 0.04	0.94 ± 0.16	0.95 ± 0.12
LVIDs, mm	2.34 ± 0.30	2.45 ± 0.35	3.04 ± 0.47*	2.48 ± 0.25†
PWs, mm	1.10 ± 0.06	1.10 ± 0.06	1.16 ± 0.08	1.08 ± 0.15
FS, %	38.7 ± 3.5	35.3 ± 4.3	32.4 ± 7.7	32.9 ± 5.1

Values are presented as mean ± SD.

\*  $P < 0.05$  control vs. sham-control. †  $P < 0.05$  control vs. isoflurane.

Awd = anterior wall thickness in diastole; AWs = anterior wall thickness in systole; FS = fractional shortening; LVIDd = left ventricular internal cavity diameter in diastole; LVIDs = left ventricular internal cavity diameter in systole; PWd = posterior wall thickness in diastole; PWs = posterior wall thickness in and systole.

### Cardiac Catheterization

Table 3 provides left ventricular pressure data measured during cardiac catheterization after 2 weeks of reperfusion. Left ventricular systolic function as determined by end-systolic pressure and maximum  $dP/dt$  were significantly increased in the isoflurane group when compared with the control group. In addition, minimum  $dP/dt$ , a measure of left ventricular diastolic function, was improved by isoflurane treatment.

### Apoptosis

Control mice showed a trend toward increased proapoptotic gene expression when compared with mice treated with isoflurane (fig. 3A). No major changes were observed for antiapoptotic genes. Cleaved caspase-3, an activated form of the protease caspase-3,<sup>22</sup> was increased significantly after 2 weeks of reperfusion compared with mice exposed to isoflurane before ischemia and 2 weeks of reperfusion (figs. 3B and C). Finally, apoptosis was assessed by TUNEL staining. The frequency of TUNEL-positive cells, expressed as the percent of total nuclei, was reduced significantly by exposure to isoflurane before ischemia and 2 weeks of reperfusion (figs. 3D and E).

### Discussion

We have confirmed previous findings in other species by showing that isoflurane acutely protects mouse myocardium against ischemia-reperfusion injury and that the mitochondrial  $K_{ATP}$  channel is involved in this acute protection.<sup>3,23</sup> This is the first demonstration of isoflurane-induced cardiac protection *in vivo* in mice, a species with significant potential to study molecular mechanisms of cardiac protection *via* genetic engineering. In addition, we show that the cardiac protection afforded by isoflurane is sustained for at least 2 weeks after ischemia-reperfusion, resulting in improved cardiac function. This is the first indication that cardioprotective agents not only limit cardiac injury but also prevent its subsequent progression.

The *in vivo* mouse model of cardiac ischemia-reperfusion was first described by Michael *et al.*<sup>24,25</sup> We chose a commonly used time frame of 30 min of ischemia.<sup>20,24,26</sup> In our control mice, after 30 min of ischemia, the infarct size as a percent of the AAR was 41.7% at 2 h of reperfusion. After 2 weeks of reperfusion, this was decreased to 28.8%. The time-dependent difference in control infarct size is likely the result of an inability of myocytes to metabolize the 2,3,5-triphenyltetrazolium chloride stain at early reperfusion times as has been suggested by other studies.<sup>27-29</sup> With a longer duration of reperfusion, the myocytes likely regain their capacity to metabolize 2,3,5-triphenyltetrazolium chloride, accounting for the reduced infarct size detected after 2

**Table 3. Cardiac Catheterization**

	Sham-Control	Sham-Isflurane	Control	Isflurane
n	8	8	8	8
ESP, mmHg	78 ± 6	79 ± 6	73 ± 9	83 ± 6†
EDP, mmHg	8 ± 2	7 ± 4	11 ± 4	9 ± 6
dP/dtmax, mmHg/s	4,880 ± 727	4,561 ± 912	3,836 ± 655*	5,087 ± 611†
dP/dtmin, mmHg/s	-4,684 ± 797	-4,762 ± 1,075	-3,582 ± 751*	-4,951 ± 608†

Values are presented as mean ± SD.

\*  $P < 0.05$  control vs. sham-control. †  $P < 0.05$  control vs. isoflurane.

EDP = end-diastolic pressure; ESP = end-systolic pressure; dP/dtmax = maximum dP/dt; dP/dtmin = minimum dP/dt.

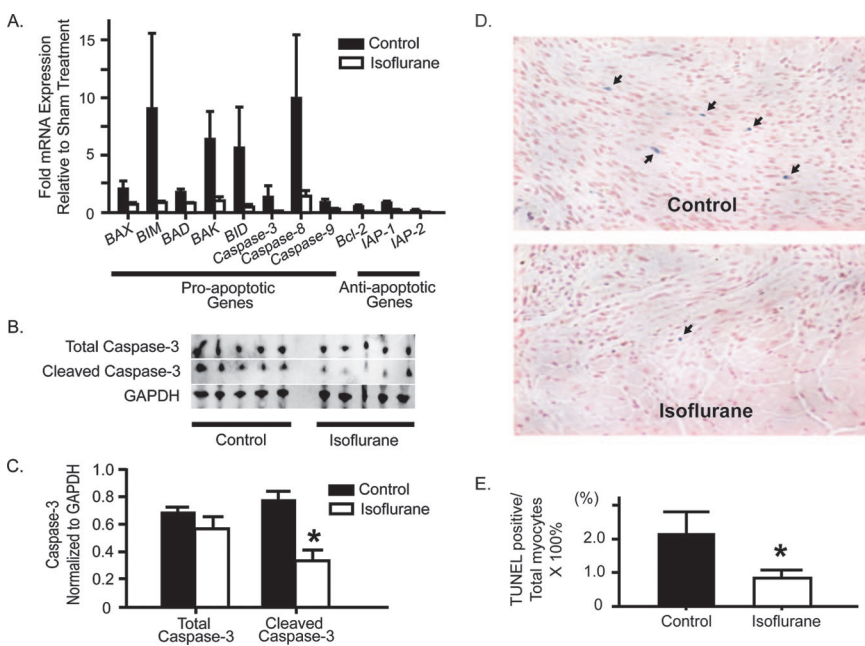
weeks of reperfusion.<sup>27-29</sup> However, when isoflurane-induced infarct size reduction is expressed as a percent of control infarct size, both 2 h and 2 weeks of reperfusion produced a similar reduction (34% and 32%, respectively), indicating that isoflurane prevented further progression of ischemia-reperfusion injury in the mouse.

We show that cardiac protection produced by isoflurane is associated with an acute memory period similar to that observed during ischemic preconditioning. In brain, isoflurane-induced reduction in cerebral infarct size observed at 2 days was no longer apparent after 2 weeks of reperfusion, indicating that isoflurane delays but does not ultimately prevent cerebral infarction.<sup>17</sup> This brings into question the utility of volatile anesthetic agents in permanently limiting ischemia-reperfusion injury. However, as shown in figure 2, isoflurane-induced cardiac protection in mice is sustained over an extended recovery period.

Since the first description of APC conferred by isoflurane against myocardial ischemia-reperfusion injury,<sup>1,2</sup>

numerous studies have attempted to characterize the mechanisms involved. These studies implicate adenosine receptors,<sup>6,7,30</sup> inhibitory guanine nucleotide binding proteins,<sup>8,31</sup> protein kinase C,<sup>7,31,32</sup> reactive oxygen species,<sup>9,33,34</sup> and  $K_{ATP}$  channels,<sup>1,23,35-37</sup> but the precise mechanism responsible for APC remains undefined. It has been shown that isoflurane preserves cardiac myocyte viability in an *in vitro* model of ischemia, and this protective effect was attenuated by 5-HD, a relatively selective mitochondrial  $K_{ATP}$  channel blocker, but not HMR-1098, the sarcolemmal  $K_{ATP}$  channel antagonist.<sup>31</sup> Opening of the mitochondrial  $K_{ATP}$  channel or a mitochondrial site of action seems to be an important mediator of APC-induced cardiac protection.<sup>3,38</sup> The current investigation confirms the importance of the mitochondrial  $K_{ATP}$  channel, but not the sarcolemmal  $K_{ATP}$  channel, in acute isoflurane-induced cardiac protection because 5-HD attenuated the protective effects.

The mechanisms by which isoflurane ultimately limits infarct size are not known. Apoptosis<sup>39-42</sup> and inflam-



**Fig. 3. Apoptosis studies.** (A) Real-time polymerase chain reaction analysis of proapoptotic and antiapoptotic gene expression in area at risk after 2 weeks of reperfusion. The data shown are representative of five independent experiments. mRNA = messenger RNA. (B) Expression of total caspase-3 and cleaved caspase-3 was visualized by Western blot analysis in 2-week reperfusion samples with and without isoflurane (n = 5 in each group). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoblot was performed to determine equal protein loading. (C) Densitometry of immunoblots in B was normalized to the GAPDH values. No differences were observed in total caspase-3 levels, but isoflurane had significantly reduced cleaved caspase-3 expression compared with controls. \* Significant ( $P < 0.05$ ) difference from control. (D) Detection of apoptotic myocytes in area at risk was determined using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) staining in 2-week reperfusion hearts in the absence or presence of pretreatment with isoflurane. Blue staining indicates TUNEL-positive cells. Magnification  $\times 400$ . (E) Nuclei staining positive for TUNEL were quantified as a percent of total nuclei. TUNEL-positive nuclei were significantly decreased in isoflurane-treated hearts as compared with control. The data shown are representative of four independent experiments. \*  $P < 0.05$  versus control.



mation<sup>42-45</sup> have been implicated in ischemia-reperfusion injury in the heart. Apoptosis has been evaluated in ischemic preconditioning at relatively short times of reperfusion<sup>46-49</sup> and in APC only at the gene level in the heart.<sup>50</sup> We found after 2 weeks of reperfusion that apoptosis in myocardium at risk of ischemia is attenuated by isoflurane exposure before ischemia and 2 weeks of reperfusion. Although histologic assessment of apoptosis may be inconclusive,<sup>51,52</sup> we showed using three different methodologies—real-time polymerase chain reaction analysis of apoptotic gene expression, cleaved caspase-3 expression, and TUNEL staining—that the increased apoptosis in control mice is abrogated by isoflurane. Gene profile showed less expression of proteins critical to the induction and progression of apoptosis, including BAX, BIM, BAD, BAK, BID, and caspase-3, -8, and -9<sup>22</sup> in isoflurane-treated mice. In addition, activation *via* cleavage of the terminal caspase (*i.e.*, caspase-3) was attenuated by isoflurane treatment. Previously, it has been shown, in agreement with our finding, that the expression of caspase-3 is reduced by APC.<sup>50</sup> It has been shown that overexpression of the interleukin-1 receptor antagonist results in cardiac protection by attenuating inflammation and is associated with reduced apoptosis.<sup>42</sup> This suggests that inflammatory components (likely released and recruited to the myocardium at reperfusion) affect necrosis and apoptosis. Volatile anesthetics have been shown to reduce ischemia-reperfusion injury by limiting inflammation in the heart.<sup>53</sup> The relative contribution of antiapoptotic and antiinflammatory mechanisms of APC in the mouse heart remain to be elucidated.

The current findings should be interpreted within the constraints of potential limitations. Hemodynamic and blood gas analysis were not performed concurrently with infarct studies; however, our hemodynamic findings in response to isoflurane were consistent with previous studies in C57B/6 mice.<sup>54</sup> Because mice have a blood volume of approximately 2 ml,<sup>55</sup> we decided to perform hemodynamic measurements in separate mice to maximize survival and produce stable baseline hemodynamics before ischemia-reperfusion. Separate hemodynamic studies resulted in no differences in blood pressure and heart rate between experimental groups at any time points. Therefore, the reductions in myocardial infarct size produced by isoflurane most likely occurred independent of hemodynamic determinants of myocardial oxygen consumption. Percent fractional shortening determined by echocardiography was not different between any group of mice studied despite significant improvements in  $dP/dt$  in mice treated with isoflurane. Because fractional shortening is determined in a single plane usually above the region of myocardial infarction, cardiac contractility determined by  $dP/dt$  may represent a more global measure of ventricular function. Although  $dP/dt_{min}$  was also improved after isoflurane, this mea-

sure may be a relatively unreliable measure of global ventricular relaxation.<sup>56</sup> In addition, our finding of sustained cardiac protection with isoflurane was determined in mice, a species weighing approximately 25 g with resting heart rates of approximately 600 beats/min. Studies in large animal models are warranted before extrapolation of our results to the clinical setting.

In summary, cardiac protection produced by isoflurane is associated with an acute memory period that is sustained for at least 2 weeks after ischemia-reperfusion. In addition, isoflurane before ischemia-reperfusion limits left ventricular dilation and improves cardiac function 2 weeks after ischemia-reperfusion in mice *in vivo*. Therefore, isoflurane may serve as a potent cardiac protective agent that not only limits reperfusion injury but more importantly limits progression. Further, myocardial ischemia-reperfusion in mice represents a novel model with significant potential for the study of molecular mechanisms of acute and sustained isoflurane-induced cardiac protection.

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