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Inhaled Sevoflurane Produces Better Delayed Myocardial Protection at 48 Versus 24 Hours After Exposure

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Ischemia preconditioning produces a delayed window of cardioprotection against subsequent ischemia and reperfusion injury. Contradictory results have been reported regarding the ability of inhaled anesthetics to produce similar effects. Our investigation was designed to test whether inhaled sevoflurane is capable of producing a delayed window of anesthetic preconditioning and to compare the differences at 24 and 48 h after exposure. Male Fischer-344 rats, 2–4 mo old, were exposed to sevoflurane (2.5% for 60 min). Twenty-four or 48 h after exposure, the hearts were isolated and perfused for 30 min (equilibration) followed by 25 min of ischemia and then 60 min of reperfusion. Control hearts received no treatment before ischemia. Left ventricular (LV) function, creatine kinase (CK), and infarct size (IS) were measured. Nuclear magnetic resonance was used to measure Na^+_i, $[Ca^{2+}]_i$, and pH_i. There was improved LV function and significant reduction in IS and CK and in both the 24- and 48-h delayed groups compared with the controls. There was also a significant recovery of LV function and reduction in IS and CK in the 48-h group when compared with the 24-h group. There was significant adenosine triphosphate preservation in both the 24- and 48-h groups, as well as a significant reduction in acidosis, $[Ca^{2+}]_I$, and Na^+_i in response to ischemia in both the groups versus the control. Sevoflurane is capable of producing a delayed window of preconditioning, and it takes more than 24 h to produce maximal protective effects.

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Preconditioning triggers endogenous defense mechanisms in tissue, rendering it resistant to irreversible injury from subsequent ischemia and reperfusion (I/R) (1). Several triggers have been identified, including multiple brief episodes of sublethal ischemia, adenosine agonists, nitric oxide donors, mitochondrial K_{ATP} channel openers, opiates, and volatile anesthetics (2,3). Preconditioning of myocardial tissue decreases infarct area, improves recovery of contractile function, and decreases the incidence of postischemic arrhythmias (4). Preconditioning can produce two temporal windows of effect. An acute window arises immediately after the stimulus and may last for up to 3 h. A delayed window arises 18–24 h after the stimulus and may last for up to 72 h (5).

Properly induced ischemia can produce a delayed window of preconditioning. However, there are few studies regarding the ability of volatile anesthetics to produce a delayed window of preconditioning in myocardial tissue, and the results of those few are conflicting (6,7). Recently, it was demonstrated that emulsified sevoflurane can produce both acute and delayed preconditioning in rabbit myocardium (8). However, there are no reported data on whether inhaled sevoflurane, at a clinically relevant concentration, can produce a delayed window of anesthetic preconditioning (APC).

The primary goal of this study was to investigate whether inhaled sevoflurane is capable of producing a delayed window of APC in rat myocardium. The secondary goal of this study was to determine if there was a difference between 24 and 48 h postexposure.

Methods

Our study was approved by the Animal Care Committee of the University of California, Davis, and all the experiments were performed within the guidelines of animal care from the National Institutes of Health (Bethesda, MD).

Male Fisher-344 rats, 2–4 mo old, were anesthetized with intraperitoneal pentobarbital (65 mg/kg) and

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heparin (1000 IU/kg) (9). Once an adequate depth of anesthesia was achieved, the hearts were then harvested and perfused using a Langendorff perfusion system at a pressure of 140 ± 20 cm H_2O at 37°C with modified Krebs-Henseleit bicarbonate solution equilibrated with humidified 95%O_2/5%CO_2 to produce a pH value of 7.40 ± 0.05. The perfusate consisted of 127 mM of NaCl, 4.75 mM of KCl, 1.25 mM of $MgCl_2$, 2.5 mM of $CaCl_2$, 2.5 mM of $NaHCO_3$, and 10 mM of glucose. A balloon pressure catheter was placed in the left ventricle (LV), and left ventricular end-diastolic pressure (LVEDP) was set to 10 cm H_2O. The hearts were paced at 5 Hz. Global ischemia was achieved by turning off the perfusion to the hearts. Once the hearts were properly prepared, they were all allowed a 30-min perfusion period for equilibration followed by 25 min of global ischemia. Pacing was stopped during the ischemic period. After ischemia, the hearts were reperfused and paced for 60 min.

APC rats were exposed to 2.5% sevoflurane delivered via a Sevotec5 variable bypass vaporizer (Datex-Ohmeda, Milwaukee, WI) for 1 h. The respiratory rate, O_2 concentration, and end-tidal CO_2 were monitored during the exposure and arterial blood gas tension analysis performed (iSTAT, Princeton, NJ) at the end of exposure to ensure that there was no hypoxia or hypercarbia (7). Delivered and exhaled gas concentrations were confirmed with an Ohmeda Rascal II (Ohmeda, Salt Lake City, UT) anesthetic drug monitor. After exposure, the rats were recovered and returned to their cages. Rats were randomly divided in one of three experimental groups: control, 24-h delayed APC, and 48-h delayed APC. In the 24-h group, the hearts were prepared and perfused 24 h after the sevoflurane exposure. In the 48-h group, the hearts were prepared and perfused 48 h after exposure. Control rats received no sevoflurane exposure.

At the end of reperfusion, the hearts were sliced into 2-mm sections. They were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) and incubated for 20 min at 37°C. After incubation, the slices were rinsed with H_2O and their images scanned into a computer using Adobe Photoshop software (Adobe, San Jose, CA). NIH image 1.62 (National Institutes of Health) was used to measure the percent area of infarction. Infarcted myocardium is unable to reduce TTC and appears pale compared with viable myocardium, which appears red secondary to the reduction of TTC. The total areas of infarct were divided by the total area of myocardium to determine the percent area of infarction (10).

A water-filled balloon connected to a pressure transducer (Medex, Dublin, CA) was placed in the LV. LVEDP was adjusted to approximately 10 cm H_2O at the beginning of the equilibrium phase of perfusion to insure that all hearts had equal preload. LV pressures were continuously recorded with Powerlab 4/20 (AD-Instruments, Colorado Springs, CO). The variables of LV performance were LV-developed pressure (LVDP), calculated as LV systolic pressure minus LVEDP for systolic function and LVEDP as a measurement of diastolic dysfunction (10).

Creatine kinase (CK) was used as a measure of myocardial injury. Coronary sinus effluent was collected in 10-min aliquots starting in the equilibrium phase and continuing throughout the 60 min of reperfusion. CK was determined using the CK-10 kit (Sigma Diagnostics, St. Louis, MO) and Shimadzu UV-VIS photospectrometer (Shimadzu, Columbia, MD) and expressed as Unit per gram of dry weight (10).

To measure $[Ca^{2+}]_I$, hearts were loaded for 30 min with perfusate containing the acetoxymethyl ester of 5F-1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (FBAPTA) at 2.5 μM during equilibration. FBAPTA was washed out of the extracellular space with control solution before measurement of $[Ca^{2+}]_i$. ^{19}F spectra were generated from the summed-free induction decays of 1500, 45-degree excitation pulses using 2000 word data files, and ±5000 Hz sweep width. $[Ca^{2+}]_i$ was calculated by using $[Ca^{2+}]_i$ = Kd [bound]/[free] where the ratio of Ca^{2+}-bound and -free FBAPTA concentrations was equal to the ratio of their corresponding peak areas, and Kd is 300 nM in our experiments (10).

^{31}P spectra were generated from the summed-free induction decays of 148, 60-degree excitation pulses using 4000 word data files, and ±4000 Hz sweep width. The pH_i value was calculated from the shift in inorganic phosphorus (Pi) resonance calibrated at 37°C with reference to control phosphocreatine (PCr). High-energy phosphates were determined as percent of control peak intensity (10).

To measure Na_i, 7.5 mM of dysprosium triethylenetetraminehexaacetic acid was substituted isoosmotically for NaCl in the perfusate, and Ca^{2+} was added to reach a perfusate concentration of 2.5 mM, as measured by calcium electrode. ^{23}Na spectra were generated from the summed-free induction decays of 1000 with excitation pulses at 90 degrees using 2000 word data files and ±4000 Hz sweep widths. Na_i (mEq/kg of dry weight) was calculated from the calibrated area under the unshifted peak of the ^{23}Na spectra after subtracting the extracellular peak. At the end of each experiment, the hearts were weighed wet and dried (at least 48 h) at 65°C to determine the dry weight (10).

Animals were randomly assigned to each group (n = 6 in each group); data are presented as mean ± SD. Analysis of variance for repeated measures was used to test the differences among groups. Two-tailed Student-Newman-Keuls posttest was used if the analysis of variance was significant. A value of $P < 0.05$ was considered statistically significant.

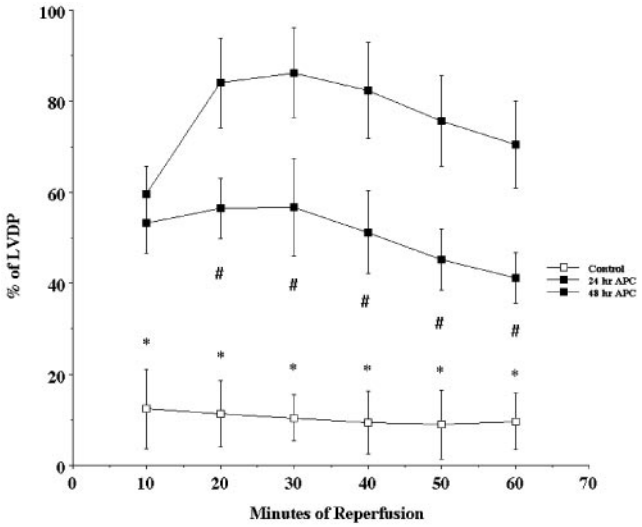

Figure 1. The recovery of left ventricular developed pressure (LVDP) during reperfusion. The LVDP was measured throughout reperfusion as a percentage compared with the baseline LVDP during the equilibration phase before ischemia. Both the 24-h (closed square) and 48-h (closed triangle) anesthetic preconditioning (APC) groups had significant ($P < 0.05$) recovery of LVDP versus control (open square). There was also a greater recovery of LVDP in the 48-h delayed APC group versus 24-h delayed APC group ($P < 0.05$). *Twenty-four-hour and 48-h delayed APC versus control ($P < 0.05$); #24 h versus 48 h ($P < 0.05$). Data presented as mean ± SD; $n = 6$ in each group.

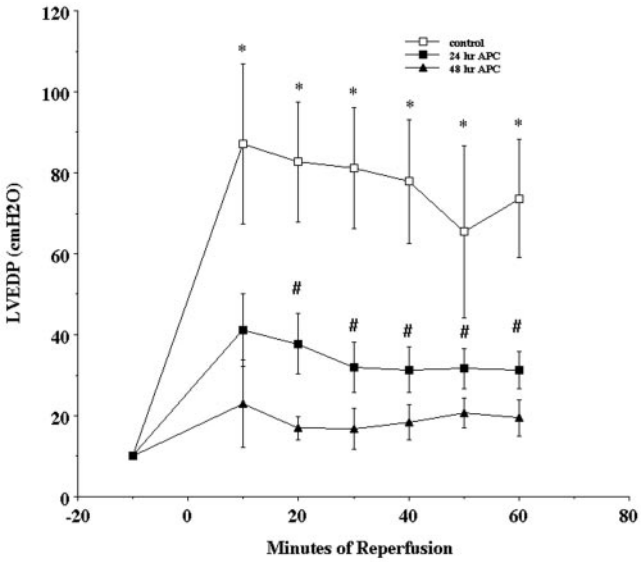

Figure 2. Results of left ventricular end-diastolic pressure (LVEDP) during 60 min of reperfusion. Units are in cm H_2O. There were significantly lower diastolic pressures in both 24-h (closed square) and 48-h (closed triangle) delayed APC groups versus control (open square) ($P < 0.05$). There was also significantly lower diastolic pressure in the 48-h delayed APC versus the 24-h delayed APC group ($P < 0.05$). All hearts were preloaded at 10 cm H_2O diastolic baseline pressure before ischemia. *Twenty-four-hour and 48-h delayed APC versus control ($P < 0.05$); #24 h versus 48 h ($P < 0.05$). Data presented as mean ± SD; $n = 6$ in each group.

Results

The 24-h delayed APC group had a significant decrease in infarct size (IS) versus control (15 ± 3 versus 31 ± 6; $P < 0.05$). The 48-h delayed APC group also had a significant decrease in IS versus control (10 ± 2 versus 31 ± 6; $P < 0.05$). There was also a significant decrease in IS in the 48-h delayed APC group versus the 24-h delayed APC group (10 ± 2 versus 15 ± 3; $P < 0.05$).

The recovery of LV performance as quantified at the end of 60 min of reperfusion versus the baseline phase before ischemia is summarized in Figure 1. The percentage recovery of LVDP in the 24-h delayed APC group was significantly improved versus control (41 ± 6 versus 10 ± 3; $P < 0.05$). The percentage recovery of LVDP in the 48-h delayed APC group was significantly improved 70 ± 9 versus control (10 ± 3; $P < 0.05$). There was also a significant recovery of LVDP in the 48-h group versus the 24-h delayed APC group ($P < 0.05$). In Figure 2, the development of diastolic dysfunction was significantly decreased in the 24- and 48-h delayed APC groups versus control (LVEDP; 31 ± 5 cm H_2O in the 24-h delayed APC group and 19 ± 5 cm H_2O in 48-h delayed APC group versus 74 ± 15 of control; $P < 0.05$). Figure 2 also demonstrates significantly less diastolic dysfunction in the 48-h delayed APC group versus the 24-h delayed APC group (19 ± 5 versus 31 ± 5; $P < 0.05$).

The results of total CK released as an indicator of myocardial injury during 60 min reperfusion are expressed as international units per gram of dry weight. Twenty-four-hour delayed APC hearts released significantly less CK than those of control rats (541 ± 22 versus 1429 ± 399; $P < 0.05$). Forty-eight-hour delayed APC hearts released significantly less CK (178 ± 45) than control hearts ($P < 0.05$), and 48-h delayed APC hearts released significantly less CK than 24-h delayed APC rats ($P < 0.05$).

Ischemia caused a significant decrease in adenosine triphosphate (ATP) in all groups (Fig. 3). APC hearts had better ATP preservation at the end of ischemia (22% ± 1% versus 36 ± 3%; 24-h group versus 48-h group, respectively) and at the end of reperfusion (32% ± 3% versus 32 ± 5%; 24-h group versus 48-h group, respectively) when compared with the control group (6% ± 4% at the end of ischemia, and 22% ± 4% at the end of reperfusion). In the 48-h group, the ATP level was higher at the end of ischemia compared with the 24-h group ($P < 0.05$), but there were no differences at the end reperfusion ($P > 0.05$). Similarly, PCr, a substrate of ATP synthesis, recovered better in both the 24- and 48-h groups (48% ± 4% versus 63% ± 2%) compared to the control group (32% ± 11%; $P < 0.05$) at the end of reperfusion, and the 48-h delayed APC group recovered better than the 24-h group ($P < 0.05$). Pi, a product of ATP breakdown, was significantly lower at the end of ischemia in both the 24- and 48-h

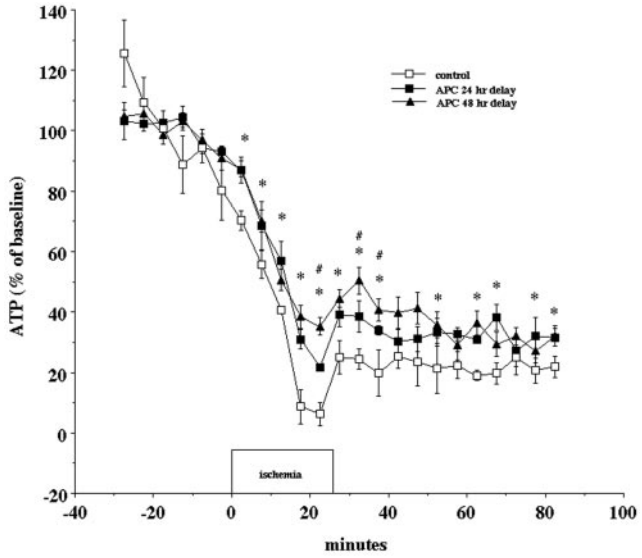

Figure 3. Ischemia decreased adenosine triphosphate (ATP) levels during the experiment. The ATP was better preserved in anesthetic preconditioning (APC) hearts than control hearts (open square) during ischemia and early reperfusion. In the delayed APC hearts, the 48-h group (closed triangle) had a higher ATP level at the end of ischemia and early reperfusion compared with the 24-h group (closed square) and no differences at the end of reperfusion. *Twenty-four-hour and 48-h delayed APC versus control ($P < 0.05$); #24 h versus 48 h ($P < 0.05$). Data presented as mean ± SD; $n = 6$ in each group.

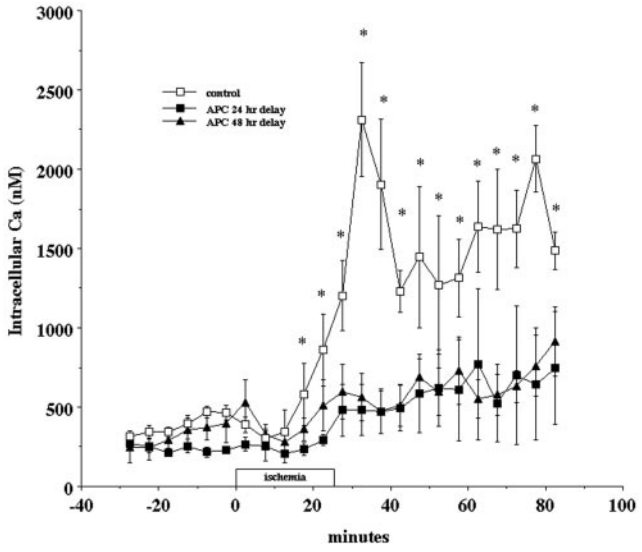

Figure 4. Ischemia causes an increase in $[Ca^{2+}]_i$ in control hearts (open square). Both 24-h (closed square) and 48-h (closed triangle) delayed anesthetic preconditioning (APC) groups limited the increases in $[Ca^{2+}]_i$ during ischemia and reperfusion (I/R) ($P < 0.05$). But there were no differences between the 24-h and 48-h groups ($P > 0.05$). *Twenty-four-hour and 48-h delayed APC versus control ($P < 0.05$). Data presented as mean ± SD; $n = 6$ in each group.

groups (597% ± 54% and 554% ± 48%) when compared to the control group (779% ± 45%; $P < 0.05$), but there were no significant differences between the 24- and 48-h delayed APC groups ($P > 0.05$).

I/R caused increases in intracellular $[Ca^{2+}]_i$ during I/R in all groups (Fig. 4). In the control hearts, $[Ca^{2+}]_i$ increased from 333 ± 33 nM of baseline to 860 ± 227 nM at the end of ischemia and to 1487 ± 117 nM at the end of reperfusion ($P < 0.05$). APC attenuated the increase in $[Ca^{2+}]_i$ in both delayed APC groups (294 ± 134 nM at the end of ischemia and 746 ± 354 nM at the end of reperfusion in the 24-h group; 511 ± 162 nM at the end of ischemia and 914 ± 220 nM at the end of reperfusion in the 48-h group). The decreases in $[Ca^{2+}]_i$ in both the 24-h and 48-h groups were significant when compared with the control group ($P < 0.05$), but there were no statistical differences between the 24-h and 48-h groups.

Ischemia caused a significant increase in Na_i (from 29 ± 4 at baseline to 120 ± 6 mEq/gm of dry weight at the end of ischemia; $P < 0.05$) in control hearts (Fig. 5). APC blunted the increase in Na_i during ischemia in both the 24-h and 48-h groups, and the Na_i was lower in the 48-h group than the 24-h group (79 ± 3 versus 86 ± 2 mEq/gm of dry weight; $P < 0.05$) at the end of ischemia. There were no statistical differences among the three groups during reperfusion.

At the end of the 25-min ischemia, the pH_i value decreased in both the control (5.91 ± 0.06) and the

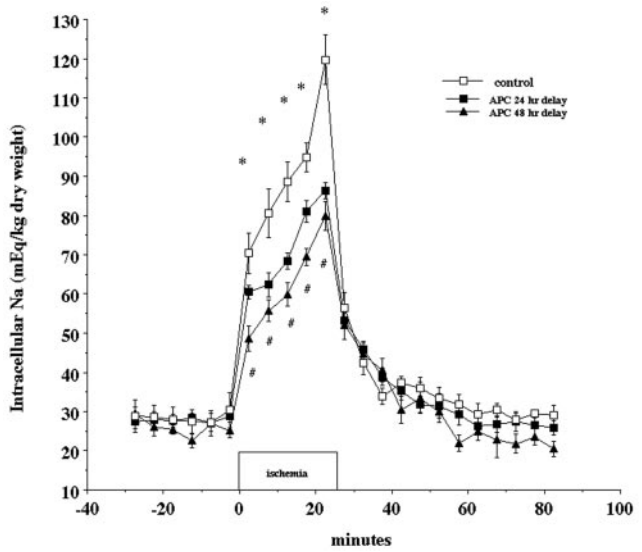

Figure 5. Ischemia caused increases in Na^+_i compared with baseline in control hearts (open square) and the 24-h (closed square) and 48-h (closed triangle) delayed anesthetic preconditioning (APC) groups during ischemia. However, the increases in Na^+_i were limited in both the delayed APC groups. Forty-eight-hour delayed APC hearts had less Na^+_i than that of the 24-h delayed APC hearts ($P < 0.05$) during ischemia. *Twenty-four-hour and 48-h delayed APC versus control ($P < 0.05$); #24 h versus 48 hr ($P < 0.05$). Data presented as mean ± SD; $n = 6$ in each group.

APC groups (24-h, 6.20 ± 0.15; 48-h, 6.23 ± 0.06) (Fig. 6). There was a statistical difference between the control group and APC groups ($P < 0.05$). There was no difference between the 24-h and 48-h groups at the end of ischemia. There were no statistical differences

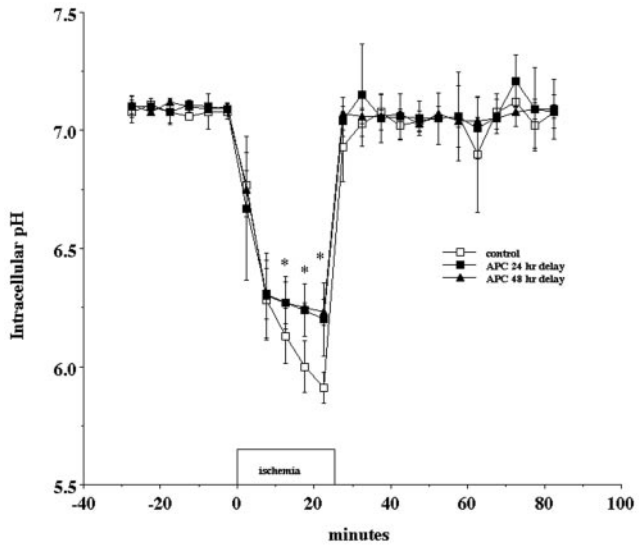

Figure 6. Ischemia caused decreases in pH_i values compared with baseline in the control group (open square) and both the 24-h (closed square) and 48-h (closed triangle) delayed APC groups during ischemia and reperfusion (I/R). There was a statistical difference between the control group and the delayed APC groups during ischemia ($P < 0.05$). There were no statistical differences among the three groups during reperfusion. There was no statistical difference between the 24-h and 48-h delayed APC groups ($P > 0.05$). *Twenty-four-hour and 48-h delayed APC versus control ($P < 0.05$). Data presented as mean ± SD; $n = 6$ in each group.

in the recovery of pH_i after 60 min of reperfusion in the control group (7.08 ± 0.07) and both delayed APC groups (24-h, 7.09 ± 0.13; 48-h, 7.08 ± 0.03).

Discussion

APC offers the exciting possibility of improving patient outcomes with drugs used in everyday practice. This may be of particular importance in patients undergoing coronary artery surgery, as well as those with known coronary artery disease for noncardiac surgery. However, many obstacles to the routine clinical use of ischemia preconditioning have become apparent because research in this area has progressed. Our data support the hypothesis that volatile anesthetics can produce a delayed window of preconditioning and also demonstrate the increased protective effect of APC with sevoflurane from 24 hours to 48 hours after the exposure to the anesthetic.

Reduction in the area of infarction is considered as the "gold standard" in the determination the effectiveness of myocardial preconditioning (11). We demonstrated not only a significant reduction in infarct area with the 24-hour delayed APC group versus control, but also a significant reduction in IS area when comparing the 48-hour delayed APC group to the 24-hour group. There was also a significant recovery of LV function in the 24-hour delayed APC group versus control and a significant improvement in the recovery of LV function in the 48-hour delayed APC versus the 24-hour group, as manifested by improved LVDP and decreased diastolic dysfunction. CK releases were decreased in the 48-hour delayed APC group when compared with the 24-hour group. This provides additional evidence that it takes more than 24 hours to develop the maximal protective effect.

Delayed APC significantly blunted the intracellular Ca^{2+} increase during I/R. This can be explained by the activity of Na^+/H^+ exchanger (NHE) located in the cell membrane. Ischemia results in intracellular acidosis that activates the NHE to maintain the pH_i and further acts to increase Na^+ influx intracellularly via the NHE. This sodium influx acts to increase $[Ca^{2+}]_i$ concentration by two main mechanisms. Increased Na^+_i concentration decreases the resting membrane potential opening voltage-gated Ca^{2+} channels and directly driving Ca^{2+} intracellularly via the Na^+/Ca^{2+} exchanger (12). Ca^{2+} is a tightly regulated second messenger of cell metabolism. Intracellular Ca^{2+} overload and the subsequent increase in cell metabolism in an oxygen-depleted environment, as well as mitochondria disruption, are among the mechanisms of cell injury associated with ischemia (13). Acute preconditioning acts via multiple complex signaling mechanisms that are under active investigation to reduce Ca^{2+} overload by activating protein kinase C, priming and opening mitochondrial and sarcolemmal ATP-regulated K-channels, and attenuation of NFκB activation (9,14–18). Although there are limited publications on the delayed window of preconditioning, the mechanisms involved could include the cyclooxygenase, protein kinase C, K-ATP channels, further down-regulation of gene transcriptions, and eventually decreases in Ca^{2+} overload and cell death (7,18). Intracellular Ca^{2+} and Na^+ levels in response to ischemia were reduced in both delayed APC groups compared with the control. We were not able to distinguish a difference in the Ca^{2+} reduction between the 24-hour and 48-hour delayed APC groups. However, we were able to show a further significant decrease in Na_i concentrations in the 48-hour delayed APC group versus the 24-hour group.

There was also significant blunting of the decrease in pH_i associated with ischemia in both of the APC groups as compared with the control. We were unable to detect a significant difference between the 24-hour and 48-hour delayed APC groups in the blunting of the pH_i decrease at the end of ischemia, but this was not unexpected, because there are a number of intracellular housekeeping mechanisms to manage acidosis that may not be altered by APC (19,20).

I/R injury profoundly disrupts mitochondrial energy production capabilities, and numerous studies have shown that mitochondria isolated from hearts

after I/R manifest reduced function, decreased membrane potential, and decreased impairment of cell respiration (21,22). Myocardial Ca^{2+}-dependent processes are highly dependent on ATP production. Decreased $[Ca^{2+}]_i$ has been reported to reduce depletion of myocardial ATP in both newborn and adult hearts (22,23). APC has been reported to preserve high-energy phosphates, and we hypothesized that APC could preserve ATP levels during I/R as a result of the relative decrease in intracellular $[Ca^{2+}]_i$ and therefore mitochondrial $[Ca^{2+}]_m$ (24). In particular, a decrease in $[Ca^{2+}]_m$ and $[Ca^{2+}]_i$ accumulation during I/R would decrease Ca^{2+}-dependent energy-consuming processes (e.g., Na^+/K^+ ATPase and Ca^{2+}-ATPase) in the cytosol and mitochondria (25,26). In our study, ATP metabolism during and after ischemia was preserved to a larger extent in the APC groups than the control. ATP levels, as well as PCr, recovered to a larger extent in the APC groups after I/R. There was also a decrease of Pi, the breakdown product of ATP metabolism, in the APC groups versus the control. Both are indirect evidence of an APC effect.

We were able to demonstrate evidence of improved protection by APC with an increased time from 24 to 48 hours after sevoflurane exposure. Reduced percent area of infarction, improved recovery LV function, and decreased CK levels were all demonstrated to a larger extent in the 48-hour delayed APC group more than the 24-hour delayed APC group. This may illustrate the vital role of the increased production of a wide variety of cytoprotective proteins, such as heat shock proteins, as well as the down-regulation of pro-apoptotic protein families, such as the Bcl-x family, in delayed APC. Sergeev et al. (27) demonstrated an acute profound change in myocardial cell phenotype in response to ischemia and exposure to isoflurane. However, Sergeev et al. (27) did not look at how long this change in phenotype lasted. The fact that we demonstrated a better protection at 48 versus 24 hours after exposure implies that it requires a certain period of time to express more proteins from certain up- and down-regulated genes. The data suggest that there could be an optimal time window for APC.

There were several limitations in this study. We only used inhaled sevoflurane, and we do not know whether all the volatile anesthetics are equal in efficacy concerning APC. Second, the NMR we used was unable to measure the $[Ca^{2+}]_m$. Because APC may act at the mitochondrial level, this could be an important measurement. However, numerous studies have reported that increased $[Ca^{2+}]_i$ has a positive correlation with increased $[Ca^{2+}]_m$. Third, the three nuclei measured with NMR were in three separate groups of hearts. Fourth, although we were unable to separate the differences between the 24- and 48-hour groups in $[Ca^{2+}]_i$, Pi, and PCr, our results demonstrated significant differences between control hearts and APC hearts. This could mean that the NMR method is unable to detect the differences at lower levels.

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