Delayed cardioprotection with isoflurane: role of reactive oxygen and nitrogen

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Experimental animals. Male Sprague-Dawley rats (Rattus norvegicus) at 8–9 wk of age maintained on a standard diet were used for these studies. Rats used in this study received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revised 1996).

Drugs and chemicals. Isoflurane was purchased from Abbott Laboratories (Abbott Park, IL). Manganese (III) tetrakis (4-benzoic acid)porphyrin chloride (MnTBAP), a superoxide scavenger (15 mg/kg ip), or N5-nitro-L-arginine methyl ester (L-NAME), a general nitric oxide synthase inhibitor (15 mg/kg ip), 15 min before isoflurane treatment abolished the delayed cardioprotective effects of isoflurane. MnTBAP and L-NAME had no effect on delayed cardioprotection in untreated hearts. Perfusion of isolated hearts with hydroethidine, a fluorescent probe for superoxide, after isoflurane treatment resulted in a twofold increase in ethidine staining of isoflurane-treated hearts compared with untreated controls, which was attenuated by myxothiazol, an inhibitor of the mitochondrial electron transport chain (0.2 mg/kg ip) and L-NAME (15 mg/kg ip). Nitrite and nitrate content in isoflurane-treated hearts was 1.5-fold higher than in untreated hearts, whereas myocardial reduced glutathione levels were decreased by 13% in 0.8% but not in 1.0% isoflurane-treated hearts. We conclude that isoflurane confers delayed cardioprotection in the adult rat, triggered by ROS and RNS.

VOLATILE ANESTHETICS immediately protect the heart against the deleterious effects of ischemia (13, 14, 37). The volatile anesthetic isoflurane confers immediate cardioprotection against stunning (45) and infarction (21). This immediate protection against myocardial ischemia by volatile anesthetics is equivalent in magnitude to that conferred by ischemic preconditioning. The observation that volatile anesthetics protect ischemic myocardium may be clinically important for patients with coronary artery disease. Indeed, clinical data support the concept that patients with coronary artery disease undergoing cardiac surgery benefit from the cardioprotective actions of these drugs (5, 33, 41).

Recently, we showed that the volatile anesthetic isoflurane conferred delayed cardioprotection in infant rabbits (42). Therefore, previous exposure to a volatile anesthetic can protect against perioperative myocardial infarction that may occur after the first 24 h after anesthesia and surgery. However, the underlying mechanisms responsible for increasing resistance to ischemia and whether delayed cardioprotection by isoflurane is independent of age and species remain unknown. Reactive oxygen species (ROS) derived from superoxide can act to initiate the synthesis of cardioprotective genes that protect the heart against subsequent ischemia (10). Mitochondria are one cellular source of superoxide. Reactive nitrogen species (RNS) such as nitric oxide (NO) generated by NO synthase (NOS) also act as a trigger for delayed cardioprotection (7, 8). Furthermore, NOS is also a cellular source of the superoxide anion under normal physiological conditions (36). We therefore reasoned that both ROS and RNS generated in hearts treated with isoflurane might act as triggers for delayed cardioprotection with isoflurane. The objectives of our study were to determine 1) the concentration of isoflurane that confers delayed cardioprotection in the adult rat, 2) the role of ROS and RNS in the induction of delayed cardioprotection, and 3) the cellular sources of ROS and RNS responsible for the induction of delayed cardioprotection by isoflurane.

MATERIALS AND METHODS

Experimental animals. Male Sprague-Dawley rats (Rattus norvegicus) at 8–9 wk of age maintained on a standard diet were used for these studies. Rats used in this study received humane care in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Revised 1996).

Drugs and chemicals. Isoflurane was purchased from Abbott Laboratories (Abbott Park, IL). Manganese (III) tetrakis (4-benzoic acid)porphyrin chloride (MnTBAP), a superoxide scavenger (12), was from AG Scientific (San Diego, CA). N5-nitro-L-arginine methyl ester (L-NAME), a general NOS inhibitor (34); myxothiazol, an inhibitor of complex III in the mitochondrial electron transport chain (39); hydroethidine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB); diethylenetriaminepentaacetic acid (DTPA); EDTA; 5-sulfosalicylic acid (SSA); NADPH; glutathione reductase; and 2,3,5-triphenyltetrazolium chloride (TTC) were all from Sigma (St. Louis, MO). Polyclonal rabbit anti-NOS1 was obtained from Transduction Labs (catalog no. 610310). Polyclonal rabbit anti-NOS2 and -NOS3 were obtained from Santa Cruz Biotechnology (catalog nos. sc-650 and sc-654). Poly-
clonal rabbit anti-Mn superoxide dismutase (SOD) was from Upstate (catalog no. 06-984, Waltham, MA), and the antibody for polyclonal sheep Cu-Zn SOD antibody was from CalBiochem (catalog no. 57-4597, San Diego, CA).

**Isolated heart perfusion.** Isolated rat hearts were perfused in the Langendorff mode at 37°C at a constant perfusion pressure equal to 108 mmHg. The standard perfusate was modified Krebs-Henseleit bicarbonate buffer, containing (in mM): 118.5 NaCl, 25.0 NaHCO3, 4.8 KCl, 0.6 MgSO4, 1.2 H2O2, and 1.2 KH2PO4 (pH 7.4 when gassed with 95% O2-5% CO2), in which the calcium content was reduced to 1.8 mM. Glucose (11.1 mM) was added to the perfusate. Before use, all perfusion fluids were filtered through cellulose acetate membranes with a pore size of 5.0 μm to remove particulate matter. Hearts were immersed in thermostatically controlled glass chambers to maintain the myocardial temperature at 37°C. A water-filled latex balloon was positioned in the cavity of the left ventricle attached to a pressure transducer via a rigid water-filled catheter and inflated so that the end-diastolic pressure was 5 mmHg (4).

**Delayed cardioprotection with isoflurane.** We performed the following experiments in random order to determine whether isoflurane induces delayed cardioprotection in the adult rat. Rats (n = 8 rats/group) were exposed to 0.5, 0.8, 1.0, or 2.0% (vol/vol) isoflurane-100% O2 for 2 h. Rats exposed to 2 h of 100% O2 served as untreated controls. The ambient oxygen levels during isoflurane exposure were supplemented with 100% O2 to counter any possible anesthetic-induced respiratory and cardiac depression. The control group was also supplemented with 100% O2. Thus both isoflurane and control groups were treated with oxygen in an identical manner. The animals were then allowed to recover in room air (21% O2). Twenty-four hours later, delayed cardioprotection was determined using the isolated perfused heart model. A three-way tap, located immediately above the site of cannulation, allowed the entire coronary perfusate to be diverted away from the heart to produce global, no-flow ischemia. Reperfusion was achieved by repositioning the tap to allow perfusate to be delivered to the heart. Hearts were subjected to 25-min global ischemia followed by 3-h reperfusion. Recovery of left ventricular developed pressure (LVDP) at 1-h reperfusion and infarct size/area at risk (percentage of left ventricle) at 3-h reperfusion were used to assess resistance to ischemia (42).

**Recovery of hemodynamic function and infarct size determination.** All hearts that were entered into the study were included in the analysis. Recovery of developed pressure was expressed as a percentage of their preischemic drug values. After 3-h reperfusion, 1.0% (wt/vol) TTC was delivered to the isolated heart via a syringe attached to the three-way stopcock. The latex balloon was removed from the left ventricle before the hearts were stained. TTC was administered at a rate of 1 ml/min for 10 min. Hearts were then rapidly removed from the perfusion apparatus and sliced along the long axis of the left ventricle from apex to base into 1-mm-thick transverse sections, transferred into a vial containing 10% formaldehyde, and stored at 4°C for 24 h. In the globally ischemic heart, the entire ventricle is at risk of infarction and therefore measurement of collateral flow and the area at risk is not required. The infarcted tissue was then separated from the noninfarcted tissue and measured as a percent (% infarct = infarct area/area at risk) × 100 using a digital color camera with a 55-mm lens (Nikon). Imaging analysis software (Imaging Research; St. Catharines, Ontario, Canada) was programmed to recognize infarcted tissue in proportion to the entire left ventricle.

**Role of ROS.** To determine whether delayed cardioprotection by isoflurane is induced by RNS, rats (n = 8 rats/group) were treated in random order with MnTBAP (15 mg/kg ip) or physiological saline vehicle for 15 min before 2-h treatment with 0.8% (vol/vol) isoflurane-100% O2 or 100% O2 alone. After this treatment, isolated hearts (n = 3 hearts/group) were perfused for 20 min with bicarbonate buffer containing hydroethidine (10 μM) to detect superoxide generation. At the end of the perfusion, hearts were frozen in optimal cutting temperature compound and sectioned. Ten-micrometer frozen sections were cut and thaw mounted on slides. A coverslip was applied to the sections on the slides, and images were obtained with a Nikon E600 microscope equipped with epifluorescence (excitation wavelength 488 nm, emission wavelength 610 nm) and a digital camera. The fluorescent intensity of nuclei in 40 cells from each heart was measured, corrected for background fluorescence in nonnuclear regions using MetaMorph software, and expressed as means ± SD (in arbitrary units of fluorescence).

**Role of RNS.** To determine whether delayed cardioprotection by isoflurane is induced by RNS, rats (n = 8 rats/group) were treated with L-NAME (15 mg/kg ip) dissolved in physiological saline vehicle 15 min before 2-h treatment with 0.8% (vol/vol) isoflurane-100% O2 or 100% O2 alone. After this treatment, isolated hearts (n = 3 hearts/group) were perfused for 20 min with bicarbonate buffer containing hydroethidine (10 μM) to detect superoxide generation. At the end of the perfusion, hearts were frozen in optimal cutting temperature compound and sectioned. Ten-micrometer frozen sections were cut and thaw mounted on slides. A coverslip was applied to the sections on the slides, and images were obtained with a Nikon E600 microscope equipped with epifluorescence (excitation wavelength 488 nm, emission wavelength 610 nm) and a digital camera. The fluorescent intensity of nuclei in 40 cells from each heart was measured, corrected for background fluorescence in nonnuclear regions using MetaMorph software, and expressed as means ± SD (in arbitrary units of fluorescence).

**Arterial blood gas and buffer measurements.** An indwelling catheter was inserted in the femoral artery, tunneled subcutaneously, and exteriorized at the back of the neck as described previously (27), and the animals were allowed to recover for 48 h before the study. Rats were then exposed to room air (21% O2), 100% oxygen alone, or 0.8% (vol/vol) isoflurane-100% O2 for 2 h, and arterial blood was withdrawn for gas and buffer analysis.

**Blood isoflurane concentration.** One milliliter of blood was withdrawn after 2-h exposure to 0.8% (vol/vol) isoflurane-100% O2. The isoflurane concentration in the blood was then determined by gas chromatography using flame ionization detection in the analytic core laboratory of the Department of Anesthesiology.
network was perfused via the cannulated aorta with cold (4°C) bicarbonate buffer to wash out blood. The free wall of left ventricle was excised and frozen in liquid nitrogen. The tissue was pulverized in a stainless steel mortar and pestle precooled with liquid nitrogen. The pulverized tissue was then homogenized in 5% SSA containing 1 mM DTPA (5 ml SSA/g tissue) and then centrifuged for 10 min at 5,000 g at 4°C. The supernatant was removed and kept frozen in liquid nitrogen. The thawed supernatant was processed for GSH/GSSG analysis using a recycling assay. An aliquot (10 μl) of the supernatant and 15 μl SSA was added to a reaction mixture of 700 μl of 0.3 mM NADPH (in 0.125 M phosphate-6.3 mM EDTA; pH 7.5), 100 μl of 6 mM DTNB (in the same buffer), and 175 μl distilled water kept at 30°C in the cuvette of spectrophotometer. The assay was initiated with 10 μl of 185 U/ml glutathione reductase. The absorbance at 412 nm was continuously recorded for 60 s. The total GSH level was established from the initial rate, and quantification was based on responses obtained with a GSH standard. For GSSG determination, 2 μl 2-vinylpyridine was added to 100 μl supernatant, and the pH was adjusted to 6–7 by adding triethanolamine. After 1-h incubation at room temperature, a 25-μl aliquot was added to a reaction mixture of 700 μl of 0.3 mM NADPH (in 0.125 M phosphate-6.3 mM EDTA; pH 7.5), 100 μl of 6 mM DTNB (in the same buffer), and 175 μl distilled water maintained at 30°C in the cuvette of spectrophotometer, and the assay was completed as described above. GSSG levels were quantified based on responses of standards. GSH levels were calculated as the difference of total GSH and GSSG.

Statistics. Data are expressed as means ± SD. Statistical analysis was performed with the use of repeated-measures ANOVA with the Greenhouse-Geisser adjustment used to correct for the inflated risk of a type I error (3), and, where this proved significant, the Mann-Whitney test was used as a second step to identify which groups were significantly different. After ANOVA, the data were reanalyzed for differences related to multiple comparisons (3). Significance was accepted at a level of P < 0.05.

RESULTS

Delayed cardioprotection studies. Rats (n = 8 rats/group) were exposed to 0.5, 0.8, 1, or 2% (vol/vol) isoflurane-100% oxygen for 2 h. Rats exposed for 2 h to 100% oxygen served as untreated controls. Rats regained sternal recumbency within 10 min after discontinuation of 2 h of treatment with isoflurane. We visually monitored for signs of respiratory distress, including changes in rate and/or depth of respirations and any increased work of breathing. None were noted. Twenty-four hours later, delayed cardioprotection was determined using an isolated perfused heart model. Aerobic cardiac function before ischemia was unaffected by isoflurane treatment (0.5, 0.8, 1.0, or 2.0% (vol/vol)) 24 h previously compared with untreated controls (heart rate = 258 ± 10 beats/min; coronary flow rate = 9 ± 1 ml/min-1·g-1; LVDP = 117 ± 5 mmHg). Thus there were no changes in preischemic function as a consequence of isoflurane treatment that could predict postischemic recovery. Hearts were then subjected to 25-min global ischemia followed by 3-h reperfusion. Recovery of LVDP at 1-h reperfusion and infarct size/area at risk (percentage of left ventricle) at 3-h reperfusion were used to assess delayed cardioprotection. Administration of isoflurane conferred delayed cardioprotection 24 h later at a concentration of 0.8% (vol/vol) with infarct size (mean ± SD) reduced by 47% (8 ± 3%) vs. untreated controls (15 ± 4%). Isoflurane [0.8% (vol/vol)] also increased the recovery of postischemic LVDP by 36% (64 ± 3%) versus untreated controls (47 ± 7%) (Fig. 1). Blood isoflurane concentration in rats exposed to 0.8% isoflurane was 0.25 ± 0.02 mM. Exposure of rats to 0.5, 1.0, or 2% isoflurane did not have any effect on delayed cardioprotection.

To determine whether inspired oxygen levels exerted any affect upon delayed cardioprotection, an additional control group of rats breathing room air (21% O2) alone was studied. Treatment of rats with 100% O2 for 2 h followed by recovery in room air for 24 h resulted in increased recovery of postischemic LVDP (47 ± 7%) compared with rats exposed to 21% O2 (38 ± 8%). However, there was no difference in infarct size between hearts previously treated with 100% O2 or 21% O2 (Fig. 1).

Role of ROS. We determined whether ROS generation is responsible for inducing delayed cardioprotection with isoflurane. MnTBAP, administered to rats 15 min before isoflurane treatment [0.8% (vol/vol)] completely abolished the delayed cardioprotective effects of isoflurane (infarct size: 17 ± 5% and recovery of LVDP: 40 ± 15%). MnTBAP had no effect on cardioprotection in untreated hearts (Fig. 2).

Cellular source of ROS. We then determined whether the mitochondrial electron transport chain and NOS are cellular
sources of ROS production resulting from isoflurane treatment. Isolated hearts were perfused with ethidium, a fluorescent probe for superoxide, after treatment of rats for 2 h with 0.8% (vol/vol) isoflurane-100% O₂ or 100% O₂ alone, resulting in a 1.8 ± 0.4-fold increase in ethidium staining for the isoflurane-treated hearts compared with untreated controls. Myxothiazol administered to rats 15 min before isoflurane-100% O₂ or 100% O₂ treatment attenuated superoxide generation by 42% and 14%, respectively (Fig. 3).

**Role of RNS.** We then determined whether RNS formation would act to induce delayed cardioprotection in rats treated with isoflurane. Rats treated with L-NAME (15 mg/kg) 15 min before isoflurane-100% O₂ completely abolished the delayed cardioprotective effects of isoflurane (infarct size: 17 ± 5% and recovery of LVDP: 43 ± 6%). L-NAME had no effect on cardioprotection in rats treated with 100% O₂ alone (Fig. 5).

**Isoflurane-induced NO production.** To determine the extent to which isoflurane treatment increased NO production, hearts were analyzed for nitrite plus nitrate content after 2-h treatment with isoflurane-100% O₂ and 100% O₂ alone. Tissue levels for nitrite and nitrate were increased 1.5-fold in hearts previously treated with isoflurane-100% O₂ compared with 100% O₂ alone (Fig. 6).

**ROS and RNS enzymatic response to isoflurane treatment.** To determine whether 2-h isoflurane treatment triggers increased expression of enzymes responsible for ROS metabolism and RNS production, we examined protein levels in isoflurane-treated and untreated hearts for Cu-Zn SOD, Mn SOD, and NOS (NOS1, NOS2 and NOS3) by Western blot analysis. With the use of standard Western blot analysis, the only NOS isoform detected was NOS3 (data for NOS1 and NOS2 not shown). Isoflurane did not alter expression of Cu-Zn SOD, Mn SOD, or NOS3 in the heart (Fig. 7).

**Isoflurane-induced glutathione reduction.** To determine whether isoflurane affects the reducing capacity of the heart,
glutathione levels were measured after 2-h treatment and 24 h after treatment and compared with untreated controls. Exposure of rats to 100% O2 alone for 2 h resulted in a slight increase in total and reduced myocardial glutathione above values present in untreated rats maintained in room air (21% O2), although this increase was not significant (Fig. 8). Exposure of rats to 0.8% isoflurane-100% oxygen for 2 h decreased total and reduced glutathione levels by 9% and 11% compared with exposure to 100% oxygen alone, respectively (Fig. 8). Twenty-four hours after exposure to isoflurane, myocardial glutathione levels returned to room air control values. In contrast, exposure of rats to 1.0% isoflurane-100% oxygen for 2 h did not change myocardial glutathione content from 100% oxygen-treated values. However, 24 h after exposure to isoflurane, total and reduced myocardial glutathione content were both decreased by 13% (Fig. 8).

Isoflurane-induced changes in blood gas and buffer status. To determine whether exposure of rats to isoflurane resulted in metabolic changes as a result of imbalances in arterial blood gas and buffer status that could provoke increased ROS and

RNS production, arterial blood was analyzed after exposure to room air (21% O2), 100% O2, and 0.8% (vol/vol) isoflurane-100% oxygen for 2 h. The time course we used matched the experimental protocol used for treating the rats for the isoflurane and delayed cardioprotection studies. Arterial oxygen pressures increased from 84 ± 1106 ± 16 mmHg in rats breathing room air to 223 ± 56 mmHg in rats breathing 100% oxygen for

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Fig. 4. Iso and superoxide production from nitric oxide (NO) synthase (NOS). Data are means ± SD; n = 3 hearts/group. A: representative images showing ethidine staining in nuclei of hearts treated with Iso-100% O2 and 100% O2 in the presence and absence of N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME; 15 mg/kg ip). B: bar graph showing the mean fluorescent intensity of ethidine staining after correction for background fluorescence. *P < 0.05, Iso-100% O2 vs. Iso-100% O2 + L-NAME; †P < 0.05, Iso-100% O2 vs. 100% O2; ‡P < 0.05, Iso-100% O2 vs. 100% O2 + L-NAME.

Fig. 5. NO generation and delayed cardioprotection by Iso. Data are means ± SD; n = 8 hearts/group. *P < 0.05, 0.8% (vol/vol) Iso-100% O2 treated vs. 100% O2; †P < 0.05, 0.8% (vol/vol) Iso-100% O2 treated with L-NAME (15 mg/kg ip) vs. without L-NAME.

Fig. 6. Iso-induced NO\textsubscript{2} + NO\textsubscript{3} formation in the heart. Data are means ± SD; n = 9 hearts/group. *P < 0.05, 100% O2 vs. Iso + 100% O2.
Arterial oxygen pressures increased from 80 ± 10 mmHg in rats breathing room air to 215 ± 23 mmHg in rats breathing 0.8% (vol/vol) isoflurane-100% oxygen for 2 h (Table 1). There were no changes in arterial buffering capacity as a result of isoflurane treatment (Table 1).

**DISCUSSION**

Volatile anesthetics exert an immediate protective effect against ischemia in adult myocardium. However, their ability to confer delayed cardioprotection and the underlying mechanisms that may trigger delayed cardioprotection with isoflurane are unknown. Our studies demonstrate that isoflurane confers delayed cardioprotection in the adult rat at a concentration of 0.8% (vol/vol) as manifested by a decrease in postischemic infarct size and an increase in recovery of LVDP. The magnitude of delayed cardioprotection conferred by isoflurane is equivalent to that conferred by ischemic preconditioning (30, 47, 48). Thus isoflurane exerts a profound effect on the heart long after anesthetic treatment has been discontinued. In our previous study, we speculated that isoflurane acts as a trigger to initiate a signal transduction pathway that results in delayed cardioprotection. In the present study, we provide evidence that isoflurane triggers increased ROS and RNS production, because delayed cardioprotection with isoflurane is abolished by the superoxide scavenger MnTBAP and the general NOS inhibitor L-NAME. Furthermore, isoflurane depletes reduced glutathione levels. Taken together, our results suggest that isoflurane may confer delayed cardioprotection by triggering an oxidative stress. Cellular sources of ROS generated by isoflurane include the mitochondrial electron transport chain, with NOS being a cellular source for both ROS and RNS. There were no changes in arterial blood gas or buffer status as a result of isoflurane treatment that could cause increased NO or superoxide formation. Our findings have implications for the postoperative management of patients with cardiac and noncardiac disease undergoing general anesthesia.

**Delayed cardioprotection with isoflurane.** Our previous study (42) has shown that isoflurane induces delayed cardioprotection in the infant rabbit. However, in a recent study (20), it was concluded that isoflurane does not confer delayed cardioprotection in the adult dog. This study prompted us to look for changes in experimental protocols that might explain the apparent differences in our findings. In the experimental protocol utilized by Kehl et al. (20), only a single dose of isoflurane [1 minimum alveolar concentration (MAC)] was evaluated and in an adult animal from a different species. On the basis of this information, we concluded that a systematic dose-response study might reveal new facts about the delayed cardioprotection afforded by isoflurane.

Cardioprotection in our study was measured using two independent end points: postischemic infarct size and recovery of LVDP. Isoflurane treatment resulted in a reduction in postischemic infarct size and an increase in recovery of LVDP compared with untreated controls. Isoflurane conferred delayed cardioprotection at a concentration of 0.8% but not 0.5, 1.0, and 2.0% (vol/vol). Patients undergoing cardiac and noncardiac surgery are routinely exposed to 0.8% isoflurane to maintain levels of general anesthesia. In contrast, immediate protection against myocardial ischemia conferred by isoflurane exists over the range of 0.25–1.25 MAC as manifested by a
reduction in infarct size (19). The reason for the presence of such a narrow therapeutic window is not known. However, our findings may explain in part the inability of Kehl et al. (20) to demonstrate delayed cardioprotection with isoflurane in the adult dog. In their study, a single dose of isoflurane [1.28% (vol/vol), 1 MAC] was evaluated, with cardioprotection assessed using only a single end point, infarct size. We believe that the study by Kehl et al. (20), which evaluated only a single concentration of isoflurane, underscores the need to perform pharmacological dose-response studies when investigating the ability of a drug to protect the ischemic myocardium. In the study by Kehl et al., there was no positive control study included to demonstrate delayed cardioprotection in their dog model. However, delayed cardioprotection by ischemic preconditioning does occur in the dog (23). We therefore considered the possibility that delayed cardioprotection by a volatile anesthetic may not occur in the dog. Analysis of a study of Davis et al. (11) revealed that permanent coronary artery occlusion in the dog before 12-h treatment with halothane [0.5–1.0% (vol/vol)] followed by 24-h recovery resulted in a reduction in infarct size compared with untreated dogs. Thus halothane can confer delayed cardioprotection in the dog after the onset of ischemia.

**ROS and delayed cardioprotection with isoflurane.** Our study demonstrates that the xenobiotic isoflurane increases superoxide generation in the heart from NOS as manifested by an increase in ethidine staining of heart tissue that is attenuated by L-NAME. Because NOS3 protein is the only NOS isoform detectable in the untreated rat heart by Western blot analysis, we attributed NO production by isoflurane to the NOS3 isoform. NOS3-dependent superoxide generation occurs in aerobically perfused hearts not subjected to stress as part of the normal physiological function of this enzyme (36). The superoxide anion can be generated by NOS3 at either the NADPH reductase domain or the arginine oxidase domain (44). Increased superoxide production after isoflurane treatment is also attenuated by myxothiazol, an inhibitor of complex III in the mitochondrial electron transport chain, indicating that the mitochondrion is a significant source of isoflurane-induced superoxide production. Pretreatment of rats with MnTBAP, a scavenger of the superoxide anion, before isoflurane treatment, abolished delayed cardioprotection conferred by isoflurane, suggesting a role for ROS in inducing delayed cardioprotection. Thus superoxide production from both NOS and mitochondria appears to act as to induce delayed protection of the heart with isoflurane.

In our study, increased superoxide production after isoflurane treatment was not accompanied by an increase in tissue levels of Cu-Zn SOD and Mn SOD protein as measured by Western blot analysis, suggesting that the heart does not increase its enzymatic defense systems for the dismutation of superoxide in the face of increases in superoxide production over the 2-h isoflurane treatment period. Our results for Mn SOD are in agreement with those of Sergeev et al. (35), who reported no increase in mRNA for Mn SOD after exposure of rat hearts to isoflurane.

Exposure of hearts to 0.8% (vol/vol) isoflurane for 2 h resulted in a significant decrease in glutathione, suggesting that isoflurane elicits a stress on the reducing capacity of the cell. Basal levels of GSH were close to previous reports (32). Interestingly, the loss of GSH was not accompanied by an increase in tissue glutathione disulfide (GSSG) as would be expected for a stress involving an increase in hydrogen peroxide, through the action of glutathione peroxidase. Although the reason for this is unclear, it is possible that isoflurane can

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Data are means ± SD; n = 6 hearts/group. Iso, isoflurane. *P < 0.05, 21% O₂ vs. 100% O₂.
deplete GSH through a hydrogen peroxide-independent mechanism, suggesting that glutathione depletion is a cause rather than a consequence of increased oxidative stress. Regardless of the mechanism, loss of GSH indicates that the heart has a diminished capacity to deal with oxidant production during isoflurane treatment. This suggests that a low-level oxidative stress occurring during anesthetic preconditioning may contribute to the protective effects observed upon later ischemia-reperfusion.

Our results suggest there may be a mechanistic link among the concentration of isoflurane, myocardial glutathione content, and delayed cardioprotection. Decreased myocardial glutathione content 2 h after exposure to 0.8% isoflurane and a return to normal values 24 h later result in delayed cardioprotection. In contrast, maintained levels of glutathione after exposure to 1.0% isoflurane and a decreased glutathione content 24 h later do not result in delayed cardioprotection. We propose that exposure to isoflurane increases superoxide and/or NO generation resulting in an oxidative stress, manifested as a decrease in glutathione content, that initiates the activation of nuclear transcription factors to induce or upregulate the synthesis of genes that confer delayed cardioprotection. In support of this idea, volatile anesthetics, including isoflurane, induce expression of early response genes in several tissues, including the heart (15, 35), that can act as transcription factors. Our proposed mechanism for triggering delayed cardioprotection with isoflurane is similar to that which may exist for induction of cardioprotective genes that confer delayed cardioprotection, with brief periods of ischemia or pharmacological agents acting to induce the transcription and translation of proteins that act as mediators of delayed cardioprotection. In late preconditioning, chemical signals released by the stress of ischemia, such as ROS, are transduced by signaling elements including protein kinase C and NF-kB to the nucleus where they initiate the transcription of cardioprotective genes such as NOS, cyclooxygenase (COX)-2, and aldose reductase (6). Further studies are needed to identify the mechanisms mediating delayed cardioprotection by isoflurane.

**RNS and delayed cardioprotection with isoflurane.** Isoflurane treatment also increases -NO generation in the heart from ROS as manifested by a 1.5-fold increase in nitrite plus nitrate content in heart tissue that was not accompanied by an increase in NOS3 protein, as determined by Western blot analysis. Our findings indicate that after isoflurane treatment, the NOS3 enzyme is turning over faster to generate both -NO and superoxide compared with untreated hearts. In a previous study in the rabbit heart, we (36) also found NOS3 generates both -NO and superoxide. Taken together, these results support the notion that superoxide and -NO are constitutively produced by NOS3 as part of the normal physiological function of the enzyme and that isoflurane increases coupled and uncoupled NOS3 activity. Our observation of isoflurane increasing -NO bioavailability in the heart supports the findings of Ashwal et al. (3), who showed that treatment of rats with isoflurane increased NOS activity in the brain. Pretreatment of rats with 1-NAME, a general inhibitor of NOS, before anesthesia abolishes delayed cardioprotection conferred by isoflurane, demonstrating a role for RNS in inducing delayed cardioprotection. However, 1-NAME also attenuated isoflurane-induced superoxide generation in our study. Thus we acknowledge the possibility that abolishing isoflurane-induced delayed cardioprotection with 1-NAME may be attributed to both inhibition of superoxide as well as NO. The role of RNS in inducing immediate cardioprotection by sevoflurane has been shown by Novalija et al. (29), who reported that treatment of guinea pig hearts with 1-NAME abolished the cardioprotective effects of sevoflurane. Our study shows that NO and superoxide generated from NOS3 appear to confer delayed protection of the heart with isoflurane. NOS3 has been shown to confer delayed protection against myocardial ischemia conferred by ischemic preconditioning, whereas NOS2 acts as a mediator in delayed ischemic preconditioning (8).

The cellular mediator(s) of delayed cardioprotection with isoflurane remain to be identified. COX-2 has been suggested to be a mediator of delayed cardioprotection by isoflurane (38). The selective COX-2 inhibitor celecoxib, when given after isoflurane treatment, abolished delayed cardioprotection. However, several limitations exist in the design and execution of this study that may impact on the conclusions made. Celecoxib was dissolved in 20% DMSO. The effect of solvent alone on cardioprotection was not reported. Tanaka et al. (38) state that “COX-2 is normally absent from cells,” yet their data reveal that the protein is constitutively expressed with a strong signal present in control hearts. Positive controls for the Western blot studies were not included in this study. Neither COX-1 nor COX-2 protein expression was affected by isoflurane treatment. In addition, the enzymatic activity of COX-2 and its products were never measured. Furthermore, there are other mechanisms through which celecoxib could mediate its effects. Celecoxib can induce apoptosis through suppression of Akt activation (2, 16) and through perturbation of intracellular calcium by inhibiting endoplasmic reticulum Ca2+-ATPases (17). These reports suggest that the ability of celecoxib to mediate delayed cardioprotection by isoflurane with respect to inhibiting COX-2 remains unclear. ATP-sensitive potassium (KATP) channels have been shown to mediate delayed cardioprotection by isoflurane (42). We previously showed that blockade of the sarcolemmal and mitochondrial KATP channels with HMR-1098 and hydroxydecanoate (5-HD) abolished delayed cardioprotection by isoflurane. In these studies, the concentrations of HMR-1098 and 5-HD were lower than those used to abolish the cardioprotective effects of ischemic preconditioning and chronic hypoxia (22), suggesting that the mechanisms by which KATP channels are activated by isoflu-
rane may be quite different from cardioprotection conferred by ischemic preconditioning and adaptation to chronic hypoxia. In an elegant study, Sergeev et al. (35) recently showed increased mRNA expression of NF-κB and seven ribosomal proteins after isoflurane treatment. NF-κB has been shown to be important in the development of delayed cardioprotection by ischemic preconditioning. Thus an oxidative stress triggered by isoflurane may initiate the synthesis of early response genes, including NF-κB, that signal the expression of proteins that confer delayed cardioprotection by activation of KATP channels.

Increased ROS production triggered by the volatile anesthetic sevoflurane immediately activates the epsilon isoform of protein kinase C (28). Isoflurane also translocates protein kinase C isoforms to subcellular targets (25, 43). The immediate cardioprotective effects of volatile anesthetics are blocked by protein kinase C inhibitors (9, 43). Furthermore, protein kinase C has been shown to activate the sarcoplasmic reticular KATP channel (24). These protein kinases are also essential components of signaling pathways involved in delayed cardioprotection conferred by ischemic preconditioning and adaptation to chronic hypoxia. However, the role protein kinases play in signaling pathways activated by isoflurane to confer delayed cardioprotection remains to be defined.

**Cellular sources of superoxide and NO.** We have shown that isoflurane triggers superoxide production from both NOS and the mitochondrial electron transport chain. Isoflurane also triggers NO production from NOS. There may be other cellular sources for increased superoxide production triggered by isoflurane. The mitochondrial KATP channel has been suggested to be a source of superoxide (31). Metabolism of isoflurane induces the expression of the 2E1 isoform of cytochrome P-450 oxidase, which can generate superoxide. The CYP2E1 isoform is expressed in the heart (40). Xanthine oxidase, located in the vascular endothelium, is an enzymatic source of superoxide, as is NADPH oxidase. We have depicted the potential contribution of these other cellular sources to superoxide production in Fig. 9. Further studies are needed to determine the cellular sources of superoxide production triggered by isoflurane and whether superoxide and NO are part of the same mechanisms or act in parallel as two independent triggers.

**Translation to clinical application.** Cardiovascular disease continues to be a global health care problem (46). The prevalence of this disease affects the outcome of both cardiac and noncardiac surgery, with perioperative cardiac morbidity one of the leading causes of death after anesthesia and surgery. Immediate anesthetic-induced protection of the human heart against ischemia has been demonstrated in the setting of coronary artery bypass graft (CABG) surgery. In patients undergoing CABG surgery, sevoflurane conferred protection against myocardial injury as manifested by a reduction in brain natriuretic peptide, a marker of myocardial contractile dysfunction (18). Our studies suggest that delayed anesthetic-induced protection of the human heart may be possible in the postoperative period. Patients with coronary artery disease who undergo general anesthesia for noncardiac surgery have the greatest severity of postoperative myocardial ischemia during the first 48 h after surgery (26). Isoflurane may be able to confer delayed cardioprotection in these patients. In addition, patients with preexisting coronary artery disease who are to undergo elective cardiac ischemia in the setting of cardiac surgery, percutaneous transluminal coronary angioplasty, and preservation of the heart for transplantation may benefit from delayed cardioprotection conferred by isoflurane.

The isolated heart is a constantly deteriorating preparation. For measurement of ventricular function, hearts were perfused for a total of 90 min to remain within the stability limits of the preparation. Hearts were reperfused for a total of 3 h before being stained with TTC to ensure that infarct size was not underestimated with recovery of ventricular function not assessed beyond 60 min of reperfusion. Coronary flow is increased compared with the in vivo situation and the perfusion medium has a low oxygen carrying capacity; to overcome this deficiency, the buffer is gassed with mixtures containing 95% oxygen. The use of the hydroethidine method to detect superoxide generation by image analysis, although capable of detecting between-group differences with 3 hearts/group, is a semiquantitative technique.

We conclude that isoflurane confers delayed cardioprotection in the adult rat 24 h after treatment, which is manifested as a decrease in infarct size and an increase in recovery of postischemic developed pressure. The initiating event that triggers delayed cardioprotection with isoflurane appears to be an oxidative stress resulting from increased generation of ROS and RNS. Further studies are needed to establish whether volatile anesthetics can confer delayed cardioprotection in diseased or aged hearts.

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