Mechanism of Preconditioning by Isoflurane in Rabbits: A Direct Role for Reactive Oxygen Species

Katsuya Tanaka, M.D.,* Dorothee Weihrauch, D.V.M. Ph.D.,† Franz Kehl, M.D. D.E.A.A.,* Lynda M. Ludwig, B.S.,‡ John F. LaDisa, Jr., M.S.,‡ Judy R. Kersten, M.D.,§ Paul S. Pagel, M.D. Ph.D.,‖ David C. Warltier, M.D. Ph.D.#

Background: Reactive oxygen species (ROS) contribute to myocardial protection during ischemic preconditioning, but the role of the ROS in protection against ischemic injury produced by volatile anesthetics has only recently been explored. We tested the hypothesis that ROS mediate isoflurane-induced preconditioning *in vivo*.

Methods: Pentobarbital-anesthetized rabbits were instrumented for measurement of hemodynamics and were subjected to a 30 min coronary artery occlusion followed by 3 h reperfusion. Rabbits were randomly assigned to receive vehicle (0.9% saline), or the ROS scavengers N-acetylcysteine (NAC; 150 mg/kg) or N-2-mercaptopropionyl glycine (2-MPG; 1 mg · kg^{-1} · min^{-1}), in the presence or absence of 1.0 minimum alveolar concentration (MAC) isoflurane. Isoflurane was administered for 30 min and then discontinued 15 min before coronary artery occlusion. A fluorescent probe for superoxide anion production (dihydroethidium, 2 mg) was administered in the absence of the volatile anesthetic or 5 min before exposure to isoflurane in 2 additional groups (n = 8). Myocardial infarct size and superoxide anion production were assessed using triphenyltetrazolium staining and confocal fluorescence microscopy, respectively.

Results: Isoflurane ($P < 0.05$) decreased infarct size to 24 ± 4% (mean ± SEM; n = 10) of the left ventricular area at risk compared with control experiments (43 ± 3%; n = 8). NAC (43 ± 3%; n = 7) and 2-MPG (42 ± 5%; n = 8) abolished this beneficial effect, but had no effect on myocardial infarct size (47 ± 3%; n = 8 and 46 ± 3; n = 7, respectively) when administered alone. Isoflurane increased superoxide anion production as compared with control experiments (28 ± 12 *vs.* −6 ± 9 fluorescence units; $P < 0.05$).

Conclusions: The results indicate that ROS produced following administration of isoflurane contribute to protection against myocardial infarction *in vivo*.

LARGE quantities of reactive oxygen species (ROS) released during reperfusion after coronary artery occlusion damage proteins responsible for intracellular homeostasis, produce tissue injury,[1–3] depress contractile function, and increase myocardial infarct size. In contrast, small quantities of ROS may exert beneficial effects during ischemia and reperfusion when released before a prolonged ischemic event.[4] ROS derived from mitochondria during a brief ischemic episode produce preconditioning.[5,6] Free radical scavengers administered during ischemic preconditioning (IPC) markedly attenuate the protective effect of the preconditioning stimulus on infarct size.[5,7] These data suggest that IPC is mediated in part by small quantities of ROS released during preconditioning. Volatile anesthetics protect myocardium against infarction through a signal transduction pathway that includes adenosine type 1 receptors,[8–10] protein kinase C,[9,11] inhibitory guanine regulatory proteins,[12] and mitochondrial and sarcolemmal adenosine triphosphate-regulated potassium (K_{ATP}) channels.[13–16] A recent investigation by Müllenheim *et al.* provides compelling evidence that ROS also mediate myocardial protection produced by volatile anesthetics.[17] We sought to confirm and extend these important results by examining the hypothesis that ROS scavengers inhibit isoflurane-induced protection against irreversible ischemic injury. We further tested the hypothesis that isoflurane directly generates ROS in rabbit ventricular myocardium *in vivo* using a confocal microscopic technique combined with the superoxide anion-specific fluorescent probe dihydroethidium.

Methods

All experimental procedures and protocols used in this investigation were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin. Furthermore, all conformed to the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C., 1996).

General Preparation

Male New Zealand White rabbits weighing between 2.5 and 3.0 kg were anesthetized with intravenous sodium pentobarbital (30 mg/kg). Additional doses of pentobarbital were titrated as required to assure that pedal and palpebral reflexes were absent throughout the experiment. A tracheostomy was performed through a ventral midline incision, and the trachea was cannulated. The rabbits were ventilated (model 683, Harvard, Holliston, MA) with positive pressure using a room air-oxygen mixture ($F_{IO_2} = 0.33$). Arterial blood gas tensions and acid-base status were maintained within a normal physiologic range (pH 7.35–7.45, Pa_{CO_2} 25–40 mmHg, and Pa_{O_2} 90–150 mmHg) by adjusting the respiratory rate or tidal volume throughout the experiment. Body temperature was maintained with a heating blanket. Heparin-
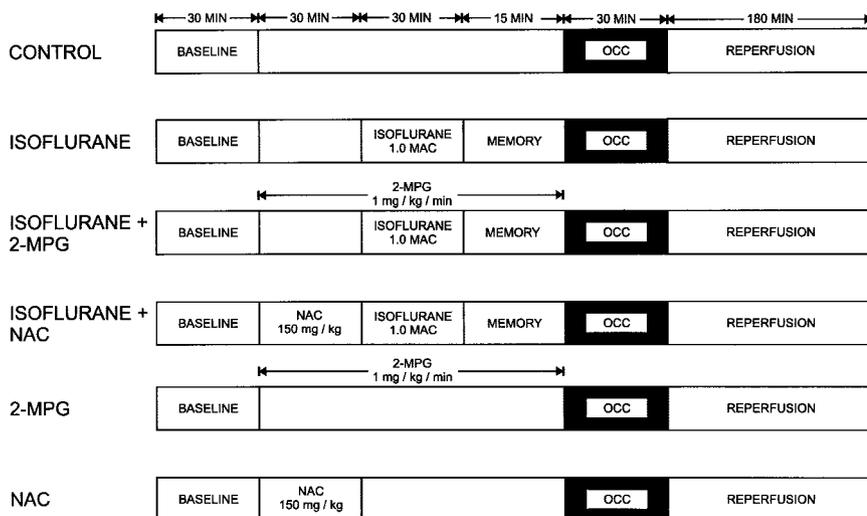
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Fig. 1. Schematic illustration of the experimental protocol. ISO = isoflurane; 2-MPG = *N*-2-mercaptopropionyl glycine; NAC = N-acetylcysteine.

filled catheters were inserted into the right carotid artery and the left jugular vein for measurement of arterial blood pressure and fluid or drug administration, respectively. Maintenance fluids consisted of 0.9% saline $(15 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{h}^{-1})$ that were continued for the duration of the experiment. A left thoracotomy was performed at the fourth intercostal space, and the heart was suspended in a pericardial cradle. A heparin-filled catheter was inserted into the left atria for the subsequent administration of dihydroethidium used to detect ROS production. A prominent branch of the left anterior descending coronary artery (LAD) was selected, and a silk ligature was placed around this artery approximately halfway between the base and apex for the production of coronary artery occlusion and reperfusion. Each rabbit was anticoagulated with 500 U of heparin immediately before LAD occlusion. Coronary artery occlusion was verified by the presence of epicardial cyanosis and regional dyskinesia in the ischemic zone, and reperfusion was confirmed by observing an epicardial hyperemic response. Hemodynamics were continuously recorded on a polygraph (Grass model 7) throughout experimentation.

Experimental Protocol

The experimental design used in the present investigation is illustrated in figure 1. Thirty minutes after instrumentation was completed and calibrated, baseline systemic hemodynamics were recorded. Rabbits were randomly assigned to one of six experimental groups using a Latin square design. All rabbits underwent a 30 min LAD occlusion followed by 3 h reperfusion. Rabbits received intravenous vehicle (0.9% saline), N-acetylcysteine (NAC; 150 mg/kg over 30 min),[18] or N-2-mercaptopropionyl glycine (2-MPG; $1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ over 75 min)[5] in the presence or absence of 1.0 minimum alveolar concentration (MAC) isoflurane. The end-tidal MAC value used for rabbits in the present investigation was 2.1%.[19] End-tidal concentrations of isoflurane were measured at the tip of the tracheostomy tube with an infra-

red anesthetic analyzer that was calibrated with known standards before and during experimentation.

Determination of Myocardial Infarct Size

Myocardial infarct size was measured as previously described.[20] Briefly, the left ventricular (LV) area at risk for infarction (AAR) was separated from surrounding normal areas, and the two regions were incubated at 37°C for 20 to 30 min in 1% 2,3,5-triphenyltetrazolium chloride in 0.1 M phosphate buffer adjusted to pH 7.4. After overnight storage in 10% formaldehyde, infarcted and noninfarcted myocardium within the AAR were carefully separated and weighed. Infarct size was expressed as a percentage of the AAR. Rabbits that developed intractable ventricular fibrillation and those with an AAR less than 15% of LV mass were excluded from subsequent analysis.

ROS Detection

Dihydroethidium is oxidized by intracellular ROS to produce fluorescent ethidium that subsequently binds to DNA (Eth-DNA) further amplifying its fluorescence.[21] The fluorescence observed after activation of the Eth-DNA complex is generally stable, but may be reduced by the presence of hydroxyl radicals.[22,23] Thus, an increase in dihydroethidium oxidation to Eth-DNA and the subsequent increase in fluorescence are highly suggestive of superoxide anion generation. In two additional groups of rabbits (n = 8), dihydroethidium (2 mg) was rapidly injected into the left atrium 5 min before the administration of isoflurane (1 MAC) or at the corresponding time point in rabbits that were not subsequently exposed to the volatile anesthetic. Isoflurane was discontinued after 30 min, and the rabbits were euthanized after 1 h with a lethal dose of pentobarbital. The heart was rapidly excised. The LV was isolated, divided into four sections of equal size, and frozen in liquid nitrogen. Kryostat sections (20 μm) of the LV were mounted on standard microscope slides. Using a laser fluorescence

Table 1. Systemic Hemodynamics

	Baseline	Isoflurane	Preocclusion	30 min CAO	Reperfusion (h)		
					1	2	3
HR (min^{-1})							
CON	259 ± 11	-	266 ± 11	264 ± 11	260 ± 10	265 ± 10	268 ± 12
ISO	260 ± 10	273 ± 8	260 ± 5	240 ± 9*	242 ± 9*	235 ± 10*	238 ± 9*
ISO+2-MPG	255 ± 9	282 ± 6*	269 ± 7	275 ± 13*	249 ± 8	241 ± 6	242 ± 2
ISO+ NAC	251 ± 7	276 ± 7*	275 ± 6*	270 ± 8	265 ± 8	264 ± 4	270 ± 7
2-MPG	243 ± 9	-	259 ± 8	263 ± 13	234 ± 5	230 ± 9	236 ± 12
NAC	255 ± 9	-	261 ± 7	255 ± 11	248 ± 11	251 ± 13	247 ± 13
MAP (mmHg)							
CON	88 ± 4	-	85 ± 3	75 ± 2*	83 ± 4	81 ± 6	78 ± 5*
ISO	89 ± 3	67 ± 5*	82 ± 3	73 ± 4*	74 ± 5*	77 ± 4*	79 ± 4*
ISO + 2-MPG	89 ± 2	51 ± 5*	79 ± 4	68 ± 6*	75 ± 6	76 ± 5	74 ± 4
ISO + NAC	90 ± 4	62 ± 2*	82 ± 4	77 ± 4*	82 ± 5	83 ± 5	83 ± 3
2-MPG	90 ± 3	-	85 ± 2	69 ± 6*	72 ± 6*	74 ± 7*	74 ± 7*
NAC	87 ± 3	-	86 ± 2	75 ± 4*	75 ± 2*	75 ± 3*	76 ± 3*
RPP (bpm · mmHg · 10^{-3})							
CON	26.3 ± 1.9	-	26.6 ± 1.6	23.0 ± 1.5*	25.0 ± 2.1	24.7 ± 2.3	23.6 ± 2.3*
ISO	26.3 ± 1.5	22.8 ± 1.4*	25.3 ± 1.0	20.5 ± 1.3*	20.9 ± 1.8*	20.8 ± 1.4*	21.5 ± 1.6*
ISO + 2-MPG	25.7 ± 1.0	20.4 ± 1.7*	25.6 ± 1.0	22.0 ± 1.6*	21.8 ± 1.9*	20.7 ± 1.6*	20.2 ± 0.9*
ISO + NAC	26.0 ± 1.4	23.2 ± 1.1	27.0 ± 1.3	24.3 ± 1.6	24.8 ± 1.4	24.9 ± 1.4	25.5 ± 0.7
2-MPG	24.4 ± 0.9	-	25.3 ± 1.1	20.5 ± 1.6*	18.9 ± 1.2*	19.2 ± 1.8*	19.8 ± 1.7*
NAC	25.0 ± 1.6	-	25.3 ± 1.1	21.8 ± 1.4*	21.2 ± 1.4*	21.8 ± 1.9*	21.7 ± 1.9*

Data are mean ± SEM.

* Significantly ($P < 0.05$) different from baseline.

CAO = coronary artery occlusion; HR = heart rate; MAP = mean arterial blood pressure; RPP = rate-pressure product; CON = control; ISO = isoflurane; 2-MPG = N-2-mercaptopropionyl glycine; NAC = N-acetylcysteine.

imaging system (MRC 600 Laser Scanning Confocal Microscopic Imaging System; Bio-Rad Laboratories; Philadelphia, PA) mounted on a Microscope (Optiphot; Nikon Corporation; Tokyo, Japan) images were recorded and stored for subsequent offline analysis on a computer workstation equipped with image analysis software (MetaMorph; Universal Imaging Corporation; Downingtown, PA). Use of the 40× objective yielded a 400× end magnification on a 292 × 195 μm^2 digital image (768 × 512 pixels). The signal-to-noise ratio was enhanced using the Kalman method. Excitation was produced using a Krypton-Argon laser at a wavelength of 488 nm, and emitted fluorescence measured at 550 nm after long pass filtering. The pixel intensity of each myocyte nucleus was determined. Background was identified as an area without cells or with minimal cytosol fluorescence. In each rabbit, 20 Kalman-averaged images were obtained and approximately 6 to 8 dihydroethidium-stained myocardial cells were analyzed by subtraction of background fluorescence from the pixel intensity of the myocardial nuclei.

Statistical Analysis

Statistical analysis of data within and between groups was performed with analysis of variance (ANOVA) for repeated measures followed by Student Newman–Keuls test. Changes within and between groups were considered statistically significant when the P value was less than 0.05. Student t test was used to compare differences of pixel intensities in ROS detection experiments.

Statistical significance was defined as $P < 0.05$. All data are expressed as mean ± SEM.

Results

Sixty-one rabbits were instrumented to obtain 48 successful myocardial infarct size experiments. Eight rabbits were excluded because the AAR/LV did not exceed 15% (1 control; 2 isoflurane alone; 1 isoflurane + 2-MPG; 3 isoflurane + NAC; and 1 NAC alone). Five rabbits were excluded because of intractable ventricular fibrillation (1 control; 2 isoflurane alone; 1 isoflurane + 2-MPG; and 1 2-MPG alone).

Systemic Hemodynamics

No differences in hemodynamics were observed between experimental groups under control conditions (table 1). Isoflurane significantly ($P < 0.05$) decreased mean arterial pressure and rate-pressure product in the presence or absence of ROS scavengers. Hemodynamics returned to baseline values 15 min after isoflurane had been discontinued. Coronary artery occlusion and reperfusion produced similar decreases in mean arterial pressure and rate-pressure product in each experimental group.

Myocardial Infarct Size

The body weight, LV weight, AAR weight, and AAR/LV were similar between groups (table 2). Isoflurane reduced myocardial infarct size (24 ± 4% of the AAR; n =

Table 2. Area at Risk

	Body Weight (g)	LV WT (g)	AAR WT (g)	AAR/LV (%)
CON	2960 ± 50	3.16 ± 0.11	0.94 ± 0.13	30 ± 4
ISO	3050 ± 80	3.16 ± 0.10	1.04 ± 0.09	33 ± 3
ISO + 2-MPG	3000 ± 70	3.10 ± 0.13	1.04 ± 0.13	33 ± 4
ISO + NAC	2780 ± 50	2.99 ± 0.11	0.88 ± 0.10	29 ± 3
2-MPG	2900 ± 40	3.10 ± 0.08	1.08 ± 0.12	34 ± 3
NAC	2790 ± 80	2.88 ± 0.08	0.83 ± 0.07	29 ± 3

Data are mean ± SEM.

LV = left ventricle; WT = weight; AAR = area at risk; CON = control; ISO = isoflurane; 2-MPG = N-2-mercaptopropionyl glycine; NAC = N-acetylcysteine.

10; fig. 2) as compared to control experiments (43 ± 3%; n = 8). NAC and 2-MPG alone did not affect infarct size (47 ± 3%; n = 8 and 46 ± 3%; n = 7, respectively), but these ROS scavengers blocked the protective effects of isoflurane (43 ± 3%; n = 7 and 42 ± 5%; n = 8, respectively).

Reactive Oxygen Species Production

Ethidium-DNA fluorescence was detected in all images examined (fig. 3). Rabbits pretreated with 1.0 MAC isoflurane demonstrated enhanced fluorescence in myocardial nuclei compared with the cytosol, in contrast to the findings in rabbits that did not receive the volatile agent (fig. 4). Fluorescence intensity in myocardial nuclei was significantly greater (28 ± 12 *vs.* −6 ± 9 fluorescence units) in isoflurane-pretreated compared with untreated rabbits.

Discussion

Mitochondria have been shown to produce small quantities of ROS during brief periods of ischemia that cause preconditioning.[5,6] The protective effects of the selective mitochondrial K_{ATP} channel opener, diazoxide, were blocked by pretreatment with ROS scavengers in isolated rabbit hearts.[24] Diazoxide also increased oxidation of the ROS probe mitotracker orange *in vitro*, an action that was attenuated by pretreatment with the selective mitochondrial K_{ATP} channel antagonist 5-hydroxydecanoate (5-HD) or the ROS scavengers NAC and 2-MPG.[25] Diazoxide directly increased ROS production as measured by the hydrogen peroxide probe 2',7'-dichlorofluorescein diacetate[26] in rat ventricular myocytes and isolated hearts. Whether volatile anesthetics increase ROS production or modulate the actions of oxygen-derived free radicals as potential mechanisms by which these agents produce myocardial protection remains unclear. Sevoflurane has been shown to impair endothelium-dependent relaxation of canine mesenteric arteries by an oxygen-derived free radical mechanism.[27] Isoflurane reduced hydroxyl radical production in the ischemic rat heart.[28] In contrast, Müllenheim *et al.*[17] very recently demonstrated that 2-MPG and another ROS

scavenger Mn(III)tetrakis(4-benzoic acid)porphyrine chloride abolished the protective effect of isoflurane in rabbit hearts. These data were the first to implicate a role for ROS in anesthetic-induced preconditioning, and suggested that volatile agents may be capable of producing small quantities of ROS that serve as mediators of cardioprotection.

The results of the present investigation confirm and extend the findings of Müllenheim *et al.*[17] and indicate that the ROS scavengers NAC and 2-MPG blocked the reduction in myocardial infarct size produced by isoflurane when these drugs were administered during exposure to isoflurane. The results further demonstrate that intensity of Eth-DNA fluorescence measured with confocal microscopy is significantly increased during a 30 min pretreatment with 1.0 MAC isoflurane. These data indicate that isoflurane directly increases superoxide anion generation independent of coronary artery occlusion and reperfusion and strongly imply that this ROS production mediates protection against irreversible ischemic injury. Mitochondria are a likely source for the production of the superoxide anion.[29] Superoxide anion generated experimentally from the enzyme complex hypoxanthine–xanthine oxidase mimics the protective effects of preconditioning.[30] Thus, it appears likely that superoxide anion produced by mitochondria may activate intracellular signaling responsible for the protective effect of isoflurane. However, we did not specifically determine the source of ROS production by isoflurane or whether the generation of this oxygen-derived free radical species is linked to mitochondrial K_{ATP} channel opening in the present investigation. These objectives are important goals of future research.

The specific oxygen-derived free radicals responsible for activation of the signal transduction of preconditioning are unknown. The Cu,Zn-superoxide dismutase (SOD) inhibitor diethyldithiocarbamic acid is a selective inhibitor of the cytosolic conversion of superoxide anion
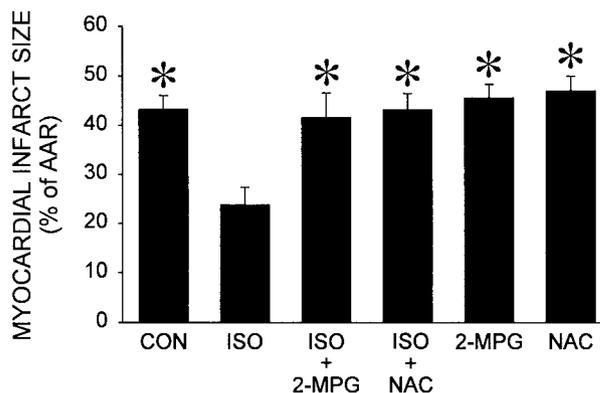

Fig. 2. Myocardial infarct size expressed as a percentage of the left ventricular area at risk (AAR) in rabbits receiving saline (CON), N-2-mercaptopropionyl glycine (2-MPG), or N-acetylcysteine (NAC) in the presence and absence of 1.0 MAC isoflurane (ISO). *Significantly (*P* < 0.05) different from ISO.
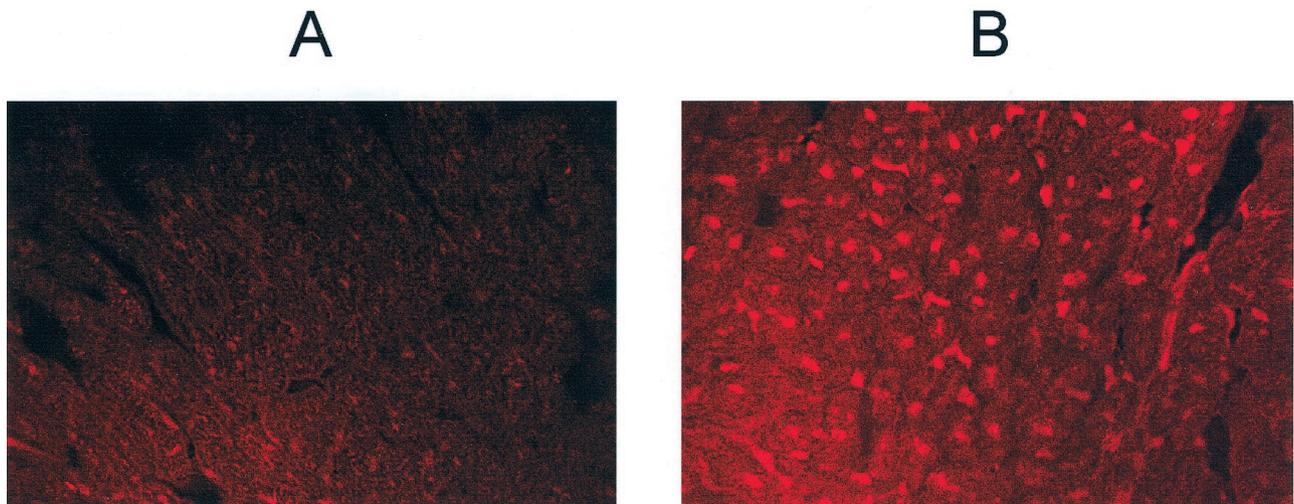
A

B

Fig. 3. Representative photomicrographs demonstrating enhanced production of superoxide anion by the expression of fluorescent ethidium bound to nuclear DNA. The fluorescence in myocardial nuclei in rabbits treated with 1.0 MAC isoflurane for 30 min (*B*) was more intense than that observed in rabbits that were not exposed to isoflurane (*A*).

to hydrogen peroxide and abolished protection produced by hypoxic preconditioning in embryonic cardiac myocytes.[6] These results suggested that conversion of superoxide anion to hydrogen peroxide may be an important step for oxidant induction of hypoxic preconditioning. N-acetylcysteine is a sulfhydryl-containing glutathione precursor that exerts antioxidant effects by contributing to glutathione synthesis, serving as a glutathione peroxidase substrate, and directly scavenging several oxygen-derived free radical species primarily by the actions of reduced glutathione.[31] Mitochondria contain large quantities of SOD, and the vast majority of superoxide anion generated as a consequence of mitochondrial electron transport is enzymatically dismutated to hydrogen peroxide and oxygen by mitochondrial Mn-SOD and cytosolic Cu,Zn-SOD. Glutathione peroxidase contained in the cytosol subsequently catalyzes the reduction of hydrogen peroxide to water. Reduced glutathione acts as the electron donor of glutathione peroxidase in this reaction. Thus, NAC maintains the cytosolic concentration of reduced glutathione and facilitates metabolism of hydrogen peroxide produced by the univalent reduction of superoxide anion. 2-MPG also acts as a sulfhydryl donor to glutathione peroxidase, and several studies indicate that 2-MPG may be more specific for mitochondrial activity than NAC.[32-34] We did not specifically identify the particular ROS scavenged by NAC or 2-MPG that was responsible for isoflurane-induced preconditioning. However, the present results indicate that exposure to isoflurane is accompanied by the generation of superoxide anion in ventricular myocytes, most likely in the mitochondria, in the absence of ischemia and reperfusion. Thus, it appears likely that the superoxide anion or another ROS generated by the superoxide anion dismutation pathway is involved in the signal transduction of isoflurane-induced preconditioning.

The present results should be interpreted within the constraints of several potential limitations. The area of the left ventricle at risk for infarction and coronary collateral blood flow are important determinants of the extent of myocardial infarction. The AAR was similar between experimental groups. Minimal coronary collateral blood flow has been previously reported in rabbits.[35] Thus, it is unlikely that the present results were affected by the size of the AAR or magnitude of coronary collateral blood flow.

Isoflurane caused similar hemodynamic effects in the presence or absence of ROS scavengers, and there were no differences in hemodynamics between groups after discontinuation of isoflurane. Thus, the present results occurred independent of the hemodynamic effects of isoflurane and/or the ROS scavengers. Nevertheless, coronary venous oxygen tension and myocardial oxygen consumption were not directly quantified in the present investigation, and differences in myocardial oxygen me-
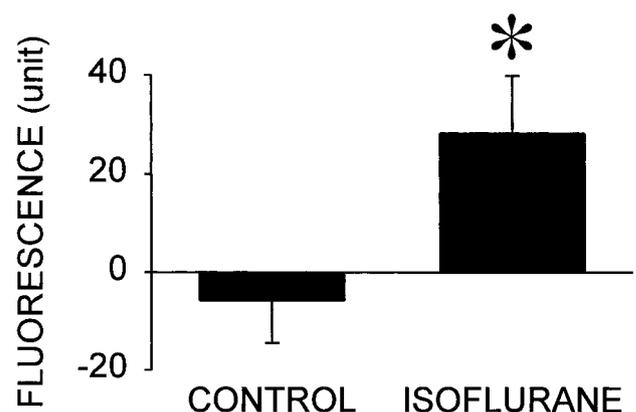

Fig. 4. Histogram depicting the effects of isoflurane on superoxide anion production measured using dihydroethidium staining. Fluorescence of ethidium bound to nuclear DNA was significantly (*$P < 0.05$) increased by administration of isoflurane.

tabolism during the administration of isoflurane with or without ROS scavengers cannot be completely excluded from the analysis. However, no differences in primary hemodynamic determinants of myocardial oxygen consumption were observed, and an indirect indicator (*i.e.*, rate-pressure product) of myocardial oxygen consumption was also similar between experimental groups.

Experiments using dihydroethidium as an indicator of superoxide anion production provide primarily qualitative results. Dihydroethidium can catalyze the dismutation of superoxide anion, thus, rates of superoxide anion production may be underestimated using this technique.[36] In addition, cytochrome *c* can oxidize dihydroethidium.[36] It is unlikely that cytochrome *c* release occurred in experiments during which dihydroethidium was used because animals were not subjected to ischemia and reperfusion. Isoflurane is unlikely to cause cytochrome *c* release because this anesthetic has previously been demonstrated to decrease apoptosis and inhibit caspase activation in ventricular myocytes.[37]

In summary, the present results provide direct evidence that isoflurane generates ROS in rabbit ventricular myocardium *in vivo* using a laser fluorescence confocal microscopic imaging technique. The present findings further indicate that scavenging of these ROS by NAC and 2-MPG abolishes myocardial protection produced by isoflurane. These findings suggest that generation of ROS by volatile anesthetics is an essential part of the signaling pathway of anesthetic-induced preconditioning.

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