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Anesthetic Effects on Mitochondrial ATP-sensitive K Channel

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Background: **Volatile anesthetics show an ischemic preconditioning–like cardioprotective effect, whereas intravenous anesthetics have cardioprotective effects for ischemic–reperfusion injury. Although recent evidence suggests that mitochondrial adenosine triphosphate–regulated potassium (mitoK$_{ATP}$) channels are important in cardiac preconditioning, the effect of anesthetics on mitoK$_{ATP}$ is unexplored. Therefore, the authors tested the hypothesis that anesthetics act on the mitoK$_{ATP}$ channel and mitochondrial flavoprotein oxidation.**

Methods: **Myocardial cells were isolated from adult guinea pigs. Endogenous mitochondrial flavoprotein fluorescence, an indicator of mitochondrial flavoprotein oxidation, was monitored with fluorescence microscopy while myocytes were exposed individually for 15 min to isoflurane, sevoflurane, propofol, and pentobarbital. The authors further investigated the effect of 5-hydroxydeanoate, a specific mitoK$_{ATP}$ channel antagonist, on isoflurane- and sevoflurane-induced flavoprotein oxidation. Additionally, the effects of propofol and pentobarbital on isoflurane-induced flavoprotein oxidation were measured.**

Results: **Isoflurane and sevoflurane induced dose-dependent increases in flavoprotein oxidation (isoflurane: R2 = 0.71, n = 50; sevoflurane: R2 = 0.86, n = 20). The fluorescence increase produced by both isoflurane and sevoflurane was eliminated by 5-hydroxydeanoate. Although propofol and pentobarbital showed no significant effects on flavoprotein oxidation, they both dose-dependently inhibited isoflurane-induced flavoprotein oxidation.**

Conclusions: **Inhalational anesthetics induce flavoprotein oxidation through opening of the mitoK$_{ATP}$ channel. This may be an important mechanism contributing to anesthetic-induced preconditioning. Cardioprotective effects of intravenous anesthetics may not be dependent on flavoprotein oxidation, but the administration of propofol or pentobarbital may potentially inhibit the cardioprotective effect of inhalational anesthetics.**

BRIEF periods of cardiac ischemia and reperfusion exert a protective effect against subsequent, more prolonged ischemia, a phenomenon termed ischemic preconditioning.[1] Preconditioning-like effects after administration of various pharmacologic agents have also been reported. Specifically, it has been demonstrated that volatile anesthetics have a cardioprotective effect on ischemic–reperfusion injury.[2–10]

Although the mechanisms of ischemia- and anesthetic-induced preconditioning have not been clearly elucidated, evidence indicates involvement of adenosine triphosphate–regulated potassium (K_{ATP}) channels. Based on previous investigations, the beneficial effects of K_{ATP} channel openers have been attributed entirely to modulation of sarcolemmal K_{ATP} channels.[7,8] However, it is known that the inner membrane of mitochondria has a highly selective channel for potassium that is also sensitive to ATP.[11] Recent evidence has shown a poor correlation between sarcolemmal K_{ATP} currents and cardioprotection by K_{ATP} channel openers.[12] Additionally, mitochondrial K_{ATP} (mitoK$_{ATP}$) activation preserves cardiac mitochondria during hypoxia.[13] Therefore, it is possible that mitoK$_{ATP}$ channel opening may be an important mechanism of preconditioning.[12,14–17] It is unknown whether anesthetics affect mitoK$_{ATP}$ currents leading to cardioprotection, although indirect evidence has been provided in dogs.[18]

Although it has been shown that propofol has a cardioprotective effect in an isolated heart preparation, the mechanism seems to be different from that by which inhalational anesthetics protect from ischemia. Additionally, intravenous anesthetics have been shown to have inhibitory effects on the mitochondrial respiratory chain.[19] Therefore, we also examined the direct effects of the intravenous agents propofol and pentobarbital on mitochondrial redox state and possible interaction with inhalational anesthetics.

We reasoned that anesthetics affect the mitoK$_{ATP}$ channel and mitochondrial respiratory chain, and the current study was designed to test this hypothesis. To confirm that alteration of inhalational anesthetic-induced flavoprotein oxidation depends on the mitoK$_{ATP}$ channel, we used the highly specific mitoK$_{ATP}$ channel antagonist 5-hydroxydecanoate (5-HD)[15] and investigated the effects of propofol and pentobarbital alone and on the changes in mitochondrial redox state induced by isoflurane.

Materials and Methods

This study was conducted according to US National Institutes of Health standards[20] and was approved by the institutional Animal Care Committee (Medical College of Wisconsin, Milwaukee, WI).

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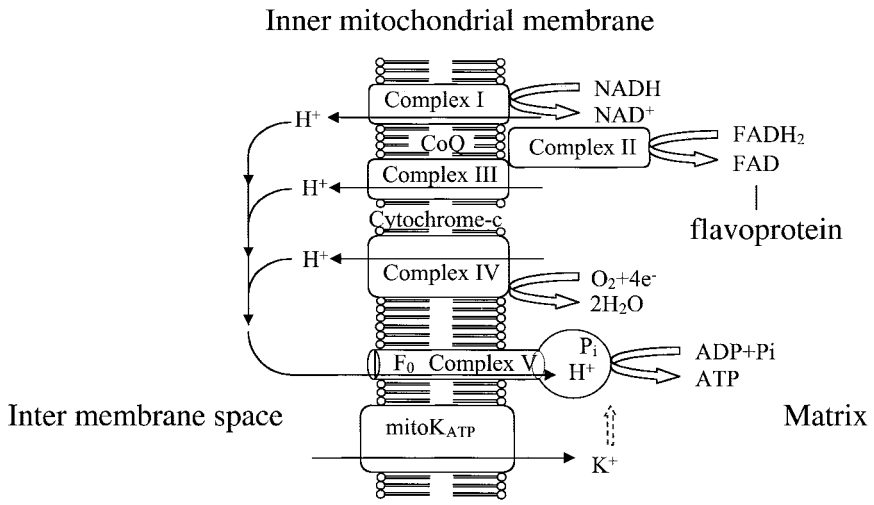

Fig. 1. Schematic representation of the relationship between mitochondrial respiratory chain and mitochondrial adenosine triphosphate–regulated potassium (mitoK$_{ATP}$) channel. MitoK$_{ATP}$ opening induces K^+ inflow and mitochondrial membrane depolarization. Subsequently, the driving force of H^+ to produce ATP at site V is weakened, and compensatory mitochondrial respiration is activated. As a result, NADH and FADH$_2$ are oxidized. (Modified from Coffee CJ: Metabolism. Madison, Connecticut, Fence Creek, 1998, pp 92.)

Preparation of Guinea Pig Cardiac Myocytes

Single cardiac myocytes were isolated from ventricles of guinea pigs weighing 200–300 g. The cell isolation procedure has been described previously.[21] Guinea pigs were first injected intraperitoneally with sodium pentobarbital (70 mg/kg) and 1,000 U heparin. During deep anesthesia, the thoracic cavities were opened, and the hearts were quickly excised. The hearts were then mounted on a Langendorff apparatus and perfused in retrograde fashion *via* the aorta with an oxygenated buffer solution containing Joklik minimum essential medium (Gibco, Life Technologies, Gaithersburg, MD). After blood was cleared from the hearts, they were perfused for approximately 14 min in an enzyme solution containing Joklik medium, 0.4 mg/ml collagenase (type II; Gibco), and 0.17 mg/ml protease (type XIV; Sigma, St. Louis, MO). The digested ventricular tissue was then chopped coarsely into small fragments and shaken in a water bath for further dispersion. The dispersed cells were filtered, centrifuged, and washed in a recovery solution containing Joklik medium, 1 mM $CaCl_2$, and 1 g/100 ml bovine albumin fraction V (Serologicals, Milwaukee, WI). Additional washing in Tyrode solution was performed before the cells were ready for experiments. Only rod-shaped cells with clear borders and striations were selected for experiments, and they were used within 12 h of isolation.

Flavoprotein Fluorescence Measurements

Because of the effect on the mitochondrial redox state, mitoK$_{ATP}$ currents may be indirectly measured by fluorescent determination of the oxidation of flavoprotein, a flavin adenine dinucleotide–linked enzyme.[15,16,22] Cells were superfused with a modified glucose-free Tyrode solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, and 2 mM $CaCl_2$ (adjusted to pH 7.4 with NaOH) at room temperature (21°C). Autofluorescence of flavin adenine dinucleotide–linked enzymes (flavoprotein fluorescence) in the mitochondria[15] (fig. 1) was excited every 30 s with light from a xenon laser bandpass filtered to 488 ± 20 nm. Emitted fluorescence was passed through a 515-nm-long pass filter, and the relative fluorescence was averaged during the excitation.

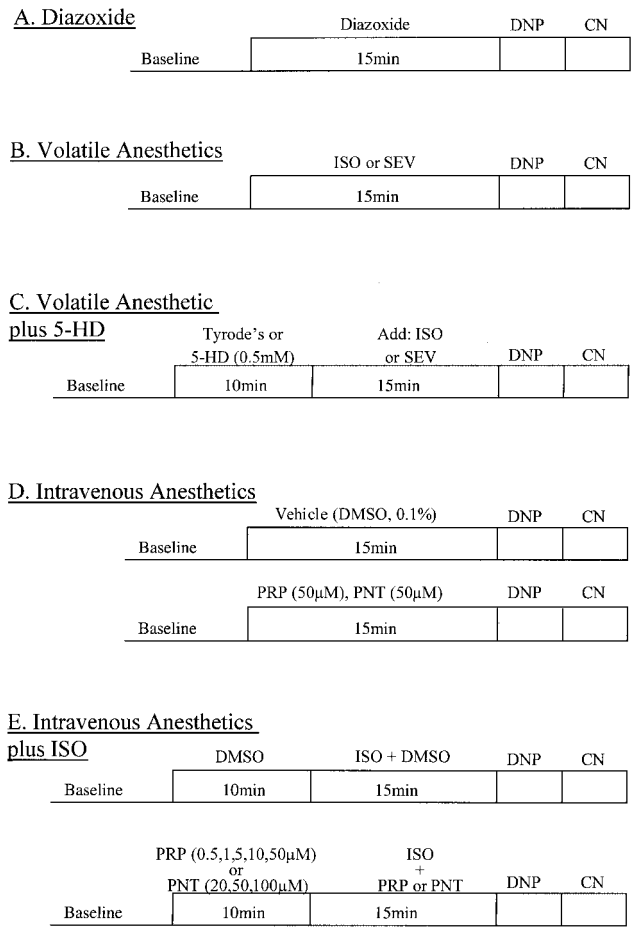

Fig. 2. (*A–E*) Experimental protocols. Baseline indicates a period of no experimental intervention. In all studies, the fluorescence level was calibrated by dinitrophenol (DNP) and cyanide (CN) at the end of each protocol. 5-HD = 5-hydroxydecanoic acid; ISO = isoflurane; SEV = sevoflurane; DMSO = dimethyl sulfoxide; PRP = propofol; PNT = pentobarbital.

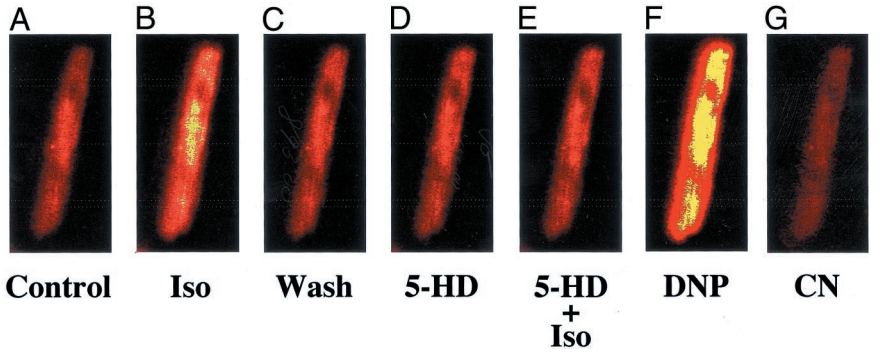

Fig. 3. Fluorescence images of mitochondrial flavoprotein in a cardiac myocyte: (*A*) baseline (23% fluorescence), (*B*) oxidation by 1.6 mM (44%) isoflurane (ISO), (*C*) wash (23%), (*D*) exposure to 5-hydroxydecanoic acid (5-HD; 24%), (*E*) combined exposure to isoflurane and 5-hydroxydecanoic acid (26%), (*F*) maximal oxidation with dinitrophenol (DNP; 100% by definition), and (*G*) minimal oxidation with cyanide (CN; 0% by definition).

Fluorescence images were obtained with ×40 oil immersion objective lenses on a Nikon inverted microscope (Nikon, Inc., Melville, NY). The values of fluorescence intensity were expressed as arbitrary units (range 0–255 using Meta Morph version 2, Universal Imaging Corp., Downingtown, PA). At the end of each protocol, flavoprotein oxidation was calibrated[15,16] with dinitrophenol (100 μM), an uncoupler of oxidative phosphorylation that releases protons in the mitochondrial matrix, and with cyanide (4 mM), which blocks mitochondrial respiration distally at the level of cytochrome-c oxidase.[23]

Experimental Protocols

Effects of Diazoxide on Flavoprotein Fluorescence. After stabilization in glucose-free Tyrode solution for 10 min, 100 μM diazoxide was administered for 15 min as shown in figure 2A. Every 20 s, an image was taken and average fluorescence intensity was calculated. The peak effect was recorded.

Effects of Inhalational Anesthetics on Flavoprotein Fluorescence. After stabilization in glucose-free Tyrode solution for 10 min, isoflurane or sevoflurane was administered for 15 min as shown in figure 2B. Every 30 s, an image was taken and average fluorescence intensity was calculated. Concentrations of isoflurane and sevoflurane in the recording chamber were measured by gas chromatography (GC-8A; Shimadzu, Columbia, MD).

Effects of 5-HD on Isoflurane- and Sevoflurane-induced Flavoprotein Oxidation. Myocytes were initially equilibrated for 10 min with glucose-free Tyrode buffer. After pretreatment with 5-HD (500 μM) or drug vehicle (glucose-free Tyrode buffer) for 10 min, isoflurane or sevoflurane plus vehicle or 5-HD (500 μM) were administered for 15 min (fig. 2C).

Direct Effects of Propofol and Pentobarbital on Flavoprotein Fluorescence and on Isoflurane-induced Flavoprotein Fluorescence. We tested whether the vehicle dimethyl sulfoxide (DMSO), propofol, or pentobarbital alone have any direct effect on flavoprotein oxidation (fig. 2D). In addition, after equilibration with glucose-free Tyrode buffer, propofol (0.5, 1, 5, 10, and 50 μM) or pentobarbital (20, 50, and 100 μM) were administered for 10 min, followed by simultaneous administration of isoflurane (1.1–1.3 mM) plus propofol or pentobarbital (fig. 2E).

Materials

The following drugs and chemicals were used in this study: Joklik modified minimum essential medium, type II collagenase (Gibco, Grand Island, NY); bovine serum albumin (Bayer, Kankakee, IL); protease, pentobarbital, dinitrophenol, cyanide (Sigma, St. Louis, MO); propofol, 5-hydroxydecanoic acid (Research Biochemicals International, Natick, MA); isoflurane (Abbott Laboratories, Madison, IL); and sevoflurane (Maruishi, Osaka, Japan).

Statistical Analysis

Data are presented as mean ± SD. Paired or unpaired *t* tests were used to verify differences in fluorescence data. Regression analysis was used for the effects of isoflurane and sevoflurane on flavoprotein oxidation.

Results

Effect of Diazoxide on Flavoprotein Fluorescence

The effects of diazoxide, a specific mitoK$_{ATP}$ agonist, were examined in 10 myocytes. Diazoxide increased

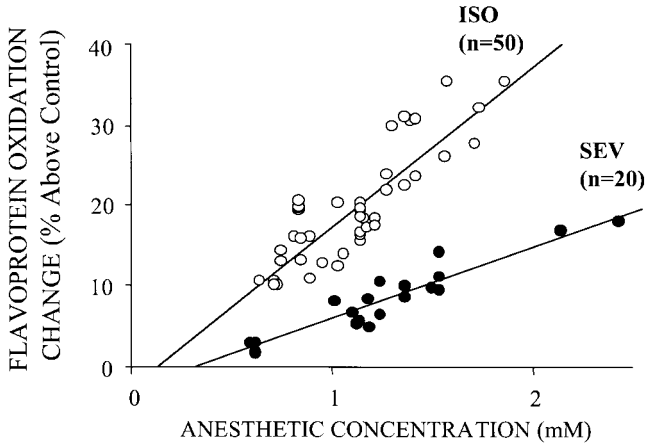

Fig. 4. The relation between relative flavoprotein fluorescence change from control and anesthetics concentration (*open circles*, isoflurane [ISO]: n = 50; *closed circles*, sevoflurane [SEV]: n = 20). The effect of isoflurane is significantly stronger than that of sevoflurane ($P < 0.01$).

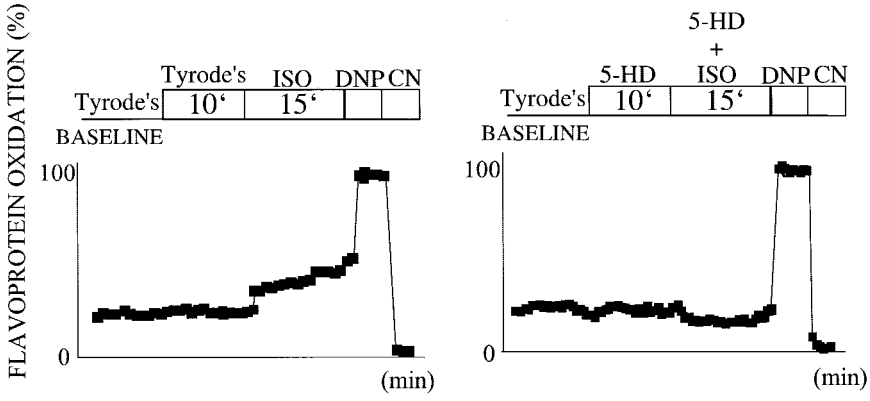

Fig. 5. Inhibitory effect of 5-hydroxydecanoic acid (5-HD) on isoflurane (ISO)- and sevoflurane (SEV)-induced flavoprotein oxidation. Representative tracing for effect of isoflurane- and 5-HD-plus-isoflurane–induced flavoprotein oxidation. Glucose-free Tyrode solution was used as vehicle for isoflurane alone. DNP = dinitrophenol; CN = cyanide.

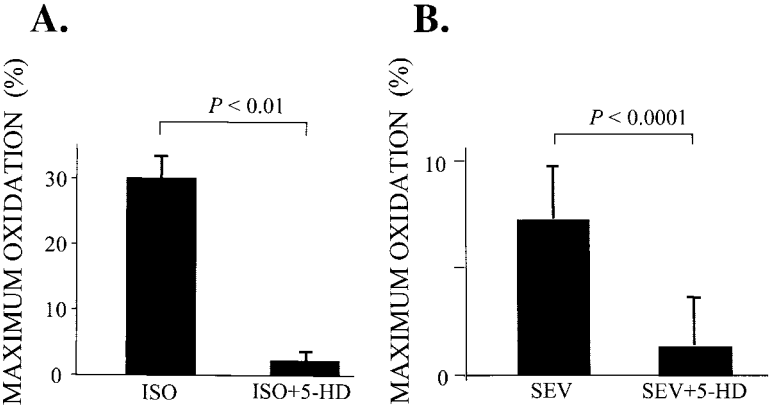

Fig. 6. The maximum flavoprotein oxidation changes from control by (*A*) isoflurane (ISO) and (*B*) sevoflurane (SEV) were significantly ($P < 0.01$, both) inhibited by 5-hydroxydecanoic acid (5-HD).

flavoprotein fluorescence from 6 ± 4% to 22 ± 14% ($P < 0.01$).

Effects of Isoflurane and Sevoflurane on Flavoprotein Fluorescence

Isoflurane administration increased flavoprotein fluorescence (fig. 3). Both isoflurane and sevoflurane administration for 15 min induced dose-dependent flavoprotein oxidation (isoflurane: $Y = -2.4 + 19.7X$, $r^2 = 0.71$; sevoflurane: $Y = -2.4 + 8.8X$, $r^2 = 0.86$, fig. 4). The effect of isoflurane was significantly stronger than that of sevoflurane ($P < 0.01$).

Inhibitory Effect of MitoK_{ATP} Blocker 5-HD on Inhalational Anesthetic-induced Flavoprotein Oxidation

5-Hydroxydecanoic acid did not have any direct effect on flavoprotein fluorescence (fig. 3). 5-HD inhibited the effect of isoflurane and sevoflurane on flavoprotein fluorescence (figs. 5 and 6). Measured anesthetic concentrations were the same during control (isoflurane, 1.3–1.9 mM; sevoflurane, 0.9–1.2 mM) and 5-HD administration (isoflurane, 1.4–1.9 mM; sevoflurane, 0.7–0.8 mM).

Effect of Propofol and Pentobarbital on Flavoprotein Oxidation and on Isoflurane-induced Flavoprotein Oxidation Change

Neither propofol (50 μM) nor pentobarbital (50 μM) showed a significant effect on flavoprotein oxidation ($P = 0.48$ and 0.86, respectively). Both propofol and pentobarbital inhibited isoflurane (1.1–1.3 mM)-induced flavoprotein oxidation dose-dependently (fig. 7). Isoflurane concentrations were not significantly different during control and drug administration. The vehicle (DMSO) did not show any effect on isoflurane-induced flavoprotein oxidation (21.8 ± 8.6 *vs.* 21.7 ± 8.1 arbitrary units, control *vs.* DMSO, $P = 0.98$).

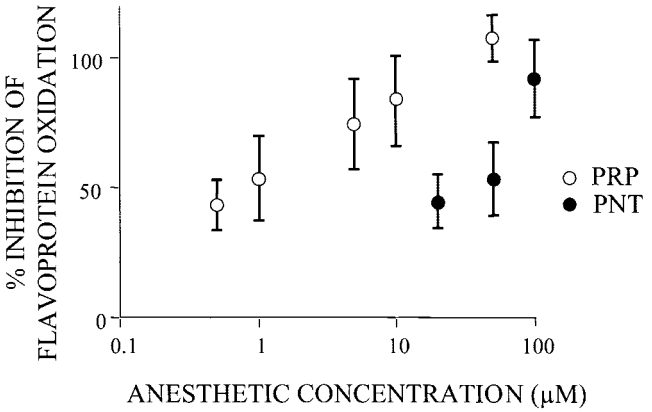

Fig. 7. Dose-dependent inhibition of isoflurane-induced flavoprotein oxidation by propofol (PRP) and pentobarbital (PNT). Data are presented as percent control. Values are mean ± SD. n = 5 for 20 μM pentobarbital; n = 6 for 50 μM pentobarbital; n = 7 for 100 μM pentobarbital; n = 6 for 0.5, 1, and 50 μM propofol; n = 5 for 5 and 10 μM propofol.

Discussion

This study examined the effects of various anesthetics on mitochondrial flavoprotein oxidation in guinea pig myocytes. The principal findings are that administrations of inhalational anesthetics isoflurane and sevoflurane produced an increase in flavoprotein oxidation through mitoK_{ATP} opening. Although the intravenous anesthetics propofol and pentobarbital had no direct effect on mitoK_{ATP} opening, they did inhibit isoflurane-induced flavoprotein oxidation.

Recent evidence shows that mitoK_{ATP} channel openers may produce cardiac preconditioning.[14,15] A mitochondrial site of action is supported by the recent finding that diazoxide preserves function and morphology of isolated cardiac mitochondria during hypoxia.[13] However, the exact mechanisms of the protective effects of mitoK_{ATP} channel opening are not yet clear. It is argued that activation of mitoK_{ATP} channels dissipates the inner mitochondrial membrane potential, weakening the driving force for ATP synthesis at site V, thus triggering a compensatory activation of the mitochondrial respiratory chain and mitochondrial flavoprotein oxidation (fig. 1).[15,16,24] However, other data indicate that the dominant effect of opening mitoK_{ATP} channels is an increase in mitochondrial matrix volume with minimal direct effect on membrane potential.[25] Additional uncertainty is due to the complex and incompletely resolved actions of agents used to study these phenomena. Diazoxide is clearly a selective opener of mitoK_{ATP} channels, but it has additional and possibly important effects on mitochondrial substrate metabolism.[26] In this context, our demonstration that diazoxide induces mitochondrial oxidation is supportive of a role of mitoK_{ATP} channels but cannot be conclusive.

We used a mitochondrial-specific K_{ATP} channel blocker 5-HD[16,27,28] to demonstrate that anesthetic-induced flavoprotein oxidation is mediated by mitoK_{ATP} channel opening. In an intact animal model of myocardial infarction, 5-HD has been found to limit isoflurane-induced preconditioning.[29] In combination, these observations strongly point to a role for mitoK_{ATP} channel activation and mitochondrial oxidation in the protective effects of isoflurane. However, it should be recognized that additional effects of 5-HD on sarcolemmal K_{ATP} channels may contribute to its preconditioning effects[30] but would not affect our findings. Inhalational anesthetics are also known to uncouple mitochondrial respiration from ATP generation.[31] Anesthetic-induced mitoK_{ATP} channel opening, as we observed, may be the mechanism of this uncoupling effect.[2,3,8]

Our data further show that propofol and pentobarbital have no significant effect on the baseline flavoprotein oxidation. Mathur *et al.*[32] reported that propofol provides cardioprotection for ischemic–reperfusion injury through a mechanism not mediated by the K_{ATP} channel, in accordance with our findings. The mechanism of their interference with isoflurane-induced flavoprotein oxidation is not known.

From previously published data on inhalational anesthetic preconditioning, anesthetic administration (15 min) is effective in producing cardiac preconditioning *in vitro*[32] and *in vivo*.[2,7] Although we used the same time interval for the administration of anesthetics in our preparation of isolated nonbeating myocytes at room temperature, we can not be certain that the mitochondrial redox states in previous studies with beating intact hearts are comparable.

In summary, we have determined that volatile anesthetics activate mitoK_{ATP} channels and induce flavoprotein oxidation. This may be a process contributing to volatile anesthetic-induced cardiac protection. Although the intravenous anesthetics studied have no effect on flavoprotein oxidation, they inhibit the flavoprotein oxidation induced by isoflurane. It is possible that this interaction is clinically important and that intravenous agents may block volatile anesthetic protection. Also, consideration should be given to the choice of background anesthetic during studies of volatile anesthetic-induced preconditioning.

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