 

# *Contribution of Reactive Oxygen Species to Isoflurane-induced Sensitization of Cardiac Sarcolemmal Adenosine Triphosphate–sensitive Potassium Channel to Pinacidil*

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***Background:*** **Myocardial protection by volatile anesthetics involves activation of cardiac adenosine triphosphate–sensitive potassium ($K_{ATP}$) channels. The authors have previously shown that isoflurane enhances sensitivity of the sarcolemmal $K_{ATP}$ channel to the opener, pinacidil. Because reactive oxygen species seem to be mediators in anesthetic preconditioning, the authors investigated whether they contribute to the mechanism of the sensitization effect by isoflurane.**

***Methods:*** **Ventricular myocytes were isolated from guinea pig hearts for the whole cell patch clamp recordings of the sarcolemmal $K_{ATP}$ channel current ($I_{KATP}$). Free radical scavengers *N*-acetyl-L-cysteine, carnosine, superoxide dismutase, and catalase were used to investigate whether reactive oxygen species mediate isoflurane facilitation of the channel opening by pinacidil. A possible role of the mitochondrial $K_{ATP}$ channels was tested using a blocker of these channels, 5-hydroxydecanoate.**

***Results:*** **The mean density (± SEM) of $I_{KATP}$ elicited by pinacidil (20 $\mu$M) was 18.9 ± 1.8 pA/pF (n = 11). In the presence of isoflurane (0.55 mM), the density of pinacidil-activated $I_{KATP}$ increased to 38.5 ± 2.4 pA/pF (n = 9). Concurrent application of isoflurane and *N*-acetyl-L-cysteine decreased the sensitization effect by isoflurane in a concentration-dependent manner, whereby the densities of $I_{KATP}$ were 32.6 ± 1.4 (n = 6), 26.2 ± 2.3 (n = 6), and 19.4 ± 2.1 pA/pF (n = 8) at 100, 250, and 500 $\mu$M *N*-acetyl-L-cysteine, respectively. Concurrent application of isoflurane and carnosine (100 $\mu$M), superoxide dismutase (100 U/ml), or catalase (100 U/ml) attenuated the densities of $I_{KATP}$ to 27.9 ± 2.6, 27.2 ± 2.9, and 25.9 ± 2.2 pA/pF, respectively. None of the scavengers affected activation of $I_{KATP}$ by pinacidil alone. 5-Hydroxydecanoate (100 $\mu$M) did not alter the sensitization effect by isoflurane, and the density of $I_{KATP}$ in this group was 37.1 ± 3.8 pA/pF (n = 6).**

***Conclusion:*** **These results suggest that reactive oxygen species contribute to the mechanism by which isoflurane sensitizes the cardiac sarcolemmal $K_{ATP}$ channel to the opener, pinacidil.**

THE cardiac sarcolemmal (sarc) and mitochondrial (mito) adenosine triphosphate–sensitive potassium ($K_{ATP}$) channels are thought to mediate the protection afforded by ischemic preconditioning (IPC) against the consequences of a prolonged ischemic injury.[1,2] The preconditioning by volatile anesthetics (APC) seems as effective as IPC in protecting the heart against ischemia–reperfusion injury by decreasing myocardial infarct size and improving postischemic functional recovery.[3,4] Importantly, both the sarc and mito $K_{ATP}$ channels have been indicated in the mechanism of APC.[3–6]

The reactive oxygen species (ROS) contribute to the pathogenesis of ischemia and reperfusion injury.[7,8] In electrophysiologic studies on isolated myocytes, ROS alter the ionic currents and the action potential profile, leading to reperfusion arrhythmias.[9–11] On the other hand, a brief exposure of myocytes to oxygen radicals generated by the xanthine/xanthine oxidase reaction elicits both the early and the late protection against hypoxia–reoxygenation injury.[12] Minute amounts of ROS may exert beneficial effects during ischemia and reperfusion when released before a prolonged ischemic episode.[13] In addition, ROS contribute to APC by improving myocardial function, reducing infarct size, and decreasing dityrosine on reperfusion, and these protective effects are attenuated when bracketing APC with the free radical scavengers.[14]

The scavengers of ROS attenuate isoflurane protection against irreversible ischemic injury.[15] It has been postulated that volatile anesthetics generate small quantities of ROS that could serve as mediators of cardioprotection.[16] The scavengers of ROS, *N*-acetyl-L-cysteine (NAC) and N-2-mercaptopropionyl glycine abolish myocardial protection by isoflurane, suggesting that generation of ROS by volatile anesthetics may play a role in the mechanism of APC.[15,17] In addition, ROS have been reported to directly activate the sarc $K_{ATP}$ channel, possibly by modifying its sensitivity to adenosine triphosphate.[18,19] However, whether ROS contribute to modulation of the cardiac sarc $K_{ATP}$ channel by volatile anesthetics is unknown.

Recently, we demonstrated that isoflurane alone does not elicit opening of the sarc $K_{ATP}$ channel in isolated guinea pig ventricular myocytes under whole cell recording conditions.[20] However, when applied extracellularly, isoflurane sensitizes the sarc $K_{ATP}$ channel to its opener, pinacidil, facilitating the pinacidil-activated whole cell $I_{KATP}$.[21] The purpose of the current study was to determine whether ROS contribute to the mechanism of the sarc $K_{ATP}$ channel sensitization by isoflurane.

## Materials and Methods

The experimental procedures of this study were approved by the Animal Use and Care Committee of the Medical College of Wisconsin, Milwaukee, Wisconsin.

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*Myocyte Isolation*

Single ventricular myocytes were enzymatically isolated from the hearts of adult Hartley guinea pigs (weight, 170–300 g) using a modified procedure of Mitra and Morad,[22] as previously reported.[20] Animals were anesthetized with pentobarbital sodium (240 mg/kg intraperitoneal) and injected with 1,000 U/ml heparin. After thoracotomy, the hearts were excised, mounted on a Langendorff apparatus, and perfused retrogradely *via* the aorta with oxygenated Joklik medium (Gibco BRI; Invitrogen, Grand Island, NY) containing 2.5 U/ml heparin. After clearing blood from the heart, this medium was replaced with an enzyme solution containing 0.4 mg/ml collagenase type II (Gibco BRI), 0.1 mg/ml protease XIV (Sigma-Aldrich, St. Louis, MO), and 1 mg/ml bovine serum albumin (Serologicals, Kankakee, IL) in the Joklik medium at a pH of 7.23. All solutions were continuously bubbled with a 95% $O_2$–5% $CO_2$ gas mixture and maintained at 37°C. After 14 min of enzyme treatment, the ventricles were excised, minced, and incubated in the enzyme solution for an additional 3–10 min in a shaker bath. The cell suspension was filtered through a 200-$\mu$m mesh and centrifuged. The cells were washed twice and stored in a Tyrode solution at 20–22°C for experiments that were completed within 8–10 h.

*Solutions*

The modified Tyrode solution contained 132 mM NaCl, 4.8 mM KCl, 1.2 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose at a pH of 7.4 with NaOH. The intracellular/pipette solution contained 60 mM K-glutamate, 50 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$, and 0.5 mM $K_2$ATP at a pH of 7.4 with KOH. The extracellular/bath solution contained 132 mM *N*-methyl-D-glucamine, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES at a pH of 7.4 with HCl. Nisoldipine (Miles-Pentex, West Haven, CT) was added to the external solution at 200 nM to block the L-type Ca channels. The 10-mM stock of pinacidil, a potassium channel opener, was prepared in 0.1 N HCl. The 1-mM stock of glyburide, a $K_{ATP}$ channel blocker, was prepared in dimethyl sulfoxide. A blocker of mito $K_{ATP}$ channel, 5-hydroxydecanoate (5-HD), was used in the bath solution at 100 $\mu$M. The ROS scavengers—NAC, carnosine ($\beta$-alanyl-L-histidine), superoxide dismutase, and catalase—were applied in the extracellular solution. The stocks of NAC (25 mM) and carnosine (5 mM) were prepared in distilled water. Superoxide dismutase and catalase were added directly to the extracellular solution immediately before use at a final (100 U/ml) concentration of each enzyme. All chemicals were purchased from Sigma-Aldrich. Isoflurane (Abbott Laboratories, North Chicago, IL) was sonicated into the bath solution that was delivered to the recording chamber from the airtight glass syringe reservoirs. The concentrations of isoflurane in the recording chamber were measured by the head space analysis method using a Schimadzu GC-8A gas chromatograph (Schimadzu, Kyoto, Japan). The average concentration of isoflurane in this study was 0.55 ± 0.07 mM (n = 35), equivalent to 1.0 ± 0.1 vol% at 22°C.

*Patch Clamp Recordings*

The sarc $K_{ATP}$ current was measured in the whole cell configuration of the patch clamp technique[23] using the EPC-7 amplifier (List, Darmstadt-Eberstadt, Germany), Digidata 1322A interface (Axon Instruments, Foster City, CA) and a personal computer. pClamp8 software (Axon Instruments) was used for data acquisition and analysis. Pipettes were pulled form borosilicate glass tubing (Garner Glass, Claremont, CA) with a multistage PC-84 puller (Sutter, Novato, CA) and were heat polished with a microforge MF-83 (Narishige, Tokyo, Japan). The resistance of patch pipettes ranged from 2 to 3 MΩ. The experiments were conducted in the recording chamber mounted on the stage of an inverted IMT2 microscope (Olympus, Tokyo, Japan). Only quiescent, rod-shaped myocytes with distinct striations were used for patch clamp recordings. After forming a gigaohm seal, the whole cell configuration was established by cell membrane rupture. The series resistance was then adjusted electronically to give the fastest possible capacitance transient without producing noise.

Whole cell $I_{KATP}$ was monitored over time during a 100-ms depolarizing voltage step to 0 mV from a holding potential of −40 mV applied every 15 s. Current amplitude was measured at the end of each voltage step. Current was normalized to cell capacitance to determine current density (pA/pF). Four sets of experimental protocols were used. Protocol A consisted of (1) 30 min of baseline control to allow for diffusion of the pipette solution containing 0.5 mM adenosine triphosphate to the cell; (2) 5–10 min of exposure to pinacidil (20 $\mu$M) to reach a steady state of current activation; and (3) 5 min of exposure to pinacidil plus glyburide (1 $\mu$M) to block the current and thus confirm $I_{KATP}$ identity. Protocol B consisted of (1) 20 min of baseline control as above; (2) 10 min of cell exposure to isoflurane (0.55 mM); (3) 5–10 min of exposure to isoflurane plus pinacidil (20 $\mu$M); and (4) 5 min of exposure to isoflurane plus pinacidil plus glyburide. In protocol C, after a 20 min baseline control, the cells were exposed for 10 min to a ROS scavenger alone before application of pinacidil together with a scavenger. In protocol D, the ROS scavengers were applied in the extracellular solution concurrently with isoflurane and were present during all remaining steps of the protocol. NAC was used at 100, 250, and 500 $\mu$M; carnosine was used at 100 $\mu$M; superoxide dismutase was used at 100 U/ml; and catalase was used at 100 U/ml.

*Statistical Analysis*

Data were analyzed using Clampfit 8 software (Axon Instruments) and Origin 6 software (OriginLab, Northampton, MA). Results are expressed as mean ±

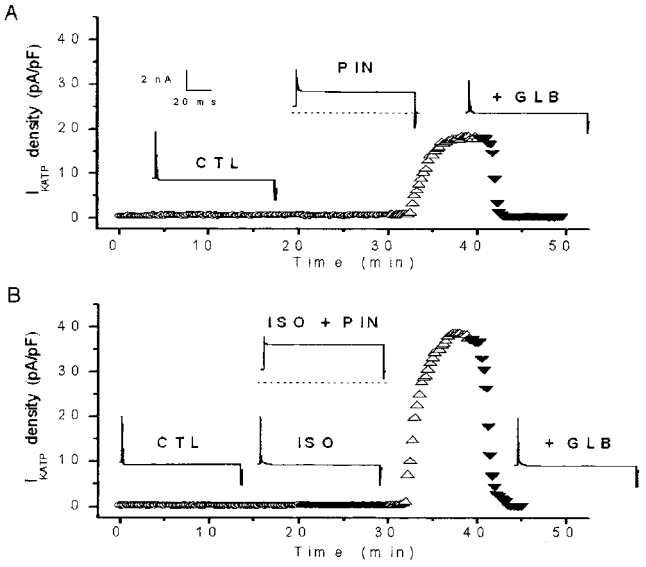


**Fig. 1. Effect of isoflurane on activation of the sarcolemmal adenosine triphosphate–sensitive potassium channel current ($I_{KATP}$) by pinacidil at 0.5 mM intracellular adenosine triphosphate. (*A*) Example traces of whole cell $I_{KATP}$ monitored during a 100-ms voltage pulse to 0 mV from the holding potential of −40 mV in the absence (CTL) and presence of 20 μM pinacidil (PIN) and corresponding time course of $I_{KATP}$ elicited by pinacidil (protocol A). Pinacidil-activated current was blocked by 1 μM glyburide (GLB). (*B*) Time course of $I_{KATP}$ and example traces of current elicited by pinacidil in the presence of 0.55 mM isoflurane (protocol B). Compared with *A*, the pinacidil-elicited $I_{KATP}$ was increased in the presence of isoflurane (ISO + PIN). Glyburide, 1 μM, blocked current elicited by pinacidil. *Dotted lines* = zero current level.**

SEM. Statistical analysis was performed using the Student *t* test and analysis of variance for repeated measures. Differences were considered significant when the *P* value was less than 0.05.

## Results

### *Effect of Isoflurane on Sensitivity of Sarcolemmal $K_{ATP}$ Channel to Pinacidil*

Figure 1A shows the representative traces and a time course of the whole cell $I_{KATP}$ activated by pinacidil. Under conditions of our study, whereby the concentration of intracellular adenosine triphosphate was nominally 0.5 mM, the whole cell $I_{KATP}$ did not activate spontaneously in the absence of the $K_{ATP}$ channel opener, pinacidil. When pinacidil (20 μM) was added to the extracellular solution (protocol A, as described in Materials and Methods), a time-independent outward current with a mean density of 18.95 ± 1.83 pA/pF (n = 11) was elicited. Current was blocked by 1 μM glyburide. Using protocol B, where isoflurane (0.55 mM) was present in the external solution before and during application of pinacidil, the enhancement of pinacidil-activated whole cell $I_{KATP}$ was evident (fig. 1B). Compared with the isoflurane-free control, the density of $I_{KATP}$ activated by pinacidil was markedly increased in the presence of isoflurane (38.5 ± 2.4 pA/pF; n = 9). This confirmed our previous findings[21] that suggested that isoflurane sensitizes the sarc $K_{ATP}$ channel to pinacidil.

### *Effects of NAC on Isoflurane Sensitization*

To test the hypothesis that ROS mediate the sensitization effect by isoflurane, a free radical scavenger, NAC, was applied in the extracellular solution at 100, 250, and 500 μM. Using protocol C, we first determined whether at a high, 500 μM concentration NAC affects the activation of $I_{KATP}$ by pinacidil alone. Figure 2A shows a time course of current activation by pinacidil in the presence of 500 μM NAC. The mean current density measured in these experiments was 18.85 ± 1.8 pA/pF (n = 6), indicating that NAC does not affect activation of $I_{KATP}$ by pinacidil. However, the results from protocol D showed that NAC altered in a concentration-dependent manner the sensitization effect by isoflurane. As shown in figure 2B, NAC at 100 and 250 μM attenuated the effects of isoflurane, and NAC at 500 μM abolished isoflurane sensitization. The mean current densities were 32.6 ± 1.4 pA/pF with 100 μM NAC (n = 6), 26.2 ± 2.3 pA/pF with 250 μM NAC (n = 6), and 19.4 ± 2.1 pA/pF with 500 μM NAC (n = 8). These results suggested that ROS

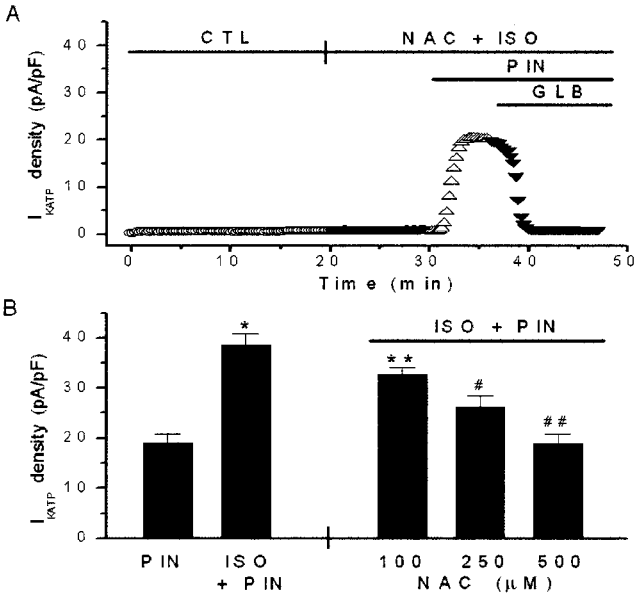


**Fig. 2. *N*-acetyl-L-cysteine (NAC) abolishes the sensitization effect by isoflurane. (*A*) Time course of sarcolemmal adenosine triphosphate–sensitive potassium channel current ($I_{KATP}$) to show that 500 μM NAC did not affect current activation by pinacidil alone (protocol C). (*B*) Mean values for density of $I_{KATP}$ elicited by pinacidil in the absence of isoflurane (PIN), in the presence of isoflurane (ISO + PIN), and in the presence of NAC and isoflurane. NAC inhibited the sensitization effect by isoflurane in a concentration-dependent manner. * $P < 0.05$, ISO + PIN *versus* other experimental groups. ** $P < 0.05$, 100 μM NAC *versus* other groups. # $P < 0.05$, 250 μM NAC *versus* other experimental groups. ## $P < 0.05$, 500 μM NAC *versus* other groups except PIN.**

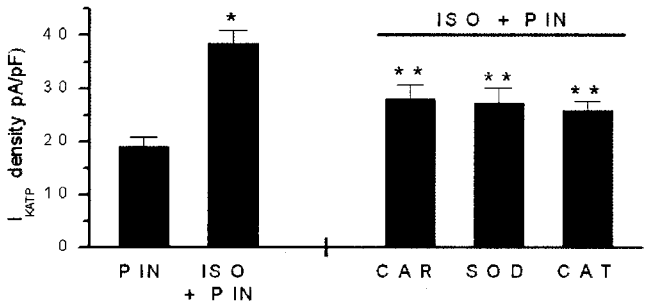


**Fig. 3. Effects of carnosine, superoxide dismutase (SOD), and catalase on isoflurane sensitization. Mean values for density of sarcolemmal adenosine triphosphate–sensitive potassium channel current ($I_{KATP}$) activated by pinacidil alone (PIN), pinacidil in the presence of isoflurane (ISO + PIN), and pinacidil in the presence of scavengers plus isoflurane. Carnosine (CAR) at 100 μM, SOD at 100 U/ml, and catalase (CAT) at 100 U/ml attenuated the sensitization effect by isoflurane. * $P$ < 0.05, ISO + PIN *versus* PIN. ** $P$ < 0.05, CAR, SOD, or CAT *versus* ISO + PIN.**

might play a role in the mechanism by which isoflurane sensitizes the sarc $K_{ATP}$ channel to pinacidil.

### *Effects of Carnosine, Superoxide Dismutase, and Catalase on Isoflurane Sensitization*

To confirm the above observations, three other ROS scavengers were tested: carnosine, superoxide dismutase, and catalase. These compounds are known to be involved in the cellular protection against damage induced by free radicals. Using protocol C, we found that, similar to NAC, these scavengers do not affect $I_{KATP}$ activation by pinacidil alone (data not shown). Results obtained with protocol D showed that carnosine at 100 μM, superoxide dismutase at 100 U/ml, and catalase at 100 U/ml all attenuated the sensitization effect by isoflurane when applied in the extracellular solution. The mean current densities were 27.9 ± 2.6 pA/pF (n = 6) with carnosine, 27.2 ± 2.9 pA/pF (n = 7) with superoxide dismutase, and 25.9 ± 2.2 pA/pF (n = 7) with catalase. The results summarized in figure 3 support in part the hypothesis of ROS mediating the sensitization effect by isoflurane.

### *Effects of 5-HD on Isoflurane Sensitization*

One metabolic pathway known to generate ROS is the mitochondrial electron transport system. Because isoflurane as well as pinacidil may activate the mito $K_{ATP}$ channels[24,25] and because opening of mito $K_{ATP}$ channels can trigger preconditioning by a mechanism involving generation of ROS,[26] we tested whether the mito $K_{ATP}$ channels play a role in the sensitization effect by isoflurane. In protocol D, 5-HD (100 μM) was applied in the extracellular solution before and during myocyte exposure to isoflurane and isoflurane plus pinacidil. 5-HD did not affect the activation of sarc $I_{KATP}$ by pinacidil alone. The current density in this group was 18.2 ± 2.6 pA/pF (n = 6) (data not shown). Interestingly, 5-HD did not alter the sensitization effect by isoflurane. As shown in figure 4, in the presence of both 5-HD and isoflurane, the mean density of pinacidil-elicited $I_{KATP}$ was 37.1 ± 3.8 pA/pF (n = 6), similar to that measured in the absence of 5-HD.

## Discussion

The sarc and mito $K_{ATP}$ channels are both involved in the mechanism of APC. Their respective roles, however, are not fully determined. Although recently the sarc $K_{ATP}$ channels have received relatively less attention than the mito $K_{ATP}$ channels, several research groups have demonstrated their importance for cellular protection.[27–32] Previously, we reported that exposure of isolated myocytes to isoflurane enhances sensitivity of the sarc $K_{ATP}$ channels to pinacidil and facilitates $K_{ATP}$ current activated by this opener. Furthermore, we demonstrated that the mechanism of isoflurane sensitization may involve multiple cellular pathways, including adenosine and phospholipid signaling.[21] The main finding from the current study is that ROS seems to be one of the mediators in the mechanism of isoflurane actions on the cardiac sarc $K_{ATP}$ channel that involve modulation of channel sensitivity to pinacidil.

Reactive oxygen species, such as superoxide radical anion, singlet oxygen, hydroxyl radical, hydrogen peroxide, and hypochlorous acid, are by-products of oxidative metabolism.[33] ROS play a role in many pathologic conditions. However, ROS may also exert beneficial effects during ischemia and reperfusion when released before a prolonged ischemic injury.[13] ROS generated by mitochondria during a brief ischemic episode produce preconditioning.[17,34,35] Volatile anesthetics are reported to generate ROS in the cardiac and vascular tissues.[16,36,37] Furthermore, ROS scavengers NAC and N-2-mercaptopropionyl glycine abolish myocardial protection by isoflurane, thus implicating a role for ROS in APC.[15]

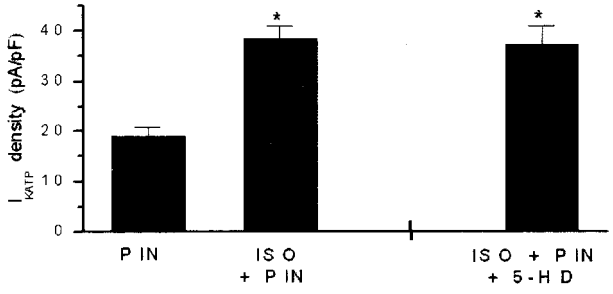


**Fig. 4. Effect of 5-hydroxydecanoate (5-HD) on isoflurane sensitization. Mean values for density of sarcolemmal adenosine triphosphate–sensitive potassium channel current ($I_{KATP}$) activated by pinacidil alone (PIN), pinacidil in the presence of 0.55 mM isoflurane (ISO + PIN), and pinacidil in the presence of 100 μM 5-HD and isoflurane (ISO + PIN + 5-HD). * $P$ < 0.05, ISO + PIN and ISO + PIN + 5-HD *versus* PIN.**

*N*-acetyl-L-cysteine is a sulfhydryl-containing glutathione precursor and endogenous substrate for glutathione peroxidase. NAC exerts its antioxidant effects *via* synthesis of glutathione and is capable of directly scavenging several oxygen-derived free radical species.[38] In our study on isolated myocytes, the activation of $I_{KATP}$ by pinacidil alone was not altered by NAC, suggesting that this compound does not interfere with pinacidil binding at the SUR2A subunit of the $K_{ATP}$ channel. However, NAC inhibited the sensitization effect by isoflurane in a concentration-dependent manner. In the presence of NAC, the pinacidil-activated $I_{KATP}$ was no longer enhanced by isoflurane. This finding suggested that ROS is involved in the sensitization effect by isoflurane.

Carnosine, an endogenous dipeptide, is present in the mammalian cardiac cells at high concentrations of up to 40 mM. Carnosine improves cardiac contractility and protects the heart against ischemia–reperfusion injury,[39–41] but the exact mechanism of this protection is unknown. In *in vitro* studies, carnosine has been shown to effectively quench singlet oxygen,[40] suggesting that it may function as a ROS scavenger. Carnosine, however, has a number of other cellular functions, such as modulation of intracellular calcium and contractility of the cardiac muscle.[42] Because of the experimental conditions of the current study, *i.e.*, whole cell configuration and very long recordings of up to 50 min, it is likely that cell perfusion with the pipette solution substantially decreased the endogenous carnosine and other scavengers in the cytosol. When applied in the extracellular solution at a relatively low (100 $\mu$M) concentration, carnosine attenuated the sensitization effect but did not abolish it. It is unlikely that this effect was due to the scavenging properties of extracellular carnosine. However, we cannot exclude the possibility that extracellular carnosine may interact directly with the sarc $K_{ATP}$ channel or other cell membrane proteins.

Superoxide dismutase and catalase are endogenous enzymes involved in removal of superoxide and hydrogen peroxide, respectively. Because of a very high molecular weight, these enzymes have limited cellular accessibility and, in addition, are rather unstable in the solution. Applied in the extracellular solution, superoxide dismutase and catalase both attenuated the sensitization effect by isoflurane, similar to carnosine. The mechanism of these effects by superoxide dismutase and catalase is unclear because under the whole cell patch clamp conditions and with continuous buffer perfusion of myocytes in the recording chamber, the extracellular accumulation of ROS that could potentially be released from the cells is unlikely. However, as with carnosine, other actions of these enzymes on the cell membrane are possible.

Opening of the mito $K_{ATP}$ channel is thought to trigger preconditioning by a mechanism involving generation of ROS.[26] Using a ROS-sensing microprobe, Obata *et al.*[43] demonstrated that openers of the mito $K_{ATP}$ channels increase the hydroxyl radical formation in the rat myocardium, and this effect is blocked by 5-HD, a putative blocker of mito $K_{ATP}$ channels.[15] Recently, it has been shown that the mito $K_{ATP}$ channel may be a trigger for APC by generating ROS in rabbit hearts *in vivo*.[17] We tested whether ROS generated *via* isoflurane activation of the mito $K_{ATP}$ channels could be involved in the mechanism of enhanced sensitivity of the sarc $K_{ATP}$ channel to pinacidil. Because 5-HD did not abolish the effects of isoflurane, this pathway seems to play a minor role, if any, in sensitization to pinacidil. However, our negative results could be related to the limitations of the experimental design. As reported recently, a local release of mitochondrial ROS causes activation of the $I_{KATP}$, indicating a coupling of the mitochondrial metabolism to cardiac electrical excitability.[44] On the other hand, it is well known that, except mitochondria, several other metabolic pathways, such as the xanthine oxidase system, the cyclooxygenase pathway of the arachidonic acid metabolism, the neutrophil system, and the amyloid $\beta$ protein system, may generate ROS.[33] Therefore, it is possible that isoflurane sensitization involves generation of ROS *via* another metabolic pathway unrelated to the opening of the mito $K_{ATP}$ channels.

In summary, the current study reports that scavengers of ROS attenuate or abolish the isoflurane sensitization effect, whereby opening of the cardiac sarc $K_{ATP}$ channel by pinacidil is enhanced in the presence of the anesthetic, suggesting that ROS mediate isoflurane effects. This study further suggests that ROS generated during APC can modulate activity of the cardiac sarc $K_{ATP}$ channel.

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