Isoflurane Activates Human Cardiac Mitochondrial Adenosine Triphosphate-Sensitive K^+ Channels Reconstituted in Lipid Bilayers

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BACKGROUND: Activation of the mitochondrial adenosine triphosphate (ATP)-sensitive K^+ channel (mitoK$_{ATP}$) has been proposed as a critical step in myocardial protection by isoflurane-induced preconditioning in humans and animals. Recent evidence suggests that reactive oxygen species (ROS) may mediate isoflurane-mediated myocardial protection. In this study, we examined the direct effect of isoflurane and ROS on human cardiac mitoK$_{ATP}$ channels reconstituted into the lipid bilayers.

METHODS: Inner mitochondrial membranes were isolated from explanted human left ventricles not suitable for heart transplantation and fused into lipid bilayers in symmetrical potassium glutamate solution (150 mM). ATP-sensitive K^+ currents were recorded before and after exposure to isoflurane and H_2O_2 under voltage clamp.

RESULTS: The human mitoK$_{ATP}$ was identified by its sensitivity to inhibition by ATP and 5-hydroxydecanoate. Addition of isoflurane (0.8 mM) increased the open probability of the mitoK$_{ATP}$ channels, either in the presence or absence of ATP inhibition (0.5 mM). The isoflurane-mediated increase in K^+ currents was completely inhibited by 5-hydroxydecanoate. Similarly, H_2O_2 (200 μM) was able to activate the mitoK$_{ATP}$ previously inhibited by ATP.

CONCLUSIONS: These data confirm that isoflurane, as well as ROS, directly activates reconstituted human cardiac mitoK$_{ATP}$ channel *in vitro*, without apparent involvement of cytosolic protein kinases, as commonly proposed. Activation of the mitoK$_{ATP}$ channel may contribute to the myocardial protective effect of isoflurane in the human heart.

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Ischemic preconditioning (IPC) refers to the phenomenon that brief periods of myocardial ischemia protect the heart against subsequent ischemia (1). Similarly, brief exposure to volatile anesthetics was also found to induce myocardial protection against subsequent ischemia acutely or after 24 h (2–6). The latter phenomenon is referred to as anesthetic-induced preconditioning (APC). Subsequent studies have shown that the mitochondrial adenosine triphosphate (ATP) sensitive K^+ channel (mitoK$_{ATP}$), located within the inner mitochondrial membrane (7), is a critical effector/mediator of both protective mechanisms (8–12). This channel is thought to be regulated by cytosolic protein kinase C (PKC) translocated to the mitochondria during preconditioning, as blockade of the PKC translocation prevents both IPC and APC in animals and humans (10,13–15). In this proposed scheme, PKC (or other cytosolic kinases) are required to traverse the physical barrier of the outer mitochondrial membranes (OMM) and to interact directly with the mitoK$_{ATP}$ located in the inner mitochondrial membrane (IMM). No evidence is available thus far to support this assertion. Findings from our recent study (16) suggest that the mitoK$_{ATP}$ channel is regulated by a local control mechanism whereby IMM-associated PKC activates the mitoK$_{ATP}$ without involvement of cytosolic components. This model is corroborated by recent evidence showing the mitoK$_{ATP}$ channel in a functional complex with PKC in the IMM (17).

Volatile anesthetics such as isoflurane, because of their lipid solubility, can cross the OMM easily and potentially interact with the mitoK$_{ATP}$ or other targets in the IMM, without the requirements of cytosolic kinases. We have shown previously that isoflurane can directly activate rat mitoK$_{ATP}$ channels reconstituted in lipid

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bilayers (18). Other studies have shown that volatile anesthetics interact with the respiratory chain in the IMM and produce reactive oxygen species (ROS) (19–22). ROS may in turn activate downstream targets (cytosolic or mitochondrial), such as PKC (20,23) or the mitoK$_{ATP}$ (24).

Several studies have shown that APC can protect human hearts against ischemia/reperfusion injury (4,25–27), but few studies have addressed the cellular mechanisms of APC in human hearts. The purpose of this study was to investigate the direct effect of isoflurane, as well as ROS, on the human cardiac mitoK$_{ATP}$ channel after reconstitution in artificial lipid bilayers. Our results suggest that both isoflurane and ROS activate the human cardiac mitoK$_{ATP}$ channels, which may contribute to isoflurane-induced myocardial preconditioning.

METHODS

Mitochondrial Isolation

The IRB for clinical studies at the Medical College of Wisconsin approved this study. The investigation conforms to the principles outlined in the Declaration of Helsinki. Human ventricular muscles were obtained from two donor hearts not suitable for transplant from brain-dead patients, with informed consent from family members, as previously described (28). Detailed patient information was previously published (28). The left ventricles in cardioplegic solution were frozen in liquid nitrogen and stored at −80°C until use. Cardiac mitochondria were isolated according to the procedure of Solem and Wallace (29) with modifications as described previously (18).

Preparation of Inner Mitochondrial Membranes

The submitochondrial fraction enriched with IMM was prepared as previously reported (18). The mitochondrial pellet was osmotically shocked by incubation in 10 mM phosphate buffer (pH 7.4) for 20 min, and then in 20% sucrose for another 15 min. Membranes were sonicated (Dual Horn for Model 550, Fisher Scientific, Hanover Park, IL) 3 times for 30 s, and centrifuged at 8000*g* for 10 min. The supernatant, containing submitochondrial particles, was fractionated using a continuous sucrose gradient (30%–60%), and then centrifuged at 80,000*g* overnight. The heavy fraction was resuspended with the isolation medium without glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) and centrifuged at 380,000*g* for 30 min. The final pellet enriched in IMM was resuspended in the isolation medium without EGTA and bovine serum albumin, and then stored at −80°C in small aliquots until use.

Reconstitution of the MitoK$_{ATP}$ Channels Into Lipid Bilayers

The IMM was reconstituted into lipid bilayers made with L-α-phosphatidylethanolamine and L-α-phosphatidylserine (Avanti Polar-Lipid, Alabaster, AL) as reported previously (18). Briefly, IMMs were

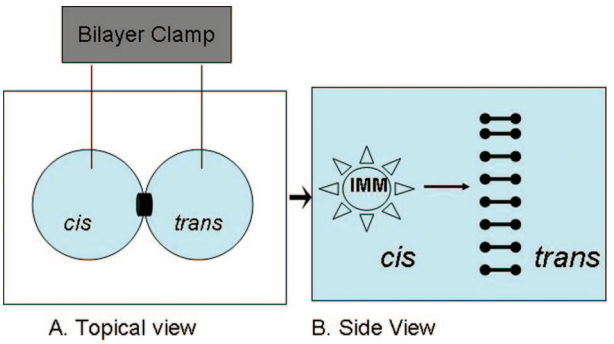

Figure 1. Schematic diagrams of a planar bilayer setup. (A) Topical view. A Delrin cup (right chamber, *trans*) with a aperture (pore size of 250 μM) was held in place in a two-chamber Delrin cutout. The left chamber is the *cis*. Phospholipids were painted across the aperture to form artificial bilayers. The chambers were connected to the headstage of an Axon amplifier via salt bridges. Voltage was applied in reference to the *cis* chamber. Membrane vesicles of inner mitochondrial membrane (IMM) and agents were added to *cis* in the presence of symmetrical 150 mM K glutamate (pH 7.2). (B) Expanded side view of the setup after bilayer formation.

added to the *cis* chamber of the artificial bilayer setup (Fig. 1) in a symmetrical solution containing: 30 mM MOPS [3-(N-morpholino)propanesulfonic acid] (pH 7.4), 150 mM potassium glutamate, 1 mM EGTA, 1.03 mM $CaCl_2$ (free Ca^{2+} 10 μM), 0.05 mM K_2ATP, and 0.5 mM $MgCl_2$. Ag/AgCl electrodes were placed into each chamber via agar salt (0.5 M KCl) bridges and the *trans* chamber was connected to the head stage of a bilayer clamp amplifier (BC-525C, Warner Instrument, Hamden, CT). The *cis* chamber was held at virtual ground, and the experiments were performed at room temperature at a holding potential of +30 or +40 mV (*trans*/*cis*, −30 or −40 mV by convention). Successful fusion was indicated by the appearance of K^+ conducting currents. The channel current measurements were digitized using an Axon Digidata 1332 AD/DA (Axon Instruments, Union City, CA) converter and collected on a PC with pClamp software (version 8.01, Axon instruments). The currents were filtered at 0.5 kHz with an 8-pole Bessel filter and digitized at 2.5 kHz. The channel activity accumulated over 2–4 min was expressed as cumulative channel open probability (NPo), where N is the apparent number of channels, and Po is the mean open-state probability. NPo was determined from amplitude histograms after multiple Gaussian curve fitting (Origin 6.0, Microcal Software, Northampton, MA). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Effect of Isoflurane and H_2O_2 on the MitoK$_{ATP}$ Channel Reconstituted in Lipid Bilayers

A stock solution of isoflurane (14.5 mM) (Abbott Laboratories, Chicago, IL) was prepared by mixing excess isoflurane with the identical buffer used for channel reconstitution. The isoflurane concentrations in the stock solution and *cis* chamber were measured

by gas chromatography (GC-8A, Shimadzu, Columbia, MO).

MitoK_{ATP} channels fused into the lipid bilayer were divided into two experimental groups. In the first group, the effect of isoflurane on the mitoK_{ATP} activities was examined. Isoflurane mitoK_{ATP} regulation was investigated after the channels were blocked by high ATP concentration (0.5 mM), as well as without prior ATP inhibition. In the second experimental group, we tested the effect of H_2O_2 (200 μM) on the mitoK_{ATP} activity. After the addition of either isoflurane or H_2O_2, the mitoK_{ATP} currents were monitored for up to 10 min. At the end of the observation, the identity of the mitoK_{ATP} channels was confirmed by their inhibition with 5-hydroxydecanoate (5-HD). HMR-1098, a sarcolemmal K_{ATP} channel inhibitor, was used to distinguish the sarcolemmal K_{ATP} channel from mitoK_{ATP} channels when necessary. All modulators were added to the *cis* chamber and vigorously stirred for at least 30 s with a submersible stirrer (Model 230, VER Scientific, and West Chester, PA).

Statistical Analysis

Statistical analysis was conducted with NCSS software (Kaysville, UT). Sample size was estimated based on a difference of 50% in NPo between groups and a power of 0.80, which required a minimum of four observations to achieve the desired power of analysis. Data are presented as mean ± SD except in Figure 3B, where one representative experiment was presented. Data were analyzed after square root transformation to eliminate the significant difference in variances between groups, followed by ANOVA. *Post hoc* tests were done with Duncan's range test. A value of $P \leq 0.05$ was considered significant.

RESULTS

Figure 2A shows the modulation of the mitoK_{ATP} channel activity by ATP, isoflurane, and 5-HD in sequence. A cluster of channels were active under control conditions, with a peak K^+ current of approximately 3 pA at a holding potential of +40 mV (*trans/cis*). The addition of 0.5 mM ATP inhibited these openings, indicating that the recorded channels were mitoK_{ATP} channels. We then tested the effect of isoflurane (0.8 mM) on the channel activity. Within 4 min of exposure, isoflurane increased the peak current beyond that observed at control. The mitoK_{ATP} channel inhibitor 5-HD (200 μM) completely abolished the K^+ fluxes, providing further confirmation that the recorded current represented the mitoK_{ATP} channel activity. A summary of data collected from six experiments are presented in Figure 2B. These findings demonstrate that isoflurane activated the human mitoK_{ATP} channels that were previously inhibited by ATP. In a separate set of experiments, we tested the effect of isoflurane on the mitoK_{ATP} channels without prior inhibition by

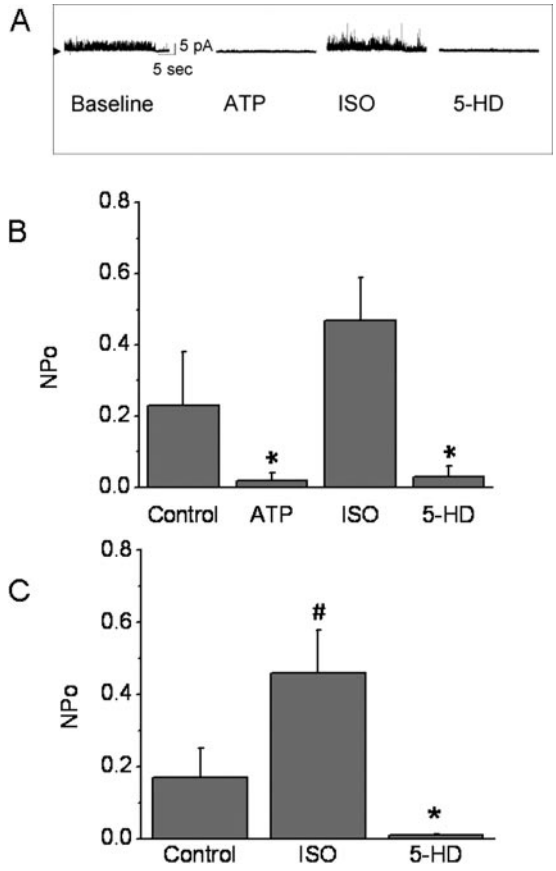

Figure 2. Regulation of human cardiac mitoK_{ATP} channel by adenosine triphosphate (ATP), isoflurane (ISO) and 5-hydroxydecanoate (5-HD). Human inner mitochondrial membranes (IMM) were fused into lipid bilayers in symmetrical 150 mM potassium glutamate (pH 7.2). Holding potential +40 mV (*trans/cis*). (A) A cluster of mitoK_{ATP} channels were active under control conditions. Addition of 0.5 mM ATP inhibited these openings and subsequent addition of ISO (0.8 mM) to the *cis* chamber increased the peak current. 5-HD (200 μM) completely inhibited the mitoK_{ATP} activities. Upward deflection represents channel openings. Arrow indicates channels closing. (B) Data summarized from several observations. *$P < 0.05$ versus control or ISO ($n = 6$). (C) A summary of several recordings in which the effect of ISO on mitoK_{ATP} channels was examined without prior inhibition by ATP. #$P < 0.05$ versus control. *$P < 0.05$ versus control or ISO ($n = 5$).

ATP. As shown in Figure 2C, isoflurane significantly increased the NPo from that seen at baseline ($P < 0.05$). The subsequent addition of 5-HD suppressed the NPo to below the control level ($P < 0.05$). These observations confirm that the effects of isoflurane on human mitoK_{ATP} are qualitatively similar to our previous findings in rat mitoK_{ATP} (18).

The time course of the effects of ATP, isoflurane, and 5-HD on mitoK_{ATP} channels reconstituted into lipid bilayers is displayed in Figure 3A. ATP (0.5 mM) reduced the mitoK_{ATP} activities seen at control within 2 min of application. A subsequent addition of isoflurane (0.8 mM) increased the channel activities peaking at 5 min. The increased activities were then completely suppressed by 5-HD. Figure 3B shows the isoflurane concentration in the bilayers

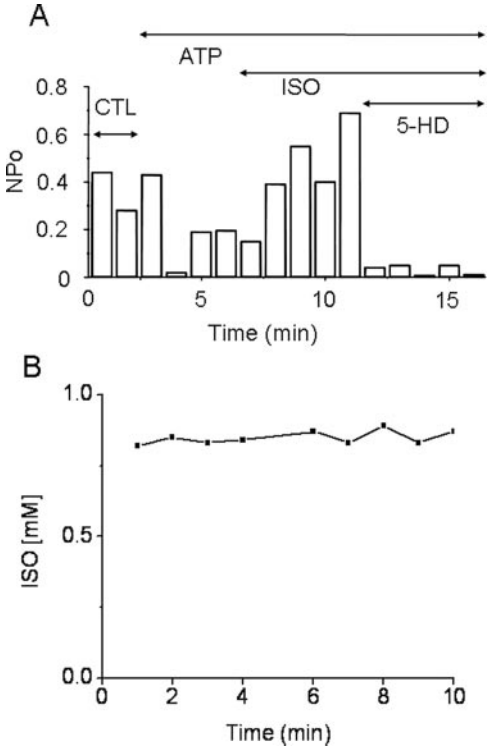

Figure 3. (A) The time course of adenosine triphosphate (ATP), isoflurane (ISO), and 5-hydroxydecanoate (5-HD) effects on the mitoK$_{ATP}$ channels fused into lipid bilayers. ATP (0.5 mM) reduced the mitoK$_{ATP}$ activities recorded at control within 2 min of application. Addition of ISO (0.8 mM) increased the channel openings, which peaked at 5 min. 5-HD (200 μM), inhibited the channels completely. (B) ISO concentrations were assessed in the bilayers chamber at 1 min interval, and remained stable for at least 10 min. Representative of 2 observations.

chamber sampled at a 1 min interval from a representative experiment. The isoflurane level remained stable for at least 10 min, as measured by gas chromatography. This concentration is equivalent to approximately 1.5 MAC (minimum alveolar anesthetic concentration) at room temperature. Thus, the effect of 5-HD inhibition was not complicated by the potential evaporation of the volatile anesthetic during the course of observations.

Several studies have demonstrated that volatile anesthetics, including isoflurane and sevoflurane, interact with the mitochondrial electron transfer chain (30–32) and can produce ROS. To explore the influence of ROS on the human mitoK$_{ATP}$ channel, we investigated the effect of exogenous H_2O_2 in our bilayer system. As shown in Figure 4A, a cluster of mitoK$_{ATP}$ channels active at baseline were first suppressed by ATP (0.5 mM). Addition of H_2O_2 (200 μM) reactivated these channels, despite the continued presence of ATP. The K^+ current was then abolished by 5-HD (200 μM). Data from several experiments ($n = 4$) are summarized in Figure 4B. Thus, H_2O_2 can directly activate the mitoK$_{ATP}$ channels, similar to the observation made previously in bovine cardiac mitoK$_{ATP}$ channels (24).

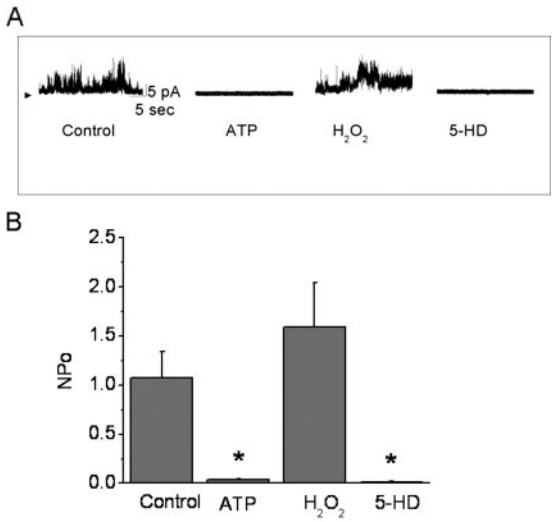

Figure 4. The effect of exogenous H_2O_2 on the human mitochondrial adenosine triphosphate (ATP)-sensitive K^+ channel (mitoK$_{ATP}$) channels reconstituted into lipid bilayers. (A) Original recordings of the mitoK$_{ATP}$ activities recorded at baseline (control), after application of adenosine triphosphate (ATP) (0.5 mM) and then H_2O_2 (200 μM). H_2O_2 reactivated mitoK$_{ATP}$ channels in the continued presence of ATP, which were sensitive to 5-hydroxydecanoate (5-HD) inhibition (200 μM). Arrow indicates channels closing. (B) Data summary. *$P < 0.05$ versus control or H_2O_2 ($n = 4$).

DISCUSSION

Activation of the mitoK$_{ATP}$ channel is considered a critical step in APC (11,12). In the present study, we have shown that isoflurane can directly activate the human cardiac mitoK$_{ATP}$ channel *in vitro*, similar to our original observation in rat cardiac mitoK$_{ATP}$ (18). Furthermore, we have provided evidence that H_2O_2, an end product of ROS generated in mitochondria, can also activate the mitoK$_{ATP}$. Therefore, isoflurane could induce APC via direct activation of the mitoK$_{ATP}$ and/or through ROS generation.

Isoflurane and MitoK$_{ATP}$ Channels

Our observations of direct activation of the mitoK$_{ATP}$ by isoflurane in humans and rats (18) are likely due to an increased number of channels being open as well as an increased open frequency of each channel. We have speculated previously that isoflurane likely induces a disruption of the allosteric interaction between the mitoK$_{ATP}$ subunits, reducing their sensitivity to ATP inhibition. Since no cytosolic components were present in the bilayer chamber, these findings imply that isoflurane can regulate the mitoK$_{ATP}$ channel without the participation of cytosolic PKC (or other enzymes) translocation. It remains to be seen if mitochondrial PKC is involved in the activation of mitoK$_{ATP}$ by isoflurane.

MitoK$_{ATP}$ Channels and APC

It is unclear how increased mitoK$_{ATP}$ channel activity might protect against ischemic myocardial damage in APC. MitoK$_{ATP}$ opening by sevoflurane reduced the cytosolic (33) and mitochondrial Ca^{2+} (34) loading

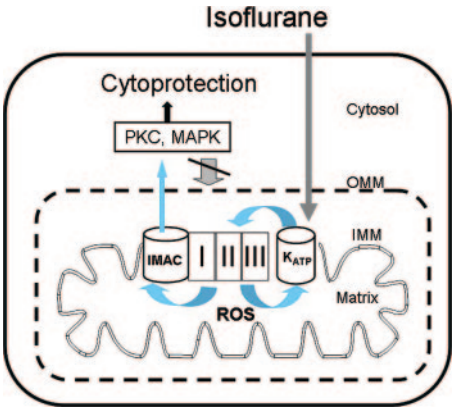

Figure 5. Modified signal cascade of mitochondrial K_{ATP} activation in isoflurane-induced preconditioning (APC) in cardiac myocytes. Isoflurane activates the K_{ATP} channel (K_{ATP}) by direct activation and via generation of reactive oxygen species (ROS) by inhibiting complexes I and/or III in the respiratory chain. ROS exits the mitochondria via the inner membrane anion channel (IMAC) and permeates freely into the cytosol across the outer mitochondrial membrane (OMM). It activates cytosolic kinases such as protein kinase C (PKC) and p38 mitogen-activated protein kinases (MAPK), as well as other cardioprotective mechanisms. Cytosolic kinases are probably too large to cross the outer mitochondria membrane (crossed arrow) during APC.

during reperfusion, and conferred a better preservation of mitochondrial bioenergetics (35). Other studies suggest that opening of the mitoK$_{ATP}$ with diazoxide may generate ROS (36). Similarly, volatile anesthetics may also generate ROS by inhibition of complexes I and/or III in electron transfer chain (22,30,32,37) and induce APC. Scavengers of ROS were shown to prevent isoflurane-induced preconditioning (19). ROS generated inside the mitochondrial matrix may exit the IMM via inner membrane anion channel (38). Once inside the cytosol, ROS can activate PKC (20) or other cytosolic kinase, such as p38 mitogen-activated protein kinase, and confer myocardial protection. On the other hand, ROS may directly activate the mitoK$_{ATP}$ inside the mitochondria, forming a positive feedback mechanism. A simplified scheme is illustrated in Figure 5.

Isoflurane and APC Signal Cascade—A Modified Scheme

The current proposal for the signal cascade in APC usually puts the mitoK$_{ATP}$ channel distal to the activation and translocation of cytosolic PKC to mitochondria, as originally proposed for IPC. Although cytosolic PKC or other kinases such as p38 mitogen-activated protein kinase or tyrosine protein kinase were proposed to activate the mitoK$_{ATP}$ during APC (39), there is still no evidence that cytosolic kinases can actually cross the physical barrier of the OMM and interact with the mitoK$_{ATP}$ in IMM during IPC or APC. The OMM is considered freely permeable to a mass smaller than 5000 Da. To circumvent this potential barrier in the hypothesized signal pathway during IPC, Costa et al. (40) proposed that protein kinase G may phosphorylate some target protein on the OMM, which then transmits the cardioprotective signals from cytosol to the IMM via PKC-ϵ located in the intermembrane space. Alternatively, we proposed a local regulatory model of the mitoK$_{ATP}$ channel by PKC(s) (16), based upon the observation that the activation of local PKC associated with human IMM opens the mitoK$_{ATP}$. In the setting of APC, isoflurane or other volatile anesthetics, because of their lipid solubility, can interact directly with the mitoK$_{ATP}$ or other channels on the IMM, without the requirement of cytosolic kinases. The mitoK$_{ATP}$ can also be regulated locally by ROS, nitric oxide, kinases, or other cytosolic messengers that can permeate the OMM during APC. In view of the findings that activation of the mitoK$_{ATP}$ channel and ROS generation are likely upstream to PKC activation during APC (22), and that isoflurane and H_2O_2 directly activate of the mitoK$_{ATP}$, we have proposed a modified scheme of APC signal cascade (Fig. 5). In this bottom-up (instead of top-down) scheme, activation of the mitoK$_{ATP}$ channel and/or ROS generation by volatile anesthetics are proximal to activation of cytosolic protective mechanisms, unlike that which was previously proposed (39).

Potential Limitation of the Study

The role of the mitoK$_{ATP}$ channel in the ischemic stress response is well known. The mitochondria isolated from the donor human hearts that were harvested from brain-dead patients may have been subjected to various stress stimuli or medications that might have affected their responses to isoflurane *in vitro*. Despite these potential limitations, we observed similar activation of the human mitoK$_{ATP}$ channel by isoflurane to that seen in rat mitochondria (18). Also, the activation of the human mitoK$_{ATP}$ channel by H_2O_2 is similar to that seen in mitochondria isolated from bovine hearts obtained from the slaughter house (24). It should be noted that the concentration of isoflurane used may be higher than clinical dosage. We did observe similar activation of the mitoK$_{ATP}$ channel at concentrations close to clinical application, i.e., 0.4 mM isoflurane in rats (18) and 0.2 mM sevoflurane in human (Jiang et al., manuscript in preparation).

Mechanistically, the myocardial protective effect of volatile anesthetics is not limited to their regulation of mitoK$_{ATP}$ channels. APC may also involve other targets, such as the permeability transition pore in mitochondria (41). The voltage-dependent anion channel, part of the mitochondrial permeability transition pore, is obviously a more accessible target for cytosolic PKC (42) or other cytosolic enzymes than ion channels embedded in the IMM.

In summary, we have characterized the effect of isoflurane on the human cardiac mitoK$_{ATP}$ channel reconstituted into the lipid bilayers. Our data indicate that isoflurane, as well as H_2O_2, increase the activity of the human mitoK$_{ATP}$. Thus, direct activation of

mitoK$_{ATP}$ channel by isoflurane and/or ROS may contribute to APC induced by isoflurane.

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