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Distinct Roles for Sarcolemmal and Mitochondrial Adenosine Triphosphate–sensitive Potassium Channels in Isoflurane-induced Protection against Oxidative Stress

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Background: **Cardiac preconditioning, including that induced by halogenated anesthetics, is an innate protective mechanism against ischemia–reperfusion injury. The adenosine triphosphate–sensitive potassium (K_{ATP}) channels are considered essential in preconditioning mechanism. However, it is unclear whether K_{ATP} channels are triggers initiating the preconditioning signaling, and/or effectors responsible for the cardioprotective memory and activated during ischemia–reperfusion.**

Methods: **Adult rat cardiomyocytes were exposed to oxidative stress with 200 μM H_2O_2 and 100 μM $FeSO_4$. Myocyte survival was determined based on morphologic characteristics and trypan blue exclusion. To induce preconditioning, the myocytes were pretreated with isoflurane. The involvement of sarcolemmal and mitochondrial K_{ATP} channels was investigated using specific inhibitors HMR-1098 and 5-hydroxydecanoic acid. Data are expressed as mean ± SD.**

Results: **Oxidative stress induced cell death in 47 ± 14% of myocytes. Pretreatment with isoflurane attenuated this effect to 26 ± 8%. Blockade of the sarcolemmal K_{ATP} channels abolished the protection by isoflurane pretreatment when HMR-1098 was applied throughout the experiment (50 ± 21%) or only during oxidative stress (50 ± 12%), but not when applied during isoflurane pretreatment (29 ± 13%). Inhibition of the mitochondrial K_{ATP} channels abolished cardioprotection irrespective of the timing of 5-hydroxydecanoic acid application. Cell death was 42 ± 23, 45 ± 23, and 46 ± 22% when 5-hydroxydecanoic acid was applied throughout the experiment, only during isoflurane pretreatment, or only during oxidative stress, respectively.**

Conclusion: **The authors conclude that both sarcolemmal and mitochondrial K_{ATP} channels play essential and distinct roles in protection afforded by isoflurane. Sarcolemmal K_{ATP} channel seems to act as an effector of preconditioning, whereas mitochondrial K_{ATP} channel plays a dual role as a trigger and an effector.**

CARDIAC preconditioning is an innate protective mechanism against injury by ischemia and reperfusion.[1] In addition to the extent of cardioprotection, one of the most remarkable characteristics of preconditioning is the memory phase, when the cardioprotective effects persist despite removal of the preconditioning stimulus. To date, numerous studies have investigated the mechanism of preconditioning, and many crucial components underlying cardioprotection have been identified. Adenosine triphosphate–sensitive potassium (K_{ATP}) channels have long been considered essential components of cardioprotection by ischemic and pharmacologic preconditioning. Acting as metabolic sensors, regulated by intracellular metabolic factors such as adenosine triphosphate, adenosine diphosphate, and cytosolic pH, they are attractive candidates as the major contributors to the mechanism of cardioprotection. Their critical role for cardiac preconditioning, including that induced by halogenated anesthetics, has been demonstrated in a number of studies.[2–5]

There are two populations of K_{ATP} channels in cardiac myocytes: the mitochondrial (mitoK_{ATP}) channel located in the inner mitochondrial membrane and the sarcolemmal (sarcK_{ATP}) channel located in the plasma membrane. Initially, before discovery of mitoK_{ATP} channels, the cardioprotective effects of preconditioning were attributed to the sarcK_{ATP} channels.[2] The protective effects of K_{ATP} channel opening were ascribed to action potential shortening and the resulting decrease in Ca^{2+} overload during ischemia and reperfusion.[6] However, later studies demonstrated that cardioprotective actions of K_{ATP} channel openers are independent of the action potential shortening.[7,8] After a more recent discovery of the mitoK_{ATP} channels in the inner mitochondrial membrane and development of selective mito and sarcK_{ATP} channel inhibitors, evidence suggested that mitoK_{ATP} channels rather than the sarcK_{ATP} channels play a more important role in cardioprotection.[9,10] HMR-1098, a specific inhibitor of sarcK_{ATP} channel, has often failed to abolish protection by ischemic and pharmacologic preconditioning, whereas cardioprotection was elicited by the mitoK_{ATP} channel activator diazoxide.[4,10] This led to a widespread opinion that cardioprotective effects of preconditioning depend mostly on the mitoK_{ATP} channel. However, some of the recent studies that involved the use of sarcK_{ATP}-specific inhibitors and genetic models of disrupted or knocked-out sarcK_{ATP} channel subunits indicated that the role of sarcK_{ATP} channel in cardioprotection should not be ignored.[11–13]

The involvement of sarcK_{ATP} channels in cardioprotection by anesthetic-induced preconditioning (APC) was demonstrated in a limited number of studies.[14,15] More studies demonstrated a predominant role of the mitoK_{ATP} channels in APC, while finding no apparent involvement of the sarcK_{ATP} channel.[16–19] In addition to the conflicting results regarding the relative importance of sarcK_{ATP} and mitoK_{ATP} channels, their specific roles remain unclear. It is uncertain whether they are triggers, important for initiating the preconditioning signaling

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cascade, or effectors, the endpoints of the preconditioning cascade, responsible for the cardioprotection memory and activated during ischemia–reperfusion.[4,5,20]

Therefore, the goal of the current study was to test whether $sarcK_{ATP}$ and $mitoK_{ATP}$ channels contribute to isoflurane preconditioning and to investigate their exact role in protection afforded by isoflurane. We found that both $sarcK_{ATP}$ and $mitoK_{ATP}$ channels are essential components of APC and that each channel plays a distinct role: The $sarcK_{ATP}$ channel acts as an effector, whereas the $mitoK_{ATP}$ channel acts both as a trigger and as an effector of preconditioning.

Materials and Methods

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

Cell Isolation

Ventricular myocytes were isolated from hearts of adult male Wistar rats (150–250 g) by enzymatic dissociation. The rats were injected with heparin (1,000 U intraperitoneally) and anesthetized with thiobutabarbital (Inactin, 100 mg/kg intraperitoneally; Sigma-Aldrich, St. Louis, MO). After thoracotomy, the hearts were excised, mounted on a Langendorff apparatus, and retrogradely perfused with heparinized Joklik medium (Gibco BRI; Invitrogen, Grand Island, NY) at the flow rate of 7 ml/min. After the blood had been washed out, the perfusate was replaced with an enzyme solution containing Joklik medium supplemented with 0.5 mg/ml collagenase type II (Invitrogen, Carlsberg, CA), 0.25 mg/ml protease XIV (Sigma-Aldrich), and 1 mg/ml bovine serum albumin (Serologicals, Kankakee, IL) at pH 7.23. All solutions were continuously gassed with a mixture of 95% O_2–5% CO_2 and were kept at 37°C. After 25 min of enzyme digestion, the ventricles were excised, minced, and incubated in the enzyme solution for additional 8–10 min in a shaker bath at 37°C. The cell suspension was filtered through 200 μm mesh and centrifuged. The cell pellet was then washed twice in modified Tyrode solution (132 mM NaCl, 10 mM HEPES, 5 mM glucose, 5 mM KCl, 1 mM $CaCl_2$, and 1.2 mM $MgCl_2$, adjusted to pH 7.4). After isolation, the myocytes were stored in Tyrode solution at room temperature and allowed to recover for 1 h before the cell survival experiments. All experiments were performed within 5 h after the cell isolation procedure.

Experimental Protocol

The suspension of isolated cardiomyocytes (1 ml) was placed in a chamber on the stage of inverted microscope (Diaphot 300; Nikon, Tokyo, Japan), and cells were allowed to settle for 10 min. The myocytes were then stained with 1 ml trypan blue solution, 0.4% (Sigma-Aldrich), for 2 min followed by a dye washout with glucose-free Tyrode solution (132 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, and 1.2 mM $MgCl_2$, adjusted to pH 7.4). Cells that were rod-shaped and excluded trypan blue were considered living[21] and were counted. The counting time was monitored and was kept uniform in all experiments (10 min). In each experiment, approximately 250 myocytes were counted. After cell counting, perfusion with glucose-free Tyrode was started. Thirty minutes into the experiment, the myocytes were exposed to oxidative stress by perfusion with 200 μM H_2O_2 and 100 μM $FeSO_4 \cdot 7H_2O$ (both from Sigma-Aldrich) for 17 min. The mixture of H_2O_2 and Fe^{2+} yields a highly reactive hydroxyl radical (OH·) *via* the Fenton reaction.[22] The 17-min duration was chosen because it was optimal for damaging approximately 50% of myocytes. After oxidative stress, the myocytes were reperfused with glucose-free Tyrode solution for another 20 min and stained with trypan blue, after which the remaining living cells were counted. The percentage of cell death was calculated from the cell count before and after oxidative stress. The location of the myocytes was monitored using a chamber bottom with a labeled grid. This enabled counting of the same myocytes before and after oxidative stress.

The time between cell counts before and after stress was exactly 67 min in all experiments. In the experimental groups that underwent APC, the myocytes were exposed to isoflurane for 20 min and 5 min anesthetic washout before oxidative stress. To investigate the effects of the $sarcK_{ATP}$ and $mitoK_{ATP}$ channels on cell survival, the specific inhibitor HMR-1098 (50 μM; a gift from Aventis Pharma, Frankfurt am Main, Germany) or 5-hydroxydecanoic acid (5-HD, 200 μM; Sigma-Aldrich, St. Louis, MO) was added to the superfusing solution at different time points. In all experimental groups, n indicates the number of animals. The protocols for all experimental groups are illustrated in figure 1.

Drugs

Isoflurane (Abbott Laboratories, North Chicago, IL) was dispersed in glucose-free Tyrode solution by sonication and delivered to cardiomyocytes from the airtight glass syringes. At the end of each experiment, samples of isoflurane-containing solution were collected from the chamber, and the concentrations of isoflurane were analyzed by gas chromatography (Shimadzu, Kyoto, Japan). The average concentration of isoflurane used in this study was 0.51 ± 0.09 mM, equivalent to 1.2 vol% at 22°C. The 5-min washout period was sufficient to remove all isoflurane from the chamber as confirmed by isoflurane measurements after the washout. HMR-1098 and 5-HD were kept as stock solution in double-distilled water. All stock solutions were diluted to required con-

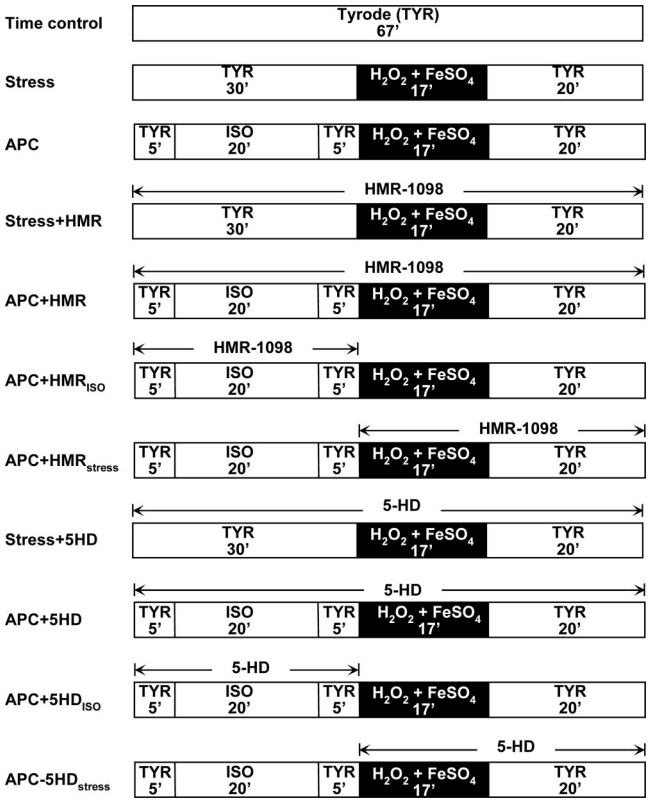

Fig. 1. Schematic illustration of experimental protocols used in the study. 5-HD = 5-hydroxydecanoic acid; APC = anesthetic-induced preconditioning; HMR = HMR-1098; ISO = isoflurane; TYR = perfusion with Tyrode solution.

centration in the superfusing buffer immediately before application.

Statistical Analysis

Results are expressed as mean ± SD. Data were analyzed using Origin 7 software (OriginLab, Northampton, MA). Statistical analysis was performed using one-way analysis of variance with Scheffé *post hoc* test. Differences were considered significant when the two-tailed *P* value was less than 0.05.

Results

Isoflurane Protects Isolated Cardiomyocytes from Oxidative Stress

Oxidative stress was used to mimic the reperfusion injury and investigate isoflurane-induced myocyte protection. In the time control group, 67 min of perfusion with glucose-free Tyrode solution had no significant effect on cell death (7 ± 5%, n = 5; fig. 2, TC group). During exposure to 200 μM H_2O_2 and 100 μM $FeSO_4$, some of the otherwise nonbeating cardiomyocytes started contracting, which resulted in cell hypercontracture and death in 47 ± 14% of cardiomyocytes (n = 11, stress group). When cardiomyocytes were pretreated with isoflurane before oxidative stress (APC group), cell

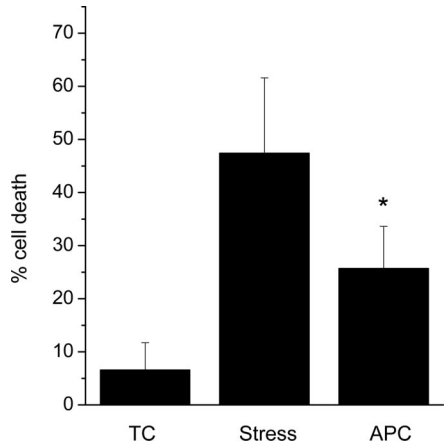

Fig. 2. Isoflurane pretreatment protects cardiac myocytes from damage by oxidative stress. Shown are the mean data expressed as percentage of myocytes that died during the course of the experiment. Experimental groups are time control (TC), oxidative stress (stress), and isoflurane-pretreated myocytes (APC). * *P* < 0.05 *versus* stress. All other values were significantly different *versus* TC group.

death was markedly attenuated to 26 ± 8% ($P < 0.05$, n = 10). These results demonstrated that *in vitro* pretreatment with isoflurane protects the cardiomyocytes from damage by oxidative stress.

Sarcolemmal K_{ATP} Channel Blockade during Oxidative Stress, but Not during Isoflurane Pretreatment, Abolishes Protection by Isoflurane

To investigate the role of $sarcK_{ATP}$ channels in protection by isoflurane, the selective $sarcK_{ATP}$ channel inhibitor HMR-1098 was used. HMR-1098 (50 μM) applied throughout the experiment had no effect on myocyte death in the time control group (5 ± 3%, n = 6; fig. 3A, TC + HMR group). Application of HMR-1098 did not potentiate the deleterious effects of oxidative stress, and cell damage in this group was 48 ± 13% (n = 9; fig. 3A, stress + HMR group). However, inhibition of the $sarcK_{ATP}$ channel completely abolished the protective effects of isoflurane, and cell death after isoflurane pretreatment in the presence of HMR-1098 was 50 ± 21% (n = 9; APC + HMR group).

To determine the exact time period in which the $sarcK_{ATP}$ channel activation is protective, HMR-1098 was applied only during isoflurane pretreatment or only during stress. When $sarcK_{ATP}$ channel inhibitor was applied during isoflurane exposure, the cardioprotective effects of isoflurane were still present and cell damage by oxidative stress was lower than without isoflurane pretreatment (29 ± 13%, n = 7; fig. 3B, APC + HMR_{ISO} group). However, when HMR-1098 was applied only during oxidative stress, the protective effects of isoflurane were completely abolished and cell death was 50 ± 12% (n = 7; APC + HMR_{stress} group). These results show that activation of the $sarcK_{ATP}$ channel during the stress period is necessary for isoflurane-induced myocyte protection.

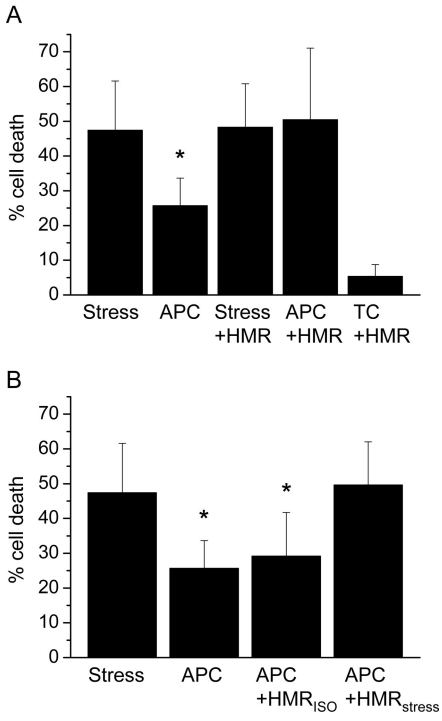

Fig. 3. Activation of sarcolemmal adenosine triphosphate–sensitive potassium channel during oxidative stress, but not during isoflurane pretreatment, is essential for the protective effects of isoflurane preconditioning. (*A*) Protection by isoflurane (APC *vs.* stress) was abolished in the presence of HMR-1098 (APC + HMR), whereas blockade of sarcolemmal adenosine triphosphate–sensitive potassium channel had no effect on cell damage by oxidative stress (stress + HMR) or cell death in time control (TC + HMR). * $P < 0.05$ *versus* stress, stress + HMR, and APC + HMR. All values were significantly different *versus* TC + HMR group. (*B*) Isoflurane-induced protection (APC *vs.* stress) was abolished when HMR-1098 was applied during the stress period (APC + HMR$_{stress}$), but not during isoflurane pretreatment (APC + HMR$_{ISO}$). * $P < 0.05$ *versus* stress and APC + HMR$_{stress}$.

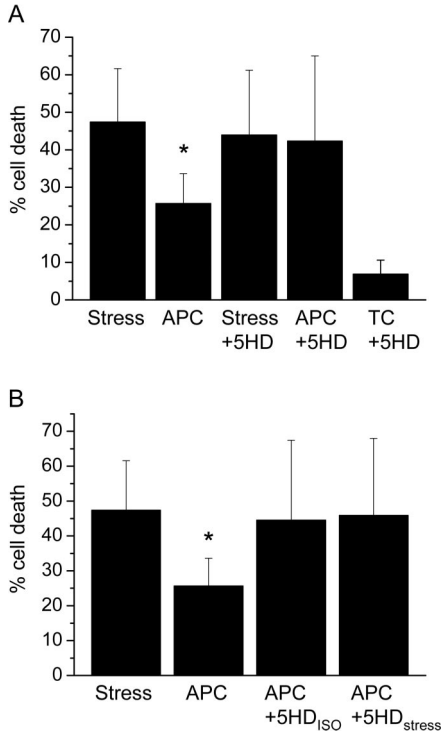

Fig. 4. Activation of mitochondrial adenosine triphosphate–sensitive potassium channel is important during both preconditioning by isoflurane and exposure to oxidative stress. (*A*) Myocyte protection by isoflurane (APC *vs.* stress) was abolished in the presence of 5-hydroxydecanoic acid (APC + 5-HD). 5-HD alone did not affect cell death in nonpreconditioned myocytes (stress + 5-HD) or in time control (TC + 5-HD). * $P < 0.05$ *versus* stress, stress + 5-HD, and APC + 5-HD. All values were significantly different *versus* TC + 5-HD. (*B*) Blockade of mitochondrial adenosine triphosphate–sensitive potassium channel during both isoflurane pretreatment (APC + 5-HD$_{ISO}$) and oxidative stress (APC + 5-HD$_{stress}$) completely abolished isoflurane-induced protection. * $P < 0.05$ *versus* stress, APC + 5-HD$_{ISO}$, and APC + 5-HD$_{stress}$.

Mitochondrial K_{ATP} Channel Inhibition during Both Isoflurane Pretreatment and Oxidative Stress Abolishes Isoflurane-induced Protection

To assess contribution of the mitoK_{ATP} channel to isoflurane-induced protection against oxidative stress, 5-HD (200 μM), a selective inhibitor of the mitoK_{ATP} channels, was used. 5-HD was applied either throughout the experiment or during specific parts of the experiment as described previously. 5-HD did not affect cell damage in the time control group (7 ± 4%, n = 6; fig. 4A, TC + 5-HD group). In addition, inhibition of mitoK_{ATP} channel had no effect on myocyte damage by oxidative stress, and cell death in the stress + 5-HD group was 44 ± 17% (n = 9; fig. 4A). However, when applied throughout the experiment, 5-HD completely abolished cellular protection by isoflurane, and cell death in APC + 5-HD group was 42 ± 23% (n = 8). When 5-HD was included during only the isoflurane exposure or only the stress period, cell death was 45 ± 23% and 46 ± 22% in the APC + 5-HD$_{APC}$ and APC + HD$_{stress}$ groups, respectively (both n = 6; fig. 4B). These results indicate that activation of the mitoK_{ATP} channel during both isoflurane pretreatment and oxidative stress plays a role in protection by isoflurane.

Discussion

In this study, freshly isolated adult cardiomyocytes were used to investigate specific roles of sarcK_{ATP} and mitoK_{ATP} channels in cardioprotective effects of APC. It was found that both sarcK_{ATP} and mitoK_{ATP} channels are essential for the protection of myocytes from damage by oxidative stress because their inhibition completely abolished the protective effects of isoflurane preconditioning. Specifically, activation of the sarcK_{ATP} channel was required for the cardioprotective effects of APC during the stress period but not during the preconditioning period. In contrast, activation of mitoK_{ATP} channel was found to be necessary during both isoflurane preconditioning and during exposure to oxidative stress. From these results, it seems that although activation of both channels is important for the cardioprotective effects of preconditioning, each channel plays a distinct role: The

sarcK_{ATP} channel acts as an effector, whereas the mitoK_{ATP} channel plays a dual role as a trigger and an effector.

In our study, we found that isoflurane protects isolated cardiomyocytes from oxidative stress *via* both sarcK_{ATP} and mitoK_{ATP} channel activation, because channel inhibition by HMR-1098 and 5-HD, respectively, abolished cardioprotective effects of APC. Similar results were reported by Toller *et al.*,[14] who demonstrated in a dog model *in vivo* that infarct size reduction achieved by desflurane preconditioning is abolished in the presence of HMR-1098 and 5-HD. Further, using the same inhibitors, both channels were found to mediate ketamine-induced protection of the force of contraction of human right atrial trabeculae during hypoxia and reoxygenation.[15] In contrast to these studies that demonstrated an equally important role for both sarcK_{ATP} and mitoK_{ATP} channels, there are studies that demonstrate a more significant role of the mitoK_{ATP} channels. For example, Zaugg *et al.*[16] found that mitoK_{ATP} channel inhibition by 5-HD, but not sarcK_{ATP} channel inhibition by HMR-1098, completely abolished the cardioprotective effects of isoflurane and sevoflurane in isolated adult rat cardiomyocytes exposed to ischemia. Similarly, Uecker *et al.*[17] showed that 5-HD completely blocked the cardioprotection by ischemic or isoflurane-induced preconditioning in isolated perfused rat hearts, whereas HMR-1098 had no effect. MitoK_{ATP} activation was found to be essential, whereas sarcK_{ATP} channel was found to play no role or have only a partial role in desflurane- and sevoflurane-induced preconditioning of isolated human right atria, respectively.[18,19]

Pronounced differences in findings from these studies that investigated the role of the sarcK_{ATP} channel in APC may result from differences in the experimental models used (isolated cardiomyocytes, isolated hearts, atrial trabeculae, *in vivo* preparation). There are also differences in the insults on the heart (ischemia and reperfusion *in vivo*, simulated ischemia without reperfusion, hypoxia, oxidative stress, metabolic inhibition). Moreover, the measured endpoints of cardiac injury also differ (cell death and infarcted area *vs.* functional parameters such as developed force of contraction). Finally, there are differences in the time and duration of application of pharmacologic inhibitors (application during preconditioning stimulus *vs.* stress period). In our preparation, the timing of application of HMR-1098, but not of 5-HD, was found to be crucial. If HMR-1098 was applied only during the preconditioning stimulus period (isoflurane exposure), cytoprotection was still present, but when HMR-1098 was applied during oxidative stress, cytoprotection was abolished. Therefore, in evaluating the studies that test the role of the sarcK_{ATP} channel in preconditioning, it is important to consider the timing of application of the inhibitors. Further, the endpoint variable used to evaluate the myocardial injury is also an important factor. For example, the sarcK_{ATP} channel was found not to have a role in the infarct size reduction by adenosine-enhanced ischemic preconditioning, but it was important for the improvement of functional recovery.[23] In the same study, the mitoK_{ATP} channel had effect primarily on the infarct size without affecting the functional recovery. Another important condition is the type of insult. Both sarc and mitoK_{ATP} channels were shown to have distinct roles in ischemia–reperfusion injury. Activation of the mitoK_{ATP} channel mediates the phorbol 12-myristate 13-acetate–induced protection against cell death during chemically induced hypoxia, but not during reoxygenation.[11] In contrast, activation of the sarcK_{ATP} channel was protective only during reoxygenation.[11] Therefore, it is possible that if only hypoxia is used as an insult,[16] involvement of the sarcK_{ATP} channel in protection against ischemia–reperfusion injury can be overlooked. In addition, the effectiveness of the inhibitors can be altered during application of certain insults. For example, HMR-1098 was found to be ineffective in blocking the sarcK_{ATP} currents during metabolic inhibition by NaCN and iodoacetate.[24] This may also explain the often reported lack of HMR-1098 effects on the cardioprotection by ischemic and pharmacologic preconditioning.

One of the most remarkable characteristics of the preconditioning phenomenon is the memory phase, when cardioprotection persists despite withdrawal of the preconditioning stimulus (such as anesthetic in APC). In the complex mechanism of preconditioning, one key element of the preconditioning pathway is triggers, activated during preconditioning stimulus and responsible for initiating the downstream signaling cascade. Another key component is the effectors, which are at the end of the preconditioning signaling pathway and are directly responsible for cardioprotective effects of preconditioning during the memory phase. Some of the identified triggers are Gi-coupled receptors, such as adenosine receptors and a small burst of reactive oxygen species.[10,25] The downstream signaling cascade includes activation of intracellular kinases, with protein kinase C playing a central role. Kinase activation results in phosphorylation and priming of the effectors and may potentially result in establishing the cardioprotection memory.[26] Both sarcK_{ATP} and mitoK_{ATP} channels have been implicated as triggers and effectors of preconditioning, but studies have yielded controversial results. In our study, application of HMR-1098 during the stress phase, but not during the preconditioning phase, completely abrogated cardioprotective effects of APC. This suggests that the sarcK_{ATP} channel acts as an effector, but not as a trigger of APC, being activated and acting protectively only during the memory phase. These results support our previous studies, which demonstrated that opening of the sarcK_{ATP} channel is potentiated during the memory phase of APC and that protein kinase C-δ mediates

this effect.[27] In the current study, application of 5-HD during both the preconditioning phase and the stress phase reversed cardioprotective effects of APC to the same extent, indicating that mitoK_{ATP} channel acts as both trigger and effector. The findings were similar to those of Fryer *et al.*,[28] who demonstrated that the mitoK_{ATP} channel acts as both trigger and effector of preconditioning by ischemia, because 5-HD administration before or after the preconditioning phase completely abolished cardioprotection in the rat hearts. In contrast, Pain *et al.*[29] demonstrated that the mitoK_{ATP} channel acts only as a trigger of ischemic preconditioning in isolated rabbit hearts. Similarly, the mitoK_{ATP} channel opening was found to trigger isoflurane-induced preconditioning of rabbit hearts by generating reactive oxygen species.[30] Interestingly, the mitoK_{ATP} channel was found to be an effector, but not a trigger, whereas the sarcK_{ATP} channel was shown to act as a trigger of the delayed cardioprotection by opioid-induced preconditioning in rats.[31] Furthermore, the role of the sarcK_{ATP} channel as a trigger was demonstrated in the delayed ischemic preconditioning of the rat heart.[32] However, these studies investigated the delayed phase of preconditioning 24 h after the preconditioning stimulus, whereas our study was focused on the early phase of preconditioning.

The role of the mitoK_{ATP} channel as both a trigger and an effector and the role of the sarcK_{ATP} channel as the effector of preconditioning could be explained by the following sequence of events. Exposure to isoflurane can directly activate mitoK_{ATP} channels.[33] Opening of the mitoK_{ATP} channel results in changes in the mitochondrial bioenergetics, which may result in a small burst of reactive oxygen species,[29] that can further affect mitochondrial bioenergetics and activate cytosolic mediators such as protein kinase C.[34–37] Activated protein kinase C translocates to the sarcolemma and can then phosphorylate the sarcK_{ATP} channel and sensitize it to opening.[27,38] The primed sarcK_{ATP} channel opens sooner and/or more during subsequent metabolic stress (ischemia–reperfusion, oxidative stress, metabolic inhibition, and others), resulting in greater K^+ efflux, a more rapid repolarization of the cell membrane, and action potential shortening with subsequent contractile failure, which may thereby decrease cytosolic Ca^{2+} loading during ischemia–reperfusion.[12,13,39] A similar sequence of events occurs in quiescent cardiomyocytes, where sarcK_{ATP} channel opening prevents diastolic depolarization of the cell membrane and decreases Ca^{2+} loading.[40] This can reduce or prevent mitochondrial Ca^{2+} overload, a major trigger of the cell death pathway.[41] The mitoK_{ATP} channel can also open during ischemia–reperfusion, which may result in depolarization of the inner mitochondrial membrane and thus further decrease driving force for the mitochondrial Ca^{2+} entry and loading during ischemia–reperfusion.[42] Evidence that pretreatment with the mitoK_{ATP} channel opener diazoxide causes early activation of the sarcK_{ATP} channel during metabolic inhibition and protects the cells from Ca^{2+} loading *via* indirect mechanism supports in part such a sequence of events.[39] From ours as well as other studies, it seems that both the sarcK_{ATP} and mitoK_{ATP} channel are crucial components of cardioprotection that can interact and potentiate each other's protective effects. For example, Tanno *et al.*[43] showed that HMR-1098 can partially block the protection of isolated rabbit hearts from ischemia–reperfusion injury induced by diazoxide. In the same study, 5-HD completely abolished the cardioprotective effects of pretreatment with a low-dose pinacidil (10 μM) that is considered to open primarily sarcK_{ATP} channels but not mitoK_{ATP} channels.

It is important to acknowledge some limitations of our study. This study relies on specificity of pharmacologic openers and blockers of the sarcK_{ATP} and mitoK_{ATP} channels. For example, both 5-HD and diazoxide were shown to have the mitoK_{ATP} channel-independent effects in mitochondria,[44] and we cannot exclude the possibility that abolishment of APC by 5-HD in our study could have been due to the nonspecific effects. In addition, certain limitations are inherent to all *in vitro* models, including the isolated myocyte model. Disaggregated myocytes are in an artificial environment that is different from that in the whole organ, and *in vitro* conditions cannot replicate all of the complexity of the *in vivo* setting. However, one advantage of our model, which contains only myocytes without the presence of other cell types, is that we are able to isolate the effects of drugs (isoflurane, the channel inhibitors) on the cardiomyocytes without vascular and neuronal influences. Also, although the use of oxidative stress does not strictly mimic the conditions during ischemia–reperfusion, both H_2O_2 and OH· were shown to be a relevant component of ischemia–reperfusion injury *in vivo*. Therefore, keeping in mind the limitations of the isolated myocyte model, this model might still provide information relevant to the *in vivo* setting.

From our results, we conclude that both the sarcK_{ATP} and the mitoK_{ATP} channel play essential and distinct roles in APC in rat heart. The sarcK_{ATP} channel acts as an effector of anesthetic preconditioning, whereas the mitoK_{ATP} channel plays a dual role as a trigger and an effector.

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