Isoflurane preconditioning uncouples mitochondria and protects against hypoxia-reoxygenation

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Ljubkovic M, Mio Y, Marinovic J, Stadnicka A, Warltier DC, Bosnjak ZJ, Bienengraeber M. Isoflurane preconditioning uncouples mitochondria and protects against hypoxia-reoxygenation. Am J Physiol Cell Physiol 292: C1583–C1590, 2007. First published January 10, 2007; doi:10.1152/ajpcell.00221.2006.—Ischemic cardiac injury can be substantially alleviated by exposing the heart to pharmacological agents such as volatile anesthetics before occurrence of ischemia-reperfusion. A hallmark of this preconditioning phenomenon is its memory, when cardioprotective effects persist even after removal of preconditioning stimulus. Since numerous studies pinpoint mitochondria as crucial players in protective pathways of preconditioning, the aim of this study was to investigate the effects of preconditioning agent isoflurane on the mitochondrial bioenergetic phenotype. Endogenous flavoprotein fluorescence, an indicator of mitochondrial redox state, was elevated to 195 ± 16% of baseline upon isoflurane application in intact cardiomyocytes, indicating more oxidized state of mitochondria. Isoflurane treatment also elicited partial dissipation of mitochondrial transmembrane potential (tetramethylrhodamine fluorescence intensity declined to 83 ± 3 and 81 ± 7% of baseline during isoflurane exposure and washout, respectively). Mild uncoupling, with preserved ATP synthesis, was also detected in mitochondria that were isolated from animals that had been previously preconditioned by isoflurane in vivo, revealing its memory nature. These mitochondria, after exposure to hypoxia and reoxygenation, exhibited better preserved respiration and ATP synthesis compared with mitochondria from non preconditioned animals. Partial mitochondrial depolarization was paralleled by a diminished Ca2+ uptake into isoflurane-treated mitochondria, as indicated by the reduced increment in rhod-2 fluorescence when mitochondria were challenged with increased Ca2+ (180 ± 24 vs. 258 ± 14% for the control). In conclusion, isoflurane preconditioning elicits partial mitochondrial uncoupling and reduces mitochondrial Ca2+ uptake. These effects are likely to reduce the extent of the mitochondrial damage after the hypoxic stress.

cardioprotection; uncoupling

BENEFICIAL EFFECTS OF INHALATIONAL ANESTHETICS on ischemic myocardium were demonstrated nearly 20 years ago (30, 53). They have been mainly attributed to an improved myocardial oxygen supply-and-demand relation due to the negative inotropic action of anesthetics (40). However, more recently, volatile anesthetics were also shown to induce pharmacological preconditioning, whereby a transient exposure to an anesthetic protects the heart against subsequent ischemic damage (4, 24). This cardioprotective effect, which was termed anesthetic pre-conditioning (APC), persists even after discontinuation of the anesthetic. It affords the protection similar to that elicited by ischemic preconditioning, and the two modes of preconditioning share a number of signaling pathways (4, 24, 39). Cardiac protection by APC has also been validated in several clinical studies (1, 13, 28).

Mitochondria emerged as the pivotal players in the complex signaling pathways leading to cardioprotection by volatile anesthetics, which also involve reactive oxygen species (ROS), G protein-coupled receptors, protein kinases, and ATP-sensitive potassium (KATP) channels (3). Besides the evidence suggesting better preservation of mitochondrial function after ischemia-reperfusion in the preconditioned heart, studies have also revealed that mitochondria are active contributors to the cardiac protection by APC. It is well known that drugs with anesthetic properties influence the mitochondrial function in the cell. For example, halothane, isoflurane, and sevoflurane have been shown to inhibit the electron transport chain at the level of NADH:ubiquinone oxidoreductase (complex I) (19), which potentially may lead to a small rise in ROS production. In that respect, a brief and limited burst of ROS originating from the mitochondria was reported to be mandatory for triggering the preconditioning mechanism (12, 46). Furthermore, activation of some mitochondrial inner membrane ion channels [such as ATP-sensitive (mitoKATP) or Ca2+-activated K+ channels (mitoKCa)] and subsequent cationic influx have been proposed to elicit cellular protection through matrix swelling and mild uncoupling, possibly by affecting the mitochondrial Ca2+ homeostasis (7, 56).

Therefore, the aim of the present study was to investigate the effect of preconditioning by volatile anesthetic isoflurane on mitochondrial bioenergetic parameters. Our hypothesis was that APC elicits mild mitochondrial uncoupling, paralleled by a reduced Ca2+ uptake, leading to a better preservation of the mitochondrial function after hypoxic stress.

MATERIALS AND METHODS

This study was conducted according to U.S. National Institutes of Health standards (NIH Publication 95-23, revised 1996) and was approved by the Institutional Animal Use and Care Committee.

Isolation of rat cardiac myocytes. Single ventricular myocytes were obtained from hearts of adult male Wistar rats weighing between 200 and 350 g by enzymatic digestion as previously described (31). After isolation, cells were stored at room temperature in a Tyrode solution containing (in mM) 140 NaCl, 5.5 KCl, 0.1 CaCl2, 2 MgCl2, 5
HEPES, 5 glucose, and 20 taurine (pH 7.4) and were used for the experiments within 5 h.

Laser scanning confocal microscopy. Isolated cardiomyocytes were visualized using an inverted laser scanning confocal microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) with a ×40/1.3 oil-immersion objective (Nikon). Fluorescent probes were excited at 488 nm with an argon laser and at 543 nm with a green helium-neon laser, and a set of filters (ND4 and ND8) was used to minimize dye bleaching. Data were analyzed using MetaMorph 6.1 software (Universal Imaging, West Chester, PA). All fluorescence intensities are expressed relative to the baseline fluorescence F0 measured at the beginning of the experiment (F/F0 × 100).

Analysis of mitochondrial redox state. Mitochondrial oxidation-reduction state was assessed through the monitoring of native autofluorescence of mitochondrial flavoprotein (5, 43). Isolated cardiomyocytes were placed in the recording chamber and superfused with modified glucose-free Tyrode solution containing (in mM) 140 NaCl, 5.5 KCl, 1 CaCl2, 1 MgCl2, and 10 HEPES (pH 7.4) at room temperature. Flavoprotein fluorescence was measured at 515 nm following excitation at 488 nm.

Analysis of mitochondrial membrane potential in isolated cardiomyocytes. Isolated cells were incubated with the mitochondrial membrane potential indicator tetramethylrhodamine (TMRE; 100 nM; Invitrogen, Carlsbad, CA) for 30 min. After TMRE loading, the sarcolemma was permeabilized by the addition of saponin (0.05 mg/ml) to avoid the artifacts produced by redistribution of the dye from the mitochondria to the cytosolic compartment (35). Permeabilized cells were superfused with the solution containing (in mM) 135 KCl, 0.5 EGTA, 1 MgCl2, 10 HEPES, 5 glutamate, 5 succinate, and 1 K2ATP (pH 7.2). TMRE (100 nM) was included in the superfusing solution throughout the experiments. TMRE fluorescence intensity was recorded at 590 nm (excitation by green helium-neon laser), and the changes in mitochondrial membrane potential (ΔΨm) were monitored by calculating relative TMRE fluorescence.

Anesthetic preconditioning of rats. Male Wistar rats were anesthetized with intraperitoneal thiobutabarbital sodium (100–150 mg/kg). A tracheotomy was performed, and the trachea was cannulated. Rats were ventilated with positive end-expiratory pressure using an air-oxygen mixture. Isoflurane was administered for 30 min and discontinued 15 min (memory period) before the heart was excised. Control animals did not receive isoflurane. End-tidal concentrations of isoflurane were measured at the tip of the tracheotomy tube by using an infrared gas analyzer that was calibrated with known standards before and during experimentation.

Isolation of mitochondria. The hearts from the preconditioned and control rats (as described above) were excised, and the left ventricles were placed into an ice-cold isolation buffer containing (in mM) 50 sucrose, 200 mannitol, 5 KH2PO4, 1 EGTA, 5 MOPS, and 0.1% bovine serum albumin (pH 7.3) and homogenized twice for 5 s with a Polytron homogenizer. The homogenate was centrifuged at 800 g, and the obtained pellet was rehomogenized (Potter-Elvehjem homogenizer) and recentrifuged. The resulting supernatants were centrifuged at 800 and 6,000 g, and the pellets were washed in isolation buffer by differential centrifugation at 800 and 6,000 g. The mitochondrial pellet was resuspended in isolation buffer without EGTA and kept on ice. Protein concentration was determined with a DC protein kit (Bio-Rad, Hercules, CA), and mitochondria were used within 4 h after isolation.

Analysis of mitochondrial membrane potential in isolated mitochondria. Mitochondrial membrane potential was monitored during states 2–4 respiration (using 5 mM pyruvate/malate as substrates) with the fluorescence indicator rhodamine 123. Mitochondria (0.05 g/l) were suspended in the respiration buffer containing 50 mM rhodamine 123, and the fluorescence was recorded by a spectrofluorometer (Photon Technology International, Birmingham, NJ) operating at excitation and emission wavelengths of 503 and 527 nm, respectively (21). After the equilibration period, state 3 respiration was evoked by the addition of ADP (250 μM). ΔΨm was expressed as the percentage of rhodamine 123 fluorescence relative to the fluorescence recorded after addition of 4 μM carbonyl cyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP), a mitochondrial uncoupler.

Mitochondrial oxygen consumption and exposure to hypoxia-reoxygenation. Mitochondrial respiration was monitored with an oxygen electrode (Hansatech Instruments, Norfolk, UK) in respiration buffer containing (in mM) 130 KC1, 5 K2HPO4, 20 MOPS, 2.5 EGTA, 0.001 Na2P2O7, 0.1% BSA (pH 7.2), and 1 g/l mitochondria. In experiments involving hypoxia and reoxygenation, EGTA was omitted and CaCl2 (5 mM) was added. State 2 respiration was stimulated with the combination of pyruvate and malate (5 mM each), succinate (5 mM) and rotenone (2 μM), or ascorbate (2.5 mM) and tetramethyl-phenylene diamine (TMPD; 0.25 mM) as substrates. ADP-stimulated state 3 respiration was measured in the presence of 250 μM ADP, and state 4 respiration after added ADP was consumed. Hypoxia was reached within 5 min as mitochondria consumed all available oxygen in the chamber. Throughout the hypoxic interval (20 min), the oxygen level within the closed chamber containing the mitochondrial suspension was at zero. After the hypoxic period, mitochondrial suspension was exposed to room air to achieve reoxygenation.

Mitochondrial ATP production. Mitochondrial ATP production rate was determined with a chemiluminescence-based method utilizing firefly luciferase and luciferin (Invitrogen), as previously described (55). Reaction solution contained respiration buffer (as described above), diadenosine pentaphosphate (0.2 μM), pyruvate and malate (each 5 mM), 0.2 g/l mitochondria, 0.1 g/l luciferin, and 1.25 mg/l luciferase. The reaction was initiated by the addition of 30 μM ADP (made ATP free by hexokinase treatment). The blank was corrected by the subtraction of the ATP blank.
Chemiluminescence was monitored in a Modulus luminometer (Turner Biosystems, Sunnyvale, CA) at room temperature for 120 s. Hypoxia was achieved by saturating the solution continuously with nitrogen for 20 min. The standard curve was obtained with defined ATP concentrations, from which the rate of mitochondrial ATP production was calculated.

Mitochondrial ROS production. The rate of mitochondrial ROS production (H₂O₂ release) was measured spectrofluorometrically, using the fluorescent probe Amplex red (12.5 μM; Invitrogen) in the presence of 0.1 U/ml horseradish peroxidase (excitation and emission wavelengths set to 530 and 583 nm, respectively). Mitochondria (0.5 mg/ml) were suspended in the respiration buffer, and the reaction was started by the addition of 5 mM succinate. The rate of H₂O₂ release was expressed as a percentage of the maximum H₂O₂ release, evoked by the addition of 1 μM antimycin A.

Monitoring of mitochondrial Ca²⁺. Isolated cardiomyocytes were incubated with the mitochondrial-specific Ca²⁺ indicator rhod-2 AM (4 μM; Molecular Probes) for at least 60 min at room temperature. After the loading, to remove cytosolic remains of the dye, cellular sarcolemma was permeabilized with saponin (0.05 mg/ml), and cells were perfused with Ca²⁺-free internal solution containing (in mM) 135 KCl, 0.5 EGTA, 1 MgCl₂, 10 HEPES, 5 glutamate, 5 succinate, and 1 K₂ATP (pH 7.2). The concentration of Ca²⁺ was subsequently increased to 100 nM, and an increase in rhod-2 fluorescence was measured. Rhod-2 fluorescence was acquired at 590 nm upon excitation at 530 nm.

Fig. 2. Isoflurane elicits partial mitochondrial depolarization. A: representative image of isolated cardiac cells loaded with the mitochondrial potentiometric dye tetramethylrhodamine (TMRE; red). Baseline TMRE fluorescence intensity was reduced upon addition of isoflurane into superfusing solution, and this effect was still present after 25 min of anesthetic washout. Administration of the mitochondrial uncoupler 2,4-dinitrophenol (DNP) substantially depleted the TMRE fluorescence. B: summarized data showing isoflurane-elicited partial mitochondrial depolarization that was sustained after discontinuation of anesthetic treatment (ISO wash group). The effect of isoflurane on TMRE fluorescence was prevented in the presence of 5-HD. C: mitochondrial membrane potential was directly assessed in isolated mitochondria with the fluorescent indicator rhodamine 123. A significant degree of depolarization was detected in mitochondria isolated from isoflurane-treated rats (anesthetic preconditioning, APC) in states 2 and 4 (S2 and S4) respiration. FCCP, carbonyl-cyanide-4-(trifluoromethoxy)-phenylhydrazone. Values are means ± SE; n = 8. *P < 0.05 vs. CTL.

Fig. 3. Effects of APC on mitochondrial respiration. A: original recordings of mitochondrial respiration during basal conditions and after hypoxic stress. Oxygen consumption was started by addition of 5 mM pyruvate/malate (pyr/mal) and S3 respiration initiated by the addition of 250 μM ADP. After all oxygen was consumed, mitochondria were left in the hypoxic environment for 20 min, followed by reoxygenation (5 min) and another addition of ADP. B: under baseline conditions, mitochondria that were isolated from the preconditioned animals (APC) exhibited accelerated S4 respiration vs. CTL, whereas no difference was detected in S3. This led to a decreased respiratory control ratio (RCR; inset), indicating an enhanced level of uncoupling. After exposure to hypoxic stress, S3 respiration and RCR were significantly better preserved in APC mitochondria, indicating a decreased degree of damage. C: a mild respiratory uncoupling in APC mitochondria was also detectable in succinate-supported respiration but not when TMPD-ascorbate was used as a substrate. Values are means ± SE; n = 6. *P < 0.05 vs. CTL.
tion with green helium-neon, and changes in mitochondrial Ca\(^{2+}\) were expressed as relative rhod-2 fluorescence (F/F\(_{0}\) × 100).

Chemicals. Isoflurane was dispersed in the experimental solution by sonication, and the concentration was analyzed at the end of each experiment by gas chromatography (Shimadzu, Kyoto, Japan). The average concentration of isoflurane was 0.61 ± 0.1 mM (1.6 vol% at 22°C), which is equivalent to ~1 MAC (minimum alveolar concentration).

Statistical analysis. Data are presented as means ± SE, and the number of experiments is shown as n. In each experimental group, cardiomyocytes or mitochondria were obtained from at least six different rats. Statistical comparisons were performed using one-way analysis of variance with Bonferroni’s post hoc test. Differences at P < 0.05 were considered significant.

RESULTS

Isoflurane increases mitochondrial oxidation. Partial mitochondrial uncoupling has been hypothesized to play an important role in the mechanism of cardioprotection (22). The effect of isoflurane on ∆Ψ\(_{m}\) was indirectly examined by monitoring the mitochondrial redox state. Mitochondrial uncoupling stimulates mitochondrial oxidation, which can be assessed through native flavoprotein autofluorescence (42, 43). Application of isoflurane resulted in an increase of flavoprotein fluorescence to 195 ± 16% of baseline vs. 97 ± 56% for the control group that was not exposed to anesthetic (Fig. 1). This effect was, however, significantly attenuated when isoflurane was coadministered with the reported mitoK\(_{ATP}\) channel inhibitor 5-hydroxydecanoic acid (5-HD; 200 μM). These data suggest that isoflurane affects the mitochondrial oxidative state.

Isoflurane evokes partial mitochondrial depolarization. To further investigate the effect of isoflurane on ∆Ψ\(_{m}\), we incubated isolated cardiomyocytes with the positively charged mitochondrial potentiometric dye TMRE. As displayed in Fig. 2A, perfusion with the isoflurane-containing solution elicited a decrease in TMRE fluorescence, which also persisted during anesthetic washout. At the end of the experiment, the ∆Ψ\(_{m}\) was substantially dissipated by the mitochondrial uncoupler 2,4-dinitrophenol (DNP; 100 μM). The data are summarized in Fig. 2B. Observed reduction in TMRE fluorescence was abolished in the presence of 5-HD (200 μM). These findings indicate that isoflurane partially depolarizes mitochondrial membrane potential. Prolonged mitochondrial depolarization, sustained for at least 25 min after the anesthetic was removed from experimental solution, revealed a memory effect of isoflurane treatment on ∆Ψ\(_{m}\). To further characterize this memory effect, we also directly measured ∆Ψ\(_{m}\) in mitochondria isolated from isoflurane-treated and control rats, utilizing the ∆Ψ\(_{m}\)-sensitive dye rhodamine 123. Obtained data (Fig. 2C) revealed a significant degree of depolarization in preconditioned mitochondria in ADP-independent state 2 (55 ± 5 vs. 48 ± 3% of FCCP-evoked depolarization) and state 4 (54 ± 5 vs. 49 ± 2%) but not in state 3 respiration.

Preconditioning with isoflurane evokes prolonged effects on mitochondrial coupling. To assess the memory effect of isoflurane treatment on mitochondrial coupling, we analyzed the oxygen consumption in mitochondria isolated from control as well as preconditioned (APC) rats. As shown in Fig. 3, A and B, in the presence of pyruvate and malate as substrates, the rate of ADP-stimulated respiration (state 3) was comparable in both control and APC cardiac mitochondria (145 ± 17 vs. 158 ± 5 nmol O\(_2\)·min\(^{-1}\)·mg protein\(^{-1}\), respectively). However, after all ADP was phosphorylated to ATP, state 4 respiration was accelerated in mitochondria from APC rats (39 ± 2 vs. 28 ± 3 nmol O\(_2\)·min\(^{-1}\)·mg protein\(^{-1}\); control vs. APC). Similarly, the uncoupling effect was also observed when the complex II substrate succinate was used but not when the respiration was measured in the presence of TMPD-ascorbate, which donates its electrons to complex IV via cytochrome c (Fig. 3C). To link the observed isoflurane-elicited effect on mitochondrial bioenergetics with cardioprotective preconditioning, we also subjected the mitochondria to hypoxia and reoxygenation (38). As shown in Fig. 3, A and B, the ADP-stimulated oxygen consumption (state 3) was better preserved after hypoxic stress in cardiac mitochondria isolated from isoflurane-treated rats compared with control mitochondria (96 ± 7 vs. 73 ± 9 nmol O\(_2\)·min\(^{-1}\)·mg protein\(^{-1}\), respectively). In fact, the RCR after hypoxia was higher for mitochondria from preconditioned rats (4 ± 0.8 vs. 2.5 ± 0.4 for control), implicating a decreased degree of damage.

Measurements of ATP production and ROS generation. An impact of preconditioning on mitochondrial ATP synthesis was also assessed. As shown in Fig. 4A, the rate of ATP production under basal conditions was similar in mitochondria isolated from both animal groups (132 ± 23 for control vs. 139 ± 18 nmol ATP·min\(^{-1}\)·mg protein\(^{-1}\) for APC). However, after exposure to hypoxic stress, ATP generation maintained substantially higher level in mitochondria obtained from the preconditioned animals (89 ± 10 vs. 31 ± 12 nmol ATP·min\(^{-1}\)·mg protein\(^{-1}\) for control vs. APC).
ATP·min⁻¹·mg protein⁻¹ for control), thus confirming the results obtained by oxygen consumption measurements.

We further tested whether isoflurane preconditioning in vivo has any effect on mitochondrial ROS production. With the use of succinate as substrate, no significant difference in the release of H₂O₂ was detected between mitochondria isolated from APC and control rats (29 ± 5 vs. 28 ± 4% of maximum release of ROS, evoked by antimycin A, Fig. 4B).

**Isoflurane treatment diminishes mitochondrial Ca²⁺ uptake.** Increased mitochondrial Ca²⁺ uptake that occurs during stress has a detrimental effect on mitochondrial and cellular viability (33). Isoflurane-evoked mitochondrial depolarization may attenuate mitochondrial Ca²⁺ loading by reducing the driving force for Ca²⁺ influx. On the basis of findings that treatment with isoflurane elicits partial mitochondrial uncoupling and depolarization, we investigated the influence of isoflurane preconditioning on mitochondrial Ca²⁺ uptake. Mitochondrial Ca²⁺ was monitored using rhod-2 fluorescent dye that accumulates preferentially into mitochondria (41, 50). Figure 5A demonstrates a substantial increase in rhod-2 fluorescence intensity when the superfusing solution was switched from Ca²⁺-free to 100 nM Ca²⁺-containing solution. However, when cardiomyocytes were transiently exposed to isoflurane, followed by the washout of the anesthetic (before Ca²⁺ challenge), the increase in mitochondrial rhod-2 intensity was attenuated (Fig. 5B). Data are summarized in Fig. 5C. Exposure to 100 nM Ca²⁺-containing solution elevated rhod-2 fluorescence to 258 ± 14% of baseline, whereas this increase was significantly blunted in preconditioned cells (180 ± 24% of baseline), indicating reduced mitochondrial Ca²⁺ uptake. This effect was also present when isoflurane was coadministered with the mitochondrial uniporter as a principal pathway for mitochondrial Ca²⁺ influx.

**DISCUSSION**

The present study investigated the effect of the preconditioning agent isoflurane on cardiac mitochondrial bioenergetics. Our results indicate that isoflurane treatment elicits a partial depolarization of cardiac mitochondria. This uncoupling effect, observed during exposure to isoflurane, was also present following the anesthetic removal, revealing some form of “memory.” Isoflurane-preconditioned mitochondria also exhibited better preservation of functional parameters after hypoxia-reoxygenation, as well as attenuated Ca²⁺ uptake, which may play an important protective role during cellular stress.

Since mitochondria are proposed to play an essential role in the mechanism of cardioprotection, the aim of this study was to examine how the treatment with the potent preconditioning agent isoflurane, both in vivo and in vitro, affects the mitochondrial energetic phenotype. Mitochondrial properties were analyzed in the whole cell as well as at the level of isolated organelles. Monitoring of the mitochondrial transmembrane potential with the fluorescent indicator TMRE in cardiomyocytes revealed its partial dissipation upon the addition of isoflurane. The mitochondrial uncoupling was also present following the anesthetic washout in cardiomyocytes as well as in the mitochondria isolated from previously preconditioned rats, thus implying that mitochondrial depolarization triggered during preconditioning is sustained after the withdrawal of the preconditioning agent.

Isoflurane effect on mitochondrial membrane potential was also investigated by monitoring the mitochondrial oxidative state via native fluorescence of flavoproteins (43). In various studies, dissipation of the mitochondrial electrochemical gradient enhanced flavoprotein fluorescence (29, 42), similar to our findings after isoflurane administration. These results indirectly confirmed our findings obtained with potentiometric dyes and measurements of the respiratory control ratio of mitochondria. Interestingly, increased flavoprotein oxidation upon exposure to isoflurane was recently also observed in the whole heart, and this effect was sustained after cessation of isoflurane treatment (52).
The mechanism(s) whereby isoflurane elicits mitochondrial uncoupling are not completely clear. An increased activity of cationic channels located in inner mitochondrial membrane, namely, mitoK\textsubscript{ATP} and mitoK\textsubscript{Ca}, was linked to alterations in $\Delta \Psi_m$ (35, 42). It was also recently proposed that an increased activity of the mitochondrial K\textsuperscript{+} channels, initiated during preconditioning, persists during the washout period and during reperfusion when it becomes important for the manifestation of the protective mechanism (16). This parallels our findings obtained in mitochondria isolated from isoflurane-preconditioned animals, which exhibited an increased level of uncoupling despite the absence of isoflurane in the experimental solution. Indeed, the addition of putative mitoK\textsubscript{ATP} channel inhibitor 5-HD into experimental solutions reduced the effects of isoflurane on mitochondrial TMRE and flavoprotein fluorescence. This suggests the possibility that the mitoK\textsubscript{ATP} activation may lead to an increased mitochondrial K\textsuperscript{+} influx and observed depolarization. Also, previous work from our laboratory (37) has demonstrated that isoflurane directly activates mitoK\textsubscript{ATP} channels reconstituted in planar lipid bilayers. However, the specificity of 5-HD was heavily questioned in recent publications (17, 18). Therefore, the observed effect of 5-HD could also be contributed to its actions on alternative, K\textsubscript{ATP} channel-unrelated pathways such as the fatty acid metabolism. Moreover, some investigators have suggested that the size of K\textsuperscript{+} fluxes catalyzed by mitoK\textsubscript{ATP} channels is too small to induce significant changes in mitochondrial membrane potential (26, 27).

Furthermore, a contribution of another mitochondrial K\textsuperscript{+} channel implicated in cardioprotection, the mitoK\textsubscript{Ca}, is unlikely in our experiments, since the mitoK\textsubscript{Ca} channel blocker pacflarin had no effect on Ca\textsuperscript{2+} influx.

Another potential explanation for isoflurane effects on mitochondrial bioenergetics comes from the recent studies demonstrating an increased activity of cardiac UCPS following ischemic preconditioning (36). Since the activity of UCPS in inner mitochondrial membrane is ROS inducible (6, 11), and isoflurane, similarly to other drugs that interact with the respiratory chain, may increase ROS production (20, 23), it is feasible that the observed mitochondrial uncoupling occurs via this pathway. This possibility is supported by the findings that transient ROS production during preconditioning stimulus constitutes a critical step of the preconditioning protective mechanism (34, 45, 48, 51). Despite the plausibility of this mechanism, addition of the UCP blocker GDP in our experiments did not affect the alterations in mitochondrial RCR identified in preconditioned animals. Possibly other mitochondrial carriers such as the ADP/ATP exchanger could be involved.

Since mitochondrial membrane potential is a major driving force for the H\textsuperscript{+} flux back to the matrix and thus for ATP production, we investigated whether the rate of ATP production is reduced in the mitochondria from isoflurane-treated rats. Our results indicate that the mild mitochondrial uncoupling elicited by isoflurane does not significantly affect the ATP synthesis. Moreover, the rate of ATP production after the mitochondrial exposure to hypoxic stress was maintained at significantly higher level in cardiac mitochondria isolated from isoflurane-treated animals, revealing their successful protection by preconditioning. These findings were corroborated by the measurements of mitochondrial respiration, which was also better preserved after stress in mitochondria from APC animals. Interestingly, these findings suggest that mitochondria themselves harbor an endogenous protective mechanism triggered by isoflurane exposure of the heart.

A number of studies demonstrated the beneficial effect of mitochondrial uncoupling on cellular viability during the conditions of metabolic challenge (2, 22, 47). One of the most detrimental pathological events that occurs during stress involves an enhanced mitochondrial uptake of increased cytosolic Ca\textsuperscript{2+}, which enters the mitochondria due to their negative potential (9, 33). This uptake is associated with the opening of the mitochondrial permeability transition pore, collapse of mitochondrial function, and finally, the cell death (8, 54). Mitochondrial depolarization induced by preconditioning could attenuate the Ca\textsuperscript{2+} accumulation by diminishing the driving force for Ca\textsuperscript{2+} influx. Our findings indicate that mitochondrial Ca\textsuperscript{2+} uptake is indeed reduced in isoflurane-pre-treated cardiomyocytes. In addition, an increase in matrix volume due to influx of water and P\textsubscript{i} (14) could also lead to a better preservation of mitochondrial architecture and oxidative phosphorylation under stress conditions (10, 15).

A correlation between $\Delta \Psi_m$ and ROS production has also been described previously (25, 32). During ischemia-reperfusion injury, the generation of toxic amounts of ROS is mostly linked to a sharp increase in mitochondrial membrane potential that occurs at the reflow (49). Mitochondrial uncoupling that is elicited by preconditioning could counteract this strong buildup of transmembrane potential during reperfusion and help to prevent a deleterious burst of ROS (44).

In summary, the present study demonstrated that preconditioning by isoflurane affects mitochondrial bioenergetical properties such as membrane potential and oxygen utilization. Despite their partial uncoupling, mitochondrial ability for ATP production is not impaired. Moreover, isoflurane-treated mitochondria exhibited a reduced potential for Ca\textsuperscript{2+} accumulation, which may attenuate the deleterious effects of Ca\textsuperscript{2+} overload. This effect likely contributes to improved tolerance to hypoxia-reoxygenation, which was observed in mitochondria of preconditioned animals.

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**REFERENCES**


50. Trollinger DR, Cascio WE, Lemasters JJ. Selective loading of Rhod 2 into mitochondria shows mitochondrial Ca\(^{2+}\) transients during the contractile cycle in adult rabbit cardiac myocytes. *Biochem Biophys Res Commun* 236: 738–742, 1997.


