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Isoflurane modulates cardiac mitochondrial bioenergetics by selectively attenuating respiratory complexes

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ABSTRACT

Mitochondrial dysfunction contributes to cardiac ischemia–reperfusion (IR) injury but volatile anesthetics (VA) may alter mitochondrial function to trigger cardioprotection. We hypothesized that the VA isoflurane (ISO) mediates cardioprotection in part by altering the function of several respiratory and transport proteins involved in oxidative phosphorylation (OxPhos). To test this we used fluorescence spectrophotometry to measure the effects of ISO (0, 0.5, 1, 2 mM) on the time-course of interlinked mitochondrial bioenergetic variables during states 2, 3 and 4 respiration in the presence of either complex I substrate K^+-pyruvate/malate (PM) or complex II substrate K^+-succinate (SUC) at physiological levels of extra-matrix free Ca^{2+} (~200 nM) and Na^+ (10 mM). To mimic ISO effects on mitochondrial functions and to clearly delineate the possible ISO targets, the observed actions of ISO were interpreted by comparing effects of ISO to those elicited by low concentrations of inhibitors that act at each respiratory complex, e.g. rotenone (ROT) at complex I or antimycin A (AA) at complex III. Our conclusions are based primarily on the similar responses of ISO and titrated concentrations of ETC. inhibitors during state 3. We found that with the substrate PM, ISO and ROT similarly decreased the magnitude of state 3 NADH oxidation and increased the duration of state 3 NADH oxidation, $\Delta\Psi_m$ depolarization, and respiration in a concentration-dependent manner, whereas with substrate SUC, ISO and ROT decreased the duration of state 3 NADH oxidation, $\Delta\Psi_m$ depolarization and respiration. Unlike AA, ISO reduced the magnitude of state 3 NADH oxidation with PM or SUC as substrate. With substrate SUC, after complete block of complex I with ROT, ISO and AA similarly increased the duration of state 3 $\Delta\Psi_m$ depolarization and respiration. This study provides a mechanistic understanding in how ISO alters mitochondrial function in a way that may lead to cardioprotection.

1. Introduction

Mitochondria are primary mediators of cardiac ischemia–reperfusion (IR) injury [1–4]; they are also important targets for volatile anesthetic (VA)-induced cardioprotection against IR injury [3,5–12]. Cardioprotection by a VA can be instituted either before the onset of ischemia (anesthetic pre-conditioning: APC) or at the onset of reperfusion (anesthetic post-conditioning: APOC) [13,14]. Regardless of the cardioprotective strategy, VAs exert effects on mitochondrial function.

Mitochondrial failure consequent to bioenergetic dysfunction leads to cell damage during IR but specific components of the mitochondrial bioenergetic mechanisms may be targets for the actions of VAs that underlie APC or APOC against IR injury. Volatile anesthetics, including isoflurane (ISO) are known to mediate cardioprotection against IR injury, in part, by attenuating function of the electron transport chain (ETC) protein complex I (NADH oxidoreductase) and by triggering mild ROS production, a critical mediator for cellular protection [15–18]. The mechanisms of ISO and other VAs to alter mitochondrial function and confer cardioprotection via their actions on ETC complexes and other mitochondrial transport proteins, however, are not well understood.

It may seem incongruous at first that IR-induced injury ultimately leads to dysfunction of mitochondrial respiration but, on the other hand, attenuation of mitochondrial respiration might minimize mitochondrial injury and reduce cell death during cardiac IR injury [19,20]. Thus, an understanding of ISO-induced actions on selective mitochondrial proteins, including the ETC complexes and transport proteins that are crucial for oxidative phosphorylation (OxPhos), is essential in elucidating the mechanisms of ISO-induced cardioprotection against IR injury.

In our recent study in isolated mitochondria [8] in which we demonstrated that ISO attenuates Ca^{2+} extrusion by the mitochondrial Na^+/Ca^{2+} exchanger, we also found that ISO caused a concentration-dependent decrease in the rate of state 3 NADH oxidation and ADP phosphorylation with the complex I substrate pyruvate/malate (PM).

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Complex I was first suggested as a site for VA effects [21,22]; this was supported by later findings [23,24]. Our initial observed effects of ISO [8] to increase the duration of state 3 NADH oxidation and $\Delta\Psi_m$ depolarization also supported complex I as a site of action, but this could alternatively have been due to inhibition of other ETC. complexes downstream of complex I, e.g., complexes III (ubiquinol cytochrome *c* oxidoreductase), IV (cytochrome *c* oxidase) and V (F_1F_0 ATP synthase), or complex II (succinate dehydrogenase) and transport proteins (e.g. adenine nucleotide transporter, ANT); alternatively, as has been reported, ISO might act as an uncoupling agent by promoting a proton leak [25] that could lead to mild $\Delta\Psi_m$ depolarization.

Because our understanding of the mechanisms of ISO-targeted mitochondrial interventions is not well defined, we compared the ISO effects to those elicited by low concentrations of known site-specific inhibitors of mitochondrial proteins. That is, we explored the potential mitochondrial targets of ISO by comparing its concentration-dependent effects with the concentration-dependent effects of known inhibitors of ETC. complexes and mitochondrial transport proteins to identify, and also rule out, possible ISO targets. In this way we hoped to better understand how VA-induced modification of mitochondrial bioenergetics can contribute to cardioprotection. To carry out our aims we used isolated cardiac mitochondria energized with substrates K^+-pyruvate/malate (PM), K^+-succinate (SUC) or SUC + Rotenone (ROT, 1 μM). Low concentrations of ROT antimycin A (AA), oligomycin (OMN), and atractyloside (ATR) were used to stepwise attenuate complexes I, III, V and ANT function, respectively; low concentrations of malonate (MAL) and potassium cyanide (KCN) were used in some experiments to attenuate functions of complexes II and IV, respectively.

2. Materials and methods

2.1. Mitochondrial isolation

Mitochondria were freshly isolated from hearts of Wistar rats (300–350 g) using protocols approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC) as described previously [8]. Briefly, rats were anesthetized with an intraperitoneal injection of inactin (150 mg/kg), and the ventricles were excised and placed in an ice-cold isolation buffer that contained (in mM): 200 mannitol, 50 sucrose, 5 KH_2PO_4, 5 MOPS, 1 EGTA and 0.1% BSA, with pH adjusted to 7.15 with KOH. The ventricles were minced in the presence of 5 U/ml protease (*Bacillus licheniformis*, Sigma) followed by differential centrifugation. All isolation procedures were carried out at 4 °C. Mitochondrial protein content was determined by the Bradford method [26]. The final mitochondrial pellet was resuspended in isolation buffer and kept on ice. All chemicals were procured from Sigma (St. Louis, MO, USA), unless stated otherwise.

2.2. Measure of mitochondrial redox state (NADH)

NADH autofluorescence was measured in the mitochondrial suspension during states 2, 3 and 4 respiration using fluorescence spectrophotometry at excitation wavelength 350 nm and emission wavelengths 395 nm and 456 nm (Photon Technology International, Birmingham, NJ), as we have described before [8,27–30]. At the end of each experiment (390 s), maximal (fully reduced) NADH (high NADH/NAD^+) was determined with 1 μM ROT, which completely blocks electron transport via FeS centers in complex I at the ubiquinone (Q) binding site so that proton pumping from NADH cannot occur; minimal (fully oxidized) NADH (low NADH/NAD^+) was determined with 20 μM trifluorocarbonylcyanidephenylhydrazone (FCCP), which induces maximal proton leak, and causes an increase in proton pumping and electron transfer between ETC complexes. The ratio of emission fluorescence (456/395) was normalized between the fully oxidized (with FCCP) state to the fully reduced (with ROT) state to represent % NADH as the total pool of NADH + NAD^+.

2.3. Measure of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was measured in the mitochondrial suspension during states 2, 3 and 4 respiration by fluorescence spectrophotometry using the fluorescent dye tetramethyl-rhodamine methyl ester (1 μM TMRM; Molecular Probes, Eugene, OR) at excitation wavelengths 546 nm and 573 nm and emission wavelength 590 nm [31], as we have described [29]. TMRM is a ratiometric, membrane potential sensitive, cationic fluorophore that equilibrates across the inner mitochondrial membrane (IMM) based on its electrochemical potential. $\Delta\Psi_m$ measurements followed the same time-line protocol as NADH measurements except that the respiratory uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (4 μM CCCP) was used to fully depolarize $\Delta\Psi_m$. The ratio of excitation wavelengths (546/573) at any time point was plotted as a percent of maximum fluorescence (100%) obtained by maximal depolarization with CCCP.

2.4. Measure of mitochondrial O_2 consumption

Mitochondrial O_2 consumption rate (respiration) was measured using a Clark-type O_2 electrode (System S 200A; Strathkelvin Instruments, Glasgow, UK) as we have described before [8,30,32,33]. Functional integrity of mitochondria was determined by the respiratory control index (RCI), defined here as the ratio of state 3 (after added ADP) to state 4 respiration (after complete phosphorylation of the added ADP). Only mitochondrial preparations with RCIs ≥ 6, measured with PM, were used to conduct further experiments.

2.5. Protocol

Measures of the three bioenergetic variables (NADH, $\Delta\Psi_m$, and respiration) were conducted with mitochondria (0.5 mg/ml) suspended in respiration buffer that contained (in mM): 130 KCl, 10 NaCl, 0.5 $CaCl_2$, 5 K_2HPO_4, 20 MOPS, 1 EGTA and 0.1% BSA at pH 7.15 adjusted with KOH, at room temperature (25 °C). NADH, $\Delta\Psi_m$, and respiration were monitored under complex I-linked substrate (10 mM PM) or complex II-linked substrate (10 mM SUC) during respiratory states 2 (before adding ADP), 3 (during phosphorylation of 250 μM ADP), and 4 (after complete phosphorylation of the ADP). Along with drug free controls (0.1% DMSO), either of three concentrations of ISO (0.5, 1, 2 mM) or several titrated low concentrations of respiratory complex inhibitors (e.g. 30, 50, 80, 120, 150 nM ROT; 25, 75, 85, 100, 110 nM AA) were added to the buffer after adding the substrate, followed by ADP. All drugs were dissolved in DMSO (0.1%). The appropriate volumes of ISO stock solutions dissolved in DMSO were prepared in tightly sealed glass vials and specific volumes from these vials were added to the experimental buffer (1 ml) to obtain the desired ISO concentrations (i.e. 0.5, 1 and 2 mM). ISO, 0.5 mM, is equivalent to approximately 1.4% atm at 25 °C with an estimated 1.3 minimum alveolar concentration (MAC) for rats [23]. To confirm the actual concentrations of ISO in each mitochondrial suspension, an aliquot of isoflurane in solution from each vial was obtained and measured by gas chromatography at the beginning (100 s) and end of each experiment (500 s). For added ISO, the measured values varied within ±10% of their initial prepared values of 0.5, 1, 1.5 and 2 mM. The time-course protocol consisted of the following: (1) 0 s, addition of mitochondrial sample into the respiration buffer and start of recording; (2) 30 s, introduction of the substrate (PM or SUC or SUC + ROT) (1 μM; high concentration that completely blocks electron flow from complex I) (state 2); (3) 90 s, addition of either of DMSO, ISO or other mitochondrial protein inhibitors, and (4) 150 s, addition of ADP (state 3).

Adding substrate PM or SUC provided for forward electron transfer from complex I or II through complex IV, respectively, and only reverse electron transfer from complex II to I with SUC. Substrate SUC with a complete block of complex I by ROT (1 μM) allowed for forward electron transfer only from complex II through IV. Sites of inhibition by