

# Isoflurane Differentially Modulates Mitochondrial Reactive Oxygen Species Production *via* Forward versus Reverse Electron Transport Flow

## Implications for Preconditioning

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### ABSTRACT

**Background:** Reactive oxygen species (ROS) mediate the effects of anesthetic precondition to protect against ischemia and reperfusion injury, but the mechanisms of ROS generation remain unclear. In this study, the authors investigated if mitochondria-targeted antioxidant (mitotempol) abolishes the cardioprotective effects of anesthetic preconditioning. Further, the authors investigated the mechanism by which isoflurane alters ROS generation in isolated mitochondria and submitochondrial particles.

**Methods:** Rats were pretreated with 0.9% saline, 3.0 mg/kg mitotempol in the absence or presence of 30 min exposure to isoflurane. Myocardial infarction was induced by left anterior descending artery occlusion for 30 min followed by reperfusion for 2 h and infarct size measurements. Mitochondrial ROS production was determined spectrofluorometrically. The effect of isoflurane on enzymatic activity of mitochondrial respiratory complexes was also determined.

### What We Already Know about This Topic

- Anesthetic preconditioning and protection from myocardial ischemic injury involves reactive oxygen species, but the mechanisms for this are unknown

### What This Article Tells Us That Is New

- In rats, isoflurane preconditioning reduced subsequent myocardial infarction size by 40% and generated reactive oxygen species primarily through inhibition of complex I activity in mitochondria

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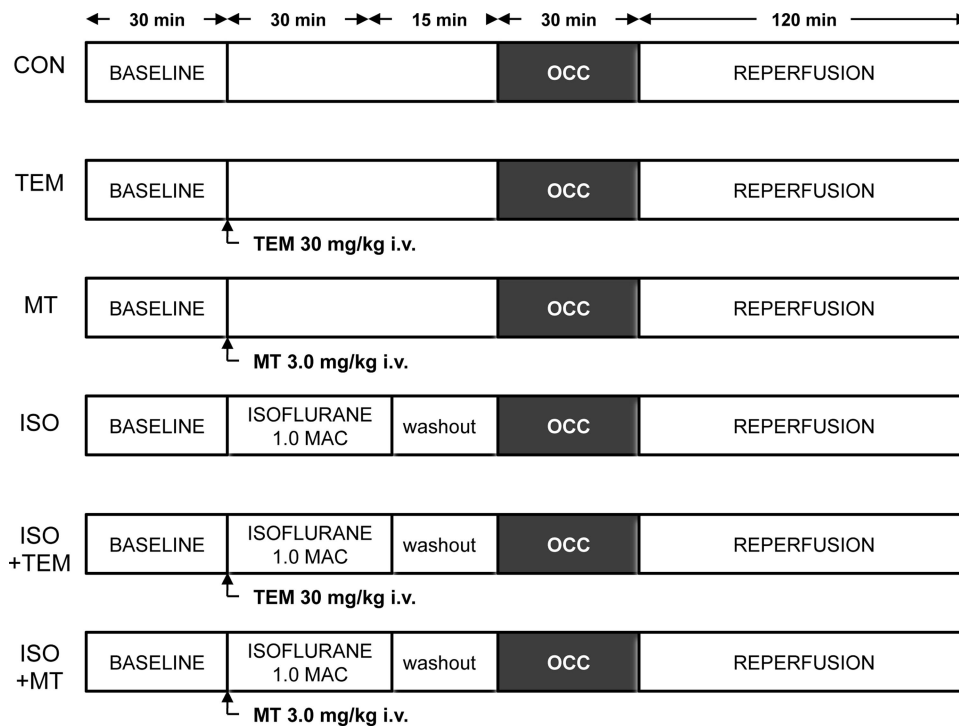
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chondrial ROS production was determined spectrofluorometrically. The effect of isoflurane on enzymatic activity of mitochondrial respiratory complexes was also determined.

**Results:** Isoflurane reduced myocardial infarct size ( $40 \pm 9\%$  = mean  $\pm$  SD) compared with control experiments ( $60 \pm 4\%$ ). Mitotempol abolished the cardioprotective effects of anesthetic preconditioning ( $60 \pm 9\%$ ). Isoflurane enhanced ROS generation in submitochondrial particles with nicotinamide adenine dinucleotide (reduced form), but not with succinate, as substrate. In intact mitochondria, isoflurane enhanced ROS production in the presence of rotenone, antimycin A, or ubiquinone when pyruvate and malate were substrates, but isoflurane attenuated ROS production when succinate was substrate. Mitochondrial respiratory experiments and electron transport chain complex assays revealed that isoflurane inhibited only complex I activity.

**Conclusions:** The results demonstrated that isoflurane produces ROS at complex I and III of the respiratory chain *via* the attenuation of complex I activity. The action on complex I decreases unfavorable reverse electron flow and ROS release in myocardium during reperfusion.

**V**OLATILE anesthetics such as isoflurane have cardioprotective effects when administered before a period of myocardial ischemia and reperfusion, and this phenomenon is referred to as anesthetic preconditioning (APC).<sup>1,2</sup> It is accepted that mitochondria play a crucial role in the mech-



**Fig. 1.** Experimental protocols used for the production of coronary artery occlusion (OCC) and reperfusion in rats treated with saline vehicle (CON), tempol (TEM), mitotempol (MT), and with and without 1.0 minimum alveolar concentration (MAC) isoflurane (ISO).  $n = 8/\text{group}$ .

anism of APC.<sup>3–5</sup> Volatile anesthetics increase signaling quantities of reactive oxygen species (ROS) production that serve as a mandatory step during triggering of APC, and scavengers of ROS block APC-induced cardioprotection.<sup>6–8</sup> Mitochondrial  $K^+$  influx through mitochondrial adenosine triphosphate-sensitive  $K^+$  channels<sup>9</sup> and/or  $Ca^{2+}$ -activated  $K^+$  channels,<sup>10</sup> uncoupling,<sup>3,5</sup> mild depolarization<sup>11</sup> and inhibition of mitochondrial respiration<sup>12,13</sup> have been suggested as possible mechanisms responsible for mitochondrial ROS generation during APC. On the other hand, a burst of ROS generation during reperfusion exacerbates ischemic injury, and this may be ameliorated by APC or by application of anesthetic at the onset of reperfusion (anesthetic postconditioning).<sup>7,14</sup> Thus, volatile anesthetics have apparent directionally opposite effect on mitochondrial ROS production when applied before ischemia or after reperfusion. The major sites responsible for mitochondrial ROS generation are complex I and complex III of the electron transport chain (ETC).<sup>15</sup> Our previous study has demonstrated that ROS could be generated at complex III during APC,<sup>16</sup> whereas direct inhibitory effect of volatile anesthetics on complex I has also been elucidated.<sup>12,13</sup> Thus, the detailed molecular mechanism and sites of ROS generation during APC are controversial.

In the current study, we investigated if mitotempol, a mitochondria-targeted superoxide dismutase mimetic, abolished APC. We hypothesized that inhibition of ETC complex I by isoflurane accounts for an increase of signaling ROS both at complex I and at complex III, and for a decrease of

detrimental ROS at the time of reperfusion through inhibition of reverse electron flow.

## Materials and Methods

All experimental procedures and protocols used in the current study were reviewed and approved by the Animal Care and Use Committee of the Medical College of Wisconsin, Milwaukee, Wisconsin, and all were in conformance with the *Guiding Principles in the Care and Use of Animals* of the American Physiologic Society and were in accordance with the *Guide for the Care and Use of Laboratory Animals*.

### Surgical Procedure and Experimental Protocol

Male Wistar rats (270–320 g) were randomly assigned to pretreatment with 0.9% saline (control) or superoxide dismutase mimetics, tempol (30 mg/kg) or mitotempol (3.0 mg/kg). Mitotempol was synthesized by Joy Joseph, Ph.D., Associate Professor of Biophysics, Medical College of Wisconsin.

The surgical procedure was performed as previously described.<sup>17</sup> Briefly, after rats were anesthetized, the trachea was intubated for artificial ventilation. A thoracotomy was performed and a suture was placed around the left anterior descending artery for coronary artery occlusion and reperfusion. Systemic hemodynamics were recorded continuously on a polygraph and digitized using a computer connected to an analog-to-digital converter.

The experimental design for *in vivo* experiments is shown in figure 1. Baseline hemodynamics and arterial blood gas tensions were recorded for 30 min. All rats underwent 30 min of left anterior descending artery occlusion followed by 2 h of reperfusion, in the absence or presence of 30 min exposure to 1 minimum alveolar concentration of isoflurane in six separate experimental groups (n = 8 per group). After reperfusion, myocardial infarct size was measured as previously described.<sup>18</sup> Infarct size was expressed as percentage of the left ventricular area at risk.

### Isolation of Rat Heart Mitochondria

Mitochondria were isolated from rat heart by differential centrifugation as previously described.<sup>3</sup> Hearts were excised and washed in isolation buffer (200 mM mannitol, 50 mM sucrose, 5 mM KH<sub>2</sub>PO<sub>4</sub>, 5 mM 3-[N-morpholino] propanesulfonic acid, 1 mM EGTA, 0.1% bovine serum albumin [BSA], pH 7.3 at 25°C adjusted with 5 M KOH) and minced into small pieces. The suspension was homogenized in the presence of 5 U/ml protease (from *Bacillus licheniformis*) by using a T 25 dispenser (IKA-Werke, Staufen, Germany). The mitochondrial pellets were resuspended in isolation buffer at a concentration of 10–20 mg/ml, stored on ice and used for experiments within 4 h. Protein concentration was determined using a modified Lowry assay kit (Bio-Rad, Hercules, CA).

### The ROS Scavenging Effects of Mitotempol and Tempol

To assess the effectiveness of mitotempol and tempol to scavenge ROS, superoxide anion generation was measured using the chemiluminescent probe 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- $\alpha$ ]pyrazin-3-one in the presence of mitotempol or tempol. 0.5 mg mitochondria were added to 1 ml respiration buffer containing 5  $\mu$ M 2-methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- $\alpha$ ]pyrazin-3-one and 5 mM pyruvate and malate were added to initiate superoxide generation. Antimycin A (10  $\mu$ M) was used to maximize ROS production rate. Chemiluminescence was measured in a Modulus luminometer (Turner Biosystems, Sunnyvale, CA) at room temperature.

### Measurement of Mitochondrial ROS Production

Mitochondrial ROS production was measured spectrofluorometrically using the fluorescent probe amplex red (12.5  $\mu$ M) in the presence of 0.1 U/ml horseradish peroxidase at 30°C. Amplex red reacts with H<sub>2</sub>O<sub>2</sub> in the presence of peroxidase and produces the red-fluorescent product resorufin. Resorufin fluorescence was measured using a spectrofluorometer (Photon Technology International, Birmingham, NJ) with excitation and emission wavelengths set at 530 and 583 nm, respectively. Measurements were performed in respiration buffer (130 mM KCl, 5 mM K<sub>2</sub>HPO<sub>4</sub>, 20 mM 3-(N-morpholino) propanesulfonic acid, 2.5 mM EGTA, 1  $\mu$ M Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, and 0.1% BSA, pH 7.4) containing mitochondria at a final concentration of 0.5 mg protein/ml. ROS

generation was stimulated with complex I substrates pyruvate (5 mM) and malate (5 mM) or complex II substrate succinate (5 mM) in the presence and absence of isoflurane (0.5 mM). Rotenone (1  $\mu$ M) and antimycin A (1  $\mu$ M) were used to inhibit the activities of complex I and complex III, respectively. Decylubiquinone (50  $\mu$ M) was used to estimate whether an increase of the ubiquinone/ubiquinol ratio affects ROS production.<sup>19</sup>

### Measurement of ROS Production in Submitochondrial Particles

Submitochondrial particles (SMPs) were prepared by sonicating mitochondria on ice (10 bursts of 10 s at 20 watt). The sonicated suspension was centrifuged at 10,000 g for 10 min (4°C) and the supernatant was then centrifuged for 1 h at 100,000 g (4°C). The pellet was resuspended in mitochondrial isolation buffer, and protein content was determined. Measurements were performed in respiration buffer containing SMPs at a final concentration of 0.5 mg protein/ml. ROS generation was stimulated with complex I substrate nicotinamide adenine dinucleotide (reduced form) (NADH, 0.5 mM) or complex II substrate succinate (5 mM) in the presence and absence of isoflurane. Superoxide dismutase (100 units/ml) treatment was required when NADH was used as substrate to reduce the substrate-dependent background rate of the assay.<sup>20</sup>

### Measurement of Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption was measured in the presence or absence of isoflurane (0.5 mM) by using an oxygen electrode (Hansatech Instruments, Norfolk, United Kingdom) maintained at 30°C, and experiments were conducted in respiration buffer containing 0.5 mg/ml mitochondrial protein. State 2 respiration was initiated with complex I substrates pyruvate (5 mM) and malate (5 mM) or complex II substrate succinate (5 mM) in the presence and absence of complex I inhibitor rotenone (1  $\mu$ M). State 3 respiration was measured in the presence of 250  $\mu$ M adenosine diphosphate, and state 4 was monitored after complete adenosine diphosphate consumption.

### Electron Transport Chain Assay

ETC enzyme activity was measured spectrophotometrically as specific donor-acceptor oxidoreductase activity.<sup>21,22</sup> Isolated mitochondria were solubilized with 2% cholic acid and diluted to a final concentration of 100  $\mu$ g/ml in the experimental buffer (220 mM D-mannitol, 70 mM sucrose, 5 mM 3-(N-morpholino) propanesulfonic acid, 2 mM EDTA, and 0.2% BSA [pH 7.4]). In the isoflurane-treated group, isoflurane (0.5 mM) dissolved in dimethylsulfoxide was added just before the reaction.

### Complex I (NADH-Ubiquinone Oxidoreductase) Activity Assay

Complex I activity was determined by the rotenone-sensitive reduction of NADH absorbance using decylubiquinone as

acceptor. The assay mixture contained 20  $\mu\text{g/ml}$  mitochondrial protein, 50 mM  $\text{KH}_2\text{PO}_4$ , 0.1 mM EDTA, 0.1% BSA, 0.15 mg/ml aroclorin, 2 mM antimycin A, and 0.2 mM NADH in a spectrophotometer cuvette. The reaction was initiated by adding 75 mM decylubiquinone, and the change in absorbance of NADH was measured at 340 nm ( $\epsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$ ).

### Complex II (Succinate-Ubiquinone Oxidoreductase)

#### Activity Assay

The activity of complex II was determined by dichlorophenolindophenol reduction. The assay mixture contained 20  $\mu\text{g/ml}$  mitochondrial protein, 50 mM  $\text{KH}_2\text{PO}_4$ , 0.1 mM EDTA, 0.1% BSA, 5 mM  $\text{NaN}_3$ , 0.5 mM duroquinone, and 25 mM dichlorophenolindophenol. The reaction was initiated by adding 20 mM succinate, and the change in absorbance of dichlorophenolindophenol was measured at 600 nm ( $\epsilon = 21 \text{ mM}^{-1}\text{cm}^{-1}$ ).

### Complex III (Ubiquinol-Cytochrome C Reductase)

#### Activity Assay

The activity of complex III was determined by antimycin A-sensitive reduction of cytochrome c in the presence of decylubiquinol. Decylubiquinol was synthesized according to the method described by Trumpower and Edwards.<sup>23</sup> The assay mixture contained 5  $\mu\text{g/ml}$  mitochondrial protein, 50 mM  $\text{KH}_2\text{PO}_4$ , 0.1 mM EDTA, 0.1% BSA, 3 mM  $\text{NaN}_3$ , and 60  $\mu\text{M}$  oxidized cytochrome c. The reaction was initiated by adding 100  $\mu\text{M}$  decylubiquinol, and the change in absorbance of cytochrome c was measured at 550 nm ( $\epsilon = 18.5 \text{ mM}^{-1}\text{cm}^{-1}$ ).

### Complex IV (Cytochrome C Oxidase) Activity Assay

The activity of complex IV was assessed by the decrease in the rate of absorbance of reduced cytochrome c at 550 nm ( $\epsilon = 18.5 \text{ mM}^{-1}\text{cm}^{-1}$ ). The assay mixture contained 50 mM  $\text{KH}_2\text{PO}_4$ , 0.15 mg/ml aroclorin, and 40  $\mu\text{M}$  reduced cytochrome c, and the reaction was initiated by adding 1  $\mu\text{g/ml}$  mitochondrial protein.

### Administration of Isoflurane In Vitro Experiments

The appropriate volume of isoflurane stock solution dissolved in dimethylsulfoxide was added to the experimental buffer to obtain the desired concentration (0.5 mM corresponding to approximately 1 minimum alveolar concentration for rats). In control experiments, we confirmed that our final dimethylsulfoxide concentration (less than 0.2% v/v) had no effect on mitochondrial ROS production, oxygen consumption, and ETC assays. At the end of each experiment, the isoflurane concentration was analyzed by gas chromatography. The concentration of isoflurane varied  $\pm 10\%$  from the reported value.

### Data Analysis

All data are presented as mean  $\pm$  SD. Statistical analysis was performed with Origin 7 (OriginLab, Northampton, MA)

software. Comparisons were performed using two-tailed hypothesis testing. One-way ANOVA was used for statistical analysis of differences between groups, with Bonferroni correction *post hoc* test. Student *t* test was used to test for a difference between two groups. Heart rate, mean arterial pressure, and rate-pressure product were analyzed by one-way repeated measures of ANOVA. A *P* value less than 0.05 was considered statistically significant.

## Results

### Effects of Mitotempol on Infarct Size In Vivo

There were no differences in baseline heart rate, mean arterial pressure, and rate pressure product between groups (table 1). Mitotempol and tempol had no effect on hemodynamics. Because of its cardiodepressant effects, heart rate, mean arterial pressure, and rate pressure product were briefly decreased by isoflurane; however, these values returned to baseline before coronary artery occlusion. Isoflurane (fig. 2) significantly decreased infarct size ( $40 \pm 9\%$  of area at risk) compared with control experiments ( $60 \pm 4\%$ ). Mitotempol and tempol alone had no effect on infarct size ( $58 \pm 7\%$  and  $59 \pm 5\%$ , respectively) but abolished the cardioprotection afforded by isoflurane ( $60 \pm 9\%$  and  $58 \pm 9\%$ , respectively). The actions of mitotempol and tempol to scavenge mitochondrial ROS were confirmed in isolated mitochondria subjected to antimycin A. ROS production was significantly reduced in the presence of mitotempol ( $3.7 \pm 1.3$  relative light units/mg protein/s) and tempol ( $3.4 \pm 1.2$  relative light units/mg protein/s) compared with the absence ( $59.5 \pm 6.4$  relative light units/mg protein/s) of antioxidants.

### ROS Production in Isolated Mitochondria and Submitochondrial Particles

Isoflurane-dependent changes in resorufin fluorescence observed during state 2 respiration in isolated mitochondria initiated by complex I-linked substrates pyruvate and malate or complex II-linked substrate succinate are shown in figure 3A–D and figure 4A–C, respectively. Rotenone, antimycin A, and ubiquinone alone increased ROS generation when complex I-linked substrates were used (fig. 3A–C). Although isoflurane alone did not alter ROS production rate with complex I-linked substrates, it enhanced ROS generation in the presence of rotenone ( $27.6 \pm 6.9$  and  $39.9 \pm 9.7$  pmol  $\text{H}_2\text{O}_2/\text{mg protein/min}$  for the control and isoflurane-treated groups, respectively), antimycin A ( $105.7 \pm 6.0$  and  $169.3 \pm 9.8$  pmol  $\text{H}_2\text{O}_2/\text{mg protein/min}$ ), or ubiquinone ( $30.6 \pm 9.5$  and  $44.2 \pm 7.8$  pmol  $\text{H}_2\text{O}_2/\text{mg protein/min}$ ) (fig. 3D). However, when electrons were delivered to complex II by succinate, isoflurane alone decreased ROS production rate ( $400.9 \pm 18.3$  and  $135 \pm 21.7$  pmol  $\text{H}_2\text{O}_2/\text{mg protein/min}$  for the control and isoflurane-treated groups, respectively), whereas isoflurane had no effect on rotenone, antimycin A, and ubiquinone-modulated ROS production (fig. 4C).

When using SMP instead of intact mitochondria, ROS production rate significantly increased in the presence of iso-



**Table 1.** Systemic Hemodynamics

	Baseline	ISO	Preocclusion	30 min CAO	Reperfusion (h)	
					1	2
HR (beats/min)						
CON	384 ± 46	—	351 ± 23	383 ± 43	362 ± 39	346 ± 36
TEM	374 ± 40	—	343 ± 48	337 ± 48	314 ± 33	305 ± 47
MT	401 ± 40	—	380 ± 28	382 ± 36	347 ± 30	345 ± 25
ISO	386 ± 42	306 ± 65*	341 ± 41	377 ± 52	350 ± 57	346 ± 56
TEM + ISO	383 ± 28	294 ± 32*	344 ± 23	369 ± 35	369 ± 38	365 ± 46
MT + ISO	368 ± 19	296 ± 17*	361 ± 26	373 ± 32	362 ± 31	342 ± 33
MAP (mmHg)						
CON	103 ± 17	—	108 ± 18	107 ± 18	80 ± 14	76 ± 15
TEM	114 ± 16	—	102 ± 19	91 ± 20	72 ± 8	67 ± 12
MT	126 ± 10	—	115 ± 8	101 ± 16	77 ± 7	69 ± 5
ISO	114 ± 15	63 ± 13*	106 ± 13	105 ± 12	75 ± 20	73 ± 24
TEM + ISO	114 ± 10	64 ± 6*	102 ± 8	102 ± 8	85 ± 8	79 ± 13
MT + ISO	119 ± 15	69 ± 8*	117 ± 15	117 ± 16	98 ± 21	86 ± 20
RPP (min/mmHg/10 <sup>-3</sup> )						
CON	47.8 ± 11.7	—	45.2 ± 8.8	46.7 ± 8.4	36.3 ± 5.4	33.2 ± 5.8
TEM	51.3 ± 10.6	—	42.4 ± 11.4	36.8 ± 10.2	29.6 ± 5.0	26.9 ± 6.7
MT	60.1 ± 7.4	—	49.3 ± 8.5	46.4 ± 7.4	35.6 ± 4.8	33.4 ± 3.7
ISO	52.3 ± 10.4	26.3 ± 9.5*	43.2 ± 8.8	45.9 ± 9.3	33.2 ± 10.2	32.7 ± 13.0
TEM + ISO	51.9 ± 7.5	25.8 ± 4.6*	42.5 ± 4.3	42.7 ± 6.4	38.9 ± 6.3	36.7 ± 7.2
MT + ISO	54.4 ± 9.0	28.7 ± 3.0*	52.2 ± 6.9	50.9 ± 8.6	43.5 ± 8.4	37.4 ± 8.9

Data are presented as mean ± standard deviation (SD). n = 8/group.

\*  $P < 0.05$  vs. baseline.

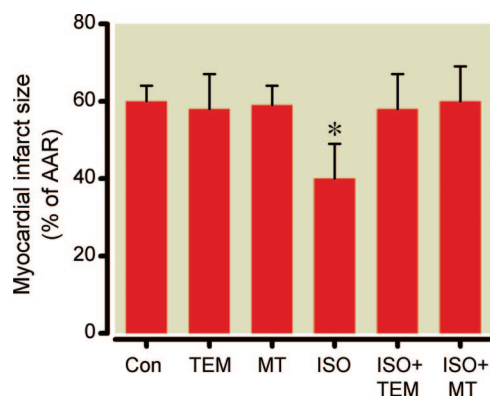
CAO = coronary artery occlusion; CON = control; HR = heart rate; ISO = isoflurane; MAP = mean arterial pressure; MT = mitotempol; RPP = rate pressure product; TEM = tempol.

flurane alone with NADH as substrate ( $15.5 \pm 6.4$  and  $35.4 \pm 8.4$  pmol  $H_2O_2$ /mg protein/min for the control and isoflurane-treated groups, respectively), but not with succinate ( $6.1 \pm 1.1$  and  $6.4 \pm 1.0$  pmol  $H_2O_2$ /mg protein/min) (fig. 5A and B).

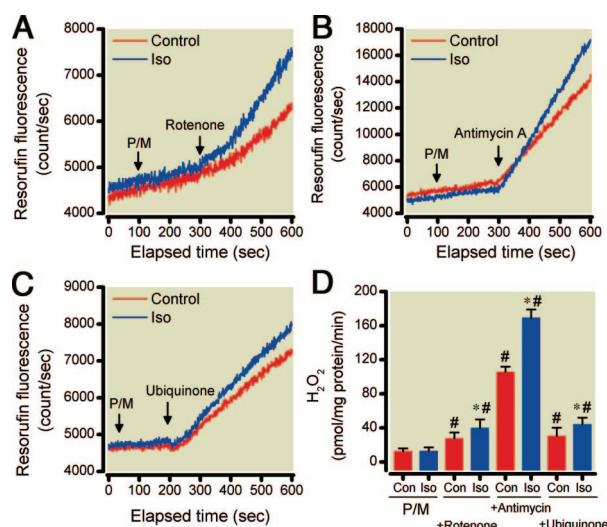
### The Effects of Isoflurane on Mitochondrial Respiration

The effects of isoflurane on mitochondrial respiration initiated by pyruvate and malate or succinate in the absence and presence of rotenone are shown in figure 6A–C. Isoflurane significantly decreased state 3 respiration ( $72 \pm 11$  nmol

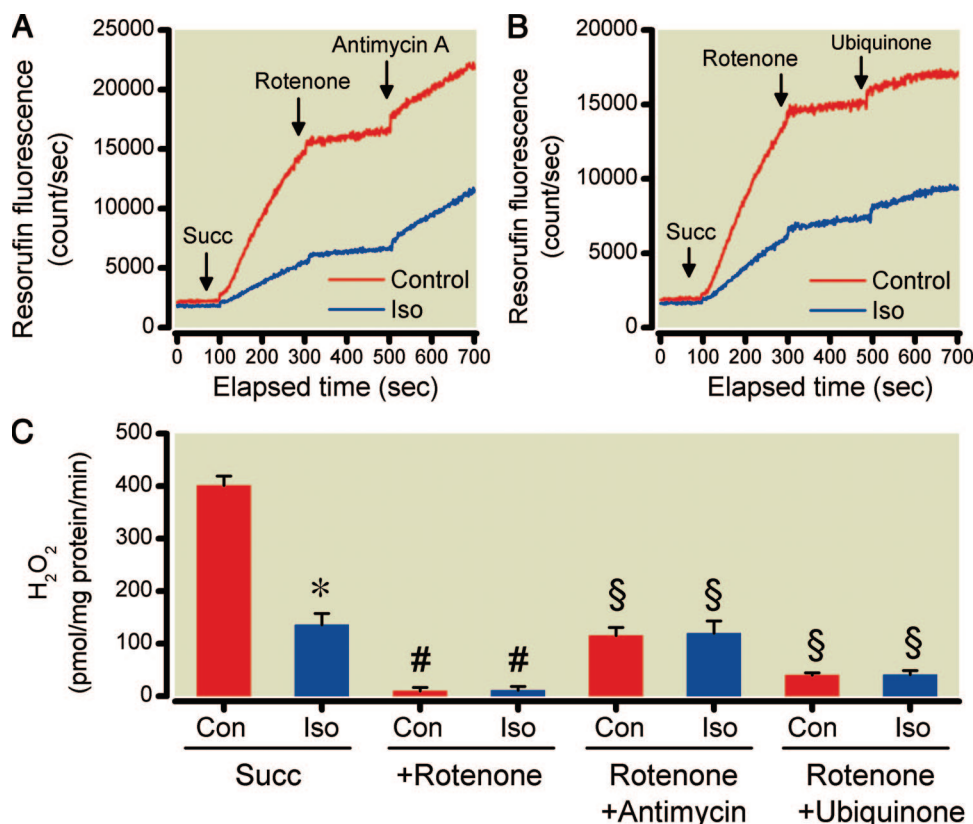
$O_2$ /min/mg protein) in comparison with the control group ( $116 \pm 12$  nmol  $O_2$ /mg protein/min) when pyruvate and malate were used (fig. 6D). When succinate was used in the



**Fig. 2.** Myocardial infarct size expressed as a percentage of the left ventricular area at risk (AAR) in rats pretreated with saline vehicle (Con), tempol (TEM), and mitotempol (MT), and in the absence or presence of isoflurane (ISO). Data are presented as mean ± SD. n = 8/group. \*  $P < 0.05$  versus Con.



**Fig. 3.** Reactive oxygen species (ROS) production in isolated mitochondria with pyruvate and malate (P/M) as substrates. Representative traces show that rotenone (A), antimycin A (B), and ubiquinone (C) induced ROS production to a larger extent in the presence compared with the absence (Con) of isoflurane (Iso). (D) Summary of the recordings. Summary data are mean ± SD. P/M alone; n = 10/group. Rotenone, antimycin A, and ubiquinone; n = 8/group. \*  $P < 0.05$  versus corresponding Con; #  $P < 0.05$  versus respective Con with P/M alone.



**Fig. 4.** Reactive oxygen species (ROS) production in isolated mitochondria with succinate (Succ) as substrate. The effects of antimycin-A (A) and ubiquinone (B) on complex II-linked ROS production in the presence or absence (Con) of isoflurane (Iso) were analyzed after reverse electron flow induced-ROS was blocked by rotenone. Iso reduced reverse electron flow induced-ROS production in the absence of rotenone; however, Iso had no effect on the presence of rotenone, antimycin A, or ubiquinone. (C) Summary of recordings. Data are presented as mean  $\pm$  SD,  $n = 8/\text{group}$ . \*  $P < 0.05$  versus corresponding Con; #  $P < 0.05$  versus the respective Con with Succ alone; §  $P < 0.05$  versus respective Con with rotenone.

absence of rotenone, isoflurane significantly increased state 3 respiration ( $124 \pm 10$  nmol O<sub>2</sub>/mg protein/min) compared with the control group ( $109 \pm 13$  nmol O<sub>2</sub>/mg protein/min) (fig. 6E). On the other hand, isoflurane had no significant

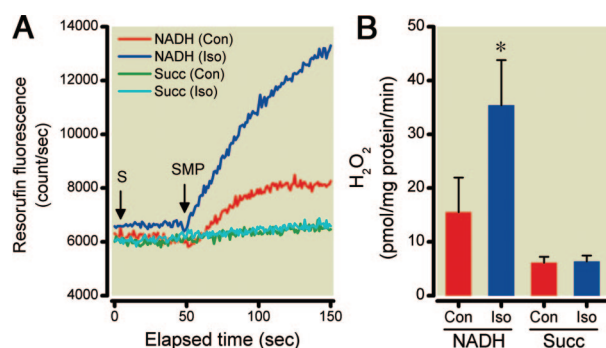
effect on mitochondrial respiration when succinate was used in the presence of rotenone (fig. 6F).

### ETC Complex Activity Assay in Solubilized Mitochondria

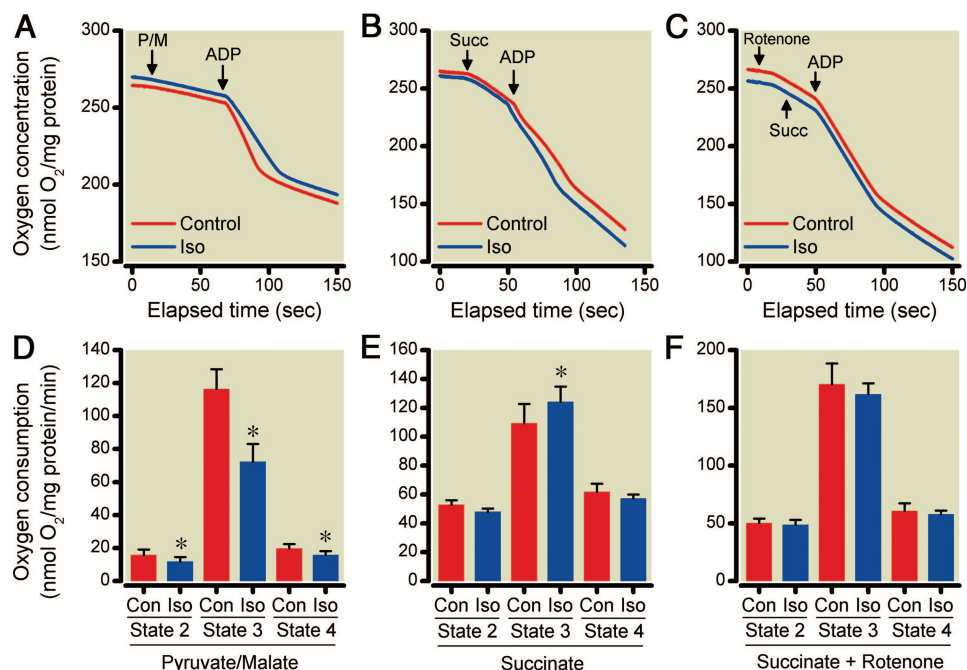
In order to further pinpoint the complexes targeted by isoflurane, the relative activities of the ETC complexes were quantified in cholic acid-solubilized mitochondria (fig. 7). Isoflurane significantly reduced only complex I activity to  $86 \pm 5\%$  ( $356 \pm 18$  nmol NADH/mg protein/min) of control ( $410 \pm 30$  nmol NADH/mg protein/min); no changes in the activities of other complexes were observed ( $99 \pm 5$ ,  $97 \pm 3$  and  $97 \pm 5\%$  of control for complex II, III, and IV activities, respectively).

### Discussion

The current study showed that (1) mitochondrial-targeted antioxidant abolished the cardioprotection of APC; (2) isoflurane enhanced ROS production in SMPs when complex I-linked substrates were used, but this occurred in intact mitochondria only in the presence of rotenone, antimycin A, or ubiquinone; (3) with a complex II-linked substrate, iso-



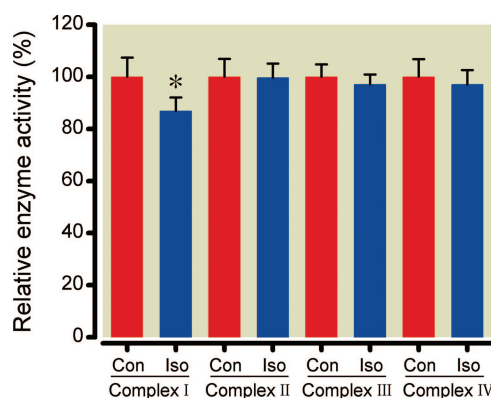
**Fig. 5.** Reactive oxygen species (ROS) production in submitochondrial particles (SMP) with complex I (NADH) and complex II (succinate) substrates in the presence or absence (Con) of isoflurane (Iso). Representative traces (A) show the effects of Iso on ROS production using the different substrates (S). Iso induced ROS generation only when NADH was used as substrate, but had no effect when succinate (Succ) was used. (B) Summary data are mean  $\pm$  SD,  $n = 8/\text{group}$ . \*  $P < 0.05$  versus Con.



**Fig. 6.** Effect of isoflurane (Iso) on mitochondrial respiration using substrates pyruvate/malate (P/M), succinate (Succ), and adenosine diphosphate (ADP) in the absence or presence of Iso. Representative traces demonstrate the effect of Iso on oxygen consumption using P/M (A), Succ (B), and Succ + rotenone (C). Iso decreased mitochondrial respiration compared with the control group (Con) when P/M were used (D). When Succ was used in the absence of rotenone, Iso significantly increased mitochondrial respiration (E). Iso had no significant effect on mitochondrial respiration when Succ was used in the presence of rotenone (F). Summary data are mean  $\pm$  SD. P/M; n = 10/group. Succinate and Succinate + Rotenone; n = 8/group. \*  $P < 0.05$  versus Con.

flurane did not alter ROS production in SMPs but decreased ROS production in intact mitochondria; and (4) isoflurane directly attenuated complex I activity.

Low concentrations of ROS<sup>24,25</sup> have been demonstrated to activate preconditioning pathways. Volatile anesthetics such as sevoflurane<sup>26</sup> and isoflurane<sup>6,16</sup> have also been shown to activate cardioprotective signaling through ROS-sensitive pathways. ROS produced by volatile anesthetics activate a variety of proteins such as protein kinase C, Akt, and glycogen synthase kinase- $\beta$ , which ultimately causes a delay of



**Fig. 7.** The effects of (Iso) on the activity of the electron transport chain complexes. Enzymatic activity for each complex during Iso is expressed relative to the absence (Con) of Iso. Data are mean  $\pm$  SD, n = 8/group. \*  $P < 0.05$  versus (Con).

permeability transition pore opening, thereby protecting the myocardium against ischemia and reperfusion injury.<sup>27</sup> Previous studies demonstrated that ROS scavengers abolished volatile anesthetic-induced reduction in myocardial infarct size.<sup>6–8</sup> Although mitochondria have been suggested as the source of protective signaling ROS in APC,<sup>27</sup> direct evidence for this was lacking. Piperidine nitroxides such as tempol are well established antioxidants *in vitro* and *in vivo*.<sup>28</sup> The antioxidant effect is based on their ability to catalyze the dismutation of superoxide and to detoxify redox-reactive forms of transition metal ions. Mitotempol, accumulated in mitochondria due to its cationic triphenylphosphonium moiety, is an effective antioxidant because of its proximity to ETC-generated ROS.<sup>29</sup> The current results demonstrate that scavenging of ROS with tempol and, more importantly, mitotempol abolishes isoflurane-induced cardioprotection. Although mitotempol is specific in mostly scavenging mitochondria-originating ROS, tempol indifferently scavenges all sources of cellular ROS. This directly implicates mitochondria as the source ROS responsible for protective signaling.

Complex I and complex III are the major sites of ROS generation in mitochondria<sup>15</sup>; however, the site responsible for volatile anesthetic-induced ROS generation remains undefined. The following evidence supports the contention that complex I is a source of isoflurane-induced ROS: isoflurane increases ROS generation with complex I- but not com-

plex II-linked substrates in SMPs (fig. 5); and isoflurane only alters activity of complex I, but not of the other complexes in solubilized mitochondria. However, isoflurane-induced ROS generation in isolated mitochondria was not detected in our experiments when complex I-linked substrates were used. This apparent discrepancy observed in isolated mitochondria compared with submitochondrial particles may have occurred because ROS generated at complex I site are released into the mitochondrial matrix where they rapidly react with manganese superoxide dismutase before interacting with detection reagent in the buffer.<sup>15</sup> Interestingly, in the presence of rotenone, antimycin A, or ubiquinone, isoflurane enhanced ROS production with complex I-linked substrates. In the case of complex I inhibitor rotenone, the inhibitory effect of isoflurane on complex I is enhanced and thereby allows isoflurane-induced  $H_2O_2$  detection in the buffer. The contribution of antimycin A (inhibitor of complex III) and ubiquinone (donates electrons to complex III in its reduced form) to isoflurane-enhanced ROS generation suggests a possible involvement of complex III. It is generally assumed that ROS at complex III is generated by oxygen reduction *via* electron transfer from ubiquinol, which is formed at the ubiquinol oxidation center. Inhibition of electron flow downstream of this center, *e.g.*, by antimycin A, increases ROS generation.<sup>15</sup> However, this mechanism is not able to explain isoflurane-enhanced ROS generation at complex III because isoflurane did not affect electron flow at complex III as demonstrated by the ETC complex assay. Recently, Dröse and Brandt<sup>19</sup> have proposed an alternate mechanism for ROS generation at complex III: they observed that ROS generation depends on the redox state of the ubiquinone pool. An increased ubiquinone/ubiquinol ratio enhances ROS generation at complex III.<sup>19</sup> According to this mechanism, an electron is transferred to oxygen from reduced downstream cytochrome  $b_L$  to oxygen in a reverse reaction *via* oxidized ubiquinone. We confirmed that exogenous ubiquinone enhanced ROS generation with complex I substrates. Interestingly, isoflurane further enhanced ROS generation in the presence of ubiquinone. Inhibition of complex I by isoflurane may further increase the ubiquinone/ubiquinol ratio by decreasing the electron flow complex I to form reduced ubiquinol, thereby enhancing ROS generation at the complex III site. Similarly, diazoxide, independent of its putative effect on mitochondrial adenosine triphosphate-sensitive  $K^+$  channels, attenuates ubiquinol concentration *via* inhibition of complex II, causing an increase in ROS at the ubiquinol oxidation.<sup>30</sup> Thus, inhibition of electron flow at upstream complexes could contribute to ROS generation at complex III through changes in the ubiquinone/ubiquinol ratio. We suggest a mechanism whereby isoflurane interacts with complex I and causes an increase in ROS generation at the complex III site.

Ischemic or pharmacologic preconditioning-induced mitochondrial  $K^+$  influx through mitochondrial adenosine triphosphate-sensitive  $K^+$  channels and/or  $Ca^{2+}$ -activated

$K^+$  channels and subsequent matrix alkalization was previously suggested as underlying mechanism of increased ROS production.<sup>31,32</sup> For example, diazoxide, a putative mitochondrial adenosine triphosphate-sensitive  $K^+$  channel agonist, produced pharmacologic preconditioning by generating small quantities of ROS.<sup>33</sup> However, several studies have demonstrated  $K^+$ -independent action of diazoxide, specifically interaction with the ETC that might also be related to ROS production as mentioned previously.<sup>30,34,35</sup> APC has been associated with mild mitochondrial depolarization, possibly through mitochondrial  $K^+$  channel opening,<sup>10,36</sup> and mild mitochondrial uncoupling has been shown to be cardioprotective.<sup>3,37,38</sup> Although a parallel increase in ROS production and depolarization during APC seems counterintuitive in view of the general assumption that mitochondrial depolarization decreases ROS production,<sup>11</sup> it may be the result of multiple actions of isoflurane on mitochondrial bioenergetics. Future studies will elucidate the interactive mechanism between volatile anesthetic-induced ROS generation *via* ETC interaction and  $K^+$  flux-induced or otherwise induced mitochondrial depolarization.

In agreement with our results from complex activity measurements, isoflurane inhibited oxygen consumption in the presence of complex I-linked substrates, but not when succinate, as complex II linked substrate, was used in the presence of rotenone. When succinate was used in the absence of rotenone, isoflurane in fact increased the oxygen consumption. This again points to complex I as the target of isoflurane. Succinate is converted to fumarate and malate and then to oxaloacetate in the Krebs cycle without rotenone.<sup>39</sup> Oxaloacetate participates in a direct feedback inhibition of complex II that results in decreased oxidation of succinate. When complex I activity is inhibited, NADH oxidation and, thereby, the levels of  $NAD^+$  are decreased, which in turn impairs the oxidation of malate to oxaloacetate.<sup>40</sup> Therefore, inhibitory effects of isoflurane on complex I could increase complex II-linked respiration *via* attenuation of oxaloacetate generation.

Although a limited increase in ROS generation is critical for initiating prosurvival signaling pathways during APC, oxidative stress contributes to mitochondrial and cellular injury during reperfusion. With succinate as substrate, electrons entering the ETC at complex II can generate ROS by reverse electron flow to complex I. Rotenone that blocks complex I largely attenuates reverse flow-induced ROS generation.<sup>41</sup> Similarly, isoflurane significantly reduces ROS generation induced by reverse electron flow when a complex II substrate is used. This action may be particularly relevant during myocardial ischemia, because succinate concentration significantly increases during hypoxia.<sup>42,43</sup> Isoflurane applied at the onset of reperfusion (anesthetic postconditioning) may attenuate reverse electron flow-induced ROS generation, thereby delaying the opening of mitochondrial permeability transition pore and increasing cell survival.<sup>14</sup> Thus, the source and mech-



anism of ROS generation observed during reperfusion is likely to be fundamentally different from that of "triggering" ROS elicited during APC.

The current results should be interpreted within the constraints of several limitations. Experiments were conducted in an established model of myocardial infarction in rats; thus, these results may not be directly comparable with those obtained in humans. We were unable to measure the effect of tempol and mitotempol on the small amount of ROS generated by isoflurane directly with *in vitro* experiments. Tempol and mitotempol convert superoxide anion to hydrogen peroxide similarly to superoxide dismutase. Therefore, the scavenging properties of those superoxide dismutase mimetics cannot be detected with the very sensitive amplex red-based assay that detects hydrogen peroxide. On the other hand, the methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2- $\alpha$ ]pyrazin-3-one-based assay is not sensitive enough for this purpose.<sup>44</sup> Nevertheless, we are confident that tempol and mitotempol would scavenge isoflurane-induced ROS as they attenuated the large amount of ROS induced by antimycin A in our measurements. In addition, measurements of ROS production and other mitochondrial parameters were performed *in vitro* because it is virtually impossible to evaluate subcellular regulation of ROS generation in the intact heart. We also did not assess the interplay between mitochondrial depolarization and ROS generation and this is the aim of ongoing studies in the laboratory.

In summary, our results confirmed the critical role of isoflurane to modulate mitochondrial ROS. The interaction of isoflurane with complex I of the ETC is responsible for generation of signaling ROS at complex I and, indirectly, at complex III. Isoflurane also attenuates reverse electron flow-induced damaging ROS production that occurs during reperfusion.

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