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Attenuation of Mitochondrial Respiration by Sevoflurane in Isolated Cardiac Mitochondria Is Mediated in Part by Reactive Oxygen Species

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Background: **Anesthetic preconditioning protects against cardiac ischemia/reperfusion injury. Increases in reduced nicotinamide adenine dinucleotide and reactive oxygen species during sevoflurane exposure suggest attenuated mitochondrial electron transport as a trigger of anesthetic preconditioning. The authors investigated the effects of sevoflurane on respiration in isolated cardiac mitochondria.**

Methods: **Mitochondria were isolated from fresh guinea pig hearts, and mitochondrial oxygen consumption was measured in the presence of complex I (pyruvate) or complex II (succinate) substrates. The mitochondria were exposed to 0, 0.13, 0.39, 1.3, or 3.9 mM sevoflurane. State 3 respiration was determined after adenosine diphosphate addition. The reactive oxygen species scavengers manganese(III) tetrakis (4-benzoic acid) porphyrin chloride and *N*-tert-Butyl-a-(2-sulfophenyl)nitrone sodium (10 μM each), or the K_{ATP} channel blockers glibenclamide (2 μM) or 5-hydroxydecanoate (300 μM), were given alone or before 1.3 mM sevoflurane.**

Results: **Sevoflurane attenuated respiration for both complex I and complex II substrates, depending on the dose. Glibenclamide and 5-hydroxydecanoate had no effect on this attenuation. Both scavengers, however, abolished the sevoflurane-induced attenuation for complex I substrates, but not for complex II substrates.**

Conclusion: **The findings suggest that sevoflurane-induced attenuation of complex I is mediated by reactive oxygen species, whereas attenuation of other respiratory complexes is mediated by a different mechanism. The opening of mitochondrial K_{ATP} channels by sevoflurane does not seem to be involved in this effect. Thus, reactive oxygen species formation may not only result from attenuated electron transport by sevoflurane, but it may also contribute to complex I attenuation, possibly leading to a positive feedback and amplification of sevoflurane-induced reactive oxygen species formation in triggering anesthetic preconditioning.**

ANESTHETIC preconditioning (APC) is the phenomenon whereby temporary exposure to a volatile anesthetic, followed by its complete washout, attenuates cardiac ischemia/reperfusion injury *via* a memory effect.[1–3] The exact signaling cascade from anesthetic exposure to attenuated ischemia/reperfusion injury, however, is not yet fully understood. Reversal of APC by scavengers of reactive oxygen species (ROS)[4–7] implicates the formation of ROS as part of the triggering mechanism of APC. We previously reported an increase in reduced nicotinamide adenine dinucleotide (NADH)[8] and ROS fluorescence[7] during preconditioning by sevoflurane in intact hearts. These findings suggest a central role for the temporary alteration of mitochondrial function in triggering APC. Reversible attenuation of mitochondrial electron transport by volatile anesthetics could cause increased formation of ROS at complex I and/or complex III of the electron transport chain (ETC).[9] ROS could then act on downstream effectors, like tyrosine kinase or protein kinase C cascades,[1,10,11] and mitochondrial (m) adenosine-triphosphate (ATP) sensitive K^+ channels[12] that are normally closed at physiologic ATP levels. It is still unclear how and where ROS are formed during the anesthetic exposure. It is also unclear if altered ETC function is a direct effect of sevoflurane or if it is mediated by mK_{ATP} channel opening[13,14] or by ROS, because various ROS have been shown to affect electron transport.[15–18]

In the present study, we used intact cardiac mitochondria to investigate the effects of sevoflurane on mitochondrial respiration. Substrates for complex I or complex II of the ETC were given to help elucidate possible sites of altered ETC function. We further used different ROS scavengers and mK_{ATP} channel blockers to test if ROS formation or mK_{ATP} channel opening by sevoflurane mediate altered ETC function.

Materials and Methods

All investigations conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health No. 85–23, revised 1996), and were approved by the Institutional Animal Care and Use Committee (Med-

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ical College of Wisconsin, Milwaukee, Wisconsin). Thirty mg of ketamine and 1,000 U of heparin were injected intraperitoneally into 20 albino English shorthaired guinea pigs (weight, 250–300 g). The animals were decapitated 15 min later when unresponsive to noxious stimulation. Immediately after a thoracotomy, the heart was taken out and immersed in a 4°C cold isolation buffer of 200 mM mannitol, 50 mM sucrose, 5 mM KH_2PO_4, 1 mM EGTA, 5 mM 3-(n-morpholino)propanesulfonic acid, and 0.1% bovine serum albumin (pH 7.15, adjusted with potassium hydroxide). The atria were discarded, and the ventricles were minced into 1-mm pieces. The tissue was rinsed, transferred to a glass Potter-Elvehjem homogenizing vessel (Kontes Glass Co., Vineland, NJ) on ice, and gently homogenized with a Teflon (DuPont, Wilmington, DE) pestle for 30 s in the presence of 1 mg/ml protease. This was followed by another 30 s of homogenization after 10-fold dilution of the protease. The mitochondria were then isolated by differential centrifugation at 4°C.[19] The tissue suspension was centrifuged at 8,000*g* for 10 min to remove the protease. The resulting pellet was then resuspended in 28 ml isolation buffer, and the suspension centrifuged at 700*g* for 10 min to remove cellular debris. The supernatant containing the mitochondrial fraction was further centrifuged at 8,000*g* for 10 min. The pellet was resuspended in 7 ml isolation buffer without EGTA and centrifuged at 8,000*g* for 10 min. The final mitochondrial pellet was resuspended in 500 μl cold isolation buffer without EGTA. The total protein concentration was determined[20] with bovine serum albumin as a standard. Anatomic integrity of the isolated mitochondria was verified by electron microscopy.

Measurement of Mitochondrial Oxygen Consumption

The 500-μl mitochondrial suspension was kept at 4°C. Immediately before each experiment, an aliquot of the concentrated mitochondria was added to 27°C respiration buffer[21] (110 mM KCl, 5 mM $K_2HPO_4 \cdot 3\ H_2O$, 10 mM 3-[n-morpholino]propanesulfonic acid, 10 mM Mg-acetate, 1 mM EDTA, 1 μM tetrasodium pyrophosphate, 0.1% BSA; pH 7.15, adjusted with potassium hydroxide) to yield 500 μl with a concentration of 500 μg protein/ml. Mitochondria from one heart were sufficient for approximately 15 experiments. Mitochondrial oxygen concentration was measured polargraphically with a Clark-type oxygen electrode (Model 1302; Strathkelvin Instruments, Glasgow, Scotland) in a water-jacketed 500-μl chamber (Model MT200A; Strathkelvin Instruments), equipped with a Teflon-coated magnetic stirring bar and monitored by an oxygen meter (Model 782; Strathkelvin Instruments). The oxygen electrode was calibrated with air-saturated water ($Po_2 \sim 150$ mmHg) and sodium sulfite (Na_2SO_3) solution (Sigma, St. Louis, MO; to achieve near zero Po_2) at the same temperature as the buffer to be used. The rate of state 3 mitochondrial respiration was determined as the maximum rate of oxygen decrease after addition of substrate and adenosine-diphosphate,[21] as shown in figures 1 and 2. Data were stored on-line using the manufacturer's software (Strathkelvin Instruments). Microsoft Excel software (Microsoft Corp., Redmond, WA) was later used for analysis.

Experimental Protocol

After sealing the chamber with a Plexiglas plug (time, t = 0 min), drugs, substrates, and adenosine-diphosphate (5 μl each) were subsequently injected into the chamber according to the protocol displayed in figure 1 (all of the following concentrations are final). The superoxide dismutase mimetic manganese(III) tetrakis (4-benzoic acid) porphyrin chloride (TBAP; OxisResearch, Portland, OR; 10 μM),[22] the negatively-charged spin trap *N*-tert-Butyl-a-(2-sulfophenyl)nitrone sodium (SPBN; Sigma; 10 μM),[23] the mK_{ATP} channel blocker 5-hydroxydecanoic acid (5-HD; Sigma; 300 μM), the nonspecific K_{ATP} channel blocker glibenclamide (Sigma; 2 μM),[24] or their respective vehicle (buffer) was added at t = 1 min to test for possible antagonism of a sevoflurane-induced effect on mitochondrial ETC. Pyruvate with malate (Sigma; 10 mM each dissolved in buffer) or succinate (Sigma; 10 mM dissolved in buffer) with the complex I blocker rotenone (Sigma; 10 μM dissolved in dimethyl sulfoxide) was added at t = 2 min as a substrate for complex I or complex II of the ETC, respectively. Sevoflurane (Abbott Laboratories, North Chicago, IL; 0.13, 0.39, 1.3, or 3.9 mM) or its vehicle dimethyl sulfoxide was added at t = 3 min to test for a sevoflurane-induced alteration of mitochondrial respiration. Adenosine-diphosphate (Sigma; 250 μM) in buffer was added at t = 4 min to start state 3 respiration. In some experiments, 2,4-dinitrophenol (Sigma; 100 μM in dimethyl sulfoxide) as an uncoupler, or antimycin A (Sigma; 100 μM in dimethyl sulfoxide) as a blocker of complex III, were given at t = 3 min to verify mitochondrial function using the chosen protocol (fig. 2A). Chamber oxygen concentration in μM was monitored for up to 12 min or until the oxygen concentration was 0. Representative tracings of 1.3 mM sevoflurane and sevoflurane plus TBAP are displayed in figure 2B. All experiments were performed at 27°C. Experiments with mitochondria from the same animal were randomized to one of the above treatment groups with at least three control experiments interspersed. All state 3 respiration rates from experiments of one heart were normalized and expressed as a percentage of control experiments from the same heart.

Statistical Analysis

All data were expressed as mean ± standard error of the mean. Group data were compared by analysis of variance to determine significance (Super ANOVA 1.11 software for Macintosh; Abacus Concepts, Berkeley,

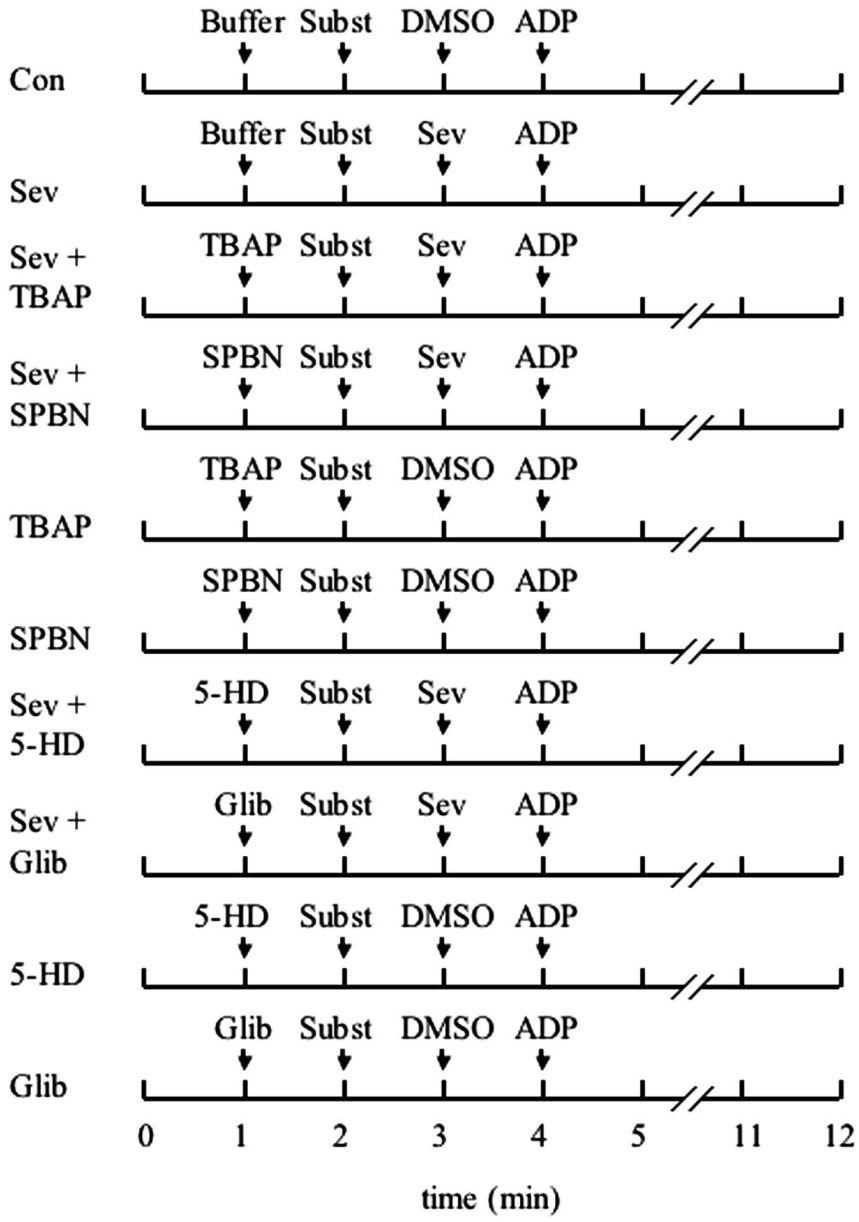

Fig. 1. Experimental protocol used to measure mitochondrial state 3 respiration. After stabilization of the mitochondrial suspension for 1 min, one of several putative antagonists (manganese[III] tetrakis [4-benzoic acid] porphyrin chloride [TBAP; 10 μM], *N*-tert-Butyl-a-[2-sulfophenyl]nitrone sodium [SPBN; 10 μM], glibenclamide [Glib; 2 μM], 5-hydroxydecanoate [5-HD; 300 μM]), or their respective vehicle was injected into the chamber. Substrate (Subst; 10 mM pyruvate with malate for complex I, or 10 mM succinate for complex II, with 10 μM rotenone to block complex I) was given 1 min later. Sevoflurane (Sev; 0.13, 0.39, 1.3, or 3.9 mM) or its vehicle dimethyl sulfoxide (DMSO) was added 1 min later. After another min, adenosine diphosphate (ADP; 250 μM) was injected to initiate state 3 respiration. Each drug and substrate was given as a 5-μl bolus to yield final concentrations. Oxygen concentration in μM was monitored for up to 12 min or until 0. All experiments were performed at 27°C.

CA). If *F* values ($P < 0.05$) were significant, *post hoc* comparisons of means tests (Student-Newman-Keuls) were used to compare the groups. Differences among means were considered statistically significant when $P < 0.05$ (two-tailed). Regression analysis was used to determine the relationships between sevoflurane concentration and the change in state 3 respiration for both substrate combinations. Statistical symbols used were * (*vs.* Con) and † (*vs.* 1.3 mM sevoflurane).

Results

Sevoflurane attenuated state 3 respiration in a dose-dependent manner when compared to vehicle controls for both substrate combinations (fig. 3). Regression analysis showed exponential relationships between sevoflurane concentration and attenuation of state 3 respiration: $y = 77.0 \pm 2.7 - (10.8 \pm 5.2 \cdot \log x)$ for pyruvate with malate ($R^2 = 0.11$; $P < 0.05$), and $y = 86.2 \pm 1.2 - (15.7 \pm 2.0 \cdot \log x)$ for succinate with rotenone ($R^2 = 0.67$; $P < 0.05$), with y being the percentage of state 3 respiration compared to control experiments and x being the sevoflurane concentration.

Neither the ROS scavengers TBAP or SPBN (fig. 4), nor the putative K_{ATP} channel blockers glibenclamide or 5-HD (fig. 5), had an effect on state 3 respiration when given alone. Both TBAP and SPBN abolished the attenuation of state 3 respiration by 1.3 mM sevoflurane when pyruvate with malate was given (fig. 4A). This attenuation of state 3 respiration was not observed when succinate with rotenone was given (fig. 4B). Neither glibenclamide nor 5-HD reversed the sevoflurane-induced

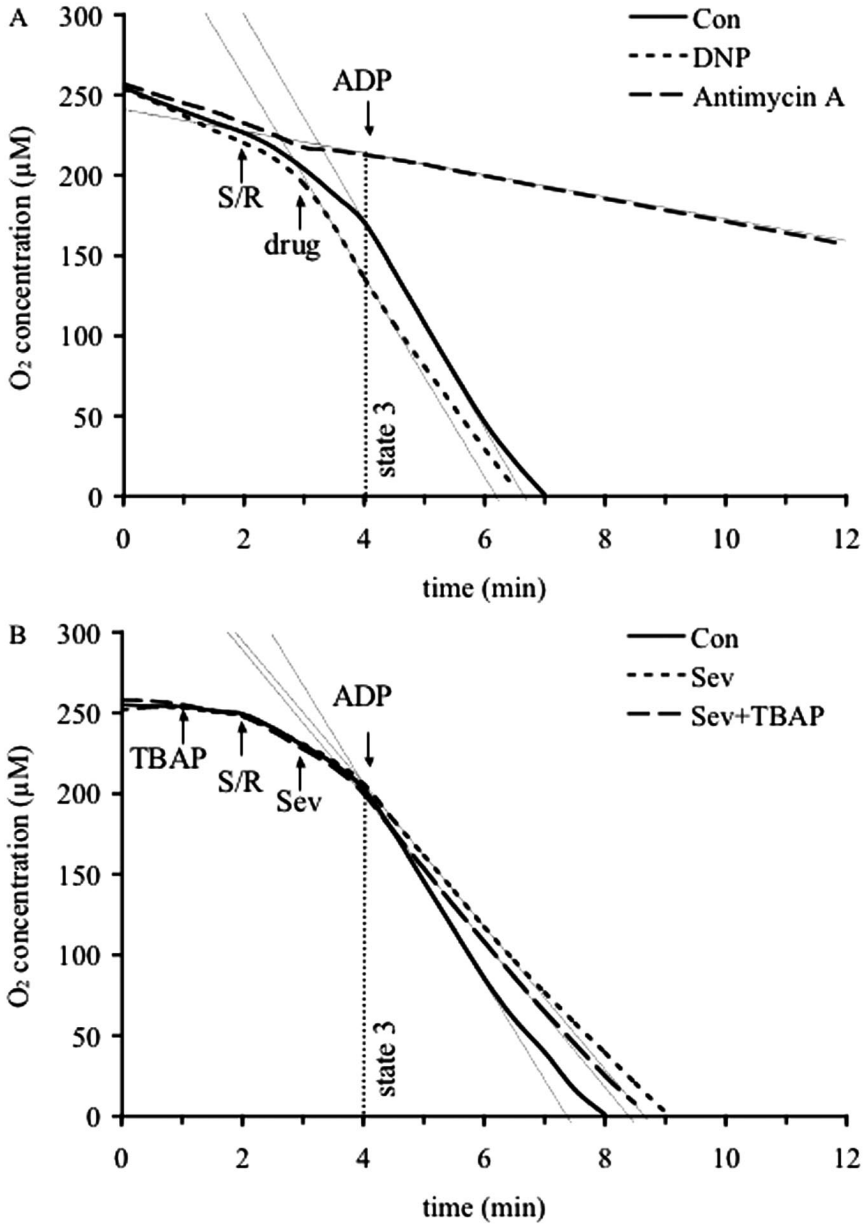

Fig. 2. Representative oxygen recordings when vehicle (Con; *A* and *B*), the complex III inhibitor antimycin A (100 μM; *A*), the uncoupler 2,4-dinitrophenol (DNP; 100 μM; *A*), or sevoflurane (Sev; 1.3 mM; *B*) were added at t = 3 min. A substrate of 10 mM succinate with 10 μM rotenone (S/R) was given at t = 2 min. The reactive oxygen species scavenger manganese(III) tetrakis (4-benzoic acid) porphyrin chloride (TBAP; 10 μM; *B*) was given at t = 1 min. Note the differences in state 3 respiration (*slope of straight lines*) after addition of 250 μM adenosine diphosphate (ADP) at t = 4 min.

attenuation of mitochondrial respiration for either substrate combination (fig. 5).

Discussion

The present study shows that (1) sevoflurane exposure causes a dose-dependent attenuation of mitochondrial state 3 respiration in isolated intact mitochondria; (2) the attenuated electron transport is not limited to complex I; (3) the results with 5-HD and glibenclamide as K_{ATP} channel antagonists suggest that this effect is independent of mK_{ATP} channel opening; and (4) the reversal by the ROS scavengers TBAP and SPBN for complex I substrate (pyruvate with malate), but not for complex II substrate (succinate with rotenone), suggests that attenuated electron transport at complex I, but not at other sites, is mediated by superoxide.

Usually, cardioprotection by APC in different models is assessed by a reduction in infarct size[2–6,8,11,25–28] and improved return of mechanical or metabolic function[8,28,29] after ischemia/reperfusion injury compared to nonpreconditioned hearts. Reversal of this cardioprotection by antagonists of suspected signaling pathways or receptors is used to identify some of the mechanisms involved.[2,5,25–27,30]

Another approach is to measure expected intracellular changes during the preconditioning stimulus, *i.e.*, exposure to the volatile anesthetic, in the presence or absence of known antagonists of APC to identify those cellular or subcellular effects of the anesthetic exposure that are relevant for triggering APC. For example, in addition to increased ROS fluorescence,[7] we recently showed that temporary exposure to a preconditioning

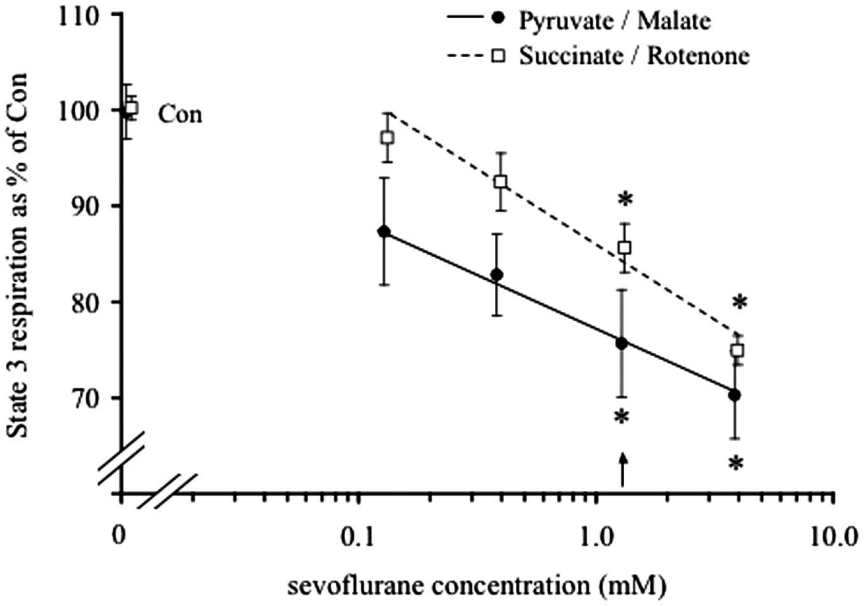

Fig. 3. Concentration-dependent attenuation of state 3 mitochondrial respiration by 0.13, 0.39, 1.3, and 3.9 mM sevoflurane as a percentage of vehicle control when substrates for complex I (10 mM pyruvate with 10 mM malate) or for complex II (10 mM succinate with 10 μM rotenone) were given. Regression analysis showed exponential relationships (*lines*) between the sevoflurane concentration as independent variable (x), and the percentage of state 3 respiration as a dependent variable (y) for both substrate groups: $y = 77.0 \pm 2.7 - (10.8 \pm 5.2 \cdot \log x)$ for pyruvate with malate ($R^2 = 0.11$; $P < 0.05$) and $y = 86.2 \pm 1.2 - (15.7 \pm 2.0 \cdot \log x)$ for succinate with rotenone ($R^2 = 0.67$; $P < 0.05$). The *arrow* indicates the concentration used for experiments with potential antagonists. All values are mean ± SEM, n = 8 per experimental group. * $P < 0.05$ *versus* Con.

concentration of sevoflurane caused a reversible increase in NADH fluorescence in intact hearts, and that this was reversed by 5-HD.[28]

Without the on-line NADH measurement, our findings would have confirmed the findings of other studies that volatile anesthetics such as sevoflurane induce preconditioning by opening mK_{ATP} channels. The observed increase in NADH fluorescence during the anesthetic exposure, however, suggested attenuation of mitochondrial respiration, *i.e.*, a more reduced mitochondrial state,[31] rather than a more oxidized state as expected with mK_{ATP} channel opening.[12,32,33] Furthermore, the paradoxic reversal of this NADH increase by 5-HD casts some doubt on the specificity of 5-HD as a mK_{ATP} channel blocker. Hanley *et al.,* using submitochondrial preparations, suggested that 5-HD, a fatty acid, could serve as a substrate to feed electrons into the ETC at the level of coenzyme Q.[34,35] This could provide a bypass for ETC sites that are attenuated by lipophilic drugs,[36] like the K_{ATP} channel openers diazoxide and pinacidil[13,34] or volatile anesthetics.[35]

Taken together, these findings offer not only the possibility that volatile anesthetics may attenuate ETC function to induce APC, but also that blockade by 5-HD does not necessarily furnish direct evidence of a mK_{ATP} channel opening.[37,38] This has already been acknowledged in the more recent literature.[14,39,40] Moreover, there is an ongoing debate about whether volatile anesthetics open mK_{ATP} channels under physiologic conditions (no shortage of substrate, oxygen, or adenosine-diphosphate to make enough ATP) and this opening triggers APC,[14] or whether they open mK_{ATP} channels faster and to a greater extent under nonphysiologic conditions, like ischemia, and mK_{ATP} channels hence serve as an effector of APC.[12,41]

Increasing evidence implies that ROS play a central role in triggering cardiac APC. Not only was APC abolished when different ROS scavengers were given,[4–7] but exposure to a preconditioning concentration of a volatile anesthetic also led to a measurable, albeit temporary, increase in ROS formation as assessed by ethidium-fluorescence.[7,14] This transient increase in ROS during anesthetic exposure could subsequently lead to protein kinase C activation[11] and mK_{ATP} channel opening,[42] and thus result in a memory effect that lasts beyond the discontinued anesthetic exposure.

How and where ROS are formed during anesthetic exposure remains unclear. As a consequence of opening of mK_{ATP} channels, increased mitochondrial electron transport[32] could result in increased constitutive formation of superoxide and thus lead to preconditioning.[43] This would implicate mK_{ATP} channel opening as a trigger of preconditioning. Attenuated electron transport, on the other hand, can also increase ROS formation.[44–46] The present study clearly demonstrates a dose-dependent attenuation of mitochondrial respiration during anesthetic exposure in isolated cardiac mitochondria under state 3 conditions. Together with our previous reports of an increase in reduced NADH fluorescence[8,28] and a decrease in oxidized flavoprotein fluorescence[47] during anesthetic exposure in intact beating hearts, we suggest that anesthetic-induced attenuation of electron transport is the likely mechanism for ROS formation.

Mitochondrial swelling, as would be expected with K^+ influx during K_{ATP} channel opening, might also indirectly attenuate ETC function.[13] We chose the two chemically different K_{ATP} channel blockers glibenclamide and 5-HD at otherwise effective concentrations (2 μM and 300 μM, respectively)[24,29] to test this hypothesis. Both failed to prevent the sevoflurane-induced attenuation of ETC function, suggesting that mK_{ATP} channel opening does not initiate this effect.

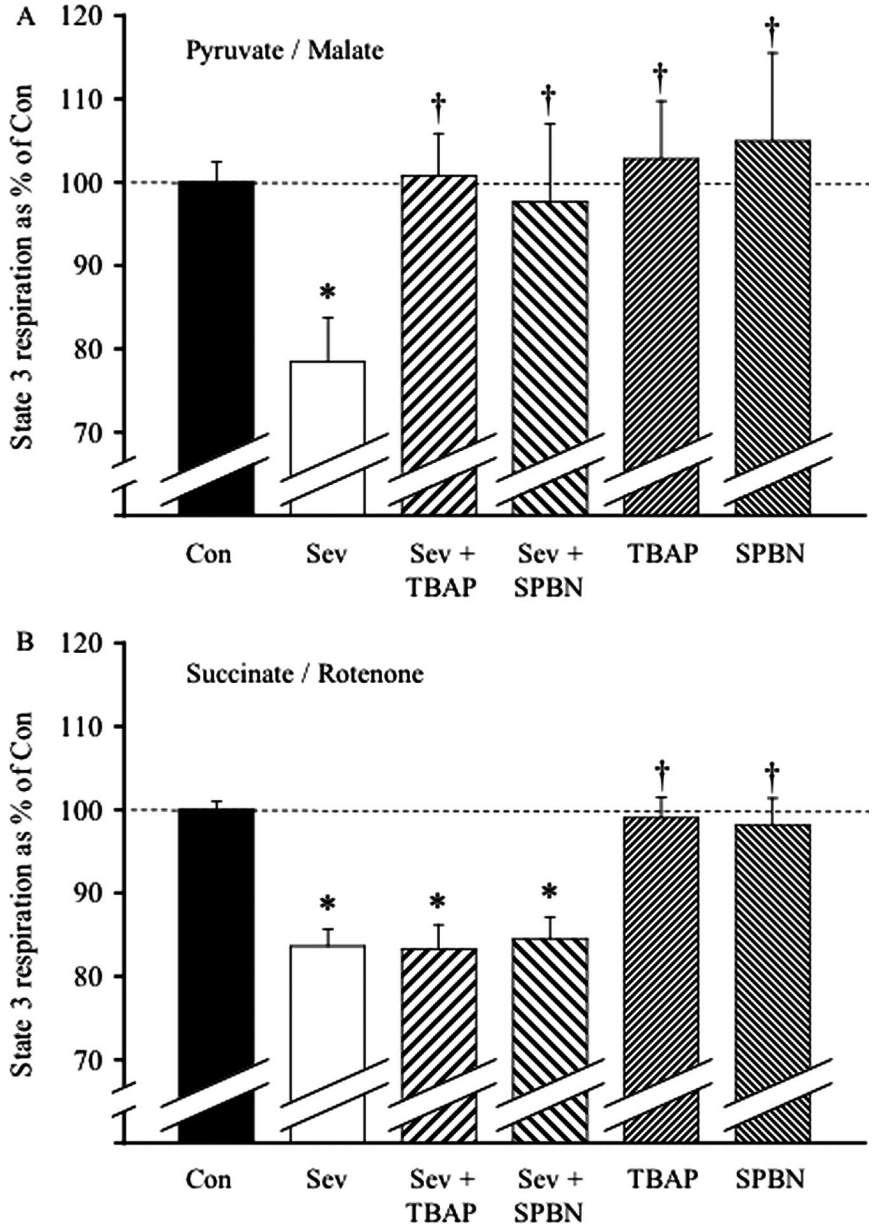

Fig. 4. Effects of the two different reactive oxygen species scavengers manganese(III) tetrakis (4-benzoic acid) porphyrin chloride (TBAP; 10 μM) and *N*-tert-Butyl-a-(2-sulfophenyl)nitrone sodium (SPBN; 10 μM) on the attenuation of state 3 mitochondrial respiration by 1.3 mM sevoflurane (sev) when substrates for complex I (10 mM pyruvate with 10 mM malate; *A*) or for complex II (10 mM succinate with 10 μM rotenone; *B*) were given. Note the substrate-dependent differences. All values are mean ± SEM, n = 8 per experimental group. * $P < 0.05$ *versus* Con. † $P < 0.05$ *versus* sevoflurane (two-tailed).

Altered ETC function may not only result in increased ROS generation but may also be caused by ROS.[15–18] Previous studies on ROS in triggering APC[4–7,14] did not examine a possible mediating role of ROS in altering ETC function during anesthetic exposure. We used two different ROS scavengers to test this possibility: TBAP as a mimetic of superoxide dismutase ($O_2^{\bullet} \rightarrow H_2O_2$) and SPBN as a free-radical spin-trap. Our findings in isolated cardiac mitochondria show a differential role for ROS in sevoflurane-induced ETC attenuation. When succinate (in the presence of rotenone) was used as the substrate to feed electrons to oxygen *via* complexes II, III, and IV of the ETC, none of the ROS scavengers affected the sevoflurane-induced attenuation of state 3 respiration. When pyruvate with malate was used as the substrate to produce mainly NADH feeding electrons to oxygen *via* complexes I, III, and IV of the ETC, both scavengers abolished the sevoflurane-induced attenuation of state 3 respiration. Taken together with our preliminary findings[48] that scavenging ROS abolished the increase in NADH fluorescence during anesthetic exposure in intact beating hearts, we suggest that complex I function is attenuated by superoxide formed during sevoflurane exposure, whereas the effect of sevoflurane on other complexes is apparently not mediated by superoxide. This also offers the interesting possibility of a positive feedback mechanism of ROS likely formed at complex III[9] to attenuate complex I function, and thus leading to more ROS formation.

In summary, the present results indicate that sevoflurane attenuates mitochondrial electron transport in isolated cardiac mitochondria. This effect appears to be

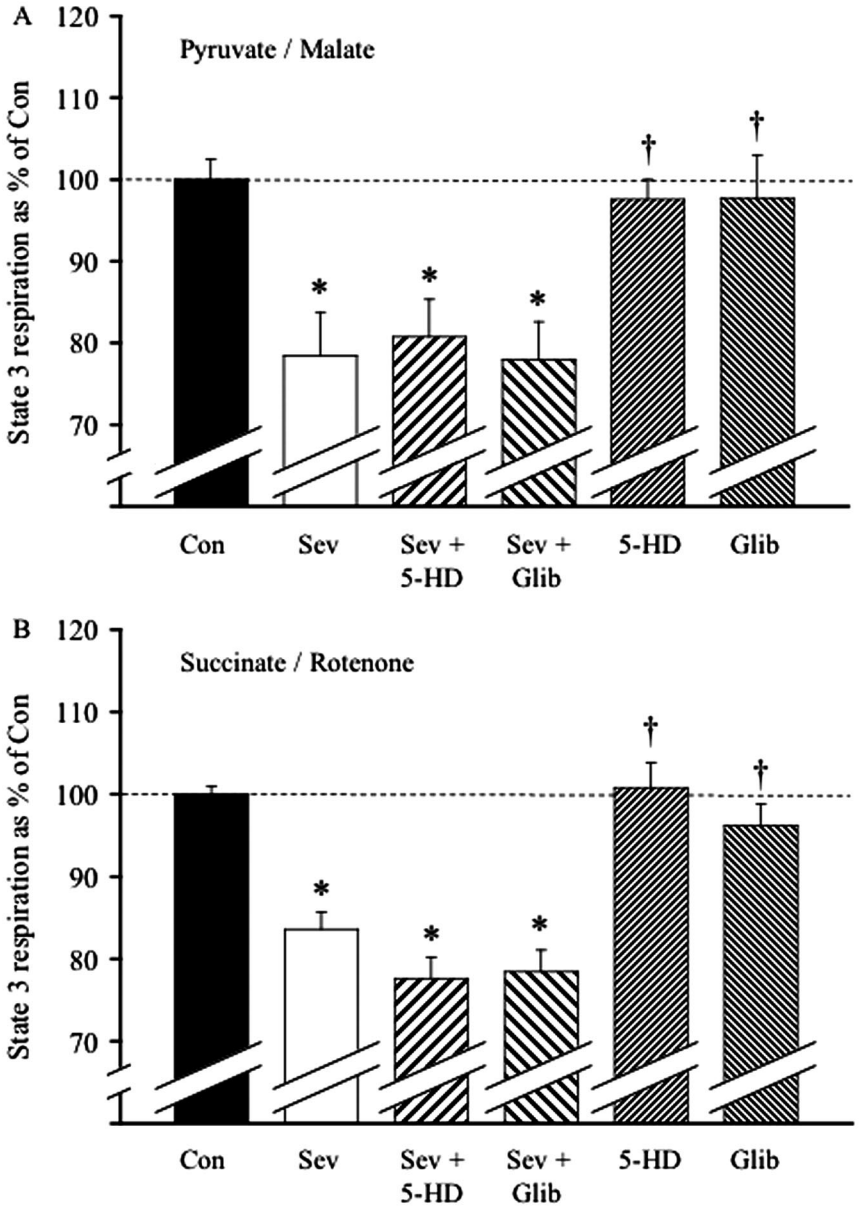

Fig. 5. Effects of the two different adenosine triphosphate-sensitive K^+ channel blockers glibenclamide (Glib; 2 μM) and 5-hydroxydecanoate (5-HD; 300 μM) on attenuation of state 3 mitochondrial respiration by 1.3 mM sevoflurane (Sev) when substrates for complex I (10 mM pyruvate with 10 mM malate; *A*) or for complex II (10 mM succinate with 10 μM rotenone; *B*) were given. All values are mean ± SEM, n = 8 per experimental group. * $P < 0.05$ *versus* Con. † $P < 0.05$ *versus* sevoflurane (two-tailed).

partially mediated by ROS, but independent of mK_{ATP} channel opening. Understanding altered mitochondrial function as a trigger for cardiac preconditioning by volatile anesthetics may provide an important component in deciphering the complexity of preconditioning as a means to attenuate ischemia/reperfusion injury.

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