Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels

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Anesthesiology Research Laboratories, Departments of 1Anesthesiology and 2Physiology and 3Cardiovascular Research Center, Medical College of Wisconsin, 4Zablocki Department of Veterans Affairs Medical Center Research Service, and 5Department of Biomedical Engineering, Marquette University, Milwaukee, Wisconsin; and 6Laboratory of Experimental Intensive Care and Anesthesiology, University of Amsterdam, Amsterdam, the Netherlands

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Heinen A, Aldakkak M, Stowe DF, Rhodes SS, Riess ML, Varadarajan SG, Camara AK. Reverse electron flow-induced ROS production is attenuated by activation of mitochondrial Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channels. Am J Physiol Heart Circ Physiol 293: H1400–H1407, 2007. First published May 18, 2007; doi:10.1152/ajpheart.00198.2007.—Mitochondria generate reactive oxygen species (ROS) dependent on substrate conditions. O\textsubscript{2}\textsuperscript{-} concentrations increase mildly on activation of the putative big mitochondrial Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channel (mtBK\textsubscript{Ca}) by low concentrations of 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS-1619). In the present study we examined effects of NS-1619 on mitochondrial O\textsubscript{2} consumption, membrane potential (\(\Delta$$\Psi$$\textsubscript{m}\)), H\textsubscript{2}O\textsubscript{2} release rates, and redox state in isolated guinea pig heart mitochondria respiring on succinate but without rotenone. NS-1619 (30 \(\mu\)M) increased state 2 and state 4 respiration by 26 \(\pm\) 4% and 14 \(\pm\) 4%, respectively; this increase was abolished by the BK\textsubscript{Ca} channel blocker paxilline (5 \(\mu\)M). Paxilline alone had no effect on respiration. NS-1619 did not alter \(\Delta$$\Psi$$\textsubscript{m}\) or redox state but decreased H\textsubscript{2}O\textsubscript{2} production by 73% vs. control; this effect was incompletely inhibited by paxilline. We conclude that opening of mtBK\textsubscript{Ca} channels increases flavoprotein electron flow, matrix K\textsuperscript{+} influx through mtBK\textsubscript{Ca} channels reduces mitochondrial H\textsubscript{2}O\textsubscript{2} production by accelerating forward electron flow. Our prior study showed that NS-1619 induced an increase in H\textsubscript{2}O\textsubscript{2} production with blocked reverse electron flow. The present results suggest that NS-1619-induced matrix K\textsuperscript{+} influx increases forward electron flow despite the high reverse electron flow, and emphasizes the importance of substrate conditions on interpretation of effects on mitochondrial bioenergetics.

mitochondria; reactive oxygen species; potassium channels

Mitochondria are known to generate reactive oxygen species (ROS), which include superoxide radical (O\textsubscript{2}\textsuperscript{-}), hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and hydroxyl radical (OH) as by-products of aerobic metabolism. Excess release of ROS has been shown to play a role in the etiology of various pathological disorders including cardiovascular disease, degenerative changes in aging, Alzheimer disease, and diabetes, as well as in ischemia-reperfusion injury. ROS are key elements in a variety of cellular signaling pathways, including cardioprotection against I/R induced by ischemic and pharmacological preconditioning.

O\textsubscript{2}\textsuperscript{-} can be generated at several sites along the mitochondrial electron transport chain (ETC) including complex III (12, 36) and complex I (8, 18, 20). Complex III generates O\textsubscript{2}\textsuperscript{-} through the oxidation of ubisemiquinone, a radical intermediate formed through the cycle in the complex. The Q\textsubscript{o} site of the cycle is a major source of O\textsubscript{2}\textsuperscript{-} production, and it is close to the intermembrane space. In contrast, O\textsubscript{2}\textsuperscript{-} generated from complex I is released into the matrix. One study (20) suggests that the primary site of O\textsubscript{2}\textsuperscript{-} generation in the mitochondrial ETC is flavin mononucleotide (FMN) of complex I via reverse electron flow, not forward electron flow via ubiquinone of complex III. Regardless of the source of O\textsubscript{2}\textsuperscript{-}, the mechanism and quantity of O\textsubscript{2}\textsuperscript{-} generated are dependent on the experimental substrate and energetic conditions. When FADH\textsubscript{2}-related substrates are used and electrons enter the ETC at complex II (succinate dehydrogenase), O\textsubscript{2}\textsuperscript{-} can be generated by reverse electron flow to complex I (21). The resulting large increase in O\textsubscript{2}\textsuperscript{-} generation is dependent on a high inner mitochondrial membrane potential (\(\Delta$$\Psi$$\textsubscript{m}\)) and is sensitive to complex I blockade by rotenone, which prevents reverse electron flow as a source of O\textsubscript{2}\textsuperscript{-} generation (18, 21). It is believed that in the presence of a high proton motive force electrons are passed to NAD\textsuperscript{+} until the pool is fully reduced to NADH; once this occurs semiquinone can only lose its unpaired electron to O\textsubscript{2} because all upstream redox centers are fully reduced (18).

K\textsuperscript{+} channels located in the IMM appear to play an important role in regulating mitochondrial function, but the mechanism remains unclear. Xu et al. (41) found evidence for big mitochondrial Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} (mtBK\textsubscript{Ca}) channels in the IMM of guinea pig ventricular cells. Sato et al. (32) demonstrated that opening of mtBK\textsubscript{Ca} channels increases flavoprotein oxidation in ventricular myocytes placed in glucose-free Tyrode solution, indicating an increase in electron transport in oxidized mitochondria. Recently, we investigated the effects of mtBK\textsubscript{Ca} channel opening and closing on function of mitochondria isolated from guinea pig hearts. We reported that putative mtBK\textsubscript{Ca} channel opening with low concentrations of 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS-1619) accelerated state 2 and state 4 respiration (electron flow) and H\textsubscript{2}O\textsubscript{2} genera-
ation at a stable polarized $\Delta \Psi_m$ in the presence of succinate and rotenone (16). In the present study we investigated effects of NS-1619 on respiration, $\Delta \Psi_m$, redox state (NADH and FAD), and $\text{H}_2\text{O}_2$ generation using succinate alone, which can induce ROS generation via reverse electron flow. We proposed that under these conditions $\text{H}_2\text{O}_2$ production would decrease because of a relative increase in forward electron flow, induced by matrix K$^+$ influx, thus countering the larger reverse electron flow caused by succinate with subsequent O$_2$$^{\cdot-}$ formation at complex I.

MATERIALS AND METHODS

All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Pub. No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Mitochondrial isolation. Heart mitochondria were isolated from ketamine-anesthetized guinea pigs (250 – 300 g) by differential centrifugation as described previously (30) with moderate modifications. Briefly, ventricles were excised, placed in an isolation buffer (in mM: 200 mannnitol, 50 sucrose, 5 K$_2$HPO$_4$, 5 3-(N-morpholino)propanesulfonic acid (MOPS), and 1 EGTA, with 0.1% bovine serum albumin (BSA), pH 7.15 (adjusted with KOH), and minced into 1-mm$^3$ pieces. The suspension was initially homogenized for 15 s in 2.5 ml of isolation buffer containing 5 U/ml protease and for another 15 s after addition of 17 ml of isolation buffer. The suspension was centrifuged at 8000 g for 10 min; the pellet was resuspended in 25 ml of isolation buffer and centrifuged again at 750 g for 10 min. Next, the supernatant was centrifuged at 8000 g for 10 min, and the final pellet was suspended in 0.5 ml of isolation buffer and kept on ice. The protein content was determined by the Bradford method (4). All isolation procedures were conducted at 4°C.

Mitochondrial O$_2$ consumption. Oxygen consumption was measured polarographically at 27°C with a respirometry system (System S 200A; Strathkelvin Instruments, Glasgow, UK). Mitochondria (0.25 mg protein/ml) were suspended in respiration buffer containing (in mM) 130 KCl, 5 K$_2$HPO$_4$, 20 MOPS, and 2.5 EGTA, with 1 $\mu$M Na$_4$P$_2$O$_7$ and 0.1% BSA, pH 7.15 adjusted with KOH. Buffer Ca$^{2+}$ concentration ([Ca$^{2+}$]) was <100 nM as assessed by the fluorescent dye indo-1. Respiration was initiated by administration of the complex II substrate succinate (10 mM). State 3 respiration was determined after addition of 250 $\mu$M ADP, and state 4 respiration was measured after complete phosphorylation of ADP to ATP. The respiratory control index (RCI) was calculated as the ratio of mean slopes during state 3 and state 4 respiration (state 3 slope/state 4 slope).

Mitochondrial $\text{H}_2\text{O}_2$ release rate. Rates of mitochondrial $\text{H}_2\text{O}_2$ release were measured spectrophotometrically [QM-8, Photon Technology International (PTI)] at 27°C after oxidation of Amplex Red (25 $\mu$M; Molecular Probes) to the highly fluorescent product resorufin in the presence of 0.1 U/ml horseradish peroxidase. Excitation and emission wavelengths ($\lambda_{ex}$ and $\lambda_{em}$) were set to 530 and 583 nm, respectively. Mitochondria (0.5 mg/ml) were suspended in respiration buffer. Time controls received 0.3% dimethyl sulfoxide (DMSO). Maximal $\text{H}_2\text{O}_2$ production was stimulated in some experiments by addition of the complex III blocker antimycin A (5 $\mu$M). Antimycin A is believed to inhibit cytochrome $b$ oxidation by cytochrome $c_1$ to cause accumulation of ubisemiquinone, which is oxidized by molecular O$_2$ to generate O$_2$$^{\cdot-}$ and $\text{H}_2\text{O}_2$ (7). Catalase (300 U/ml) was added to confirm $\text{H}_2\text{O}_2$ production by attenuating the $\text{H}_2\text{O}_2$ signal, since catalase converts $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$; $\text{H}_2\text{O}_2$ release rates were expressed as arbitrary fluorescence units (see Figs. 4 and 5A) or as percentage of time control experiments (see Fig. 5B). Baseline $\text{H}_2\text{O}_2$ levels were calibrated from a mean of three standard curves of photon counts over a range of 10–200 nM $\text{H}_2\text{O}_2$ (added to assay medium in the presence of reactants Amplex Red and horseradish peroxidase); each regression was linear ($R > 0.99$).

Mitochondrial redox state. Mitochondrial redox state was measured by the autofluorescence that arises from compounds endogenous to the mitochondrion. The NAD$^+$ signal is not fluorescent, but an increase in NADH fluorescence signal reflects an increase in the ratio of NADH to NAD$^+$, i.e., a net shift in the pyridine nucleotide pool to the reduced state. In contrast to NADH, a decrease in the FAD signal (flavoprotein fluorescence) occurs when the carrier binds to electrons. Thus a decrease in FAD reflects an increase in the ratio of reduced to oxidized flavoprotein (14). NADH and FAD were measured from the same aliquot of mitochondrial suspension (0.5 mg/ml) in respiratory buffer with the aid of an electronic chopper that switched between the excitations for NADH and FAD so that the time resolution for the three NADH and FAD emission signals was 7 s. NADH was determined by exciting at 350-nm $\lambda_{ex}$ and recording at 460-nm and 405-nm $\lambda_{em}$ (the latter reference wavelength is less sensitive to NADH changes). The fluorescence ratio, $F_{460}/F_{405}$, is interpreted as a measure of NADH. Mitochondrial FAD fluorescence was recorded at 540-nm $\lambda_{em}$ from light filtered at 480-nm $\lambda_{em}$.

Mitochondrial membrane potential. $\Delta \Psi_m$ was monitored at 27°C in a cuvette-based spectrophotometer (QM-8, PTI) operating at 503-nm $\lambda_{ex}$ and 527-nm $\lambda_{em}$, respectively, in the presence of the fluorescent dye rhodamine 123 (50 nM). Mitochondria (0.5 mg/ml) were suspended in respiration buffer. $\Delta \Psi_m$ was expressed as the percentage of rhodamine 123 fluorescence in the presence of fully coupled mitochondria relative to the fluorescence after addition of the uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP) at 4 $\mu$M. To verify the functional integrity of mitochondria, 250 $\mu$M ADP was added and repolarization of $\Delta \Psi_m$ after complete phosphorylation of ADP was measured.

Chemicals and reagents. Rhodamine 123, Amplex Red, and indo-1 were purchased from Molecular Probes (Eugene, OR) and high-purity KCl from EMD Chemicals (Gibbstown, NJ). All other chemicals were purchased from Sigma. NS-1619, paxilline, and Amplex Red were dissolved in DMSO before being added to the experimental buffer.

Statistical analyses. Group data were compared by analysis of variance. If $F$ values ($P < 0.05$) were significant, post hoc comparisons of means tests (Student-Newman-Keuls) were considered statistically significant when $P < 0.05$ (2-tailed). Data are presented as means ± SE.

RESULTS

Mitochondrial integrity after isolation. The morphological integrity of mitochondria after the isolation procedure was verified by electron microscopy. Figure 1 shows isolated guinea pig heart mitochondria with intact inner and outer membranes suspended in isolation buffer. A RCI of 2.5 ± 0.1 in the control group with succinate as substrate demonstrated strong coupling of respiration and oxidative phosphorylation. This indicates functioning mitochondria with integrity of the respiratory complexes in the IMM after the isolation procedure.

Mitochondrial respiration. The experimental procedure and sample traces for respiration experiments are shown in Fig. 2. Opening of putative mtBK$\text{Ca}$ channels by 30 $\mu$M NS-1619 significantly increased state 2 respiration by 26 ± 4% over control and state 4 respiration by 14 ± 4% over control (Fig. 3, A and C). To verify that the increase in state 2 and state 4 respiration was due to mtBK$\text{Ca}$ channel opening by NS-1619, 5 $\mu$M paxilline was added in the absence or presence of 30 $\mu$M NS-1619 (Fig. 3). Preadministration of paxilline significantly blocked the NS-1619 induced increase in state 2 (to 4 ± 3% from 26 ± 4%) and state 4 (to −1 ± 3% from 14 ± 4%).
respiration. Paxilline alone had no significant effect on respiration (0 ± 2%), which indicates that mtBKCa channels were closed under these experimental conditions. State 3 respiration was decreased statistically by NS-1619 (~12 ± 3% vs. control), but coadministration of paxilline reduced but did not significantly block this effect (~21 ± 2%) (Fig. 3B), which may indicate a mtBKCa channel-independent effect. These data demonstrate that a low concentration of NS-1619 increases succinate-supported respiration, but only during the resting states when the basal respiratory rate is low.

Reverse electron flow-induced $\text{H}_2\text{O}_2$ generation. To verify that the major mechanism of ROS production with the complex II substrate succinate is due to reverse electron flow, we measured mitochondrial $\text{H}_2\text{O}_2$ release rate. In representative tracings, Fig. 4 shows that succinate initiated a large increase in the $\text{H}_2\text{O}_2$ release rate that was abolished by either the mitochondrial uncoupler CCCP (Fig. 4A) or the complex I blocker rotenone (Fig. 4B). This verified in our model that reverse electron flow into complex I of the ETC is the main mechanism by which $\text{O}_2^{•−}$ is generated with succinate alone as the substrate. Moreover, the rate of $\text{H}_2\text{O}_2$ release in succinate-supported respiration was ~35 times higher than the rate attained by the complex I substrate pyruvate (Fig. 4C). Antimycin A, a complex III blocker, increased the $\text{H}_2\text{O}_2$ release rate much more after uncoupling with CCCP than after blocking of complex I with rotenone.

Effect of mtBKCa channel opening on $\text{H}_2\text{O}_2$ generation. To investigate the effect of mtBKCa channel opening on reverse electron flow-induced $\text{H}_2\text{O}_2$ production, we measured the $\text{H}_2\text{O}_2$ release rate after addition of NS-1619 in succinate-supported mitochondria. Results are expressed as percentage of time controls. Figure 5 shows that addition of NS-1619 significantly decreased the $\text{H}_2\text{O}_2$ release rate from 85 ± 2% to 12 ± 1%. Preadministration of paxilline statistically attenuated this reduction in ROS generation (28 ± 5% vs. 12 ± 1%). Paxilline alone had no significant effect on mitochondrial $\text{H}_2\text{O}_2$ release rate (79 ± 2% vs. 85 ± 2%).

Effect of NS-1619 on mitochondrial redox state. In this in vitro preparation, oxidation of succinate and reduction of molecular $\text{O}_2$ involve, in part, reverse electron flow, specifi-
released into the matrix or cytosol at complex III and into the matrix at complex I (12). In turn, $O_2^{•−}$ is dismutated by superoxide dismutase in the matrix (SOD2) or cytosol (SOD1) to form $H_2O_2$, most of which is detoxified to $H_2O$ by the glutathione system. However, $H_2O_2$ is also a progenitor of the highly reactive $•OH$ in the presence of a reduced transition metal such as $Fe^{2+}$. Since $H_2O_2$ is highly permeable through the IMM, the $H_2O_2$ measured in this study likely reflects its generation in the matrix.

Several studies have reported that $O_2^{•−}$ generation is greater during respiration supported with $FADH_2$-linked substrates than with complex I substrates (21, 37). $O_2^{•−}$ generation with the complex II substrate succinate is caused by reverse electron flow into complex I of the ETC and is largely dependent on a high $\Delta\Psi_m$ (20, 21). This is supported by our observation that uncoupling of mitochondria with CCCP, which collapses $\Delta\Psi_m$ by allowing proton reentry through the IMM, halted $H_2O_2$ measured in this study likely reflects its generation in the matrix.

Possible significance of reverse electron flux. Significant $O_2^{•−}$ generation occurs via forward electron flow in the presence of complex I Q site inhibitors like rotenone, but much more is generated during reverse electron flow through complex I. Lambert and Brand (18) argue that the site of generation by complex I is likely a ubisemiquinone-binding site rather than upstream flavin or FeS centers. Reverse electron flow may be a significant factor in I/R injury. Physiologically, succinate is synthesized at low concentrations (0.2–0.4 mM) inside mitochondria in vivo and is not a natural substrate. However, it rises substantially during ischemia or hypoxia (up to 4–7 mM) (20). It is possible that during early ischemia, when NADH levels are high, and during initial reperfusion, the oxidation of accumulated succinate generates the high $\Delta\Psi_m$ and reduces power necessary for reversal of electron transfer and $O_2^{•−}$ generation at complex I. In phosphorylating mitochondria respiration is controlled by both ATP turnover and electron supply. Adenine nucleotide translocase (ANT) is an important site of control in oxidative phosphorylation (14) because it catalyzes the one-for-one exchange of adenine nucleotides. In energized mitochondria, ANT preferentially ejects more ATP than ADP brought into the matrix. This would lead to a greater extramitochondrial ATP-to-ADP ratio, which could lead to activation of succinate dehydrogenase (complex II) and stimulation of reverse electron flow.

**Effects of NS-1619 and paxilline on BKCa channel**. NS-1619, a benzimidazole derivative, promotes opening of high-conductance (300 pS) BKCa channels in membranes of a wide variety of cell types (34). NS-1619-induced effects on smooth muscle can be blocked by charybotoxin or paxilline, but not by glibenclamide, which indicates that the action of NS-1619 in plasma membranes is predominantly on the BKCa channel. The rapid effect of NS-1619 suggests that its mechanism of action is either directly on the channel protein itself or on a closely

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**Fig. 3.** Summarized data for the effects of 30 μM NS-1619 and the antagonist effects on big mitochondrial Ca$^{2+}$-sensitive K$^+$ channel (mtBKCa) opening by 5 μM paxilline on mitochondrial respiration in the presence of the complex II substrate succinate (10 mM). *P < 0.05 vs. control; #P < 0.05 vs. NS-1619 (n = 10 for each group).
associated modulatory protein (28). The absence of intracellular Ca\(^{2+}\) prevents BK\(_{Ca}\) channel activation by NS-1619, and the drug may increase channel activation by making the channel more sensitive to intracellular Ca\(^{2+}\) (28). The BK\(_{Ca}\) channels are tetramers of a pore-forming \(\alpha\)-subunit of the slo gene family and a regulatory \(\beta\)-subunit, which is structurally unique and transmembrane spanning; the \(\alpha\)-subunit, encoded by a single gene, is comprised of seven transmembrane segments and four intracellular hydrophobic domains (17, 23, 39). Several groups are attempting to more clearly identify and characterize these channels in cardiac IMM.

**Modulation of mitochondrial function by mtBK\(_{Ca}\) channels.** Xu et al. (41) first reported evidence for mtBK\(_{Ca}\) channels in the IMM of guinea pig ventricular cells. Patch-clamp recordings from mitoplasts of these cells showed Ca\(^{2+}\)-dependent, large-K\(^{+}\) conductance channels in the IMM, and immunoblots of cardiac mitochondria with antibodies against the COOH-terminal part of BK\(_{Ca}\) channel identified a 55-kDa protein as part of this putative channel. The \(\beta_{1}\)-subunit of the mtBK\(_{Ca}\) channel, as tentatively identified in the IMM, interacts with the cytochrome-c oxidase subunit I (26). The binding sites for charybdotoxin and NS-1619 are likely in the cytosolic compartment, whereas sites for Ca\(^{2+}\) are likely on the matrix side of the IMM (40).

The ultimate bioenergetic modulating effects of K\(^{+}\) influx into the mitochondrial matrix through K\(^{+}\) channels, including both mtBK\(_{Ca}\) and mitochondrial ATP-sensitive K\(^{+}\) channels (mtK\(_{ATP}\)), however, remains unclear. Sato et al. (32) demonstrated that NS-1619 increases flavoprotein oxidation in ventricular myocytes placed in glucose-free Tyrode solution; this suggested an increase in electron transport in oxidized mitochondria. Recently, we reported (16) on the concentration-dependent effects of NS-1619 on respiration, \(\Delta \Psi_m\) and H\(_2\)O\(_2\) generation in isolated guinea pig heart mitochondria respiring on the complex II substrate succinate in the presence of the complex I blocker rotenone to prevent reverse electron flow. NS-1619 increased state 2 and state 4 respiration, effects that were inhibited by paxilline. These findings are in agreement with the hypothesis of O’Rourke (25), who suggested that mitochondrial K\(^{+}\) channels function as energy (stored as the proton gradient, \(\Delta \Psi_m\))-dissipating channels by expending \(\Delta \Psi_m\) in part, to eject K\(^{+}\) that enters the matrix through activated K\(^{+}\).
channels via an electroneutral K\(^{+}\)/H\(^{+}\) exchanger. This decrease in H\(^{+}\) would stimulate respiration to compensate for net proton leak, with the consequence of a maintained m\((16)\). In the present report we demonstrated during succinate-supported respiration that putative mtBK\(_{Ca}\) channel opening by 30 \(\mu M\) NS-1619, and by inference by lower concentrations, again increased state 2 and state 4 respiration and had no effect on redox state or m. Cancherini et al. (9) reported recently that NS-1619 stimulated nonphosphorylating respiration (state 4) and inhibited ADP-stimulated respiration (state 3) in isolated rat heart mitochondria. These effects of NS-1619 were also described previously by Debska et al. (13) and by our group (16) and are confirmed again in this study in guinea pig heart mitochondria. However, in the former study (9) evidence was presented that NS-1619 does not specifically transport K\(^{+}\) via a channel or cation transporter. In the presence of the complex V inhibitor oligomycin they reported that NS-1619 depolarized m in K\(^{+}\)-containing as well as K\(^{+}\)-free buffer, that the respiratory effects were not blocked by paxilline, that NS-1619-induced matrix swelling occurred also in a tetraethylammonium-based buffer, and that the latter effect was not blocked by paxilline. On the basis of these findings Cancherini et al. (9) suggested that NS-1619 promotes nonselective permeabilization of the IMM to ions rather than acting on a specific IMM K\(^{+}\) channel. However, in this and our prior study (16), the effect of NS-1619 to enhance state 4 respiration was inhibited by paxilline, a known BK\(_{Ca}\) channel inhibitor, and uncoupling did not occur at less than 30 \(\mu M\) NS-1619.

From these pharmacological results, we conclude that the NS-1619-induced increase in state 4 respiration, and the state- and substrate-dependent effects on H\(_{2}O\)\(_{2}\) production, are likely mtBK\(_{Ca}\) channel mediated. We cannot reconcile differences between these studies, but we agree that the specificity of NS-1619 for the putative mtBK\(_{Ca}\) channel remains speculative. Moreover, the putative mtBK\(_{Ca}\) channel will need to be better identified and characterized in the IMM to substantiate its role in modulating mitochondrial bioenergetics. What is evident to us, however, is that NS-1619 clearly initiates pharmacological preconditioning against cardiac I/R injury and that this protective effect is effectively blocked by a O\(_{2}^{\cdot-}\) dismutase mimetic as well as by paxilline (33).

Effect of putative mtBK\(_{Ca}\) channel opening on reverse electron flow-induced H\(_{2}O\)\(_{2}\) production. Under physiological conditions reverse electron flow does not occur, because forward electron flow through complex I via NADH prevents it. However, under pathophysiological conditions in which NADH is depleted, reverse electron flow may lead to O\(_{2}^{\cdot-}\) generation at complex I (6, 35, 37). In succinate-supported isolated mitochondria O\(_{2}^{\cdot-}\) generation due to reverse electron flow to complex I is dependent on a fully charged m under state 4.
conditions; reverse flow is blocked by the complex I blocker rotenone. Our results suggest that reverse electron flow-induced H$_2$O$_2$ production can be modulated by matrix K$^+$ flux. The H$_2$O$_2$ release rate during enhanced state 4 respiration by complex I (i.e., no pyruvate to increase the NADH-to-NAD$^+$ ratio) is enhanced by the presence of succinate because of a compensatory outward flux of K$^+$ and influx of protons via K$^+$/H$^+$ exchange, which accelerates forward electron flow, thus reducing the impact of reverse flow on O$_2^-$ generation. Clearly, the consequences of altered matrix K$^+$ flux likely alter the flux of other cations in addition to H$^+$, for example, Na$^+$ and Ca$^{2+}$ by Na$^+$/H$^+$ and Na$^+$/Ca$^{2+}$ exchange in the IMM.

In summary, we report that in fully membrane-polarized and reduced mitochondria matrix K$^+$ influx can either increase or decrease O$_2^-$ generation depending on substrate conditions. This work emphasizes the impact of experimental substrate conditions when analyzing mitochondrial bioenergetics, and may help to explain some of the conflicting results in the literature regarding the effect of K$^+$ channel activation and matrix K$^+$ flux on modulating mitochondrial function and H$_2$O$_2$ production. Moreover, caution must be taken on the effect of NS-1619 to open mtBK$_{Ca}$ channels or of paxilline to block them because although paxilline completely reversed the increase in respiration, it only incompletely blocked the decrease in H$_2$O$_2$ production. We must have a precise identification of the drugs used to explore these mechanisms. Much research remains to be done to understand the physiological (cell conditioning) and pathological (cell damage) conditions by which matrix K$^+$ flux modulates matrix pH, respiration, and O$_2^-$ generation.

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