Potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation

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Received 25 June 2001; accepted in final form 27 September 2001

Ozcan, Cevher, Martin Bienengraeber, Petras P. Dzeja, and Andre Terzic. Potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation. Am J Physiol Heart Circ Physiol 282: H531–H539, 2002; 10.1152/ajpheart.00552.2001.—K+ channel openers have been recently recognized for their ability to protect mitochondria from anoxic injury. Yet the mechanism responsible for mitochondrial preservation under oxidative stress is not fully understood. Here, mitochondria were isolated from rat hearts and subjected to 20-min anoxia, followed by reoxygenation. At reoxygenation, increased generation of reactive oxygen species (ROS) was associated with reduced ADP-stimulated oxygen consumption, blunted ATP production, and disrupted mitochondrial structural integrity coupled with cytochrome c release. The prototype K+ channel opener diazoxide markedly reduced mitochondrial ROS production at reoxygenation with a half-maximal effect of 29 μM. Diazoxide also preserved oxidative phosphorylation and mitochondrial membrane integrity, as indicated by electron microscopy and reduced cytochrome c release. The protective effect of diazoxide was reproduced by the structurally distinct K+ channel opener nicorandil and antagonized by 5-hydroxydecanoic acid, a short-chain fatty acid derivative and presumed blocker of mitochondrial ATP-sensitive K+ channels. Opener-mediated mitochondrial protection was simulated by the free radical scavenger system composed of superoxide dismutase and catalase. However, the effect of openers on ROS production was maintained in nominally K+-free medium in the presence or absence of the K+ ionophore valinomycin and was mimicked by malonate, a modulator of the mitochondrial redox state. This suggests the existence of a K+ conductance-independent pathway for mitochondrial protection targeted by K+ channel openers. Thus the cardioprotective mechanism of K+ channel openers includes direct attenuation of mitochondrial oxidant stress at reoxygenation.

Diazoxide; nicorandil; anoxia; ATP; reactive oxygen species; cardioprotection; energy metabolism; heart

MITOCHONDRIA PLAY A CENTRAL role in cell energetics, and determine cellular life and death under stress (24, 47, 49). In the heart, conditions associated with metabolic and oxidative challenge severely compromise mitochondrial functions, and precipitate lethal cell injury (5, 6, 46). Preserving the functional and structural integrity of mitochondria is, therefore, a prerequisite for successful cardioprotection (29, 50). To increase myocardial tolerance and reduce injury, a number of strategies that modulate mitochondrial respiration, ATP production, ion transport and/or free-radical generation have been considered (16, 20, 31, 32, 35, 43). In particular, potassium channel openers that preferentially target mitochondrial functions have emerged as powerful cardioprotective agents (3, 5, 11, 13, 38, 51, 53). Prototype drugs such as diazoxide and nicorandil reduce infarct size, salvage the myocardium, and enhance survival of cardiomyocytes (1, 5, 8, 12, 28). A direct effect of potassium channel openers on the mitochondrial metabolic state appears essential in the preservation of heart muscle against anoxic injury (5, 18, 27, 40). However, the mechanism responsible for the cardioprotective effect of potassium channel openers under oxidant stress is not fully understood.

It has been proposed that in mitochondria potassium channel openers target an ATP-sensitive K+ (mitochondrial KATP) channel implicated in the regulation of mitochondrial ion and volume homeostasis (8, 13, 51). Yet, the molecular identity of mitochondrial KATP channels and their role in cytoprotection remains elusive (27, 50, 53). Moreover, the effects of potassium channel openers depend on the metabolic substrates utilized by mitochondria and could be associated with regulation of dehydrogenase activity and the redox state of the respiratory chain (10, 39, 45, 48). Indeed, components of the mitochondrial respiratory chain, in particular the succinate dehydrogenase-coenzyme Q complex, are sites of free radical production contributing to oxidative injury in cardiomyocytes (54). At the whole heart or cellular level, reduction in intracellular free-radical production may contribute to the protection afforded by potassium channel openers (14, 30, 34, 54). Alternatively, a free radical-dependent signaling mechanism has also been proposed as a trigger of cardioprotection mediated by potassium channel openers (7, 41). At present, it remains unknown whether protection by potassium channel openers is associated with modulation of reactive oxygen species (ROS) generation in mitochondria.

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Here, we provide evidence that potassium channel openers suppress ROS generation and reduce cytochrome c release in isolated cardiac mitochondria at reoxygenation. Free radical scavengers mimicked the protective effect of potassium channel openers on oxidative phosphorylation. This suggests that direct attenuation of oxygen stress contributed to reduction of mitochondrial injury after anoxia-reoxygenation.

METHODS

The investigation conformed to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication No. 5377-3, 1996) and was approved by the Institutional Animal Care and Use Committee.

Isolation of heart mitochondria. Mitochondria were isolated from hearts of pentobarbital sodium-anesthetized (100 mg/kg ip) rats (Harlan Sprague Dawley; Indianapolis, IN) as previously described (15, 40). Ventricles were removed into an ice-cold buffer composed of (in mmol/l) 50 sucrose, 200 mannitol, 5 KH2PO4, 1 EGTA, 5 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.3, and 0.2% bovine serum albumin (BSA) and homogenized (PT 10/35 Polytron, Brinkman Instruments; Westbury, NY), and the mitochondrial fraction was obtained by differential centrifugation (Sorvall RCSC, Kendro Laboratory Products; Newtown, CT). Mitochondria were washed, suspended in isolation buffer (without EGTA and BSA) and kept on ice. Mitochondrial protein concentration was determined with a DC protein kit (Bio-Rad; Hercules, CA).

Anoxia-reoxygenation protocol. Mitochondria (2 mg of protein) were placed and stirred (30°C) in an airtight closed multichannel ESON-6CH chamber (19, 40) filled with 1 ml of incubation media composed of (in mmol/l) 110 KCl, 5 K2HPO4, 2.5 pyruvate, 2.5 malate, and 10 MOPS, pH 7.3. Anoxia was induced within 5 min because mitochondria consumed all available oxygen within the chamber (40), which was monitored by an oxygen-sensitive electrode (Microelectrodes; Bedford, NH). Anoxia was maintained for 20 min, and then reoxygenation produced by exposure of the stirred mitochondrial suspension to room air was maintained for an additional 20 min. Mitochondrial function and structure were evaluated throughout the anoxia-reoxygenation protocol.

Mitochondrial oxygen consumption and ATP production. Mitochondrial oxygen consumption was monitored with an oxygen-sensitive electrode, and data were processed with the use of Bioquest software (15). Mitochondrial respiration was determined in the absence (state 2) or presence (state 3 and V3) of 50 μM ADP. The rate of ATP production was determined by measuring ATP levels in mitochondrial perchloric acid extracts with the use of high-performance liquid chromatography (Hewlett-Packard; Waldbronn, Germany) as previously described (40).

ROS generation. Mitochondria were preloaded with 10 μM of the ROS-sensitive dye 2,7-dichlorofluorescein (DCFH) diacetate (Sigma; St. Louis, MO). ROS produced by mitochondria cause oxidation of DCFH, yielding the fluorescent product dichlorofluorescein (DCF) (54). Production of ROS was measured in a potassium solution composed of (in mmol/l) 110 KCl, 5 K2HPO4, 2.5 pyruvate, 2.5 malate, and 10 MOPS, pH 7.3, or nominally potassium-free incubation medium composed of (in mmol/l) 110 sucrose, 5 NaHPO4, 2.5 pyruvate, 2.5 malate, and 10 MOPS, pH 7.3. Mitochondrial aliquots (50 μl) were obtained at the onset, at 10 min, and at 20 min of reoxygenation and were diluted with 1 ml of H2O. DCF fluorescence in the mitochondrial suspension was measured using a spectrofluorometer (Gilford Fluoro IV, Ciba Corning; Oberlin, OH) at an excitation and emission wavelengths of 500 and 530 nm, respectively. DCF fluorescence was normalized to maximal values obtained by exposing mitochondrial suspension to 1 mM H2O2. To obtain independent measurements of ROS generation, mitochondrial suspension was incubated with a distinct ROS-sensitive probe, p-hydroxyphenylacetate (PHPA; 500 μM), in the presence of 4 units of horseradish peroxidase (HRP). Oxidation of PHPA, coupled to the enzymatic reduction of H2O2 by HRP, produces the PHPA fluorescence dimer (26). With the use of a fluorometer, the rate of mitochondrial ROS production was followed by the monitoring of PHPA fluorescence at an excitation of 320 nm and emission of 400 nm.

Cytochrome c release. Mitochondrial suspension was centrifuged, and the supernatant was separated from the pellet to assess the amount of cytochrome c released after anoxia-reoxygenation. The supernatant underwent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4–20%), and proteins were transferred to nitrocellulose membranes by semidyke blotting. After incubation with the blocking reagent (5% fat-free milk powder in phosphate-buffered saline) nitrocellulose membranes were probed with a mouse anti-cytochrome c monoclonal antibody (1:1,000 dilution; Biosource International; Camarillo, CA) and then exposed to an HRP-conjugated secondary antibody (Jackson ImmunoResearch Laboratories; West Grove, PA). The signal was revealed by an enhanced chemiluminescence detection system (Pierce; Rockford, IL). Quantification was performed with the use of gel densitometry.

Electron microscopy. Mitochondria were fixed using Trump's buffer (1% glutaraldehyde, 4% formaldehyde, and 0.1 M phosphate buffer, pH 7.2), rinsed with phosphate-buffered sucrose, and postfixed in phosphate-buffered 1% osmium tetroxide as described (40). Samples were en bloc stained with 2% uranyl acetate for 30 min at 60°C, rinsed, dehydrated, and embedded in Spurr's resin. Twelve thin sections for each sample were cut on an ultramicrotome (Ultracut E, Reichelt-Jung; Wien, Austria), placed on copper grids, and stained with lead citrate. Mitochondria were micrographed with an electron microscope (model EM-1200 EX II; Jeol; Peabody, MA). Mitochondrial structure was defined as intact, swollen, or damaged using previously established parameters (40).

Chemicals. Diazoxide was purchased from RBI (Natick, MA) and dissolved as a concentrated stock solution in dimethyl sulfoxide (DMSO). The maximal concentration of DMSO within the incubation medium was kept under 0.5%, and control experiments were performed with corresponding DMSO concentrations. Nicorandil (Chugai Pharmaceuticals; Tokyo, Japan), a kind gift from Dr. G. J. Gross (44), was dissolved in incubation medium. 5-Hydroxydecanoic acid (5-HD) was purchased from ICN Biomedicals (Aurora, OH) and dissolved in the incubation medium. All other chemicals were obtained from Sigma.

Statistical analysis. Data are presented as means ± SE, and n represents the number of mitochondrial preparations used for functional studies or the actual number of imaged mitochondria in electron micrographs. Group comparisons were performed by ANOVA with the Student-Newman-Keuls post hoc correction procedure or with the Student’s t-test. A P value of <0.05 was considered statistically significant.

RESULTS

Potassium channel openers suppress mitochondrial ROS generation at reoxygenation. Mitochondria are a major intracellular site generating ROS, an inevitable
byproduct of oxidative metabolism (42). ROS production by mitochondria critically depends on the redox state of the respiratory chain (4, 55). Here, oxidative stress imposed on cardiac mitochondria by anoxia-reoxygenation was associated with a significant burst in ROS generation (Fig. 1A). On average, after 20 min of anoxia, ROS-sensitive DCF fluorescence increased from 61 ± 3% at the onset of reoxygenation to 76 ± 2% and 99 ± 2% at 10-min and 20-min reoxygenation, respectively (P < 0.05; Fig. 1A). The prototype mitochondrial potassium channel opener diazoxide (100 μM) significantly suppressed ROS production throughout reoxygenation (Fig. 1A). Specifically, ROS generation was 27 ± 3%, 37 ± 4%, and 45 ± 5% at the onset, 10-min, and 20-min reoxygenation in diazoxide-treated mitochondria (n = 18), values significantly lower compared with untreated postanoxic controls (P < 0.05; Fig. 1A). The effect of diazoxide was concentration dependent, with an apparent half-maximal effect (IC50) at 29 ± 1 μM (n = 3; Fig. 1B). The structurally distinct potassium channel opener nicorandil (100 μM) also significantly reduced mitochondrial ROS production at each time point after reoxygenation (n = 6; Fig. 1C). The effect of both diazoxide and nicorandil on ROS production was antagonized by 5-HD, a presumed blocker of mitochondrial KATP channels (n = 6, Fig. 1, A and C). In fact, in the presence of 5-HD (500 μM), ROS production in opener-treated mitochondria was similar to that of untreated postanoxic controls (P > 0.05; Fig. 1, A and C). The ability of potassium channel openers to reduce ROS generation was confirmed by measuring mitochondrial H2O2-dependent oxidation of PHPA (Fig. 1D). After 20-min anoxia, H2O2 generation was 63 ± 2%, 83 ± 2%, and 100% versus 38 ± 3%, 50 ± 2%, and 56 ± 5% at the onset, 10-min, and 20-min reoxygenation in untreated and diazoxide-treated mitochondria, respectively (n = 6, P < 0.05; Fig. 1D). Thus two independent methods of ROS detection indicate that potassium channel openers reduce oxidant stress in cardiac mitochondria challenged by anoxia-reoxygenation.

Fig. 1. Potassium channel openers prevent mitochondrial reactive oxygen species (ROS) generation at reoxygenation. A: in isolated cardiac mitochondria exposed to 20-min anoxia, followed by 0.5-, 10-, or 20-min of reoxygenation, ROS production was measured by means of dichlorofluorescein (DCF) fluorescence in the absence of diazoxide (untreated), in the presence of 100 μM diazoxide (Diaz), or in the presence of 100 μM diazoxide + 500 μM 5-hydroxydecanoic acid (5-HD). B: concentration-response curve for diazoxide-mediated inhibition of mitochondrial ROS production is presented at 20-min reoxygenation. Half-maximal effect (IC50) was achieved at 29 ± 1 μM. Data points were fit with the Hill equation. C: mitochondrial ROS production in the absence of nicorandil (untreated) and in the presence of 100 μM nicorandil (Nicor), or in the presence of 100 μM nicorandil + 500 μM 5-HD. D: ROS generation independently measured by H2O2-dependent oxidation of p-hydroxyphenylacetate (PHPA) in untreated (–Diaz) and treated (+Diaz) mitochondria.
ROS scavengers preserve oxidative phosphorylation at reoxygenation. Established ROS scavengers, such as superoxide dismutase (SOD) and catalase, can abrogate oxidant stress (21). Here, anoxiareoxygenation dramatically suppressed mitochondrial respiration and ATP synthesis (Fig. 2). Mitochondrial oxygen consumption decreased from 311 ± 14 (n = 16) to 76 ± 4 (n = 18) ng atoms O$_2$·min$^{-1}$·mg protein$^{-1}$ (after the addition of 250 μM ADP, P < 0.05; Fig. 2A), whereas ATP production dropped from 738 ± 14 to 208 ± 9 nmol ATP·min$^{-1}$·mg protein$^{-1}$ (n = 6, P < 0.05; Fig. 2B). Accordingly, the respiratory control ratio, an index of oxidative phosphorylation coupling, decreased from 8.8 ± 0.3 (n = 16) in normoxia to 1.6 ± 0.1 (n = 18) after anoxiareoxygenation (P < 0.05). Treatment with SOD (1,000 units) and catalase (100 μM), which were present throughout the anoxiareoxygenation challenge, protected mitochondrial oxidative phosphorylation (Fig. 2). In SOD-catalase-treated mitochondria, ADP-stimulated respiration was 207 ± 14 ng atoms O$_2$·min$^{-1}$·mg protein$^{-1}$ (n = 14; Fig. 2A), the respiratory control ratio was 3.4 ± 0.2 (n = 14), and ATP production was 432 ± 17 nmol ATP·min$^{-1}$·mg protein$^{-1}$ (n = 7; Fig. 2B) after anoxiareoxygenation. These values were significantly higher compared with those from nontreated mitochondria (P < 0.05; Fig. 2).

Thus the conventional free radical scavengers SOD and catalase protect mitochondrial function at reoxygenation indicating that oxidant stress is a major contributing factor to injury.

Potassium channel openers protect mitochondria at reoxygenation. We evaluated whether the antioxidant effect of potassium channel openers (Fig. 1) also translates into a “mitoprotective” effect under the same anoxiareoxygenation protocol. Indeed, mitochondria treated with diazoxide (100 μM) or nicorandil (100 μM) maintained a vigorous respiratory response to ADP (250 μM) after an anoxiareoxygenation challenge (Fig. 3A). On average, in diazoxide- and nicorandil-treated mitochondria, ADP-stimulated respiration was 237 ± 7 (n = 24) and 203 ± 8 (n = 12) ng atoms O$_2$·min$^{-1}$·mg protein$^{-1}$ (Fig. 3A), with a respiratory control ratio of 5.0 ± 0.4 (n = 24) and 3.65 ± 0.3 (n = 12), respectively. These values were significantly higher than those obtained in untreated mitochondria (P < 0.05; Fig. 3A). Potassium channel openers also maintained the mitochondrial rate of ATP production, which was 497 ± 18 nmol·min$^{-1}$·mg protein$^{-1}$ in diazoxide-treated (n = 6) and 436 ± 48 nmol ATP·min$^{-1}$·mg protein$^{-1}$ in nicorandil-treated (n = 3) mitochondria after anoxiareoxygenation (Fig. 3B). These values are also significantly higher than those from untreated controls (n = 6, P < 0.05; Fig. 3B). The protective effect of openers on mitochondrial respiration and ATP production was antagonized by 5-HD (Fig. 3, A and B). On average, in mitochondria subjected to anoxiareoxygenation, respiration in the presence of 5-HD was 80 ± 7 (n = 13) and 84 ± 8 (n = 11) ng atoms O$_2$·min$^{-1}$·mg protein$^{-1}$ despite treatment with diazoxide and nicorandil, respectively (Fig. 3A). Accordingly, the ATP production rate was 275 ± 19 (n = 4) and 264 ± 23 (n = 3) nmol ATP·min$^{-1}$·mg protein$^{-1}$ in the presence of 5-HD in diazoxide- and nicorandil-treated anoxiareoxygenated mitochondria (Fig. 3B). These values were not significantly different from those recorded in untreated mitochondria (P > 0.05; Fig. 3, A and B). In addition to functional protection, potassium channel openers also preserved mitochondrial structural integrity (Fig. 3C). In normoxia, 83% of isolated mitochondria displayed intact morphology, with 10% being swollen and 7% damaged (n = 504). After anoxiareoxygenation, 91% of mitochondria were damaged or swollen with 9% remaining intact (n = 230; Fig. 3, C and D). On the other hand, diazoxide-treated mitochondria had a rather preserved morphology after anoxiareoxygenation (Fig. 3C), with 66% remaining intact limiting the swollen and damaged mitochondrial population to 13% and 21%, respectively (n = 158; Fig. 3D). Thus potassium channel openers protect cardiac mitochondria from anoxiareoxygenation injury, mimicking the protective efficacy of conventional free radical scavengers.

Potassium channel openers and ROS scavengers prevent cytochrome c release at reoxygenation. Release of cytochrome c is associated with mitochondrial damage and initiation of signaling events leading to cell death (24). Here, anoxiareoxygenation induced marked release of a mitochondrial protein recognized by the
specific anti-cytochrome c antibody as a 12-kDa band, corresponding to the molecular mass of cytochrome c (n = 3; Fig. 4A). Treatment of mitochondria with potassium channel openers diazoxide (100 μM) or nicorandil (100 μM) throughout the anoxia-reoxygenation insult significantly reduced release of cytochrome c (Fig. 4A). At reoxygenation, cytochrome c released in diazoxide- and nicorandil-treated mitochondria amounted respectively to 20 ± 3% (n = 4) and 28 ± 3% (n = 4) compared with untreated controls (P < 0.05; Fig. 4B). Protection afforded by potassium channel openers was mimicked by the free radical scavenging system, SOD + catalase, which reduced cytochrome c release to 43 ± 8% (n = 3) of the value in untreated mitochondria (Fig. 4, A and B). Conversely, the effect of openers on cytochrome c release was antagonized by 5-HD, a short-chain fatty acid derivative (n = 4; Fig. 4, A and B). Thus potassium channel openers prevent cytochrome c release, indicating reduction of oxidative stress-induced mitochondrial damage through a 5-HD-sensitive mechanism.

Reduction of ROS generation by potassium channel openers is potassium independent and mimicked by an inhibitor of succinate dehydrogenase. The molecular identity of the putative mitochondrial K<sub>ATP</sub> channel, and the mechanism by which potassium channel openers afford protection of mitochondrial function, is unknown so far (3, 11, 13, 38, 51, 53). In fact, it appears difficult to directly relate a change in mitochondrial potassium conductance with regulation of ROS production (27). Alternatively, mitochondrial protection could be a consequence of opener-mediated modulation of the mitochondrial redox state and dehydrogenase activity, critical in ROS generation (48, 53). To test this hypothesis, the effect of diazoxide on mitochondrial ROS production after anoxia-reoxygenation was here measured in nominally potassium-free medium. Diazoxide (100 μM), present throughout the anoxia-reoxygenation insult, reduced ROS production in the absence of added potassium, as monitored by changes in DCF fluorescence (Fig. 5A). At the onset of reoxygenation, production of ROS was reduced from 47 ± 3% in untreated to 30 ± 1% in diazoxide-treated mitochondria (n = 3, P < 0.05; Fig. 5A). Similarly, at 10- and 20-min reoxygenation, ROS generation was reduced by diazoxide treatment from 79 ± 4 to 54 ± 2% and from 101 ± 3 to 72 ± 2%, respectively (n = 3, P < 0.05; Fig. 5A). Moreover,
 protection afforded by the potassium channel opener was sustained in the presence of the potassium ionophore valinomycin (50 ng/mg protein), used to shunt any remaining potassium conductance (Fig. 5A). Valinomycin, on its own did not produce significant change in ROS generation (*n* = 3; *P* > 0.05; Fig. 5A). However, diazoxide-induced suppression of ROS production remained significant even in the presence of valinomycin, i.e., 35 ± 2%, 62 ± 2%, and 78 ± 2% at the onset and 10- and 20-min reoxygenation, respectively (*n* = 3), values lower than those in untreated controls (*P* < 0.05; Fig. 5A). Because diazoxide affects mitochondrial succinate-supported respiration and succinate dehydrogenase activity (30, 31), the ability of malonate, an inhibitor of succinate dehydrogenase, to modulate ROS production was evaluated (Fig. 5B). Malonate (5 mM), used at a concentration that inhibits mitochondrial succinate dehydrogenase, also significantly reduced ROS generation (Fig. 5B). On average, malonate treatment decreased generation of ROS from 54 ± 3 to 37 ± 1% at the onset, from 92 ± 6 to 54 ± 1% at 10 min, and from 106 ± 6 to 66 ± 3% after 20 min of reoxygenation (*n* = 6, *P* < 0.05; Fig. 5B). Thus reduction of ROS generation by potassium channel openers at reoxygenation is potassium conductance independent and is mimicked by modulators of the mitochondrial redox state.

**DISCUSSION**

Cardiac mitochondria are highly vulnerable to injury induced by oxidative stress, which precipitates myocardial dysfunction in a number of disease conditions. Here we demonstrate that reoxygenation-induced generation of ROS reduces ADP-stimulated oxygen consumption, blunts ATP production, and disrupts mitochondrial integrity promoting cytochrome c release. Oxidant stress at reoxygenation was relieved by the emerging class of cardioprotective agents, known as the potassium channel openers, which suppressed ROS generation and prevented cytochrome c release, thus preserving the structural and functional integrity of mitochondria. Opener-mediated protection was apparently potassium independent and was mimicked by free radical scavengers or regulators of the mitochondrial redox state. Thus mitochondria-targeted attenuation of oxidant stress provides a mechanistic basis for increased tolerance to injury and cardioprotection.

ROS-mediated oxidant injury damages proteins, lipids, and nucleic acids and eventually routes myocytes through necrotic and/or apoptotic pathways of cell death (4, 24). The surge of ROS production observed here at reoxygenation could be the result of disrupted mitochondrial electron transport, associated with a
drop in ATP production and cytochrome c release, which have been recognized as early features in cellular death (9, 42, 54). The burst in ROS could have also resulted from dehydrogenase-catalyzed oxidation of lactate and succinate, known to accumulate in heart tissue during anoxia (33). Therefore, modulators of mitochondrial ROS production are actively being considered for enhanced mitochondrial protection against injury (27, 54).

This includes potassium channel-opening drugs, such as diazoxide and nicorandil, which preferentially target mitochondrial functions and have been recognized for their strong cardioprotective properties (8, 40, 44, 51, 54). Here, potassium channel openers effectively preserved mitochondrial respiration and ATP production and limited mitochondrial damage, preventing cytochrome c release at reoxygenation. These findings are in accord with previous reports showing the ability of potassium channel openers to sustain oxidative phosphorylation after anoxia (40) and prevent apoptotic cell death in the myocardium (1, 37, 52). However, it has remained controversial whether protection afforded by openers is mediated through suppression or increase in ROS generation (7, 27, 34, 41, 54). Such controversy may relate to the multifactorial nature of ROS generation in the cell, implicating several regulators of the cellular redox potential (20, 25, 35, 55). The present study demonstrates at the mitochondrial level that the protective effect of potassium channel openers involves reduction of ROS generation, which ultimately limits oxidative damage and maintains the integrity of the inner and outer mitochondrial membranes. This finding, along with previous studies (30, 34, 44, 54) performed at the cellular or whole heart level, suggests an “antioxidant” effect of potassium channel openers. Indeed, SOD-catalase, a ROS-neutralizing system, produced protection of mitochondria similar to that observed with potassium channel openers. Thus, in addition to the previously recognized effects of potassium channel openers on mitochondrial volume regulation and Ca\(^{2+}\) loading (13, 17, 19), the present study identifies that modulation of ROS generation contributes to opener-mediated mitochondrial protection.

Although SOD-catalase could scavenge ROS that cross mitochondrial membranes as well as ROS directly from generating sites in injury-permeabilized mitochondria (4, 26, 42, 55), the mechanism responsible for reduction of ROS generation by potassium channel openers remains unknown. The conventional site of action for this class of agents has been the plasma-membrane K\(_{ATP}\) channel responsible for setting membrane excitability in response to metabolic stress (2, 56). Although overexpression of recombinant K\(_{ATP}\) channel subunits promotes cytoprotection during metabolic stress (22, 23), in the heart regulation of plasmalemmal channel activity appears not to be a prerequisite for opener-mediated protection (8). Therefore, subcellular sites for potassium channel openers, including those in mitochondria, have been considered (13, 28). In fact, diazoxide was originally found to inhibit mitochondrial dehydrogenase activities and dehydrogenase-dependent flavoprotein reduction (45, 48). More recently, a nucleotide-regulated potassium conductance, the mitochondrial K\(_{ATP}\) channel, was identified as a target for potassium channel openers (8), although the intimate mechanism relating channel opening with mitochondrial protection remains unclear (12, 13, 27, 53). Here, the protective effect of potassium channel openers was antagonized by 5-HD, a short-chain fatty acid, known to counteract the action of openers on dehydrogenase-dependent flavoprotein reduction and mitochondrial K\(_{ATP}\) channel opening (28). In principle, regulation of the mitochondrial redox state and/or potassium membrane permeability may have contributed to opener-mediated suppression of ROS production. The effect of potassium channel openers was potassium independent and was sustained despite shunting the mitochondrial potassium conductance with valinomycin, a potassium ionophore. This would suggest that opener-mediated attenuation of oxidant stress in mitochondria might have occurred independently of mitochondrial K\(_{ATP}\) channels. Indeed, reduction in ROS production was here achieved with an inhibitor of succinate dehydrogenase, a respiratory chain enzyme previously shown to exhibit high sensitivity to diazoxide (45, 48). Like diazoxide, the succinate dehydrogenase inhibitor malonate is known to prevent reduction of flavoproteins and electron flow into the coenzyme Q cycle, a critical component in ROS generation (20, 33). Short-term treatment of metabolically active tissues with inhibitors of succinate dehydrogenase has been associated with cytoprotection (36). Thus a redox-based mechanism appears to contribute to reduction of ROS generation, and the protective action of potassium channel openers on mitochondria in the setting of anoxia-reoxygenation.

In summary, this study demonstrates that potassium channel openers protect cardiac mitochondria by attenuating oxidant stress at reoxygenation. Suppression of excessive ROS production induced by potassium channel openers preserved electron transport, maintained oxidative phosphorylation, and prevented cytochrome c release, indicating a generalized protection of the organelle from anoxia-reoxygenation injury. Opener-mediated mitochondrial protection was simulated by a free radical scavenging system. Although the effects of openers were antagonized by 5-HD, reduction of ROS production was sustained in the absence of potassium and mimicked by regulators of the mitochondrial redox state, suggesting the existence of a mitochondrial K\(_{ATP}\) channel-independent protection pathway. Thus suppression of mitochondrial ROS generation at reoxygenation provides a paradigm for improved cardiac bioenergetics and increased tolerance to oxidative stress.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-64822, the American Heart Association, Miami Heart Research Institute, the Bruce and Ruth Rappaport Program in Vascular Biology and Gene Delivery, and the Marriott Foundation. A. Terzic is an Established Investigator of the American Heart Association.
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