Original Article
Critical role of mitochondrial ROS is dependent on their site of production on the electron transport chain in ischemic heart

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Abstract: Reactive oxygen species (ROS) generation has been implicated in many pathologies including ischemia/reperfusion (I/R) injury. This led to multiple studies on antioxidant therapies to treat cardiovascular diseases but paradoxically, results have so far been mixed as ROS production can be beneficial as a signaling mechanism and in cardiac protection via preconditioning interventions. We investigated whether the differential impact of increased ROS in injury as well as in protection could be explained by their site of production on the mitochondrial electron transport chain. Using amplex red to measure ROS production, we found that mitochondria isolated from hearts after I/R produced more ROS than non-ischemic when complex I substrate (glutamate/malate) was used. Interestingly, the substrates of complex II (succinate) and ubiquinone (sn-glycerol 3-phosphate, G3P) produced less ROS in mitochondria from I/R hearts compared to normal healthy hearts. The inhibitors of complex I (rotenone) and *complex* III (antimycin A) increased ROS production when glutamate/malate and G3P were used; in contrast, they reduced ROS production when the complex II substrate was used. Mitochondrial calcium retention capacity required to induce mitochondrial permeability transition pore (mPTP) opening was measured using calcium green fluorescence and was found to be higher when mitochondria were treated with G3P and succinate compared to glutamate/malate. Furthermore, Langendorff hearts treated with glutamate/malate exhibited reduced cardiac functional recovery and increased myocardial infarct size compared to hearts treated with G3P. Thus, ROS production by the stimulated respiratory chain complexes I and III has opposite roles: cardio-deleterious when produced in complex I and cardio-protective when produced in complex III. The mechanism of these ROS involves the inhibition of the mPTP opening, a key event in cell death following ischemia/reperfusion injury.

Keywords: Reactive oxygen species, electron transport chain, ischemia, reperfusion, mitochondrial permeability transition pore

Introduction

Oxidative stress generated by dysfunctional mitochondria in ischemia/reperfusion (I/R) injury contributes to heart failure and other major cardiovascular diseases [1-3]. The generation of reactive oxygen species (ROS) and their by-products by the defective mitochondrial electron transport chain (ETC) activates cytotoxic mechanisms responsible for cell death via apoptosis and necrosis [1]. Cardiovascular research has for a long time been focused on antioxidant therapies like vitamin E and CoQ_{10} based on promising experiments but clinical trials were inconclusive at best, showed no improvement versus placebo, and were even harmful in some trials [2-6]. Speculation about why these trials failed includes questioning the doses used, disease progression at the time of administration, length of the trials as well as the antioxidants tested, but a more plausible argument has been the recognition that such therapies need to be more targeted towards mitochondria and the respiratory chain, which are the main sources of ROS in cardiac cells [2, 7]. From this focus, new treatments have been proposed such as MitoQ which has shown positive results in experiments but unfortunately again, the clinical data is not definitive [8, 9].

Adding to the confusing data, ROS are also known to be second-messenger molecules involved in growth factor and cytokine signal-
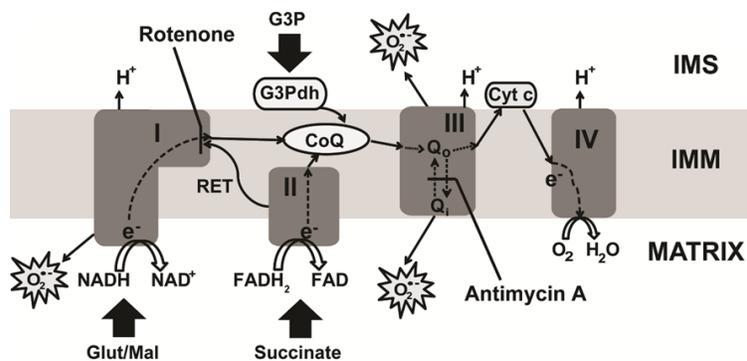
Figure 1. The electron transport chain within the inner mitochondrial membrane (IMM). Illustration showing electron (e⁻) transport chain from complex I to IV (dashed and solid lines) and highlighting different sites of reactive oxygen species (ROS) generation as well as indicating the sites of inhibitor action. Glutamate/malate (Glut/Mal) is issued as substrate of complex I; Succinate, as substrate of complex II; and sn-glycerol 3-phosphate (G3P) as, activator of the CoQ pool. G3Pdh: glycerol 3-phosphate dehydrogenase; RET: reverse electron transfer; Cyt c: cytochrome *c*; CoQ: ubiquinone; and IMS: intermembrane space.

and protected states. To this end, we proposed an extension to the quantity theory that narrows down the focus to the mitochondrial respiratory chain. We hypothesized that it is not only the *quantity* of ROS that determines the outcome, but it is the quantity *and* specific ETC *source* of that ROS that matters.

In this study, our focus was to determine whether ROS generated by mitochondrial ETC complexes I and III (**Figure 1**), the main sources of ROS, had differential impacts on the myocardium when sequentially stimulated. We provide evidence suggesting that ROS from complex III mediates cardio-protective effects of delaying the opening of the mitochondrial permeability transition pore (mPTP) in isolated mitochondria, as well as improving functional recovery and reducing infarct size in isolated hearts following I/R stress. Oppositely, our data suggests that ROS from complex I is damaging to mitochondria and whole-heart tissue as judged by the same measures of transition pore opening, functional recovery and infarct size after I/R.

Materials and methods

Animals

Male adult mice (C57BL/6J), 8-16 weeks old, were used. Protocols received approval from the UT Health Science Center at San Antonio Institutional Animal Care and Use Committee. The protocols conformed to the Guide for the Care and Use of Laboratory Animals: Eighth Edition published by the National Research Council. The investigation conformed to the *Guide for the Care and Use of Laboratory Animals,* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Langendorff preparation and heart perfusion

All materials used were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stat-

ing, initiation of mitogen-activated protein kinase (MAPK) pathways and communication vital for cell adhesion [10]. In particular, ROS are known to be upregulated in ischemic and pharmacological preconditioning studies to protect the myocardium against I/R injury by activating pathways involving p38 MAPK, MAPKAP kinase 2 and NFκB [11-14]. Paradoxically, ROS inhibition has been shown to induce cardioprotection against I/R injury in post-conditioning studies [15, 16], but ROS upregulation has also been observed as being necessary to activate mechanisms for limiting reperfusion injury [17]. In all, there still has yet to be proposed a unified theory that satisfactorily explains both the cardio-beneficial and cardio-deleterious effects of ROS in I/R.

As many studies have shown the opposing ROS impacts on cardiac tissue, it has become common to attribute the differences in outcomes of protection or damage to the *quantity* of ROS produced by mitochondria. In this theory, low quantities of ROS are responsible for the cell survival mechanisms while excess amounts lead to cell and tissue death. Appealing as this may be, it fails to account for the fact that an increase in ROS is observed in both protection and damage, thus, despite low/physiological quantities of ROS being generated naturally for signaling purposes, abnormal quantities are needed in *both* diseased

ed. Mice were anesthetized by intraperitoneal injection of pentobarbital (60 mg/kg), and heparin (200 UI/kg) was used to prevent blood coagulation. Hearts were surgically removed and immediately arrested in cold (4°C) Krebs Henseleit bicarbonate buffer (KH) solution (mM): glucose 11, NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and $CaCl_2$ 3, pH 7.4. The aorta was rapidly cannulated and the heart was retrograde-perfused at a constant rate (3 ml/min) in the Langendorff mode using KH buffer bubbled with 95% O_2/5% CO_2 at 37°C. After 30 minutes of equilibration, global normothermic ischemia was induced by stopping KH buffer supply to the aorta for 30 minutes followed by 20 minutes of reperfusion for mitochondrial isolation. For myocardial infarct size imaging studies, hearts were reperfused for 120 minutes to allow for the development of I/R damage. We preferentially considered good hearts the ones that reached a minimum left ventricular developed pressure (LVDP) of 80 mmHg at the end of the basal perfusion (before ischemia). In the mouse model, this I/R protocol typically results in ~50% of infarct size. Sham hearts were not subjected to I/R but only were perfused for the same duration as the I/R protocol. To determine the role of the stimulation of different ETC complexes in myocardial infarction and cardiac functional recovery, hearts were perfused with the KH buffer supplemented with 3 mM of each of the substrates, glutamate/malate, succinate or sn-glycerol 3-phosphate.

Functional measurements

The heart function was recorded throughout the experiments using a catheter (1.4F SPR-671; Millar Instruments, Colorado Springs, CO) connected to a MAC LAB (Powerlab) acquisition system from ADInstruments (Sydney, Australia) as previously described in our articles [18, 19]. The catheter was directly inserted into the left ventricle (LV) after a left atrial incision was made to expose the mitral annulus; the LV end-systolic pressure (LVSP), the LV end-diastolic pressure (LVEDP), and heart rate (HR) were directly obtained from LabChart5.5 (ADInstruments) software as described in our article [20]. The LV developed pressure (LVDP = LVSP - LVEDP) and Rate-Pressure Product (RPP = LVDP × HR) were calculated from the recordings.

Myocardial infarct size

The heart was removed from the Langendorff apparatus at the end of the reperfusion period and cut into four transverse slices parallel to the atrio-ventricular groove as previously described [18]. Slices were incubated for 7 minutes in 2% triphenyltetrazolium chloride (TTC) at 37°C followed by fixation with 4% paraformaldehyde. This staining differentiated the infarcted (pale) from viable (red) myocardial tissue. The slices were photographed using digital microscopic imaging (Moticam 5 MP camera). The area of necrosis was quantified by computerized planimetry with Adobe Photoshop CS6, and the total area of necrosis was calculated and expressed as a percentage of the total heart area.

Preparation of isolated mitochondria

Mitochondria were isolated from fresh, ischemic (20-minute reperfusion) and non-ischemic (sham) hearts at 4°C as previously described in [20]. Myocardial sections (approximately 0.15-0.22 g) were placed in isolation buffer A (mM): sucrose 70, mannitol 210, EDTA 1 and Tris-HCl 50, pH 7.4. The tissue was finely minced and homogenized in the same Buffer A (0.1 g of tissue/ml of buffer). The homogenate was centrifuged at 3,000 rpm for 3 minutes in a Galaxy 20R centrifuge (VWR, Radnor, PA); the supernatant was centrifuged at 13,000 rpm for 10 minutes. The mitochondrial pellet was resuspended in isolation Buffer B (mM): sucrose 150, KCl 50, KH_2PO_4 2, succinic acid 5 and Tris/HCl 20, pH 7.4). Additional isolation buffers C and D were prepared with 5 mM glutamate/malate and 5 mM sn-glyecerol 3-phosphate respectively, instead of 5 mM succinate as used in Buffer B. Protein concentration was estimated using the Bradford method assay kit (Bio-Rad, Hercules, CA).

Mitochondrial H_2O_2 measurement

Mitochondrial ROS generation was measured spectrofluorometrically (560 nm excitation and 590 nm emission) in 100 μg mitochondrial protein incubated in a solution containing: 20 mM Tris, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, and 0.15% bovine serum albumin adjusted to pH 7.4 at 30°C with continuous stirring. Superoxide generated in mitochondria has a short half-life and does not diffuse easily across
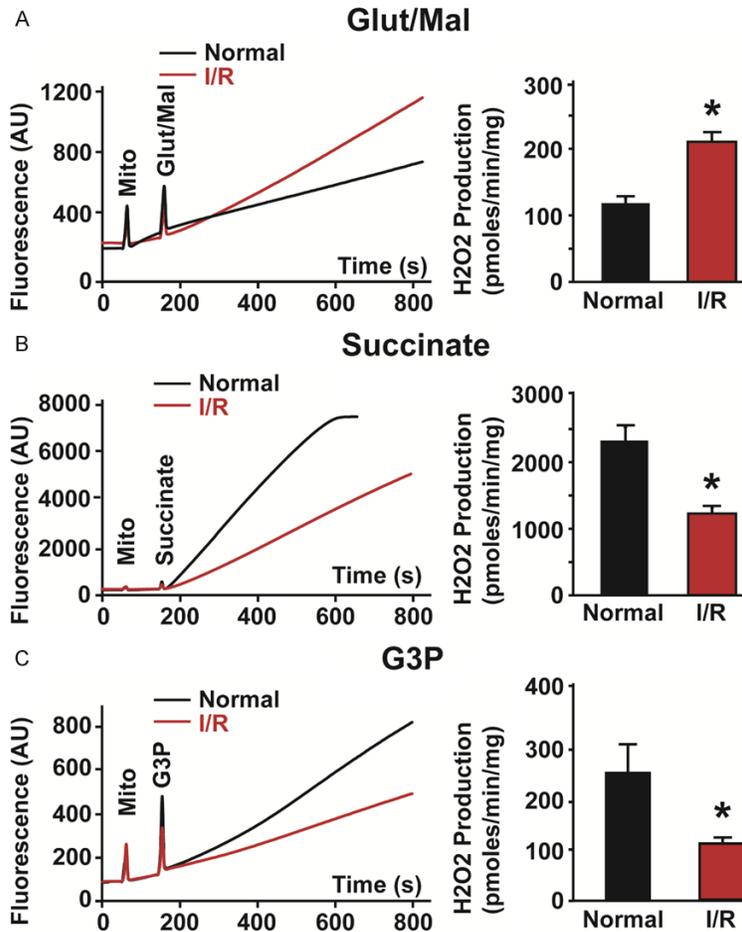
Figure 2. ROS production from activated ETC complexes measured as H_2O_2 release in normal mitochondria and mitochondria following ischemia/reperfusion (I/R) injury. Typical measurements of H_2O_2 release from normal (black trace) and I/R (red trace) mitochondria when complex I (3 mM glutamate/malate, Glut/Mal) (A), complex II (3 mM succinate) (B) and CoQ (3 mM sn-glycerol 3-phosphate, G3P) (C) were stimulated in the presence of amplex red and horseradish peroxidase (Left). Graphs showing how the rate of H_2O_2 production measured for a 60 second period after addition of substrate increased in I/R mitochondria after Glut/Mal was added but decreased after succinate and G3P were added (Right). Values are mean ± SEM; *p < 0.05 normal *versus* I/R mitochondria, n = 5-6/group.

vate complexes I and II, as well as the coenzyme Q pool, respectively. Complex I inhibitor, rotenone (2 µM) and complex III inhibitor, antimycin A from Streptomyces sp. (20 µM) were also used.

Ca^{2+}-induced mitochondrial permeability transition

The installation of mitochondrial permeability transition pores (mPTP) was assessed following in vitro Ca^{2+} overload as previously described [23, 24]. Free Ca^{2+} concentration outside the mitochondria was recorded using 0.1 µM calcium green-5N (Thermo Fisher) which binds reversibly to Ca^{2+}, using excitation and emission wavelengths set at 500 and 530 nm, respectively. Isolated mitochondria (500 µg of protein) were suspended in 2 ml isolation Buffer B, C or D and pre-incubated for 90 seconds in a spectrofluorometer (Hitachi F-2710) set at 30°C. $CaCl_2$ pulses (10 µmoles or 10 µL of 1 mM stock solution) were applied every 60 seconds to the cuvette leading to 20 nmol Ca^{2+} (per mg of mitochondrial protein). The Ca^{2+} pulses induce a peak of extra-mitochondrial Ca^{2+} concentration that returns to near-baseline levels as Ca^{2+} enters the mitochondrial matrix via uptake by the Ca^{2+} uniporter. With increasing calcium loading, the extra-mitochondrial Ca^{2+} concentration starts accumulating, thereby reflecting a lower capacity for mitochondrial Ca^{2+} uptake. This is followed by a sustained Ca^{2+} increase, indicating a massive release of the mitochondria Ca^{2+} by the mPTP opening. The Ca^{2+} retention capacity (CRC) was defined as the amount of Ca^{2+} required to trigger this massive Ca^{2+} release which is used here as an indicator of the mPTP sensitivity to Ca^{2+}. CRC is expressed as nmol of $CaCl_2$ per mg of mitochondrial protein.

the membranes, but the H_2O_2 derived from dismutation easily diffuses through the membranes [21, 22]. As a result, generation of ROS can be monitored as a function of H_2O_2 levels. Hydrogen peroxide was measured with the H_2O_2-sensitive dye amplex red reagent (10 µM) (Thermo Fisher, Waltham, MA) in the presence of horseradish peroxidase (0.345 U/mL) and H_2O_2 levels were calculated from a calibration curve obtained from fluorescence emission intensity as a function of H_2O_2 concentration. The sodium salts of glutamate/malate (3 mM), succinate (3 mM) and *sn*-glycerol 3-phosphate (G3P) (3 mM) were used to acti-

Figure 3. ROS production from activated ETC complexes measured as H_2O_2 release in normal mitochondria after insult with pro-apoptotic drugs. Typical measurements of H_2O_2 release in healthy mitochondria from control (black trace), with complex I inhibitor, rotenone (Rot, orange trace) and with complex III inhibitor, antimycin A (AA, purple trace) when complex I (3 mM glutamate/malate, Glut/Mal) (A), complex II (3 mM succinate) (B) and CoQ (3 mM *sn*-glycerol 3-phosphate, G3P) (C) were stimulated in the presence of amplex red and horseradish peroxidase (Left). Bar graphs showing changes in the rate of H_2O_2 production measured for a 60 second period after addition of each inhibitor (Right). Values are mean ± SEM; *$p < 0.05$ control *versus*+inhibitor, n = 5-6/group.

Statistical analysis

The data shown in bar graphs are expressed as means with error bars that are the standard errors of the mean (± SEM) for a minimum of five independent hearts (n ≥ 5). The Student's t-test and one-way ANOVA with post-

hoc Dunnett's, Tukey's and Sidak's corrections for multiple comparisons were appropriately used to assess the differences observed. Analysis was performed using Prism 6 (Graphpad Software, La Jolla, CA). A difference of $p < 0.05$ was considered to be statistically significant.

Results

Following I/R, stimulation of complex I increases ROS production but stimulation of complex II and CoQ decreases it

We first sought to ascertain the production of ROS by stimulated complexes in isolated mitochondria from both sham and ischemic myocardium using commonly used [25-27] *de novo* ETC substrates, glutamate/malate (complex I) and succinate (complex II), as well as CoQ substrate, G3P. In line with past studies, following ischemic injury, ROS production increases in complex I [28]. Activating complex I (glutamate/malate) resulted in an increase in ROS production in mitochondria isolated from I/R hearts, as compared to mitochondria isolated from non-ischemic hearts (**Figure 2A**). Surprisingly, treatment of I/R mitochondria with the complex II substrate, succinate or the Q-pool substrate, G3P resulted in reduced ROS production rate in I/R mitochondria when compared to non-ischemic tissue (**Figure 2B**, **2C**). These results show opposite ROS production when complex I is activated, compared to when complex II and CoQ are activated in mitochondria isolated from ischemic hearts and in normal mitochondria. We also noted the marked difference in rates when each complex is stimulated, with succinate/complex II stimulation resulting in
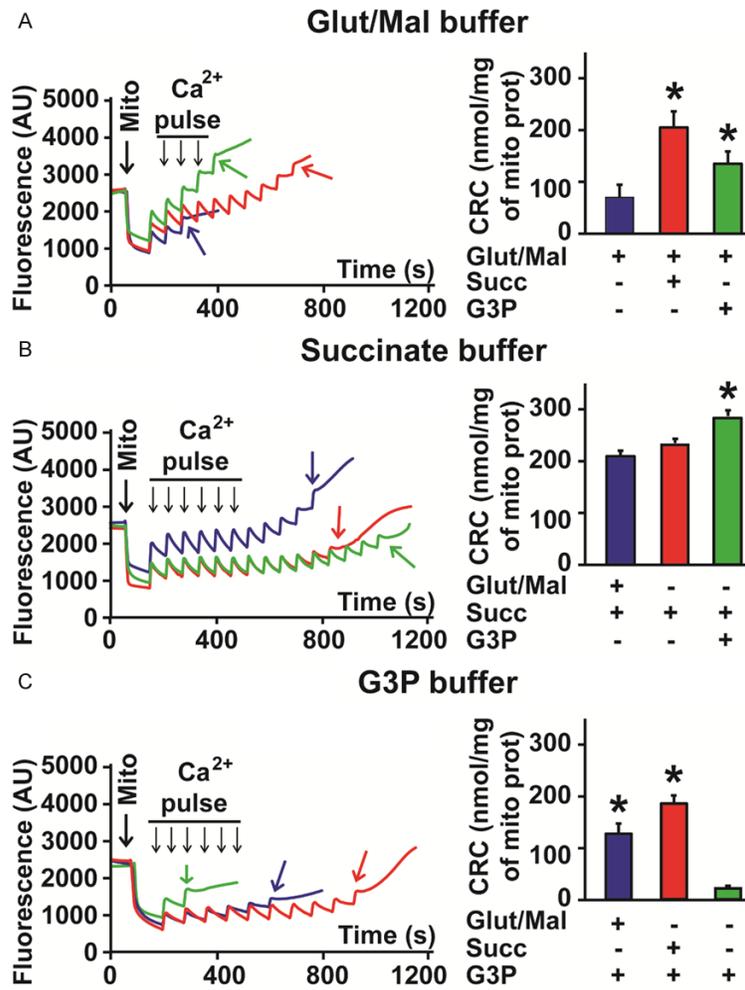
Figure 4. Mitochondrial Ca^{2+} induced opening of the mPTP for buffers of each substrate. A. Left: Example of recordings showing the calcium overload required to induce the mPTP opening (colored arrows) of isolated mitochondria re-suspended in a buffer containing 5 mM glutamate/malate (Glut/Mal) as substrate (blue trace) and after supplementation with 3 mM succinate (Succ, red trace) and 3 mM *sn*-glycerol 3-phosphate (G3P, green trace). Right: Bar graph showing improved calcium retention capacity (CRC) when Glut/Mal is supplemented with other substrates. Values are mean ± SEM; *p < 0.05 supplements *versus* control (Glut/Mal), n = 6/group. B. Left: Example of recordings showing the calcium overload required to induce the mPTP opening (colored arrows) of isolated mitochondria re-suspended in a buffer containing 5 mM succinate (Succ) as substrate (red trace) and after supplementation with 3 mM glutamate/malate (Glut/Mal, blue trace) and 3 mM *sn*-glycerol 3-phosphate (G3P, green trace). Right: Bar graph showing calcium retention capacity (CRC) when succinate is supplemented with other substrates. Values are mean ± SEM; *p < 0.05 supplements *versus* control (Succ), n = 6/group. C. Left: Example of recordings showing the calcium overload required to induce the mPTP opening (colored arrows) of isolated mitochondria re-suspended in a buffer containing 5 mM *sn*-glycerol 3-phosphate (G3P) as substrate (green trace) and after supplementation with 3 mM succinate (Succ, red trace) and 3 mM glutamate/malate (Glut/Mal, blue trace). Right: Bar graph showing improved calcium retention capacity (CRC) when G3P is supplemented with other substrates. Values are mean ± SEM; *p < 0.05 supplements *versus* control (G3P), n = 6/group.

Pro-apoptotic drugs increase ROS production when complex I and the Q pool are stimulated, but reduce ROS production when complex II is stimulated in healthy mitochondria

Next, we studied if pro-apoptotic drugs resulted in similar responses to complex stimulation. As shown in **Figure 3**, addition of complex I blocker, rotenone showed an increase in ROS generation by activated complex I and a modest increase on the activated Q cycle. The addition of antimycin A, which blocks the Q_o site of complex III led to dramatic increase in ROS generation when both complex I and CoQ were activated (**Figure 3A, 3C**). However, when complex II was stimulated, the addition of both inhibitors resulted in reduction of ROS generation. In fact, in the presence of rotenone, a complex I blocker and hence a blocker of reverse electron transfer (RET), ROS rate slowed down the most (succinate+rotenone, 1010 ± 139 *versus* succinate, 2274 ± 225 pmoles/min/mg, n = 5, p = 0.001) when compared to antimycin A (succinate+antimycin A, 1538 ± 88 pmoles/min/mg), suggesting that the electron transfer from the stimulated complex II to complex I (RET) is a larger source of ROS production than the forward transfer to complex III. Together, these findings indicate that pro-apoptosis ETC inhibitors induce opposite ROS production when complex I or II is stimulated. These results also show that these pro-apoptotic drugs that affect the ETC [29] have similar effects on ROS pro-

tremendously more ROS production than complex I and CoQ.

duction to those observed following I/R injury (**Figure 2**), and therefore, can be used to
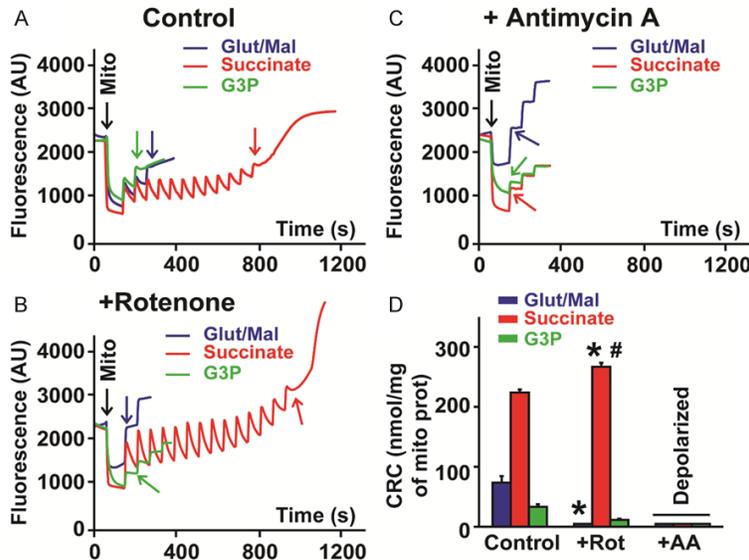
Figure 5. Mitochondrial Ca^{2+} induced opening of the mPTP for buffers of each substrate with pro-apoptotic inhibitors. A. Typical recording showing the calcium overload required to induce the mPTP opening (colored arrows) of isolated mitochondria re-suspended in a buffer containing 5 mM glutamate/malate (Glut/Mal, blue trace), 5 mM succinate (Succ, red trace) and 5 mM sn-glycerol 3-phosphate (G3P, green trace) as substrate (control). B. Typical recording showing the calcium overload required to induce the mPTP opening (colored arrows) of isolated mitochondria re-suspended in a buffer containing 5 mM glutamate/malate (Glut/Mal, blue trace), 5 mM succinate (Succ, red trace) and 5 mM sn-glycerol 3-phosphate (G3P, green trace) and treated with rotenone (Rot, 2 µM). C. Typical recording showing the calcium overload required to induce the mPTP opening (colored arrows) of isolated mitochondria re-suspended in a buffer containing 5 mM glutamate/malate (Glut/Mal, blue trace), 5 mM succinate (Succ, red trace) and 5 mM sn-glycerol 3-phosphate (G3P, green trace) and treated with antimycin A (AA, 20 µM). D. Bar graphs showing mitochondrial calcium retention capacity (CRC) measured of mitochondria re-suspended in a buffer containing each substrate and supplemented with either rotenone or antimycin A. Values are mean ± SEM; *p < 0.05 inhibitor treated *versus* respective control (same substrate), #p < 0.05 Succ+Rot *versus* other substrates+Rot, n = 6/group.

pared the sensitivity of mPTP opening as a function of calcium retention capacity in isolated fresh mitochondria treated with each complex's substrate to stimulate ROS production. **Figure 4** shows the time course of addition of free Ca^{2+} pulses in the solution surrounding mitochondria. The initial Ca^{2+} concentration in the buffer was set to Ca^{2+} contaminant that declined after adding mitochondria due to a global reduction of the solution's optical transmittance as a result of the increase in turbidity after loading mitochondria. The following slower phase prior to the addition of the Ca^{2+} pulses reflects the rate of initial Ca^{2+} uptake by the mitochondria.

In isolated mitochondria, using buffers of *de novo* ETC substrates, glutamate/malate and succinate, we found that the control mitochondrial calcium retention capacity (CRC) measurements were 73 ± 11 and 240 ± 5 nmol/mg of mitochondrial protein (n = 6), respectively. These results indicate that application of glutamate/malate, the substrate of complex I, to mitochondria increases the sensitivity of the mPTP opening to calcium overload when compared to succinate, the substrate of complex II. We also found that when glutamate/malate was used to prepare the isolation buffer, addition of either succinate (3 mM) or the ETC intermediate, G3P (3 mM) rescued the mitochondrial CRC, with succinate showing a greater recovery (glutamate/malate, 73 ± 11 *versus* glutamate/malate+succinate, 183 ± 24 nmol/mg of mitochondrial protein, n = 6, p = 0.002). This recovery was greater than that for G3P (glutamate/malate+succinate, 183 ± 24 *versus* glutamate/malate+G3P, 150 ± 20 nmol/mg of mitochondrial protein, n = 6) (**Figure 4A**). On the other hand, when succinate, the substrate of complex II, was used to

approximate I/R effects when the different complexes are being studied.

Activation of complex II and the Q-pool result in increased mitochondrial calcium retention capacity while activation of complex I shows a decrease in capacity

We then asked what impact the increases in ROS from each complex would have on the opening of the mPTP, a pivotal event known to cause cell death in I/R injury [30] and who's inhibition was recently shown by our group to be necessary in cardioprotection against I/R injury via the G-protein coupled estrogen receptor 1 (GPER1) [31]. As the mPTP opening may be induced by calcium overload [32], we com-

Figure 6. Measure of cardiac functional recovery in control and after addition of substrates after I/R. Cardiac function was recorded throughout the experiments in isolated perfused hearts treated with KH buffer for control and after addition of different substrates (3 mM). A. Bar graph showing an improvement of the cardiac functional recovery (Rate-Pressure Product, RPP) at 120 min reperfusion (end of reperfusion) in *sn*-glycerol 3-phosphate (G3P) group compared to control (Ctrl) and glutamate/malate (Glut/Mal) groups. B. Bar graph indicating that the left ventricular developed pressure (LVDP) at 120 min reperfusion did not change in control or the supplements of each substrate. C. Bar graph showing an improvement of the heart rate (HR) at 120 min reperfusion (end of reperfusion) in *sn*-glycerol 3-phosphate (G3P) group compared to control and glutamate/malate (Glut/Mal) groups. Values are mean ± SEM; *p < 0.05 substrate *versus* control; #p < 0.05 G3P *versus* Glut/Mal, n = 5-6/group.

prepare the isolation buffer, addition of glutamate/malate (3 mM), slightly decreased the CRC (succinate, 240 ± 5 *versus* succinate+glutamate/malate, 217 ± 11 nmol/mg of mitochondrial protein, n = 6, not significant), while the addition of G3P (3 mM) led to a statistically significant increase in CRC (succinate, 240 ± 5 nmol/mg of mitochondrial protein *versus* suc-

cinate+G3P, 293 ± 8 nmol/mg of mitochondrial protein, n = 6, p = 0.001) (**Figure 4B**). Use of the less traditional substrate, G3P as buffer (5 mM) resulted in poor CRC (G3P, 33 ± 4 nmol/mg of mitochondrial protein), which was only aided by addition of 3 mM of the more commonly used ETC substrates glutamate/malate (G3P+glutamate/malate, 120 ± 20 nmol/mg of mitochondrial protein, n = 6, p = 0.002 *versus* G3P control) and succinate (G3P+succinate, 193 ± 16 nmol/mg of mitochondrial protein, n = 6, p < 0.0001 *versus* G3P control) (**Figure 4C**).

Furthermore, we compared effects of ETC inhibitors rotenone and antimycin A when glutamate/malate, succinate or G3P were used as ROS substrate for mitochondrial CRC. As shown in **Figure 5**, addition of antimycin A resulted in mitochondrial depolarization when all three substrates were used. In fact, we found that addition of antimycin A to the cuvette containing pre-incubated mitochondria immediately led to the opening of the mPTP. Interestingly, when complex I (glutamate/malate buffer) was activated, addition of rotenone led to similar mitochondrial depolarization with immediate mPTP opening even before addition of Ca^{2+} pulses commenced (n = 6, p < 0.001). When CoQ (G3P buffer) was activated, addition of rotenone did not show any change in CRC (G3P+rotenone, 27 ± 4 *versus* G3P, 33 ± 4 nmol/mg of mitochondrial protein, n = 5-6, not significant). Interestingly, when complex II (succinate buffer) was stimulated, addition of rotenone remarkably enhanced CRC (succinate+rotenone, 267 ± 7 *versus* succinate, 240 ± 5 nmol/mg of mitochondrial protein, n = 6, p = 0.02) suggesting a delay of the mPTP opening. Together, these results indicate that the ROS production in complex I promotes the mPTP opening, while ROS produced in complex III induces delay of the mPTP opening and presumably may lead to cardioprotection.

Activation of CoQ in contrast to complexes I and II improved cardiac functional recovery and reduced myocardial infarct size following I/R

Finally, we asked whether resultant differences in ROS produced by each complex could be observed in an *ex vivo* animal model and possibly highlight clinically relevant effects. Using Langendorff retrograde perfusion, we studied myocardial infarct size and cardiac functional
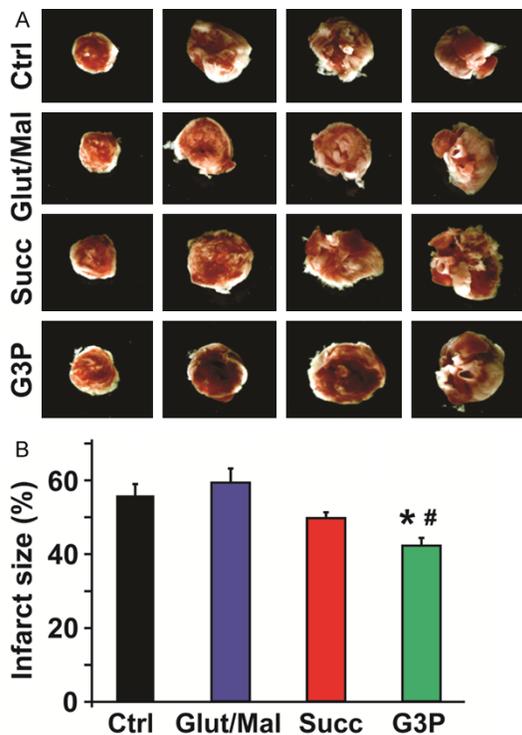
Figure 7. Measure of myocardial infarct size in control and after supplement of substrates after I/R. Myocardial infarct size was measured at the end of 120 min reperfusion in isolated perfused hearts treated with KH buffer for control and after addition of different substrates. A. Representative images from 4 slices of the same heart in each condition. White areas correspond to the infarcted zone. B. Bar graphs showing reduction of myocardial infarct size in *sn*-glycerol 3-phosphate (G3P) group compared to control (Ctrl) and glutamate/malate (Glut/Mal) groups. Values are mean ± SEM of % infarct size; *p < 0.05 substrate treated *versus* control; #p < 0.05 G3P *versus* Glut/Mal, n = 6-7/group.

recovery following I/R stress, with reduced infarct size and higher rate-pressure product (RPP) indicating cardioprotection. We compared these parameters in isolated hearts perfused with KH buffer that was enriched with each of the three substrates to stimulate ROS production. With regards to the heart regaining cardiac function following I/R, the RPP values obtained at 120 minutes of reperfusion, as a percent of RPP before ischemia, were in the order: G3P (72 ± 9%), succinate (43 ± 7%), and glutamate/malate (36 ± 1%); compared to control (36 ± 4%) (n = 5-7) (**Figure 6A**). As shown in **Figure 6B**, **6C**, the resultant RPP at the end of reperfusion was mostly driven by changes in heart rate, as the LVDP readings were fairly similar across all conditions. Incitement of ROS

production at complex III by activating the Q-pool with G3P resulted in smaller infarct sizes (G3P, 42 ± 2%) as compared to the control (56 ± 4%, n = 5-7, p = 0.01). However, activating complex I with glutamate/malate increased the infarct size from that exhibited by controls (glutamate/malate, 59 ± 4% *versus* control, 56 ± 4%, n = 5-7, not significant) (**Figure 7A**, **7B**). Comparing G3P (complex III ROS) to glutamate/malate (complex I ROS) showed statistically significant benefits of G3P (**Figures 6**, **7**; Infarct size: p = 0.001 and RPP: p = 0.04; n = 5-7). These results suggest that complex III ROS induce cardioprotective effects against ischemia reperfusion injury, while complex I ROS appear to promote myocardial injury.

Discussion

In this study, we demonstrated that not only is the quantity of ROS important, but the quantity and mitochondrial ETC source of that ROS in mitochondria is pertinent to its impact as either cardio-protective or cardio-deleterious in a heart I/R model. Specifically, we showed that ROS from complex I results in a reduction of mitochondria's ability to sequester Ca^{2+} ions in healthy mitochondria, greater myocardial infarct size, and less functional recovery in the whole heart following I/R injury. Conversely, we revealed how ROS from complex III leads to an improvement in mitochondrial Ca^{2+} buffering, as highlighted by the delay in the mPTP opening, and reduces the overall heart infarct size while improving recovery after I/R. Our results show that stimulating complex III either from complex II or Q-pool substrates leads to ROS production that is cardio-beneficial, as opposed to that from stimulating complex I, which is detrimental to both mitochondria and the stressed heart as a whole.

ROS production at complexes I and III

The increase in production of mitochondrial ROS has been demonstrated using cell labeling methods as being a relevant process in many pathological conditions like heart attack, diabetes and cancer [33-35], and it also participates in necrosis and apoptosis associated with cardiac I/R injury (reviewed in detail in [36, 37]). Complex I is a major source of electron leakage which results in ROS production in the ETC. Previous studies have debated the exact site of ROS generation on complex I [36, 38],

but the ubiquinone-binding domain appears to be main site during succinate-driven reverse electron transfer (RET) from complex II [39] and this also happens to be the same site at which rotenone blocks transfer of electrons to CoQ [40]. Following ischemic damage, the iron sulfur clusters proximal to this ubiquinone binding site are thought to be the locations for ROS production which result in the increase in ROS that we also observed [38] (**Figure 2A**). ROS production from complex I is more dependent on ΔpH than ΔΨ (membrane potential) [39, 41] and thus less reliant on mitochondrial energization. Thus, I/R injury and rotenone increase ROS generation from the same region on complex I when this complex is activated by blocking electron escape, as we observed (**Figures 2A** and **3A**).

Complex II influences ROS production at complex I (via RET) [28, 29] and complex III [36] and although some evidence has suggested complex II to be a source of ROS under certain conditions [42], it is only complexes I and III that are globally accepted as main generators. Following I/R insult or application of rotenone to complex I, we observed a decrease in succinate-driven ROS (**Figures 2B** and **3B**) which are in line with a loss of RET to complex I. For complex II, our findings further highlight its dependence on its neighboring complexes for ROS production. From this, it may be inferred that following I/R injury, activated complex II no longer influences ROS production at complexes I or III, hence the observed decrease (**Figure 2**); while rotenone and antimycin A also lead to diminished ROS formation as complex II's access to the adjacent enzymatic units is diminished (**Figure 3**).

Complex III has two known sites of ROS production, with one releasing ROS into the inter-membrane space and the other into the matrix [43]. Complex III works by transferring electrons from ubiquinol to ferricytochrome c by utilizing hemes b_L and b_H, and the Rieske iron-sulfur protein [36]. The contribution of complex III to ROS production in normal physiology remains uncertain with some groups suggesting it to be smaller [44, 45] while others postulate it to be larger [38, 46, 47] than complex I involvement. However, there is consensus on complex III being a major site of ROS production after I/R [36, 38]. Ischemic damage reduced ROS production from G3P stimulation (**Figure 2C**) contrary to the expectation of an increase. I/R disrupts complex III function [48] and G3P/CoQ stimulation probably would have been impaired from affecting ROS from this complex. Accordingly, downstream application of antimycin A, which blocks electron transfer from the Q_0 site of complex III increased ROS production when complex I and complex III were stimulated (**Figure 3**). And despite complex III ROS relying more on ΔΨ than ΔpH [49], application of the transfer blocker, antimycin A results in ROS production. As succinate influences ROS at *both* complexes I and III, we opted to include G3P in order to gain further insight into ROS production from complex III. G3P, also known as L-α-glycerophosphate is thought to be an inadequate substrate by itself for studying H_2O_2 production in heart mitochondria [50] but when coupled with glutamate or succinate can augment ROS production at complex III. Mracek et al. have shown that glycerophosphate dehydrogenase (G3Pdh in **Figure 1**) is a source of ROS particularly in brown adipose tissue but that in heart mitochondria, stimulating the dehydrogenase with G3P results in the bulk of ROS being made at complex III [51]; hence, we opted to use it in our studies to influence ROS generation at complex III.

Opposing effects of ROS from complex I versus complex III

Kalogeris et al. summed up current opinion that the "double-edged sword" impact of ROS prompted by I/R may be traced back to i) the type of ROS produced ii) the amount and time when the ROS is produced and iii) the cellular and subcellular loci of creation [52]. First, reactive species known to cause damage to cells and tissue include the superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($^\bullet OH$), hydrogen peroxide (H_2O_2), and peroxynitrite ($ONOO^-$) [53]. In cardiovascular tissue, the most important and abundant form of ROS is $O_2^{\bullet-}$ [54] and within this tissue, it is known that mitochondria, and specifically the respiratory chain are the dominant sources of $O_2^{\bullet-}$ [2, 7, 36]. With regards to the heart, I/R damage results in increased $O_2^{\bullet-}$ production by both subsarcolemmal and interfibrillary mitochondria [55, 56], which were the focus of our study.

Second, the amount of ROS and when it is produced are critical to the effect it has. The amount of ROS following I/R overwhelms the

hitherto, tightly-regulating antioxidant machinery of the cell and the ROS can thus attack DNA, proteins and subcellular structures [52]. In our experiments, we used the ETC substrates glutamate/malate, succinate and G3P, at non-physiological concentrations of 3 mM to instigate this increase in ROS. As evidenced by the increase in ROS in pre- and postconditioning, the time when ROS increase commences is also vital [11-14, 17]. During ischemia, cells are starved of both nutrients and oxygen and when reperfusion begins, it is the accumulation of Ca^{2+} in the matrix, depletion of adenine nucleotide, increased inorganic phosphate concentration, and oxidative stress that leads to the opening of mPTP [32] whose effects include depolarizing mitochondria, uncoupling the ETC, allowing for efflux of Ca^{2+} and intermembrane proteins such as the pro-apoptotic cytochrome *c*, and causing swelling of the mitochondrial matrix [57].

Thirdly, the cellular/subcellular location of ROS generation is pertinent to the outcomes of I/R. This idea of compartmentalization for the control of ROS is both logical and supported by a large body of evidence (reviewed in [58-60]). In normal physiology, the cell highly regulates the amount of ROS, particularly through superoxide dismutase (SOD) which degrades ROS to H_2O_2, which can be further degraded to water and molecular oxygen. The mitochondrial matrix contains manganese-SOD (Mn-SOD or SOD-2) while the cytoplasm and the intermembrane space in mitochondria contain copper/zinc-SOD (Cu/Zn-SOD or SOD-1) [52, 61]. Insults like I/R ignite overwhelming quantities of ROS that overpower these safeguards and leads to senescence, autophagy and apoptotic pathways associated with cardiovascular diseases [1].

In the present study, we observed that not only is it the subcellular compartment key to ROS impact, but the mitochondrial ETC complex generating that ROS is crucial as well. By studying isolated mitochondria's ability to sequester calcium before opening the mPTP, we found that the impact of ROS produced by activated complexes I and III on the opening of the mPTP is contradictory (**Figure 4**). The mPTP opening is known to be associated with an increase in ROS and its opening is a trademark event of mitophagy following I/R injury [20]. Our data indicate that the treatment of fresh and healthy

mitochondria with succinate buffer (5 mM) (for complex II) delays the opening of the mPTP as the calcium retention capacity (CRC) increases, while the stimulation of complex I (glutamate/malate buffer, 5 mM) actually reduces mitochondrial CRC (**Figure 4A**, **4B**). For each of these substrates, addition of the other substrate either aids or impedes the CRC effect. As shown in **Figure 4**, when glutamate/malate buffer (5 mM) is supplemented with either succinate or G3P (3 mM), this serves to improve the mitochondria's CRC; on the contrary, addition of glutamate/malate (3 mM) to succinate buffer (5 mM) solution slightly reduces CRC and, as can be deduced from our hypothesis, addition of 3 mM G3P (for complex III) to succinate buffer (5 mM) increases CRC. As discussed earlier, G3P by itself is a poor cardiac ROS substrate, and this was also shown by its low CRC values when it was used as substrate buffer (5 mM) (**Figure 4C**); however, it was improved significantly in the presence of the natural substrates, glutamate/malate (3 mM) and succinate (3 mM).

Following our hypothesis that the ETC source of ROS is critical in cardioprotection, we studied the CRC effects of increased ROS production when complexes I and III were sequentially blocked by use of rotenone and antimycin A as blockers. Rotenone inhibits oxidation of the iron-sulfur clusters in complex I while also blocking RET from complex II [36, 62] (**Figure 1**). Antimycin A blocks the Q_i site of complex III resulting in increased ROS production at the complex's Q_0 site, with this ROS being expelled into the intermembrane space from the Q_0 site [43, 51, 63, 64]. Application of antimycin A, a drug with no biological equivalent, to mitochondria results in the collapse of membrane potential, opening of the mPTP [62, 65] and hence, the inability to absorb Ca^{2+} as we also found (**Figure 5**). Furthermore, addition of rotenone to activated complex I, which increases ROS at this site also depleted mitochondrial CRC when using glutamate/malate; but rotenone's blocking of RET improved CRC when complex II was activated probably due to more "good" ROS coming from complex III. We found that limiting ROS from resting state complex I by applying rotenone and stimulating complex II using succinate, served to enhance the cardio-positive effects of the ROS from complex III by increasing CRC (**Figure 5D**). The fact that the stimula-

tion of the complexes II and III (while inhibiting influence of complex I) increases the mitochondrial CRC indicate that ROS produced under these conditions are cardioprotective since it is well established that the inhibition of mPTP opening protects the heart during pre- and post-conditioning, and controlled reperfusion [37].

The differential effects of ROS from complexes I and III in isolated mitochondria suggested opposite roles and we sought to determine if these roles extended to intact myocardium. For this, we compared cardiac functional recovery as well as myocardial infarct size in isolated hearts perfused with KH buffer versus KH with supplementation of 3 mM of each substrate. Although our previous experiments had been in isolated mitochondria with substrates easily accessing the inner membrane of mitochondria, all three substrates are found in the cytosol and can be transported across cell membranes from exogenous sources [66-69].

The rate-pressure product (RPP), or cardiovascular product, used in hemodynamic stress studies in cardiac physiology, is used to predict myocardial oxygen consumption, or quite simply, myocardial workload [70]. As the product of heart rate and systolic blood pressure, RPP can be used to monitor the functional impact of I/R injury on the heart [18, 71, 72] and in our studies, the RPP was used to determine the level of functional recovery following I/R. In this study, we found that G3P showed the greatest recovery with glutamate/malate showing the least, further evidence for the opposing complexes' ROS. The left ventricular developed (LVDP) was similar in all groups and the improvement of the RPP with G3P infusion was mostly due to the increase in the heart rate (HR) (**Figure 6A-C**), suggesting that G3P may also directly modulate pace maker activity.

Myocardial infarct size was measured using histological staining and planimetry and the results showed significantly different outcomes following I/R (**Figure 7**). Following the trends, we observed in mitochondrial CRC, applying the substrates to whole tissue further cemented our proposition that complex III ROS are cardioprotective and complex I ROS are cardio-deleterious: G3P resulted in reduced infarct size compared to glutamate/malate. As KH buffer already contains a mitochondrial stimulant and

Krebs cycle driver in the form of glucose, we did not expect dramatically pronounced differences between the samples. This was because glucose was stimulating complexes I and II, as well as supporting an active tricarboxylic acid cycle, which is necessary for the heart to survive *ex vivo*. Nevertheless, we did observe significant differences between glutamate/malate and G3P treatment, enough to support the notion of opposed impacts of ROS. With regards to succinate, we also did observe smaller infarct sizes compared to glutamate/malate but not to the same extent as those for G3P (**Figure 7**). As succinate/complex II feeds electrons to both complexes I and III, this intermediate result was not surprising. Furthermore, Chouchani and colleagues have shown that *in vivo*, the accumulation of succinate during ischemia is the main driver for ROS generation by complex I via RET during reperfusion and that a reduction of this RET is adequate enough to mitigate I/R injury in murine models [73].

Impact on cardioprotection and antioxidant therapy

Based on our results, a fundamental question that arises is how *exactly* cardiomyocytes, or specifically, mitochondria recognize or delineate between "good" versus "bad" ROS in times of stress? As ROS in normal conditions and controlled quantities features as a signaling conduit [10], it may be possible that under such circumstances neither complex I nor complex III ROS are good or bad. In pathophysiological conditions resulting from I/R or exogenous inhibitors, we have shown that ROS from complexes I and III are *not* the same, but the mechanism or even the sites of action of these ROS leading to cardiac protection or damage are the subject of future studies. Drose et al. have suggested a mechanistic model to explain 'signaling ROS' by studying diazoxide effects on mitochondrial ROS generation [63]. They proposed that diazoxide, a complex II blocker, could stimulate the production of 'signalling ROS' at the Q_o site of complex III by inhibition of the succinate-ubiquinone oxidoreductase (complex II) thereby partially oxidizing the Q-pool. They hypothesized that following stress, 'signaling ROS' activates mitochondrial signaling pathways that during reoxygenation eventually reduce the production of 'deleterious' ROS by NADH-ubiquinone oxidoreductase (complex I) via RET and that in addition, diazox-

ide can directly reduce oxidative damage evoked by RET at complex I by inhibition of complex II. However, studies in H9c2 myoblasts by other investigators have suggested complex I being a source of signaling ROS for muscle differentiation [74]. In our study, our data agree with the Drose group in suggesting that the source of this 'signaling' ROS in heart mitochondria to be complex III, while also highlighting the deleterious effects of ROS from complex I. We extend this premise into the impact that both ETC sources have on cardiac tissue recovery following ischemic insult. Despite this model offering some insights into ROS classification, much is still needed to fully elucidate how cells respond to ROS from different ETC sites. Multiple factors need consideration when defining such pathways including the varying needs and responses of mitochondria in different organs [25, 50] and how, for example, in the brain, glutamate is both an excitatory neurotransmitter and a cause of neurotoxicity [75]. Understanding these mechanisms will be vital to designing targeted therapeutic agents for cardioprotection as current models of coenzyme Q_{10} treatment [6] or applying indiscriminant antioxidants as one-size fits all panaceas have proven to be somewhat fruitless as both good and bad ROS are destroyed [3].

In conclusion, we demonstrated the opposite impact of ROS generated by stimulation of complexes I, II and III after ischemia-reperfusion and also application of pro-apoptotic drugs in fresh mitochondria. We propose an extension to the concept that the *quantity* of ROS is important by showing that the mitochondrial ETC *source* complex of that ROS is also pivotal. We went on to demonstrate the positive impact of the ROS from mitochondrial complex III in cardioprotection via the inhibition of the mPTP opening, and following I/R, reduction of myocardial infarct size and improvements in cardiac functional recovery, while showing the cardio-deleterious effects of ROS from complex I.

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Disclosure of conflict of interest

None.

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