Reactive Oxygen Species Production in Energized Cardiac Mitochondria During Hypoxia/Reoxygenation

Modulation by Nitric Oxide

Paavo Korge, Peipei Ping, James N. Weiss

Abstract—Mitochondria are an important source of reactive oxygen species (ROS), implicated in ischemia/reperfusion injury. When isolated from ischemic myocardium, mitochondria demonstrate increased ROS production as a result of damage to electron transport complexes. To investigate the mechanisms, we studied effects of hypoxia/reoxygenation on ROS production by isolated energized heart mitochondria. ROS production, tracked using Fe²⁺-catalyzed, H₂O₂-dependent H₂DCF oxidation or Amplex Red, was similar during normoxia and hypoxia but markedly increased during reoxygenation, in proportion to the duration of hypoxia. In contrast, if mitochondria were rapidly converted from normoxia to near-anoxia ([O₂], <1 μmol/L), the increase in H₂DCF oxidation rate during reoxygenation was markedly blunted. To elicit the robust increase in H₂DCF oxidation rate during reoxygenation, hypoxia had to be severe enough to cause partial, but not complete, respiratory chain inhibition (as shown by partial dissipation of membrane potential and increased NADH autofluorescence). Consistent with its cardioprotective actions, nitric oxide (NO) abrogated increased H₂DCF oxidation under these conditions, as well as attenuating ROS-induced increases in matrix [Fe²⁺] and aconitase inhibition caused by antimycin. Collectively, these results suggest that (1) hypoxia that is sufficient to cause partial respiratory inhibition is more damaging to mitochondria than near-anoxia; and (2) NO suppresses ROS-induced damage to electron transport complexes, probably by forming NO-Fe²⁺ complexes in the presence of glutathione, which inhibit hydroxyl radical formation. (Circ Res. 2008;103:873-880.)

Key Words: mitochondria ■ reactive oxygen species ■ hypoxia/reoxygenation ■ nitric oxide

Reactive oxygen species (ROS) generated during prolonged ischemia and subsequent reperfusion are known to contribute to ischemia/reperfusion injury (reviewed elsewhere), although, paradoxically, transient ROS generation is also an essential trigger for cardioprotection by ischemic (IP) and pharmacological preconditioning (reviewed elsewhere). In cardiac cells, mitochondria are the major source of ROS, although intracellular NADPH oxidase, xanthine oxidase, monoamine oxidase, etc, may become important ROS sources under pathophysiological conditions. Mitochondria energized with physiologically relevant NADH-related substrates produce very little ROS, unless other factors, such as respiratory chain inhibitors (reviewed elsewhere) or mitochondrial ATP-sensitive K channel activators, are also present. Experiments with isolated cardiac myocytes have demonstrated that hypoxia increases ROS production and implicate mitochondria as the major source. ROS are known to damage electron transport complexes, potentially leading to a positive-feedback cycle during ischemia/reperfusion or hypoxia/reoxygenation, in which increased ROS toxicity impairs respiration, leading to further increases in ROS production, further respiratory dysfunction, and so forth. If this hypothesis is correct, then anoxia should be less effective in supporting this positive-feedback cycle, because ROS production requires the presence of some O₂. To test the importance of hypoxia versus near-anoxia on ROS production, we compared ROS production by isolated energized cardiac mitochondria exposed to graded levels of hypoxia, followed by reoxygenation. We find that ROS production, measured from Fe²⁺-dependent H₂DCF oxidation rate, is markedly increased after reoxygenation preceded by hypoxia, but not near-anoxia ([O₂], <1 μmol/L).

A second goal of this study was to test how nitric oxide (NO) impacts hypoxia-induced ROS production, because NO plays an important role in redox-based cell signaling and is cardioprotective against ischemia/reperfusion injury. Both ROS and NO interact with thiol groups, and NO also binds Fe²⁺ with high affinity, preventing its participation in hydroxyl radical formation from H₂O₂ by the Fenton reaction. Consistent with this idea, we find that NO markedly suppresses reoxygenation-induced H₂DCF oxidation, ROS-
induced matrix Fe$^{2+}$ increase, and aconitase inactivation, which may contribute to its cardioprotective actions.

**Materials and Methods**

This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academic Press, Washington DC, 1996). All procedures were approved by UCLA Animal Research Committee. All measurements were carried out using a Fiber Optic Spectrofluorometer (Ocean Optics) in a partially open continuously stirred cuvette at room temperature (22 to 24°C). Mitochondria were isolated from rabbit hearts as described previously$^9$ and added (0.5 to 1.0 mg/mL) to incubation buffer containing 100 mmol/L KCl, 10 mmol/L Hapes, pH 7.4 with Tris, followed by addition of 2.5 mmol/L potassium phosphate (P$_i$) and substrates as indicated. In some experiments, ADP was added in combination with hexokinase, glucose, and MgCl$_2$ to ensure a continuous ADP load to mitochondria, similarly to that in active cardiac cells.

O$_2$ delivery was regulated by adjusting stirring speed (settings 1 to 10, corresponding to 60 to 1100 rpm). The effect of stirring speed on the rate of O$_2$ diffusion into buffer, and how this was balanced by O$_2$ consumption by respiring mitochondria to regulate buffer [O$_2$] levels, is shown in the online data supplement, available at http://circres.ahajournals.org. Alternatively, O$_2$ delivery could be increased rapidly and significantly by injecting a small amount of compressed air into the buffer.

Mitochondria O$_2$ consumption was measured continuously by monitoring buffer O$_2$ content via a fiber optic oxygen sensor FOXY-AL300 (Ocean Optics). The O$_2$ sensor responded to O$_2$ changes at the level of >1 μmol/L (0.1 kPa) (see the online data supplement).

Mitochondrial membrane potential ($\Delta \psi_m$) was estimated using tetramethylrhodamine methyl ester (TMRM) (200 nmol/mL) in the buffer solution.$^9$

ROS production was monitored from reduced dichlorofluorescin (H$_2$DCF) oxidation (excitation/emission, 490/525 nm) after incubating mitochondria with H$_2$DCF diacetate (10 μmol/mL) and washing away extramitochondrial dye. Alternatively, O$_2^-$ production was measured using MitoSOX red (excitation/emission, 510/580 nm).$^{10}$

H$_2$O$_2$ release from mitochondria was measured using Amplex Red Hydrogen Peroxide Assay Kit (Invitrogen) (excitation/emission, 560/590 nm) (see Figure IV in the online data supplement).

Mitochondrial NADH autofluorescence was recorded at excitation/emission (340/460 nm) wavelengths. The NADH signal was calibrated by making mitochondria anoxic with N$_2$ to fully reduce NAD and subsequently adding O$_2$ and FCCP to fully oxidize NAD.

Mitochondrial iron uptake or release of bound iron in the matrix was determined by monitoring quenching of Phen Green or calcein fluorescence by chelatable iron.$^{11}$ Mitochondria were incubated with 10 μmol/L Phen Green FL or 5 μmol/L calcein-acetoxyethyl ester (calcein-AM) for 15 minutes at room temperature and washed, and fluorescence was measured at 490 nm excitation/520 nm emission. “Dequenching” was accomplished with 200 μmol/L 1,10-phenanthroline.

Aconitase activity was determined by an increase in NADPH fluorescence after adding reaction mixture and permeabilizing inner membrane with alamethicin, as described.$^{12}$

Changes in buffer NO level released from diethylamine NONOate sodium salt (DETA-NONO) or β-nitroso-N-acetylpenicillamine were monitored electrochemically using a NO electrode (World Precision Instruments, Sarasota, Fla).

**Results**

**Graded Hypoxia in Isolated Mitochondria**

We hypothesized that when hypoxia becomes severe enough to limit electron transport rate at complex IV (ie, near the K$_d$ of cytochrome c oxidase for O$_2$), increased O$_2^-$ production at complexes I and III may result.$^3$ To create a controllable level of hypoxia in a range sufficient to cause respiratory inhibi-

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Balancing O$_2$ supply relative to O$_2$ consumption to create graded hypoxia in isolated mitochondria. A, Isolated TMRM-loaded mitochondria, energized with malate/glutamate, decreased buffer [O$_2$] to several micromolar (upper trace, see the online data supplement for O$_2$ calibration). Mitochondria were able to support $\Delta \psi_m$ (lower trace) until the O$_2$ supply was further decreased by reducing stirring speed in stages from 6.5 to 4.5 as indicated, which was reversed by increasing stirring speed. Anoxia (N$_2$) further decreased O$_2$ to 0 and fully dissipated $\Delta \psi_m$. B, Mitochondria, respiring in the presence of malate/glutamate, P$_i$, ADP, and an ATP-consuming system (glucose [G], Mg$^{2+}$, hexokinase [H]) maintained low NADH fluorescence until buffer O$_2$ was decreased to a critically low level, at which point, $\Delta \psi_m$ (monitored in parallel experiments) dissipated partially (arrow) and NADH fluorescence increased. NADH fluorescence could be regulated by changing the stirring speed to decrease and increase O$_2$ supply. Maximum NADH fluorescence was measured by making mitochondria anoxic with N$_2$ and minimum fluorescence by providing air and FCCP (1 μmol/L).

dition, we carefully balanced O$_2$ supply rate against O$_2$ consumption rate by isolated mitochondria in a cuvette. Figure 1A shows simultaneous traces of buffer [O$_2$] and TMRM fluorescence measuring $\Delta \psi_m$ in isolated mitochondria energized with malate/glutamate. When added to a cuvette equilibrated with room air ($pO_2$=160 mm Hg or [O$_2$]=220 μmol/L), mitochondria gradually depleted [O$_2$] to very low levels. $\Delta \psi_m$ remained fully polarized, consistent with the ability of mitochondria to downregulate O$_2$ consumption to match supply at low [O$_2$].$^{13}$ However, when the speed of the stirring bar was lowered from 7 to 6.5 to reduce O$_2$ diffusion into the cuvette, $\Delta \psi_m$ depolarized slightly and stabilized at a new level. Further reductions in stirring speed caused graded $\Delta \psi_m$ depolarization, which reversed immediately when the stirring speed was increased back to 7. Replacing air with N$_2$, on the other hand, depolarized $\Delta \psi_m$ rapidly and completely.
To document that hypoxia sufficient to depolarize $\Delta \Psi_m$ was attributable to respiratory inhibition, we also measured NADH autofluorescence (Figure 1B). NADH fluorescence increased significantly when O$_2$ was depleted to the level causing partial $\Delta \Psi_m$ depolarization. Further decreases in O$_2$ supply by decreasing the stirring speed increased NADH fluorescence reversibly. The maximum value of NADH fluorescence was ascertained by replacing air with N$_2$, and the minimum value by adding FCCP in the presence of O$_2$ (Figure 1B).

These findings demonstrate that the level of hypoxia to which isolated mitochondria are subjected can be finely regulated over a range causing graded respiratory inhibition, as manifested by partial $\Delta \Psi_m$ depolarization and increased NADH levels.

**ROS Production During Hypoxia/Reoxygenation Versus Anoxia/Reoxygenation**

Our next goal was to examine how graded hypoxia versus anoxia affects ROS production by isolated mitochondria. As described in detail in the online data supplement, we found that O$_2$ and subsequent H$_2$O$_2$ production in isolated mitochondria is severely underestimated by H$_2$DCF oxidation rate unless Fe$^{2+}$ is added, so that its rapid uptake into the matrix by iron transporters generates OH radicals and/or other reactive intermediates associated with Fenton chemistry to catalyze H$_2$DCF oxidation. Using this method, we compared how substrates and [O$_2$] affected mitochondrial ROS production. In Figure 2A, isolated mitochondria loaded with H$_2$DCF were energized with malate/glutamate and incubated at the indicated stirring speeds to regulate O$_2$ supply. The amount of mitochondria and stirring speed were selected to allow a gradual decrease in buffer [O$_2$] to a few micromolar (see the online data supplement for calibration). Addition of 5 $\mu$mol/L Fe$^{2+}$ to hypoxic mitochondria increased H$_2$DCF oxidation (FDCF) (lower trace), similar to the normoxic rate in Figure IC, trace a. B, Same as A, except that 5 $\mu$mol/L Fe$^{2+}$ was added at the time of reoxygenation, resulting in a much greater increase in H$_2$DCF oxidation rate. C, Isolated mitochondria were added to anoxic buffer (equilibrated with N$_2$) containing malate/glutamate and 2.5 mmol/L P$_i$. Addition of 5 $\mu$mol/L Fe$^{2+}$ caused a modest increase in H$_2$DCF oxidation (attributable to thyl radicals; see the online data supplement), which accelerated after reoxygenation comparably to Figure IC, trace a. D, Average increases in H$_2$DCF oxidation rate (mean±1 SD) after addition of 5 $\mu$mol/L defer-oxamine (desf) to glutamate/malate-energized mitochondria during normoxia (IC), hypoxia (3A), hypoxia/reoxygenation (3B), anoxia (3C), and anoxia/reoxygenation (3C).
slow H$_2$DCF oxidation (Figure 2C), possibly because of the small amount of O$_2$ added with the mitochondria or to the reaction of thiyl radicals with H$_2$DCF in the absence of O$_2$ (supplemental Figure III). With full oxygenation, H$_2$DCF oxidation rate increased modestly but to a much lesser extent than in mitochondria exposed to hypoxia/reoxygenation (Figure 2D). These findings indicate that hypoxia at a level causing partial respiratory inhibition primes mitochondria to produce excessive ROS on reoxygenation, whereas total anoxia does not.

We also studied ROS production by succinate-energized mitochondria, which consume O$_2$ at a higher rate and can therefore deplete buffer oxygen more rapidly and completely, even in state 2. Figure 3A shows that when isolated mitochondria energized with succinate were added to buffer equilibrated with room air, they depleted O$_2$ much more rapidly and completely than an equivalent amount of mitochondria energized with complex I substrates (compare the rates of O$_2$ depletion in Figure 3A to Figures 1A, 2A, and 2B). The final level of O$_2$ depletion was below the O$_2$ electrode sensitivity (<1 μmol/L), as seen at the end of the trace in Figure 3A when room air was replaced with N$_2$. At this near-anoxic level, H$_2$DCF oxidation rate increased modestly. As shown in the online data supplement, these changes are attributable to H$_2$DCF oxidation by thiyl radicals under anoxic conditions, which is masked in the presence of O$_2$. Consistent with this interpretation, the increase in H$_2$DCF oxidation rate was inhibited by transient reoxygenation with an air bolus and then resumed after buffer O$_2$ was again depleted. Under these conditions, addition of Fe$^{2+}$ had little effect on H$_2$DCF oxidation rate (Figure 3A). Moreover, when Fe$^{2+}$ was added at the time of reoxygenation, the increase in H$_2$DCF oxidation rate was also very modest (Figure 3B). The average changes in H$_2$DCF oxidation rate are summarized in Figure 3D. Note that compared to the same amount of identically H$_2$DCF-loaded isolated mitochondria energized with complex I substrates, the increase in H$_2$DCF oxidation rate in succinate-energized isolated mitochondria after reoxygenation in the presence of Fe$^{2+}$ was markedly reduced (compare to Figure 2D). In all of the succinate experiments, the standard amount of mitochondria was sufficient to rapidly deplete extramitochondrial O$_2$ level below the sensitivity of the O$_2$ electrode (near-anoxia), resulting in rapid Δψ$_m$ depolarization (data not shown). Thus, the time window during which isolated mitochondria were exposed to a level of hypoxia sufficient to partially inhibit respiratory chain activity, but not yet at the near-anoxia level associated with complete Δψ$_m$ dissipation, was relatively short. After reoxygenation, mitochondrial O$_2$ consumption rate showed no signs of respiratory chain inhibition, averaging 90±3% of the prehypoxic rate.

We postulated that the lesser ROS production on reoxygenation in succinate-energized mitochondria compared to complex I-energized mitochondria might be attributable to their higher rate of O$_2$ consumption causing more severe O$_2$ depletion. To test this possibility, we added half the amount of succinate-energized mitochondria to the cuvette, to avoid severe O$_2$ depletion and marked Δψ$_m$ dissipation (Figure 3C).
The thyl radical-related increase in H$_2$DCF oxidation rate was avoided (consistent with hypoxic rather than near-anoxic conditions), and addition of Fe$_{2}^+$ at the time of reoxygenation now markedly accelerated H$_2$DCF oxidation rate (Figure 3C and 3D) to a level comparable to complex I–energized mitochondria (Figure 2D), when corrected for the lesser amount of mitochondria.

Together, these findings demonstrate that hypoxia sufficient to cause partial respiratory chain inhibition results in significant Fe$_{2}^+$-dependent ROS production on reoxygenation, whereas exposure to an equivalent period of anoxia or near-anoxia does not have this effect.

**Nitric Oxide Inhibits Fe$_{2}^+$-H$_2$O$_2$–Induced H$_2$DCF Oxidation After Hypoxia/Reoxygenation**

NO interacts with ROS signaling and, when given at the time of reperfusion, is known to protect the heart from ischemia/reperfusion injury (reviewed elsewhere). NO also protects cells against damaging effects of ROS, and 1 possible mechanism may be inhibition of the Fenton reaction caused by NO binding to Fe$_{2}^+$ coordination sites. We therefore tested the effects of NO on hypoxia-induced ROS production. In Figure 4A, complex I–energized mitochondria depleted buffer [O$_2$] to a low level, after which, they were challenged with reoxygenation and 3 Fe$_{2}^+$ pulses, which significantly and progressively increased in H$_2$DCF oxidation rate. When 10 μmol/L DETA-NONO was added just before reoxygenation, however, the increase in Fe$_{2}^+$-dependent H$_2$DCF oxidation was markedly suppressed (Figure 4B). The middle NO electrode trace in Figure 4B documents that DETA-NONO spontaneously released NO, which was accompanied by transient inhibition of mitochondria O$_2$ consumption in the upper trace. The finding that the third Fe$_{2}^+$ pulse was delivered after buffer NO level had decreased to back to 0, but H$_2$DCF oxidation rate remained low, may also indicate some suppression of ROS production by NO at the level of respiratory complexes. Figure 4C summarizes the average increase in H$_2$DCF oxidation rate after the first, second, and third Fe$_{2}^+$ additions, illustrating the marked suppression by pretreatment with DETA-NONO.

**Nitric Oxide Attenuates ROS-Induced Increases in Matrix [Fe$_{2}^+$] and Depression of Aconitase Activity**

In isolated heart, a substantial portion of ischemia/reperfusion damage is thought to be related to free radical production, catalyzed by Fe$_{2}^+$ with open coordination sites (required for Fenton-based O$_{2}^•$ generation). In calcein-loaded mitochondria exposed to hypoxia-reoxygenation, however, we did not detect significantly increased calcein quenching, as an indicator of increased matrix Fe$_{2}^+$. To test the hypothesis that NO inhibits the Fenton reaction by binding to Fe$_{2}^+$ coordination sites exposed by ROS, we needed a more robust method to generate ROS and, so, used antimycin to increase H$_2$O$_2$ production in energized mitochondria.

Figure 5A shows that when energized calcein-loaded mitochondria were exposed to antimycin to cause a sustained increase in H$_2$O$_2$ production (see supplemental Figure IVB), calcein fluorescence was progressively quenched. The quenching was attributable to iron release, because it was reversed with the iron chelator phenanthroline. In Figure 5B, the NO donor DETA-NONO was added after antimycin. Whereas NO (middle trace) remained elevated, the quenching of calcein fluorescence ceased and then subsequently resumed at a slower rate after NO levels had returned to 0 and
transport complexes, possibly by forming dinitrosyl-dithiolate–Fe complexes in the presence of Fe$^{2+}$ and matrix glutathione (GSH). Our major conclusions are as follows: (1) matrix ROS production is similar during hypoxia as during normoxia, despite decreased O$_2$ availability in the former situation; (2) following hypoxia, reoxygenation results in a marked increase in ROS production, which is associated with reduced O$_2$ consumption reflecting electron transport dysfunction; (3) for reoxygenation to cause a marked increase in ROS production, the degree of preceding hypoxia must be severe enough to cause partial respiratory inhibition, as reflected by modest ΔΨ$_m$ dissipation and increased NADH autofluorescence; (4) in contrast, following anoxia or near-anoxia severe enough to induce major ΔΨ$_m$ dissipation, reoxygenation does not increase ROS to nearly the same extent, and O$_2$ consumption returns to preanoxic levels, indicating preserved electron transport function; and (5) the increase in Fe$^{2+}$-H$_2$O$_2$–dependent H$_2$DCF oxidation after hypoxia/reoxygenation is persistently attenuated by NO, most likely as a result of NO binding to free coordination sites of iron, with subsequent inhibition of hydroxyl radical formation by the Fenton reaction.

The role of partial, in contrast to nearly complete, ΔΨ$_m$ dissipation in accelerating O$_2$ production on reoxygenation deserves further comment. In succinate-energized mitochondria, in which O$_2$ production by reverse electron transport requires a maintained proton gradient, ΔΨ$_m$ dissipation inhibits reverse electron transport, thereby suppressing O$_2$ production. In complex I–energized mitochondria, however, reverse electron transport should not play a significant role in O$_2$ generation, so full ΔΨ$_m$ dissipation during near-anoxia may not be the protective mechanism. It is more likely that O$_2$ production is just too severely limited by the near absence of O$_2$ to impair electron transport.

In general, 2 conditions are required for “primary” ROS (O$_2$) production by the respiratory chain: reduced electron transport centers (in complexes I or III) and the presence of O$_2$ to allow 1 electron reduction to O$_2$. A sustained O$_2$ supply to mitochondria with inhibited respiratory chain promotes a large increase in ROS production, which can further damage electron transport. Thus, our findings are consistent with a scenario in which partial respiratory inhibition during hypoxia maintains significant ROS production in the matrix despite reduced O$_2$ availability, causing progressive and persistent electron transport dysfunction, such that when reoxygenation delivers O$_2$ in excess, ROS production markedly accelerates. In general these results support conclusions by Schumacker and colleagues that were made by using cultured cardiomyocytes and novel redox-sensitive fluorescence resonance energy transfer probes. It could be argued that in our experimental setup, hypoxia is not really uniform, because isolated mitochondria are intermittently exposed to high O$_2$ when they swirl near the air-water interface at the top of the cuvette. However, this same process would have occurred in succinate-energized mitochondria, yet they did not show the marked increase in H$_2$DCF oxidation on reoxygenation, unless the concentration of mitochondria in the cuvette was decreased. This suggests that O$_2$ gradients in the cuvette are unlikely to account for our findings, although we cannot...
exclude the possibility that ROS generation via succinate-mediated reverse electron transport is inhibited because of severe \( \Delta V_m \) depolarization.

Our observation that anoxia or near-anoxia did not result in comparably high ROS production or persistent respiratory suppression on reoxygenation suggests that the ongoing ROS production during hypoxia plays a key role in impairing the electron transport chain. Complex I is the most sensitive to ischemia/reperfusion injury\(^2\) and also contains at least nine Fe/S clusters that are used to transfer electrons between active sites. Interestingly, these clusters are not sensitive to exogenous H\(_2\)O\(_2\)\(^{25}\) suggesting that generation of O\(_2^-\) in the immediate vicinity of Fe-S clusters in complex I may be required to disrupt function during ischemia.\(^7\) A highly localized interaction might also account for our inability to detect matrix iron release with calcine during relatively brief hypoxia/reoxygenation, in contrast to antimycin–induced ROS production into the matrix, which released iron from aconitate Fe/S clusters and inhibited aconitase activity. Regarding Fe release, antimycin may better mimic the sustained ROS production during prolonged hypoxia/reoxygenation.

It is important to distinguish the putative deleterious effects of ROS after prolonged ischemia/reperfusion from the protective effects of transient mitochondrial ROS generation during ischemic and pharmacological preconditioning.\(^2\) In the latter case, stimulation of mitochondrial ROS production, typically triggered by interventions that open mitochondrial ATP-sensitive K channels, activates downstream signaling via protein kinase pathways to protect mitochondria from injury during prolonged ischemia/reperfusion or hypoxia/reoxygenation.\(^6\) In triggering cardioprotection, one protective action of ROS signaling is to prevent subsequent excessive ROS production during prolonged ischemia/reperfusion.\(^6\) The mechanisms by which ROS-mediated protein kinase signaling impacts hypoxia/reoxygenation-induced ROS production in isolated mitochondria will be an interesting area for future investigation using the approaches developed in the present study.

**Effects of NO**

NO administered before ischemia triggers cardioprotection by activating mito-K\(_{ATP}\) channels via protein kinase G, stimulating mitochondrial ROS production to activate downstream cardioprotective pathways (reviewed elsewhere\(^26\)). However, NO administered at the time of reoxygenation also protects the heart from injury.\(^15\) We found that an NO bolus markedly suppressed the hypoxia-reoxygenation induced increase in H\(_2\)DCF oxidation rate. This effect could be explained by high-affinity binding of NO to Fe\(^{2+}\) limiting Fe\(^{2+}\) oxidation by Fenton reaction.\(^18\) Binding of NO to released Fe\(^{2+}\), or iron in [4Fe-4S] clusters exposed to H\(_2\)O\(_2\), provides simplest explanation why NO attenuated ROS-induced matrix iron increase and aconitase inhibition. It may seem surprising that NO protects cells exposed not only to exogenous H\(_2\)O\(_2\), but also to enzymatic systems generating O\(_2^-\)\(^{16,17}\) because NO can react with O\(_2^-\) to generate peroxynitrite radicals. However, peroxynitrite rapidly reacts with GSH inside cells and mitochondria. Because relatively low [GSH] (IC\(_{50}\) = 10 μmol/L) is sufficient to prevent inhibition of complexes I to III caused by 200 μmol/L peroxynitrite,\(^27\) 5 to 10 μmol/L glutathione in the matrix should effectively avoid peroxynitrite toxicity. In addition, the high catalytic activity of manganese superoxide dismutase in the matrix that keeps O\(_2^-\) levels low can also limit peroxynitrite formation. Because peroxynitrite is capable of directly inducing H\(_2\)DCF oxidation\(^28\) and Fe\(^{2+}/H_2O_2\)-induced oxidation was inhibited by NO, we assume that relatively little peroxynitrite was generated.

In our experiments the inhibitory effect of NO on H\(_2\)DCF oxidation rate persisted after extramitochondrial NO returned to 0 levels (Figure 5A), suggesting that a longer-lasting interaction, such as matrix protein S-nitrosation, may also be involved. Recent data indicates that reversible inhibition of complex I by S-nitrosation reduces ROS generation at reperfusion, with less oxidative inactivation of respiratory chain, aconitase activity, and inhibition of PTP opening.\(^29,30\) S-Nitrosation of mitochondrial thiols by NO also blocks thiol oxidation, inhibiting cell death induced by GSH depletion.\(^31\) Recently, it was reported that NO can directly activate mitochondrial protein kinase Cε, which inhibits permeability transition pore opening.\(^32\)

**Caveats in Using H\(_2\)DCF As an ROS Indicator**

Despite well-established experimental documentation that Fe\(^{2+}\) or Fe\(^{2+}\)-containing catalysts are required to measure H\(_2\)O\(_2\)-induced H\(_2\)DCF oxidation, recent analysis of literature suggests that in most studies, the role of the catalyst has been ignored,\(^33\) leading to serious underestimation of matrix ROS production. However, mitochondria have a Fe\(^{2+}\) carrier in the inner membrane,\(^34\) making it possible to increase matrix [Fe\(^{2+}\)] simply by adding a known amount Fe\(^{2+}\). Fe\(^{2+}\) uptake into the matrix had no direct effect on O\(_2^-\) production but promoted H\(_2\)DCF oxidation by H\(_2\)O\(_2\) with greatly increased sensitivity. As shown in the online data supplement and documented recently by others,\(^14\) in the absence of Fe\(^{2+}\), ROS production generates thyl radicals from glutathione or cysteine, which do not oxidize H\(_2\)DCF in the presence of O\(_2\) but do in its absence. In intact cells, H\(_2\)DCF oxidation is also promoted by cytochrome c release, independent of ROS production.\(^35\) These technical limitations make it difficult to prove unequivocally in intact cells that mitochondria are the source of ROS, especially during hypoxia/reoxygenation and emphasize the importance of isolated mitochondria studies.

**Conclusions**

We have further elucidated the source of ROS production during ischemia/reperfusion by showing that in isolated mitochondria exposed to a degree of hypoxia sufficient to cause partial, but not complete, respiratory inhibition, electron transport chain is persistently depressed by ongoing matrix ROS generation. As a result, during reoxygenation, ROS production markedly accelerates, causing further suppression of electron transport, which promotes greater ROS production, etc., in a vicious cycle. NO inhibits this scenario, potentially contributing to its cardioprotective effects when administered at the time of reoxygenation in intact heart.

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H$_2$DCF oxidation depends on both ferrous iron and H$_2$O$_2$.

Oxidation of H$_2$DCF can occur by reaction with H$_2$O$_2$, but it is very slow, especially at low [H$_2$O$_2$]. H$_2$O$_2$–induced H$_2$DCF oxidation is significantly accelerated by presence of iron or iron-containing catalyst, such as a heme group, horseradish peroxidase or cytochrome c $^{1-3}$. By using H$_2$DCF prepared from H$_2$DCF diacetate by alkaline hydrolysis $^4$, we were able to confirm previously published results, indicating that under our conditions both iron-containing catalysts (all of those listed above were tested) or iron and H$_2$O$_2$ are required for H$_2$DCF oxidation. Interestingly, glutathione peroxidase, a selenium containing peroxidase normally present in the matrix, had no significant effect on H$_2$DCF oxidation, even when used in 10 times higher concentrations than those at which horseradish peroxidase had significant effects (data not shown). Iron-containing proteins are also known to increase H$_2$DCF oxidation independent of ROS. One reported anomaly for H$_2$DCF oxidation in the presence of horseradish peroxidase (plant enzyme) is a generation of secondary radicals that can after reacting with O$_2$ further oxidize H$_2$DCF $^5$. Therefore, incubation conditions, as we demonstrate below, are extremely important for rational use of H$_2$DCF. In control experiments, we also established that neither rotenone nor antimycin had any direct effect on H$_2$DCF fluorescence.

Fig. S1A demonstrates that in vitro oxidation of deacetylated H$_2$DCF to its fluorescent form DCF requires both Fe$^{2+}$ and H$_2$O$_2$. Addition of Fe$^{2+}$ (2.5 μM) promoted a slight increase in DCF fluorescence probably related to formation of oxidizing iron species like ferryl or FeO$_2^{2+}$. However, subsequent addition of H$_2$O$_2$ induced much more rapid and significant increase in H$_2$DCF oxidation (Fig. S1A, trace a). H$_2$O$_2$ alone had no significant effect, but H$_2$DCF was rapidly oxidized after Fe$^{2+}$ addition (Fig. S1A, trace b), but not after addition of oxidized iron (10 μM Fe$^{3+}$, Fig. S1A, trace c).
These results indicate that \( \cdot \text{OH} \) formation via the Fenton reaction is required to oxidize H\(_2\)DCF.

Consistent with this interpretation, the known \( \cdot \text{OH} \) scavengers mannitol and dimethyl sulfoxide (DMSO) both suppressed H\(_2\)DCF oxidation by H\(_2\)O\(_2\) plus Fe\(^{2+}\) (Fig. S1A, traces d & e). However, by increasing iron concentration the importance of Fe\(^{2+}\)-oxygen interaction in generating ferryl ions or even hydroxyl radicals \(^6\) and subsequent oxidation of H\(_2\)DCF by these species is increasing.

In isolated mitochondria loaded with H\(_2\)DCF, the catalytic importance of Fe\(^{2+}\) is shown in Fig. S1B-C. Fig. S1B demonstrates that energized calcein-loaded mitochondria rapidly accumulate exogenous Fe\(^{2+}\), but not Fe\(^{3+}\), as indicated by quenching of calcein fluorescence, attributable to an inner membrane carrier which preferentially transports Fe\(^{2+}\) into the matrix \(^7\), \(^8\). Similar findings were also obtained with another iron indicator, Phen Green FL (data not shown). Fig. S1C shows that in H\(_2\)DCF-loaded mitochondria energized with Complex I substrates malate and glutamate, H\(_2\)DCF oxidation rate was very low until 5 \( \mu \)M Fe\(^{2+}\) was added to promote \( \cdot \text{OH} \) (and/or ferryl) formation in the matrix. Subsequent addition of Fe\(^{2+}\), together with rotenone to increase \( \cdot \text{O}_2 \) release into the matrix, further accelerated H\(_2\)DCF oxidation. In contrast, if rotenone was added before 5 \( \mu \)M Fe\(^{2+}\) (Fig. S1C, inset), it had only a modest effect on H\(_2\)DCF oxidation rate, which was significantly accelerated by adding Fe\(^{2+}\). Thus, although the respiring mitochondria were generating \( \cdot \text{O}_2 \) and H\(_2\)O\(_2\), ROS production was underestimated by H\(_2\)DCF oxidation in the absence of a catalyst primarily promoting the Fenton reaction. Similar results were obtained with diazoxide (50 \( \mu \)M), which only modestly increased H\(_2\)DCF oxidation unless Fe\(^{2+}\) was also added (Fig. S1D).

Matrix Fe\(^{2+}\) uptake is accompanied by matrix alkalinization \(^9\), which could accelerate mitochondrial \( \cdot \text{O}_2 \) production \(^10\). To examine this possibility, mitochondria energized with NADH-related substrates were loaded with the \( \cdot \text{O}_2 \) indicator MitoSOX and challenged with diazoxide or antimycin to increase \( \cdot \text{O}_2 \) production \(^10\), \(^11\). As shown in Fig. S1E, MitoSOX fluorescence slightly
increased upon addition of diazoxide, but was not accelerated by subsequent addition of Fe\(^{2+}\). When antimycin was substituted for diazoxide, similar results were obtained (Fig. S1F). Antimycin promoted a much higher rate of MitoSOX oxidation compared to diazoxide. This increase was inhibited by adding a low concentration of the mitochondrial superoxide dismutase mimetic manganese(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTPyT) (Fig S1F, trace a). Notably, MitoSOX oxidation reflecting O\(_2^*\) production rate was not significantly affected by addition of Fe\(^{2+}\) or subsequent binding of iron with deferoxamine (Fig. S1F, trace b). These findings indicate that Fe\(^{2+}\) by itself does not accelerate O\(_2^*\) production, excluding this as a possible explanation for increased H\(_2\)DCF oxidation.

Based on these results, we conclude that O\(_2^*\) and H\(_2\)O\(_2\) production in isolated mitochondria is severely underestimated by H\(_2\)DCF oxidation rate unless Fe\(^{2+}\) is added to catalyze ROS-dependent H\(_2\)DCF oxidation by generating \(^{•}\)OH radicals. Similar conclusion has been reached earlier regarding H\(_2\)DCF oxidation in endothelial cells that required uptake of extracellular iron by the transferrin-dependent iron transport mechanism\(^{12}\).

**Fig. S1.** H\(_2\)DCF oxidation requires both Fe\(^{2+}\) and H\(_2\)O\(_2\). **A.** Deacetylated H\(_2\)DCF in vitro. **Trace a:** Addition of Fe\(^{2+}\) (2.5 \(\mu\)M) to deacetylated H\(_2\)DCF (5 \(\mu\)M ) in KCl-buffer only mildly increased fluorescence until H\(_2\)O\(_2\) (20 \(\mu\)M ) was added, causing a marked increase. **Trace b** and **c:** H\(_2\)O\(_2\) (20 \(\mu\)M) alone had no significant effect on DCF fluorescence until Fe\(^{2+}\) (2.5 \(\mu\)M) was subsequently added (trace b), unlike Fe\(^{3+}\) (10 \(\mu\)M) which was ineffective (trace c). **Trace d-e:** Same protocol as trace b, but in the presence of \(^{•}\)OH scavengers mannitol (100 mM, trace d) or DMSO (15\% (v/v), trace e). Although baseline fluorescence was similar in traces a-e, traces c-e were offset for clarity. **B.** Demonstration of Fe\(^{2+}\) uptake by mitochondria. Calcein-loaded
mitochondria were incubated in the presence of 2.5 mM Pi and 5 mM each of Complex I substrates malate and glutamate (mal, glu). Addition of ferric iron Fe$^{3+}$ (5 μM) had no effect, but ferrous iron Fe$^{2+}$ (5 μM) quenched calcein fluorescence due to Fe$^{2+}$ uptake into the matrix. C. H$_2$DCF-loaded mitochondria were energized with malate/glutamate, in the presence of 2.5 mM Pi. **Trace a (black):** Addition of 5 μM Fe$^{2+}$ increased fluorescence, indicating the requirement of catalyst Fe$^{2+}$ for H$_2$DCF oxidation by endogenous H$_2$O$_2$ generated by energized mitochondria. Rotenone (10 μM) and 5 μM Fe$^{2+}$ further accelerated H$_2$DCF oxidation rate due to enhanced O$_2^{-}$ and H$_2$O$_2$ production by rotenone. **Trace b (blue):** 10 μM rotenone added first to increase mitochondrial ROS production accelerated H$_2$DCF oxidation rate only slightly (see expanded trace in inset). After 5 μM Fe$^{2+}$ was added, however, H$_2$DCF oxidation rate increased markedly and comparably to that in trace a. D. Under the same conditions, 50 μM diazoxide, which promotes mitochondrial ROS production by opening mitoK$_{ATP}$ channels, caused only a slight increase H$_2$DCF oxidation rate until Fe$^{2+}$ was added. Note that after Fe$^{2+}$, H$_2$DCF oxidation was comparable to that after rotenone + Fe$^{2+}$ in panel C, both much greater than in the absence of drugs. (As recommended, the buffer contained 2 mM MgCl$_2$ and 0.2 mM ATP with diazoxide present). E-F. Demonstration that Fe$^{2+}$ has no direct effect on mitochondrial O$_2^{-}$ production. In panel E, mitochondria were loaded with MitoSOX red (2 μM) to monitor O$_2^{-}$ under the same conditions as in panel D. Diazoxide increased MitoSOX oxidation rate, which was unchanged by subsequent addition of Fe$^{2+}$ (5 μM). In panel F, trace a (black), malate/glutamate-energized mitochondria loaded with 2 μM MitoSOX red were exposed to 2.5 μM antimycin, which markedly increased MitoSOX oxidation rate. Additions of the mitochondrial superoxide dismutase mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP, 0.15 μM) decreased MitoSOX oxidation rate. In trace b (blue), energized mitochondria from the same
preparation as in trace a were challenged with 5 μM Fe$^{2+}$ before 2.5 μM antimycin was added to increase MitoSOX oxidation. Subsequent addition deferoxamine (200 μM) to chelate Fe had no significant effect on MitoSOX oxidation.

**Calibration of buffer [O$_2$]**

**Fig. S2. A.** Dependence of O$_2$ diffusion rate into the buffer on the speed of the stirring bar. After depleting O$_2$ in 2 ml buffer with N$_2$, O$_2$ repletion recorded with the O$_2$ sensor was more rapid at stirring speed 7 than 5. **B.** Calibration of O$_2$ sensor at low [O$_2$]. After O$_2$ depletion with N$_2$ in 2 ml buffer, mixtures of N$_2$ and O$_2$ yielding final O$_2$ concentrations of 0.1, 0.5, 1, 2, 3, 5 and 10 μM were applied successively. The O$_2$ sensor’s limit of detection was around 1-2 μM.

*NO directly oxidizes H$_2$DCF.*

Fig. S3A shows that the *NO donor SNAP directly oxidizes H$_2$DCF when *NO is released from SNAP. When added directly to deacetylated H$_2$DCF (20 μM) in KCl-buffer, 100 μM SNAP did not significantly increase buffer [*NO] (top trace) or H$_2$DCF oxidation (lower trace) until 10 μM Fe$^{2+}$ was added to release *NO from SNAP. In this regard, S-nitrosothiols such as SNAP behave differently from DetaNONO, which rapidly releases *NO when added to a mitochondrial suspension (Fig. 4 & 5). Catalytic breakdown of S-nitrosothiols is known to be stimulated by transition metals (reduced copper, iron).

DETA-NONO similarly increased H$_2$DCF oxidation without the need to add Fe$^{2+}$ (not shown). However, the oxidant is thought to be not *NO, but either *NO$_2$ and/or possibly N$_2$O$_3$ and antioxidants, most notably GSH can compete with the probe for oxidation by *NO-derived
oxidants. This could also be one explanation why *NO inhibited Fe²⁺/ROS promoted H₂DCF oxidation in the matrix.

H₂DCF oxidation during anoxia is due to thiyl radicals.

Mitochondria contain glutathione and other thiol groups that can react nonenzymatically with all reactive oxygen species. In these reactions, thiols that are efficient antioxidants which protect mitochondria from oxidative damage, undergo one-electron oxidation to form thiyl radicals. Therefore, thiols and H₂DCF (reduced dye) compete for oxidation by ROS. Here we show that in the absence of catalyst (Fe²⁺), the rate of H₂DCF oxidation in the matrix during normoxia is low, implying that thiol oxidation to thiyl radicals prevails. Fig. S2A shows that after succinate-energized mitochondria had depleted buffer [O₂] to zero, there was distinct increase in H₂DCF oxidation rate. Applying a stream of argon (Arg) onto the surface of the buffer had no significant effect on H₂DCF oxidation rate, but replacing the Argon stream with air rapidly and reversibly inhibited H₂DCF oxidation. This is explained by oxidation of H₂DCF by thiyl radicals in the absence of O₂, since O₂ is well-known to inhibit this reaction. During each consecutive air bolus, more ROS were generated which, in the absence of catalyst, did not oxidize H₂DCF, but oxidized thiols into thiyl radicals instead. During Argon application, the absence of O₂ allowed the thiyl radicals thus formed to oxidize H₂DCF.

Further evidence that thiol→thiyl→H₂DCF oxidation is responsible for the observed increase in H₂DCF oxidation rate during anoxia was obtained in Fig. S3C by decreasing the number of thiol groups available for oxidation by ROS during normoxia. This was accomplished using SNAP as a transnitrosating agent to directly transfer NO⁺ groups to reduced thiols. In Fig. S3C, mitochondria energized with succinate were treated with 100 μM SNAP to
protect thiol groups from oxidation. As buffer O$_2$ was depleted, the rate of H$_2$DCF oxidation now decreased, rather than increased, whereas reoxygenation accelerated H$_2$DCF oxidation, exactly opposite to the response in Fig. S3B. The simplest explanation is that S-nitrosation decreased the thiol group oxidation to thiyl radicals, so that H$_2$DCF was oxidized preferentially by ROS when O$_2$ was present, and very few thiyl radicals were present to oxidize H$_2$DCF when O$_2$ was absent.

**Fig. S3.** A. The $^\cdot$NO donor SNAP oxidizes H$_2$DCF when $^\cdot$NO is released from SNAP. SNAP (100 µM) was added to deacetylated H$_2$DCF (20 µM) in KCl-buffer, which did not significantly increase buffer $[^\cdot$NO] (top trace) or H$_2$DCF oxidation (lower trace) until 10 µM Fe$^{2+}$ was added to release $^\cdot$NO from SNAP. B. Succinate (10 mM) energized mitochondria depleted buffer O$_2$, which increased H$_2$DCF oxidation rate. A stream of Argon gas was then applied at the surface of the buffer and alternated with a stream of air. Argon had initially no effect on increased H$_2$DCF oxidation, but air suppressed H$_2$DCF oxidation, consistent the known inhibitory effect of O$_2$ on H$_2$DCF oxidation by thiyl radicals.$^{14}$ This suppressed oxidation was rapidly accelerated with argon that can be explained by H$_2$DCF oxidation with thiyl radicals. C. Mitochondria were incubated in the presence of 10 mM succinate and 100 µM SNAP. After buffer O$_2$ was depleted, H$_2$DCF oxidation rate decreased, rather than increased as in Fig. 3B. Mitochondria were reoxygenated three times, during which H$_2$DCF oxidation rate transiently increased. N$_2$ indicates zero [O$_2$] measured by the O$_2$ sensor. The ROS scavenger MPG- N-(2-
mercaptopropionyl)glycine (2.5 mM) added at the end of the trace completely inhibited H$_2$DCF oxidation.

**H$_2$O$_2$ release from mitochondria during hypoxia/reoxygenation.**

Fig. S4A demonstrates the effect of extramitochondrial O$_2$ level on H$_2$O$_2$ release from mitochondria energized with NAD-related substrates. To reduce uncoupling caused by free fatty acids and ensure that mitochondria are able to maintain steady state O$_2$ consumption at low O$_2$ levels buffer contained also bovine serum albumin (BSA, 0.5 mg/ml). BSA has reported to have no significant effect on fluorescence signal in the Amplex red assay. Lower trace shows that increase in resorufin fluorescence, reflecting H$_2$O$_2$ release from mitochondria, was initially relatively slow (at high O$_2$, 200-100 μM) and further decreased in parallel with buffer pO$_2$ decrease. At low pO$_2$ values observed decrease in H$_2$O$_2$ release was further suppressed by decreasing a stirring speed from 8 to 5 that resulted in buffer pO$_2$ decrease leading to inhibition of mitochondrial O$_2$ supply and respiratory chain activity. Here, despite an increase in respiratory chain reduction, H$_2$O$_2$ release remained depressed because of limited O$_2$ availability. However, H$_2$O$_2$ release rapidly increased (lower trace) after buffer pO$_2$ was increased by increasing the stirring speed back to 8 (upper trace). This manipulation of pO$_2$ level was repeated with the same result on H$_2$O$_2$ release. These results show that the amount of superoxide/ H$_2$O$_2$ generated in reduced mitochondria at low O$_2$ concentration was only slightly less than that at high O$_2$ concentration at the start of incubation where factors inhibiting respiratory chain were absent. Furthermore, reoxygenation of mitochondria with reduced respiratory chain induced a burst of H$_2$O$_2$ release. For comparison Fig. S4B shows antimycin-induced H$_2$O$_2$ release in the same preparation and under similar conditions as in Fig. S4A, except buffer O$_2$
depletion was avoided. This relatively high and sustained H$_2$O$_2$ release requires significant (70%) inhibition of complex III activity$^{16}$ that is unlikely to happen during hypoxia/reoxygenation in Fig. S4A.

Although changes in mitochondrial H$_2$O$_2$ release during hypoxia/reoxygenation were expectedly small they support our results with matrix loaded dye, H$_2$DCF, i.e. both methods demonstrated dependence of dye oxidation on respiratory chain reduction and O$_2$ supply. Because 100% of superoxide derived from complex I and about 50% of superoxide derived from complex III is released into the matrix$^{17}$ H$_2$O$_2$ changes in that compartment are likely to be more sensitive indicator of mitochondrial ROS production, especially after decrease in matrix glutathione content. H$_2$O$_2$ diffusing out of isolated mitochondria could be assumed to represent a small part of H$_2$O$_2$ that has escaped reduction by matrix glutathione peroxidase, but primarily superoxide/ H$_2$O$_2$ produced by complex III into intermembrane space.

Compared with hypoxia/reoxygenation effect on mitochondrial H$_2$O$_2$ release antimycin promoted much higher and sustained increase in mitochondrial H$_2$O$_2$ efflux (Fig. S4B). This substantial increase in ROS production was utilized to study ROS effect on aconitase activity, matrix [Fe$^{2+}$] and modulation of ROS/Fe$^{2+}$ effect by•NO.

**Fig. S4A** Malate/glutamate (5 mM both) energized mitochondria were incubated in KCl buffer in the presence of 2.5 mM Pi, 12.5 µM Amplex red, 0.05U of horseradish peroxidase and 0.5 mg/ml bovine serum albumin (BSA). Upper trace shows changes in buffer O$_2$ concentration and lower trace changes in resorufin fluorescence depending on buffer O$_2$ supply regulated by stirring speed and
reoxygenation with compressed air. Calibration bar shows fluorescence increase in response to addition of 50 nM H$_2$O$_2$.

**S4B.** Experiment in S4A was repeated with addition of 5 μM antimycin before buffer O$_2$ level was significantly decreased with mitochondria respiration.

**References**


Fig. S2
Fig. S3

A

SNAP Fe²⁺ NO↑ 5 min F_{DCF} 100 U

B

O₂=220 μM Mito Pi Suc 6 Arg Air

C

O₂=220 μM Mito SNAP Pi Suc 5 Air N₂ O₂=0

Air

MPG

10 min F_{DCF} 100 U
Fig. S4