Cell death during ischemia: relationship to mitochondrial depolarization and ROS generation

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Levraut, Jacques, Hirotaro Iwase, Z.-H. Shao, Terry L. Vanden Hoek, and Paul T. Schumacker. Cell death during ischemia: relationship to mitochondrial depolarization and ROS generation. Am J Physiol Heart Circ Physiol 284: H549–H558, 2003. First published October 10, 2002; 10.1152/ajpheart.00708.2002.—Ischemia-reperfusion injury induces cell death, but the responsible mechanisms are not understood. This study examined mitochondrial depolarization and cell death during ischemia and reperfusion. Contracting cardiomyocytes were subjected to 60-min ischemia followed by 3-h reperfusion. Mitochondrial membrane potential (ΔΨm) was assessed with tetramethylrhodamine methyl ester. During ischemia, ΔΨm decreased to 24 ± 5% of baseline, but no recovery was evident during reperfusion. Cell death assessed by Sytox Green was minimal during ischemia but averaged 65 ± 7% after 3-h reperfusion. Cyclosporin A, an inhibitor of mitochondrial permeability transition, was not protective. However, pharmacological antioxidants, such as thiourea, an inhibitor of mitochondrial permeability transition, were attenuated by mitochondrial electron transport inhibitors, suggesting that the ROS are generated by oxidants that are generated during ischemia and cell death after reperfusion and decreased lipid peroxidation as assessed with C11-BODIPY. Cell death was also attenuated when residual O2 was scavenged from the perfusate, creating anoxic ischemia. These results suggested that reactive oxygen species (ROS) were important for the decrease in ΔΨm during ischemia. Finally, 143B-m osteosarcoma cells lacking a mitochondrial electron transport chain failed to demonstrate a depletion of ΔΨm during ischemia and were significantly protected against cell death during reperfusion. Collectively, these studies identify a central role for mitochondrial ROS generation during ischemia in the mitochondrial depolarization and subsequent cell death induced by ischemia and reperfusion in this model.

reactive oxygen species; hypoxia; oxidants

REACTIVE OXYGEN SPECIES (ROS) have been implicated as participants in the myocardial damage induced by ischemia-reperfusion (I/R) (1, 4, 9, 17, 21, 25). Most studies have focused on the importance of oxidant stress generated during reperfusion, when a burst of ROS is generated after oxygen is reintroduced into the system after a prolonged period of ischemia (32, 39). However, growing evidence suggests that oxidant stress begins during ischemia before reperfusion. For example, in cardiomyocytes subjected to simulated I/R, we observed (32, 33) an increase in ROS generation during ischemia followed by a large burst of oxidant production during the first few minutes after reoxygenation. In that model, antioxidants were more protective when given throughout the experiment than when given only at reperfusion, which supports the idea that oxidants generated during the ischemic phase contribute to cell injury and are important determinants of cell survival and recovery of function (2, 35).

ROS generation cannot occur during ischemia unless some residual O2 is still present. Previous studies using cardiomyocytes revealed that trace levels of O2 are still detectable during simulated ischemia (Po2 = 5–7 mmHg). During ischemia, indexes of oxidant stress were attenuated by mitochondrial electron transport inhibitors, suggesting that the ROS are generated by oxidants that are generated during ischemia. Collectively, observations support the notion that superoxide is generated during ischemia despite the conditions of low O2 concentration ([O2]) (11), and they suggest that these oxidants may play an important role in determining cell survival during I/R.

Although previous studies indicate that oxidants generated during ischemia may contribute to cell damage, the specific mechanism by which these ROS disrupt cellular function is not known. The present study sought to clarify the physiological consequences of oxidants generated during ischemia before reperfusion. We hypothesized that oxidant stress generated at the mitochondria during ischemia could contribute to a loss of mitochondrial membrane potential (ΔΨm), which in turn could contribute to the overall cellular injury and survival.

MATERIALS AND METHODS

Cell culture and perfusion system. Embryonic chick cardiomyocytes were prepared as previously described (35) and were grown on glass coverslips in a humidified incubator. Experiments were performed on spontaneously contracting cells at 3–5 days after isolation, under controlled O2-CO2 conditions at 37°C on an inverted microscope. A flow-through chamber was created by clamping a stainless steel spacer ring between two coverslips, allowing perfusion of the space...

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between with buffered salt solutions (BSS; 0.5 ml/min) equilibrated with O₂-CO₂ gas mixtures in a water-jacketed column. Stainless steel tubing connecting the column to the chamber prevented diffusive entry of ambient O₂ through the tubing wall.

II/R model. Cells were equilibrated for 30 min by superfusion with BSS (in mM: 120 NaCl, 18 NaHCO₃, 4 KCl, 1 MgSO₄, 0.8 NaH₂PO₄, 1.4 CaCl₂, and 5.6 glucose; 5% CO₂, pH 7.35). During simulated ischemia, cells were superfused with a variation of BSS containing 20 mM 2-deoxyglucose (2-DOG) to inhibit glycolysis, zero glucose, 8 mM KCl with a variation of BSS containing 20 mM 2-deoxyglucose (2-DOG) to inhibit glycolysis, zero glucose, 8 mM KCl and low [O₂] and hypercarbia (pH = 6.8) obtained by bubbling with 80% N₂-20% CO₂. During hypoxic acidosis, cells were superfused with BSS bubbled with 80% N₂-20% CO₂. In other experiments, complete anoxia was achieved in the ischemic or hypoxic medium by adding EC-Oxyrase (10 μl/ml), an oxidase mixture that reduces O₂ to H₂O. Anoxia was confirmed with an optical phosphorescence quenching method using a porphyrin probe in solution to measure pH₂ within the perfusion chamber (Oxyspot) (24). After ischemia or hypoxia/anoxia (1 h), reperfusion was carried out with normoxic (21% O₂, 5% CO₂) gas. TMRE loading properties in cardiomyocytes, and the nonquenching characteristics of this fluorophore in assessing membrane potential under nonquenching conditions were reported previously (6). Stable levels of fluorescence were typically observed under baseline conditions (Fig. 1A). This fluorescence was rapidly dissipated on addition of the protonophore FCCP, consistent with the expected loss of ΔΨₘ induced by this uncoupling agent.

To assess the effects of ischemia on ΔΨₘ, cells loaded with TMRE were studied for 30 min under baseline normoxic conditions followed by 60 min of simulated ischemia. During ischemia, a progressive decrease in TMRE fluorescence was observed, reaching 24.0 ± 5.5% of the initial intensity after 1 h. Minimal recovery of TMRE fluorescence was seen after return to normoxia (reperfusion; Fig. 1B). Cell death within the same field of cells was minimal (<5%) at the end of ischemia but increased significantly during 3-h reperfusion (Fig. 1C). Inspection of TMRE fluorescence images revealed that the majority of cells lost all fluorescence during ischemia and some cells retained some fluorescence at an attenuated level. Reperfusion was not associated with significant recovery of fluorescence in either case.

Mitochondrial depolarization could conceivably be caused by activation of the mitochondrial permeability transition (MPT) pore, a high-conductance putative channel in the inner mitochondrial membrane (10). The opening of the MPT pore has been suggested to contribute to the decrease in ΔΨₘ during I/R injury (18). Cyclosporin A inhibits the opening of this pore and was therefore used to evaluate its contribution to membrane depolarization and cell death during I/R. Cyclosporin A (0.2 μM) had no significant effect on membrane depolarization during simulated ischemia (Fig. 2A) and had no significant effect on cell death after 3-h reperfusion (Fig. 2B). Additional studies using a higher concentration (0.5 μM) also failed to abolish the fall in TMRE fluorescence (data not shown). These results suggest that opening of the MPT pore does not contribute significantly to the depolarization and cell death in this model.
We previously found (33) evidence of mitochondrial oxidant stress during ischemia, before the start of reperfusion. To determine whether ROS generation during ischemia contributed to the fall in $\Delta \psi_m$ and subsequent cell death in this model, pharmacological antioxidants were added to the perfusate throughout the experiment and the effects on depolarization and cell death were assessed (Table 1). The thiol reductants 2-mercaptopropionyl glycine (2-MPG; 400 $\mu$M) and pyrrolidine dithiocarbamate (PDTC, 10 $\mu$M) significantly

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<th>Fall in $\Delta \psi_m$ (end of ischemia), %</th>
<th>Cell Death (end of reperfusion), %</th>
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<tr>
<td>Control</td>
<td>9 76 ± 5.5</td>
<td>66 ± 7</td>
</tr>
<tr>
<td>2-MPG (0.4 mM)</td>
<td>4 31 ± 13</td>
<td>18 ± 15</td>
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<tr>
<td>NAC (0.5 mM)</td>
<td>4 34 ± 15</td>
<td>15 ± 5</td>
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<tr>
<td>PDTC (10 $\mu$M)</td>
<td>4 31 ± 10</td>
<td>17 ± 5</td>
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<tr>
<td>1,10-Phenanthroline (10 $\mu$M)</td>
<td>4 20 ± 10</td>
<td>6 ± 1</td>
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Values are means ± SE for $n$ fields of cells. $\Delta \psi_m$, mitochondrial potential; 2-MPG, 2-mercaptopropionyl glycine; NAC, N-acetylcysteine; PDTC, pyrrolidine dithiocarbamate.
attenuated the fall in ΔΨm at the end of ischemia, as did N-acetyl-L-cysteine (NAC; 0.5 mM). Likewise, the metal chelator 1,10-phenanthroline significantly attenuated the decrease in ΔΨm during ischemia. These antioxidant compounds also significantly lessened cell death after 3-h reperfusion. Collectively, these studies suggested that oxidant stress during ischemia contributes to the fall in ΔΨm.

ROS generated from the mitochondrial electron transport chain can induce cardiolipin oxidation within the inner mitochondrial membrane (29). This oxidative damage could contribute to the observed membrane depolarization by compromising the integrity of the inner membrane. To detect lipid peroxidation, cardiomyocytes loaded with the lipophilic probe C11-BODIPY were subjected to simulated I/R. During ischemia, a significant increase in fluorescence was observed, consistent with an increase in the oxidation of this fluorophore. If H2O2 is required for the oxidative damage during ischemia, then lipid peroxidation should be attenuated if SOD is inhibited. Accordingly, the Cu,Zn-SOD inhibitor diethyldithiocarbamate (DDC; 1 mM) was used to attenuate SOD activity. This caused a significant attenuation in C11-BODIPY fluorescence (Fig. 3A). In replicate experiments, the rate of increase in fluorescence during ischemia (0.77 ± 0.19 arbitrary units (a.u./min); n = 6) was significantly greater (P < 0.01) than during baseline (0.04 ± 0.09 a.u./min; n = 6). Administration of DDC significantly decreased the slope of this relationship (−0.43 ± 0.13, n = 3; P < 0.01), suggesting that H2O2 contributes to lipid peroxidation. If free iron in the cell contributes to hydroxyl radical generation by the Fenton reaction, then iron chelation should also attenuate lipid peroxidation. Addition of the chelator 1,10-phenanthroline (10 μM) during ischemia caused a significant decrease in the fluorescence signal (Fig. 3B). In replicate experiments, 1,10-phenanthroline administration significantly decreased the rate of fluorescence increase (−0.63 ± 0.13 a.u./min, n = 3; P < 0.01) compared with ischemia. To determine whether mitochondria are the source of these oxidants, the electron transport inhibitor myxothiazol was added during ischemia to inhibit ROS generation by complex III (30). Myxothiazol blocks electron transfer from ubiquinol to the Rieske iron-sulfur center in complex III, thereby preventing the generation of ubisemiquinone, which is a major source of superoxide generation. Myxothiazol (2 μM) significantly attenuated the rate of increase in fluorescence during ischemia (−0.026 ± 0.106 a.u./min, n = 3). Addition of DMSO, the solvent used in myxothiazol experiments, produced no detectable effect.

ROS generation during ischemia most likely begins with univalent electron transfer to O2, thereby generating superoxide. This process requires that some residual O2 must still be present to provide substrate for that reaction. In previous studies (33) we found that low levels of O2 were present during ischemia in this model. In the present study, O2 tension within the flow-through chamber was assessed with a phosphorescence quenching technique previously shown to be accurate at low [O2] (24, 37). During simulated ische-
The O₂ tension decreased progressively, reaching a value of \( \sim 7 \) mmHg within \( \sim 10 \) min (Fig. 4). Therefore, it is possible that superoxide could be generated using residual O₂ that is present during ischemia.

If residual O₂ contributes to the generation of superoxide during ischemia, and if these oxidants contribute to mitochondrial depolarization and cell death, then significant protection should ensue if residual O₂ is scavenged from the system during ischemia. To test this, an enzymatic O₂ scavenger was added to the perfusate to create anoxic conditions during ischemia, thereby limiting the availability of O₂ as an electron acceptor. Anoxic conditions (\( PO_2 = 0 \) mmHg) were created by adding EC-Oxyrase to the ischemia buffer after it had been equilibrated with 80% N₂-20% CO₂. Measurements confirmed that this decreased the \( PO_2 \) within the chamber from \( \sim 7 \) to \( <0.1 \) mmHg during ischemia. Heat inactivation of EC-Oxyrase (100°C for 15 min) abolished its O₂-scavenging properties (data not shown). During anoxic ischemia (\( PO_2 = 0 \) mmHg), the decrease in \( \Delta \psi_m \) was attenuated (43 ± 7% decrease; Fig. 5A) and cell death after reperfusion was lessened (12 ± 6%; \( P < 0.001 \)) compared with standard hypoxic ischemia (66 ± 7%; \( PO_2 = 7 \) mmHg) (Fig. 5C). Heat treatment of EC-Oxyrase abolished its protective effects on membrane potential and cell death (data not shown). These findings suggested that residual O₂ contributes to cell death and the irreversible decline in \( \Delta \psi_m \) during I/R.

During reperfusion after anoxic ischemia, TMRE fluorescence increased progressively, indicating a resto-
ration of $\Delta \Psi_m$ (Fig. 5A). By contrast, minimal evidence of recovery was seen during reperfusion after standard hypoxic ischemia. This suggested that the mechanism responsible for loss of $\Delta \Psi_m$ may be different between hypoxic and anoxic ischemia. Normally, $\Delta \Psi_m$ reflects a balance between the rate of proton extrusion from the mitochondrial matrix (a function of the rate of electron transport) and the rate at which protons reenter the matrix (a function of ATP synthase activity and/or ion leaks). During anoxia, electron flux should cease. Therefore, $\Delta \Psi_m$ should decrease unless glycolytic ATP is available to maintain $\Delta \Psi_m$ through reverse operation of the ATP synthase. During ischemia in our model, inhibition of glycolysis by 2-DOG may have prevented reverse operation of the ATP synthase. To explore the mechanism responsible for the fall in $\Delta \Psi_m$ during anoxia, cells were subjected to anoxia under the same acidic conditions used for ischemia (20% CO$_2$), except that glucose was added to the perfusate and 2-DOG was omitted to permit glycolysis to continue. During anoxic acidosis, no decrease in $\Delta \Psi_m$ was observed (Fig. 5B) and minimal cell was evident after reperfusion (Fig. 5C), which suggested that $\Delta \Psi_m$ was maintained during anoxia by reverse operation of the ATP synthase. To confirm that reverse operation of the ATP synthase was responsible for sustaining $\Delta \Psi_m$ during anoxia when glycolysis remained functional, oligomycin (10 $\mu$M) was added to inhibit the ATP synthase during anoxic acidosis. Under those conditions, $\Delta \Psi_m$ decreased significantly (Fig. 5B). During reperfusion (21% O$_2$, 5% CO$_2$) without oligomycin, clear evidence of mitochondrial repolarization was evident because proton pumping was restored when electron transport resumed and the mitochondria membrane integrity was not compromised. The decrease in $\Delta \Psi_m$ caused by anoxic acidosis with oligomycin was associated with relatively low cell death (Fig. 5C). These findings indicate that low levels of residual O$_2$ during ischemia are injurious because they contribute to an irreversible decline in $\Delta \Psi_m$. By contrast, the decline in $\Delta \Psi_m$ caused by anoxia plus glucose deprivation is reversible and associated with minimal cell death.

Preliminary studies of standard hypoxic ischemia suggested that a correlation may exist between the magnitude of the decrease in $\Delta \Psi_m$ and subsequent cell death. To determine whether such a dose-response relationship exists, we experimentally varied the severity of ischemia by adjusting the residual level of O$_2$ during ischemia without changing its duration (1 h). In these experiments the Po$_2$ during ischemia was increased from 7 mmHg (hypoxic ischemia) to ~15 mmHg. Subsequent cell death was measured after 3 h reperfusion. As shown in Fig. 6, the extent of cell death during reperfusion was significantly attenuated when the fall in $\Delta \Psi_m$ during ischemia was less severe. This suggested that the magnitude of mitochondrial depolarization during ischemia might contribute mechanistically to the cell death measured at the end of reperfusion.

To determine the significance of mitochondrial ROS generation for the depolarization and cell death during I/R, $\rho^0$-cells were loaded with TMRE and studied during simulated ischemia. Despite a lack of electron transport, $\rho^0$-cells maintain $\Delta \Psi_m$ by ATP/ADP exchange via the adenine nucleotide translocator in the inner membrane (5). Wild-type 143B osteosarcoma cells demonstrated a marked depletion of $\Delta \Psi_m$ during ischemia that was qualitatively similar to that seen in cardiomyocytes (Fig. 7). However, ischemia failed to produce a similar depletion of TMRE fluorescence in $\rho^0$-cells. Cell death in wild-type cells averaged 82.2 \pm 9.9% vs. 28.7 \pm 7.5% in the mitochondria-deficient cells ($P < 0.001$). Thus the $\rho^0$-cells were protected against mitochondrial depolarization and subsequent cell death compared with wild-type cells.

**DISCUSSION**

These studies demonstrate that mitochondria undergo a significant and irreversible decrease in potential during ischemia. The degree of depolarization correlates with the extent of cell death during reperfusion. However, mitochondrial depolarization by itself does not cause cell death, because administration of anoxia plus oligomycin caused a reversible mitochondrial depolarization without causing significant cell death. Mitochondrial depolarization during ischemia was triggered by ROS generated from the mitochondrial electron transport chain despite the low [O$_2$] conditions. These oxidants appear to initiate a cascade of lipid peroxidation that disrupts the integrity of the inner mitochondrial membrane, thereby preventing repolarization during reperfusion. Activation of the MPT pore apparently did not contribute to this process, because attempts to inhibit the activation of that pore failed to prevent depolarization or cell death. By contrast, a variety of pharmacological antioxidant compounds attenuated both the fall in $\Delta \Psi_m$ and subsequent cell death. Furthermore, scavenging of residual O$_2$ during ischemia prevented the depletion of $\Delta \Psi_m$.
and significantly protected cells. Finally, 143B cells lacking a mitochondrial electron transport chain failed to demonstrate a depletion of $\Delta \Psi_m$ during ischemia and were significantly protected against cell death during reperfusion. Collectively these studies identify a central role for mitochondrial oxidant generation during ischemia in the irreversible mitochondrial depolarization and subsequent cell death induced by ischemia and reperfusion in this model (Fig. 8).

Role of ROS during I/R. ROS have long been associated with I/R injury. It is increasingly evident that ROS play diverse roles in I/R, ranging from protective effects at one extreme to damage-inducing effects at the other. For example, low levels of oxidants appear to function as signaling agents during the induction of ischemic preconditioning (13, 26, 31). The activation of preconditioning confers significant protection against subsequent lethal ischemia. In contrast, higher levels of oxidant stress are observed at the start of reperfusion, when a transient burst of ROS generation is observed that correlates with subsequent cell death (32). During ischemia before reperfusion, ROS are generated by the electron transport chain of mitochondria (33, 34), although the significance of these oxidants in cell injury is not fully understood. The present study focused on the relationship between oxidant stress during ischemia, cell survival, and the fall in $\Delta \Psi_m$ in a cardiomyocyte model.

Relationship between ischemic ROS and $\Delta \Psi_m$. $\Delta \Psi_m$ is normally maintained by proton pumping, which is linked to the rate of electron transport. If the O$_2$ tension in the cell falls below a critical level of 1–4 mmHg, $\Delta \Psi_m$ should decrease because electron transport becomes limited by the availability of O$_2$ at cytochrome oxidase. In our study, large decreases in TMRE fluorescence were observed during ischemia, indicating that a significant fall in $\Delta \Psi_m$ must have occurred. However, the fall in $\Delta \Psi_m$ could not be explained by a lack of O$_2$, because the O$_2$ tension did not fall below the critical level during the ischemic exposure (19). Moreover, $\Delta \Psi_m$ failed to show significant recovery during reperfusion, which suggests that the mitochondria sustained an irreversible injury during the ischemic exposure. The extent of cell death after 3-h reperfusion correlated significantly with the extent of the loss in $\Delta \Psi_m$, which suggests that the mitochondrial injury sustained during ischemia could contribute to cell death during reperfusion.

Several observations support the conclusion that ROS contribute importantly to the decline in $\Delta \Psi_m$.
during ischemia. First, a variety of chemically dissimilar antioxidant compounds given during ischemia were able to attenuate the fall in ΔΨ\textsubscript{m} and to significantly lessen cell death after reperfusion. Second, the scavenging of residual O\textsubscript{2} during anoxic ischemia abrogated both the fall in ΔΨ\textsubscript{m} and later cell death. Both the enzymatic activity of EC-Oxyrase and its protective effects were abolished by heat denaturation, which indicates that the protection it provided was due to its O\textsubscript{2} scavenging properties. The studies with anoxic ischemia indicate that low residual levels of O\textsubscript{2} during ischemia are important for mitochondrial depolarization and cell death because they act as a substrate for the generation of ROS. Third, the results with C11-BODIPY indicate that lipid peroxidation occurs during ischemia and that ROS originate from the mitochondrial electron transport chain. Fourth, the ρ\textsuperscript{0}-cells lacking an electron transport chain failed to exhibit mitochondrial depolarization and were significantly protected against cell death during reperfusion. We conclude that mitochondrial ROS generation during ischemia contributes importantly to the fall in ΔΨ\textsubscript{m}.

This response is likely due to the formation of lipid peroxides, which could undermine ΔΨ\textsubscript{m} by destabilizing the inner membrane. A loss of membrane integrity could also explain why ΔΨ\textsubscript{m} failed to recover when normal O\textsubscript{2} levels were restored during reperfusion.

This conclusion is consistent with the findings of Lesniewski et al. (23), who demonstrated that cardiolipin levels decrease during ischemia in subsarcolemmal mitochondria. Cardiolipin is a membrane phospholipid found in high abundance within mitochondria that interacts with electron transport proteins (14, 15) and may also be important for maintaining the integrity of the inner membrane in terms of its ability to support the transmembrane potential. Mitochondrial oxidant generation during ischemia may explain the loss of cardiolipin, which could contribute to the mitochondrial dysfunction associated with I/R (28). Alternatively, oxidants could contribute to mitochondrial damage through direct oxidation of other lipids and proteins or by promoting the opening of the MPT pore (3). Each of these mechanisms could contribute to mitochondrial depolarization because of their effects on electron transport and/or mitochondrial membrane integrity. However, our findings suggest that the decrease in ΔΨ\textsubscript{m} was not a result of MPT pore opening, because cyclosporin A treatment had no significant effect on the decrease in membrane potential during ischemia or the extent of cell death. This conclusion is consistent with previous studies suggesting that MPT pore opening is unlikely to occur under the low pH conditions of ischemia and is more likely to occur after reperfusion (18, 20); indeed, we observed a fall in ΔΨ\textsubscript{m} during ischemia before reperfusion. However, other investigators have used higher (10, 16) or lower (38) concentrations of cyclosporin A to inhibit the opening of that pore, so it is not clear whether protection would have been observed at different concentrations or with other inhibitors of the MPT pore.

The data suggest that H\textsubscript{2}O\textsubscript{2} and hydroxyl radicals, rather than superoxide, are responsible for the oxidative damage to mitochondria. Normally, superoxide degradation by SOD is an important step in preventing oxidant injury by that radical, so it is surprising that SOD inhibition is protective during ischemia. One explanation is that H\textsubscript{2}O\textsubscript{2} may pose an unusual threat to the cell under conditions of ischemia, when release of iron from sites where it is normally chelated could facilitate hydroxyl radical generation via the Fenton reaction. Relative to hydroxyl radical, superoxide is far less reactive and may be less injurious during relatively short periods of ischemia.

**Relationship between mitochondrial depolarization and cell death.** We observed a significant correlation between the fall in TMRE fluorescence and cell death. When the magnitude of the fall in ΔΨ\textsubscript{m} was manipulated by adjusting the severity of the ischemia, cell survival was found to be worse in experiments where the fall in ΔΨ\textsubscript{m} was larger. Previous studies in cardiomyocytes demonstrated that ROS generation tends to increase as O\textsubscript{2} tension is lowered from 35 to 7 mmHg (6). We therefore suggest that milder ischemia was protective in the present study because of the lesser oxidant stress it generated and the associated decrease in oxidant damage to membranes. An oxidant-mediated disruption of mitochondrial inner membrane integrity during ischemia could conceivably lead to cell death by promoting matrix swelling, release of cytochrome c to the cytosol, and activation of the cell apoptotic machinery (36). However, mitochondrial depolarization by itself was not lethal to these cells, as evidenced by the minimal extent of death observed when ΔΨ\textsubscript{m} was depleted with anoxic acidosis plus oligomycin. For a given degree of depolarization, it is conceivable that matrix swelling caused by lipid peroxidation and loss of membrane integrity is more severe than when caused simply by electron transport inhibition. In the former case, reoxygenation would not promote recovery of ΔΨ\textsubscript{m} because the loss of membrane integrity would defeat the effects of proton pumping. In the latter case, a restoration of electron transport during reoxygenation would allow ΔΨ\textsubscript{m} to recover.

Interestingly, hypoxia alone (PO\textsubscript{2} \textapprox 7 mmHg) was not sufficient to induce significant cell death in this study. Likewise, hypercapnic acidosis (20% CO\textsubscript{2}) was well tolerated under normoxic conditions. However, when the two conditions were combined, >60% cell death resulted. Acidosis may increase cell death by causing the release of Fe\textsuperscript{2+} from intracellular sites where it is normally chelated. The ROS released in response to hypoxia alone appear to be well tolerated by cells (7). However, in a setting where acidosis causes release of iron ions, these ROS may lead to generation of hydroxyl radicals as a consequence of Fenton interactions. The resulting loss of membrane integrity could explain the observed decrease in ΔΨ\textsubscript{m} and subsequent failure to recover during reoxygenation. This interpretation is consistent with our observation that an iron chelator was most protective of ΔΨ\textsubscript{m} and cell viability. Although the importance of the Fenton reaction in I/R...
injury is not new (1, 4, 9, 17, 21, 25), its potential involvement in cellular injury during ischemia before reperfusion has not been explored previously, to our knowledge.

In summary, these studies reveal that ROS generated during ischemia contribute to the irreversible loss of $\Delta V_m$. The extent of this damage correlates with the extent of cell death during reperfusion, and interventions that minimize the oxidative stress during ischemia also attenuate the loss of $\Delta V_m$. These findings therefore suggest that oxidant generation during ischemia before reperfusion plays a significant role in determining cell death during reperfusion.

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