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Reactive oxygen species, but not Ca$^{2+}$ overloading, trigger pH- and mitochondrial permeability transition-dependent death of adult rat myocytes after ischemia-reperfusion

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reactive oxygen species (ROS), Ca$^{2+}$, and the mitochondrial permeability transition (MPT) in pH-dependent ischemia-reperfusion injury to adult rat myocytes. Myocytes were incubated in anoxic Krebs-Ringer-HEPES buffer at pH 6.2 for 3 h to simulate ischemia. To simulate reperfusion, myocytes were reoxygenated at pH 6.2 or 7.4 for 2 h. Some myocytes were treated with MPT blockers (cyclosporin A and N-methyl-4-isoleucine cyclosporin) and antioxidants (desferal, diphenylhexylene diamine, and 2-mercapto propionyl glycin). Mitochondrial membrane potential, inner membrane permeabilization, and ROS formation were imaged with tetramethylrhodamine methyl ester, calcein, and chloromethyl dichlorofluorescin diacetate, respectively. For Ca$^{2+}$ imaging, myocytes were coloaded with rhod-2 and fluo-4 to evaluate mitochondrial and cytosolic Ca$^{2+}$, respectively. After 10 min of reperfusion at pH 7.4, calcein redistributed across the mitochondrial inner membrane, an event preceded by mitochondrial ROS formation and accompanied by hypercontracture, mitochondrial depolarization, and then cell death. Acidotic reperfusion, antioxidants, and MPT blockers each prevented the MPT, depolarization, hypercontracture, and cell killing. Antioxidants, but neither MPT blockers nor acidic reperfusion, inhibited ROS formation after reperfusion. Furthermore, anoxic reperfusion at pH 7.4 prevented cell death. Both mitochondrial and cytosolic Ca$^{2+}$ increased during ischemia but recovered in the first minutes of reperfusion. Mitochondrial and cytosolic Ca$^{2+}$ overloading again occurred late after reperfusion. This late Ca$^{2+}$ overloading was blocked by MPT inhibition. Intramitochondrial Ca$^{2+}$ chelation by cold loading/warm incubation of BAPTA did not prevent cell death after reperfusion. In conclusion, mitochondrial ROS, together with normalization of pH, promote MPT onset and subsequent myocyte death after reperfusion. In contrast, Ca$^{2+}$ overloading appears to be the consequence of bioenergetic failure after the MPT and is not a factor promoting MPT onset.

cyclosporin A; lactate dehydrogenase; mitochondrial membrane potential; hypercontracture

Tissue ischemia causes anoxia, ATP depletion, glycolytic substrate exhaustion, and acidosis. The last of these, acidosis, protects against the onset of necrotic cell death (5, 6, 23, 51). However, upon reperfusion, recovery of normal intracellular pH is a stress that precipitates cell death, a “pH paradox” (26). In neonatal myocytes and blood-perfused rabbit papillary muscles, such a pH paradox is directly associated with reperfusion-induced cell death (5, 6, 23). However, the precise mechanisms in pH-dependent reperfusion injury remain incompletely understood.

One mechanism prominent in pH-dependent ischemia/reperfusion injury is an onset of the mitochondrial permeability transition (MPT) (45). Opening of high-conductance permeability transition (PT) pores in the mitochondrial inner membrane causes an onset of the MPT, leading to ATP depletion and necrotic cell death (oncosis) (10, 38). The MPT also causes large-amplitude mitochondrial swelling, outer membrane rupture, and release of cytochrome-c and other factors from the intermembrane space into the cytosol that initiate apoptotic signaling (30, 52).

Cyclosporin A (CsA) and its immunosuppressive analogs, like N-methylvaline cyclosporin and N-methyl-4-isoleucine cyclosporin (NIM811), block opening of PT pores (50). These compounds prevent both apoptotic and necrotic cell death after ischemia-reperfusion and other toxic stresses in various models (14, 44, 45). Pharmacological protection by CsA does not necessarily prove to be a key role of the MPT in reperfusion injury, because CsA also inhibits calcineurin, a calcium-dependent protein phosphatase (20). Moreover, CsA has a relatively narrow concentration range of efficacy and becomes toxic at higher doses. Direct monitoring of mitochondrial inner membrane permeability from mitochondrial entrapment of 2-[3H]-deoxyglucose, however, confirms that mitochondrial inner membrane permeabilization actually occurs after ischemia-reperfusion to myocardium (15). Deoxyglucose trapping to monitor the MPT has some practical limitations, particularly the time required to isolate mitochondria and the inability to monitor changes of PT pore activity continuously after reperfusion. To detect the MPT in living cells, our group (41) developed an approach using laser-scanning confocal microscopy to visualize movement of the green fluorescent dye calcein across the mitochondrial inner membrane as PT pores open. With the use of this technique, a CsA-sensitive MPT was directly documented after oxidative stress and reperfusion of ischemic rat hepatocytes (39, 45).

The mechanisms contributing to ischemia-reperfusion injury to myocardium are incompletely understood. Antioxidants protect ischemic myocardium against ischemia-reperfusion injury, suggesting a cytotoxic role of reactive oxygen species (ROS)

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(4), whereas other studies also suggest that ROS play trigger roles in cardioprotection conferred by ischemic preconditioning (2, 48). Ca\textsuperscript{2+} overloading has been suggested to play a causative role in ischemic myocardial dysfunction (1, 37), but Ca\textsuperscript{2+} homeostasis after reperfusion, particularly in mitochondria, remains unclear (28, 36, 46). Accordingly, the aims of the present study were to investigate the role of the MPT in ischemia-reperfusion injury in adult rat myocytes and to characterize the roles of ROS and Ca\textsuperscript{2+} in MPT-mediated lethal reperfusion injury. Our results indicate that mitochondrial ROS formation, but not Ca\textsuperscript{2+} overloading, upon reperfusion induces the MPT and bioenergetic failure, leading to Ca\textsuperscript{2+} dysregulation and necrotic death.

**MATERIALS AND METHODS**

**Isolation and culture of adult rat cardiac myocytes.** Animals received humane care according to the protocols approved by the Institutional Care and Use Committee of the University of North Carolina. Adult rat ventricular cardiac myocytes were isolated by collagenase digestion, as described previously (42). Briefly, male Sprague-Dawley rats (250–350 g) were given heparin sodium (100 U) and pentobarbital sodium (15 mg) for anesthesia by intraperitoneal injection. The heart was then removed and mounted on a Langendorff perfusion apparatus using nonrecirculating retrograde perfusion with Krebs-Henseleit bicarbonate buffer (KHB) containing (in mM) 118 NaCl, 25 NaHCO\textsubscript{3}, 4.7 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.25 CaCl\textsubscript{2}, and 10 glucose (pH 7.4), saturated with 95% O\textsubscript{2}-5% CO\textsubscript{2} at 37°C from a height of 100 cm at a rate of 15 ml/min. After 5 min, the heart was perfused with calcium-free KHB for 5 min. Collagenase type 2 (3 U/ml, Worthington Biochemical, Lakewood, NJ) was then added to the calcium-free KHB buffer, and the heart was perfused in a recirculating mode. After 15 min, the heart was removed from the apparatus, and the ventricles were separated below atrioventricular junction. After four incisions toward the apex, the tissue was placed into a beaker containing calcium-free KHB buffer. The ventricles were agitated in a shaking bath (37°C) at a rate of 50 rpm for 30 min to release rod-shaped myocytes. The cells were filtered through nylon mesh and allowed to settle by gravity for 5 min. The cell pellet was subsequently suspended in an incubation buffer containing (in mM) 118 NaCl, 25 NaHCO\textsubscript{3}, 4.7 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 10 glucose, 30 HEPES, 60 taunire, and 20 creatine and 1% bovine serum albumin, vitamins (Sigma Chemicals, St. Louis, MO), and amino acids (Sigma Chemicals) at pH 7.4, 37°C. Calcium concentration was gradually increased to 1.2 mM over 15 min. After centrifugation at 20 g for 30 s, myocytes were resuspended in medium 199 containing 10 units/ml penicillin, 10 μg/ml streptomycin, 5% fetal calf serum, and 100 nM insulin. With the use of this method, each heart yielded 0.5–1×10\textsuperscript{6} rod-shaped myocytes with viability >80%, as determined by trypan blue exclusion. For viability assays, aliquots (1 ml) of 3×10\textsuperscript{6} cells were plated onto 24-well microtiter plates (Falcon, Lincoln Park, NJ). For confocal microscopic studies, 9×10\textsuperscript{4} cells were cultured on 42-mm round glass coverslips in 60-mm culture dishes. All plates and coverslips were coated with 0.25 mg/ml laminin. Myocytes were allowed to attach for 1 h in humidified 5% CO\textsubscript{2}-95% air at 37°C and then washed once to remove unattached cells. Cells were subsequently cultured in medium 199 for 4–12 h. All experiments were conducted within 18 h after plating.

**Simulation of ischemia/reperfusion in cultured adult rat cardiac myocytes.** To simulate the anoxia, substrate depletion, and acidosis of ischemia, myocytes were incubated in Krebs-Ringer-HEPES buffer (KRH) containing (in mM) 115 NaCl, 5 KCl, 1 CaCl\textsubscript{2}, 1 KH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, and 25 HEPES buffer (pH 6.2) in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) for 3 h. Anoxia in the anaerobic chamber was maintained under an atmosphere of 90% N\textsubscript{2}-10% H\textsubscript{2} in the presence of a heated palladium catalyst to convert residual oxygen to water vapor. Oxygen tension in the chamber was <0.001 Torr. To simulate the reoxygenation and return to physiological pH of reperfusion, anaerobic KRH at pH 6.2 was replaced with aerobic glucose-free KRH at pH 7.4 or 6.2. This model is widely used to study mechanisms of ischemia-reperfusion injury in cells cultured from heart and other tissues (6, 25, 45). For simplicity and directness of expression, we refer to “simulated ischemia-reperfusion” simply as “ischemia-reperfusion.” Some myocytes were treated 20 min before and then continuously after reperfusion with CsA; tacrolimus, a calcineurin inhibitor; or Nim811, a nonimmunosuppressive MPT blocker. In other experiments, myocytes were treated with antioxidans, including 0.5 mM desferal, 1 μM diphenylphhenylenediamine (DPPD), and 5 mM 2-mercapto propionyl glycine (2-MPG), beginning 20 min before reperfusion and continuously thereafter.

**Assay for cell death.** Cell death was assessed by propidium iodide (PI) fluorometry using a multiwell fluorescence reader (BMG Labtechnologies, Offenburg, Germany), as previously described (40). Briefly, myocytes were incubated in KRH containing 50 μM PI. Fluorescence from each well was measured at excitation and emission wavelengths of 530 nm and 590 nm, respectively. For each well, fluorescence at 20 min after addition of PI (A) was measured before ischemia and then at given times thereafter (X). Experiments were terminated by permeabilizing plasma membranes with 375 μM digitonin. After 40 min, final fluorescence (B) was measured. The percentage of nonviable cells (D) was calculated as D = 100(X - A)/(B - A). PI fluorometry reflects necrotic cell death and correlates closely with trypan blue uptake and enzyme release (25, 40). To examine anoxic reperfusion, myocytes cultured on 24-well microtiter plates were exposed to 3 h of ischemia at pH 6.2, and extracellular pH was increased to 7.4 without reoxygenation. To prevent oxygen back diffusion during the cell death assay, plates were sealed with vacuum tape (3M, Minneapolis, MN) inside the anaerobic chamber.

**Assay for lactate dehydrogenase release.** Release of lactate dehydrogenase (LDH) into the incubation medium was measured spectrophotometrically (40). Total activity of LDH before ischemia-reperfusion was determined after permeabilization of myocytes with 375 μM digitonin. LDH release in the incubation medium was expressed as the percentage of total LDH activity.

**Loading with tetramethylrhodamine methyl ester, calcein, and chloromethyl dihydrodichlorofluorescein.** Myocytes cultured on glass coverslips were loaded with tetramethylrhodamine methyl ester (TMRM) and calcein in KRH to monitor mitochondrial membrane potential and inner membrane permeability, respectively, by modification of procedures described earlier (45). Briefly, myocytes were incubated at pH 6.2 in KRH containing 3 μM PI in the anoxic chamber for 3 h. During the last 20 min of ischemic incubation, 100 nM TMRM was added. Reperfusion was then instituted with aerobic KRH containing 30 nM TMRM and 3 μM PI.

For calcein loading, myocytes were incubated with 1 μM calcein-AM at room temperature for 30 min in aerobic medium 199 containing 20 mM HEPES. The cells were then incubated in medium 199 without HEPES in humidified 5% CO\textsubscript{2}-95% room air at 37°C for 3 h and then subjected to 3 h of ischemia in KRH. With this cold loading/warm incubation procedure, most calcein localized to mitochondria (32, 47).

To evaluate ROS formation, myocytes were loaded with 10 μM chloromethyl dihydrodichlorofluorescein (cmHDCF) diacetate (cmH2DCF-DA) 30 min before reperfusion by modification of earlier procedures using dihydrodichlorofluorescein diacetate (39), cmHDCF fluorescence was measured with a fluorescence plate reader and confocal microscope.

**Loading of myocytes with Ca\textsuperscript{2+} indicators rhod-2 and fluo-4.** To monitor mitochondrial Ca\textsuperscript{2+}, myocytes on glass coverslips were loaded with 10 μM rhod-2 AM by the cold loading/warm incubation, as described for calcein loading. To monitor cytosolic Ca\textsuperscript{2+}, cells were coloaded with 10 μM fluo-4 AM in medium 199 at 37°C for 30 min before onset of ischemia. For confocal imaging of Ca\textsuperscript{2+} during...
ischemia, myocytes were incubated in anoxic KRH at pH 6.2 in the presence of exogenous oxygen-consuming respiratory complexes (3.3% Oxyrase, Oxyrase, Mansfield, OH) to prevent back diffusion of oxygen into the gas-tight chamber (18, 33). After 3 h of ischemia, reperfusion was instigated by infusing air-saturated KRH at pH 7.4.

**Intramitochondrial loading of myocytes with Ca\textsuperscript{2+}-chelator BAPTA.** To chelate intramitochondrial Ca\textsuperscript{2+} during ischemia-reperfusion, myocytes cultured on 24-well microtiter plates were incubated with 1 to 50 μM BAPTA-AM, a Ca\textsuperscript{2+} chelator, by the cold loading/warm incubation protocol (see above), as described previously (9). Myocytes were then subjected to 3 h of ischemia, and cell death was fluorometrically assessed during 2 h of reperfusion.

**Laser-scanning confocal microscopy.** The red fluorescence of TMRM and PI and the green fluorescence of calcein and cmDCF were imaged by using an inverted Zeiss 510 laser-scanning confocal microscope equipped with a ×63 numerical aperture, 1.4 oil-immersion planapochromat lens. Temperature on the microscope stage was maintained at 37°C. Green and red fluorescence were excited with 488 nm and 543 nm light, respectively. Emission was separated by a 545 nm dichroic mirror and directed through 500- to 530-nm band-pass (green) and 560-nm long-pass (red) barrier filters. Pinholes were set to Airy units of 1.0 in both channels.

**Statistics.** Differences between means were compared by the Student’s t-test or ANOVA using P < 0.05 as the criterion of significance. Data were expressed as means ± SE, except when noted otherwise. All experiments are representative of at least three different cell isolations.

**RESULTS**

**pH-dependent ischemia-reperfusion injury to myocytes.** To simulate the anoxia, substrate depletion, and acidosis of myocardial ischemia, cultured myocytes were incubated in anerobic KRH at pH 6.2 for 3 h. During 3 h of ischemia, most cells shortened (data not shown) but remained viable, as judged by LDH release (Fig. 1A). These results are consistent with previous findings that acidic pH strongly protects against hypoxic cell injury in neonatal myocytes and other cell types (6, 45). To simulate reperfusion, myocytes were reoxygenated with KRH at pH 7.4. Cell death then occurred in a time-dependent manner and increased to 60% after 2 h of reperfusion (Fig. 1, A and B). However, when myocytes were reoxygenated with KRH at pH 6.2, virtually no cell death occurred (Fig. 1B). These results show that a return to normal pH is required to induce reperfusion-induced death in this model. The results of Fig. 1, A and B, also illustrate that cell death measured by the PI fluorometry gives results virtually identical to LDH release.

**Protection by cyclosporin A and NIM811 against pH-dependent reperfusion injury.** To test whether the onset of the MPT contributes to pH-dependent reperfusion injury, ischemic myocytes were reperfused in the presence of CsA, an MPT blocker. Lower concentrations of CsA (0.5-1 μM) were cyttoprotective, whereas a higher concentration (5 μM) was cytotoxic (Fig. 1C). This dose-response relationship for CsA was similar to earlier findings (14, 16, 45).

CsA also inhibits calcineurin, a calcium-dependent protein phosphatase (20). Thus suppression of cell death by CsA might be the consequence of calcineurin inhibition. Accordingly, myocytes after 3 h of ischemia were reperfused with 1 μM tacrolimus, an immunosuppressive agent that inhibits calcineurin but does not block the MPT (20). Tacrolimus did not prevent cell death (Fig. 1D). To further test the role of the MPT, myocytes were also reperfused in the presence of NIM811, a nonimmunosuppressive CsA analog that blocks the MPT but does not inhibit calcineurin (49). NIM811, in the concentration range between 0.5 and 5 μM, protected myocytes against ischemia/reperfusion (Fig. 1D). Unlike CsA, the dose dependence of NIM811 was not biphasic, and protection was not lost at a higher dosage.

**Confocal imaging to monitor onset of MPT.** To reveal directly the onset of the MPT after reperfusion, we imaged calcein by confocal microscopy. Calcein is a green-fluorescing, 623-Da polyanionic fluorophore that is normally impermeant directly the onset of the MPT after reperfusion, we imaged calcein by confocal microscopy. Calcein is a green-fluorescing, 623-Da polyanionic fluorophore that is normally impermeant to the mitochondrial inner membrane. Only after PT pores had opened during ischemia (Fig. 2). Although myocytes shortened during ischemia, they still excluded PI, an indication that cell viability had not been lost.

After 10 min of reperfusion at pH 7.4, calcein began to redistribute and equilibrate between the cytosol and the mitochondrial matrix space (41, 45). After cold ester loading/warm incubation, most calcein localized exclusively to mitochondria (data not shown), and this mitochondrial loading persisted after 3 h of ischemia, indicating that PT pores had not opened during ischemia (Fig. 2). Although myocytes shortened during ischemia, they still excluded PI, an indication that cell viability had not been lost.

After 20 min, calcein equilibration between mitochondria and cytosol was close to complete, although the exact time course varied from cell to cell. Notably, total calcein fluorescence increased as the fluorophore redistributed from mitochondria into the cytosol. Presumably, some calcein fluorescence was quenched inside the mitochondria, and this fluorescence quenching was lost after calcein release (3). In the myocyte shown in Fig. 2A, all cellular calcein was lost after 60 min of
reperfusion, and its nucleus was stained with PI, indicating a loss of viability. In contrast, when myocytes were reperfused at pH 6.2 (Fig. 2B) or with CsA (Fig. 2C), calcein was retained by mitochondria and viability was maintained, although some partial calcein redistribution was evident after 60 min following reperfusion with CsA (Fig. 2C). This latter effect may reflect the fact that CsA protection against PT pore opening may not be permanent with transient pore opening occurring late after reperfusion with CsA (8, 17, 26).

Confocal imaging of mitochondrial membrane potential. When PT pores open, mitochondria depolarize. TMRM is a red-fluorescing cationic fluorophore that accumulates electrophioreically into mitochondria in response to the negative mitochondrial membrane potential ($\Delta \Psi$). To examine changes of $\Delta \Psi$ in relation to the MPT after reperfusion, TMRM fluorescence was monitored by confocal microscopy. After 3 h of ischemia, red fluorescence of TMRM was barely detectable because of mitochondrial depolarization during ischemia. However, after reperfusion, mitochondria repolarized quickly within 5 min (data not shown). This repolarization was sustained for tens of minutes afterward (Fig. 3). For the myocyte shown in Fig. 3, mitochondria began to lose $\Delta \Psi$ after 52 min. At this time, the myocytes began to shorten further. Time-lapse video playback revealed that as depolarization

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Fig. 2. MPT and cell death after ischemia and reperfusion to myocytes. Adult rat cardiac myocytes were cold-loaded with calcein and subjected to 3 h of ischemia at pH 6.2 followed by reperfusion at pH 7.4 (A), reperfusion at pH 6.2 (B), and reperfusion at pH 7.4 with 1 $\mu$M CsA (C). Red-fluorescing PI (3 $\mu$M) was present to detect loss of cell viability. Confocal images were collected. In all 3 experiments, green calcein fluorescence was retained by mitochondria, and viability was maintained after reperfusion at pH 6.2. C: calcein redistribution was inhibited, and cell death did not occur after reperfusion at pH 7.4 with CsA. After 60 min, partial release of calcein from mitochondria into cytosol was evident.

Fig. 3. Hypercontracture and mitochondrial depolarization in reperfused myocytes. Myocytes were subjected to 3 h of ischemia and reperfused at pH 7.4, as described in Fig. 2. Tetramethylrhodamine methyl ester (TMRM) (100 nM) was added 20 min before reperfusion followed by 30 nM in reperfusion buffer. After 3 h of ischemia, TMRM labeling of myocyte was virtually absent, indicative of mitochondrial depolarization. After 35 min of reperfusion, mitochondria accumulated TMRM, indicating repolarization. At 52 min of reperfusion, myocyte shortened and began losing TMRM fluorescence. After 80 min, nearly all TMRM fluorescence was lost. Subsarcolemmal mitochondria consistently were the last to depolarize.
began, individual mitochondria would oscillate, releasing and then reaccumulating TMRM (data not shown). After a few cycles of such oscillation, depolarization became sustained. After 80 min, virtually no TMRM fluorescence remained, although the progression of depolarization in the myocyte shown in Fig. 3 was somewhat slower than the mean time to depolarization after reperfusion of 36.7 min (SD = 16.1; n = 20 animals). Consistently, interfibrillar mitochondria depolarized in advance of subsarcolemmal mitochondria (Fig. 3).

When cells were reoxygenated at pH 7.4 in the presence of CsA to block PT pore opening, mitochondria again repolarized early, but the late phase of depolarization and hypercontracture did not occur (see Fig. 6B). Reperfusion at pH 6.2 also prevented the late-phase mitochondrial depolarization and hypercontracture (see Fig. 6C). Together, these results with calcine and TMRM directly showed that reperfusion caused CsA- and pH-sensitive inner membrane permeabilization and depolarization that then led to cell death.

Protection by antioxidants against pH- and MPT-dependent reperfusion injury. We investigated the effects of antioxidants on cell death after ischemia-reperfusion to understand the role of ROS in pH- and MPT-dependent reperfusion injury to myocytes. Myocytes cultured in 24-well microtiter plates were subjected to ischemia for 3 h and reperfused at pH 7.4. Some myocytes were reperfused in the presence of 0.5 mM desferal, an iron chelator. Desferal has antioxidant effects by virtue of its inhibition of iron-mediated hydroxyl radical generation (39). Desferal prevented nearly all cell killing occurring between 5 min and 2 h of reperfusion (Fig. 4A), DPPD, a lipid radical scavenger (35, 39), and 2-MPG, a cell-permeable synthetic glutathione analog (4), protected to a similar degree. Taken together, cytoprotection by all three antioxidants strongly suggested a direct involvement of ROS in MPT-mediated myocyte death after ischemia-reperfusion.

Inhibition of ROS formation by antioxidants but not by CsA. To investigate the cause-and-effect relationship between ROS generation and the MPT, myocytes cultured in multiwell plates were loaded with 10 μM cmH$_2$DCF-DA for 30 min before reperfusion. cmDCF fluorescence was then evaluated using a fluorescence plate reader. As normoxic controls, some myocytes were incubated aerobically in K RH at pH 7.4 for 3 h. The cells were then further incubated normoxically for 2 h. In normoxic cells, cmDCF fluorescence increased slowly, representing basal ROS formation associated with normal cellular metabolism (Fig. 4B) (39). By contrast, reperfusion after ischemia produced a marked increase of cmDCF fluorescence. After 5 min of reperfusion, cmDCF fluorescence increased >220%, compared with normoxic cells, indicating a rapid increase of ROS formation after reperfusion (Fig. 4B). ROS generation after ischemia-reperfusion was biphasic with a rapid increase during the first 60 min of reperfusion and a slower increase thereafter. After 2 h of reperfusion, cmDCF fluorescence was nearly 230% greater than that in normoxic myocytes never subjected to ischemia. Desferal and 2-MPG each blocked ROS formation to levels associated with normoxic perfusion (Fig. 4B).

The effect of CsA on ROS generation was also evaluated. As shown in Fig. 4B, reperfusion with CsA did not prevent increased cmDCF fluorescence. Because CsA prevented cell death (Fig. 1) but did not block the increase of cmDCF fluorescence, ROS generation is likely upstream to the MPT after reperfusion.

Protection against cell death by anoxic reperfusion. To further investigate the importance of ROS in cell death, we restored pH to 7.4 without reoxyg enation after 3 h of ischemia at pH 6.2. When extracellular pH was normalized without reoxygenation, cell death remained <10% for up to 5 h, which was not different from myocytes reperfused anoxically at pH 6.2 (Fig. 5A). Thus reoxygenation was needed to trigger lethal reperfusion injury. Because normalization of pH was also needed to trigger reperfusion injury, we used cmDCF fluorometry to assess whether reoxygenation at acidic pH prevented cell killing by suppressing ROS formation. However, reoxygenation at pH 6.2 did not suppress ROS formation (Fig. 5B). Thus restoration of pH and ROS formation must both occur to trigger lethal reperfusion injury.

Mitochondrial ROS generation after reperfusion. To characterize further a causative role of ROS in MPT induction, myocytes were coloaded with cmH$_2$DCF-DA and TMRM to monitor ROS generation and ΔΨ, respectively. After 3 h of ischemia, TMRM fluorescence (Fig. 6, A–C, top rows) was lost due to lack of oxygen (Ischemia, Fig. 6A). Within 20 min of reperfusion, mitochondria repolarized and took up TMRM (Fig. 6A, first row, second column). Subsequently, beginning at ~40 min, the myocyte in Fig. 6A hypercontracted, depolarized, and then lost viability after 120 min, as judged by PI staining of the nucleus. Confocal imaging of cmDCF showed that ROS increased progressively after reperfusion.
Myocytes with rhod-2 and fluo-4 loaded into the mitochondrial and cytosolic compartments, respectively, were exposed to 3 h of ischemia. To image Ca\(^{2+}\) under anaerobic conditions on the microscope stage, myocytes were incubated in anoxic KRH at pH 6.2 containing respiratory complexes as 3.3% Oxyrase before placement in gas-tight chambers to maintain anoxia throughout a prolonged ischemia (19, 22). After 5 min of ischemia, both rhod-2 and fluo-4 fluorescence were weak (Fig. 7A). After 60 min of ischemia, rhod-2 and fluo-4 fluorescence increased by 130% and 188%, respectively, compared with values at 5 min of ischemia (Figs. 7A and 8A). After 2 and 3 h of ischemia, rhod-2 fluorescence increased to 754% and 1,130% of the 5 min value, respectively (Figs. 7A and 8A), and fluo-4 fluorescence increased to 849% and 1,103%, respectively. Overlay images confirmed that rhod-2 fluorescence was localized to mitochondria, whereas fluo-4 was in the cytosol. These data indicated substantial Ca\(^{2+}\) overloading in both the mitochondrial and cytosolic compartments during ischemia.

After 3 h of ischemia, myocytes were reperfused with aerobic KRH at pH 7.4. Within 1 min of reperfusion, rhod-2 fluorescence decreased from 1,130% to 781% and fluo-4 decreased from 1,103% to 439%, suggesting that cytosolic Ca\(^{2+}\) decreased somewhat faster than mitochondrial Ca\(^{2+}\) during the early phase of reperfusion (Figs. 7A and 8A). Ca\(^{2+}\) in both compartments continued to decrease. After 30 min, rhod-2 and fluo-4 fluorescence decreased to 304% and 224%, respectively, of the 5-min ischemic value. After 60 min, both mitochondrial and cytosolic Ca\(^{2+}\) increased slightly, and at the same time, the myocyte began to shorten further. Marked Ca\(^{2+}\) overloading then occurred in both compartments after 70 min. This Ca\(^{2+}\) overloading persisted for about 10 min after which membrane integrity was lost and the Ca\(^{2+}\) fluorophores were released into the medium (Figs. 7A and 8A).

Both rhod-2 and fluo-4 fluorescence were also imaged after reperfusion in the presence of 1 μM CsA. As in the absence of CsA, cytosolic and mitochondrial Ca\(^{2+}\) recovered substantially in the first minute after reperfusion (Figs. 7B and 8B). However, in contrast to what occurred in the absence of CsA, the late phase of Ca\(^{2+}\) overloading did not occur, at least up to 120 min of reperfusion. Moreover, cell shortening and hypercontraction did not occur. Reperfusion at pH 6.2, a condition blocking the MPT, also prevented the late phase of Ca\(^{2+}\) overloading and cell death (data not shown). Taken together, these results with and without CsA indicate that Ca\(^{2+}\) overloading late after reperfusion was the consequence rather than the cause of the MPT in myocytes.

Although late Ca\(^{2+}\) overloading appeared to be a consequence rather than a cause of the MPT, mitochondrial Ca\(^{2+}\) might nonetheless be permissive in MPT onset. To assess this issue, myocytes cultured on 24-well plates were loaded with 1 to 50 μM BAPTA-AM by cold ester loading/warm incubation to chelate mitochondrial Ca\(^{2+}\) before ischemia-reperfusion. Cold ester loading causes uptake of BAPTA into both cytosol and mitochondria, whereas subsequent warm incubation permits release of BAPTA from cytosol but not from the mitochondria (9, 24). Loading of 1 to 10 μM BAPTA-AM failed to suppress cell death after reperfusion, whereas higher BAPTA accelerated cell death (Fig. 9). These results suggest that mitochondrial Ca\(^{2+}\) is not permissive for onset of MPT-dependent cell death after reperfusion.

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**DISCUSSION**

Ischemia causes tissue anoxia, exhaustion of glycolytic substrates, ATP depletion, and acidosis. Acidosis is a consequence of hydrolysis of high-energy phosphates, accumulation of lactic acid from anaerobic metabolism, and release of protons sequestered in acidic organelles (26). Although tissue acidosis is frequently considered detrimental, numerous studies (6, 45, 51) demonstrate that the naturally occurring acidosis of ischemia confers protection to myocardium and other tissues. Paradoxically, the recovery of normal pH after reperfusion is an independent event aggravating injury and causing irreversible cell death, a phenomenon called the “pH paradox” (31). Restoration of pH represents a major and independent factor contributing to cell killing, because reoxygenation at acidic pH prevents cell death (45). Consistent with previous findings (6) in neonatal cardiomyocytes, the present study demonstrated that such a pH paradox also occurs in cultured adult rat myocytes. After 3 h of ischemia at pH 6.2, most myocytes remained viable, although the cells were shortened (Fig. 1). Hypercontracture and subsequent necrotic cell death occurred after reoxygenation at pH 7.4 but not at pH 6.2 (Figs. 1 and 2).

The MPT has been implicated as occurring after many forms of cellular stress leading to lethal injury, including anoxia, hypoxia, oxidative stress, drug-induced injury, death receptor activation, photodamage injury, and reperfusion injury to cells from heart and other tissues (14, 16, 21, 25, 38, 45). Reperfusion at pH 7.4 in the presence of CsA, an MPT blocker, prevented myocyte death (Fig. 1C). Because tacrolimus, an immunosuppressive agent that does not block the MPT, did not suppress reperfusion-induced death (Fig. 1D), cytoprotection of CsA was directly associated with its ability to block the MPT. Moreover, NIM811, a nonimmunosuppressive MPT blocker, also protected against cell death (Fig. 1D). Confocal imaging of calcine (Fig. 2) and TMRM (Figs. 3 and 6) showed directly that the MPT and mitochondrial depolarization preceded myocyte death after reperfusion, events that were prevented by reperfusion at pH 6.2 and 7.4 in the presence of CsA. These results support the conclusion that MPT induction accounts for pH-dependent myocyte death after reperfusion.

Reperfusion with antioxidants also prevented pH-dependent death (Fig. 4), suggesting an important role of ROS in inducing the MPT. A causative role of ROS in MPT induction was
Further supported by observations that 1) reperfusion stimulated ROS formation (Fig. 6); 2) antioxidants prevented ROS formation after reperfusion (Fig. 6); 3) mitochondria were the major source of ROS generation (Fig. 6); 4) antioxidant treatment prevented mitochondrial ROS formation and the MPT, depolarization, and hypercontracture that followed mitochondrial ROS formation (Fig. 6); 5) CsA prevented the MPT and cell death after reperfusion but did not inhibit mitochondrial ROS formation (Figs. 1, 4, and 6); and 6) anoxic reperfusion prevented cell death (Fig. 5). These results support the conclusion that mitochondrial ROS generation triggers the MPT after reperfusion, leading to myocyte death (Fig. 10). However, protection by acidic pH was not mediated by suppression of ROS formation, because acidic reperfusion did not prevent accelerated ROS formation but did block the MPT and cell death (Figs. 1, 2, and 5B). Thus both ROS formation and normalization of pH after reperfusion were needed to trigger cell death.

CsA protected in a biphasic fashion (Fig. 1C). At a concentration between 0.5 and 1 μM, CsA was cytoprotective, whereas higher CsA did not protect. This dose-response relationship is consistent with previous studies (14, 45) in heart and other tissues. NIM811, the nonimmunosuppressive analog of CsA, also protected, but unlike CsA, NIM811 did not lose efficacy at higher concentrations. Because NIM811 inhibits the MPT in isolated mitochondria with virtually the same affinity as CsA and because CsA but not NIM811 inhibits calcineurin, loss of protection by CsA at high concentrations may be due to an effect on calcineurin (49). After reperfusion, depolarization of interfibrillar mitochondria consistently preceded depolarization of subsarcolemmal mitochondria (Fig. 3). The basis for the relative resistance of subsarcolemmal mitochondria to the MPT is unknown.

A recent study in isolated perfused rat hearts suggests that ischemia alone without reperfusion is sufficient to induce the MPT (7). In the present work, however, the MPT occurred only after reperfusion, because mitochondria loaded with calcinein before ischemia retained the fluorophore during 3 h of ischemia (Fig. 2). Calcinein was released from mitochondria into the cytosol only after reperfusion (Fig. 2). The difference between the prior work in perfused rat hearts and the present study of myocytes may be related to the importance of pH in MPT induction. Recovery to pH is an important permissive step in MPT induction after ischemia-reperfusion. In the prior study of
perfused rat hearts, mitochondria were isolated from ischemic hearts by standard procedures at pH 7.2–7.4. Thus the MPT may have occurred during the isolation procedure, because CsA (0.1 μM), used in the isolation buffer to block the MPT, may have been inadequate to block fully MPT onset (see Fig. 1), especially during the first homogenization step when the ratio of mitochondria to CsA is greatest. Alternatively, the difference between our work in myocytes and that in perfused hearts may be due to differences in the models. However, previous work has shown that CsA treatment at reperfusion blocks apoptosis and/or necrosis in models of global or reperfusion (WE Cascio, TA Johnson, CL Engle, RT Currin, and JJ Lemasters, unpublished data).

The molecular composition of PT pores remains controversial. In one model, the PT pore consists of the voltage-dependent anion channel, the adenine nucleotide translocator (ANT), cyclophilin D, and various ancillary proteins (26, 34). A more recent model suggests that PT pores form from misfolded integral membrane proteins that cluster and enclose aqueous channels (17). In the latter model, chaperones, including cyclophilin D, block conductance through the nascent pores until matrix Ca2+ increases to open the pores. Because formation of misfolded protein clusters can exceed the number of chaperones required to regulate the aqueous channels within the clusters, the misfolding model explains empirical observations that the MPT progresses from a CsA-sensitive and Ca2+-dependent regulated activity to a CsA-insensitive and Ca2+-independent unregulated one as the time and strength of MPT induction increases (17, 29, 43). The model is also consistent with the recent observation (27) that the MPT occurs even in ANT-deficient liver mitochondria isolated from conditional ANT-double knockout mice, because membrane proteins other than ANT may become misfolded and participate in PT pore formation. In the present study, we observed that partial calcein redistribution began to occur late after reperfusion, even in the presence of CsA (Fig. 2). This effect may reflect progression of the MPT from a CsA-sensitive regulated conductance to a CsA-insensitive unregulated conductance after prolonged reperfusion. The observation underscores the need to validate protection by CsA and NIM811 in long-term recovery experiments in a fully in vivo model.

Increased ROS formation can contribute to cellular dysfunction, as has been described in many models (4, 11, 13, 39). A moderate increase of ROS, however, can lead to protection against injury, especially in ischemic preconditioning of heart (2, 48). Mitochondrial ROS formation increased immediately after initiation of reperfusion. After longer reperfusion, the rate of ROS formation gradually decreased. The antioxidants desferal, DPPD, and 2-MPG reverted ROS formation after reperfusion to levels associated with normoxic incubation and blocked onset of the MPT (Figs. 4 and 6). CsA and acidotic reperfusion each blocked the MPT but did not inhibit ROS formation, which implies that mitochondrial ROS formation is
not the consequence of the MPT. Taken together, the results support the conclusion that mitochondria are both a source of ROS and a target of ROS toxicity after reperfusion.

How reperfusion stimulates mitochondrial ROS formation in ischemic myocytes remains unknown. In hepatocytes exposed to tert-butyl hydroperoxide, early oxidation of mitochondrial pyridine nucleotides leads to increased ROS formation, onset of the MPT, and cell death (9). Thus oxidation of reduced pyridine nucleotides and glutathione may be one factor promoting mitochondrial ROS after reperfusion.

Ca\textsuperscript{2+} overloading is also suggested to play a key role in myocardial dysfunction after ischemia-reperfusion (1, 37). However, the present data do not support a causative role of Ca\textsuperscript{2+} overloading in ischemia-reperfusion injury to myocytes. Confocal imaging of rhod-2 and fluo-4 showed that mitochondrial and cytosolic Ca\textsuperscript{2+} substantially increased during ischemia (Figs. 7 and 8). This Ca\textsuperscript{2+} dysregulation during ischemia recovered rapidly after reperfusion, and both mitochondrial and cytosolic Ca\textsuperscript{2+} remained low during the initial phase of reperfusion. Later, Ca\textsuperscript{2+} from both compartments increased markedly. Importantly, MPT blockade with CsA prevented this late phase of Ca\textsuperscript{2+} overloading (Figs. 7 and 8), and intramitochondrial Ca\textsuperscript{2+} chelation with BAPTA did not suppress cell killing after reperfusion (Fig. 9). These results indicate that Ca\textsuperscript{2+} overloading is the consequence rather than the cause of the MPT after ischemia-reperfusion of myocytes.

After the MPT, mitochondria uncouple and lose their ability to synthesize ATP. With uncoupling, futile hydrolysis of ATP occurs, catalyzed by the mitochondrial ATP synthase operating in reverse. Thus cellular Ca\textsuperscript{2+} overloading after MPT onset is likely the result of ATP depletion and inhibition of ATP-driven Ca\textsuperscript{2+} pumps and secondary ion exchangers that serve to translocate Ca\textsuperscript{2+} out of the cytosol across the plasma membrane and into the sarcoplasmic reticulum. Frequently, we observed a phase of shortening before full loss of TMRM fluorescence, which was accompanied by transient depolarization of individual mitochondria (Fig. 3). This phase of shortening was also accompanied by a small increase of Ca\textsuperscript{2+} (Figs. 7A and 8A). Both shortening and the accompanying small increase of Ca\textsuperscript{2+} were prevented by CsA (Figs. 7B and 8B). Thus transient PT pore opening was likely causing a partial depletion of ATP, which then inhibited cellular Ca\textsuperscript{2+} pumps to cause an increase of Ca\textsuperscript{2+} and shortening. Because PT pore opening became sustained, ATP was fully depleted, leading to maximal Ca\textsuperscript{2+} overload, hypercontracture, and, ultimately, cell death. Consistent with this interpretation, we observed the beginning of calcine release from mitochondria at time points earlier than full and sustained mitochondrial depolarization (Fig. 2 and data not shown).

In conclusion, the present study shows that normalization of pH after reperfusion initiates mitochondrial ROS formation, onset of the MPT, and necrotic cell death in isolated adult rat myocytes (Fig. 10). Reperfusion with the MPT blockers CsA and NIM811 prevents this MPT-dependent cell death. Antioxidants, but not CsA, block mitochondrial ROS formation after reperfusion. Antioxidants also prevent the MPT and cell death. Taken together, these findings strongly support the conclusion that ROS formation and normalization of pH are both necessary to trigger PT pore opening after reperfusion. With PT pore opening, ATP levels likely decline, leading to inhibition of Ca\textsuperscript{2+} and other ion pumps. As a consequence, overloading of Ca\textsuperscript{2+} in mitochondria and cytosol occurs after PT pore opening, which is prevented by MPT blockade. Because the MPT is a causative factor precipitating reperfusion injury to myocytes, strategies applied at reperfusion to prevent ROS generation and MPT onset show promise for decreasing infarction and improving cardiac function after myocardial ischemia.

REFERENCES


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