Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes

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Qian, Ting, Anna-Liisa Nieminen, Brian Herman, and John J. Lemasters. Mitochondrial permeability transition in pH-dependent reperfusion injury to rat hepatocytes. Am. J. Physiol. 273 (Cell Physiol. 42): C1783–C1792, 1997.—To simulate ischemia and reperfusion, cultured rat hepatocytes were incubated in anoxic buffer at pH 6.2 for 4 h and reoxygenated at pH 7.4. During anoxia, intracellular pH (pHi) decreased to 6.3, mitochondria depolarized, and ATP decreased to <1% of basal values, but the mitochondrial permeability transition (MPT) did not occur as assessed by confocal microscopy from the redistribution of cytosolic calcine into mitochondria. Moreover, cell viability remained >90%. After reperfusion at pH 7.4, pHi returned to pH 7.2, the MPT occurred, and most hepatocytes lost viability. In contrast, after reperfusion at pH 6.2 or with Na+-free buffer at pH 7.4, pHi did not rise and cell viability remained >80%. After acidic reperfusion, the MPT did not occur. When hepatocytes were reperfused with cyclosporin A (0.5–1 µM) at pH 7.4, the MPT was prevented and cell viability remained >80%, although pHi increased to 7.2. Reperfusion with glycine (5 mM) also prevented cell killing but did not block recovery of pHi or the MPT. Retention of cell viability was associated with recovery of 30–40% of ATP. In conclusion, preventing the rise of pHi after reperfusion blocked the MPT, improved ATP recovery, and prevented cell death. Cyclosporin A also prevented cell killing by blocking the MPT without blocking recovery of pHi. Glycine prevented cell killing but did not inhibit recovery of pHi or the MPT.

Cyclosporin A; dimethyl amiloride; glycine; ischemia; pH paradox

Mitochondrial inner membrane initiates onset of the MPT (20). These pores conduct both positively and negatively charged solutes of up to 1,500 Da. Pore opening induces mitochondrial depolarization, swelling, and uncoupling of oxidative phosphorylation. Cyclosporin A, an immunosuppressive cyclic oligopeptide, specifically blocks conductance of the permeability transition pore (20) and has been shown to prevent cell injury caused by anoxia and oxidative stress in a number of models (7, 14, 18, 19, 24, 25, 28, 32, 36, 38, 42). The MPT pore is also strongly inhibited by pH <7 (3, 20, 34). Thus the pH dependency of reperfusion injury may be the consequence of the pH dependency of the MPT.

Glycine is a cytoprotective amino acid that protects renal tubular cells and hepatocytes against lethal hypoxic injury (29, 33, 46). Recently, glycine was also shown to protect against pH-dependent posthypoxic injury to renal tubular cells and endothelial cells of livers stored for transplantation surgery (2, 11, 47). Importantly, glycine protected when used only during reperfusion. However, the mechanism of cytoprotection by glycine remains controversial.

Importantly, our data show that a Na+-dependent pH paradox linked to pHi plays a key role in reperfusion-induced killing of hepatocytes. Onset of the MPT is a crucial step in this cell-killing process. Overall, our results indicate that the protection by acidic pH and cyclosporin A against reperfusion injury to hepatocytes involves inhibition of the pH-dependent onset of the MPT, whereas glycine exerts its protective effect downstream to the MPT.

MATERIALS AND METHODS

Hepatocyte isolation and culture. Hepatocytes were isolated from 24-h-fasted male Sprague-Dawley rats (200–300 g) by collagenase digestion, as described previously (23). Viability of isolated hepatocytes routinely exceeded 95%, as determined by trypan blue exclusion. For culture, hepatocytes were resuspended in Waymouth's medium MB-752/1 containing 2 mM l-glutamine, 27 mM NaHCO3, 10% fetal calf serum, 100 nM insulin, and 100 nM dexamethasone. For cell viability assay, aliquots (1 ml) of 1.5 × 106 cells were plated onto 24-well microtiter plates (Falcon, Lincoln Park, NJ) or glass coverslips, both coated with type I rat tail collagen. For ATP measurement, cell culture density was 1.5 × 106 cells in 60 × 15 mm tissue culture dishes (Falcon). Hepatocytes were used after overnight incubation in 5% CO2-95% air at 37°C. All experiments were carried out in Krebs-Ringer-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)
buffer (KRH) containing (in mM) 115 NaCl, 5 KCl, 2 CaCl$_2$, 1 KH$_2$PO$_4$, 1.2 MgSO$_4$, and 25 NaHEPES (pH 7.4 or 6.2). In some experiments, Na$^+$ was substituted with choline or CI$^-$ was substituted with gluconate in the buffer.

Model of ischemia and reperfusion in cultured hepatocytes. To simulate the anoxia and acidosis of tissue ischemia, hepatocytes were incubated in KRH at pH 6.2 in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). To simulate the reoxygenation and return to physiological pH after reperfusion, anaerobic KRH at pH 7.4 was replaced with aerobic KRH at pH 7.4. In many experiments, the conditions of this simulated reperfusion were varied by changing the composition of the reperfusion buffer. Anoxia in the anaerobic chamber was maintained in an atmosphere of 90% N$_2$-10% H$_2$. Any oxygen entering the chamber by back diffusion was continuously converted to water vapor by reaction with hydrogen catalyzed by a heated palladium catalyst. Oxygen tension in the chamber was monitored by a gas analyzer (model 10; Coy Laboratory Products) and was routinely less than one part per million (<0.001 Torr).

Cell viability assay. Cell viability was assessed by propidium iodide-digitalization using a multiwell fluorescence scanner (CytoFluor 2300; Millipore, Bedford, MA), as described previously (35). Briefly, hepatocytes were incubated in KRH containing 30 µM propidium iodide. Fluorescence from each well was measured using excitation and emission wavelength of 546 nm (40-nm band pass) and 620 nm (50-nm band pass), respectively. For each experiment, an initial fluorescence measurement (A) was made 20 min after addition of propidium iodide-containing buffer and then at intervals thereafter. Individual experiments were terminated by addition of 375 µM digitonin to permeabilize all cells, and a final fluorescence measurement (B) was obtained 20 min later. The percentage of viable cells (V) was calculated as $V = 100(B - X)/(B - A)$, where $X$ is the fluorescent intensity at any given time.

pH$_i$ measurement. Carboxysemiphenothorhodofluor-1 (SNARF-1) was used to measure pH$_i$. SNARF-1 is a dual-emission fluorophore with a large emission spectral shift in response to pH changes (10, 41). To load SNARF-1, overnight cultured hepatocytes were incubated at 37°C for 30 min in KRH containing 10 µM SNARF-1-acetoxymethyl ester (SNARF-1-AM) diluted from a 1 mM stock solution in dimethyl sulfoxide. The loading buffer was then removed and replaced with fresh KRH. Before each measurement of SNARF-1 fluorescence, the incubation buffer was replaced. This washing removed any extracellular fluorophore that may have leaked from the cells, leaving only intracellular fluorophore as an indicator of pH$_i$. Fluorescence was measured with a multiwell fluorescence scanner using 530-nm (30-nm band pass) excitation light. Emission at 590 nm (40-nm band pass) and 620 nm (50-nm band pass) were collected consecutively in two scans ~18 s apart. After subtracting background fluorescence from wells not loaded with SNARF-1, the 590/620 nm fluorescence ratio was calculated. A calibration curve was generated for each experiment by incubating SNARF-1-loaded cells with 10 µM nigericin to equilibrate pH$_i$ and pH$_o$. The calibration buffer contained (in mM) 135 KCl, 15 NaCl, 1 CaCl$_2$, 1 mM KH$_2$PO$_4$, 0.5 mM MgSO$_4$, and 10 mM HEPES, pH 5.6–7.5. Because all fluorescence measurements were performed outside the anaerobic chamber, pH$_i$ values during the anoxic period were obtained from separate plates to avoid oxygen interference. At each time point during anoxia, a plate was tightly sealed with 3M sealing tape (Model 471; 3M, St. Paul, MN) after the buffer was first replaced with fresh anaerobic medium and fluorescence was then measured immediately (within 1 min) after the plate was removed from the anaerobic chamber. In this way, cells were maintained anaerobic while SNARF-1 fluorescence was measured.

ATP measurements. Hepatocytes were incubated at a density of 1.5 × 10$^6$ cells/dish in KRH buffer. To extract ATP, KRH buffer was replaced with 1 ml of cold 0.6 M HClO$_4$, and cells were scraped with a disposable cell scraper (Baxter Healthcare, McGow Park, IL). After centrifugation (9,000 g, 1 min), 0.8 ml of the supernatants was neutralized with 5 M KOH and 0.4 M imidazole. After one more centrifugation, supernatants were diluted 200-fold with deionized distilled water. ATP was then measured with a commercial luciferin/luciferase kit (Promega Enliten, Madison, WI) using an MGM instruments Optocomp I luminometer (Hamden, CT) and was converted to units of nanomoles per 10$^6$ cells.

Laser-scanning confocal microscopy. The green fluorescence of calcein and red fluorescence of tetramethylrhodamine methyl ester (TMRM) and propidium iodide were excited with the 488- and 568-nm lines of an argon-krypton laser. Fluorescence was divided by a 560-nm emission dichroic reflector and was measured by separate photomultipliers through 515- to 565-nm band-pass and 560-nm long-pass barrier filters using a Zeiss LSM-410 inverted laser-scanning confocal microscope (Carl Zeiss, Oberkochen, Germany). A Zeiss numerical aperture 1.4, 63 × Planapochromat objective lens was used, and pinholes were set to Airy units of 0.9 in both channels.

Cell loading and incubation. Hepatocytes cultured on glass coverslips were loaded in KRH with 500 nM TMRM and 1 µM calcein-AM for 15 min at 37°C as described in Ref. 36. To simulate ischemia, hepatocytes were incubated at pH 6.2 in KRH containing 100 nM TMRM and 3 µM propidium iodide in the anoxic chamber for 4 h. During the last 15 min of anoxic incubation, cells were reloaded with 0.5 µM calcein-AM to improve cellular calcein loading, since preliminary experiments showed a nonspecific leakage of calcein during the 4 h of anoxic incubation. Inside the anoxic chamber, coverslips containing cultured hepatocytes were mounted in a closed gas-tight FCS2 chamber (Bioptechs, Butler, PA). After 4 h, the sealed FCS2 chamber was mounted on the microscope stage to view the anoxic cells. Subsequently, reperfusion was simulated by infusion of aerobic KRH containing 500 nM TMRM and 3 µM propidium iodide.

Materials. SNARF-1-AM, calcein-AM, and TMRM were purchased from Molecular Probes (Eugene, OR); the luciferase/luciferin ATP measurement kit was from Promega Enliten (Madison, WI); propidium iodide and type I collagen were from Sigma (St. Louis, MO); HEPES and collagenase D were from Boehringer Mannheim Biochemicals (Indianapolis, IN); Waymouth’s medium MB-252/1 was from Gibco Laboratories (Grand Island, NY); insulin was from Squibb-Novo (Princeton, NJ); and dexamethasone sodium phosphate was from Lyphomed (Rosemont, IL). Cyclosporin A, a cyclic endopeptidase, was the gift of Sandz Pharmaceuticals (East Hanover, NJ). Other chemicals of analytical grade were obtained from the usual commercial sources.

RESULTS

pH dependency of ischemia-reperfusion injury to cultured hepatocytes. To study ischemia-reperfusion injury to cultured hepatocytes under conditions relevant to
tissue ischemia in vivo, we simulated the anoxia and acidosis of tissue ischemia by placing sealed microtiter plates of aerobic cultured hepatocytes in an anaerobic chamber and then replacing the still aerobic KRH at pH 7.4 with preequilibrated anaerobic KRH at pH 6.2. Under these conditions, cell viability remained >85% after 4 h (Fig. 1, A and B). This result is consistent with our previous findings showing that acidic pH strongly protects against loss of hepatocyte viability in models of hypoxia (16, 17). After subjecting cultured hepatocytes to simulated ischemia, we simulated the reoxygenation and restoration of normal pH after reperfusion by replacing anaerobic KRH at pH 6.2 with aerobic KRH at pH 7.4. After reperfusion in this way, cell viability decreased to <40% in 2 h (Fig. 1A). By contrast, when the hepatocytes were reoxygenated with KRH at pH 6.2, little additional cell killing occurred (Fig. 1A).

After 4 h of anoxia at pH 6.2, we also raised pH without reoxygenation. When pH was increased without reoxygenation, cell viability decreased to ~30% within 2 h (Fig. 1B). In contrast, viability remained >75% after a total of 6 h of anoxia at pH 6.2. Thus lethal reperfusion injury was initiated by increased pH rather than by reoxygenation, namely, reperfusion injury in this model was a pH paradox rather than an oxygen paradox. These results confirmed our previous findings in perfused rat livers (12) and established a model to study intracellular events during pH-dependent ischemia-reperfusion injury.

Protection by Na\(^+\)-free buffer and lack of protection by Ca\(^{2+}\)-free buffer and dimethyl amiloride against pH-dependent reperfusion injury. Previous experiments in cardiac myocytes showed that protection by acidotic pH against reperfusion injury was mediated by maintenance of intracellular acidosis after reperfusion. In particular, when the Na\(^+\)/H\(^+\) exchanger, a major pH regulator, was inhibited with dimethyl amiloride, pH did not rise after reperfusion at pH 7.4, and lethal reperfusion injury was prevented (4, 21, 27). In contrast to this earlier finding in myocytes, dimethyl amiloride (50 µM) did not prevent lethal reperfusion injury to cultured hepatocytes (Fig. 2). Indeed, dimethyl amiloride in a concentration range between 5 and 1,000 µM failed to improve viability of reperfused hepatocytes (data not shown). However, substitution of Na\(^+\) with choline in the reperfusion buffer protected completely against cell killing after reoxygenation at pH 7.4 (Fig. 2). To test the hypothesis that pH-dependent injury was mediated by Ca\(^{2+}\) influx as suggested by Carini et al. (9), we also reoxygenated hepatocytes with Ca\(^{2+}\)-free KRH. Ca\(^{2+}\)-free buffer failed to produce any protective effect (Fig. 2).

pH during reperfusion injury. To measure pH during simulated ischemia and reperfusion, we loaded hepatocytes with SNARF-1 and used an emission fluorescence ratio technique to monitor pH. Under the conditions employed, the 590/620 nm emission ratio was almost...
linear with pH$_i$ when pH$_i$ was clamped to pH$_o$ using nigericin in the pH range of 5.6–7.5 (Fig. 3, inset). Although SNARF-1 was well retained by aerobic hepatocytes, we replaced the buffer before each fluorescence measurement to avoid interference by dye that may have leaked into the extracellular medium from cells damaged by ischemia-reperfusion. From ratiometric measurements of SNARF-1 fluorescence, we observed a progressive recovery of pH$_i$ during reoxygenation at pH 7.4 after 4 h of anoxia at pH 6.2. Half-maximal recovery occurred after ~30 min, and full recovery was achieved after 2 h. Reoxygenation with buffer at pH 6.2 or with Na$^+$-free buffer at pH 7.4 blocked the recovery of pH$_i$. By contrast, reoxygenation with Ca$^{2+}$-free buffer or with dimethyl amiloride-containing buffer at pH 7.4 did not prevent recovery of pH$_i$ (Fig. 3).

Protection by cyclosporin A against reperfusion injury. To test the hypothesis that onset of a cyclosporin A-sensitive MPT was contributing to reperfusion injury, we reoxygenated hepatocytes with buffer containing cyclosporin A. Because cyclosporin A may not penetrate cells rapidly, we added cyclosporin A to the cells during the last 20 min of 4 h of anoxia at pH 6.2. As shown in Fig. 4, cyclosporin A protected against lethal reperfusion injury in a biphasic fashion. Lower concentrations (0.5–1 µM) protected, whereas higher concentrations (2 µM) did not. Maximal protection occurred with ~0.5 µM cyclosporin A.

Protection by glycine against reperfusion injury. To assess the effect of glycine, a cytoprotective amino acid, on reperfusion injury to cultured hepatocytes, we subjected hepatocytes to 4 h of anoxia at pH 6.2 followed by reoxygenation at pH 7.4 in the presence of various concentrations of glycine. Glycine at low concentrations (0.05–0.5 mM) partially prevented lethal reperfusion injury (data not shown), whereas 5 mM glycine blocked essentially all reperfusion-induced cell killing (Fig. 5). To test the hypothesis that the cytoprotection of glycine was mediated by regulation of Cl$^-$ influx as suggested by Schnellmann and associates (30, 31, 45), we reperfused cells in Cl$^-$-free KRH buffer with and without glycine. As shown in Fig. 5, Cl$^-$-free reperfusion did not...
protect against reperfusion injury to cultured hepatocytes or alter the cytoprotection by glycine. Similarly, cell killing and glycine protection against cell killing were also not changed when Cl− concentration in the reperfusion buffer was titrated to 25% and 50% of values in KRH (data not shown). We also determined the effects of cyclosporin A and glycine on the recovery of pHi after reperfusion. Although both agents prevented cell killing, neither cyclosporin A nor glycine prevented the recovery of pHi to pH 7.1–7.2 after reperfusion (Fig. 6). Also, dimethyl amiloride in the presence of glycine did not prevent recovery of pHi during reperfusion (Fig. 6). Similarly, reperfusion in Ca2+-free buffer in the presence of glycine did not alter pHi recovery (data not shown).

Onset of the MPT after reperfusion. Because cyclosporin A prevented pH-dependent reperfusion injury to hepatocytes, we applied laser-scanning confocal microscopy to directly monitor the changes of mitochondrial membrane permeability and membrane potential (ΔΨ) in relation to cell killing during reperfusion. Hepatocytes were coloaded with TMRM and calcein. TMRM is a cationic fluorophore that accumulates electrophoretically in mitochondria in response to their highly negative ΔΨ. Calcein is taken up into the cytosol when loaded at 37°C but is excluded by normal mitochondria. Thus, during normal aerobic incubation, confocal microscopy reveals mitochondria as bright spots in the red fluorescence channel and as dark voids in the green fluorescence channel, as described previously (36).

After 4 h of simulated ischemia (anoxia at pH 6.2), TMRM labeling of mitochondria was almost completely lost, indicating mitochondrial depolarization (Fig. 7, top; compare with the baseline image in Fig. 4 of Ref. 36). By contrast, the calcein image was unchanged and mitochondria remained as dark voids, indicating that they continued to exclude the cytosolic fluorophore. Additionally, the calcein image showed development of plasma membrane blebbing and general cellular swelling, characteristic features of ATP depletion injury (23).

When hepatocytes were reperfused (reoxygenated at pH 7.4) after 4 h of simulated ischemia (anoxia at pH 6.2), mitochondria in hepatocytes began to repolarize and to take up TMRM within 5 min (Fig. 7). After 20 min of reperfusion, however, one of the two cells in the field lost TMRM fluorescence. Simultaneously, calcein fluorescence filled the dark mitochondrial voids. These events signify onset of the MPT (36). Subsequently, cell death occurred, as shown by nuclear staining with propidium iodide and release of all cytosolic calcein. In the other hepatocyte in the field, onset of the MPT and mitochondrial depolarization did not occur. Rather, mitochondrial ΔΨ continued to recover, as indicated by accumulation of TMRM fluorescence. Moreover, cell viability was retained. Overall, in 5 separate experiments, 19 of 20 cells showed onset of the MPT and subsequent cell death after reoxygenation at pH 7.4.

To test the hypothesis that acidic reperfusion and reperfusion with cyclosporin A were preventing cell killing by blocking onset of the MPT, we monitored TMRM and calcein fluorescence as ischemic hepatocytes were reoxygenated with KRH at pH 6.2 (Fig. 8) or with KRH at pH 7.4 in the presence of cyclosporin A (Fig. 9). After reperfusion at acidic pH, a repolarization occurred as indicated by TMRM uptake. Additionally, calcein did not redistribute into the mitochondria, indicating that the MPT had not occurred. Overall, in 3 experiments, only 1 of 12 cells underwent the MPT after reoxygenation at pH 6.2. This cell subsequently lost viability. Similarly, when cells were reoxygenated at pH 7.4 in the presence of cyclosporin A, mitochondria repolarized and mitochondrial impermeability to calcein was retained, an effect observed in 13 of 13 cells in 4 separate experiments. Reperpusion at pH 6.2 and at pH 7.4 with cyclosporin A also prevented loss of cell viability, as indicated by retention of cytosolic calcein fluorescence and lack of nuclear labeling with propidium iodide.

We also investigated whether glycine could prevent pH-dependent onset of the MPT after reperfusion (Fig. 10). In contrast to acidic reperfusion and cyclosporin A, reperfusion with glycine did not produce mitochondrial repolarization or block onset of the MPT, although cell viability was maintained. Overall, onset of the MPT occurred in 23 of 25 cells in 3 experiments, but only 1 cell lost viability.

ATP during reperfusion injury. We also measured cellular ATP during simulated ischemia and reperfusion. After 4 h of anoxia at pH 6.2, cellular ATP decreased from 18 to 0.1 nmol/10⁶ cells (Table 1). After reperfusion at pH 7.4, ATP initially increased to 1.4 nmol/10⁶ cells after 30 min but then decreased to about one-half that value after 60 and 120 min. When hepatocytes were reoxygenated at pH 6.2 or 7.4 in the presence of cyclosporin A or glycine, a greater and sustained recovery of ATP occurred, and 30–40% of basal ATP was restored within 2 h (Table 1).
DISCUSSION

pH paradox in ischemia-reperfusion injury to cultured rat hepatocytes. Anoxia and acidosis are cardinal features of tissue ischemia. Previously, our laboratory and others demonstrated that the naturally occurring acidosis of ischemia protects strongly against hypoxic killing of heart, kidney, and liver cells (5, 6, 12, 16, 17, 22, 29, 47). In reperfusion, however, the transition from acidosis to normal pH precipitates cell death (4, 5, 12, 21, 27, 47). Here, we documented such a pH paradox in cultured rat hepatocytes. We showed that hepatocytes retained viability for as long as 6 h of anoxic incubation at pHo 6.2 (Fig. 1B). However, when pHo was returned...
to 7.4 with reoxygenation, cell killing ensued in a majority of cells within 1–2 h. This injury was not oxygen dependent, since reperfusion with oxygenated buffer at pH 6.2 prevented cell killing (Fig. 1A). Moreover, increasing pHo to 7.4 without reoxygenation precipitated cell killing to the same extent as with reoxygenation (Fig. 1B). In experiments not reported here, we observed a nearly identical pH paradox in hepatocytes after washout of 2.5 mM cyanide (chemical hypoxia).

Previously, in our model of chemical hypoxia to hepatocytes, we showed that protection against cell killing by acidic pH was mediated by pHi. Treatments that increased pHi during chemical hypoxia, such as monensin, accelerated cell killing, whereas treatments that decreased pHi, such as the Na\(^+\)/H\(^+\) exchange inhibitor amiloride, delayed the onset of cell death (17). Similarly, in models of ischemia-reperfusion to cultured cardiac myocytes and isolated perfused papillary muscles, dimethyl amiloride delayed recovery of pHi and prevented cell killing, whereas monensin accelerated recovery of pH, and hastened cell death (21, 27). Thus our expectation was that dimethyl amiloride would prevent lethal reperfusion cell injury in the pH paradox to hepatocytes. However, dimethyl amiloride over a broad range of concentrations had no effect on reperfusion-induced cell killing (Fig. 2). Likewise, dimethyl amiloride did not prevent the recovery of pHi from acidic to normal level after reperfusion (Fig. 3), even in the presence of glycine (Fig. 6). Thus the likely explanation for the lack of cytoprotection by dimethyl amiloride is its failure to prevent the recovery of pHi after reperfusion.

Recently, Carini and co-workers (8) suggested that protection against cell death by acidosis may be mediated by suppression of Na\(^+\)/H\(^+\) exchange and Na\(^+\)-HCO\(_3\)^\(^-\) cotransport. However, we showed previously that acidosis protects against chemical hypoxia (KCN + iodoacetate), even when intracellular and extracellular Na\(^+\) are equilibrated with monens-
Table 1. ATP concentration of hepatocytes after reperfusion

<table>
<thead>
<tr>
<th>Time, min</th>
<th>pH 7.4</th>
<th>pH 6.2</th>
<th>pH 7.4 + CyA</th>
<th>pH 7.4 + Glycine</th>
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<tbody>
<tr>
<td>30</td>
<td>1.4 ± 0.78</td>
<td>3.8 ± 0.33</td>
<td>3.8 ± 1.03</td>
<td>4.0 ± 1.15</td>
</tr>
<tr>
<td>60</td>
<td>0.6 ± 0.24</td>
<td>6.7 ± 0.43*</td>
<td>4.4 ± 0.85*</td>
<td>7.3 ± 2.17*</td>
</tr>
<tr>
<td>120</td>
<td>0.72 ± 0.34</td>
<td>7.6 ± 1.56*</td>
<td>5.3 ± 1.53*</td>
<td>8.2 ± 1.18*</td>
</tr>
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Values are means ± SE in nM/n106 cells of duplicate determinations from 2–3 cell isolations. Hepatocytes were incubated in anaerobic Krebs-Ringer-HEPES (KRH) at pH 6.2 for 4 h, as described in Fig. 1. Cells were then reoxygenated at pH 6.2 or at pH 7.4 with KRH, with KRH containing 1 µM cyclosporin A (CyA), or with KRH containing 5 mM glycine. ATP (nM/n106 cells) was measured using luciferin/luciferase, as described in MATERIALS AND METHODS. Basal ATP averaged 18.6 ± 0.70 nM/n106 cells. ATP after 4 h of anoxia at pH 6.2 averaged 0.1 ± 0.07 nM/n106 cells. *P < 0.05 vs. pH 7.4.

sin in 10 mM Na+ medium. Thus acidosis is a protective factor independent of Na+ uptake. Nonetheless, this does not exclude the possibility that Na+ uptake is an additional factor promoting cell killing.

pH regulation and cell killing after reperfusion. Because dimethyl amiloride did not prevent the recovery of pHi after reperfusion, we conclude that amiloride-sensitive Na+/H+ exchange does not mediate this pHi recovery in cultured hepatocytes. In rat cardiac myocytes, the Na+-HCO3 cotransporter participates in the recovery of pHi after acidic loading (8). This occurs even in HCO3−-free medium, presumably by employing endogenously produced CO2 that is converted to HCO3− by carbonic anhydrase on the cell surface (43). Thus the recovery of pHi in hepatocytes after reperfusion may also be mediated by Na+-HCO3 cotransport. Additionally, an amiloride-insensitive and HCO3−-independent mechanism may contribute to pHi recovery, as recently shown in acid-loaded perfused rat livers (13). Future experiments will be needed to determine the exact mechanism by which pHi recovers in our model of simulated ischemia-reperfusion to cultured hepatocytes.

When recovery of pHi was blocked by reperfusion with Na+-free or acidic buffer, cell killing was prevented. To test the hypothesis that reperfusion injury was the result of cellular Ca2+ overloading mediated by concerted Na+/H+ and Na+/Ca2+ exchange (9), we also reperfused ischemic hepatocytes with Ca2+-free buffer. Ca2+-free reperfusion failed to protect hepatocytes against lethal reperfusion injury or alter the recovery of pHi. Thus we conclude that Ca2+ overload after reperfusion is not the mechanism underlying pH-dependent reperfusion injury in our model.

Contribution of the MPT to pH-dependent reperfusion injury. Recently, mitochondrial dysfunction associated with onset of the MPT was implicated in lethal cell injury after anoxia, oxidative stress, and reperfusion in heart and liver cells (7, 14, 18, 19, 24, 25, 28, 32, 36, 38, 42). In hepatocytes, onset of the MPT during oxidative stress was directly demonstrated using confocal microscopy (36). In the present study, we showed by direct confocal imaging that onset of the MPT and mitochondrial depolarization preceded cell killing after reperfusion (Fig. 7). Moreover, low pH and cyclosporin A, treatments that strongly suppress onset of the MPT in isolated mitochondria, blocked onset of the MPT and mitochondrial depolarization after reperfusion (Figs. 8 and 9). These results support our hypothesis that recovery of pHi after reperfusion causes onset of the MPT and leads to lethal reperfusion injury.

Cytoprotection by cyclosporin A showed a biphasic dose response. Protection occurred at 0.5–1 µM but was lost at higher concentrations. This dose-response relationship is similar to that reported earlier for isolated myocytes and perfused hearts (18, 19, 32). At higher concentrations, cyclosporin A becomes cytotoxic (26, 40), which may overcome its beneficial effect on the MPT.

Our results showed that rising pH, after reperfusion triggers onset of the MPT. Once the MPT occurs, mitochondria depolarize and the uncoupler-stimulated mitochondrial ATPase become activated (24, 35). Such ATP hydrolysis may promote cell killing. To test this hypothesis, we measured ATP during simulated ischemia-reperfusion to cultured hepatocytes. When ischemic hepatocytes were reperfused at pH 7.4, we observed little recovery of ATP (Table 1). By contrast, when cells were reperfused at pH 6.2 or at pH 7.4 with 1 µM cyclosporin A, 30–40% of basal ATP was restored in 2 h. Thus protection against cell killing by acidosis and cyclosporin A was associated with partial ATP recovery. Because acidosis and cyclosporin A both act to block onset of the MPT, we conclude that accelerated ATP hydrolysis caused by onset of the MPT is the likely basis for continued ATP depletion and subsequent cell killing.

Protection of glycine against reperfusion injury. Glycine is a cytoprotective amino acid that protects kidney and liver cells against hypoxia and ATP depletion-induced cell death in various models (29, 46). Glycine also protects against pH-dependent posthypoxic injury to renal tubular cells and reperfusion injury to endothelial cells of livers after cold storage for transplantation (2, 11). In the present study, glycine strongly protected against pH-dependent reperfusion injury to cultured hepatocytes. Full protection occurred when glycine was administered only during reperfusion. Thus glycine acted specifically against those mechanisms that precipitated reperfusion-induced cell death. However, glycine did not block recovery of pHi to normal levels after reperfusion (Fig. 6). This result indicated that cytoprotection by glycine was not mediated by intracellular acidification.

Several mechanisms have been suggested to explain cytoprotection by glycine, including inhibition of proteolysis (33). Studies in renal tubules also suggest that inhibition of Cl− influx through Cl− channels mediates glycine cytoprotection (31). To investigate the contribution of Cl− influx to reperfusion injury in hepatocytes, we used gluconate to substitute for Cl− in our reperfusion buffer. We found that glycine cytoprotection was not dependent on Cl− in the medium (Fig. 5) and further that cell killing was not diminished in the absence of Cl− as it was in the absence of Na+ (Fig. 2).
Two very recent studies also demonstrate that glycine cytoprotection is independent of medium Cl\(^{-}\) and that Cl\(^{-}\)-free medium is not cytoprotective (11, 44). Earlier, Miller and Schnellmann (31) reported cytoprotection to renal proximal tubular cells exposed to antimycin when 50% of medium NaCl was replaced isosmotically with mannitol. However, mannitol is an antioxidant that protects against injury caused by mitochondrial oxygen radical generation during respiratory inhibition (17). Thus protection may be due to mannitol rather than decreased Cl\(^{-}\) concentration.

Nonetheless, a number of agonists and antagonists of amino acid-gated Cl\(^{-}\) channels do protect against lethal cell injury (44, 45). As suggested by Venkatachalam et al. (44), the conductance of the putative channel involved in cell killing may not be specific only to Cl\(^{-}\). Moreover, recent work in cultured sinusoidal endothelial cells exposed to cyanide suggests that glycine protects by inhibiting an organic anion channel, which opens just before the onset of cell death (37). In any event, glycine cytoprotection did not prevent the onset of the MPT and mitochondrial depolarization (Fig. 10). Thus protection by glycine in reperfusion injury appears to occur at a point downstream to onset of the MPT.

In previous work in cells from kidney and other tissues, glycine cytoprotection was not associated with increased ATP levels (29, 44). Thus recovery of ATP after reperfusion of hepatocytes with glycine in the present work was unexpected (Table 1). One possible source for ATP after reperfusion with glycine is the glycine cleavage system, which catalyzes the tetrahydrofolate-dependent oxidation of glycine to \(\text{CO}_2\), \(\text{NH}_4^+\), and formate, the reduction of NAD\(^+\) and NADP\(^+\) to NADH and NADPH, and the phosphorylation of ADP to ATP (1, 15). Hepatocytes are particularly enriched in the enzymes of the glycine cleavage system. During reoxygenation, NADH and NADPH formed during glycine cleavage are reoxidized by mitochondrial respiration, further driving the overall reaction to completion. We cannot say from our experiments that ATP recovery is necessary for glycine cytoprotection against pH-dependent reperfusion injury, but work in other cell types indicates that glycine cytoprotection is independent of the recovery of ATP. However, when it occurs, ATP recovery is most likely beneficial.

In conclusion, our data demonstrate that the return of normal pH\(_r\) after reperfusion leads to onset of the MPT and lethal reperfusion injury to cultured hepatocytes. Treatments such as reperfusion at low pH inhibited recovery of pH\(_r\), blocked onset of the MPT, and prevented cell killing. Cyclosporin A also blocked onset of the MPT and reduced cell killing. In addition, glycine protected against reperfusion injury, but the mechanism by which glycine prevented cell killing remains obscure and may be related to inhibition of proteolysis (33) or another process occurring after recovery of pH\(_r\).

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