Physiological pH
Effects on Posthypoxic Proximal Tubular Injury
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After O2 deprivation, tissue acidosis rapidly self-corrects. This study assessed the effect of this pH correction on the induction, and pathways, of posthypoxic proximal tubular injury. In addition, ways to prevent the resultant injury were explored. Isolated rat proximal tubular segments (PTSs) were subjected to hypoxia/reoxygenation (50/30 or 30/50 minutes) under the following incubation conditions: 1) continuous pH 7.4, 2) continuous pH 6.8, or 3) hypoxia at pH 6.8 and reoxygenation at pH 7.4 (NaHCO3, or Tris base addition). Continuously oxygenated PTSs maintained under these same pH conditions served as controls. Lethal cell injury was assessed by lactate dehydrogenase (LDH) release. pH effects on several purported pathways of hypoxia/reoxygenation injury were also assessed (ATP depletion, lipid peroxidation, and membrane deacylation). Acidosis blocked hypoxic LDH release (pH 7.4, 50±2%; pH 6.8, 6±1%) without mitigating membrane deacylation or ATP depletion. During reoxygenation, minimal LDH was released (3-5%) if pH was held constant. However, if posthypoxic pH was corrected, immediate (≤5 minutes) and marked cell death (e.g., 55±3% with Tris) occurred. This was dissociated from lipid peroxidation or new deacylation, and it was preceded by a depressed ATP/ADP ratio (suggesting an acidosis-associated defect in hypoxic/posthypoxic cell energetics). Realkalinization injury was not inevitable, since it could be substantially blocked by 1) posthypoxic glycine addition, 2) transient posthypoxic hypothermia, or 3) allowing a 10-minute reoxygenation (cell recovery) period before base addition. Neither mannitol nor graded buffer Ca2+ deletion conferred protection. Acute pH correction caused no injury to continuously oxygenated PTSs. Conclusions are as follows: 1) Posthypoxic "pH shock" causes virtually immediate cell death, not by causing de novo injury but, rather, by removing the cytoprotective effect of acidosis. 2) This injury can be prevented by a variety of methods, indicating a great potential for salvaging severely damaged posthypoxic PTSs. (Circulation Research 1993;72:837–846)

KEY WORDS • oxygen radicals • phospholipase • deacylation • reperfusion injury • acute renal failure

Although acute renal ischemia can cause extensive proximal tubular necrosis, its histological appearance does not typically manifest itself until the early reperfusion period.1 For example, if rats are subjected to 60 minutes of complete renal arterial occlusion and then the kidneys are immediately fixed by immersion in formalin, no light microscopic evidence of proximal tubular necrosis is observed. However, if 60 minutes of ischemia is followed by 30 minutes of vascular reperfusion, striking tubular necrosis is apparent. Thus, it is clear that some consequence of vascular reflow is required for the full expression of ischemic tissue damage. This phenomenon has been widely referred to as "reperfusion injury."

Several potential explanations for renal reperfusion injury have been advanced and include the following: 1) The appearance of cell necrosis during vascular reflow may either represent the normal histological evolution of lethal cell injury incurred during O2 deprivation or be due to ongoing renal ischemia during "reperfusion" because of the "no-reflow" phenomenon.2 The findings that ex vivo suspensions of proximal tubules are predominantly killed during oxygen deprivation, not reoxygenation,3–5 are consistent with these possibilities. 2) In vivo reperfusion injury may be induced by an abrupt increase in tubular metabolic work. According to this hypothesis,5 sublethally damaged tubules may become necrotic because reperfusion dictates glomerular filtration, tubular Na+ reabsorption, and, hence, a drain on already compromised cellular energetics. 3) Reperfusion injury may result from postischemic oxygen-derived free radical (OFR) production, triggering a distinct new wave of tissue damage.8 Reports that several antioxidants may mitigate postischemic acute renal failure support this view.9 4) Reperfusion injury may be mediated by acute cell Ca2+ loading. Since plasma Ca2+ concentrations exceed those within cells by approximately 10,000:1 and because damaged cells may be unable to extrude or sequester an acute calcium load, reperfusion may lead to critical intracellular calcium overload, thereby inducing cell necrosis.4,10 5) The appearance of cell necrosis during reperfusion, not ischemia, could be explained by an abrupt increase in tissue pH. During ischemia, tissue pH drops to ≈6.5–7.0, primarily because of ATP hydrolysis and CO2 accumulation.11 Since many tissues, including proximal tubules,
are reportedly resistant to O2 deprivation injury within this pH range.3,10,12–21 acidosis may explain why necrosis does not develop during an ischemic insult. During reperfusion, physiological pH is restored (“pH shock”), presumably because of H+ efflux from, and HCO3 entry into, the damaged kidney. Thus, it is theoretically possible that reperfusion injury could be caused by an abrupt pH increment and/or the loss of the hydrogen ion’s cytoprotective effect. In support of this pH theory are reports that 1) energy-depleted hepatocytes and cardiac myocytes develop cell necrosis with base addition16,17,37 and 2) bicarbonate addition causes mild cytosolic Ca2+ loading and total protein decrements in posthypoxic acidic renal tubules.3 However, whether these latter changes reflected lethal tubular damage was not clearly defined.

Because of the above suggestions that pH may be a critical determinant of postischemic/posthypoxic renal tubular injury, the present study was undertaken with the following specific objectives in mind: 1) to test whether an abrupt restoration of physiological pH during a posthypoxic period triggers proximal tubular necrosis, 2) to ascertain whether this necrosis is a result of de novo alkali-induced injury (i.e., pH shock) or whether it results from the loss of a transient acidosis-mediated cytoprotective effect, 3) to assess whether purported pH effects on pathways of hypoxia/reoxygenation injury (i.e., oxidant tissue stress,18,22,23 deranged cellular energetics,24,25 transmembrane Ca2+ fluxes,26,27 and phospholipase activity28–31) are important determinants of realkalinization-induced cell death, and 4) to ascertain whether realkalinization injury is inevitable (i.e., whether the cells are irreversibly damaged such that necrosis must ensue with the removal of the protective effect of acidosis). These questions were addressed using an in vitro system, rather than in vivo renal tissues, such that pH effects on systemic hemodynamics could be avoided, the onset and amount of cell injury could be strictly defined, and proximal tubular cell–specific results could be obtained.

**Materials and Methods**

**Proximal Tubular Segment Preparations**

Male Sprague-Dawley rats (150–250 g, Charles River Laboratories, Cambridge, Mass.) were used for all proximal tubular segment (PTS) preparations, as previously described.32 In brief, rats (one or two for each preparation) were anesthetized with pentobarbital (30–40 mg/kg), the kidneys were removed, and the cortices were resected, minced, and digested in 15 ml collagenase-containing buffer for 35 minutes to release PTSs. After digestion, the tissues were sieved, centrifuged, and washed five times to remove the remaining collagenase. Viable PTSs were separated from other cortical tissues by centrifugation through 31% Percoll, followed by suspension (3–6 mg PTS protein/ml) in a nutrient buffer with either a physiological (7.4) or an acidic (6.8) pH (thereby simulating normal arterial in vivo ischemic pH conditions, respectively)3,10,15. The pH 7.4 buffer consisted of (mM) NaCl 100, KCl 2.1, NaHCO3 25, KH2PO4 2.4, CaCl2 1.2, MgCl2 1.2, MgSO4 1.2, glucose 5, sodium lactate 4, sodium butyrate 10, and alanine 1, along with 0.6% dextran T500 gassed with 95% O2–5% CO2. The pH 6.8 buffer was identical, except that its pH was reduced to 6.8 by 1N HCl addition. Before all experiments, the PTS suspensions were rewarmed from 4°C (isolation temperature) to 36°C (experimentation temperature) for 15 minutes, and then a 150-μl aliquot was removed for an assessment of baseline PTS viability, as determined by the percent lactate dehydrogenase (LDH) release (a well-recognized marker of lethal hypoxia/reoxygenation injury) (e.g., see Reference 15).

**Set 1 Experiments: Effects of Acidosis and NaHCO3, Realkalinization on Hypoxia/Reoxygenation PTS Injury**

The following experiment assessed the degree to which acidosis mitigates lethal hypoxic PTS injury and whether restoration of a physiological pH with NaHCO3 during reoxygenation causes additional cell killing. To this end, each of five separate PTS preparations was divided into two parts, one part being suspended in either the pH 6.8 or the pH 7.4 buffer. After completing the 15-minute rewarming period, each sample was subdivided, creating the following experimental groups:

**Continuous PTS oxygenation at pH 6.8 and 7.4.** Aliquots of PTS (2 ml each), one suspended in the pH 6.8 and one in the pH 7.4 buffer, were subjected to continuous oxygenation (95% O2–5% CO2) in a shaking 36°C water bath for 50 minutes, followed by removal of a 150-μl aliquot for assessment of LDH release. Then, 250 μl of 200 mM NaCl was added to each aliquot (to serve as an osmotic control for NaHCO3 addition; see below). The PTSs were reoxidized with 95% O2–5% CO2 and incubated for an additional 30 minutes. Finally, LDH release was reassessed, and the suspension pH was determined while being gassed with 95% O2–5% CO2.

**Hypoxia/reoxygenation injury at pH 6.8 and 7.4.** Aliquots of PTS (2 ml each), one suspended in the pH 6.8 and one in the pH 7.4 buffer, were subjected to 50 minutes of hypoxic incubation (gassing with 95% N2–5% CO2)3–5 after which percent LDH release was determined. Then, 250 μl of 200 mM NaCl was added, and the PTSs were reoxygened (95% O2–5% CO2) for 30 minutes. Finally, LDH release was reassessed, and the ending suspension pH was determined.

**Effect of NaHCO3 addition on acidic PTS suspensions.** Two aliquots of PTS (2 ml), previously suspended in the pH 6.8 buffer, were subjected to either continuous oxygenation or to the above hypoxia/reoxygenation protocol. After completing either the initial 50-minute oxygenation or hypoxic period, NaHCO3 (=250 μl, 200 mM; the exact amount, ±10%, being that required to change the buffer pH from 6.8 to 7.4 on a given day) was added to abruptly raise the suspension pH. Percent LDH release (at 50 and 80 minutes) and the final PTS suspension pH were determined.

**Set 2 Experiments: Effect of Realkalinization With a Nonvolatile Buffer**

To assess whether the effect of NaHCO3 during reoxygenation is HCO3 specific or whether it is secondary to an abrupt rise in pH, the influence of nonvolatile buffer (Tris base) addition was determined. To this end, four separate PTS preparations were suspended in the pH 6.8 buffer and subdivided to create the following experimental groups: 1) continuous oxygenation+NaCl...
addition: 80 minutes of continuous oxygenation, as noted above; 2) continuous oxygenation + Tris addition: 80 minutes of continuous oxygenation, with Tris base (≈250 µL, 200 mM) being added at 50 minutes to restore a physiological pH; 3) hypoxia/reoxygenation + NaCl addition: hypoxia/reoxygenation protocol, as described above; and 4) hypoxia/reoxygenation + Tris addition: repeat of the hypoxia/reoxygenation protocol, except that =250 µL Tris base was added at the start of reoxygenation. LDH release was assessed at 50 and 80 minutes, and the final suspension pH was determined.

To assess how rapidly Tris exacerbates posthypoxic injury, three PTS aliquots were subjected to hypoxia (pH 6.8), with Tris being added at the start of reoxygenation. Percent LDH release was measured 5 minutes later.

To ascertain whether Tris alters PTS viability independent of its pH effect, the following controls were performed. Three separate PTS preparations were each subdivided into two parts: one part was suspended in the standard pH 7.4 buffer, and one was suspended in the pH 6.8 buffer after its pH was titrated back to pH 7.4 by Tris addition (creating the same Tris concentrations used above). Each of these samples was subjected to continuous oxygenation or to the 50-minute hypoxia/30-minute reoxygenation protocol described above. By comparing results obtained in these two different buffers, the effects of Tris on PTS viability, independent of pH changes, could be ascertained.

Set 3 Experiments: pH Effects on Membrane Decaylation/Reacetylation During Hypoxia/Reoxygenation

To ascertain the effects of pH on membrane decaylation/reacetylation during hypoxia/reoxygenation injury, three sets of PTSs were prepared, and each was divided into six aliquots: 1) hypoxia for 50 minutes in the pH 7.40 buffer, 2) hypoxia for 50 minutes in the pH 6.8 buffer, 3) hypoxia/reoxygenation (50/30 minutes) in the pH 7.4 buffer, 4) hypoxia/reoxygenation in the pH 6.8 buffer, 5) hypoxia/reoxygenation in the pH 6.8 buffer with NaHCO3 addition at the start of reoxygenation, and 6) hypoxia/reoxygenation with Tris addition at the start of reoxygenation. At the completion of the protocols (50 or 80 minutes), percent LDH release was assessed, and the PTS aliquots were analyzed by previously described high-performance liquid chromatography (HPLC)3 for the following dominant membrane-associated fatty acids: palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2), and arachidonic (C20:4) acids. Four PTS aliquots, subjected to 80 minutes of continuous oxygenation at pH 7.4, served to establish normal free fatty acid (FFA) concentrations.

Set 4 Experiments: pH Effects on Hypoxia/Reoxygenation Lipid Peroxidation

Three PTS preparations were divided into six aliquots: 1) oxygenated incubation for 80 minutes at pH 7.4, 2) oxygenated incubation for 80 minutes at pH 6.8, 3) hypoxia/reoxygenation (50/30 minutes) at pH 7.4, 4) hypoxia/reoxygenation at pH 6.8, 5) hypoxia (pH 6.8) with pH 7.4 reoxygenation (HCO3 addition), and 6) hypoxia (pH 6.8) with pH 7.4 reoxygenation (Tris addition). PTS malondialdehyde concentrations, an index of lipid peroxidation,9,32,33 and, hence, of oxidant stress, were assessed at the 50- and 80-minute time points by a previously described thiobarbituric assay method.32,33 Percent LDH release at these times was also assessed.

Set 5 Experiments: Effects of Extracellular Ca2+ on NaHCO3-Induced Reoxygenation Injury

To determine whether an extracellular—intracellular Ca2+ flux mediates realalkalization injury, four sets of PTSs were prepared and suspended in pH 6.8 buffer with either the standard Ca2+ concentration (1.2 mM) or a low Ca2+ concentration (120 µM or 120 nM Ca2+ added to the PTS buffer, n=2 each). Each of these samples was subdivided as follows: 1) 80 minutes of continuous oxygenation with NaCl addition at 50 minutes, 2) 80 minutes of continuous oxygenation + NaHCO3 addition at 50 minutes, and 3) 50-minute hypoxia/30-minute reoxygenation with NaHCO3 addition at 50 minutes. LDH release was determined at 50 and 80 minutes.

To ascertain whether extracellular Ca2+ depletion/deletion, imposed only during reoxygenation (not during hypoxia/reoxygenation, as described above), mitigates realalkalization injury, the following experiments were performed: Two sets of PTSs, suspended in the pH 6.8 buffer with a 1.2 mM Ca2+ concentration, were each divided into five aliquots and subjected to either 80 minutes of continuous oxygenation with pH correction at 30 minutes (one aliquot) or to 50 minutes of hypoxia (four aliquots) followed by pH correction and 30 minutes of reoxygenation. Just before pH correction, the free extracellular Ca2+ concentration was either left unaffected or lowered to =0.3, 0.1, or 0 mM by adding a Ca2+ chelator, EGTA, in a concentration of 0.9, 1.1, or 1.3 mM, respectively. Percent LDH release was assessed at the completion of the 80-minute incubation.

Set 6 Experiments: Effect of NaHCO3 Addition on PTS ATP Concentrations

Each of five PTS preparations was divided into two parts, one part being suspended in the pH 7.4 and one in the pH 6.8 buffer. The pH 7.4 aliquot was subdivided as follows: 1) hypoxia for 50 minutes, 2) 50-minute hypoxia/5-minute reoxygenation, and 3) 55 minutes of continuous oxygenation. The pH 6.8 aliquot was also subdivided to yield these same three conditions; in addition, two other pH 6.8 samples were added: 1) hypoxia/5-minute reoxygenation, with NaHCO3 being added at the start of reoxygenation, and 2) continuous oxygenation for 55 minutes, with NaHCO3 being added at 50 minutes. Only a 5-minute reoxygenation period was used, such that any changes in ATP levels induced by realalkalization would precede lethal cell injury, thereby suggesting a causal relation. On completing these protocols, an aliquot was removed for assessment of LDH release, and then the samples were analyzed for ATP, ADP, and AMP by HPLC, as previously described.9 Total adenine nucleotide (ATP+ADP+AMP) concentrations and the ATP/ADP ratio, a sensitive index of cell/respiratory integrity, were also assessed.

Attempts to Mitigate Realalkalization Injury

The following experiments assessed whether realalkalization injury is inevitable (i.e., whether the cells are irreversibly damaged during hypoxia such that cell death
during realkalinization must result) or whether cell viability can be maintained despite restitution of a physiological pH. To this end, four approaches were taken: 1) addition of the cytoprotectant glycine at the time of base addition, 2) imposing transient hypothermia at the time of base addition, 3) allowing 10 minutes of reoxygenation before base addition, and 4) mannitol addition before base addition. In these experiments, a 30-minute hypoxic period was used, and base correction was done using a 50%–50% mixture of Tris and HCO₃. The hypoxic challenge was shortened to 30 minutes, such that a greater likelihood of cell salvage might be achieved; the Tris-HCO₃ mixture was used, since this was found to induce more consistent results than HCO₃ alone.

**Glycine addition.** Five PTS preparations, suspended in the pH 6.8 buffer, were each divided into three aliquots: 1) continuous oxygenation for 80 minutes with pH correction at 30 minutes, 2) hypoxia/reoxygenation (30/50 minutes) with pH correction at the start of reoxygenation, and 3) hypoxia/reoxygenation as per aliquot 2 with glycine addition (final concentration, 3 mM) at the time of pH correction (30 minutes). Percent LDH release was assessed at the completion of the experiments.

To ascertain whether posthypoxic glycine addition can block more lengthy hypoxic injury, the above experiment was repeated (n=4) using a 50-minute hypoxia/30-minute reoxygenation protocol.

**Hypothermia.** It has previously been demonstrated in vivo that imposing hypothermia at the time of vascular reperfusion mitigates the severity of postischemic acute renal failure. However, it is unclear whether it does so by mitigating some consequence of vascular reperfusion (e.g., realkalinization injury) or by protecting persistently ischemic tubules subjected to the no-reflow phenomenon. To assess whether hypothermia can, in fact, mitigate posthypoxic tubular injury, five sets of PTSs, pH 6.8, were each divided into three aliquots: 1) continuous oxygenation for 70 minutes, 2) 30-minute hypoxia/40-minute reoxygenation with base addition at the start of reoxygenation, or 3) hypoxia/reoxygenation as per aliquot 2, except that at the time of base addition the PTSs were iced for 1 minute and then held at 22°C on a shaking water bath for 8 minutes. After completing this cooling period, the PTSs were immediately rewarmed (1 minute) to the routine incubation temperature (36°C) for an additional 30 minutes. Percent LDH release was determined at the end of each incubation (70 minutes).

**Delayed realkalinization.** The following experiment assessed whether allowing 10 minutes of reoxygenation before base addition would permit a modicum of cellular repair to occur such that the PTSs could withstand realkalinization injury. To this end, six PTS aliquots, pH 6.8, were each divided into three aliquots: 1) continuous oxygenation for 70 minutes, 2) 30-minute hypoxia/40-minute reoxygenation with base addition at the start of reoxygenation, and 3) hypoxia/reoxygenation as per aliquot 2, except that the time of base addition the PTSs were iced for 1 minute and then held at 22°C for 1 minute before being held at 22°C for 1 minute.

**Mannitol addition.** It has previously been observed that mannitol, given after ischemia, can mitigate in vivo acute renal failure. To ascertain whether it can also confer direct cytoprotection on posthypoxic tubules, the following experiment was performed. Five sets of PTSs, suspended in the pH 6.8 buffer, were each divided into three aliquots: 1) continuous oxygenation for 70 minutes, 2) 30-minute hypoxia/40-minute reoxygenation with realkalinization at 10 minutes after reoxygenation, and 3) hypoxia/reoxygenation and realkalinization as per aliquot 2 but with mannitol addition (final concentration, 100 mM) at the start of reoxygenation. Percent LDH release was assessed at the end of the 70-minute incubation. (Of note, it had previously been determined that 100 mM mannitol has no direct adverse effect on PTS LDH release.)

**Calculations and Statistics**

All values are presented as mean±1 SEM. For each set of experiments, the results from paired PTS aliquots were compared by two-tailed paired Student’s t test. Where appropriate, the Bonferroni correction was applied. The percent LDH release values reported represent the actual percent LDH released minus the baseline percent LDH released (i.e., the value observed after the 15-minute rewarmed process) such that the given values reflect how much cell injury occurred during the actual period of experimentation. In addition, by so doing, slight differences in percent LDH release incurred during the rewarmed process in the pH 6.8 and pH 7.4 buffer on any particular day could be normalized (overall: pH 6.8, 7±1%; pH 7.4, 6±1% after completing rewarmed). Statistical significance was judged by a value of p<0.05. For the sake of concise data presentation, the LDH release data from identical protocols used throughout the study are depicted together (since comparable results were obtained).

**Results**

**Effect of Acidosis and Realkalinization on Hypoxia/Reoxygenation Injury: LDH Release**

As depicted in Figure 1, PTSs suspended in pH 7.4 buffer developed marked cell killing during hypoxia (50±2% LDH release) but not during reoxygenation...
TABLE 1. Individual Free Fatty Acid Concentrations After Hypoxia/Reoxygenation Injury in Isolated Rat Proximal Tubular Segments

<table>
<thead>
<tr>
<th></th>
<th>C16:0 (nmol/mg protein)</th>
<th>C18:0 (nmol/mg protein)</th>
<th>C18:1 (nmol/mg protein)</th>
<th>C18:2 (nmol/mg protein)</th>
<th>C20:4 (nmol/mg protein)</th>
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<tbody>
<tr>
<td>Normal</td>
<td>0.51±0.10</td>
<td>0.67±0.08</td>
<td>0.06±0.01</td>
<td>0.04±0.006</td>
<td>0.15±0.03</td>
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<tr>
<td>Hypoxia</td>
<td></td>
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<tr>
<td>N₂ (pH 7.4)</td>
<td>2.66±0.25</td>
<td>1.42±0.25</td>
<td>0.88±0.16</td>
<td>1.37±0.17</td>
<td>1.82±0.17</td>
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<tr>
<td>N₂ (pH 6.8)</td>
<td>2.80±0.30</td>
<td>1.39±0.25</td>
<td>0.86±0.16</td>
<td>1.27±0.12</td>
<td>1.71±0.20</td>
</tr>
<tr>
<td>Hypoxia/reoxygenation</td>
<td></td>
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<tr>
<td>N₂/O₂ (pH 7.4)</td>
<td>2.94±0.34</td>
<td>1.85±0.23</td>
<td>0.93±0.12</td>
<td>1.31±0.25</td>
<td>1.73±0.16</td>
</tr>
<tr>
<td>N₂/O₂ (pH 6.8)</td>
<td>2.40±0.35</td>
<td>1.71±0.31</td>
<td>0.64±0.07</td>
<td>0.79±0.17</td>
<td>0.96±0.12</td>
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<tr>
<td>N₂/O₂ (pH 6.8)/O₂ (HCO₃⁻)</td>
<td></td>
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<tr>
<td>N₂ (pH 6.8)/O₂ (Tris)</td>
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<td></td>
<td>3.05±0.32</td>
<td>1.80±0.17</td>
<td>0.90±0.06</td>
<td>1.31±0.19</td>
<td>1.69±0.16</td>
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FFA, free fatty acid. Values are mean±1 SEM.

Individual FFA concentrations during hypoxia and reoxygenation are shown under different pH conditions. The normal proximal tubular segment FFA concentrations were determined on four aliquots of proximal tubular segments (obtained from four separate preparations) after 80 minutes of continuous oxygenation at pH 7.4. Proximal tubular segments were subjected to hypoxia at pH 7.4 and pH 6.8, hypoxia and reoxygenation at pH 7.4 and pH 6.8, and hypoxia at pH 6.8 followed by HCO₃⁻ or Tris addition during reoxygenation. The FFA totals for these groups are presented in Figure 2.

(≈5% increment over the end-hypoxic value). In contrast, PTSs suspended in the pH 6.8 buffer appeared to be completely protected against hypoxia/reoxygenation, with just 9±1% LDH release occurring by the end of the experiment (p=NS versus continuously oxygenated pH 7.4 controls [7±1%]). However, if NaHCO₃ was added at the start of reoxygenation, the protection afforded by acidosis was largely eliminated, with the percent LDH release rising from an end-hypoxic value of 6±1% to 36±3% by the completion of the reoxygenation period. This realkalinization injury was not NaHCO₃ specific, since correction of pH during reoxygenation with Tris addition caused even greater LDH release (to 55±3%, exactly equalizing that observed in PTSs subjected to hypoxia/reoxygenation at a constant pH 7.4). This Tris effect was due to the pH correction and not to direct Tris toxicity, since the Tris control experiments (which compared pH 7.4 Tris buffer with the standard pH 7.4 buffer) revealed essentially identical percent LDH releases after hypoxia (48±1% versus 50±1%), after reoxygenation (54±1% versus 53±1%), and after 80 minutes of continuous oxygenation (5±2% versus 5±2%) (Tris buffer versus standard buffer, respectively; data not depicted). Tris-mediated posthypoxic killing was completely expressed within just 5 minutes, since by 5 and 30 minutes after Tris addition, identical percent LDH release results were observed (both 55±3%).

Effect of pH and pH Correction on Continuously Oxygenated PTSs

Under oxygenated conditions, identical percent LDH releases were observed in the pH 6.8 or 7.4 buffer (after 50 minutes, both 4±1%; after 80 minutes, both 7±1%), indicating no direct pH effect on PTS viability in the absence of hypoxia. Rapid pH correction from 6.8 to 7.4 with HCO₃⁻ or Tris addition after 50 minutes of oxygenation also did not significantly increase percent LDH release (7±1% versus 10±1%, p=NS versus 80-minute continuously oxygenated controls). Thus, no evidence for realkalinization-mediated cell killing in the absence of hypoxia was obtained.

Ending pH Assessments

The final pH for PTSs subjected to continuous oxygenation in the pH 7.4 buffer was 7.45±0.03. Those PTSs that underwent continuous oxygenation in the pH 6.8 buffer had a final pH of 7.09±0.03 (indicating H⁺ buffering during the course of the experiments). NaHCO₃ and Tris addition raised these pH values to 7.50±0.06 and 7.46±0.04, respectively. All PTSs subjected to hypoxia/reoxygenation showed a drop in the final pH, in comparison to their continuously oxygenated counterparts. How-

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**FIGURE 2. Bar graph showing total free fatty acid (FFA) concentrations (C16:0+C18:0+C18:1+C18:2+C20:4) after hypoxia and reoxygenation under different pH conditions, pH 7.4, continuous pH 7.4, pH 6.8, continuous pH 6.8; pH 6.8+HCO₃⁻ or pH 6.8+tris, hypoxia at pH 6.8+base addition at the start of reoxygenation to restore pH 7.4; NS, not significant. The shaded area represents the 95% confidence band for total FFAs under oxygenated conditions.**
ever, the decrease was threefold greater \((p<0.002)\) for those PTSs that underwent hypoxia/reoxygenation in the acid buffer \((7.09±0.03→6.93±0.2, Δ0.16±0.02)\) versus the pH 7.4 buffer \((7.46±0.04→7.41±0.03, Δ0.05±0.02)\).

**Free Fatty Acid Concentrations**

Individual free fatty acid (FFA) concentrations are given in Table I, and their totals are presented in Figure 2. Hypoxia caused marked elevations of all FFAs, the totals rising sevenfold to eightfold over the values seen in the oxygenated controls. This increment was not influenced by incubation pH. During reoxygenation, those PTSs maintained a continuous 7.4 pH did not change their FFA concentrations (i.e., in comparison to their end-hypoxic values). However, the continuously acidotic PTSs were able to decrease their FFA concentrations by \(≈20\%\) during reoxygenation \((p<0.005)\), indicating reacylation and/or FFA metabolism (and hence, a modicum of cellular protection versus their pH 7.4 posthypoxic counterparts). HCO3 or Tris, added during reoxygenation, prevented these FFA decrements (Figure 2). However, they did not induce any FFA increments (i.e., over the end-hypoxic values).

**Lipid Peroxidation During Hypoxia/Reoxygenation**

As depicted in Figure 3, reoxygenation of hypoxic PTSs did not cause lipid peroxidation, irrespective of the pH conditions. Rather, malondialdehyde values fell from their end-hypoxic values in nine of 12 instances \((p<0.05)\), despite the fact that HCO3 or Tris addition caused marked cell killing during this period \((53±4\% and 48±5\% release for these particular Tris- and HCO3-treated aliquots, respectively). Malondialdehyde concentrations for PTSs maintained under continuous oxygenated conditions for 50 and 80 minutes were 0.68±0.1 and 0.46±0.1 nmol/mg protein, respectively; the results were not influenced by the incubation pH.

**Extracellular Ca\(^{2+}\) Effects of Realalkalinization Injury**

Removing Ca\(^{2+}\) from the PTS buffer tended to exacerbate, not mitigate, HCO3-induced posthypoxic LDH release \((1.2 \text{mM Ca}^{2+}, 29±4\%; 120 \text{µM Ca}^{2+}, 28\% and 40\%); 120 \text{nM Ca}^{2+}, 33\% and 49\%). In addition, low Ca\(^{2+}\) incubation tended to increase PTS LDH release when NaHCO3 was added to oxygenated PTSs \((1.2 \text{mM Ca}^{2+}, 7±1\%; 120 \text{µM Ca}^{2+}, 11\% and 11\%; 120 \text{nM Ca}^{2+}, 28\% and 18\%). Neither the 120 \text{µM nor the 120 nM Ca}^{2+} buffer altered PTS viability under conditions of continuous oxygenation without HCO3 addition \((≤9\% \text{ LDH release})\).

Addition of EGTA before realalkalinization/reoxygenation did not mitigate the extent of LDH release \((no \text{EGTA, 56\% and 52\%; 0.9 mM EGTA, 54\% and 57\%; 1.1 mM EGTA, 54\% and 58\%; 1.3 mM EGTA, 56\% and 58\%; oxygenated controls, 9\% and 14\%)\).

**Adenine Nucleotide Assessments**

Under oxygenated conditions, pH 6.8 versus 7.4 incubation did not affect ATP levels (Figure 4), the ATP/ADP ratio (Figure 4), or the total adenine nucleotide pool (range, 9.9–11.4 nmol/mg protein; not depicted). NaHCO3 addition to oxygenated PTS at pH 6.8 did not significantly alter any of these adenylate parameters compared with the pH 7.4 controls. Hypoxia caused profound ATP depletion, the degree not being pH dependent (Figure 4). Total adenine nucleotide was better preserved at pH 6.8 versus pH 7.4 \((4.15±0.50 versus 2.14±0.20 \text{nmol/mg, } p<0.005)\), but the ATP/ADP ratio was significantly worse at the lower pH \((0.65±0.01 versus 0.89±0.03, p<0.002)\). During reoxygenation, the continuous pH 6.8 and pH 7.4 groups had virtually the same degree of ATP resynthesis, despite

**Figure 3.** Graph showing malondialdehyde (MDA) concentrations at the end of hypoxia and after 30 minutes of reoxygenation. pH 6.8, continuous pH 6.8; pH 7.4, continuous pH 7.4; pH 6.8+HCO3 or pH 6.8+Tris, base added at the start of reoxygenation. The points represent the mean values for each group of three. All SEM values are ±0.1. In no instance did the reoxygennation value exceed that for the time-matched oxygenated controls \((0.46±0.10 \text{nmol/mg protein})\).

**Figure 4.** Bar graph showing ATP and ATP/ADP ratios (left ordinate) and percent lactate dehydrogenase (LDH) release (right ordinate) under oxygenated \((O_2)\), hypoxic \((N_2)\), and 5-minute reoxygenated \((N_2→O_2)\) conditions at differing pH levels. 6.8→7.4 indicates HCO3 addition to pH 6.8→exposed proximal tubular segments.
marked differences in LDH release and despite the fact that the residual total adenine nucleotide pool was higher at pH 6.8 versus pH 7.4 (4.31±0.52 versus 2.63±0.09, p<0.03; indicating more residual nucleotide for ATP resynthesis at the lower pH). This relative lack of ATP formation in the pH 6.8 posthypoxic group was also reflected by a continuing significant depression of the ATP/ADP ratio (1.33±0.07) versus that observed in the pH 7.4 posthypoxic group (2.32±0.20, p<0.005). NaHCO₃ addition during reoxygenation did not lower ATP, the ATP/ADP ratio, or total adenine nucleotide (compared with the pH 6.8 reoxygenated group), despite its causing modest LDH release (20±6% versus 9±1% for the pH 6.8 reoxygenated group, p<0.05).

Attempts to Prevent Realkalinization Injury

Glycine. Addition of glycine significantly attenuated the realkalinization injury that followed both the 30-minute hypoxic and 50-minute hypoxic challenges (Figures 5A and 5B, respectively). Background LDH release (no hypoxia, base addition) for these experiments was 10±1%.

Hypothermia. Brief hypothermia imposed at the time of base addition virtually eliminated realkalinization/reoxygenation injury (Figure 6A). Background LDH release for these experiments (no hypoxia, base addition) was 10±1%.

Delayed realkalinization. Allowing 10 minutes of reoxygenation before base addition also conferred significant protection against realkalinization injury (Figure 6B). Background LDH release for this experiment was 12±1%.

Mannitol. Mannitol addition at the end of hypoxia paradoxically exacerbated the cell injury that resulted from base addition 10 minutes into the reoxygenation period (no mannitol, 26±1%; mannitol, 38±3%; p<0.03). Background LDH release was 11±1%.

Discussion

One of the most striking features of ischemic acute renal failure is that tubular necrosis becomes apparent not during O₂ deprivation but, rather, during reoxygenation, when early cellular repair theoretically should begin. Although multiple studies have indicated that acidosis, a constant component of tissue ischemia, may explain the maintenance of cell integrity during ATP depletion (reviewed in Reference 10), the reason for the rapid appearance of necrosis during reoxygenation has remained much more controversial. Thus, the goals of this study were to 1) ascertain to what degree acidosis protects PTs from hypoxic cell injury, 2) analyze whether an abrupt restoration of physiological pH (pH shock) during reoxygenation triggers tubular necrosis (e.g., by inducing de novo injury or by removing the...
The present study confirms that acidosis can indeed confer striking protection against O2 deprivation–induced tubular injury, since lowering the buffer pH from 7.4 to 6.8 reduced LDH release from 55 ± 2% to 9 ± 1%, with the latter value not significantly differing from that observed in continuously oxygenated controls. However, it is important to note that, although acidosis appeared to totally block cell killing, severe, albeit sublethal, cell injury still occurred. In support of this conclusion are the following findings: 1) Acidosis did not mitigate deacylation (FFA increments), a marker of cell membrane injury (e.g., see Reference 5). 2) Severe ATP depletion still resulted, and the ATP/ADP ratio, an overall marker of cellular respiratory integrity, was, in fact, worsened under acidic conditions (assessed both during hypoxia and reoxygenation). 3) With realkalinization, virtual immediate cell death ensued (e.g., 55 ± 3% LDH release within 5 minutes of Tris addition). Of note, base addition caused no cell death in the absence of prior hypoxia. Thus, the fact that massive, and essentially instantaneous, posthypoxic cell death occurred with base addition indicates that 1) acidosis does not prevent severe hypoxic injury and 2) the ability of pH shock to cause posthypoxic cell killing is due to the removal of the protective influence of acidosis, not pH shock per se, since pH correction in the absence of hypoxia caused no LDH release. An interesting observation throughout this study was that Tris consistently caused greater posthypoxic LDH release than did HCO3− (at 30 minutes of reoxygenation, 55% versus 36%; at 5 minutes of reoxygenation, 55% versus 20%) despite comparable posthypoxic pH corrections. Although the reason for this is unknown, a likely explanation is that HCO3− generates CO2 during the buffering process. Since CO2, but not HCO3−, diffuses intracellularly, this partitioning could mitigate the HCO3−-induced intracellular pH increment, thereby lessening realkalinization injury. Of note in this regard is that intracellular, not extracellular, pH is believed to be the critical determinant of the protective effect of acidosis.20

After it was demonstrated that pH correction evokes dramatic posthypoxic PTS injury, the next goal of this study was to ascertain whether several purported pH effects on pathways of O2 deprivation/reoxygenation injury were responsible. Since pH can influence OFR production18,22,23 and because OFRs have been suggested as mediators of postischemic acute renal failure,9 the influence of pH shock on reoxygenation lipid per-oxidation (malondialdehyde concentrations) was assessed. However, malondialdehyde levels fell during reoxygenation, irrespective of the treatment protocol. Thus, it appears quite unlikely that reoxygenation/realkalinization injury is OFR dependent. Several previous in vitro studies have also suggested that reoxygenation does not evoke OFR-mediated injury.36–39

However, in each of those studies, hypoxia was induced at pH 7.4. Since this pH appears to preclude “reoxygenation” injury (i.e., LDH release occurs during hypoxia, not reoxygenation; see Figure 1), negative assessments of oxidant stress under those conditions must be interpreted with caution. However, in the present study, pH adjustments (pH 6.8 hypoxia/pH 7.4 reoxygenation) shifted cell killing from the hypoxic to the posthypoxic period, and yet falling, not rising, malondialdehyde levels resulted. Thus, this finding strongly supports previous assertions that OFRs are not critical mediators of posthypoxic, or postischemic, tubular injury.

Because an extracellular—intracellular Ca2+ flux may underlie both hypoxia/reoxygenation and ischemia/reperfusion injury (e.g., see References 10 and 40) and because a transmembrane Ca2+ shift may be pH dependent (reduced at a low pH),26,27 protection against realkalinization injury was sought by Ca2+ removal from the PTS buffer. Because extreme extracellular Ca2+ depletion can have direct adverse cellular effects,10 different levels of Ca2+ depletion were tested, both by removing Ca2+ from the buffer and by variable degrees of EGTA addition. However, despite these different approaches and different degrees of Ca2+ deletion, no protection was obtained. Thus, it appears unlikely that PTS realkalinization injury is extracellular Ca2+ dependent. However, this does not exclude Ca2+, per se, as an important factor in hypoxic/reoxygenation injury, since cytosolic Ca2+ loading can result from intracellular, as well as extracellular, Ca2+ translocations and because Ca2+/calmodulin binding is pH dependent.51,42

Although a pH-dependent depression of phospholipase activity did not appear to explain the cytoprotective effect of acidosis (i.e., equal FFA increments occurred during hypoxia, irrespective of pH), the possibility that posthypoxic pH shock triggers a new wave of membrane deacylation and, hence, of cell killing required investigation. Thus, reoxygenation-induced FFA changes were quantified, and the following results were obtained: 1) Under conditions of a constant 7.4 pH, FFA concentrations remained unchanged during the posthypoxic period, suggesting no reoxygenation-induced deacylation. 2) Continuous pH 6.8–exposed PTSs developed modest posthypoxic FFA decrements (consistent with membrane reacylation and/or FFA metabolism and confirming a modicum of acidosis-induced protection). 3) Tris-HCO3− addition during reoxygenation caused no FFA increments (versus end-hypoxic values) despite their causing marked cell killing. Of note, cell death can nonspecifically cause FFA release. Thus, the failure of additional FFA generation during realkalinization strongly suggests that Tris-HCO3−-induced cell killing was not due to an acute stimulation of phospholipase activity.

Because acidosis can suppress Na+/H+ exchange,24 a process that can consume a substantial amount of proximal tubular ATP,25 it was tested whether realkalinization abruptly increases ATP consumption, thereby inducing energy depletion and, hence, cell necrosis during a posthypoxic period. To this end, adenine nucleotides were measured in the immediate (5-minute) posthypoxic period in an attempt to document ATP depletion before massive cell killing, thereby supporting a cause and effect relation. However, HCO3− addition did not compromise posthypoxic ATP concentrations despite the fact that 10% LDH release occurred. Thus, this finding suggests that realkalinization does not abruptly compromise cellular energetics. Of great interest was that the protected (pH 6.8) and
unprotected (pH 7.4) PTSSs had virtually identical ATP levels, whether assessed at the end of hypoxia or during reoxygenation. Given the dramatic differences in LDH release for these two groups (54% versus 9%), far higher ATP values would be expected in the pH 6.8-protected group, particularly during the reoxygenation period, when ATP resynthesis can occur. Indeed, factoring the reoxygenation ATP values by the number of "viable cells" (i.e., those retaining LDH) suggests markedly lower ATP concentrations in the pH 6.8-protected group. Furthermore, as noted previously, the reoxygenation ATP/ADP ratio, a sensitive index of mitochondrial/cellular respiratory integrity, was approximately 50% lower at pH 6.8 than at pH 7.4. Thus, these findings raise the intriguing possibility that, although acidaemia may confer cytoprotection, it may compromise hypoxia/reoxygenation cellular energetics, potentially predisposing to acute realkalinization injury. Of note, in this regard, is that gentamicin also exacerbates posthypoxic/postischemic tubular injury while inducing this same type of defect in cellular energetics.43

The final goal of this study was to ascertain whether acidosis merely delays cell death (i.e., that despite the absence of membrane disruption, irreversible cell injury has occurred such that cell death is inevitable) or whether prevention/rescue from realkalinization injury can be achieved. To make this assessment, the ability of glycine (a well-known cytoprotectant) to block realkalinization injury was addressed, and indeed, marked protection was achieved. This result is important for at least three reasons: 1) It has previously been indicated that glycine must be present during hypoxia to block hypoxic cell injury10,34; however, the current finding that it is also markedly protective even when added during reoxygenation indicates that this is clearly not the case. 2) The fact that glycine is able to block cell death even after the completion of the hypoxic period indicates that it must exert its beneficial influence at some distal site in the cell injury cascade. 3) Glycine-induced protection suggests that posthypoxic cell salvage may be possible. However, in regard to this last issue, a major caveat must be noted: if acidosis and glycine were to confer protection by a common pathway (e.g., membrane stabilization), then substituting glycine for acidosis at the time of realkalinization need not necessarily indicate that durable cell salvage can, in fact, be achieved (i.e., outliving the presence of the protective agent). Thus, two additional experiments were undertaken: 1) imposing transient hypothermia at the time of realkalinization and 2) delaying realkalinization for 10 minutes into the reoxygenation period such that a modicum of cell repair might occur, thereby allowing them to withstand pH shock. Indeed, both of these maneuvers conferred dramatic protection, indicating that durable posthypoxic cell salvage can, in fact, be achieved. Of interest, mannitol, an agent known to mitigate postischemic acute renal failure even when administered after ischemia,36 worsened rather than improved realkalinization injury. Thus, it may be that mannitol's ability to improve in vivo postischemic renal injury is largely due to its diuretic/vasodilatory influences rather than to a direct cytoprotective effect.

In conclusion, this study indicates the following: 1) Restoration of a physiological pH after hypoxia triggers severe and virtually instantaneous tubular cell death. This indicates that although acidosis may prevent lethal hypoxic injury, severe (albeit sublethal) cell damage still occurs. 2) pH correction triggers posthypoxic tubular cell death not by causing de novo injury but, rather, by removing the cytoprotective effect of acidosis. 3) This realkalinizationreoxygenation injury occurs in the absence of lipid peroxidation or acute deacylation, and it is extracellular Ca2+ independent. 4) Despite inducing cytoprotection, acidosis may adversely affect hypoxia/reoxygenation cellular energetics (e.g., as reflected by a depressed ATP/ADP ratio), perhaps predisposing to the realkalinization effect. 5) Realkalinization injury is not inevitable; i.e., substantial posthypoxic cell salvage can be achieved by a variety of methods. Thus, it may be possible to convert the seemingly transient cytoprotective influence of acidosis into a sustained beneficial effect.

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