Effects of ischemia-reperfusion injury on renal ammonia metabolism and the collecting duct

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Han K-H, Kim H-Y, Croker BP, Reungjui S, Lee S-Y, Kim J, Handlogten ME, Adin CA, Weiner ID. Effects of ischemia-reperfusion injury on renal ammonia metabolism and the collecting duct. Am J Physiol Renal Physiol 293: F1342–F1354, 2007. First published August 8, 2007; doi:10.1152/ajprenal.00437.2006.—Acute renal injury induces metabolic acidosis, but its specific effects on the collecting duct, the primary site for urinary ammonia secretion, the primary component of net acid excretion, are incompletely understood. We induced ischemia-reperfusion (I/R) acute renal injury in Sprague-Dawley rats by clamping the renal pedicles bilaterally for 30 min followed by reperfusion for 6 h. Control rats underwent sham surgery without renal pedicle clamping. I/R injury decreased urinary ammonia excretion significantly but did not persistently alter urine volume, Na⁺, K⁺, or bicarbonate excretion. Histological examination demonstrated cellular damage in the outer and inner medullary collecting duct, as well as in the proximal tubule and the thick ascending limb of the loop of Henle. A subset of collecting duct cells were damaged and/or detached from the basement membrane; these cells were present predominantly in the outer medulla and were less frequent in the inner medulla. Immunohistochemistry identified that the damaged/detached cells were A-type intercalated cells, not principal cells. Both TdT-mediated dUTP nick-end labeling (TUNEL) staining and transmission electron microscopic examination demonstrated apoptosis but not necrosis. However, immunoreactivity for caspase-3 was observed in the proximal tubule, but not in collecting duct intercalated cells, suggesting that mechanism(s) of collecting duct intercalated cell apoptosis differ from those operative in the proximal tubule. We conclude that I/R injury decreases renal ammonia excretion and is associated with intercalated cell-specific detachment and apoptosis in the outer and inner medullary collecting duct. These effects likely contribute to the metabolic acidosis frequently observed in acute renal injury.

Acute renal injury is a common clinical occurrence that results in abnormalities in multiple renal functions. Glomerular filtration is decreased, impairing the ability to excrete metabolic impurities (reviewed in 32, 60). Sodium, potassium, and water transport are impaired, leading to volume overload, hyperkalemia, and disorders of water balance. Mineral metabolism, including calcium and phosphorus and vitamin D metabolism, is abnormal, resulting in hypocalcemia, hyperphosphatemia, and hyperparathyroidism. In addition, acid-base disturbances are common and can contribute to impaired cardiovascular stability due to complications of metabolic acidosis on myocardial contractility, arrhythmogenicity, and vascular reactivity (1).

The mechanism by which acute renal injury induces metabolic acidosis is not well understood. Metabolic acidosis could either reflect urinary bicarbonate losses resulting from inadequate bicarbonate reabsorption or inadequate net acid excretion. The routine clinical observation that urine pH is ~6 in acute renal injury makes urinary bicarbonate losses relatively unlikely. The primary component of net acid excretion is ammonia, both under basal conditions and in response to metabolic acidosis (10, 12, 18). If there is inadequate renal ammonia metabolism in acute renal injury, then it is unlikely to be due to changes in glomerular filtration; essentially none of urinary ammonia excretion derives from glomerular filtration (20). Instead, ammonia metabolism involves intrarenal ammonia production combined with specific transport mechanisms in the proximal tubule, loop of Henle, and the collecting duct (10, 31). Thus changes in renal ammonia excretion in acute renal injury, if present, are likely to involve changes in one or more components of renal ammonia metabolism.

In the present studies, we sought to examine the effects of acute renal injury on renal ammonia metabolism. We induced acute renal injury using an ischemia-reperfusion injury model. First, we examined whether ischemia-reperfusion injury alters glomerular filtration rate (GFR), renal ammonia excretion, and other measures of renal tubular ion transport. We then examined whether ischemia-reperfusion injury damages the renal collecting duct, the site where 70–80% of urinary ammonia is secreted (20, 52), and, if so, whether ischemia-reperfusion injury has specific effects on intercalated cells, principal cells, or both. Finally, we examined whether ischemia-reperfusion injury-induced cell injury in the collecting duct involved apoptosis or cellular necrosis.

METHODS

Committee, depending on the site of the animal studies, and were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. Male Sprague Dawley rats weighing 250–350 g were obtained from Harlan Sprague Dawley (Indianapolis, IN) and maintained in a temperature-controlled room with alternating 12:12-h light-dark cycles. Animals were fed a standard diet and allowed free access to water.

Surgical procedures. Rats were anesthetized using 5% inhalant isoflurane in 100% oxygen and maintained with 1.5–2% isoflurane in 100% oxygen through a tracheostomy tube. Animals were placed on a heating pad, and body temperature was monitored using a rectal thermometer (Control Company, Friendswood, TX) and maintained at 37 ± 1°C. The left femoral vein and artery were catheterized using polyethylene tubing (PE-50, Intramedic, Clay-Adams, Parsippany, NJ) for fluid administration and blood sampling, respectively. All animals received fluid support including p-aminohippuric acid (PAH)–inulin (inulin 2.5 mg/ml; PAH 0.001 g/ml) at a rate of 3 ml/h for the duration of surgery. A polyethylene tubing T-port was constructed and attached to the arterial catheter (Transonic Systems, Ithaca, NY) during the entire duration of the experiment. Following a ventral midline celiotomy, the urinary bladder was catheterized using PE-160 (Intramedic) tubing, and the renal pedicles were isolated. Renal ischemia was induced by clamping both renal pedicles for 30 min using vascular microclamps (Accurate Technologies, Falls Church, VA) during the entire duration of the experiment. Similar results were obtained. After clamp removal, the kidneys were harvested, the abdomen was sutured closed using 4-0 polyglyconate (Maxon, Sherwood, Davis, and Geck, St. Louis, MO) for fluid administration and blood sampling, respectively. All animals received fluid support including p-aminohippuric acid (PAH)–inulin (inulin-FITC, Sigma) was 10% neutral buffered formalin for histological analysis. In some cases, a similar experimental model was used except intravenous fluids were not administered during the surgical period. Similar results were obtained.

Sham-operated control rats were treated identically, except that the renal pedicles were not clamped.

Reagents. PAH sodium salt (Sigma, St. Louis, MO) was dissolved in distilled water to form a 0.22 g/ml solution. Seventy-milligram doses of fluorescein isothiocyanate-inulin (inulin-FITC, Sigma) were then dissolved in 29.85 ml sterile saline and added to 150 μl of the 0.22 g/ml PAH solution. These solutions were prepared before each experiment and were protected from light at all times by covering with aluminum foil.

Serum and urine analysis. Blood was collected at the time of catheter placement (baseline, 1 ml), 15 minutes before ischemia (200 μl), at clamp removal (0 h, 300 μl), and 3 h (1 ml) and 6 h (2 ml) after clamp removal. Serum blood urea nitrogen (BUN) and creatinine were measured at baseline, 0, 3, and 6 h, and serum sodium and potassium were measured at baseline, 3, and 6 h. Serum inulin concentrations were measured 15 min before ischemia and 3 and 6 h posts ischemia. Urine was collected for 30 min before ischemia and collectively for 0–3 h and 3–6 h post-clamp removal. Urine volumes were recorded and used to determine urine flow rate. Urine sodium and potassium were measured from the baseline (preischemia), 0- to 3-h, and 3- to 6-h samples. Urine and serum samples were stored at −20°C until the completion of the experiment. Samples for inulin determination were transferred to −80°C until analyses were performed.

Sodium, potassium, creatinine, urea nitrogen, and HCO₃⁻ in urine were determined using the VetACE clinical chemistry system (Alfa Wassermann, West Caldwell, NJ). Urinary ammonia concentration was measured using a commercially available assay (Ammonia Reagent Set, Pointe Scientific, Canton, MI).

Antibodies. Affinity-purified antibodies to Rh B-glycoprotein (Rhbg) and Rh C-glycoprotein (Rhcg) generated in this laboratory have been characterized previously (24, 55, 56, 65, 69). Antibodies to the

<table>
<thead>
<tr>
<th>Table 1. Effect of ischemia-reperfusion injury on urine composition</th>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
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<tr>
<td>Sham</td>
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<td>Sham</td>
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Values are mean ± SE. GFR, glomerular filtration rate; Pre, 30 min time period before ischemia-reperfusion. *P < 0.05, †P < 0.001 vs. sham operated.
Effect of ischemia-reperfusion injury on renal cell histology

Table 2. Effect of ischemia-reperfusion injury on renal cell histology

<table>
<thead>
<tr>
<th>Segment/Condition</th>
<th>Normal</th>
<th>Cell Swelling</th>
<th>Vacuolization</th>
<th>BB Loss</th>
<th>Nuclear Condensation</th>
<th>Karyolysis, Karyorrhexis, Cell Sloughing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal convoluted tubule</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>0.67±0.02</td>
<td>1.67±0.42</td>
<td>2.67±0.33</td>
<td>1.50±0.50</td>
<td>0.50±0.22</td>
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</tr>
<tr>
<td>Control</td>
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<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
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<tr>
<td>Proximal straight tubule</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>0.00±0.00</td>
<td>2.50±0.34</td>
<td>3.00±0.00</td>
<td>3.00±0.00</td>
<td>2.17±0.31</td>
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<tr>
<td>Control</td>
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<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
<td>0.00±0.00*</td>
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<td>MTAL</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ischemia-reperfusion</td>
<td>1.17±0.54</td>
<td>1.33±0.42</td>
<td>N/A</td>
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<td>1.33±0.21</td>
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</tr>
<tr>
<td>Control</td>
<td>5.00±0.00*</td>
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<td>N/A</td>
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<td>0.00±0.00*</td>
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<tr>
<td>Collecting duct</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Ischemia-reperfusion</td>
<td>0.00±0.00</td>
<td>2.33±0.33</td>
<td>N/A</td>
<td>2.67±0.33</td>
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<tr>
<td>Control</td>
<td>5.00±0.00*</td>
<td>0.00±0.00*</td>
<td>N/A</td>
<td>0.00±0.00*</td>
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Values are means ± SE. Scoring system: “+0” if none were involved, “+1” if approximately 1–10%, “+2” if approximately 11–25%, “+3” if approximately 26–50%, “+4” if approximately 51–75%, and “+5” if approximately 76–100% were involved. MTAL, medullary thick ascending limb of the loop of Henle; BB loss, brush-border loss; N/A, not applicable. *P < 0.001, †P < 0.01, ‡P < 0.05 vs. ischemia-reperfusion.
labeling (TUNEL) from control and ischemia-reperfusion kidneys were obtained in the cortex, outer stripe of the outer medulla, inner stripe of the outer medulla, and the initial inner medulla by an investigator blinded to the treatment status of the kidneys using a Nikon E600 microscope equipped with DIC optics, a DXM1200F digital camera, and ACT-1 software (Nikon). The number of TUNEL-positive and TUNEL-negative intercalated cells were quantified in the least three micrographs per kidney. The number of TUNEL-positive and TUNEL-negative intercalated cells in each animal was averaged, and the average was used for statistical analysis.

Transmission electron microscopy. Glutaraldehyde-fixed kidneys were removed, and tissue from various regions of the renal medulla was cut into 1-mm³-sized blocks and postfixed with 1% osmium tetroxide in phosphate buffer for 2 h, dehydrated in a graded series of ethanol, and embedded in poly/Bed-812 resin (PolySciences, Warrington, CA). One-micrometer semithin sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate and photographed with a transmission electron microscope (HITACHI H-7650).

Caspase-3 and H⁺-ATPase double labeling. To detect caspase-3, deparaffinized tissue sections were treated in 10 mM sodium citrate buffer (pH 6.0) and placed in a microwave oven for antigen retrieval. After endogenous peroxidase quenching and serum blocking, tissue sections were incubated with antibodies to cleaved caspase-3 (Asp175) at 4°C overnight. The sections were then washed and incubated with peroxidase-conjugated donkey anti-rabbit IgG Fab fragment (Jackson ImmunoResearch laboratories, West Grove, PA) for 1 h. Sections were then incubated with peroxidase substrate solution, a mixture of 0.05% DAB and 0.01% H₂O₂, for 5 min at room temperature. The above procedure was then repeated with the substitution of a secondary primary antibody (H⁺-ATPase) and the substitution of Vector SG for DAB. A blocking peptide to activated caspase-3 was used to confirm specificity of activated caspase-3 immunoreactivity.

Statistical analysis. Data were analyzed using unpaired Student’s t-test, and P < 0.05 was taken as evidence of statistical significance. When comparing the number of TUNEL-positive and -negative intercalated cells in control and ischemia-reperfusion kidneys, we used chi-squared analysis to determine statistical significance; n refers to the number of animals studied. In all cases, tissue from six control and six ischemia-reperfusion injury animals was studied, unless otherwise noted.

RESULTS

Physiological parameters. Acute ischemia-reperfusion injury significantly decreased GFR compared with sham-operated control animals (Table 1). There were no differences in urine production rates between ischemia-reperfusion and sham-operated control rats at 0–3 h and 3–6 h; urinary Na⁺ rates were increased and K⁺ rates were decreased significantly at the 0- to 3-h time point but not at the 3- to 6-h time point when kidney samples were obtained. Since GFR was decreased, whereas Na⁺ and K⁺ excretion rates at 6 h were unchanged, fractional excretion of both Na⁺ and K⁺ was increased (data not shown). There was a tendency for urinary HCO₃⁻ excretion rates to be increased, but the difference was not statistically significant at the 3- to 6-h time point. Table 1 summarizes these results.

In contrast to the lack of sustained effects of ischemia-reperfusion on Na⁺ and K⁺ excretion, ischemia-reperfusion induced significant and persistent decreases in urinary ammonia excretion (Fig. 1). Ischemia-reperfusion injury appears to specifically alter ammonia metabolism through mechanisms independent of changes in water, sodium, and potassium excretion.
trol, sham-operated rat kidneys. In particular, there was no significant damage, as identified by any of these criteria, in any of these segments in control rat kidneys. Thus, in addition to previously identified effects of ischemia-reperfusion injury in the proximal tubule and the thick ascending limb of the loop of Henle, there is also significant damage to the collecting duct, particularly in the outer medulla.

Further examination of kidneys from ischemia-reperfusion kidneys showed that a subset of cells was detached from the basement membrane and appeared to be in the process of being extruded into the tubule lumen (Fig. 2). No detached cells were visible in sham-operated control kidneys. Detached cells, when examined using hematoxylin and eosin staining, had increased cytoplasmic density, suggesting they were intercalated cells. These changes were most dramatic in the outer medulla. In the inner medulla fewer cells were in the process of being extruded into the tubule lumen. In the cortex, there was no evidence of cellular damage in the collecting duct, connecting segment, or distal convoluted tubule.

Identification of damaged collecting duct cell-type. To identify which of the different cell types present in the collecting duct were damaged in ischemia-reperfusion injury, we performed immunohistochemical examination using a variety of intercalated cell- and principal cell-specific markers. Antibodies directed against the vacuolar H^+^-ATPase labeled cells in the outer medullary collecting duct which appeared to be in the

Fig. 3. H^+^-ATPase and AE1 immunoreactivity in the OMCD. A: control kidney. Intercalated cells in the OMCD (arrow) exhibit apical H^+^-ATPase (blue) immunoreactivity and basolateral AE1 (brown) immunoreactivity. B: ischemia-reperfusion injury kidney. Several intercalated cells in the OMCD in the ischemia-reperfusion injury kidney are either in the process of detaching from the basement membrane (black arrowhead) or have completely detached and are present in the tubule lumen (white arrowhead). C: no primary control: control kidney. No nonspecific immunoreactivity was present in control kidney sections in which the primary antibody was omitted. The asterisk (*) identifies the lumen of an OMCD. D: no primary control: ischemia-reperfusion injury kidney. No nonspecific immunoreactivity was present in ischemia-reperfusion injury kidney sections in which the primary antibody was omitted. Arrows indicate detached or intraluminal OMCD cells and verify the absence of nonspecific immunoreactivity in damaged cells.
process of being extruded into the tubule lumen. Colocalization of H^+\text{-ATPase} with AE1 demonstrated that these cells expressed both H^+\text{-ATPase} and AE1, identifying these as A-type intercalated cells (Fig. 3, A and B). In control experiments performed without primary antibody, there was no nonspecific immunoreactivity (Fig. 3, C and D).

Because ischemia-reperfusion injury decreased ammonia excretion, we examined expression of the ammonia transporter...
family members, Rhbg and Rhcg (68, 70). As shown in Figs. 4 and 5, collecting duct cells in the process of being extruded expressed intense Rhbg and Rhcg immunoreactivity, consistent with their identification as A-type intercalated cells.

The majority of cells in the collecting duct are principal cells. However, principal cells, identified by AQP-2 immunoreactivity, appeared histologically normal, were not detached from the basement membrane, and were not identified in the tubule lumen (Fig. 6). Thus ischemia-reperfusion injury appears to damage collecting duct intercalated cells but not principal cells.

To determine whether all of the detached cells present in the tubule lumen were collecting duct cells, we used double-labeling with Rhbg, which labels collecting duct intercalated cells, and AQP-2, which labels collecting duct principal cells. Both Rhbg-positive, AQP-2-negative collecting duct intercalated cells and Rhbg-negative, AQP-2-positive principal cells were also seen.
labeled cells and Rhbg-negative, AQP-2-negative, non-collecting duct cells were present in the tubule lumen (Fig. 7). No Rhbg-negative, AQP-2-positive collecting duct principal cells were identified in the tubule lumen. Thus the intraluminal cells present in the collecting duct in ischemia-reperfusion injury is a mixed population of cells that comprises both collecting duct intercalated cells and non-collecting duct cells. The latter probably includes proximal tubule and thick ascending limb of the loop of Henle cells. Accurate counting of the number of non-collecting duct cells could not be performed because they frequently were either fragmented or formed large clumps, preventing accurate enumeration.

**TUNEL staining.** Ischemia-reperfusion injury can cause cellular loss through either cellular necrosis or through induction of apoptosis (57). Histological aspects of apoptosis were examined using standard TUNEL histology combined with double-labeling with H⁺-ATPase to identify whether ischemia-reperfusion injury induces apoptosis of H⁺-ATPase-positive collecting duct intercalated cells. Many TUNEL-positive cells were observed in the ischemic kidneys both in the proximal tubule and the thick ascending limb of the loop of Henle (not shown), as well as in A-type intercalated cells in the outer medullary collecting duct and the inner medullary collecting duct (Fig. 8). TUNEL-positive, H⁺-ATPase-negative collecting duct cells, i.e., apoptotic principal cells, were, in general, not observed. Table 3 quantifies the number of TUNEL-positive and TUNEL-negative intercalated cells in ischemia-reperfusion injury and sham-operated control kidneys. At least a component of the damage to medullary collecting duct intercalated cells in response to ischemia-reperfusion injury is due to apoptosis.

**Transmission electron micrographic examination of ischemia-reperfusion injury kidneys.** To confirm the presence of apoptosis, we examined kidneys using transmission electron microscopy. In ischemia-reperfusion injury kidneys, cells in the outer medulla that were either partially or completely detached from the basement membrane had intact cellular and intracellular membranes and were not necrotic (Fig. 9). We also observed irregularly shaped nucleus and nuclear condensation, consistent with cells undergoing apoptosis.

**Caspase-3 immunoreactivity.** Caspase-3 is a key enzyme involved in the apoptotic pathway in some, but not all, cells (47). Activated caspase-3 immunoreactivity was observed in proximal tubule, but not in the collecting duct, of the ischemia-reperfusion kidney. To confirm expression in the proximal tubule, but not the collecting duct, we used colocalization with H⁺-ATPase to identify collecting duct intercalated cells (Fig. 10). No caspase-3 immunoreactivity was observed in the control kidney. To confirm specificity of caspase-3 immunoreactivity, we used HL60 promyelocytic leukemia cells in which apoptosis was induced by pretreatment with 0.5 μg/ml actinomycin D (Fig. 10E) (14). Preincubating the antibody to cleaved caspase-3 with a blocking peptide blocked immunoreactivity (Fig. 10F). Thus the cellular mechanisms underlying apoptosis appear to differ in the proximal tubule and the collecting duct.

**DISCUSSION**

The present studies are the first to examine the effects of acute renal injury induced by ischemia-reperfusion injury on renal ammonia excretion. In this model of acute renal injury, there was decreased urinary ammonia excretion associated with specific loss of A-type intercalated cells, predominantly...
from the outer medullary collecting duct, but also involving loss in the inner medullary collecting duct. The cellular damage was highly specific for intercalated cells and did not involve principal cells. Intercalated cells that were damaged appeared to be undergoing apoptosis and were in the process of being detached from the basement membrane and extruded into the lumen where they could be shed into the urine. These effects of ischemia-reperfusion injury on the collecting duct may contribute to the metabolic acidosis that frequently accompanies acute renal injury.

Acute renal injury is a commonly observed complication in hospitalized patients that results frequently in metabolic acidosis (27, 28, 43). The acidosis can be severe and can result in substantial complications, including compromised cardiac contractility, increased susceptibility to arrhythmias, and compromised cellular functions (1, 58, 60). Despite the well-known association of metabolic acidosis with acute renal injury, the mechanism by which the acidosis occurs has not been extensively studied. One proposed mechanism is an acute increase in metabolic acid production (32). However, as the present study shows, other mechanisms also contribute. Urinary ammonia excretion is decreased and likely contributes to the metabolic acidosis occurs in acute renal injury. Although ischemia-reperfusion injury frequently damages the proximal tubule and the loop of Henle, decreased bicarbonate reabsorption is unlikely to be the only cause of acidosis, as there were no significant changes in urinary bicarbonate excretion.

A second major finding of these studies is that ischemia-reperfusion injury damages the collecting duct. These effects are most prominent in the outer medulla, which is consistent with previous studies showing that the predominant effect of ischemia-reperfusion injury occurs in outer medulla. Our observation of collecting duct damage is consistent with previous observations in humans where acute renal injury was associated with both cell loss from the collecting duct and the presence of collecting duct cells in urine (46, 54). Furthermore, in humans with acute renal injury, the number of collecting duct cells in the urine exceeds the number of loop of Henle cells and is ~50% of the number of proximal tubule cells (54). The present study is both consistent with these previous observations and extends them by showing that the damaged collecting duct cells, at least in the acute renal injury model used in the present study, are intercalated cells. Moreover, the present study shows that the intratubular cellular casts observed in the collecting duct in acute renal injury (45, 59) can consist of both collecting duct intercalated cells and cells from more proximal epithelial sites, most likely the proximal tubule and the loop of Henle. Why other studies have not routinely identified the presence of collecting duct damage in ischemia-reperfusion injury is not clear.

The collecting duct intercalated cell damage present in response to ischemia-reperfusion injury likely mediates, at least in large part, the decreased rate ammonia excretion observed in these studies. A majority, ~70–80%, of the ammonia present in the urine is secreted by the collecting duct (20, 52). Ammonia secretion involves parallel proton and ammonia transport, and intercalated cells are the primary site of proton secretion (16, 19). Collecting duct ammonia transport appears to involve both diffusive and transporter-mediated components (22, 23); the transporter-mediated components appear to be mediated by Rhbg and Rhcg (22, 23), which are expressed in highest concentrations in intercalated cells (11, 21, 49, 55, 56, 65). The damage to collecting duct intercalated cells likely impairs secretion of both $H^+$ and $NH_3$, thereby resulting in decreased net acid excretion without substantial changes in urinary pH, as is observed clinically.

Although it is possible that altered ammonia metabolism in other renal epithelial cells contributes to the decreased ammonia excretion, this is unlikely, at least in the time course these studies examined. Ammonia is produced in the proximal tubule, where it is preferentially secreted into the luminal fluid,

| Table 3. Proportion of intercalated cells with TUNEL-positive staining |
|-----------------|-----------------|-----------------|
| Condition       | Sham-Operated Control | Ischemia-Reperfusion Injury |
| Cortex          | 0.0 ± 0.0         | 0.3 ± 0.03       |
| OSOM            | 0.0 ± 0.0         | 3.5 ± 0.5*       |
| ISOM            | 0.2 ± 0.2         | 10.8 ± 0.7*      |
| Inner medulla, initial | 0.0 ± 0.0       | 20.7 ± 2.2*     |

Values are mean ± SE and represent mean percentage of intercalated cells (identified by $H^+\cdot$ATPase immunoactivity) with TdT-mediated dUTP nick-end labeling (TUNEL)-positive immunoreactivity; $n$ represents no. of kidneys examined. At least 60 intercalated cells in each region in each kidney were quantified. OSOM, outer stripe of the outer medulla; ISOM, inner stripe of the outer medulla. *$P < 0.001$ vs. sham-operated control.

Fig. 9. Transmission electron microscopy in the outer medulla. A: low-power micrograph of OMCD. A detaching intercalated cell (IC; black arrow) is shown. Plasma membrane and cytoplasmic structures are intact and there is no evidence of necrosis. An adjacent principal cell (PC) is histologically normal. B: a high-power micrograph of an OMCD intercalated cell with an eccentric nucleus and nuclear condensation, a characteristic finding of apoptosis, is shown (black arrow).
and is then reabsorbed by the thick ascending limb of the loop of Henle (20, 31). We are unaware of studies reporting proximal tubule ammoniagenesis in response to ischemia-reperfusion injury, but in another model of acute renal injury, glycerol-induced acute renal injury, renal ammoniagenesis is unchanged (48). Ischemia-reperfusion injury decreases Na\(^+/\)H\(^+\) exchanger-3 (NHE-3) and Na\(^+/\)K\(^+\)-2Cl\(^-\) cotransporter (NKCC-2) expression, the transporters involved in proximal tubule and thick ascending limb of the loop of Henle ammonia transport (17, 34, 66, 67), suggesting ammonia transport may be decreased in these segments. However, this cannot be the entire cause of the acute decreased renal ammonia excretion. If it were, then intrarenal ammonia content would be decreased, whereas direct measurements have shown increased intrarenal ammonia in ischemia-reperfusion injury (15, 35, 71). This increase in intrarenal ammonia in ischemia-reperfusion injury likely reflects decreased collecting duct ammonia secretion due to intercalated cell damage.

Another important finding in the present study is that the effects of ischemia-reperfusion on the collecting duct were maximal in the outer medulla and the initial portion of the inner medulla and were highly specific for intercalated cells. The predominance of effects in the outer medulla is consistent with a wide variety of studies examining the proximal tubule and the loop of Henle (25, 26, 37, 63), and extends these studies by also demonstrating effects in the collecting duct in this region. Our observation that ischemia-reperfusion injury preferentially affected intercalated cells, with no detectable damage to adjacent principal cells, however, has not been reported previously to our knowledge. Since one mechanism underlying ischemia-reperfusion injury involves generation of oxygen free radicals, it is possible that there are higher rates of oxygen utilization by intercalated cells than in principal cells that may underlie the greater sensitivity of intercalated cells to ischemia-reperfusion injury. Consistent with this possibility is that the mitochondria density in intercalated cells is greater than in principal cells (39, 40).

Acute ischemic and nephrotoxic insults to the kidney can cause cellular damage due to either necrosis or apoptosis. In general, necrosis is the result of overwhelming and severe...
cellular ATP depletion and results in diffuse cellular damage and an acute inflammatory reaction in the surrounding tissue that is easily detected morphologically (3, 36). None of these were observed in the present study, consistent with the relatively short period, 30 min, of renal ischemia used before renal reperfusion. Instead, widespread evidence of apoptosis, both in the proximal tubule and loop of Henle, as previously reported (7, 44), and in the collecting duct was observed in these studies. In general, apoptotic cells can be identified for only short periods of time, generally 1–3 h, before undergoing phagocytosis and destruction (5, 50). The relatively short time, 6 h after reperfusion, at which tissue was obtained in the present study probably enabled identification of the apoptotic collecting duct cells. Although this is the first identification, to our knowledge, of apoptotic intercalated cells in response to acute renal injury, apoptosis is important in the remodeling of collecting duct intercalated cells during embryogenesis and early renal development (30). However, it is important to note that apoptosis is involved in the developmental removal of B-type intercalated cells, but not A-type intercalated cells (30). Thus it is likely that the mechanisms involved in apoptotic removal of the B-type intercalated cell during development differ from those involved in apoptotic removal of medullary collecting duct A-type intercalated cells in response to ischemia-reperfusion injury.

In the kidney, apoptosis is important in renal tubular injury from a variety of causes and contributes both to abnormal fluid and electrolyte transport, and possibly to the tubular repair after induction of ischemia-reperfusion injury (36, 62). Caspases are cytosolic enzymes belonging to a large protein family that are final mediators of apoptosis (42). Caspase-3 is one of several downstream executioner caspases and is important in many models of renal injury (61). The present study, by showing that ischemia-reperfusion injury activates caspase-3 in the proximal tubule but not in collecting duct intercalated cells, suggests that the cellular mechanisms of apoptosis differ in these two renal epithelial cell populations. This differential role of caspase-3 in different cell populations in the same tissue is similar to findings previously observed in other models of renal injury (6) and in ischemic injury in other tissues (8). More importantly, the present study suggests that the cellular mechanisms of ischemia-reperfusion injury-induced apoptosis may differ in different renal populations.

Acid-base and ammonia transport in the collecting duct are regulated by a wide variety of mechanisms. Changes in a protein's subcellular localization with trafficking from intracellular sites to plasma membrane is important for many proteins involved in acid-base and ammonia transport, particularly H+/ATPase and Rhcg (2, 4, 56, 64). Protein-protein interactions are also important in the regulation of acid-base transport, such as demonstrated for H+/ATPase-mediated H+ secretion (38, 53). It is possible that these, and other regulatory mechanisms, may also contribute to altered ammonia metabolism in ischemia-reperfusion injury.

In the present study, ischemia-reperfusion injury caused an acute increase in urinary sodium excretion rates that then returned to normal. Similar observations have been reported previously (17, 63). The increase in urinary sodium excretion is likely related to decreased proximal tubule and thick ascending limb of the loop of Henle sodium absorption involving changes in Na+/K+-ATPase, NHE-3, NKCC-2, and the thiazide-sensitive cotransporter (17). Since GFR was decreased in response to ischemia-reperfusion injury, the fractional excretion of sodium, and potassium, was increased (data not shown). Whether this reflects impaired single-nephron sodium and potassium transport or reflects physiological decreases in single-nephron reabsorption to maintain solute homeostasis cannot be determined from the present studies. However, the rates of intravenous sodium and potassium administration were identical in ischemia-reperfusion injury and control animals. This suggests that the appropriate renal response would be identical sodium and potassium excretion rates, which would necessitate increased fractional excretion in animals with ischemia-reperfusion injury-induced decreases in GFR. Thus, whether the increased fractional excretion of sodium and potassium represents impaired transport or physiologically regulated decreased transport cannot be determined at present.

Ischemia-reperfusion injury decreased GFR but did not significantly alter urine volume, which suggests that distal water reabsorption was decreased. Supporting this is the observation that urinary urea nitrogen and creatinine concentrations were significantly decreased in ischemia-reperfusion compared with sham-operated control rats (data not shown). These findings are consistent with previous observations of decreased collecting duct arginine vasopressin-dependent osmotic water permeability (25) and decreased AQP-2 and AQP-3 expression (13, 17, 29, 33). The present study adds to these previous studies by demonstrating that these changes in collecting duct water transport are not due to loss of principal cells and instead appear to reflect ischemia-reperfusion-induced changes in principal cell-mediated water transport.

Renal net acid excretion includes, in addition to ammonia, urinary bicarbonate and titratable acid excretion. Although there was a tendency for urinary bicarbonate excretion to increase, this did not reach statistical significance. Titratable acid excretion comprises only 20–40% of total net acid excretion, and the primary urinary buffer comprising titratable acids is phosphate (20). An almost universal finding in acute renal failure, including ischemia-reperfusion injury-induced acute renal failure, is hyperphosphatemia due to decreased urinary phosphate excretion (9, 41, 51). Thus titratable acid excretion is likely to be decreased in parallel with ammonia excretion in response to ischemia-reperfusion injury and therefore to also contribute to the development of metabolic acidosis.

In summary, acute renal injury induced by ischemia-reperfusion decreases urinary ammonia excretion. This appears to be due to selective loss of A-type intercalated cells from the outer medullary collecting duct and, to a lesser extent, the inner medullary collecting duct. This cellular loss is specific to the intercalated cell and does not involve adjacent principal cells. Intercalated cells in these regions are predominantly lost by detachment from the basement membrane and extrusion into the luminal space. These observations provide a cellular explanation for the metabolic acidosis commonly seen with acute renal injury.

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