A Possible Molecular Basis of Natriuresis During Ischemic-Reperfusion Injury in the Kidney

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Abstract. Ischemic renal injury is associated with increased fractional excretion of sodium, suggesting a Na⁺ reabsorption deficiency in renal tubules. To determine whether alterations in expression of the major Na⁺ transporter genes might contribute to the natriuresis that follows ischemic acute renal failure, the expression of these genes was analyzed in renal cortex and medulla after ischemic-reperfusion injury. Rats were subjected to 30 min of renal pedicle clamping and then sacrificed at 12, 24, or 48 h after reperfusion. Serum creatinine increased significantly at 12 and 24 h, indicative of acute renal failure, but decreased substantially by 48 h. mRNA levels for the NHE-3 Na/H exchanger of the proximal tubule, the apical Na-K-2Cl cotransporter of the thick ascending limb of Henle, the Na-CI cotransporter of the distal convoluted tubule, the epithelial Na⁺ channel of the collecting duct, and the basolateral Na⁺-K⁺-ATPase were measured by Northern hybridization. NHE-3 mRNA decreased by approximately 75% at 12 h and remained suppressed at 24 and 48 h after reperfusion. Na-K-2Cl cotransporter mRNA decreased by approximately 88% at 12 h and remained suppressed at 24 and 48 h. Na-CI cotransporter mRNA remained unchanged at 12 h, decreased by approximately 60% at 24 h, and returned to almost control levels at 48 h. mRNA levels for sodium channels (β subunit) remained unchanged. Na⁺-K⁺-ATPase mRNA in the medulla decreased by approximately 35 to 40% at 12 and 24 h and by 70% at 48 h, whereas in cortex it decreased by only <15% at 12 or 48 h after reperfusion. These results suggest that sharp reductions in expression of the NHE-3 Na/H exchanger and the apical Na-K-2Cl cotransporter are major factors in the natriuresis/diuresis that is one of the hallmarks of ischemic acute renal failure. Lasting suppression of these transporters, despite improvement in renal function, could contribute to the deranged NaCl and water excretion that often leads to volume depletion during recovery from ischemic acute renal failure. (J Am Soc Nephrol 9: 605–613, 1998)
gene is minimally suppressed in reperfusion injury. These changes may be the molecular basis of natriuresis observed during recovery from ischemic ARF.

Materials and Methods

Renal Ischemia-Reperfusion Model

Male Sprague Dawley rats (250 to 350 g) were housed two per cage, with free access to food and water. Rats were subjected to ischemic renal injury as described (16–18). Rats were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. Animals were placed on a heating pad to maintain their body temperature at a constant level. The abdomen was prepared with Betadine, a midline peritoneal incision was made, and the kidneys were exposed. A nontraumatic vascular clamp was applied across the pedicles. Completeness of ischemia was verified by the blanching of the kidneys, indicating cessation of blood flow. Blood flow to the kidneys was reestablished after 15 or 30 min by removal of the clamps. Sutures were placed at the incision site, and the animal was allowed to recover. The animals were sacrificed at 12, 24, or 48 h after reperfusion, and the kidneys were removed. Cortex and medulla were separated and snap-frozen. Frozen kidneys were stored at −70°C. For control, kidneys were removed at the time of initial surgery without clamping. Three rats were studied for each reperfusion time point after 15 or 30 min of ischemia.

RNA Isolation

Total cellular RNA was extracted from renal cortex or medulla by the method of Chomczynski and Sacchi (19). In brief, 0.5 to 1 g of tissue was homogenized at room temperature in 10 ml of Tris reagent (Molecular Research Center, Cincinnati, OH). RNA was extracted by phenol/chloroform and precipitated by isopropanol (19). RNA was quantified by spectrophotometry and stored at −80°C.

Northern Hybridization

Total RNA samples (30 μg/lane) were fractionated on a 1.2% agarose-formaldehyde gel. The samples were transferred to Magna NT nylon membranes (Micron Separations, Inc.), using 10X saline-sodium phosphate-ethylenediamine tetra-acetic acid (SSPE) as transfer buffer. Membranes were cross linked by ultraviolet light and baked for 1 h (20). Hybridization was performed according to Church and Gilbert (21). Briefly, membranes were placed for 1 h in 0.1× SSPE/1% sodium dodecyl sulfate (SDS) solution at 65°C. The membranes were then prehybridized for 1 to 3 h at 65°C with 0.5 M sodium phosphate buffer, pH 7.2, 7% SDS, 1% bovine serum albumin, 1 mM ethylenediamine tetra-acetic acid (EDTA), and 100 μg/ml sonicated salmon sperm DNA. The membranes were hybridized overnight in the above solution with 30 to 50× 10⁶ cpm of 32P-labeled DNA probe for each of the sodium transporters. The cDNA probes were labeled with 32P-deoxynucleotides, using the RadPrime DNA labeling kit (Life Technologies). The membranes were washed twice in 40 mM sodium phosphate buffer, pH 7.2, 5% SDS, 0.5% bovine serum albumin, and 1 mM EDTA for 10 min at 65°C, washed four times in 40 mM sodium phosphate buffer, pH 7.2, 1% SDS, and 1 mM EDTA for 10 min at 65°C, exposed to a PhosphorImager cassette at room temperature for 24 to 72 h, and read by PhosphorImager (Molecular Dynamics). The following rat PCR product fragments were used as probes in the Northern blot analyses: apical Na-K-2Cl, nucleotides 509 to 3237; Na-Ci, nucleotides 1436 to 2800; NHE-3, nucleotides 1883 to 2217; sodium channel β subunit, nucleotides 1012 to 1848; Na⁺-K⁺-AT-Pase α1 subunit, nucleotides 89 to 421. These probes are highly specific and under high stringency conditions do not cross react with other transporters.

Materials

32P was purchased from New England Nuclear (Boston, MA). Nitrocellulose filters and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). RadPrime DNA labeling kit was purchased from Life Technologies.

Statistical Analyses

The data are expressed as mean ± SEM where appropriate. For statistical analysis of the differences in the levels of mRNA expression, PhosphorImager readings from three separate experiments were obtained and analyzed. Equal RNA loading in various lanes was verified by nitrocellulose membrane transferred RNA. For serum creatinine measurement, blood samples from three separate animals were assayed for each time point. Statistical analysis was determined using ANOVA. P < 0.05 was considered statistically significant.

Results

Rats were subjected to renal pedicle clamping as described in Materials and Methods and according to established methods (16–18). Animals were sacrificed at 12, 24, and 48 h after reperfusion. Blood samples were obtained at the time of sacrifice and analyzed for serum creatinine. Figure 1 shows the serum creatinine values at 12, 24, and 48 h of reperfusion in rats subjected to 30 min of renal ischemia. Serum creatinine increased at 12 and 24 h but decreased sharply at 48 h after reperfusion. These results demonstrate that renal function at 48 h after reperfusion, although still impaired, is significantly improved compared with 24 h of reperfusion.

In the next series of experiments, we examined the effect of 30 min of ischemic injury on mRNA expression levels of the various sodium transporters. Equal loading of RNA samples was confirmed by ethidium bromide staining of nitrocellulose membrane transferred RNA of the various gels that were used for Northern hybridization (Figure 2). We first examined the effect of ischemia on the expression of NHE-3, the Na⁺/H⁺ exchanger isoform responsible for most of the sodium reabsorption in the proximal tubule (22). Recent studies from our laboratories showed that renal artery occlusion suppresses NHE-3 mRNA levels at 12 and 24 h after reperfusion (23). In the present study, expression of NHE-3 was examined up to 48 h after reperfusion. As shown in Figure 3, the expression of NHE-3 mRNA in the cortex and medulla decreased at 12 h and remained suppressed at 24 and 48 h after reperfusion. NHE-3 expression in the cortex decreased by 78, 84, and 81% at 12, 24, and 48 h after reperfusion, respectively (P < 0.01 versus control for each time point).

We next examined the expression of the apical Na-K-2Cl cotransporter, the major transporter for reabsorption of sodium in the medullary thick ascending limb of Henle. Figure 4 shows that in kidneys from rats subjected to 30 min of renal ischemia, expression of the apical Na-K-2Cl cotransporter at 12 h of reperfusion decreased significantly and remained low at 24 and 48 h in both cortex and medulla. Expression of the apical Na-K-2Cl cotransporter in the medulla decreased by 88, 78, and 82% at 12, 24, and 48 h after reperfusion, respectively,
Figure 1. Serum creatinine concentrations at 0, 12, 24, or 48 h of reperfusion in rats with 30 min of ischemia.

compared with control kidneys removed at time 0 ($P < 0.01$ for each time point versus control). The Na-K-2Cl cotransporter mRNA is less abundant in the cortex (Figure 4), consistent with established functional and molecular studies (8,14). The magnitude of the reduction in the expression of Na-K-2Cl cotransporter mRNA in the cortex of ischemic kidneys was less than in the medulla.

Expression of the thiazide-sensitive Na-Cl cotransporter was studied next. As shown in Figure 5, the Na-Cl cotransporter was detected at highest levels in the cortex (compare control lanes in cortex and medulla), consistent with its known expression in the distal convoluted tubules (24). Na-Cl cotransporter mRNA in the cortex at 12 h after reperfusion were only slightly reduced from control levels (88% of control, $P > 0.05$), decreased significantly at 24 h after reperfusion (41% of control, $P < 0.05$), and returned to almost normal levels at 48 h after reperfusion (94% of control, $P > 0.05$, $n = 3$), indicating recovery from IRI. NaCl cotransporter mRNA levels in the medulla of control kidneys were very low and decreased in reperfusion injury (Figure 5).

NHE-3, the Na-K-2Cl cotransporter, and the Na-Cl cotransporter are responsible for most of the sodium reabsorption occurring in the proximal tubule, ascending limb of Henle, and distal convoluted tubule, respectively (7,8,14). Sodium reabsorption in collecting duct is predominantly mediated via the amiloride-sensitive sodium channel (11,12). The purpose of the next series of experiments was to examine the expression of sodium channel mRNA in IRI. Northern blot analysis of mRNA levels for the 3 subunit of the sodium channel in ischemic-reperfusion injury is shown in Figure 6 and demonstrates that expression of the 3 subunit in the cortex and medulla remained unchanged at 12, 24, and 48 h after reperfusion ($P > 0.05$ versus control).

Transport of sodium from the cell to the blood is predominantly mediated via the basolateral Na$^+-$K$^+$-ATPase (15). Several studies have demonstrated decreased Na$^+-$K$^+$-ATPase activity in reperfusion injury (reviewed in reference 6). The purpose of the next series of experiments was to examine the expression of Na$^+-$K$^+$-ATPase in ischemic-reperfusion injury. As shown in Figure 7, mRNA levels for Na$^+-$K$^+$-ATPase in the medulla decreased by 41, 35, and 69% at 12, 24, and 48 h after reperfusion, respectively ($P < 0.05$ for each time point versus control). Expression of the Na$^+-$K$^+$-ATPase mRNA in the cortex, however, decreased minimally (11, 10, and 15% at 12, 24, and 48 h of reperfusion, respectively; $P > 0.05$ for each time point).

To determine whether decreased expression of sodium-absorbing transporters correlates with increased excretion of sodium, rats were subjected to IRI as described in Materials and
Methods and sacrificed at 24 and 48 h after ischemia. Fractional excretion of sodium (FENa) was measured based on sodium and creatinine concentrations in the urine and plasma. As indicated in Table 1, FENa was significantly higher at 24 and 48 h after ischemia compared with control rats, consistent with decreased reabsorption of sodium in renal tubules of ischemic kidneys.

Discussion
The results of the above experiments show that mRNA levels for sodium transporters are variably affected in renal ischemia-reperfusion injury (Figures 3 through 7). NHE-3 Na/H exchanger mRNA levels decreased at 12 h and remained suppressed at 24 and 48 h after reperfusion (Figure 3). The bumetanide-sensitive apical Na-K-2Cl cotransporter mRNA was suppressed at 12 h and remained low at 24 and 48 h of reperfusion in kidneys from rats subjected to 30 min of renal ischemia (Figure 4). Kidneys from rats subjected to 30 min of renal ischemia showed greater suppression of Na-K-2Cl cotransporter mRNA (Figure 4) compared with that observed after 15 min of occlusion (data not shown). The thiazidesensitive Na-Cl cotransporter mRNA remained unaltered at
12 h, decreased at 24 h, and returned to normal at 48 h after reperfusion (Figure 5). mRNA levels for sodium channel β subunit remained unchanged (Figure 6). The expression of the Na\(^+\)-K\(^+\)-ATPase mRNA in the medulla decreased at 12 h and further declined at 48 h after reperfusion (Figure 7).

Sodium reabsorption occurs in the proximal tubule, medullary thick ascending limb of Henle (mTAL), distal convoluted tubule, and collecting duct. Suppression of mRNA levels for NHE-3 (Figure 3), which is responsible for the majority of sodium reabsorption in proximal tubules (22), is consistent with decreased reabsorption of sodium and HCO\(_3^-\) in this nephron segment. This would be expected to cause both natriuresis and HCO\(_3^-\) wasting. Although increased excretion of sodium has been noted in ischemic-reperfusion injury, HCO\(_3^-\) wasting has not been a prominent feature of ARF. Recent studies have shown that suppression of NHE-3 mRNA and activity in ischemic-reperfusion injury are related to a more than eightfold increase in colonic H\(^+\)-K\(^+\)-ATPase mRNA, which is expressed in distal nephron segments (23). Increased expression of the colonic H\(^+\)-K\(^+\)-ATPase mRNA would be consistent with increased H\(^+\) secretion and, as a result, enhancement of HCO\(_3^-\) reabsorption, thereby preventing excessive losses of HCO\(_3^-\).

More than approximately 30% of the filtered load of sodium
is reabsorbed in the mTAL via the apical Na-K-2Cl cotransporter (7,8). This transporter is sensitive to inhibition by furosemide and bumetanide and has distinct binding sites for sodium, potassium, and chloride (7,8,14). Suppression of the apical Na-K-2Cl cotransporter mRNA (Figure 4) is consistent with decreased reabsorption of sodium and chloride in the mTAL, which in turn could contribute to the increased sodium and chloride excretion observed in ischemic ARF.

The distal convoluted tubule and collecting duct together reabsorb approximately 10% of the filtered load of sodium (7,8) via apical Na-Cl cotransporter (7,8,14) and the amiloride-sensitive sodium channel (7,12), respectively. Na-Cl cotransporter mRNA levels returned to normal at 48 h after reperfusion (Figure 5), and the sodium channel β subunit expression remained unchanged in IRI (mRNA levels in the medulla decreased at 48 h but did not attain significant values) (Figure 6). It should be mentioned that IRI could alter ion transport by affecting protein synthesis, protein processing, and protein localization independent of changes in mRNA levels.

Transport of sodium from renal epithelial cells to the blood is mediated predominantly by the basolateral Na⁺-K⁺-ATPase (15). Figure 7 demonstrates that mRNA levels for the Na⁺-K⁺-ATPase are decreased significantly in the medulla in IRI. These results are consistent with previous studies showing decreased Na⁺-K⁺-ATPase activity in reperfusion injury (reviewed in references 6 and 25) and suggest that decreased Na⁺-K⁺-ATPase in reperfusion injury involves both cellular ATP depletion (reviewed in reference 6) and alterations in gene expression. It has been proposed that loss of epithelial cell polarity and targeting of the Na⁺-K⁺-ATPase to the luminal membrane could be responsible for increased sodium excretion after ischemic injury (26). On the basis of the results of experiments in Figures 2 through 6, we propose that a major factor responsible for increased sodium excretion in ischemic ARF is the sharp reductions in expression of the NHE-3 Na/H exchanger and the apical Na-K-2Cl cotransporter.

To determine the effect of severity of the initial ischemic insult on ion transporters, rats were subjected to 15 min of renal pedicle clamping and studied at 12, 24, and 48 h after reperfusion. There was no rise in serum creatinine at 12, 24, or 48 h after reperfusion after 15 min of ischemia (0.4 mg/dl at time 0 versus 0.46 at 12 h, 0.4 at 24 h, and 0.4 at 48 h after reperfusion; P > 0.05 for each time point versus control). Northern hybridizations revealed that 15 min of ischemia did not affect mRNA expression of the sodium absorbing transporters significantly.

Medullary interstitial osmotic gradients are maintained to a large extent by the reabsorption of sodium and chloride in the mTAL (27). Most of the sodium and almost all of the chloride reabsorption in the mTAL is mediated via the apical Na-K-2Cl cotransporter (7,8,14). It has been shown that inhibition of the apical Na-K-2Cl cotransporter (by furosemide) could reduce the interstitial osmotic gradient in the medulla and impair the action of antidiuretic hormone on water reabsorption in medullary collecting duct cells (28). Decreased expression of the apical Na-K-2Cl cotransporter in IRI (Figure 4) could reduce reabsorption of sodium and chloride and result in suppression of the medullary osmotic gradient. This in turn could blunt the action of antidiuretic hormone and, in conjunction with the suppression of aquaporin 2 (29), result in the urinary concentrating defect that is observed in ischemic renal failure.

**Table 1.** Fractional excretion of sodium in ischemic reperfusion injury (IRI) a

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<th>Control</th>
<th>24 h after IRI</th>
<th>48 h after IRI</th>
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<td>1.1 ± 0.1</td>
<td>14.1 ± 3.7 ( ^b )</td>
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\( ^a \) Results are expressed as percentage of control (n = 3 for each group).

\( ^b \) \( P < 0.05 \) versus control.

\( ^c \) \( P < 0.03 \) versus control.

*Figure 7. Na⁺-K⁺-ATPase Northern hybridization. Representative Northern blots showing Na⁺-K⁺-ATPase transcript levels in cortex and medulla at 0, 12, 24, and 48 h after 30 min of renal pedicle occlusion. Corresponding nitrocellulose membrane-transferred RNA is shown in Figure 2. Na⁺-K⁺-ATPase transcript size was 4.2 kb.*
The apical Na-K-2Cl cotransporter can mediate the transport of NH$_4^+$ into the medullary interstitium. Ammonium is synthesized in the mitochondria of proximal tubule cells, transported to the cytosol, and excreted in part via the NHE-3 Na$^+$/H$^+$ exchanger (30,31). Once in the lumen of the proximal tubule, it is delivered to the mTAL, where it is reabsorbed predominantly via the apical Na-K-2Cl cotransporter by binding to its potassium-binding site (32). The reabsorbed NH$_4^+$ enters the interstitium, is transported to the medullary collecting duct cells, and is secreted into the collecting duct lumen (30,33). Suppression of NHE-3 and the Na-K-2Cl cotransporter in ischemic-reperfusion injury could impair urinary NH$_4^+$ excretion by decreasing its secretion into the proximal tubule and its reabsorption in the mTAL. It is therefore conceivable that the NH$_4^+$ excretion defect that has been observed in ischemic renal failure could to some extent result from suppression of NHE-3 and the Na-K-2Cl cotransporter.

The most salient feature of the present studies is sustained suppression of NHE-3 and the apical Na-K-2Cl cotransporter mRNA expression during recovery from renal failure. GFR, assessed by serum creatinine or inulin clearance as markers of renal function, is maximally decreased during the first day of reperfusion in animals subjected to 30 min of renal ischemia and improves at 48 h (Figure 1) (16–18). Decreased GFR in the first day of reperfusion would reduce the filtered load of electrolytes and could prevent huge losses of sodium and chloride that otherwise could result from suppression of the Na-K-2Cl cotransporter and NHE-3. The expression of NHE-3 and Na-K-2Cl transporters at 48 h after reperfusion still remains significantly low (Figures 3 and 4), whereas the GFR continues to improve (Figure 1). Significant suppression of NHE-3 and the Na-K-2Cl cotransporter at 48 h of reperfusion, despite noticeable improvement in the GFR, could result in severe sodium loss (Table 1) and be responsible for the “diuretic phase” of acute ischemic renal failure. We therefore suggest that massive urinary loss of sodium, and the resulting
volume depletion, in patients recovering from acute ischemic renal failure (34) is likely caused by sustained suppression of NHE-3 and the apical Na-K-2Cl cotransporter. Figure 8 is a schematic diagram comparing the expression of sodium, chloride, and HCO$_3^-$ transporters in ischemia-reperfusion injury (bottom panel) versus the normal condition (top panel). According to this proposed scheme, suppression of NHE-3 and the Na-K-2Cl cotransporter decreases reabsorption of sodium, chloride, and HCO$_3^-$ and increases their delivery to distal nephron segments. Compensatory upregulation of colonic H$^+\cdot$K$^+\cdot$ATPase (23) results in increased HCO$_3^-$ reabsorption, thereby preventing huge losses of HCO$_3^-$. Because there is no compensatory upregulation of the Na-Cl cotransporter and sodium channel, ischemia-reperfusion injury results in renal loss of sodium and chloride.

The molecular mechanism of Na-K-2Cl cotransporter and NHE-3 suppression during reperfusion injury is unknown. One possibility is that severe ischemic injury in the proximal tubule and mTAL cells causes a general decrease in transcriptional activity, which in turn leads to suppression of mRNA levels for membrane transporters, including the Na-K-2Cl cotransporter and NHE-3. However, increased expression of cortical and medullary NHE-1 (23), which is expressed in proximal tubule and mTAL cells, and lack of suppression of Na$^+\cdot$K$^+\cdot$ATPase in the cortex (Figure 7), which is heavily expressed in proximal tubule, argues against such a possibility. Another plausible explanation is that suppression of the Na-K-2Cl cotransporter and NHE-3 is secondary to cell swelling resulting from decreased Na-K-ATPase activity. Whether regulatory elements sensitive to cell swelling or hypoxemia exist within the promoter region of these two transporters needs careful examination.

The alterations in the expression of the thiazide-sensitive Na-Cl cotransporter in ischemic-reperfusion injury are intriguing. mRNA levels for the Na-Cl cotransporter remained unchanged at 12 h but decreased at 24 h after reperfusion. The Na-Cl cotransporter has been shown to be sensitive to luminal sodium concentrations (24). In support of this hypothesis, studies have shown that treatment of rats with furosemide, an inhibitor of the Na-K-2Cl cotransporter, results in increased delivery of sodium and chloride to the distal nephron and induces upregulation of the Na-Cl cotransporter (24). It is possible that decreased Na-Cl cotransporter mRNA at 24 h of reperfusion is secondary to a very low luminal sodium concentration resulting from extremely low GFR. At 48 h after reperfusion, improvement in the GFR (along with suppressed Na-K-2Cl cotransporter) could result in increased delivery of sodium to distal nephron, which in turn might cause Na-Cl cotransporter mRNA levels to return to normal.

In conclusion, expression of mRNA encoding the apical Na-K-2Cl cotransporter and NHE-3 decreased in IRI. Despite improvement in GFR, lasting suppression of these two transporters, in conjunction with reduced Na$^+\cdot$K$^+\cdot$ATPase expression, could contribute to increased sodium, chloride, and water excretion during the recovery phase from ischemic ARF. The etiology of fluid and electrolyte abnormalities after acute tubular necrosis are likely more complex than simply tubular destruction. Attempts to modulate these transporters may improve patient management in sublethal renal injury.

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