Decreased abundance of major Na\(^+\) transporters in kidneys of rats with ischemia-induced acute renal failure

TAE-HWAN Kwon,\(^1\) JØRGEN FRØKIÆR,\(^2\) JIN SUK HAN,\(^3\) MARK A. KNEPPER,\(^4\) AND SØREN NIELSEN\(^1\)

\(^1\)Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C; \(^2\)Department of Clinical Physiology, Aarhus University Hospital and Institute of Experimental Clinical Research, DK-8200 Aarhus N, Denmark; \(^3\)Department of Internal Medicine, Seoul National University Hospital, 110-744 Seoul, Korea; and \(^4\)Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Kwon, Tae-Hwan, Jørgen Frøkiær, Jin Suk Han, Mark A. Knepper, and Søren Nielsen. Decreased abundance of major Na\(^+\) transporters in kidneys of rats with ischemia-induced acute renal failure. Am J Physiol Renal Physiol 278: F925–F939, 2000.—Ischemia-induced acute renal failure (ARF) is known to be associated with significant impairment of tubular Na reabsorption. We examined whether temporary bilateral renal ischemia (30, 40, or 60 min) and reperfusion (1–5 days) affect the abundance of several renal Na transporters and urinary Na excretion (UNaV) in rats. In rats with mild ARF (30 min), immunoblotting revealed that proximal tubule type 3 Na\(^+/\)H\(^+\) exchanger (NHE-3) and type II Na-P\(_i\)-cotransporter (NaPi-II) were significantly decreased to 28 ± 6 and 14 ± 6% of sham levels, respectively, at day 1. Moreover, Na\(^+/\)K\(^+\)-ATPase levels were also significantly decreased (51 ± 11%), whereas there was no significant decrease in type I bumetanide-sensitive cotransporter (BSC-1) and thiazide-sensitive cotransporter (TSC) levels. Consistent with reduced Na transporter abundance, fractional urinary Na excretion (FeNa) was significantly increased in mild ARF (30 min) and UNaV was unchanged, despite a marked reduction in glomerular filtration rate. Na transporter levels and renal Na handling were normalized within 5 days. Severe ischemic injury (60 min) resulted in a marked decrease in the abundance of Na\(^+/\)K\(^+\)-ATPase, NHE-3, NaPi-II, BSC-1, and TSC at both days 1 and 5. Consistent with this, FeNa was significantly increased at days 1 and 5. Intravenous K-melanocyte-stimulated hormone treatment partially prevented the ischemia-induced downregulation of renal Na transporters and reduced the high FeNa to control levels. We conclude that reduced levels of Na transporters along the nephron may play a critical role in the impairment of tubular Na reabsorption, and hence increased Na excretion, in ischemia-induced ARF.

sodium transport; sodium-hydrogen exchanger; sodium-inorganic phosphate cotransporter; sodium-potassium-2 chloride transporter; sodium-chloride cotransporter

KIDNEYS INJURED BY ISCHEMIA and reperfusion manifest a variety of functional defects, prominent among which is an impairment of tubular reabsorption of sodium and water (7). The proximal tubule (especially the S3 segment) and the outer medullary thick ascending limb have been demonstrated to suffer the most severe injury after an ischemic insult (8, 48).

The proximal tubule is the site of reabsorption of approximately two-thirds of the NaCl that enters the tubular fluid by glomerular filtration. An important fraction of the sodium reabsorption in the proximal tubule occurs by the apical Na\(^+/\)H\(^+\) exchanger, resulting in a net process of sodium and bicarbonate reabsorption. Several lines of evidence, including immunolocalization of type-3 Na\(^+/\)H\(^+\) exchanger (NHE-3) to apical plasma membrane domains in the proximal tubule, have made it clear that NHE-3 is the isoform responsible for apical membrane Na\(^+/\)H\(^+\) exchanger activity (1, 4, 27, 42). The Na-K-ATPase (43) in the basolateral membrane of proximal tubule cells (20) is essential for the efficient proximal sodium reabsorption. It has been suggested that during ischemic injury the proximal tubule Na-K-ATPase is partly redistributed to the apical plasma membrane in this segment (32). Moreover, decreased transcription of Na-K-ATPase mRNA after ischemic injury (46, 50) indicates that Na-K-ATPase expression may be dysregulated and thus could play a role in the impaired proximal tubular sodium reabsorption in postischemic kidneys.

Concentration of the urine requires establishment and maintenance of a hypertonic medullary interstitium. The loop of Henle generates a high osmolality in renal medulla via the countercurrent multiplier, which is dependent on the NaCl reabsorption by the thick ascending limb (TAL) (24, 25). Furthermore, urinary dilution is also dependent on NaCl reabsorption in the TAL and distal convoluted tubule (DCT) (25). Several sodium transporters are responsible for this. The apically expressed Na-K-2Cl cotransporter (rat type 1 bumetanide-sensitive cotransporter BSC-1 or NKCC2) (14, 18, 19, 22, 34, 51), NHE-3 (2), and the Na-K-ATPase are key components responsible for sodium reabsorption by the TAL (24). The Na-Cl cotransporter (thiazide-sensitive Na-Cl cotransporter (TSC)) is expressed in the apical plasma membrane of DCT (35) and is also responsible for sodium and chloride reabsorption in this segment (15, 23).
Recent studies have revealed dysregulation of several renal sodium transporters in experimental rat model of nephrotic syndrome, i.e., adriamycin-induced nephrosis (16). Moreover, we have recently demonstrated that there are major changes in the expression of several major renal sodium transporters in rats with experimental chronic renal failure (5/6 nephrectomy) and that this was associated with the altered tubular sodium handling and decreased urinary concentration (27).

Thus it can be hypothesized that changes in the expression of major renal sodium transporters after renal ischemia-reperfusion injury may play a critical role in the impairment of tubular sodium and water reabsorption in experimental acute renal failure (ARF) as well as in the impairment of the urinary concentration seen in postischemic period. Thus, in addition to the changes in expression of renal aquaporins that we have observed in a previous study (26), changes in expression of renal sodium transporters may play a fundamental role in disturbances in extracellular fluid volume regulation seen in ARF.

In the present study, we examined 1) whether there are changes in abundance of major renal sodium transporters [Na-K-ATPase, NHE-3, type II Na-Pi cotransporter (NaPi-II), BSC-1, and TSC] in rats with experimental ARF induced by temporary bilateral ischemia (30, 40, or 60 min) and reperfusion (1, 2, or 4 days); 2) whether the changes in sodium transporter abundance are associated with alterations in urinary sodium excretion in rats with ARF; 3) whether the expression of sodium transporters to some extent is selectively affected by ischemia and reperfusion injury compared with other membrane proteins; and 4) whether reducing ischemia-reperfusion injury by treatment with an anti-inflammatory agent [i.e., α-melanocyte-stimulating hormone (α-MSH)] (11) affects the expression of sodium transporters and the changes in urinary sodium excretion after renal ischemia.

**METHODS**

Experimental Animals

Studies were performed in adult Munich-Wistar rats initially weighing 236 ± 6 g (Møllegard Breeding Centre, Eiby, Denmark). The rats were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water.

Induction of Ischemia-Induced ARF in Rats

After a period of acclimation to the metabolic cages, experimental ARF was induced by occlusion of both renal arteries for 30 or 60 min (Fig. 1). During surgery, rats were anesthetized with halothane (Halocarbon Laboratories) and placed on a heated table to maintain rectal temperature at 37–38°C. Both kidneys were exposed through flank incisions, mobilized by being dissected free from the perirenal fat. A small portion of the renal artery was gently dissected from the vein (30). The renal arteries were occluded with a smooth-surface vascular clip (60-g pressure, World Precision Instruments) for 30 or 60 min. Total ischemia was confirmed by observing blanching of the entire kidney surface. After the clips were removed, the kidneys were observed for an additional 2–5 min to ensure color change indicating blood reflow, and the wound was closed with 3–0 silk and surgical metal clamps. The rats were

---

1 Animal experiments were carried out on the basis of a license from the Department of Justice, Copenhagen, Denmark (Dyreforsøgstilsynet, Copenhagen, Denmark).
returned to metabolic cages, and 24-h urine output and water intake were measured daily according to the protocols depicted in Fig. 1.

As a control group, rats were subjected to sham operations identical to the ones used for ARF rats without occlusion of both renal arteries. Sham-operated rats were monitored parallel with rats with ARF. All rats were killed under light halothane anesthesia, during which kidneys were rapidly removed and processed for membrane fractionation on the same day.

Clearance Studies

The rats were maintained in the metabolic cages, allowing quantitative urine collections and measurements of water intake (Fig. 1). Urine volume, osmolality, creatinine, and sodium and potassium concentration were measured. Plasma was collected from the abdominal aorta at the time of death for measurement of sodium and potassium concentration, creatinine, and osmolality.

Experimental Protocol

The following protocols were performed (Fig. 1).

Protocol 1. ARF was established in rats by bilateral renal arterial occlusion for 30 min, and they were monitored for an additional 24 h (ARF 30/1d; n = 8). For sham-operated rats, n = 9.

Protocol 2. ARF was established in rats by bilateral renal arterial occlusion for 60 min and monitored for an additional 24 h (ARF 60/1d; n = 10). For sham-operated rats, n = 8.

Protocol 3. ARF was established in rats by bilateral renal arterial occlusion for 30 min and monitored for an additional 5 days (ARF 30/5d; n = 6). For sham-operated rats, n = 5.

Protocol 4. ARF was established in rats by bilateral renal arterial occlusion for 60 min and monitored for an additional 5 days (ARF 60/5d; n = 5). For sham-operated rats, n = 5.

Protocol 5. For immunocytochemistry, kidneys of rats with ARF [protocol identical to protocols 1 (n = 4), 2 (n = 4), 3 (n = 3), and 4 (n = 3)] and of sham-operated rats [protocol identical to protocols 1 (n = 2), 2 (n = 2), 3 (n = 2), and 4 (n = 2)] were perfusion fixed (see below; not depicted in Fig. 1).

Protocol 6. ARF was established in rats by bilateral renal arterial occlusion for 40 min and monitored for an additional 2 days. The animals were divided into two groups: α-MSH nontreated ARF (n = 9) and α-MSH treated ARF (n = 8). α-MSH (50 µg, iv; Phoenix Pharmaceutical, Mountain View, CA) was given at the midpoint of the ischemic period, at 6 and 18 h postperfusion, and then every 24 h thereafter, as previously described (11). For sham-operated rats treated with vehicle, n = 9.

Protocol 7. For immunocytochemistry, kidneys of rats with ARF, either treated (n = 5) or not treated (n = 5) with α-MSH and of sham-operated rats treated with vehicle (n = 5) were perfusion fixed (see below; not depicted in Fig. 1).

Membrane Fractionation for Immunoblotting

The kidneys were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 µM leupeptin, 1 mM phenylmethylsulfonyl fluoride) by using an ultra-turrax T8 homogenizer (IKA Labortechnik) at maximum speed for 10 s, and the homogenate was centrifuged in an Eppendorf centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was then centrifuged at 200,000 g for 1 h to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles (29). Gel samples (Laemmi sample buffer containing 2% SDS) were made of this pellet.

Primary Antibodies

For semiquantitative immunoblotting and immunocytochemistry, we used previously characterized monoclonal and polyclonal antibodies summarized as follows.

- **Na-K-ATPase.** A monoclonal antibody against the α1-subunit of Na-K-ATPase was previously characterized (20).
- **NHE-3 (LL546AP).** An affinity-purified polyclonal antibody to NHE-3 corresponds to one previously characterized (16, 21, 27). This antibody was raised against a peptide corresponding to amino acids 809–831 in the COOH-terminal tail of rat NHE-3 plus a cysteine added to the NH2-terminal part (NH2-6QADGPEEQLQPAPESTMH-COOH). The peptide sequence was based on the rat NHE-3 sequence reported by Orlowski et al. (37).
- **NaPi-II (LL696AP).** An affinity-purified polyclonal antibody to NaPi-II corresponding to one previously characterized (8) was raised against the final 24 amino acids of COOH-terminal sequence.

- **BSC-1 (LL320AP).** An affinity-purified polyclonal antibody to the apical Na-K-2Cl cotransporter of the TAL was previously characterized (21, 22, 34).
- **TSC (LL573AP).** An affinity-purified polyclonal antibody to the apical NaCl cotransporter of the DCT was characterized (23).

- **Sheep anti-rat gp330 (megalin).** This immune serum was previously characterized (33).
- **Folate-binding protein (FBP) immune serum.** This serum was previously characterized (13).
- **Electrogenic Na-HCO3 cotransporter (rkNBC1).** A synthetic peptide corresponding to amino acids 1021 to 1035 of the COOH-terminal of rat NBC (40) was used to generate a polyclonal antibody (31).
- **Electroneutral Na-HCO3 cotransporter (NBC3).** A synthetic peptide corresponding to amino acids 1197–1214 of the COOH terminal of human NBC3 (39) was used to generate a polyclonal antibody (28).

Electrophoresis and Immunoblotting

Samples of membrane fractions from total kidney were run on 6–16% gradient polyacrylamide minigels (BioRad Mini Protein II) for BSC-1, TSC, gp330 (megalin), rkNBC1, and NBC3 or 12% polyacrylamide minigels for Na-K-ATPase, NHE-3, NaPi-II, and FBP. For each gel, an identical gel was run in parallel and subjected to Coomassie staining to ensure identical loading (44). The other gel was subjected to immunoblotting and densitometric analysis, as described previously (26, 29).

Immunocytochemistry and Immunoelectron Microscopy

The kidneys from rats with ARF and sham-operated controls were fixed by retrograde perfusion via the aorta with periodate-labeled paraformaldehyde (PLP; 0.01 M NaO4, 0.075 M lysine, 2% paraformaldehyde, in 0.0375 M Na2HPO4 buffer, pH 6.2). For light microscopy, the frozen tissue blocks were cryosectioned (0.8–1 µm, Reichert Ultracut S Cryoultramicrotome), sections were incubated with primary antibodies, and the labeling was visualized with horseradish peroxidase-conjugated secondary antibodies (P447 or P448; 1:100, DAKO, Glostrup, Denmark), followed by incubation with diaminobenzidine (26, 27).

For the immunoelectron microscopy, frozen samples were freeze-substituted in Reichert AF5 (Reichert, Vienna, Austria) (34). Immunolabeling was performed on ultrathin Lowicryl HM20 sections (60–80 nm), which were incubated over-
night at 4°C with primary antibodies (see above). The labeling was visualized with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR.E.M10, BioCell Research Laboratories, Cardiff, UK). The sections were stained with uranyl acetate and lead citrate before examination in Philips CM100 electron microscopes.

**Statistical Analyses**

Values were presented as means ± SE. Comparisons among groups were made by unpaired t-test. P values < 0.05 were considered significant.

**RESULTS**

Temporary Bilateral Renal Ischemia and Reperfusion Injury Results in a Significant Acute Renal Insufficiency

As shown in Table 1, the plasma creatinine levels in rats with ARF 1 day after release of 30 (ARF30/1d) or 60-min (ARF60/1d) bilateral renal artery occlusion were significantly higher (P < 0.05) than those of corresponding sham-operated rats. Consistent with this, creatinine clearance (CCr) was significantly reduced 1 day after renal ischemia (Table 1).

Next, we determined the plasma creatinine levels 5 days after release of 30 (ARF30/5d) or 60-min bilateral renal artery occlusion (ARF60/5d; Table 2). In rats with mild ARF (ARF30/5d), the plasma creatinine levels and CCr normalized within day 5. In contrast, in rats with severe ARF (ARF60/5d) plasma creatinine levels were persistently increased, and CCr was significantly decreased as well (P < 0.05).

**Table 1. Changes in renal function after renal ischemia**

<table>
<thead>
<tr>
<th></th>
<th>ARF(30/1d)</th>
<th>Sham</th>
<th>ARF(60/1d)</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 9)</td>
<td>(n = 10)</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>P&lt;sub&gt;Cr&lt;/sub&gt;, µmol/l</td>
<td>109 ± 29&lt;sup&gt;±&lt;/sup&gt;</td>
<td>44 ± 3.4</td>
<td>213 ± 43&lt;sup&gt;±&lt;/sup&gt;</td>
<td>43 ± 3</td>
</tr>
<tr>
<td>C&lt;sub&gt;r&lt;/sub&gt;, mL/min</td>
<td>0.1 ± 0.1*</td>
<td>1.6 ± 0.1</td>
<td>0.1 ± 0.01*</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>F&lt;sub&gt;ENa&lt;/sub&gt;, %</td>
<td>1 ± 0.2*</td>
<td>0.4 ± 0.1</td>
<td>12.5 ± 2.5*</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>U&lt;sub&gt;o&lt;/sub&gt;V&lt;sub&gt;H2O&lt;/sub&gt;, µl/min</td>
<td>0.7 ± 0.2</td>
<td>0.9 ± 0.2</td>
<td>0.5 ± 0.1*</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Urine output, µl·min&lt;sup&gt;−1&lt;/sup&gt;·kg&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>Baseline</td>
<td>Day −2</td>
<td>Day −1</td>
<td>After operation</td>
</tr>
<tr>
<td></td>
<td>39 ± 4</td>
<td>33 ± 3</td>
<td>64 ± 10</td>
<td>54 ± 9</td>
</tr>
<tr>
<td></td>
<td>34 ± 4</td>
<td>30 ± 2</td>
<td>54 ± 8</td>
<td>54 ± 9</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>57 ± 10&lt;sup&gt;±&lt;/sup&gt;</td>
<td>28 ± 2</td>
<td>17 ± 6&lt;sup&gt;±&lt;/sup&gt;</td>
<td>48 ± 7</td>
</tr>
<tr>
<td></td>
<td>2.2 ± 0.3*</td>
<td>6.3 ± 0.5</td>
<td>2.0 ± 0.3*</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>T&lt;sub&gt;TH2O&lt;/sub&gt;, µl·min&lt;sup&gt;−1&lt;/sup&gt;·kg&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>60 ± 13&lt;sup&gt;±&lt;/sup&gt;</td>
<td>146 ± 16</td>
<td>6 ± 2*</td>
<td>181 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE from rats (n) with acute renal failure (ARF) and sham-operated control animals. Plasma creatinine (P<sub>Cr</sub>), creatinine clearance (CCr), FE<sub>Na</sub>, fractional excretion of sodium; U<sub>o</sub>V<sub>H2O</sub> rate of urinary sodium excretion; U/P<sub>am</sub>, urine-to-plasma osmolality ratio; and solute-free water reabsorption (T<sub>TH2O</sub>) were measured at day 1. ARF(30/1d) and ARF(60/1d), ARF established in rats after 30- and 60-min bilateral renal ischemia, respectively, with monitoring in the following 24 h; sham, sham-operated rats. The slightly lower CCr in ARF(30/1d) compared to the other sham control groups (ARF30/1d, ARF30/5d, and ARF60/5d) may be caused by the relatively long duration of anesthesia. *P < 0.05 when ARF rats were compared with sham-operated rats.

**Table 2. Changes in renal function after renal ischemia**

<table>
<thead>
<tr>
<th></th>
<th>ARF(30/5d)</th>
<th>Sham</th>
<th>ARF(60/5d)</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>P&lt;sub&gt;Cr&lt;/sub&gt;, µmol/l</td>
<td>34 ± 1.5</td>
<td>32 ± 1.9</td>
<td>112 ± 33&lt;sup&gt;*&lt;/sup&gt;</td>
<td>28 ± 0.6</td>
</tr>
<tr>
<td>C&lt;sub&gt;r&lt;/sub&gt;, mL/min</td>
<td>1.5 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>0.5 ± 0.1&lt;sup&gt;±&lt;/sup&gt;</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>F&lt;sub&gt;ENa&lt;/sub&gt;, %</td>
<td>0.7 ± 0.06</td>
<td>0.7 ± 0.02</td>
<td>3 ± 0.8&lt;sup&gt;±&lt;/sup&gt;</td>
<td>0.8 ± 0.05</td>
</tr>
<tr>
<td>U&lt;sub&gt;o&lt;/sub&gt;V&lt;sub&gt;H2O&lt;/sub&gt;, µl/min</td>
<td>1.4 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.4 ± 0.3&lt;sup&gt;±&lt;/sup&gt;</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>Urine output, µl·min&lt;sup&gt;−1&lt;/sup&gt;·kg&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>Baseline</td>
<td>Day −2</td>
<td>Day −1</td>
<td>After surgery</td>
</tr>
<tr>
<td></td>
<td>31 ± 3</td>
<td>30 ± 3</td>
<td>56 ± 6</td>
<td>52 ± 2</td>
</tr>
<tr>
<td></td>
<td>28 ± 2</td>
<td>29 ± 2</td>
<td>56 ± 6</td>
<td>47 ± 3</td>
</tr>
<tr>
<td></td>
<td>Day 1</td>
<td>43 ± 8&lt;sup&gt;±&lt;/sup&gt;</td>
<td>21 ± 4</td>
<td>62 ± 11</td>
</tr>
<tr>
<td></td>
<td>Day 2</td>
<td>50 ± 10</td>
<td>29 ± 1</td>
<td>121 ± 19&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>35 ± 5</td>
<td>24 ± 2</td>
<td>112 ± 19&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Day 4</td>
<td>31 ± 2</td>
<td>24 ± 3</td>
<td>147 ± 18&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Day 5</td>
<td>26 ± 2</td>
<td>26 ± 4</td>
<td>136 ± 18&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>U/P&lt;sub&gt;am&lt;/sub&gt;</td>
<td>7.3 ± 0.7</td>
<td>8 ± 1.2</td>
<td>1.5 ± 0.1&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>T&lt;sub&gt;TH2O&lt;/sub&gt;, µl·min&lt;sup&gt;−1&lt;/sup&gt;·kg&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>156 ± 3</td>
<td>161 ± 14</td>
<td>54 ± 11&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats. P<sub>Cr</sub>, C<sub>r</sub>, F<sub>ENa</sub>, U<sub>o</sub>V<sub>H2O</sub>, U/P<sub>am</sub>, and T<sub>TH2O</sub> were measured at day 5. ARF(30/5d) and ARF(60/5d) ARF established in rats after 30- and 60-min bilateral renal ischemia, respectively, with monitoring in the following 5 days. *P < 0.05 when ARF rats were compared with sham-operated rats.

Renal Sodium Handling Is Impaired in Rats with ARF

Rats with ARF induced by temporary bilateral renal ischemia for 30 or 60 min revealed a significant increase in the fractional urinary excretion of sodium (F<sub>ENa</sub>; Tables 1 and 2, Fig. 2). This indicates that ischemia-induced ARF is associated with impairment
of the tubular reabsorption of filtered sodium in postischemic kidney. Rats with mild ARF induced by 30-min ischemia had significantly increased FE\textsubscript{Na} at day 1 after renal ischemia (1 ± 0.2 in ARF30/1d vs. 0.4 ± 0.1% in sham control rats, P < 0.05), which normalized within 5 days (Table 2). Rats with severe ARF (60-min ischemia) also had markedly increased FE\textsubscript{Na} at day 1 after renal ischemic injury (12.5 ± 2.5 in ARF vs. 1.1 ± 0.1% in sham controls, P < 0.05). However, in contrast to mild ARF, rats with severe ARF had significantly increased FE\textsubscript{Na}, which persisted during the 5-day experimental period (3 ± 0.8 in ARF vs. 0.8 ± 0.05% in sham control rats, P < 0.05).

Next, we examined the changes in the rates of urinary sodium excretion (U\textsubscript{Na}V) in rats with ARF. In rats with mild ARF induced by 30-min ischemia (ARF30/1d), the U\textsubscript{Na}V was unchanged compared with sham-operated control rats (0.7 ± 0.2 in ARF vs. 0.9 ± 0.2 μmol/min in sham control rats, P > 0.05), despite a significant decrease in glomerular filtration rate (GFR), which was assessed by C\textsubscript{Cl} levels (Table 1). Consistent with this, the U\textsubscript{Na}V normalized 5 days after 30-min ischemia in ARF30/5d rats. In contrast, rats with severe ARF (either 1 day or 5 days after 60-min ischemia) had significantly decreased U\textsubscript{Na}V levels, despite a marked increase in FE\textsubscript{Na} (Tables 1 and 2). This suggests that severe ARF is associated with an impairment of urinary sodium excretion, which may result in sodium retention and volume overload, especially in the early phase of severe ARF where GFR is markedly decreased. However, rats with severe ARF (ARF60/5d) showed a marked increase in U\textsubscript{Na}V levels 5 days after 60-min ischemia, compared with ARF60/1d rats, despite a marked improvement in renal function assessed by GFR (0.5 ± 0.1 in ARF60/5d vs. 0.1 ± 0.01 ml/min in ARF60/1d, P < 0.05, Tables 1 and 2). Thus this suggests that the tubular sodium reabsorption in postischemic kidneys is significantly impaired, despite a marked improvement in renal function during the recovery phase of ARF. It may therefore be speculated that the expression of sodium transporters in postischemic kidneys may be severely affected by ischemia-reperfusion injury.

Renal Water Handling Is Impaired in Rats With ARF

As reported previously (26), rats with mild ARF (ARF30/1d) showed significantly increased urine output 1 day after release of bilateral renal artery occlusion (Table 1). Consistent with the marked increase in urine output, ARF30/1d rats had significantly decreased urine osmolality. In contrast, ARF60/1d rats, which had severe acute renal insufficiency, had markedly decreased urine output 1 day after release of bilateral renal artery occlusion (Table 1). However, these rats (ARF60/1d) also had a significant reduction in urine osmolality (659 ± 104 in ARF vs. 1,650 ± 200 mosmol/kgH\textsubscript{2}O in sham control rats, P < 0.05), despite a marked decrease in urine output. This suggests that urinary concentration is significantly impaired in rats with both oliguric and nonoliguric ARF (Table 1).

Next, we monitored the urine output in rats with ARF for 5 days after release of 30 (ARF30/5d)- or 60-min bilateral renal artery occlusion (ARF60/5d). In rats with mild ARF (ARF30/5d), the urine output was significantly increased 1 day after release of renal ischemia and then normalized progressively within 5 days (Table 2). Parallel to this, the urine osmolality was significantly decreased, and then normalized within days 4 and 5. In contrast, rats with severe ARF (ARF60/5d) had a marked polyuria during the following 5 days after the ischemic insult (Table 2). Furthermore the urine osmolality was significantly and persistently decreased during the 5-day experimental period (445 ± 33 in ARF 60/5d vs. 1,856 ± 144 mosmol/kgH\textsubscript{2}O in sham control rats at day 5, P < 0.05). Consistent with this, the urine-to-plasma osmolality ratio and solute-free water reabsorption were also significantly decreased at day 5 (Table 2), indicating an impairment of urinary concentration in ARF.

Na-K-ATPase Abundance in the Postischemic Kidney

Semiquantitative immunoblotting using membrane fractions prepared from the whole kidney of ischemia-induced ARF rats and sham-operated rats revealed that ARF was associated with a markedly reduced Na-K-ATPase abundance in postischemic kidneys (temporary 30- or 60-min bilateral renal ischemia). One day after release of 30-min renal ischemia, densitometric analysis revealed a significant decrease in Na-K-ATPase abundance in rats with ARF to 51 ± 11% of sham levels (100 ± 5%, P < 0.05, Table 3). Furthermore, the Na-K-ATPase levels were markedly decreased in rats with ARF60/1d to 22 ± 8% of sham levels (100 ± 10%, P < 0.05, Fig. 3).

Next, we examined the changes of Na-K-ATPase levels 5 days after release of 30- or 60-min renal ischemia. In ARF30/5d rats, Na-K-ATPase levels were not significantly different (69 ± 18% of sham levels (100 ± 19%), P > 0.05), indicating a partial recovery from ischemia and reperfusion injury in mild ARF. In contrast, rats with severe ARF (ARF60/5d) showed that Na-K-ATPase abundance remained very low (14 ± 7% of sham levels at day 5 after 60-min ischemic injury, Fig. 3).

NHE-3 Abundance in the Postischemic Kidney

Semiquantitative immunoblotting demonstrated a significantly reduced NHE-3 abundance 1 day after release of 30- or 60-min ischemic injury (Table 3), corresponding to 28 ± 6% of sham levels (ARF30/1d) and 20 ± 8% of sham levels (ARF60/1d).

As shown in Fig. 4, 5 days after release of 30-min bilateral renal ischemia (ARF30/5d), the NHE-3 levels were not significantly reduced (68 ± 19% of sham levels

---

2 In response to 60-min ischemia, rats had significantly reduced urine production at day 1 (identical to the observations in protocol 2). However, some of the rats had very low urine production (anuric or severely oliguric), and these rats did not survive during the following days because of renal failure. In contrast, ARF rats that had less severe oliguria (at day 1 after the 60-min ischemic insult) survived and developed severe polyuria during the following 5 days. The data shown in Table 2 are from these surviving rats only.
DECREASED ABUNDANCE OF NA TRANSPORTERS IN ARF

Table 3. Changes in the expression levels of major sodium transporters in rats with ischemia-induced ARF

<table>
<thead>
<tr>
<th></th>
<th>ARF (30/1d)</th>
<th>ARF (30/5d)</th>
<th>ARF (60/1d)</th>
<th>ARF (60/5d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na-K-ATPase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>51 ± 11%*</td>
<td>69 ± 18%</td>
<td>22 ± 6%*</td>
<td>14 ± 7%*</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 6)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100 ± 5%</td>
<td>100 ± 19%</td>
<td>100 ± 10%</td>
<td>100 ± 13%</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 5)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>NHE-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>28 ± 6%*</td>
<td>68 ± 19%</td>
<td>20 ± 8%</td>
<td>2 ± 1%*</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 6)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100 ± 9%</td>
<td>100 ± 23%</td>
<td>100 ± 19%</td>
<td>100 ± 16%</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 5)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>NaPi-II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>14 ± 6%*</td>
<td>67 ± 16%</td>
<td>19 ± 10%*</td>
<td>2 ± 0.8%*</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 6)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100 ± 13%</td>
<td>100 ± 20%</td>
<td>100 ± 3%</td>
<td>100 ± 20%</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 5)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>BSC-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>68 ± 12%</td>
<td>72 ± 8%</td>
<td>11 ± 3%*</td>
<td>7 ± 2%*</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 6)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100 ± 11%</td>
<td>100 ± 14%</td>
<td>100 ± 9%</td>
<td>100 ± 6%</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 5)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>TSC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ARF</td>
<td>91 ± 19%</td>
<td>80 ± 17%</td>
<td>17 ± 5%*</td>
<td>11 ± 7%*</td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 6)</td>
<td>(n = 10)</td>
<td>(n = 5)</td>
<td></td>
</tr>
<tr>
<td>Sham</td>
<td>100 ± 5%</td>
<td>100 ± 5%</td>
<td>100 ± 10%</td>
<td>100 ± 1%</td>
</tr>
<tr>
<td>(n = 9)</td>
<td>(n = 5)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± S.E. n, No. of rats; NHE-3, type 3 Na+/H+ exchanger; NaPi-II, type II Na-Pi cotransporter; BSC-1, type 1 bumetanide-sensitive cotransporter; TSC, thiazide-sensitive Na-Cl cotransporter. For densitometry of immunoblots, sodium transporter labeling was calculated as a fraction of the mean value from sham-operated control rats. *P < 0.05 when ARF rats were compared with sham-operated rats.

levels, P > 0.05). In contrast, rats with severe ARF (ARF 60/5d) showed that NHE-3 levels were persistently decreased to 2 ± 1% of sham levels (100 ± 16%, P < 0.05, Fig. 4).

NaPi-II Abundance in the Postischemic Kidney

Semiquantitative immunoblotting demonstrated a marked reduction in NaPi-II abundance 1 day after release of 30 (14 ± 6%) of sham levels, P < 0.05, Table 3) or 60-min ischemic injury (19 ± 10% of sham levels, P < 0.05, Fig. 5).

Five days after release of 30-min bilateral renal ischemia (ARF 30/5d), the NaPi-II levels in postischemic kidney were partly normalized to 67 ± 16% of sham levels (100 ± 20%, P > 0.05). In contrast, rats with severe ARF (ARF 60/5d) showed that NaPi-II levels were persistently and significantly decreased to very low levels, corresponding to 2 ± 0.8% of sham levels (100 ± 20%, P < 0.05, Fig. 5). This demonstrates that severe ARF is associated with a marked reduction in NaPi-II abundance.

Na-K-2Cl Cotransporter (BSC-1 or NKCC-2) Abundance in the Postischemic Kidney

Next we examined the levels of the apical Na-K-2Cl cotransporter (BSC-1), the major transporter for apical sodium reabsorption by the TAL. In contrast to the observed severe decrease in the abundance of Na-K-ATPase, NHE-3, and NaPi-II levels after mild renal ischemia (30-min ischemia), there was no significant decrease in BSC-1 abundance in rats with mild ARF. As shown in Fig. 6, semiquantitative immunoblotting demonstrated that the BSC-1 levels in ARF 30/1d rats was 68 ± 12% of sham levels (100 ± 11%, P > 0.05), which were also maintained 5 days after ischemia: 72 ± 8% of sham levels (100 ± 14%, P > 0.05). In contrast, in rats with severe ARF (60-min ischemia), BSC-1 levels were severely decreased at day 1 to 11 ± 3% of sham levels (100 ± 9%, P < 0.05, Fig. 7) and remained severely reduced at day 5 to 7 ± 2% of sham levels (100 ± 6%, P < 0.05, Fig. 7).

Na-Cl Cotransporter (TSC) Abundance in the Postischemic Kidney

Apical sodium entry into the DCT is mediated by the thiazide-sensitive Na-Cl cotransporter TSC (15, 23). We also examined whether experimental ARF is associated with the changes in abundance of TSC in postischemic kidney. In rats with mild ARF, semiquantitative immunoblotting revealed no significant decrease in TSC abundance (Table 3). The TSC levels in ARF 30/1d

---

Fig. 3. Immunoblots of membrane fractions of whole kidneys from ARF (60-min ischemia) and sham-operated rats. A and C: immunoblots were reacted with α-isofrom-specific monoclonal antibodies to Na-K-ATPase and reveal a ~96-kDa band. B: densitometric analysis of all samples from ARF 60/1d and sham-operated rats reveals a marked decrease in rat ARF with 22 ± 8% of sham levels (100 ± 10%). D: densitometric analysis of all samples from ARF 60/5d and sham-operated rats reveals that the Na-K-ATPase levels are markedly decreased in rats with ARF to 14 ± 7% of sham levels (100 ± 13%). n, No. of rats. *P < 0.05.
rats was 91 ± 19% of sham levels (100 ± 5%, P > 0.05), which remained at this level 5 days after ischemia: 80 ± 17% of sham levels (100 ± 5%, P > 0.05). In contrast, in rats with severe ARF (60-min ischemia), the TSC levels were significantly decreased to 17 ± 5% of sham levels 1 day after 60-min bilateral ischemia and remained markedly decreased corresponding to 11 ± 7% of sham levels at day 5 (Fig. 8). Electrogenic NBC (rkNBC1) and Electroneutral NBC (NBC3) Abundance in Postischemic Kidney

The electrogenic Na-HCO3 cotransporter rkNBC1 is present in the basolateral membrane domains of proximal tubule, especially S1 and S2 segments of the proximal tubules in rat kidney (41). The electroneutral NBC3 was recently cloned from the human skeletal muscle (39) and is exclusively present at the intercalated cells of connecting tubule and collecting duct in rat kidney (28). rkNBC1 abundance was significantly decreased in postischemic kidneys [ARF30/1d: 34 ± 11 (n = 8) vs. 100 ± 8% in control rats (n = 9), P < 0.05; ARF60/1d: 15 ± 9% (n = 10) vs. 100 ± 10% in control rats (n = 8), P < 0.05, Fig. 9]. Moreover, collecting duct NBC3 abundance was also decreased markedly, corresponding to 36 ± 15 in ARF30/1d (n = 8) and 13 ± 3% in ARF60/1d (n = 10) of sham levels [100 ± 6 (n = 9) and 100 ± 10% (n = 8), respectively (P < 0.05, not shown)]. Thus the abundance of these two Na-HCO3 cotransporters was also severely reduced.

Megalin (gp330) and FBP Abundance in Postischemic Kidney

To examine whether the abundance of all proximal tubule membrane proteins was reduced to the same extent as that of the sodium transporters, we examined the expression levels of other proteins that are associated with the apical plasma membrane domains in the proximal tubule, i.e., megalin and FBP. Megalin, which was described as the Heyman nephritis antigen gp330, is present at the brush-border membrane, coated pits, endocytotic vacuoles, lysosomes, and dense apical tubules in the proximal tubule (12). FBP is located at the brush-border membrane, endocytotic vacuoles, and dense apical tubules in the proximal tubule (5). Semi-quantitative immunoblotting demonstrated that megalin abundance in postischemic kidneys was marginally reduced 1 day after release of 30- or 60-min ischemic injury, corresponding to 69 ± 4 (n = 8) and 70 ± 11%
Fig. 6. Immunoblots of membrane fractions of whole kidneys from ARF (30-min ischemia) and sham-operated rats. A and C: immunoblots were reacted with affinity-purified anti-type 1 bumetanide-sensitive cotransporter (BSC-1), which recognizes a strong, broad band of molecular mass ~146–176 kDa centered at ~161 kDa. B: densitometric analysis of all samples from ARF 30/1d and sham-operated rats reveals that the BSC-1 levels are not significantly decreased in rats with ARF: 68 ± 12% of sham levels (100 ± 11%). D: densitometric analysis of all samples from ARF 30/5d and sham-operated rats reveals that BSC-1 levels are not significantly decreased in rats with ARF: 72 ± 8% of sham levels (100 ± 14%). n, No. of rats.

The proximal tubules exhibited strong Na-K-ATPase labeling of basolateral plasma membranes in sham-operated control rats (Fig. 10C, arrowheads). Significantly, the labeling of NHE-3 in the proximal tubule of postischemic kidney (ARF 30/1d, Fig. 10B, arrows) as well as of mTAL (not shown) was reduced compared with sham control rats. The NHE-3 labeling patterns were confirmed by immunoelectron microscopy (not shown).

Immunocytochemistry Confirms the Decreased Abundance of Sodium Transporters in Postischemic Kidneys

In sham-operated rats, anti-NHE-3 antibody labeled the apical plasma membrane domains (Fig. 10A, arrows) of S1 and S2 convoluted proximal tubules, with a weaker staining of the S2 and S3 straight proximal tubule segments and descending thin limb cells (not shown). The labeling was exclusively confined to the apical domains and intermicrovillar cleft of the proximal tubule cells (arrows), whereas the apical brush-border and basolateral plasma membranes were unlabeled (Fig. 10A). Furthermore, an intense labeling was also seen of the apical plasma membrane domains of medullary TAL in sham-operated rats (not shown).

In ARF 30/1d rats, light microscopical examination demonstrated some tubular damage and cell death after renal ischemia and reperfusion (not shown). Tubular necrosis was most pronounced in the proximal tubule (not shown). Necrotic cells were detached from the tubular basement membrane and were seen in the tubular lumen. Furthermore, disruption of the brush border and focal loss of individual tubular cells in the proximal tubule were also seen (not shown). Immunocytochemistry demonstrated that the labeling of NHE-3 in the proximal tubule of postischemic kidney (ARF 30/1d, Fig. 10B, arrows) as well as of mTAL (not shown) was reduced compared with sham control rats. The NHE-3 labeling patterns were confirmed by immunoelectron microscopy (not shown).

The proximal tubules exhibited strong Na-K-ATPase labeling of basolateral plasma membranes in sham-operated control rats (Fig. 10C, arrowheads). Signifi-
cant basolateral labeling was observed of collecting duct principal cells, and very intense basolateral labeling was present in the TAL cells. This strong labeling also extended into the cortical portions of the TAL and of DCT (Fig. 10C). This is consistent with previous evidence (20). In rats with ARF (ARF30/1d), the basolateral labeling of the proximal tubule was considerably weaker (Fig. 10D), consistent with a significant decrease in Na-K-ATPase levels determined by immunoblotting. Thus part of the reduced Na-K-ATPase abundance revealed by immunoblotting was ascribed to reduced levels in the proximal tubule. There was no evidence for Na-K-ATPase labeling in the apical plasma membrane in kidneys from rats with ARF (or sham-operated control rats).

In sham-operated rats, immunocytochemistry confirmed that TSC immunolabeling was seen exclusively in DCT in the apical and subapical regions (Fig. 11A). In rats with mild ARF (ARF30/1d), TSC labeling was maintained in the apical plasma membrane domains of DCT (Fig. 11B), consistent with the unchanged TSC levels determined by immunoblotting.

In sham-operated rats, an intense BSC-1 labeling was seen in apical domains of mTAL and cTAL cells (not shown), consistent with previous observations (34, 36). In rats with ARF (ARF30/1d), the morphology of TAL cells in the inner stripe of the outer medulla, as well as the labeling of BSC-1, was variable (Fig. 11D). Several mTAL cells showed normal morphology and normal BSC-1 labeling patterns (Fig. 11D, arrowhead), whereas other TAL cells exhibited tubular cell swelling into the tubular lumen and maintained normal BSC-1 labeling (Fig. 11D, inset). In contrast, some mTAL cells showed a clearly reduced labeling of BSC-1 (Fig. 11D, asterisk).

α-MSH Treatment Markedly Inhibits the Decline in Renal Functions After Renal Ischemia and Reperfusion

Several pharmacological interventions have been tried to reduce the loss of renal function after renal ischemia and reperfusion injury (45). Recently, α-MSH, which is known to inhibit both inflammatory and nitric oxide (NO) pathways (9), has been demonstrated by Chiao and colleagues (11) to be effective in reducing ischemia and reperfusion injury in rats. We therefore examined whether α-MSH treatment affects both the expression of sodium transporters and the changes in the renal functions in postischemic ARF. For this purpose, we used the same protocols as described by Chiao et al.

α-MSH treatment markedly reduced the severity of acute renal insufficiency because ARF rats treated with α-MSH had significantly lower plasma creatinine levels (68 ± 15 µmol/l, P < 0.05), compared with untreated
Fig. 10. Immunocytochemical analyses of NHE-3 and Na-K-ATPase in 0.8- to 1-µm cryosections of proximal tubules from sham-operated rats (A and C), and ARF (ARF30/1d) rats (B and D). A: In sham-operated rats, NHE-3 labeling is seen at apical domains of proximal tubule cells (arrows) but is absent in brush-border and basolateral membrane domains. B: in ARF rats, NHE-3 labeling (arrows) is also seen at apical domains of proximal tubule cell but is much weaker compared with that seen in kidneys from sham-operated rats. C: in sham-operated rats, α1 isoform Na-K-ATPase labeling is noted along basolateral plasma membranes of proximal tubule cells (arrowheads). D: in ARF rats, Na-K-ATPase labeling is also seen at basolateral plasma membrane domains (arrowheads). However, labeling density is much weaker compared with sham-operated rats. Magnification: ×1,100.

Fig. 11. Immunocytochemical analyses of TSC in distal convoluted tube (DCT) and BSC-1 in the medullary thick ascending limb (mTAL) of sham-operated (A and C) and ARF (ARF30/1d) rats (B and D). A: TSC labeling is seen at apical plasma membrane domains of DCT cells in sham-operated rats. B: in ARF rats (ARF30/1d), labeling of TSC in DCT cells is not weaker. C: abundant BSC-1 labeling is seen of apical plasma membrane domains of mTAL cells in inner stripe of outer medulla from sham-operated control rats. D: in rats with ARF (30/1d), the morphology of TAL cells in inner stripe of the outer medulla as well as labeling of BSC-1 was variable. Several mTAL cells showed remained BSC-1 labeling with normal morphology (arrowhead), and some revealed tubular cell swelling into tubular lumen but with maintained BSC-1 labeling (inset). In contrast, some mTAL cells showed decreased labeling of BSC-1 (asterisk). Magnification: ×1,100.
Changes in renal function 2 days after release of 40-min bilateral renal ischemia with or without α-MSH treatment

<table>
<thead>
<tr>
<th></th>
<th>ARF (n=9)</th>
<th>ARF + MSH (n=8)</th>
<th>Sham (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P&lt;sub&gt;Cr&lt;/sub&gt;, μmol/l</td>
<td>194 ± 45*</td>
<td>68 ± 15*</td>
<td>32 ± 1</td>
</tr>
<tr>
<td>C&lt;sub&gt;Cr&lt;/sub&gt;, ml/min</td>
<td>0.4 ± 0.1*</td>
<td>0.8 ± 0.1*</td>
<td>1.3 ± 0.06</td>
</tr>
<tr>
<td>F&lt;sub&gt;E&lt;/sub&gt;N&lt;sub&gt;a&lt;/sub&gt;, %</td>
<td>4.2 ± 0.6*</td>
<td>1.5 ± 0.6</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>Urine output, µl·min&lt;sup&gt;-1&lt;/sup&gt;·kg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>51 ± 16*</td>
<td>118 ± 18*</td>
<td>163 ± 10</td>
</tr>
</tbody>
</table>

Baseline period
- Day 1: 44 ± 1
- Day 1: 40 ± 3
- Day 2: 94 ± 13*<sup>†</sup>
- Day 2: 71 ± 8*<sup>†</sup>
- U/P<sub>osmol</sub>: 1.7 ± 0.3*<sup>†</sup>
- T<sub>2</sub>H<sub>2</sub>O, µl·min<sup>-1</sup>·kg<sup>-1</sup>: 51 ± 16*<sup>†</sup>

Values are means ± SE, n. No. of rats. P<sub>Cr</sub>, C<sub>Cr</sub>, F<sub>E</sub>N<sub>a</sub>, U/P<sub>osmol</sub>, and T<sub>2</sub>H<sub>2</sub>O were measured at day 2. α-MSH, α-melanocyte-stimulating hormone. *P < 0.05 when ARF rats, which were either treated or not treated with α-MSH, were compared with sham-operated rats. †P < 0.05 when ARF rats that were not treated with α-MSH were compared with ARF rats treated with α-MSH.

ARF rats (194 ± 45 μmol/l, Table 4). Moreover, α-MSH treatment significantly reduced the increased F<sub>E</sub>N<sub>a</sub> (4.2 ± 0.6 in ARF vs. 1.5 ± 0.6% in α-MSH-treated ARF rats, P < 0.05, Fig. 12) and the degree of polyuria (Table 4). Consistent with this, urine osmolality, urine-to-plasma osmolality ratio and solute-free water reabsorption in α-MSH-treated ARF rats were also significantly improved (Table 4). Thus α-MSH significantly inhibits the changes in renal sodium handling after renal ischemia and reperfusion.

α-MSH Treatment Reduces the Ischemia-Induced Decrease in Sodium Transporters

As shown in Fig. 13 and Table 5, α-MSH treatment dramatically prevents the decrease in the abundance of all the investigated sodium transporters after renal ischemia and reperfusion. This is consistent with marked improvement in renal sodium handling in response to α-MSH treatment of rats with acute renal failure (Fig. 12, Table 4). In contrast, in rats with ARF (ARF40/2d) that were not treated with α-MSH, the abundance of all the major sodium transporters investigated was significantly reduced in postischemic kidney compared with sham control rats. Immunocytochemistry also revealed that α-MSH treatment during reperfusion after renal ischemia is effective in inhibiting the downregulation of sodium transporter expression in postischemic kidneys (not shown).

DISCUSSION

We have demonstrated that the abundance of several major renal sodium transporters (Na-K-ATPase, NHE-3, NaPi-II, BSC-1, and TSC) in rats with ARF (2 days after 40-min bilateral renal ischemia) was severely reduced after α-MSH treatment of rats with acute renal failure (Fig. 12, Table 4). In contrast, in rats with ARF (ARF40/2d) that were not treated with α-MSH, the abundance of all the major sodium transporters investigated was significantly reduced in postischemic kidney compared with sham control rats. Immunocytochemistry also revealed that α-MSH treatment during reperfusion after renal ischemia is effective in inhibiting the downregulation of sodium transporter expression in postischemic kidneys (not shown).

**Table 4. Changes in renal function 2 days after release of 40-min bilateral renal ischemia with or without α-MSH treatment**

**Table 5. Changes in the abundance of major Na transporters by α-MSH treatment of rats with bilateral ischemia-induced ARF**

Fig. 12. Effect of α-MSH treatment on F<sub>E</sub>N<sub>a</sub> levels in rats with ARF (2 days after 40-min bilateral renal ischemia) and sham-operated control rats. Rats with ARF have significantly increased F<sub>E</sub>N<sub>a</sub> 2 days after renal ischemia (4.2 ± 0.6%). In contrast, rats with ARF that were treated with α-MSH show a markedly reduced F<sub>E</sub>N<sub>a</sub> (1.5 ± 0.6%) compared with rats with ARF that were not treated with α-MSH. F<sub>E</sub>N<sub>a</sub> in α-MSH-treated rats with ARF is similar to that in sham-operated rats (1 ± 0.1%). *P < 0.05 ARF rats compared with sham-operated rats. †P < 0.05 ARF rats compared with α-MSH-treated ARF rats.

Fig. 13. Effect of α-MSH treatment on expression of several sodium transporters (Na-K-ATPase, NHE-3, NaPi-II, BSC-1, and TSC) in rats with ARF (2 days after 40-min bilateral renal ischemia). Semi-quantitative immunoblotting demonstrates that α-MSH treatment during reperfusion in rats with ARF significantly reduces the decline in sodium transporter expressions after renal ischemia, compared with ARF rats that were not treated with α-MSH (see Table 5).
and water to the TAL segment, especially in mild ARF. α-MSH treatment significantly prevented both the ischemia-induced downregulation of sodium transporters and the increase in \( FE_{Na} \). Together, the results suggest that decreased levels of sodium transporters along the nephron in postischemic kidneys may play a critical role in the impairment of tubular sodium reabsorption and hence contribute to increased sodium and water excretion during the recovery phases of ischemia-induced acute renal failure.

Downregulation of Sodium Transporters in the Proximal Tubule

We demonstrated that the abundance of NHE-3 and NaPi-II in postischemic kidney was severely decreased at day 1 after renal ischemia. Importantly, the reduction in the proximal tubule abundance of these transporters was confirmed by immunocytochemistry. The reduction is consistent with the previous findings of significantly reduced expression of NHE-3 mRNA levels after 30-min renal ischemia (50). Furthermore, the abundance of Na-K-ATPase in postischemic kidney as well as the labeling of Na-K-ATPase in the basolateral domains of proximal tubule was also significantly reduced by ischemia. Also, this is consistent with previous demonstrations of a reduced level of Na-K-ATPase mRNA (46, 50). These findings suggest that 1) the proximal tubule is susceptible to ischemia and reperfusion injury, consistent with previous observations (7); and 2) the proximal tubule reabsorption of filtered sodium, \( HCO_3^- \), and phosphate may be severely compromised in postischemic kidney due to the significant reduction in expression of proximal tubule sodium transporters.

Sodium Transporters in the TAL and DCT

mTAL of Henle are also known to be susceptible to ischemia-induced injury. The Na-K-2Cl cotransporter BSC-1 (or NKCC2), which is localized at the apical plasma membrane domains of mTAL and cTAL segments, mediates the apical NaCl transport in these water-impermeable segments. Several factors have been demonstrated to regulate the abundance of BSC-1 levels. Increase in the delivery of NaCl to the loop of Henle or chronic administration of vasopressin is known to upregulate BSC-1 levels (14, 22, 27). Interestingly, we demonstrated that the abundance of BSC-1 was not significantly decreased either at day 1 or at day 5 after mild, temporary, bilateral ischemic injury (30-min bilateral renal ischemia). This is consistent with the lack of a substantial decrease in the abundance of BSC-1 in postischemic kidneys in rats subjected to unilateral 45-min renal ischemia and contralateral nephrectomy (17). It is possible that the observed decrease in proximal tubular sodium and water reabsorption, which was associated with a decrease in abundance of NHE-3, NaPi-II, Na-K-ATPase, and aquaporin-1 (AQP1) levels (17, 26), may result in an increased delivery of sodium and water to the TAL segment, especially in mild ARF. This may possibly and indirectly induce BSC-1 expression and thus prevent a substantial decrease in BSC-1 levels in mild ARF. In contrast, severe ischemic injury (60-min renal ischemia) significantly reduced the abundance of BSC-1.

TSC is expressed in the DCT (35) and is responsible for a large fraction of the net sodium and chloride reabsorption that occurs in the distal portion of the mammalian renal tubule (10, 15, 23, 38, 47). In the present study, we have demonstrated that the abundance of TSC was not decreased either at day 1 or at day 5 after mild (30-min) temporary, bilateral ischemic injury. The molecular mechanisms involved in maintaining BSC-1 and TSC levels after a mild ischemic injury is presently unknown. However, it may also be speculated that increased delivery of sodium and fluid to the distal nephron may have significant effects on the TSC levels as well as BSC-1 levels.

Significant Reduction in GFR Prevents the Extensive Loss of Urinary Sodium

A reduction in GFR in rats with ARF reduces the filtered load of sodium. This is consistent with the observation that the rate of urinary sodium excretion was unchanged in mild ARF or even decreased in severe ARF. Thus a significant reduction in GFR tends to prevent the extensive loss of sodium that otherwise could result from the markedly reduced abundance of sodium transporters along the nephron in postischemic kidneys. Indeed, we have demonstrated that there was a significant reduction in the abundance of sodium transporters at 1 day after 60-min bilateral ischemia. Because the GFR was increased at day 5 compared with day 1 (assessed by \( CCr \)), the threefold increase in urinary sodium excretion (Tables 1–3) is likely to be a result of the decreased abundance of several sodium transporters. Reduction of sodium transporter abundance is also likely to be responsible for the polyuria in the diuretic phases of ARF. Moreover, both the reduced abundance of sodium transporters and the marked increase in \( FE_{Na} \) were completely prevented by treatment with α-MSH during the reperfusion period. The results together strongly suggest that decreased levels of sodium transporters along the nephron in postischemic kidneys may play a critical role in the impairment of tubular reabsorption of filtered sodium and hence contribute to increased sodium and water excretion during the recovery phase of ischemia-induced ARF.

Effects of α-MSH Treatment

α-MSH is a potent anti-inflammatory agent that inhibits neutrophil migration and production of neutrophil chemokines and NO (9). Previously, our laboratory demonstrated that α-MSH treatment of rats with bilateral ischemia-induced ARF significantly reduced the downregulation of AQP1, AQP2, and AQP3 levels and that this was paralleled by functional changes (26). In the present study, both the reduced abundance of sodium transporters and the marked increase in \( FE_{Na} \) were completely prevented by treatment with α-MSH.
during the reperfusion period. This strongly supported the view that reduction in sodium transporter expression played a significant role in the altered tubular sodium handling. A potential effect of α-MSH is consistent with previous observations that α-MSH treatment in mice and rats with ischemia-induced ARF improves renal function significantly by reducing the neutrophil plugging and erythrocyte congestion in the medullary region (11). Although the exact mechanisms for the α-MSH effects are not known, inhibition of NO production and decreased production of chemokines in response to ischemic injury may be involved in preventing the down-regulation of sodium transporters and aquaporins, in association with preservation of renal functions. The exact role and signaling pathways involved in NO, endothelin, and chemokines in altering sodium transporters and expression of aquaporins remain to be identified. However, it should be emphasized that a direct tubular effect of α-MSH cannot be excluded.

Reduced Abundance of Na-HCO\textsubscript{3} Cotransporters

We demonstrated that the abundance of electronegative NBC (rKNBC1) in the proximal tubule and electroneutral NBC (NBC3) in the distal nephron was significantly reduced by the ischemia and reperfusion injury. In the proximal tubule, rKNBC1 (localized at the basolateral membrane in S1 and S2 segment of the proximal tubule) mediates basolateral proximal tubule HCO\textsubscript{3} efflux in this segment (6). Thus reduced expression of both NHE-3 and rKNBC1 in the proximal tubule is likely to play a role for the marked decrease in sodium and HCO\textsubscript{3} absorption in this segment. This may also, in turn, play a role in the increased delivery of sodium and HCO\textsubscript{3} to the distal nephron, especially in the early phase of mild ischemic injury. Recently, Wang et al. (49) reported that reduced expression of NHE-3 mRNA with increased expression of H\textsuperscript+\textendash K\textsuperscript+\textendash ATPase mRNA levels was associated with early phase of mild ischemia-induced ARF. This is consistent with our findings of decreased abundance of NHE-3 protein, and thus it may be possible that increased delivery of HCO\textsubscript{3} to distal nephron may change the expression of H\textsuperscript+\textendash K\textsuperscript+\textendash ATPase mRNA levels for the acid-base homeostasis. However, increased expression of H\textsuperscript+\textendash K\textsuperscript+\textendash ATPase mRNA levels was rapidly declined at 24 h after reperfusion (49).

In the distal nephron, we recently demonstrated that the electroneutral NBC3, which has been cloned from human skeletal muscle, is exclusively present in the intercalated cells of connecting tubule and cortical, outer medullary, and inner medullary collecting duct (28). The electroneutral NBC3 protein abundance in the intercalated cells was also reduced in response to ischemia and reperfusion injury, and this may also participate in the altered sodium handling in posts ischemic kidneys.

Reduced Abundance of Sodium Transporters Compared with Other Membrane Proteins

In contrast to the marked reduction in the expression of the examined proximal tubule sodium transporters (NHE-3, NaPi-II, Na-K-ATPase, and rKNBC1), which were reduced to less than one-third of control levels, the abundance of megalin or FBP, respectively, in proximal tubule was not severely affected or even maintained. This may intuitively suggest that there may be some selectivity in the mechanisms associated with the decreased abundance of several sodium transporters compared with other membrane proteins. However, it should be emphasized that the mechanisms underlying the changes in expression are undefined, and the potential selectivity therefore remains to be further established.

Summary

The ischemic insult is associated with a marked decrease in abundance of several sodium transporters along the nephron in posts ischemic kidneys, as well as a significant impairment of tubular reabsorption of filtered sodium. Moreover, α-MSH treatment markedly reduced the ARF-induced increase in FE\textsubscript{Na} and the decrease in sodium transporter expression levels. We conclude that decreased levels of sodium transporters along the nephron may play a critical role in the impairment of tubular reabsorption of filtered sodium and that this contributes to the increased sodium and water excretion during the recovery phase of ischemia-induced ARF, despite a marked improvement in renal function. Further studies are needed to understand the mechanistic basis of decreased transporter and aquaporin expression in ARF.

The authors thank Dr. Henrik Birn for anti-megalin antibody, Dr. Sheldon P. Rothenberg for anti-folate binding protein antibody, Dr. Christian Aalkjaer for anti-rKNBC1 antibody, and Dr. Ira Kurtz for anti-NBC3 antibody. We also thank Zhila Nikrozi, Mette Vistisen, Inger Merete Paulsen, Gitte Christensen, and Annette Stockwell for expert technical assistance.

Support for this study was provided by the Karen Elise Jensen Foundation, Novo Nordic Foundation, Danish Medical Research Council, University of Aarhus Research Foundation, University of Aarhus, Dongguk University, Commission of the European Union (EU-Biotech, and EU-TMR project), and intramural budget of the National Heart, Lung, and Blood Institute.

Present address of T.-H. Kwon: Department of Physiology, School of Medicine, Dongguk University, Kyungju 780–714, Korea.

Address for reprint requests and other correspondence: S. Nielsen, Dept. of Cell Biology, Institute of Anatomy, Univ. of Aarhus, DK–8000 Aarhus C, Denmark (E-mail: sn@ana.au.dk).

Received 28 April 1999; accepted in final form 30 December 1999.

REFERENCES

DECREASED ABUNDANCE OF NA TRANSPORTERS IN ARF


