Altered expression of renal NHE3, TSC, BSC-1, and ENaC subunits in potassium-depleted rats

MARIE-LOUISE ELKJÆR,1 TAE-HWAN KWON,1,2 WEIDONG WANG,1 JAKOB NIELSEN,1 MARK A. KNEPPER,3 JØRGEN FRØKJÆR,1 AND SØREN NIELSEN1
1The Water and Salt Research Center, University of Aarhus, DK-8000 Aarhus C, Denmark; 2Department of Physiology, School of Medicine, Dongguk University, 780-714 Kyungju, Korea; and 3Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

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Elkjær, Marie-Louise, Tae-Hwan Kwon, Weidong Wang, Jakob Nielsen, Mark A. Knepper, Jørgen Frøkjær, and Søren Nielsen. Altered expression of renal NHE3, TSC, BSC-1, and ENaC subunits in potassium-depleted rats. Am J Physiol Renal Physiol 283: F1376–F1388, 2002.—The purpose of this study was to examine whether hypokalemia is associated with altered abundance of major renal Na+ transporters that may contribute to the development of urinary concentrating defects. We examined the changes in the abundance of the type 3 Na+/H+ exchanger (NHE3), Na+-K+-2Cl– cotransporter (BSC-1), the thiazide-sensitive Na+-Cl– cotransporter (TSC), and epithelial sodium channel (ENaC) subunits in kidneys of hypokalemic rats. Semiquantitative immunoblotting revealed that the abundance of BSC-1 (57%) and TSC (46%) were profoundly decreased in the inner stripe of the outer medulla (ISOM) and cortex/outer stripe of the outer medulla (OSOM), respectively. These findings were confirmed by immunohistochemistry. Moreover, total kidney abundance of all ENaC subunits was significantly reduced in response to the hypokalemia: α-subunit (61%), β-subunit (41%), and γ-subunit (60%), and this was confirmed by immunohistochemistry. In contrast, the renal abundance of NHE3 in hypokalemic rats was dramatically increased in cortex/OSOM (736%) and ISOM (210%). Downregulation of BSC-1, TSC, and ENaC may contribute to the urinary concentrating defect, whereas upregulation of NHE3 may be compensatory to prevent urinary Na+ loss and/or to maintain intracellular pH levels.

hypokalemia; sodium transport; kidney; urine concentration

Hypokalemia, a common electrolyte disturbance frequently encountered in clinical medicine, is often associated with several distinct renal functional defects, including nephrogenic diabetes insipidus presented by polyuria and urinary concentrating defect. Consistent with this, our laboratory previously demonstrated that rats with hypokalemia have a significant vasopressin-resistant polyuria with reduced expression of inner medullary aquaporin-2 (AQP2) levels (33).

Urinary concentration and dilution depend on the presence of a distinct segmental distribution of transport properties along the renal tubule, and urinary concentration depends on 1) the hypertonic medullary interstitium, driven by active NaCl reabsorption as a consequence of countercurrent multiplication in water-impermeable nephron segments (35); and 2) osmotic equilibration in water-permeable renal tubular segments, which chiefly depends on aquaporin water channels (38). Thus defects in any of these mechanisms would be predicted to be associated with urinary concentrating defects.

Over the years, physiological and biochemical investigations have identified the major proteins involved in physiological processes of transepithelial salt and water transport along the nephron and collecting duct. Cloning of cDNAs and determination of the amino acid sequence of these proteins have made it possible to produce selective polyclonal or monoclonal antibodies to them. This has allowed investigators to use comprehensive sets of antibodies against proteins relevant to a given physiological process and to investigate entire pathways through simultaneous assessment of the regulatory state of all members of the pathway. Recently, a series of studies have identified that dysregulated sodium transporters play an important role in animal models of various disorders of sodium and water balance, including chronic renal failure (28), ischemia-induced acute renal failure (29), cirrhosis (13), lithium-induced nephrogenic diabetes insipidus (31), syndrome of inappropriate antidiuretic hormone secretion (10), primary aldosteronism (25, 34), and vitamin D-induced hypercalcemia (49). In the kidney proximal tubule, the type 3 Na+/H+ exchanger (NHE3) is expressed apically and participates in sodium reabsorption in this segment (3). The loop of Henle generates a high osmolality in the renal medulla by driving the countercurrent multiplier, which is dependent on NaCl reabsorption by the thick ascending limb (TAL). In this segment, the apically located Na+-K+-2Cl– cotransporter (rat type 1

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bimetanide-sensitive cotransporter (BSC-1 or NKCC2)]
(15–17, 19) and NHE3, in conjunction with the Na⁺-
K⁺-ATPase in the basolateral membranes, are mainly
responsible for sodium reabsorption by the TAL (3).
In the distal convoluted tubule, the thiazide-sensitive
Na⁺-Cl⁻ cotransporter (TSC or NCC) is involved in
apical sodium reabsorption by the TAL (21, 25).
Epithelial sodium channel (ENaC) subunits are known to be expressed in
the connecting tubule and collecting duct, including
the inner medullary collecting duct (18). ENaC partic-
ipates in sodium reabsorption and is regulated by the
hormones controlling sodium and water balance, e.g.,
the mineralocorticoid aldosterone and vasopressin
(10, 34).

Potassium depletion has been demonstrated to be
associated with altered sodium reabsorption in distinct
segments of the renal tubules. Soleimani et al. (44)
demonstrated that potassium depletion was associated
with an increase in luminal Na⁺/H⁺ exchange and
casualateral Na⁺-HCO₃⁻ cotransport in the proximal
tubule. When rat superficial cortical nephrons were
perfused in vivo, sodium and fluid absorption
decreased along the loop of Henle in rats fed a low-
kpotassium diet (46). Consistent with this, Northern
hybridization revealed that mRNA levels for both the
apical Na⁺-K⁺-2Cl⁻ cotransporter in the medulla and the
Na⁺-Cl⁻ cotransporter in the cortex were de-
creased in kidneys of rats with hypokalemia along with
an increased urinary chloride loss (5). Micropuncture
studies also have shown that potassium depletion was
associated with reductions in the delivery of sodium
and water to early and late regions of the distal tubule
(47). Moreover, plasma aldosterone levels, which are
critically involved in controlling sodium balance, are
suppressed by potassium depletion (27). These
findings therefore strongly indicate that changes in external
potassium balance may affect the transport of sodium
and fluid along the nephron and that this may be
caused by changes in the expression of major renal
sodium transport proteins.

Therefore, the purposes of the present study were 1)
to examine whether hypokalemia affects the protein
abundance of major renal sodium transporters and 2)
to examine whether the changes in the abundance of
sodium transporters are associated with alterations in
urinary concentration and urinary sodium excretion in
hypokalemic rats. The abundance and cellular/subcell-
ular distribution of sodium transporters were deter-
mined by semiquantitative immunoblotting and immu-
nohistochemistry using antibodies against major renal
sodium transporters (NHE3, Na⁺-K⁺-ATPase, BSC-1,
TSC, and ENaC subunits).

METHODS

Experimental Animals

Studies were performed in male Wistar rats (M & B,
Lille-Skensved, Denmark) that were kept in metabolic cages
for at least 48 h before the experiment was started and had
free access to control chow and tap water (C1000, Altromin,
Lage, Germany). During the entire experiment, the rats were
kept in individual metabolic cages, with a 12:12-h light-dark
cycle and a temperature of 21 ± 2°C.

Experimental Protocol

After a period of acclimation, the animals were random-
ized into two groups matched for body weight: the hypokale-
mia group (n = 12) and the control group (n = 12). To produce
hypokalemia, rats were fed a potassium-deficient diet
(21037, potassium content: 0.18 g/kg chow, Altromin) for 4
days. In the control group, rats were fed control chow (C1000,
potassium content: 7 g/kg chow, Altromin) and offered the
amount of food corresponding to the mean intake of food in
the hypokalemia group of rats during the previous day. Thus
the food intake was matched between the two groups. Rats in
both groups had free access to water throughout the experi-
ment.

Clearance Studies

Daily urinary output and water intake were determined
throughout the study. Urinary volume, osmolality, creati-
nine, sodium, and potassium concentration were measured.
Plasma was collected from the abdominal aorta at the time of
death for measurement of potassium, sodium, creatinine, and
plasma osmolality.

Measurement of Plasma Aldosterone

Blood samples were drawn in lithium-heparin glass vials.
Immunoactive aldosterone was measured by a radioimmu-
oassay method (Diagnostic System Laboratories, Webster,
TX). Using a rabbit anti-aldosterone antibody and ¹²⁵I-aldo-
sterone, a radioimmunoassay was performed by incubation of
plasma samples in precoated tubes.

Primary Antibodies

For semiquantitative immunoblotting and immunocyto-
chemistry, previously characterized polyclonal and monoclo-
anal antibodies were used and summarized below.

NHE3 (LL546AP). An affinity-purified polyclonal antibody
against NHE3 has previously been characterized (12, 23).

TSC (LL573AP). An affinity-purified polyclonal antibody
against apical TSC in the distal convoluted tubule has pre-
viously been characterized (25).

BSC-1 (LL320AP). An affinity-purified polyclonal antibody
against apical BSC-1 in the TAL has previously been charac-
terized (11, 23, 39).

α-ENaC (Q3560–2 and 92472). An affinity-purified polyclo-
nal antibody and polyclonal immunoserum against
α-ENaC has previously been characterized (18, 34).

β-ENaC (Q3755). An affinity-purified polyclonal antibody
against β-ENaC has previously been characterized (34).

γ-ENaC (LL550). An affinity-purified polyclonal antibody
against γ-ENaC has previously been characterized (18, 34).

Na⁺-K⁺-ATPase. A monoclonal antibody against the α1-
subunit of Na⁺-K⁺-ATPase has previously been characte-
rized (22).

Membrane Fractionation for Immunoblotting

The kidneys from six rats in the control group and seven
rats in the hypokalemia group were used for membrane
fractionation, which was followed by immunoblotting. One
kidney from each rat was used as the total kidney, and the
other kidney was dissected into the cortex/outer stripe of
the outer medulla (OSOM), inner stripe of the outer medulla
(ISOM), and inner medulla (IM). The tissue was homoge-
nized in dissection buffer (0.3 M sucrose, 25 mM imidazole, 1
mM EDTA, pH = 7.2, containing and 8.5 μM leupeptin, 0.4 mM pefabloc, and 1 mM phenylmethylsulfonyl fluoride) using an Ultraturrax T8 homogenizer (IKA Labortechnik), and the homogenate was centrifuged in an Eppendorf 5403 centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria. The supernatant was either used (ISOM) or centrifuged at 200,000 g for 1 h to produce a pellet containing membrane fractions enriched for both plasma membrane and intracellular vesicles (total kidney and cortex/OSOM). Gel samples were made using Laemmli sample buffer containing 2% SDS.

**Electrophoresis and Immunoblotting**

Samples of membrane fractions together with molecular markers were run on 9 or 12% polyacrylamide minigels (Bio-Rad Mini Protean II). For each gel, an identical gel was run in parallel and subjected to Coomassie staining to ensure identical loading. The other gel was subjected to immunoblotting. After transfer by electroelution to nitrocellulose membranes, blots were blocked with 5% milk in 80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, and 0.1% Tween 20, pH = 7.5 (PBS-T) for 1 h and incubated overnight at 4°C with affinity-purified primary antibodies (NHE3, TSC, BSC-1, Na+-K+-ATPase and α-, β-, and γ-ENaC, respectively). The labeling was visualized with horseradish peroxidase-conjugated secondary antibodies (PO447 or PO448, diluted 1:3,000, DAKO, Glostrup, Denmark) using the enhanced chemiluminescence system (ECL; Amersham).

**Quantitation of Expression Levels of Sodium Transporters**

ECL films with bands within the linear range were scanned using an AGFA scanner (Arcus II) and Microsoft software to control the scanner. The labeling density was determined on blots where samples from hypokalemic rats were run on each gel with samples from respective control rats. The labeling density was corrected by densitometry of Coomassie-stained gels.

**Preparation of Tissue for Immunohistochemistry and Immunoelectron Microscopy**

Four hypokalemic and four control rats were anesthetized with halothane inhalation, and their kidneys were fixed by retrograde perfusion through the abdominal aorta with 3% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH = 7.4). The kidneys were used for paraffin embedding for immunohistochemistry. For immunoperoxidase labeling, kidney blocks containing all kidney zones were dehydrated and embedded in paraffin. The paraffin-embedded tissue was cut at 2 μm on a rotary microtome (Leica). The staining was carried out using indirect immunoperoxidase. The sections were dewaxed and rehydrated, and the endogenous peroxidase was blocked by 0.5% H2O2 in absolute methanol for 10 min at room temperature. To reveal antigens, sections were incubated with 1 mmol/l Tris solution (pH 9.0) supplemented with 0.5 mM EGTA and heated using a microwave oven for 10 min. Nonspecific binding of immunoglobulin was prevented by incubating the sections in 50 mM NH4Cl for 30 min, followed by blocking in PBS supplemented with 1% BSA, 0.05% saponin, and 0.2% gelatin. Sections were incubated overnight at 4°C with primary antibodies diluted in PBS supplemented with 0.1% BSA and 0.3% Triton-X-100. After a rinsing with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatine for 3 × 10 min, the sections were incubated in horseradish peroxidase-conjugated secondary antibodies (PO448 goat anti-rabbit or PO447 goat anti-mouse, DAKO) diluted 1:200 in PBS supplemented with 0.1% BSA and 0.3% Triton-X-100, followed by incubation with diaminobenzidine. The light microscopy was carried out using a Leica DMRE light microscope.

**Statistical Analyses**

Values are presented as means ± SE. Comparisons between groups were made by unpaired t-test with equal or unequal variances. P values <0.05 were considered significant.

**RESULTS**

**Rats Treated with a Potassium-Deficient Diet Had Low Plasma and Urinary Potassium Levels Along with Polyuria and Decreased Urinary Concentration**

Rats treated with a potassium-deficient diet for 4 days developed significant hypokalemia with a decrease in plasma potassium levels to 2.5 ± 0.04 vs. 3.7 ± 0.06 mmol/l in controls (P < 0.05) (Table 1). Urinary excretion of potassium decreased significantly to 2.7 ± 0.3 vs. 9.5 ± 0.2 μmol/min in controls (P < 0.05), and the fractional excretion of potassium also decreased markedly to 15 ± 2.3 vs. 31 ± 1.1% in controls (P < 0.05). Along with this, the hypokalemic rats developed significant polyuria and increased water intake. In the basal period before treatment with a potassium-deficient diet, urinary output was not different between rats subjected to hypokalemia and controls: 49 ± 4 vs. 45 ± 3 μl·min⁻¹·kg⁻¹ [not significant (NS)]. After 4 days of treatment with a low-potassium diet, urinary output was significantly higher in the hypokalemic rats: 99 ± 10 vs. 53 ± 5 μl·min⁻¹·kg⁻¹ in controls (P < 0.05). In contrast, urinary output was unchanged in control rats. The marked increase in urinary output was also associated with decreased urinary osmolality as well as urinary-to-plasma osmolality ratio (P < 0.05) (Table 1).

**Table 1. Functional data**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls (n = 8)</th>
<th>HK (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt., g</td>
<td>219 ± 2</td>
<td>219 ± 1</td>
</tr>
<tr>
<td>P_{o_{2}m}}, mmol/kgH2O</td>
<td>296 ± 1.2</td>
<td>296 ± 2.0</td>
</tr>
<tr>
<td>P_{Na}, mmol/l</td>
<td>137 ± 0.4</td>
<td>137 ± 0.4</td>
</tr>
<tr>
<td>P_{K}, mmol/l</td>
<td>3.7 ± 0.06</td>
<td>2.5 ± 0.04*</td>
</tr>
<tr>
<td>P_{cr}, μmol/l</td>
<td>18 ± 0.4</td>
<td>24 ± 1.0*a</td>
</tr>
<tr>
<td>P_{urea}, mmol/l</td>
<td>4.0 ± 0.2</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>U_{o_{2}m}}, mmol/kgH2O (n = 12)</td>
<td>1,096 ± 110</td>
<td>530 ± 61*</td>
</tr>
<tr>
<td>U/P_{o_{2}m}}</td>
<td>3.6 ± 0.5</td>
<td>1.8 ± 0.3*</td>
</tr>
<tr>
<td>C_{cr}, μL·min⁻¹·kg⁻¹</td>
<td>8.2 ± 0.3</td>
<td>6.9 ± 0.3*</td>
</tr>
<tr>
<td>V_{H2O}, μL·min⁻¹·kg⁻¹</td>
<td>124 ± 13</td>
<td>54 ± 18*</td>
</tr>
<tr>
<td>U_{Na} × U_{c}, μmol·min⁻¹·kg⁻¹ (n = 12)</td>
<td>6.1 ± 0.2</td>
<td>5.9 ± 0.2</td>
</tr>
<tr>
<td>U_{K} × U_{c}, μmol·min⁻¹·kg⁻¹ (n = 12)</td>
<td>9.5 ± 0.2</td>
<td>2.7 ± 0.3*</td>
</tr>
<tr>
<td>F_{E_{Na}}, %</td>
<td>0.59 ± 0.03</td>
<td>0.56 ± 0.01</td>
</tr>
<tr>
<td>F_{E_{K}}, %</td>
<td>31 ± 1.1</td>
<td>15 ± 2.3*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; HK, hypokalemic; P_{o_{2}m}, plasma osmolality; P_{Na}, plasma Na; P_{K}, plasma K; P_{cr}, plasma creatinine; P_{urea}, plasma urea; (U/P)_{o_{2}m}, urine-to-plasma osmolality ratio; C_{cr}, creatinine clearance; V_{H2O}, solute-free water reabsorption; U_{Na} × U_{c}, rate of urinary Na excretion; U_{K} × U_{c}, rate of urinary K excretion; F_{E_{Na}}, fractional excretion of Na; F_{E_{K}}, fractional excretion of K.

*aP < 0.05 compared with control group.
1), confirming that hypokalemia is associated with reduced urinary concentration.

**Rats Treated with a Potassium-Deficient Diet Had No Changes in Plasma and Urinary Sodium Levels**

In contrast to the marked alteration in water and potassium balance, no significant changes in plasma and urinary sodium levels were observed in the hypokalemic rats. Plasma sodium levels were unchanged in both groups of rats: 137 ± 0.4 mmol/l in hypokalemic rats and 137 ± 0.4 mmol/l in controls. Urinary excretion of sodium was 5.9 ± 0.2 μmol·kg⁻¹·min⁻¹ in hypokalemic rats vs. 6.1 ± 0.2 μmol·kg⁻¹·min⁻¹ in controls (not significant), and the fractional excretion of sodium was also unchanged (0.56 ± 0.01% in hypokalemic rats vs. 0.59 ± 0.03% in controls, NS). These data are summarized in Table 1.

**Rats Treated with a Potassium-Deficient Diet Had No Changes in Blood pH or HCO₃⁻ Levels**

No significant changes were observed in the acid-base status of the hypokalemic rats. Blood pH (7.42 ± 0.01 in hypokalemic rats vs. 7.43 ± 0.1 in controls) and blood HCO₃⁻ (27.4 ± 0.2 in hypokalemic rats vs. 26.9 ± 0.3 mmol/l in controls) were not significantly different between hypokalemic and control rats. Also, no changes in blood P CO₂ (5.7 ± 0.1 kPa in hypokalemic rats vs. 5.5 ± 0.1 kPa in controls, NS) or urine pH levels (7.11 ± 0.21 in hypokalemic rats vs. 7.16 ± 0.26 in controls, NS) were observed.

**Rats with Hypokalemia Had Low Plasma Aldosterone Levels**

An identical set of hypokalemic and control rats was set up for measurement of plasma aldosterone levels, and there were significantly lower plasma aldosterone levels in the hypokalemic rats compared with controls: 140 ± 12 vs. 234 ± 40 pg/ml (P < 0.05).

Table 2. Densitometric analysis of immunoblots from HK and Con rats

<table>
<thead>
<tr>
<th>Transporter</th>
<th>n</th>
<th>Total Kidney</th>
<th>Cortex + OSOM</th>
<th>ISOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>6</td>
<td>100 ± 5</td>
<td>100 ± 15</td>
<td></td>
</tr>
<tr>
<td>HK</td>
<td>7</td>
<td>441 ± 37*</td>
<td>736 ± 45*</td>
<td>210 ± 28*</td>
</tr>
<tr>
<td>TSC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>6</td>
<td>100 ± 9</td>
<td>100 ± 20</td>
<td></td>
</tr>
<tr>
<td>HK</td>
<td>7</td>
<td>58 ± 7*</td>
<td>46 ± 6*</td>
<td></td>
</tr>
<tr>
<td>BSC-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>6</td>
<td>100 ± 16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK</td>
<td>7</td>
<td>57 ± 6*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺-K⁺-ATPase</td>
<td></td>
<td>100 ± 2</td>
<td>100 ± 3</td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>6</td>
<td>128 ± 3*</td>
<td>113 ± 2*</td>
<td>87 ± 4</td>
</tr>
<tr>
<td>HK</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as a percentage. n, No. of rats; Con, controls; HK, hypokalemic; OSOM and ISOM, outer stripe and inner stripe of outer medulla, respectively; NHE3, type 3 Na⁺/H⁺ exchange; TSC, thiazide-sensitive Na⁺-Cl⁻ cotransporter; BSC-1, type 3 bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter. *P < 0.05 vs. Con rats.

Fig. 1. Semiquantitative immunoblotting of membrane fractions of total kidney (A), cortex/outer stripe of the outer medulla (OSOM; C), and inner stripe of the outer medulla (ISOM; E). A, C, and E: immunoblots reacted with anti-type 3 Na⁺/H⁺ exchanger (NHE3) antibody revealed a single ~87 kDa band. B, D, and F: densitometric analyses revealed a marked increase in total kidney (B), cortex/OSOM (D), and ISOM (F) NHE3 abundance in kidneys from hypokalemic rats compared with control rats. Values are means ± SE. n, No. of rats. *P < 0.05.
Rats with Hypokalemia Had a Marked Increase in Renal NHE3 Abundance

Semiquantitative immunoblotting of kidneys from hypokalemic rats showed a significant increase in the protein abundance of NHE3 in the kidney cortex/OSOM (736 ± 45 vs. 100 ± 15%, P < 0.05) and in the ISOM (210 ± 28 vs. 100 ± 25%, P < 0.05) (Fig. 1, C–F, Table 2). Moreover, there was a parallel increase in total kidney NHE3 abundance in hypokalemic rats: 441 ± 37 vs. 100 ± 5% (P < 0.05) (Fig. 1, A and B, Table 2). Immunohistochemical analysis also showed profoundly increased NHE3 labeling in kidneys from hypokalemic rats (Fig. 2). In control rats, anti-NHE3 antibody labeled the apical plasma membrane domains of the proximal tubules, whereas basolateral plasma membranes were unlabeled (Fig. 2A). Furthermore, an intense labeling of the apical plasma membrane domains of cortical medullary TAL was also seen (Fig. 2, C and E). In kidney sections of hypokalemic rats, NHE3 labeling was significantly increased in the apical part of the proximal tubules in the cortex (Fig. 2B, arrow) and OSOM (not shown) as well as in the apical part of the TAL in the cortex (Fig. 2D, arrow), OSOM (not shown), and ISOM (Fig. 2F, arrow).

In contrast to the dramatic upregulation of the NHE3, small but significant changes were observed in the expression levels of the Na⁺-K⁺-ATPase, with an upregulation in total kidney to 126 ± 3 vs. 100 ± 2% (P < 0.05) (Fig. 3, A and B, Table 2) and in the cortex/OSOM to 113 ± 2 vs. 100 ± 3% in control (P < 0.05) (Fig. 3, C and D, Table 2). No significant changes were observed in ISOM (87 ± 4 vs. 100 ± 8%, NS) (Fig. 3, E and F, Table 2).

Rats with Hypokalemia Had a Marked Decrease in Renal BSC-1 and TSC Abundance

Figure 4 shows immunoblots of BSC-1 using membrane preparations from ISOM in hypokalemic rats and control rats. Affinity-purified anti-BSC-1 antibody recognized a broad band of molecular mass 146–176...
kDa centered at ∼161 kDa (Fig. 4), consistent with previous observations (11). In contrast to the significantly increased kidney levels of NHE3 in hypokalemic rats, densitometric analysis revealed a marked decrease in BSC-1 abundance in ISOM from rats with hypokalemia corresponding to 57 ± 6% of levels in control rats (100 ± 16%, *P < 0.05) (Fig. 4B, Table 2). Immunohistochemistry also showed a marked down-regulation of BSC-1 in ISOM (Fig. 5). In control rats, distinct BSC-1 labeling was seen in the apical part of TAL cells (Fig. 5, A, C, and E), whereas in hypokalemic rats the labeling in the apical part of TAL cells was much weaker (Fig. 5, B, D, and F).

As shown in Fig. 6, affinity-purified anti-TSC antibody recognized a broad band centered at ∼165 kDa. TSC abundance in the membrane fractions of cortex/OSOM corresponded to 46 ± 6% of levels in control rats (100 ± 20%, *P < 0.05) (Fig. 6D, Table 2). Consistent with this, a significant decrease in TSC abundance was also seen in membrane fractions from total kidney: 58 ± 7 vs. 100 ± 9% in controls (*P < 0.05) (Fig. 6B). This was confirmed by immunohistochemistry showing reduced labeling in the distal convoluted tubule of kidneys from potassium-deficient rats (Fig. 7, B and D), whereas control rats demonstrated intense TSC labeling in the apical part of the distal convoluted tubules (Fig. 7, A and B). The downregulation of TSC is

Fig. 4. Semiquantitative immunoblotting of membrane fractions of ISOM. A: immunoblots reacted with anti-type 1 bumetanide-sensitive cotransporter (BSC-1) antibody revealed a broad band of molecular mass (146–176 kDa) centered at ∼161 kDa. B: densitometric analyses revealed that BSC-1 abundance in thick ascending limb in ISOM is significantly decreased in hypokalemic rats compared with control rats. Values are means ± SE. n, No. of rats. *P < 0.05.
consistent with the observed reduction in plasma aldosterone.

**Rats with Hypokalemia Had a Marked Decrease in Renal α-, β-, and γ-ENaC Abundance**

As shown in Fig. 8, semiquantitative immunoblotting demonstrated that α-ENaC abundance was markedly decreased in membrane fractions from total kidneys (61 ± 5%, P < 0.05) and cortex/OSOM (61 ± 4%, P < 0.05) of hypokalemic rats (Table 3). Moreover, densitometric analysis revealed a significant reduction of β- and γ-ENaC abundance (Figs. 9 and 10, respectively). β-ENaC abundance was reduced to 45 ± 5 vs. 100 ± 8% in total kidney and 42 ± 5 vs. 100 ± 16% in cortex/OSOM (Fig. 9, Table 3). γ-ENaC abundance was reduced to 60 ± 3 vs. 100 ± 11% in total kidney and 86 ± 3 vs. 100 ± 5% in cortex/OSOM (Fig. 10, Table 3). Consistent with this, immunoperoxidase microscopy confirmed the reduction of α-, β-, and γ-ENaC labeling in collecting duct principal cells of hypokalemic rats (Fig. 11). In control rats, α-ENaC labeling was present in the apical domains of the collecting duct principal cells (arrow, Fig. 11A), consistent with previous observations (18). In contrast, there was only little α-ENaC labeling in kidneys from hypokalemic rats (Fig. 11B, arrow). β- and γ-ENaC immunolabeling was localized at the apical plasma membrane domains and dispersed in the cytoplasm in collecting duct principal cells from control rats (Fig. 11, C and E, arrows), as demonstrated earlier (18). In kidneys from hypokalemic rats, there was a marked overall reduction in the labeling of β- and γ-ENaC (Fig. 11, D and F, arrows), consistent with immunoblotting and densitometry.

**DISCUSSION**

The present study demonstrated that experimentally induced hypokalemia is associated with substantial upregulation of NHE3 in the proximal tubules and TALs of rat kidney. In contrast, the abundance of the BSC-1 in the TALs was markedly decreased. This suggests that downregulation of BSC-1 is likely to play a role in the urinary concentration defect associated with
hypokalemia, in addition to the previously demonstrated downregulation of AQP2. Moreover, the protein abundances of TSC and ENaC subunits were markedly reduced in hypokalemia. Specifically, reduced TSC and α-ENaC abundances are consistent with the reduced plasma aldosterone levels associated with hypokalemia. The reduced abundances of TSC and ENaC may well contribute to the urinary concentration defect associated with hypokalemia.

Increased NHE3 Abundance in Proximal Tubules and TALs in Hypokalemia

NHE3, which is expressed apically in proximal tubule cells, is believed to be the protein that mediates transcellular sodium and HCO₃⁻ reabsorption, in conjunction with the Na⁺-K⁺-ATPase and the electrogenic Na⁺-HCO₃⁻ cotransporter (NBC1) in the basolateral plasma membrane. NHE3 is also expressed apically in the cortical and medullary thick ascending limb cells, suggesting that the Na⁺/H⁺ exchanger may also play an important role in Na⁺ and HCO₃⁻ reabsorption in this segment. The present study revealed that NHE3 abundance in the proximal tubules and TALs of rat kidney was significantly increased. Consistent with the increased abundance of NHE3 in the proximal tubules, micropuncture studies have previously shown that potassium depletion in rats was associated with substantially reduced lithium clearance, indicating that sodium and water delivery to the end of the proximal tubules was reduced (43), possibly due to increased proximal tubular reabsorption in hypokalemia (47).

These findings are also supported by a previous observation that chronic potassium depletion was associated with increased apical Na⁺/H⁺ exchange and basolateral Na⁺-HCO₃⁻ cotransport (44). The increased apical NHE3 expression may mediate the apical component of the increased transcellular sodium transport in the proximal tubule. Transgenic mice lacking NHE3 survive but have significantly reduced HCO₃⁻ and isosmotic sodium and water reabsorption in the proximal tubules (41, 48). This condition was associated with marked compensatory upregulation of several other major sodium transporters along the nephron. This provides evidence of an important role for NHE3 in sodium reabsorption in the proximal nephron (7). Thus it is likely that NHE3 upregulation in hypokalemia represents a compensatory response preventing sodium loss due to the consequences of reduced aldosterone levels and reduced urinary concentrating capacity.

Hypokalemia also stimulates H⁺ secretion and HCO₃⁻ reabsorption (8, 9). NHE3 is thought to play an important role in the process of HCO₃⁻ reabsorption in addition to sodium reabsorption (2, 6, 41, 48). This suggests that the upregulation of NHE3 in hypokalemia may be associated not only with increased sodium reabsorption but also with increased HCO₃⁻ reabsorption. A number of studies have previously investigated changes in NHE3 mRNA and protein expression in response to chronic or subchronic hypokalemia (32). Laghmani et al. (32) recently reported that NHE3 protein and mRNA expression were not altered in rats fed a potassium-deficient diet for 2 wk compared with controls rats, and NHE3 mRNA expression was reduced when the rats were fed a potassium-deficient diet for 5 wk.

Upregulation of NHE3 in volume-contracted states has also been demonstrated (14). Hypokalemic rats in the present study had polyuria, polydipsia, and reduced urine osmolality. Although there were virtually no differences in plasma sodium and plasma osmolalities between hypokalemic and control rats, it cannot be excluded that the upregulation of NHE3 may be indirectly caused by a direct effect of volume depletion.

Finally, the previous demonstration that potassium depletion leads to intracellular acidification (1) should be mentioned, and it is possible that this could increase
the abundance of the NHE3 to maintain intracellular pH levels. Although this remains speculative, it would be consistent with previous observations that enhanced renal NHE3 protein abundance is associated with chronic metabolic acidosis (23, 28). Moreover, Amemiya et al. (4) demonstrated that exposure of opossum kidney cells (clone P) that express NHE3 to low extracellular potassium caused a transient decrease in intracellular pH, which was followed by activation and upregulation of NHE3 after 24 h of incubation. Thus it is possible that the observed increase in the expression of NHE3 in hypokalemic rats could be caused by, or is associated with, indirect effects of intracellular acidification related to hypokalemia. This may contribute to the maintenance of intracellular pH levels, in conjunction with NBC1 in the proximal tubules (37, 40) and the electroneutral NBCn1 in TALs (30).

Decreased Abundance of BSC-1 in TALs in Hypokalemia

BSC-1 (15, 51), which is localized at the apical plasma membrane domains of medullary and cortical TAL segments, is significantly decreased in hypokalemic rats compared with control rats. This decrease was observed in membrane fractions prepared from total kidney and cortex/OSOM. The table below summarizes the densitometric analysis of immunoblots from HK and Con rats.

### Table 3. Densitometric analysis of immunoblots from HK and Con rats

<table>
<thead>
<tr>
<th>Subtype</th>
<th>n</th>
<th>Total Kidney</th>
<th>Cortex + OSOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ENaC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>6</td>
<td>100 ± 9</td>
<td>100 ± 10</td>
</tr>
<tr>
<td>HK</td>
<td>7</td>
<td>61 ± 5*</td>
<td>61 ± 4*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>6</td>
<td>100 ± 8</td>
<td>100 ± 16</td>
</tr>
<tr>
<td>HK</td>
<td>7</td>
<td>45 ± 5*</td>
<td>42 ± 5*</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Con</td>
<td>6</td>
<td>100 ± 11</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>HK</td>
<td>7</td>
<td>60 ± 3*</td>
<td>86 ± 3*</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as a percentage. n, No. of rats; Con, controls; HK, hypokalemic; ENaC, epithelial sodium channel. *P < 0.05 vs. Con rats.
Takahashi et al. (45) recently demonstrated the importance of BSC-1 for overall NaCl and fluid reabsorption. They showed that transgenic mice lacking BSC-1 suffer from dehydration and have renal insufficiency and die within 2 wk after birth, with signs of severe volume depletion. This is thought to be due to salt wasting by TAL malabsorption (45). Interestingly, a small fraction of these mice could be kept alive by the administration of indomethacin, although as adults they exhibited severe polyuria (45). Thus BSC-1 is important for urinary concentration. This supports our conclusion that the decrease in BSC-1 abundance observed in response to hypokalemia in this study plays a significant role in the hypokalemia-induced polyuria and urinary concentrating defect.

Because the abundance of the vasopressin-regulated AQP2 in kidney was also decreased in hypokalemic rats...
(33), it is possible that the vasopressin-adenyl cyclase pathway is affected in both the TAL and in the collecting duct in response to hypokalemia. Indeed, Kim et al. (26) demonstrated that the increase in cAMP levels as well as adenylate cyclase activity in the isolated inner medullary collecting duct from hypokalemic rats were significantly blunted in response to vasopressin. This and the observation that BSC-1 expression is regulated by vasopressin are also consistent with the view that downregulation of BSC-1 is likely to play a role in the urinary concentration defects associated with hypokalemia (in addition to the downregulation of AQP2 abundance). However, further studies are needed to determine the underlying mechanisms involved in the downregulation of TAL sodium transporter BSC-1 expression in hypokalemia-induced nephrogenic diabetes insipidus.

Decreased Abundance of TSC and α, β-, and γ-ENaC in Hypokalemic Rats

The distal convoluted tubules reabsorb sodium mainly through apical TSC (25). ENaC subunits are known to be expressed in the connecting tubule, cortical, outer, and inner medullary collecting duct (18). TSC and ENaC mediate sodium reabsorption, which is finely regulated by the hormones that control sodium balance, e.g., the mineralocorticoid aldosterone. Consistent with this, it has recently been suggested that aldosterone stimulates sodium reabsorption by the kidney in part through its action to increase the abundance of the TSC in the distal convoluted tubules (25) and the α-subunit ENaC in collecting duct principal cells (34). Moreover, it has been demonstrated that the effect of aldosterone to stimulate renal sodium retention can be overridden by the phenomenon of aldosterone escape, resulting in a marked decrease in TSC abundance in the distal convoluted tubules, but not α-ENaC in the collecting duct (50).

Mice lacking TSC have only mild symptoms of disturbances of fluid and sodium homeostasis, but renal handling of magnesium and calcium are altered, as observed in Gitelman’s syndrome (42).

In this study, hypokalemia in rats was associated with a significantly decreased abundance of TSC and all three ENaC subunits in the kidney. Because potassium depletion is known to reduce plasma aldosterone levels (27), which was confirmed in this study, it is likely that the decreased abundance of TSC and ENaC subunits is directly caused by low plasma aldosterone levels. It is likely that the reduced abundances of TSC and ENaC subunits may contribute to the reduced urinary concentration mechanism.

Summary

Hypokalemia is associated with substantial upregulation of NHE3 in the proximal tubules and TALs of rat kidney, whereas BSC-1 in the TALs, TSC in the distal...
convoluted tubules, and ENaC subunits in the connecting segments and collecting ducts are downregulated. The decreased expression of BSC-1, TSC, and ENaC may contribute to the urinary concentrating defect in hypokalemia, in addition to the previously demonstrated decreased AQP2 levels. In contrast, upregulation of NHE3 is likely to be compensatory to prevent urinary sodium loss and/or to maintain intracellular pH levels.

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