Altered expression of renal acid-base transporters in rats with lithium-induced NDI

Young-Hee Kim,1,2 Tae-Hwan Kwon,1,3 Birgitte M. Christensen,1,2 Jakob Nielsen,1,2 Susan M. Wall,4 Kirsten M. Madsen,5 Jørgen Frøkiær,1,6 and Søren Nielsen1,2

1The Water and Salt Research Center, Institutes of 2Anatomy and 6Experimental Clinical Research, University of Aarhus, DK-8000 Aarhus C, Denmark; 3Department of Physiology, School of Medicine, Dongguk University, Kyungju 780-714, Korea; 4Renal Division, Emory University School of Medicine, Georgia 30322; 5Department of Medicine, University of Florida, Gainesville, Florida 32610-0215

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Kim, Young-Hee, Tae-Hwan Kwon, Birgitte M. Christensen, Jakob Nielsen, Susan M. Wall, Kirsten M. Madsen, Jørgen Frøkiær, and Søren Nielsen. Altered expression of renal acid-base transporters in rats with lithium-induced NDI. Am J Physiol Renal Physiol 285: F1244–F1257, 2003. First published August 26, 2003; 10.1152/ajprenal.00176.2003.—Prolonged lithium treatment of humans and rodents often results in hyperchloremic metabolic acidosis. This is thought to be caused by diminished net H+ secretion and/or excessive back-diffusion of acid equivalents. To explore whether lithium treatment is associated with changes in the expression of key renal acid-base transporters, semiquantitative immunoblotting and immunocytochemistry were performed using kidneys from lithium-treated (n = 6) and control (n = 6) rats. Rats treated with lithium for 28 days showed decreased urine pH, whereas no significant differences in blood pH and plasma HCO3− levels were observed. Immunoblot analysis revealed that lithium treatment induced a significant increase in the expression of the H+ -ATPase (B3-subunit) in cortex (190 ± 18%) and inner stripe of the outer medulla (190 ± 9%) and a dramatic increase in inner medulla (900 ± 104%) in parallel to an increase in the expression of type 1 anion exchanger (400 ± 40%). This was confirmed by immunocytochemistry and immuno-electron microscopy, which also revealed increased density of intercalated cells. Moreover, immunohistochemistry and immunocytochemistry revealed a significant increase in the expression of the type 1 electronegative Na+/HCO3− cotransporter (NBC) in cortex (200 ± 23%) and of the electroneutral NBCn1 in inner stripe of the outer medulla (250 ± 54%). In contrast, there were no changes in the expression of Na+/H+ exchanger-3 or of the Cl−/HCO3− exchanger pendrin. These results demonstrate that the expression of specific renal acid-base transporters is markedly altered in response to long-term lithium treatment. This is likely to represent direct or compensatory effects to increase the capacity for HCO3− reabsorption, NH4+ reabsorption, and proton secretion to prevent the development of systemic metabolic acidosis.

Lithium is an important drug in treating bipolar affective disorders. However, lithium treatment is associated with various serious side effects. In particular, nephrogenic diabetes insipidus (NDI; i.e., a pronounced vasopressin-resistant polyuria and inability to concentrate urine) is the most common side effect in patients (42). Moreover, chronic lithium treatment has been known to be associated with hyperchloremic metabolic acidosis and distal renal tubular acidosis (13, 31, 38). The underlying mechanisms for the impaired urinary acidification in the distal nephron and collecting duct after lithium treatment are unknown. It has been suggested, however, that the impaired acidification may be the result of (1) an inability to generate a maximum pH gradient across the distal nephron for H+ secretion (gradient defect; see Ref. 31); (2) a primary impairment of the proton pump in the collecting duct (secretory defect; see Ref. 18); or (3) an unfavorable effect of lithium on the electrical gradient promoting H+ secretion (voltage-dependent defect; see Ref. 6). The molecular basis for the above, including potential changes in the expression of key renal acid-base transporters, is currently unknown.

Renal regulation of acid-base balance involves H+ secretion and HCO3− reabsorption along the nephron and collecting duct and is mediated by several key acid-base transporters. In the proximal tubule, H+ secretion and HCO3− reabsorption occur by the apical Na+/H+ exchanger-3 (NHE3) in conjunction with the basolateral electronegative Na+/HCO3− cotransporter (NBC1), which mediates electrogenic HCO3− efflux. The functional importance of NHE3 has been demonstrated in NHE3 null mice, showing that proximal tubule HCO3− reabsorption is significantly reduced up to ~60% (40). In the thick ascending limb (TAL) of Henle’s loop, 10–15% of the filtered HCO3− is reabsorbed by combined function of the apical NHE3 and basolateral anion exchanger type 2 (AE2; see Ref. 3). Moreover, the electroneutral NBCn1 (10) is localized in the basolateral plasma membrane of the medullary thick ascending limb (mTAL) cells and in the intercalated cells of the outer and inner medullary collecting duct (IMCD; see Ref. 44). The observed

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increase in NBCn1 protein expression in mTAL in response to chronic NH4Cl loading (25) suggests that NBCn1 may play a role in transporting HCO3− into the cells across the basolateral plasma membrane, thereby increasing the reabsorption of NH4+ in the mTAL cells as an adaptive response to chronic metabolic acidosis (16).

The final regulation of acid-base balance takes place in the collecting ducts, where the intercalated cells are located. In the kidney cortex, at least two types of intercalated cells, type A and type B cells, exist in the connecting tubule (CNT) and cortical collecting duct (CCD; see Refs. 2, 8, and 43). Type A intercalated cells secrete H+ via a vacuolar H+-ATPase that is located in the apical plasma membrane and apical tubulovesicles and reabsorb HCO3− through a band 3-like Cl−/HCO3− exchanger, AE1, located in the basolateral membrane (2, 8, 22, 41). Type B intercalated cells have these transport processes in opposite membranes. They secrete HCO3− via a novel anion exchanger, pendrin, located in the apical plasma membrane and subapical vesicles (23, 39) and have the vacuolar H+-ATPase in the basolateral plasma membrane and intracellular vesicles (2, 22). Recently, mutations in ATP6B1, the gene encoding the B1-subunit of the apical H+-ATPase mediating distal nephron acid secretion, were reported to cause distal renal tubular acidosis with sensorineural deafness in human patients (20). Moreover, recent studies have demonstrated the presence of the newly discovered anion exchanger, pendrin, and the vacuolar H+-ATPase in the apical plasma membrane and subapical intracellular vesicles of a third type of intercalated cells, the non-A-non-B intercalated cells (23, 45). The function of this cell type has not been established. As the collecting ducts descend from the cortex to outer and inner medulla, type B cells gradually disappear, and only type A-like intercalated cells and principal cells remain in the inner stripe of the outer medulla (ISOM) and initial part of the inner medulla. Intercalated cells are not found in the terminal part of the inner medulla under normal conditions (28).

The purpose of this study was to explore whether there are significant changes in the protein expression of key renal acid-base transporters associated with long-term lithium treatment. The clarification of altered expression of acid-base transporters will help explore the possibility that these changes are directly involved in the development of metabolic acidosis and impaired urinary acidification in response to chronic lithium treatment or in the compensatory process. Thus we examined the expression of H+-ATPase (B1-subunit), band 3-like Cl−/HCO3− exchanger 1 (AE1), pendrin, NBCn3, electronegic NBC1, and electroneutral NBCn1 in kidneys of control rats and chronic lithium-treated rats by semiquantitative immunoblotting and high-resolution immunocytochemistry using specific antibodies.

METHOD

Experimental Animals

Studies were performed in adult male Sprague-Dawley rats (M & B Centre, Ejby, Denmark). The rats were maintained on a standard rodent diet (Altromin, Lage, Germany) with free access to water before the experiments.

Protocol 1. Lithium chloride was added to the chow to give a concentration of 40 mmol lithium/kg dry food, as previously described (26). We administered 40 mmol lithium/kg dry food for 4 wk to experimental rats without giving access to NaCl blocks. All rats had free access to water. For the final 10 days, both lithium-treated rats and control rats were maintained in metabolic cages to receive the same amount of food (a constant daily sodium intake) and for measurements of daily water intake and urine output. Urine volume, urine pH, osmolality, creatinine, and sodium and potassium concentrations were measured. Arterial blood was collected from the abdominal aorta at the time of death for measurements of blood pH, blood HCO3− concentration, and total CO2 levels. Plasma was also collected to determine concentrations of sodium, potassium, urea nitrogen, creatinine, and osmolality.

Protocol 2. To establish whether lithium treatment is associated with a defect of urinary acidification, we induced acute metabolic acidosis by gavage of 3.5 mmol NH4Cl/100 g body wt in both control rats and 4 wk lithium-treated rats (40 mmol lithium/kg dry food for 4 wk). The animals were maintained in metabolic cages for 5 days after NH4Cl loading to collect urine and measure urine pH. The arterial blood was collected from the abdominal aorta for blood-gas analysis at the time of death.

Primary Antibodies

For semiquantitative immunoblots and immunocytochemistry, we used the following polyclonal antibodies to several key acid-base transporters, which have been well characterized in previous studies: B1-subunit of vacuolar type H+-ATPase (46), band 3-like Cl−/HCO3− exchanger AE1 (kindly provided by Dr. Philip S. Low, Purdue University, West Lafayette, IN), anion exchanger pendrin (23), rat kidney electronegic NBC1 (30), electroneutral Na+/HCO3− cotransporter NBCn1 (25, 44), and Na+/H+ exchanger NHE3 (21).

Preparation of Protein for Immunoblotting

All rats were killed under light halothane anesthesia, and right kidneys were rapidly removed and processed for immunoblotting. The kidneys were dissected into cortex, ISOM, initial one-third of the inner medulla, and terminal two-thirds of the inner medulla and homogenized in dissection buffer (0.3 M sucrose, 25 mM imidazole, and 1 mM EDTA, pH 7.2, containing 8.5 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride) using an ultraturrax T8 homogenizer (IKA Labortechnik, Staufen, Germany). This homogenate was centrifuged in an Eppendorf centrifuge at 4,000 g for 15 min at 4°C to remove whole cells, nuclei, and mitochondria, and the protein concentration was determined by BSA assay (Pierce, Rockford, IL). Gel samples were made by using Laemmli sample buffer (containing 2% SDS) to this supernatant.

Electrophoresis and Immunoblotting

Protein samples of homogenates from each region of the kidney were run on 9 or 12% SDS-polyacrylamide minigels (Bio-Rad Mini Protean III). For each gel, an identical gel was run in parallel and subjected to Coomassie blue staining to
ensure identical loading. Proteins were transferred to nitrocellulose paper by electrophoresis, blocked with 5% milk in 80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 0.1 M NaCl, and 0.1% Tween 20, pH 7.5, for 1 h, and incubated overnight at 4°C with primary antibody. The labeling was visualized with horseradish peroxidase-conjugated secondary antibodies (P448; Dako, Glostrup, Denmark) using the enhanced chemiluminescence system (Amersham Biosciences, Little Chalfont, UK). Enhanced chemiluminescence films with bands were scanned within the linear range using an AGFA scanner (ARCUS II) and Corel Photopaint Software to control the scanner. The labeling density was corrected by densitometry of the Coomassie blue-stained gels.

**Immunocytochemistry**

Rats were anesthetized with halothane inhalation, and the kidneys (n = 6 in lithium-treated rats; n = 6 in control rats) were fixed by retrograde perfusion via the abdominal aorta with cold PBS for 15 s, followed by perfusion with 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 min. The kidneys were removed and cut into 1- to 2-mm-thick slices that were fixed additionally by immersion in the same fixative for 1 h at 4°C. Sections of the tissue were either (1) cryoprotected overnight in 2.3 M sucrose and freeze substituted for immunoelectron microscopy or (2) dehydrated in a graded series of ethanol and xylene for paraffin embedding. Immunoperoxidase labeling was either performed with a preembedding method (pendrin) or postembedding method (H⁻-ATPase, AE1, electrongenic NBCn1, electroneutral NBCn1, NHE3, and AE2). For the preembedding method, 50-μm-thick vibratome sections were washed with 50 mM NH₄Cl in PBS, and, before incubation with anti-pendrin antibodies, the tissue sections were incubated for 3 h with PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin (solution A). Tissue sections were then incubated overnight at 4°C with anti-pendrin antibodies in PBS containing 1% BSA (solution B). After washes with solution A, the sections were incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (P448 1:200; Dako) diluted in solution B. The tissues were then rinsed, first in solution A and subsequently in 0.05 M Tris buffer (pH 7.6). For the detection of horseradish peroxidase, the sections were incubated in 0.1% diaminobenzidine in 0.05 M Tris buffer for 5 min, after which H₂O₂ was added. After the sections were washed with 0.05 M Tris buffer, they were dehydrated in a graded series of ethanol and embedded in Epon. Semithin sections were stained with hematoxylin. For immunoperoxidase labeling using postembedding methods, paraffin-embedded tissues were sectioned (~2 μm) on a microtome (Leica), and the sections were dewaxed in xylene and rehydrated in a graded series of ethanol. The sections were incubated with 0.5% H₂O₂ in absolute methanol for 10 min at room temperature to block the endogenous peroxidase activity. To reveal antigens, sections were heated with 0.5 mM EGTA in 1 mM Tris (pH 9.0) for 10 min in a microwave oven. The sections were treated with 50 mM NH₄Cl for 30 min and blocking solution (1% BSA, 0.05% saponin, 0.2% gelatin, and PBS) to prevent nonspecific staining and then incubated overnight at 4°C with primary antibodies diluted in dilution buffer (0.1% BSA, 0.3% Triton X-100, and PBS). After being rinsed three times with PBS supplemented with 0.1% BSA, 0.05% saponin, and 0.2% gelatin for 10 min, sections were incubated in horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (1:200; Dako). The microscopic examinations were carried out using a Leica DMRE light microscope.

**Immunoelectron Microscopy**

Paraformaldehyde-fixed and frozen tissues were freeze substituted in a Reichert APS freeze substitution unit. Samples were sequentially equilibrated over 3 days in methanol containing 0.5% uranyl acetate at temperatures gradually raised from −80 to −70°C, rinsed in pure methanol for 24 h while the temperature was increased from −70 to −45°C, and infiltrated with graded Locrycyl HM 20 and methanol solutions (1:1, 2:1) and pure Locrycyl HM 20 before ultraviolet polymerization for 2 days at −45°C and 2 days at 0°C. For immunogold labeling, Locrycyl HM 20 sections were pre-treated with a saturated solution of NaOH in absolute ethanol (2–3 s), rinsed with water, and preincubated for 10 min with 0.1% sodium borohydride and 50 mM glycine in 0.5 M Tris, pH 7.4, containing 0.1% Triton X-100. Sections were rinsed in 1% BSA and 5 M Tris, pH 7.4, containing 0.1% Triton X-100 and incubated overnight at 4°C with antibody against H⁻-ATPase (B₁-subunit, 1:100). After three rinses, the sections were incubated with goat anti-rabbit IgG conjugated to 10-nm colloidal gold particles (1:50; BioCell Research Laboratories, Cardiff, UK) for 1 h at room temperature. The sections were stained with uranyl acetate and lead citrate before examination in a Philips Morgagni electron microscope.

**Statistical Analyses**

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

**RESULTS**

**Increased Urine Output and Decreased Urine pH in Rats Treated with Lithium**

The rats fed lithium-containing food for 4 wk had significantly increased urine output compared with control rats: 159 ± 12 in lithium-treated rats vs. 18 ± 2 ml/day in control rats (P < 0.05). In parallel, the increase in urine output was accompanied by a significant increase in water intake (Table 1). Consistent with this, lithium-treated rats had a markedly lower urine osmolality and a lower urine-to-plasma osmolality ratio, indicating that chronic lithium treatment was associated with a severe impairment of urinary concentrating ability (Table 1). Chronic lithium-treated rats exhibited a significantly lower urine pH compared with control rats (7.5 ± 0.1 vs. 7.8 ± 0.1 in control rats, P < 0.05), whereas blood pH and HCO₃⁻ levels were not changed (Table 1).

**Impaired Urinary Acid Excretion in Lithium-Treated Rats in Response to an Acute Acid Loading**

As shown in Fig. 1, the decrease in urine pH was significantly blunted and delayed in lithium-treated rats in response to an acute acid loading (NH₄Cl loading) compared with control rats (6.1 ± 0.15 vs. 5.6 ± 0.06 at 7 h after NH₄Cl loading, P < 0.05). Moreover, there was a 5-h delay in reaching the lowest urine pH in the lithium-treated rats. At day 3 after an acute acid loading, the urine pH of both groups returned to baseline before the acid loading. However, blood HCO₃⁻ concentration (27.2 ± 0.23 vs. 29.7 ± 0.79, P < 0.05)
Increased Expression of \( H^+ \)-ATPase in Collecting Duct of Rats Treated with Lithium

Semi-quantitative immunoblots revealed that lithium treatment for 4 wk was associated with a marked increase in \( H^+ \)-ATPase (B1-subunit) expression in cortex, inner stripe of the outer medulla, and initial part of the inner medulla (Fig. 2 and Table 2). The increase in protein expression of \( H^+ \)-ATPase was most predominant in the initial part of the inner medulla, which showed a ninefold increase by densitometric analysis.

Immunoperoxidase microscopy in the kidney cortex of both control and lithium-treated rats revealed that the \( H^+ \)-ATPase (B1-subunit) was expressed selectively in the intercalated cells of CNTs and CCDs, and there was no immunolabeling in the proximal tubules (Fig. 3, A and B), consistent with previous evidence (46). In lithium-treated rats, the \( H^+ \)-ATPase-labeled intercalated cells were generally flattened, and the overall density of intercalated cells appeared to be increased in the CNT and CCD (Fig. 3, A and B). Higher magnification revealed that, in the diluted CNT of lithium-treated rats (Fig. 3D), the intercalated cells appeared to have an altered morphology, being flattened and exhibiting a less polarized immunolabeling of \( H^+ \)-ATPase compared with that of control rats (Fig. 3C).

In the control rats, \( H^+ \)-ATPase-positive cells decreased in number as the collecting duct descends from the cortex and outer medulla toward the tip of the renal papilla, and there were only a few \( H^+ \)-ATPase-labeled cells in the initial part of the IMCD (Fig. 4A). In contrast, in lithium-treated rats, the density of \( H^+ \)-ATPase-positive intercalated cells was dramatically increased in the initial part of the IMCD (Fig. 4B) and \( H^+ \)-ATPase-positive intercalated cells were also observed in the middle part of the inner medulla (not shown). Higher magnification revealed that the intercalated cells observed in the initial (Fig. 4, C and D) and middle (not shown) part of the inner medulla exhibited apical immunolabeling of \( H^+ \)-ATPase in both control (Fig. 4C) and lithium-treated (Fig. 4D) rats. In addition, as shown by immunoelectron microscopy in Fig. 5, the intercalated cells in the IMCD of the lithium-treated rats showed the ultrastructural characteristics of type A intercalated cells, which have apical microvilli and are rich in mitochondria in the cytoplasm. At higher magnification, labeling of \( H^+ \)-ATPase was observed in both the apical plasma membrane and apical vesicles (Fig. 5, inset), with the same subcellular distribution as in the controls (not shown). Notably, intercalated cells were often seen adjacent to other intercalated cells (Figs. 3–6) in lithium-treated rats.

Table 1. Parameters of renal function

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Lithium</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>326 ± 4</td>
<td>253 ± 2†</td>
</tr>
<tr>
<td>Water intake, ml/day</td>
<td>19 ± 2</td>
<td>154 ± 13†</td>
</tr>
<tr>
<td>Urine output, ml/day</td>
<td>18 ± 2</td>
<td>159 ± 12†</td>
</tr>
<tr>
<td>Urine pH</td>
<td>7.8 ± 0.1</td>
<td>7.5 ± 0.1</td>
</tr>
<tr>
<td>( U_{\text{osm}}, \text{mosmol/kgH}_2\text{O} )</td>
<td>1,290 ± 111</td>
<td>108 ± 19†</td>
</tr>
<tr>
<td>( U_{\text{Na}}, \text{mosmol/kgH}_2\text{O} )</td>
<td>286 ± 7</td>
<td>304 ± 6</td>
</tr>
<tr>
<td>( U/P_{\text{Na}} )</td>
<td>4.5 ± 0.4</td>
<td>0.4 ± 0.1†</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.3 ± 0.02</td>
<td>7.3 ± 0.01</td>
</tr>
<tr>
<td>Plasma ( \text{HCO}_3^- ), mmol/l</td>
<td>23.6 ± 0.8</td>
<td>23.5 ± 1.1</td>
</tr>
<tr>
<td>( P_{\text{Na}}, \text{mmol/l} )</td>
<td>134.8 ± 3.4</td>
<td>144.2 ± 3.5</td>
</tr>
<tr>
<td>( P_{\text{K}}, \text{mmol/l} )</td>
<td>3.7 ± 0.1</td>
<td>4.1 ± 0.1†</td>
</tr>
<tr>
<td>( P_{\text{creat}}, \mu\text{mol/l} )</td>
<td>30 ± 2.1</td>
<td>39 ± 1.5</td>
</tr>
<tr>
<td>( U_{\text{Na}}, \mu\text{mol/l} )</td>
<td>113 ± 8</td>
<td>18 ± 1.7†</td>
</tr>
<tr>
<td>( U_{\text{K}}, \mu\text{mol/day} )</td>
<td>1.6 ± 0.06</td>
<td>4.4 ± 0.19*</td>
</tr>
<tr>
<td>( U_{\text{K}}, \mu\text{mol/day} )</td>
<td>3.5 ± 0.14</td>
<td>4.12 ± 0.20</td>
</tr>
<tr>
<td>( C_{\text{cr}}, \text{ml/min} )</td>
<td>1.7 ± 0.11</td>
<td>1.6 ± 0.12</td>
</tr>
<tr>
<td>( F_{\text{Ex}}, % )</td>
<td>0.5 ± 0.03</td>
<td>0.8 ± 0.07*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. \( U_{\text{osm}}, \) urine osmolality; \( U_{\text{Na}}, \) plasma osmolality; \( U/P_{\text{Na}}, \) urine-to-plasma osmolality ratio; \( P_{\text{Na}}, \) plasma sodium; \( P_{\text{K}}, \) plasma potassium; \( P_{\text{creat}}, \) plasma creatinine; \( U_{\text{Na}}/V, \) rate of urinary sodium excretion; \( U_{\text{K}}/V, \) rate of urinary potassium excretion; \( C_{\text{cr}}, \) creatinine clearance; \( F_{\text{Ex}}, \) fractional excretion of sodium. † \( P < 0.05 \) or ‡ \( P < 0.01 \), lithium-treated rats compared with control rats.

and total CO₂ (27.2 ± 0.23 vs. 29.7 ± 0.79, \( P < 0.05 \)) were still significantly lower in lithium-treated rats compared with controls at day 5 after an acute acid loading. Thus the results demonstrate a significantly reduced ability to excrete an acid load in lithium-treated rats.
Fig. 2. Semiquantitative immunoblotting using proteins from kidney cortex (A and B), inner stripe of the outer medulla (C and D), and initial part of the inner medulla (E and F) of control (Con) and lithium-treated (Li) rats. Immunoblots were reacted with antibody against the B1-subunit H^+ATPase and revealed a band of ~56 kDa. There was a marked increase in the protein expression the H^+ATPase in all kidney zones examined in lithium-treated rats. Especially in the initial part of the inner medulla (E and F), the abundance of H^+ATPase expression was increased dramatically, corresponding to a 9-fold increase in the lithium-treated rats compared with controls. *P < 0.05.

**Increased Expression of AE1 in Collecting Duct of Rats Treated with Lithium**

Consistent with an increase in the expression of H^+ATPase, the expression of the band 3-like Cl^-/HCO_3^- exchanger AE1 was also significantly increased in the inner medulla of kidneys from lithium-treated rats compared with those from controls (Fig. 6, A and B; Table 2). Immunocytochemistry demonstrated strong labeling of AE1 in the basolateral plasma membrane of intercalated cells, consistent with previous studies. Importantly, the density of AE1-positive cells was increased markedly in inner medulla of kidneys from lithium-treated rats (Fig. 6D) compared with controls (Fig. 6C). Thus there is a parallel change in the expression pattern of AE1 and the B1-subunit of the H^+ATPase.

**Table 2. Changes in the protein expression levels of acid-base transporters in the kidneys of lithium-treated rats**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>n</th>
<th>Lithium</th>
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<tbody>
<tr>
<td>Cortex</td>
<td></td>
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<tr>
<td>H^+ATPase</td>
<td>1.0 ± 0.14</td>
<td>6</td>
<td>1.9 ± 0.18*</td>
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<tr>
<td>Pendrin</td>
<td>1.0 ± 0.09</td>
<td>6</td>
<td>1.0 ± 0.07</td>
<td>5</td>
</tr>
<tr>
<td>Electrogenic NBC1</td>
<td>1.0 ± 0.07</td>
<td>6</td>
<td>2.0 ± 0.23*</td>
<td>6</td>
</tr>
<tr>
<td>NHE3</td>
<td>1.0 ± 0.08</td>
<td>6</td>
<td>1.4 ± 0.22</td>
<td>6</td>
</tr>
<tr>
<td>ISOM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H^+ATPase</td>
<td>1.0 ± 0.23</td>
<td>6</td>
<td>1.9 ± 0.09*</td>
<td>5</td>
</tr>
<tr>
<td>Electroneutral NBCn1</td>
<td>1.0 ± 0.28</td>
<td>6</td>
<td>2.5 ± 0.54*</td>
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<tr>
<td>NHE3</td>
<td>1.0 ± 0.16</td>
<td>6</td>
<td>0.9 ± 0.14</td>
<td>6</td>
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<tr>
<td>Initial IM</td>
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<td></td>
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<tr>
<td>H^+ATPase</td>
<td>1.0 ± 0.37</td>
<td>6</td>
<td>9.0 ± 1.04*</td>
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<tr>
<td>AE1</td>
<td>1.0 ± 0.40</td>
<td>5</td>
<td>4.0 ± 0.42*</td>
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<tr>
<td>Electroneutral NBCn1</td>
<td>1.0 ± 0.09</td>
<td>6</td>
<td>0.9 ± 0.22</td>
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</tr>
</tbody>
</table>

Values are means ± SE; n, no. of rats. ISOM, inner stripe of the outer medulla; IM, inner medulla; NBC1, electrogenic Na^+-HCO_3^- cotransporter; NHE3, type 3 Na^+ /H^+ exchanger; AE1, band 3-like Cl^-/HCO_3^- exchanger. For densitometry of immunoblots, the labeling densities were calculated as a fraction of the mean value from control rats. *Significant difference, P < 0.05.

**Unchanged Expression of Pendrin in Response to Lithium Treatment**

In contrast to the marked increase of H^+ATPase in response to chronic lithium treatment, the expression of pendrin, a novel Cl^-/HCO_3^- exchanger, was unchanged in the cortex [102 ± 7 vs. 100 ± 9% in control rats, not significant (NS), Fig. 7, A and B; Table 2]. Immunocytochemistry revealed that pendrin was expressed in the apical plasma membrane domains of type B intercalated cells and non-A-non-B cells (Fig. 7, C and D), as previously demonstrated (23). There were no changes in pendrin immunoreactivity in lithium-treated rats (Fig. 7D) compared with control rats (Fig. 7C).

**Increased Expression of Electrogenic NBC1 in Kidney Cortex in Rats Treated with Lithium**

Semiquantitative immunoblots of the outer cortex of chronic lithium-treated and control rats revealed a marked increase in electrogenic NBC1 expression [238 ± 29 vs. 100 ± 12% in control rats, P < 0.05, Fig. 8 and Table 2].

Immunocytochemistry showed abundant NBC1 immunolabeling in the basolateral plasma membrane of the proximal tubule in kidneys of both control (Fig. 9A) and lithium-treated (Fig. 9B) rats. In the controls, the most abundant immunolabeling of NBC1 was observed in the S1 segments connected to glomeruli, and weaker labeling was seen in S2 segments (Fig. 9A). However, in lithium-treated rats (Fig. 9B), the immunolabeling of electrogenic NBC1 was increased in both S1 and S2 segments of proximal tubules (Fig. 9, C and D). In contrast, there was no NBC1 labeling in the straight part (S3 segment) of the proximal tubules in either control or lithium-treated rats (not shown).

**Unchanged Expression of NHE3 in Response to Lithium Treatment**

Semiquantitative immunoblots revealed no significant changes in NHE3 expression in membrane frac-
tions prepared from the cortex (140 ± 22 vs. 100 ± 8% in controls, NS, Table 2) or the ISOM (90 ± 14 vs. 100 ± 16% in controls, NS, Table 2) of lithium-treated rat kidneys.

Increased Expression of Electroneutral NBCn1 in the ISOM of Rats Treated with Lithium

Semiquantitative immunoblots of the ISOM of lithium-treated and control rats showed a significant increase in the protein expression of the electroneutral NBCn1 (250 ± 54 vs. 100 ± 28% in controls, \( P < 0.05 \), Fig. 10, A and B). In contrast, the expression of electroneutral NBCn1 in the inner medulla was unchanged (88 ± 22 vs. 100 ± 9% in controls, NS, Table 2).

Immunocytochemistry revealed that the electroneutral NBCn1 was localized in the basolateral plasma membrane of mTAL cells of Henle's loop (Fig. 10, C and D) and intercalated cells of outer (Fig. 10, C and D) and inner (not shown) medullary collecting duct, as previously shown (44). In lithium-treated rats (Fig. 10D), the NBCn1 immunolabeling density was increased in both mTAL cells and in intercalated cells of the outer medullary collecting duct. Moreover, the density of intercalated cells in the ISOM appeared to be increased in the lithium-treated rats (Fig. 10D) compared with control rats (Fig. 10A).

DISCUSSION

In this study, we demonstrated that the protein expression levels of key renal acid-base transporters were altered markedly in rats with lithium-induced NDI. After chronic lithium treatment, there was a dramatic upregulation of the expression of the \( \text{B}_1 \)-subunit of the \( \text{H}^+ \)-ATPase in both the cortex and medulla of lithium-treated rats. In particular, the expression was upregulated dramatically (9-fold) in the inner medulla. This was paralleled by an increase in the density of \( \text{H}^+ \)-ATPase-positive intercalated cells.
lated cells. The mechanisms and signaling cascades involved in the induction of increased expression of H⁻/H₁₁₀₀₁-ATPase and the increase in the prevalence of intercalated cells are not known. However, it is possible that these changes (31, 38) represent an adaptive response to decreased H⁻/H₁₁₀₀₁-secretion in the collecting duct, which was reported in previous studies and believed to be the result of a decrease in the electric gradient for H⁻/H₁₁₀₀₁-secretion (voltage-dependent defect) or a decrease in the H⁺-ATPase activity (secretory defect) in lithium-treated rats (6, 12). This study also demonstrates a significant increase in the expression of the electrogenic NBC1 in the proximal tubule and the electroneutral NBCn1 in mTAL. The electrogenic NBC1 is involved in the reabsorption of sodium and HCO₃⁻ in the proximal tubule, whereas the electroneutral NBCn1 may play a role in NH₄⁺ reabsorption in the mTAL and facilitate the urinary excretion of total ammonia (NH₄⁺/NH₃). It is therefore conceivable that the increased expression of these transporters represents an adaptive change to the metabolic acidosis that has been reported to be associated with lithium-induced NDI (13, 31, 38). Moreover, the increased expression of electrogenic NBC1 could play a role in sodium reabsorption in the proximal tubule in response to lithium-induced urinary sodium wasting. There were no signs of systemic metabolic acidosis in the low-dose-lithium-treated animals in the present study, which would be consistent with efficient compensatory mechanisms operating. The results also demonstrate that the expression of NHE3 and pendrin was unaltered. The significance of this remains to be established.

Impaired Urinary Acid Excretion in Lithium-Treated Rats in Response to Acute Acid Loading

In the present study, low-dose-lithium-treated rats exhibited no signs of metabolic acidosis, since blood pH

Fig. 4. Light micrographs of 2-μm paraffin sections of the inner stripe of the outer medulla (ISOM) and the initial part of the inner medulla (IM) from kidneys of control (A and C) and lithium-treated (B and D) rats. The sections were labeled for H⁻-ATPase (B₁-subunit). In the control rat kidneys, the number of intercalated cells labeled with H⁻-ATPase decreased in the initial part of the inner medullary collecting duct (IMCDi; A). In contrast, there was a marked increase in the number of H⁺-ATPase-labeled intercalated cells in IMCDi of the lithium-treated rats (B). Higher magnification revealed distinct apical labeling of H⁻-ATPase in both controls (C) and lithium-treated rats. However, the density of labeled cells markedly increased in the kidneys of lithium-treated rats (D). Bars = 50 μm (A and B) and 10 μm (C and D).
and plasma HCO₃⁻ levels were unchanged. However, the expression of key acid-base transporters in kidney was altered markedly, indicating that an efficient compensatory mechanism was in operation to maintain blood pH. In protocol 2, we examined how the kidneys of lithium-treated rats and control rats handle an acute acid loading. The results showed that urinary excretion of acid was blunted markedly in lithium-treated rats compared with controls, consistent with previous reports (6, 12). Thus the increase in the expression of the H⁺-ATPase (B₁-subunit) protein and increased density of H⁺ pump-expressing cells is likely to represent a
compensatory process in response to the blunted urinary acid excretion, potentially indicating a decrease in H^+\text{-}ATPase activity. This is discussed in more detail below.

**Increased Expression of H^+\text{-}ATPase in Collecting Duct of Rats Treated with Lithium**

Immunoblotting analyses revealed that lithium-treated rats had dramatically increased H^+\text{-}ATPase expression in the cortex (1.9-fold) and outer (1.9-fold) and inner (9-fold) medulla. Moreover, immunocytochemistry revealed that the density of H^+\text{-}ATPase-positive intercalated cells was increased markedly in the initial and middle part of the inner medulla compared with control rats, as previously reported in the high-dose-lithium-treated rats (11).

Chronic lithium chloride administration in rats has been reported to be associated with hyperchloremic metabolic acidosis and distal renal tubular acidosis (31, 38). The underlying mechanisms of the inability to decrease urine pH appropriately in lithium-treated rats are poorly understood. However, it has been suggested that the distal renal tubular acidosis with lithium treatment might be because of either 1) an inability to generate a maximum pH gradient across the distal nephron for H^+ secretion (gradient defect; see Ref. 31), 2) a primary impairment of the H^+ pump in the collecting duct (secretory defect; see Ref. 18), or 3)
a detrimental effect of lithium on the electric gradient favoring H⁺ secretion by inhibition of sodium reabsorption (voltage-dependent defect; see Ref. 6). It was initially assumed to be the result of a gradient defect, because of an excessive back-diffusion of acid, as had been suggested for amphotericin B (6). However, studies of turtle urinary bladder revealed that, under open-circuit conditions, lithium caused a significant decrease in transepithelial potential difference by inhibiting sodium transport and, hence, caused a significant decrease in H⁺ secretion (6). In contrast, restoration of the potential difference to control levels in the presence of lithium caused a return of H⁺ secretion to the control value (6). This finding therefore strongly indicates that lithium causes an acidification defect, at least in part, by inhibiting sodium transport, thereby decreasing the favorable electric gradient for H⁺ secretion (a voltage-dependent impairment of proton secretion). Consistent with this, it is noteworthy that, in the collecting duct, sodium reabsorption is mainly regulated by the apical epithelial sodium channel (ENaC) under the control of vasopressin and aldosterone (29). ENaC is also characterized by a high selectivity for both sodium and lithium (33). We have demonstrated recently that lithium-induced NDI (using a protocol identical to the one used in the present study) is associated with a decrease in tubular reabsorption of filtered sodium and an increase of urinary sodium excretion (26), which was interpreted to be mainly attributed to dysregulation of ENaC subunits by both aldosterone and vasopressin (32). A reduction in sodium reabsorption through impairment of ENaC function in the collecting ducts could potentially decrease H⁺ secretion in chronic lithium-treated rats by decreasing the lumen negative transepithelial potential (27).

In addition, there is evidence that lithium has a direct inhibitory effect on the H⁺ pump. Studies by Dafnis et al. (12) demonstrated that lithium inhibits H⁺-ATPase activity in both the cortical and outer medullary collecting duct after in vivo and in vitro administration. In the present study, urine pH was not significantly decreased in lithium-treated rats compared with controls in response to acute NH₄Cl loading. This indicates that the urinary excretion of acid is blunted markedly in rats with prolonged lithium treatment. However, H⁺-ATPase (B₁-subunit) protein expression was increased significantly. Therefore, it can be spec-

Fig. 9. Immunohistochemical localization of the electronegic Na⁺-HCO₃ cotransporter, NBC1, using a 2-µm paraffin section from control (A and C) and lithium-treated (B and D) rat kidneys. Immunoreactivity of NBC1 was observed in the basolateral membrane of both S1 and S2 segments of the proximal tubules, with more intense labeling in the S1 segment in both control and lithium-treated rats. There was an increase in the intensity of NBC1 immunolabeling in lithium-treated rat kidneys (B and D). Bars = 100 µm (A and B) and 40 µm (C and D).
ulated that the significantly increased H\(^+\)-ATPase (B1-subunit) protein expression represents a compensation in response to distal renal tubular acidosis induced either by reduced lumen negative transepithelial potential or decreased H\(^+\)-ATPase activity. The main reason for the increased H\(^+\)-ATPase protein expression in the inner medulla is likely to be because of the striking increase in the number of intercalated cells of the IMCD. The mechanism behind the increased number of intercalated cells in the inner medulla in response to chronic lithium treatment is not known, and further studies are warranted.

**Increased Expression of Electrogenic NBC1 in the Proximal Tubule and Electroneutral NBCn1 in the mTAL**

We demonstrated that the expression of electrogenic NBC1 was increased significantly in kidney cortex of lithium-treated rats. Na\(^+\)-HCO\(_3\) cotransporter was localized initially by functional studies to the basolateral membrane of the proximal tubule where it plays a role in mediating electrogenic HCO\(_3\) efflux (7). Consistent with this, recent immunocytochemical analyses demonstrated that electrogenic NBC1 is present at the basolateral plasma membrane domains of predominantly S1 and S2 segments of proximal tubules in rat kidney (37). This indicates that NBC1 mediates basolateral proximal tubule HCO\(_3\) efflux, and later studies in humans with mutations in NBC1 support this view (19). Several previous studies demonstrated that systemic pH levels play an important role in the regulation of HCO\(_3\) reabsorption in the proximal tubule (4). In vitro microperfusion studies in the proximal tubule of rat kidney have shown that the activity of the basolaterally expressed Na\(^+\)-HCO\(_3\) cotransporter is up-regulated in response to metabolic acidosis (34). Thus it is possible that the increased expression of the electrogenic NBC1 in this study may serve to compensate for a systemic metabolic acidosis potentially associated with chronic lithium administration. However, the regulation of NBC1 in the proximal tubule is not well understood, and our previous study (25) demonstrated that renal protein expression of NBC1 was not altered significantly in rats with chronic metabolic acidosis induced by oral NH\(_4\)Cl loading. This finding is also consistent with a previous study demonstrating unchanged expression of renal cortical NBC1 mRNA in rats with NH\(_4\)Cl-induced metabolic acidosis (9). Therefore, the functional increase of the NBC1 in response to metabolic acidosis may be the result of posttranslational regulation.

One possible explanation for the increased expression of NBC1 may be that NBC1 could play a role in supporting sodium reabsorption in the proximal tubule in response to the sodium wasting associated with lithium-induced NDI. Lithium treatment is associated with increased renal sodium excretion in addition to the severe urinary concentrating defects despite the
activated renin-angiotensin-aldosterone system and high plasma vasopressin level (5, 15). Because ANG II is also known to stimulate the activity of basolateral Na\textsuperscript{+}-HCO\textsubscript{3}\textsuperscript{−} cotransport in the renal proximal tubule (36), it is possible that the protein expression of NBC1 could be regulated in response to high ANG II levels and contraction of extracellular fluid volume. Future studies are therefore warranted for defining the role of sodium balance in the regulation of NBC1 protein expression.

In addition to the electronegic NBC1, several other members of the NBC1 family have been identified recently and functionally characterized in vitro. These transporters could potentially play a role in mediating acid-base transport in the kidney or in maintaining intracellular pH levels (1, 10, 35). The electroneutral NBCn1, cloned from rat smooth muscle cells (10), is mainly present in the basolateral membrane of mTAL cells (44). In the present study, semiquantitative immunoblotting and immunocytochemistry revealed that the protein expression of NBCn1 is increased significantly in response to chronic lithium treatment.

It is well known that chronic metabolic acidosis is associated with a number of adaptive changes in renal tubules, which cause a significant increase in urinary net acid excretion and contribute to increased urinary acidification. These adaptations include an enhanced NH\textsubscript{4}\textsuperscript{+} production and secretion in the proximal tubule (17) and increased HCO\textsubscript{3}\textsuperscript{−} reabsorption and net NH\textsubscript{4}\textsuperscript{+} absorption in the mTAL of the rat kidney (16), in addition to an increase in the activity of Na\textsuperscript{+}/H\textsuperscript{+} exchange and HCO\textsubscript{3}\textsuperscript{−} reabsorptive capacity in the proximal tubules (4, 24, 34). We have demonstrated previously that the expression of electroneutral NBCn1 was increased markedly in response to chronic metabolic acidosis induced by oral NH\textsubscript{4}Cl loading (25). We also provided evidence that NBCn1 may play a role in supporting NH\textsubscript{4}\textsuperscript{+} reabsorption in mTAL (25). A major fraction of the NH\textsubscript{4}\textsuperscript{+} is reabsorbed at the TAL mainly through the Na\textsuperscript{+}/K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporter, presumably by substituting for K\textsuperscript{+}. Then, in TAL cells, lipid-soluble NH\textsubscript{3} from NH\textsubscript{4}\textsuperscript{+} can diffuse passively into the interstitium through the basolateral plasma membrane, whereas H\textsuperscript{+} from NH\textsubscript{4}\textsuperscript{+} combines intracellularly with HCO\textsubscript{3}\textsuperscript{−} to form H\textsubscript{2}CO\textsubscript{3} (carbonic acid). This is converted to CO\textsubscript{2} and H\textsubscript{2}O, and CO\textsubscript{2} diffuses into the interstitium where it combines with H\textsubscript{2}O to yield the H\textsuperscript{+} and HCO\textsubscript{3}\textsuperscript{−}. In the medullary interstitium, H\textsuperscript{+} combines with NH\textsubscript{3} to form NH\textsubscript{4}\textsuperscript{+}, whereas HCO\textsubscript{3}\textsuperscript{−} is transported into the cells through the basolateral NBCn1. Thus the increased expression of NBCn1 in ISOM of lithium-treated rats may be speculated to play a role in net NH\textsubscript{4}\textsuperscript{+} absorption in mTAL to increase urinary net acid excretion. Additional studies are required to fully describe the effects of chronic lithium treatment on NH\textsubscript{4}\textsuperscript{+} production and secretion in the proximal tubule.

Unchanged Pendrin Expression in the CNT and CCD

Pendrin, a novel anionic exchanger, is localized in the apical domain of type B intercalated cells and in non-A-non-B intercalated cells in the CNT and CCD of both mouse and rat kidneys (23, 45), and we observed no changes in the pendrin expression in rats with lithium-induced NDI.

The majority of filtered HCO\textsubscript{3}\textsuperscript{−} is reabsorbed by the proximal tubule. However, the final regulation of urine acidification takes place in the collecting duct, which is a site of both reabsorption and secretion of HCO\textsubscript{3}\textsuperscript{−}. In a previous study (14), we have suggested that the fine control of urinary HCO\textsubscript{3}\textsuperscript{−} excretion is achieved by the changes in pendrin expression in the CNT and CCD when changes of HCO\textsubscript{3}\textsuperscript{−} transport are required for acid-base homeostasis, for example, in metabolic acidosis or alkalosis. However, pendrin expression was not altered in rats with lithium treatment, which showed enhanced expression of electronegic NBC1 expression in the proximal tubule. The significance of this remains unestablished.

In summary, the expression of several key renal acid-base transporters was altered significantly in rats with lithium-induced NDI. There was increased expression of the electronegic NBC1 in the proximal tubule and the electroneutral NBCn1 in the mTAL. These changes might increase sodium and HCO\textsubscript{3}\textsuperscript{−} reabsorption in the proximal tubule and allow increased NH\textsubscript{4}\textsuperscript{+} reabsorption in the mTAL, thus serving to compensate for the concomitant extracellular fluid contraction and systemic and/or intracellular metabolic acidosis previously reported in chronic lithium treatment. Moreover, H\textsuperscript{+}-ATPase protein (B1-subunit) expression in the collecting duct and the prevalence of intercalated cells in the IMCD were increased dramatically despite blunted urinary acid excretion in response to acute NH\textsubscript{4}Cl loading. The mechanism of the increase in the prevalence of intercalated cells remains to be established. However, we propose that the increased expression of H\textsuperscript{+}-ATPase may represent an adaptive response to the distal renal tubular acidosis associated with chronic lithium treatment.

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REFERENCES


