Altered expression of renal AQPs and Na\(^+\) transporters in rats with Lithium-induced NDI

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1Department of Cell Biology, Institute of Anatomy, University of Aarhus, DK-8000 Aarhus C; 2School of Biomedical Sciences, University of Leeds, Leeds LS2 9NQ, United Kingdom; 3Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892; and 4Department of Clinical Physiology, Aarhus University Hospital and Institute of Experimental Clinical Research, DK-8200 Aarhus N, Denmark

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Kwon, Tae-Hwan, Ulla H. Laursen, David Marples, Arvid B. Maunsbach, Mark A. Knepper, Jørgen Frøkiaer, and Søren Nielsen. Altered expression of renal AQPs and Na\(^+\) transporters in rats with Lithium-induced NDI. *Am J Physiol Renal Physiol* 279: F552–F564, 2000.—Lithium (Li) treatment is often associated with nephrogenic diabetes insipidus (NDI). The changes in whole kidney expression of aquaporin-1 (AQP1), -2, and -3 as well as Na-K-ATPase, type 3 Na/H exchanger (NHE3), type 2 Na-Pi cotransporter (NaPi-2), type 1 bumetanide-sensitive Na-K-Cl cotransporter (BSC-1), and thiazide-sensitive Na-Cl cotransporter (TSC) were examined in rats treated with Li orally for 4 wk: protocol 1, high doses of Li (high Na\(^+\) intake), and protocol 2, low doses of Li (identical food and normal Na\(^+\) intake in Li-treated and control rats). Both protocols resulted in severe polyuria. Semiquantitative immunoblotting revealed that whole kidney abundance of AQP2 was dramatically reduced to 6% (protocol 1) and 27% (protocol 2) of control levels. In contrast, the abundance of AQP1 was not decreased. Immunoelectron microscopy confirmed the dramatic downregulation of AQP2 and AQP3, whereas AQP4 labeling was not reduced. Li-treated rats had a marked increase in urinary Na\(^+\) excretion in both protocols. However, the expression of several major Na\(^+\) transporters in the proximal tubule, loop of Henle, and distal convoluted tubule was unchanged in protocol 2, whereas in protocol 1 significantly increased NHE3 and BSC-1 expression or reduced NaPi-2 expression was associated with chronic Li treatment. In conclusion, severe downregulation of AQP2 and AQP3 appears to be important for the development of Li-induced polyuria. In contrast, the increased or unchanged expression of NHE3, BSC-1, Na-K-ATPase, and TSC indicates that these Na\(^+\) transporters do not participate in the development of Li-induced polyuria.

aquaporin; nephrogenic diabetes insipidus; sodium transport; urinary concentration mechanism; water transport

LITHIUM HAS BEEN WIDELY USED as a pharmacological agent in psychiatric therapy and is established as the drug of choice for treating bipolar affective disorders (32). However, lithium treatment is associated with various serious side effects (60). In particular, nephrogenic diabetes insipidus (NDI) is commonly associated with lithium treatment (7, 40). Hence, patients on lithium therapy often present polyuria, polydipsia, and reduced ability to concentrate urine.

Urinary concentration and dilution depend on the presence of a discrete segmental distribution of transport properties along the renal tubule, and urinary concentration depends on 1) the hypertonic medullary interstitium, which is generated by active NaCl reabsorption as a consequence of countercurrent multiplication in water-impermeable nephron segments and 2) the high water permeability (constitutive or vasopressin regulated) in other renal tubular segments for osmotic equilibration, which chiefly depends on aquaporins (AQPs). Thus defects in any of these mechanisms would be predicted to be associated with urinary concentrating defects.

AQPs are a family of membrane proteins that function as water channels (46). Aquaporin-1 (AQP1) is highly abundant in the proximal tubule and descending thin limb (48). Several studies have emphasized its critical role in the constitutive water reabsorption in these segments (36). In the kidney collecting duct principal cell, at least three AQPs are known to be expressed, and they participate in vasopressin-regulated water reabsorption. AQP2 is the apical water channel of the principal cells and is the chief target for regulation of collecting duct water permeability by vasopressin (46). Water transport across the basolateral plasma membrane of collecting duct principal cells is thought to be mediated by AQP3 (11) and AQP4 (55). Consistent with this view, transgenic mice lacking AQP3 (33) are severely polyuric, and inner medullary collecting ducts from AQP4-deficient mice have a fourfold reduction in vasopressin-stimulated water permeability (35). A series of studies has demonstrated that altered expression and apical targeting of AQP2 play a significant role in water balance disorders (46). In particular, we have demonstrated that chronic lithium treat-
ment is associated with a marked reduction in AQP2 expression in the inner medullary collecting duct principal cells of rats, concurrent with the development of severe polyuria (40). Thus lithium-induced NDI may be due, at least partly, to a reduction in AQP2 expression. However, it is still unknown whether other AQPs or sodium transporters play important roles in lithium-induced NDI.

Recently, we demonstrated that altered expression of renal sodium transporters is associated with deranged urinary concentration and urinary sodium excretion in several water and sodium balance disorders (13, 29, 31). In proximal tubule, type 3 Na\(^+\)/H\(^+\) exchanger (NHE3) and type 2 sodium-phosphate cotransporter (NaPi-2) are both expressed apically (13, 26, 31, 43), whereas the Na-K-ATPase is heavily expressed in the basolateral membrane of the renal tubule cells (24) and is responsible for sodium reabsorption. The loop of Henle generates a high osmolality in renal medulla by driving the countercurrent multiplier, which is dependent on the NaCl absorption by the thick ascending limb (TAL) (29). The apically expressed Na-K-2Cl cotransporter [rat type 1 bumetanide-sensitive cotransporter (BSC-1 or NKCC2)] and NHE3, in conjunction with basolaterally expressed Na-K-ATPase, are mainly responsible for sodium reabsorption by the TAL (29). In the distal convoluted tubule, the thiazide-sensitive NaCl cotransporter (TSC or NCC) is involved in apical sodium reabsorption.

Therefore, it could be speculated that 1) lithium-induced NDI may be associated with significant alterations in expression of collecting duct water channels AQP3, AQP4, and AQP1 in the proximal tubule and the descending limb of the loop of Henle, where it plays a critical role in the countercurrent multiplication process (in addition to AQP2); 2) lithium-induced NDI may be associated with marked alterations in the expression of major renal sodium transporters. Potentially, reductions in the expressions of sodium transporters may participate in the urinary concentrating defects, or increased expressions might reflect secondary compensatory changes to conserve water and sodium in a pathological state of polyuria and natriuresis; and 3) changes in expression of sodium transporters might influence the transcellular lithium transport and absorption and, hence the intracellular accumulation of lithium.

In the present study, we therefore examined 1) whether chronic lithium treatment affects the expression of whole kidney aquaporin levels or of major renal sodium transporters; and 2) whether the changes in abundance of AQPs and sodium transporters are associated with alterations in urinary concentration and urinary sodium excretion in lithium-treated rats using two different protocols with different sodium intake.

**METHODS**

**Experimental Animals**

Male Munich-Wistar rats were obtained from Mellegaard Breeding Centre (Ejby, Denmark). Rats were maintained on a standard rodent diet (Altromin no. 1324; sodium content 0.2%, Lage, Germany) with free access to water. For lithium treatment, we used two different protocols.

**Protocol 1.** Lithium chloride was added to the chow to give a concentration of 40 or 60 mmol lithium/kg dry food as previously described (40). Rats received food containing 40 mmol lithium/kg dry food for the first 7 days and thereafter 60 mmol lithium/kg dry food for the following 3 wk. Previously, we demonstrated that this protocol resulted in plasma lithium levels at therapeutic levels (0.8–1.3 mM) and minimized the weight loss caused by lithium (40). All rats on lithium treatment had access to a solid NaCl block for supplying adequate NaCl, preventing lithium intoxication and fatal outcome (57), and avoiding negative sodium balance. All rats had free access to water. Water intake was determined continuously for rats on lithium treatment and control rats. Urine output and urine osmolality were determined on the days before death (days 22, 24, and 27), as described below.

**Protocol 2.** To avoid the effects of high sodium intake on the expression of AQPs and sodium transporters in lithium-treated rats by using the classic protocol 1, in protocol 2 we administered only 40 mmol lithium/kg dry food for 4 wk to experimental rats without giving them access to NaCl blocks. All rats had free access to water. This protocol resulted in plasma lithium levels at 0.61 ± 0.02 mM in lithium-induced NDI. Plasma lithium concentrations were measured by flame emission photometry. For the final 10 days, both lithium-treated rats and control rats were maintained in the metabolic cages. They were supplied with the same amount of food per day [lithium-treated rats: 8 g food/100 g body wt (BW) containing 40 mmol lithium/kg dry food; control rats: 8 g food/100 g BW] to give a constant daily sodium intake. The rats were fed once daily in the morning and ate nearly all of the offered food during the course of the day (7.3 ± 0.2 in lithium-treated rats vs. 7.0 ± 0.4 g/100 g BW in control rats, not significant (NS), Table 1). Thus the estimated daily sodium intake in food\(^1\) was virtually identical between rats with lithium-induced NDI and control rats: 630 ± 15 vs. 609 ± 33 μmol·day\(^{-1}\)·100 g BW\(^{-1}\), respectively. However, the measured daily urinary sodium excretion was significantly increased in lithium-treated rats: 676 ± 40 μmol·day\(^{-1}\)·100 g BW\(^{-1}\) (P < 0.05) compared with control rats (468 ± 26 μmol·day\(^{-1}\)·100 g BW\(^{-1}\)). Thus rats treated with a low dose of lithium without additional sodium supplementation revealed a mildly negative sodium balance. This is consistent with previous observations that chronic lithium treatment is associated with significant natriuresis (58, 59).

**Clearance Studies**

**Protocol 1.** The rats were maintained in the metabolic cages at days 22, 24, and 27 after initiation of lithium treatment, allowing quantitative urine collections. 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.

**Protocol 2.** The rats were maintained in the metabolic cages for the last 10 days of lithium treatment, allowing quantitative urine collections. Urine volume, osmolality, creatinine, and sodium and potassium concentration were mea-

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\(^1\) Sample calculation: 1) daily food intake in rats: 7.0 g·day\(^{-1}\)·100 g BW\(^{-1}\); 2) Na\(^+\) content in food (Altromin no. 1324): 0.2%; 3) daily Na\(^+\) intake (7.0 g·day\(^{-1}\)·100 g of BW\(^{-1}\)) × 0.2% = 0.014 g Na\(^+\)·day\(^{-1}\)·100 g BW\(^{-1}\); 4) 1 mmol Na\(^+\) = 23 mg; 5) thus 0.014 g Na\(^+\)·day\(^{-1}\)·100 g BW\(^{-1}\) corresponds to 0.609 μmol Na\(^+\)·day\(^{-1}\)·100 g BW\(^{-1}\).
Membranes, blots were blocked with 5% milk in PBS-T. The other gel was subjected to immunoblotting using a Mini Protean II. For each gel, an identical gel was run in parallel. Samples of membrane fractions from total kidney were run on 12 or 6–16% gradient 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 (56). The other gel was subjected to immunoblotting. After transfer by electroelution to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated with primary antibodies (see below). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, enhanced chemiluminescence system, Amersham Pharmacia Biotech). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, enhanced chemiluminescence system). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, enhanced chemiluminescence system). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, enhanced chemiluminescence system). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, enhanced chemiluminescence system). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, enhanced chemiluminescence system). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, enhanced chemiluminescence system).

**Membrane Fractionation for Immunoblotting**

Whole kidneys were homogenized (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, pH 7.2, containing 8.5 μM leupeptin and 1 mM phenylmethylsulfonyl fluoride) by using an ultraturrax T8 homogenizer (IKA Labortechnik) 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. Gel samples (Laemmli sample buffer containing 2% SDS) were made of this pellet.

**Electrophoresis and Immunoblotting**

Samples of membrane fractions from total kidney were run on 12 or 6–16% gradient polyacrylamide minigels (Bio-Rad Mini Protein II). For each gel, an identical gel was run in parallel and subjected to Coomassie staining to ensure identical loading (56). The other gel was subjected to immunoblotting. After transfer by electroelution to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (80 mM Na₂HPO₄, 20 mM NaH₂PO₄, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 1 h and incubated with primary antibodies (see below). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P447 or P448, diluted 1:3,000, DAKO, Glostrup, Denmark) by using the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Primary Antibodies**

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

- **AQP1 (CHIP serum or LL266AP).** Immune serum or an affinity-purified antibody to AQP1 has previously been characterized (56).
- **AQP2 (LL127 serum or LL127AP).** Immune serum or an affinity-purified antibody to AQP2 has previously been described (9, 40).
- **AQF3 (LL128AP).** An affinity-purified monoclonal antibody to AQF3 has previously been characterized (11).
- **AQF4 (LL182AP).** An affinity-purified polyclonal antibody to AQF4 has previously been characterized (55).
- **NaPi-2 (LL696AP).** An affinity-purified polyclonal antibody to NaPi-2 that was raised against the final 24 amino acids of the COOH-terminal sequence has previously been characterized (5).
- **Na-K-ATPase.** A monoclonal antibody against the alpha-1 subunit of Na-K-ATPase has previously been characterized (24).
- **BSC-1 (LL320AP).** An affinity-purified polyclonal antibody to the apical Na-K-2Cl cotransporter of the TAL has previously been characterized (12, 26, 47).
- **TSC (LL573AP).** An affinity-purified polyclonal antibody to the apical TSC of the distal convoluted tubule has previously been characterized (28).

**Immunocytochemistry**

The kidneys from high doses of lithium-treated rats (n = 3) and control rats (n = 3, protocol 1) were fixed by retrograde perfusion via the abdominal aorta with 2% paraformaldehyde with 0.1% glutaraldehyde in 0.1M sodium cacodylate buffer. Kidneys were postfixed for 1 h, and tissue blocks were infiltrated for 30 min with 2.3 M sucrose containing 2% paraformaldehyde, mounted on holders, and rapidly frozen in liquid nitrogen. For light microscopy, the frozen tissue blocks were cryosectioned (0.8–1 μm, Reichert Ultracut S Cryoultramicrotome; Reichert, Vienna, Austria), and sections were incubated with primary antibodies (see above). The labeling was visualized with horseradish peroxidase-conjugated secondary antibody (P448 1:100, DAKO), followed by incubation with diamobenzidine.

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**Table 1. Functional data of renal function**

<table>
<thead>
<tr>
<th></th>
<th>Lithium (Protocol 1)</th>
<th>Control</th>
<th>Lithium (Protocol 2)</th>
<th>Control</th>
</tr>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>BW, g</td>
<td>260 ± 8*</td>
<td>359 ± 14</td>
<td>350 ± 6*</td>
<td>387 ± 6</td>
</tr>
<tr>
<td>Food intake, g/100 g BW</td>
<td>ND</td>
<td>ND</td>
<td>7.3 ± 0.2</td>
<td>7.0 ± 0.4</td>
</tr>
<tr>
<td>Urine output, μl·min⁻¹·kg⁻¹</td>
<td>449 ± 53†</td>
<td>28 ± 4</td>
<td>168 ± 33*</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>U Na⁺, mmol/kgH₂O</td>
<td>150 ± 8*</td>
<td>1385 ± 263</td>
<td>340 ± 57*</td>
<td>1374 ± 141</td>
</tr>
<tr>
<td>P Na⁺, mmol/kgH₂O</td>
<td>303 ± 1.1</td>
<td>309 ± 2.9</td>
<td>296 ± 1.2*</td>
<td>303 ± 1.1</td>
</tr>
<tr>
<td>(U/P) Na⁺, μmol·min⁻¹·100 g BW</td>
<td>0.5 ± 0.03*</td>
<td>4.5 ± 0.8</td>
<td>1.2 ± 0.2*</td>
<td>4.5 ± 0.5</td>
</tr>
<tr>
<td>T'H₂O, μl·min⁻¹·kg⁻¹</td>
<td>-224 ± 38*</td>
<td>92 ± 15</td>
<td>-6 ± 29*</td>
<td>96 ± 2</td>
</tr>
<tr>
<td>U Na⁺, mmol/l</td>
<td>27 ± 4*</td>
<td>143 ± 19</td>
<td>32 ± 5*</td>
<td>117 ± 8</td>
</tr>
<tr>
<td>U Na⁺, V, μmol·min⁻¹</td>
<td>1.25 ± 0.25*</td>
<td>0.37 ± 0.04</td>
<td>0.47 ± 0.03*</td>
<td>0.32 ± 0.02</td>
</tr>
<tr>
<td>FE Na⁺, %</td>
<td>1 ± 0.2</td>
<td>0.6 ± 0.06</td>
<td>0.6 ± 0.2*</td>
<td>0.4 ± 0.02</td>
</tr>
<tr>
<td>P Na⁺, mmol/l</td>
<td>139 ± 0.4</td>
<td>140 ± 0.6</td>
<td>137 ± 0.4</td>
<td>140 ± 0.4</td>
</tr>
<tr>
<td>P K⁺, mmol/l</td>
<td>5 ± 0.2</td>
<td>4.6 ± 0.1</td>
<td>5.4 ± 0.2*</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>P ara nitrogen, mmol/l</td>
<td>4.4 ± 0.2*</td>
<td>6 ± 0.2</td>
<td>6.0 ± 0.3*</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>P creat, μmol/l</td>
<td>32 ± 1*</td>
<td>38 ± 0.7</td>
<td>39.2 ± 1.5</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>C creat, ml/min</td>
<td>2.3 ± 0.3</td>
<td>1.7 ± 0.2</td>
<td>2.1 ± 0.2</td>
<td>2.5 ± 0.1</td>
</tr>
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</table>

Values are means ± SE. BW, body wt; U Na⁺, urine osmolality; (U/P) Na⁺, urine-to-plasma osmolality ratio; T'H₂O, solute-free water reabsorption; U Na⁺, V, rate of urinary sodium excretion; FE Na⁺, fractional excretion of sodium; P Na⁺, plasma sodium; P K⁺, plasma potassium; P ara creatinine; C creat, creatinine clearance; ND, not determined. *P < 0.05 or †P < 0.01 when lithium-treated rats are compared with control rats.
RENAL AQPs AND Na⁺ TRANSPORTERS IN Li-INDUCED NDI

Immunoelectron Microscopy

The frozen samples were freeze-substituted in a Reichert autofreeze-substitution unit (Reichert) (42, 47, 48). Briefly, the samples were sequentially equilibrated over 3 days in methanol containing 0.5% uranyl acetate at temperatures gradually increasing from −80 to −70°C, and then they were rinsed in pure methanol for 24 h while the temperature was increased from −70 to −45°C. At −45°C, the samples were infiltrated with Lowicryl HM20 and methanol at 1:1, 2:1, and, finally, pure Lowicryl HM20 before ultraviolet polymerization for 2 days at −45°C and 2 days at 0°C. For electron microscopy immunlabeling was performed on ultrathin Lowicryl HM20 sections (60–80 nm), which were incubated overnight at 4°C with primary antibodies. The labeling was visualized with goat-anti-rabbit IgG conjugated to 10-nm colloidal gold particles (GAR:EM10, BioCell Research Laboratories, Cardiff, UK) diluted 1:50. The sections were stained with uranyl acetate and lead citrate before examination in Philips CM100 or 208 electron microscopes.

Statistical Analyses

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

RESULTS

Chronic Lithium Treatment was Associated with Altered Renal Water and Sodium Handling

The rats fed with lithium-containing food for 4 wk by using both protocols had significantly increased urine output compared with control rats; protocol 1: 449 ± 53 in lithium-treated rats vs. 28 ± 4 μl.min⁻¹.kg⁻¹ in control rats at day 27 after onset of lithium treatment, P < 0.01 (Fig. 1A) and protocol 2: 168 ± 33 in lithium-treated rats vs. 29 ± 3 μl.min⁻¹.kg⁻¹ in control rats at day 27, P < 0.01 (Fig. 1C). In parallel, increase in urine output was accompanied by a significant increase in water intake (Fig. 1, B and D). Consistent with this, lithium-treated rats had markedly lower urine osmolality, lower urine-to-plasma osmolality ratio, and impaired solute-free water reabsorption (T²H₂O), indicating that chronic lithium treatment is associated with a severe impairment of urinary concentration (Table 1).

Chronic lithium treatment with free access to a solid NaCl block (protocol 1) caused a significant increase in rate of urinary sodium excretion: 1.3 ± 0.3 in lithium-treated rats vs. 0.4 ± 0.04 μmol.min⁻¹.100 g of BW⁻¹ in control rats, at day 27, P < 0.05 (Table 1). Rats on chronic lithium treatment, having the same sodium intake as control rats (protocol 2, 630 ± 13 in lithium-treated rats vs. 609 ± 33 μmol.day⁻¹.100 g BW⁻¹ in control rats, NS), had an increase in the rate of urinary sodium excretion, but the increase was less marked: 0.47 ± 0.03 in lithium-treated rats (676 ± 40 μmol.day⁻¹.100 g BW⁻¹) vs. 0.32 ± 0.02 μmol.min⁻¹.100 g BW⁻¹ in control rats (468 ± 26 μmol.day⁻¹.100 g BW⁻¹) at day 27, P < 0.05 (Table 1). This shows that chronic lithium treatment is associated with significant natriuresis, consistent with previous observations (58, 59).

Changes in Whole Kidney Abundance of AQP2, AQP3, and AQP1 in Response to Chronic Lithium Treatment

Previously, we demonstrated that chronic lithium treatment is associated with a marked reduction in AQP2 expression in the inner medullary collecting duct principal cells in rats, concurrent with the development of severe polyuria (40). To test whether changes in expression of other AQPs play an important role in the etiology of lithium-induced NDI, semiquantitative immunoblots were performed to examine the whole kidney abundance of AQP3 and AQP1, as well as AQP2 in both protocols. Consistent with the previous findings of a marked downregulation of AQP2 in kidney inner

Fig. 1. Urine output (A and C) and water intake (B and D) at the end of the experimental protocols: lithium-treated rats (n = 8 in protocol 1 and n = 6 in protocol 2, open bars) and control rats (n = 7 in protocol 1 and n = 6 in protocol 2, solid bars). The urine output and water intake in lithium-treated rats was significantly increased at days 22, 24, and 27 after initiation of lithium treatment. **P < 0.01.
medulla (40), chronic lithium treatment using the same protocol (protocol 1) caused a dramatic reduction in whole kidney abundance of AQP2 to 6 ± 1% of control levels (100 ± 11%, P < 0.05, Fig. 2, A and B). Moreover, in rats on low-dose lithium treatment receiving the same amount of daily sodium as the control animals (protocol 2), whole kidney AQP2 abundance was also significantly reduced to 27 ± 5% of control levels (100 ± 10%, P < 0.05, Fig. 2, C and D).

In parallel, whole kidney abundance of the basolateral collecting duct water channel AQP3 was also dramatically reduced, consistent with a significant polyuria and decreased urinary concentration (protocol 1: 6 ± 2% of control levels (100 ± 20%, P < 0.05, Fig. 3, A and B) and protocol 2: 14 ± 4% of control levels (100 ± 9%, P < 0.05, Fig. 3, C and D)). This suggests that reduced expression of both apical AQP2 and basolateral AQP3 may be etiologically important in rats with lithium-induced NDI.

As described previously (14), AQP4 abundance was not sufficient in whole kidney homogenates to assess its renal abundance by using immunoblotting. However, as described below, no major changes in AQP4 expression were observed by immunocytochemistry and immunoelectron microscopy.

In contrast, whole kidney abundance of proximal nephron water channel AQP1 was not reduced by chronic lithium treatment. After 4 wk of lithium treatment, the abundance of AQP1 was maintained at 90 ± 15% of control levels (100 ± 9%, NS, Fig. 4, A and B) in protocol 1 and at 115 ± 38% of control levels (100 ± 21%, NS, Table 2) in protocol 2. Table 2 summarizes the changes in expression of AQPs and sodium transporters.

Changes in the Whole Kidney Abundance of NHE3, NaPi-2, and Na-K-ATPase in Response to Chronic Lithium Treatment

We examined the changes in expression of major renal sodium transporters in lithium-treated rats to clarify the potential roles of sodium transporters in the
etiology of lithium-induced NDI. Semiquantitative immunoblots were made by using antibodies to NHE3 present in the proximal tubule, descending thin limb, and TAL; NaPi-2 in the proximal tubule; and α1-subunit of Na-K-ATPase found in all renal tubule segments.

In proximal tubule and TAL, NHE3 contributes to sodium and bicarbonate reabsorption. Semiquantitative immunoblotting revealed that whole kidney abundance of NHE3 was significantly increased in rats with chronic high doses of lithium treatment (protocol 1) to 185 ± 17% of control levels (100 ± 11%, P < 0.05, Fig. 5, A and B), whereas no significant changes were observed in rats treated with low doses of lithium (protocol 2), whole kidney Na-K-ATPase abundance was not altered (Fig. 7, C and D).

### Changes in Whole Kidney Abundance of BSC-1, and TSC in Response to Chronic Lithium Treatment

The apical Na-K-2Cl cotransporter (BSC-1) is the major transporter for the apical sodium reabsorption by the TAL. In contrast to the markedly reduced expression of the vasopressin-regulated water channels AQP2 and AQP3 in the collecting duct, the abundance of whole kidney BSC-1 levels was significantly increased in rats with chronic high doses of lithium treatment (protocol 1) to 202 ± 40% of control levels (100 ± 19%, P < 0.05, Fig. 8, A and B). In contrast, no changes were observed in rats treated with low doses of lithium (protocol 2), TSC abundance was not significantly changed: 151 ± 16% of control levels (100 ± 21%, NS, Fig. 9, A and D).

### Immunochemistry of AQP3 and AQP4 in Lithium-Treated Rats

Immunoelectron microscopy was performed on ultrathin Lowicryl HM20 sections from cryosubstituted kidney inner medullary tissues from lithium-treated rats (protocol 1) and control rats.

Immunoelectron microscopy demonstrated that AQP2 immunogold labeling in the inner medullary collecting duct principal cells was associated with apical plasma membranes and intracellular vesicles in the apical part of the cells (not shown). In contrast, in rats with lithium-induced NDI, AQP2 labeling in the inner medulla was extremely sparse (not shown), consistent with a previous study (40). The immunogold labeling pattern of AQP3 also confirmed the immunoblotting observations and demonstrated a distinct AQP3 immunogold labeling at the basolateral plasma membrane domains of the collecting duct principal cells in control rats (Fig. 10A), whereas AQP3 labeling was extremely

<p>| Table 2. Changes in the expression levels of renal aquaporins and major sodium transporters in lithium-treated rats |
|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Lithium (Protocol 1)</th>
<th>Control</th>
<th>Lithium (Protocol 2)</th>
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<tbody>
<tr>
<td>n</td>
<td>14</td>
<td>6</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>AQP1</td>
<td>115 ± 15%</td>
<td>115 ± 3%</td>
<td>115 ± 4%</td>
<td>100 ± 14%</td>
</tr>
<tr>
<td>AQP2</td>
<td>27 ± 11%</td>
<td>27 ± 10%</td>
<td>27 ± 10%</td>
<td>35 ± 4%</td>
</tr>
<tr>
<td>AQP3</td>
<td>14 ± 4%</td>
<td>14 ± 4%</td>
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<td>14 ± 4%</td>
</tr>
<tr>
<td>Na-K-ATPase</td>
<td>109 ± 16%</td>
<td>109 ± 8%</td>
<td>109 ± 8%</td>
<td>109 ± 8%</td>
</tr>
<tr>
<td>NHE3</td>
<td>150 ± 20%</td>
<td>150 ± 20%</td>
<td>150 ± 20%</td>
<td>150 ± 20%</td>
</tr>
<tr>
<td>NaPi-2</td>
<td>108 ± 11%</td>
<td>108 ± 11%</td>
<td>108 ± 11%</td>
<td>108 ± 11%</td>
</tr>
<tr>
<td>BSC-1</td>
<td>156 ± 16%</td>
<td>156 ± 16%</td>
<td>156 ± 16%</td>
<td>156 ± 16%</td>
</tr>
<tr>
<td>TSC-1</td>
<td>151 ± 16%</td>
<td>151 ± 16%</td>
<td>151 ± 16%</td>
<td>151 ± 16%</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats; AQP1, AQP2, AQP3: aquaporin-1, -2, -3, respectively; NHE3, type 3 Na/H exchanger; NaPi-2, type 2 Na-Pi cotransporter; BSC-1, type 1 bumetanide-sensitive Na-K-2Cl cotransporter; TSC, thiazide-sensitive Na-CI cotransporter. For densitometry of immunoblots, aquaporins and sodium transporter labeling were calculated as a fraction of the mean value from control rats. *P < 0.05 when lithium-treated rats were compared with control rats.
sparse in rats with lithium-induced NDI (Fig. 10B). In contrast to AQP3, immunogold electron microscopy revealed that there was no major reduction in AQP4 labeling in the basal plasma membrane domains of inner medullary collecting duct principal cells of rats with lithium-induced NDI (Fig. 11, A and B). Consistent with previous findings (40, 23), collecting duct principal cells from rats with lithium-induced NDI revealed a marked hypertrophy and cellular enlargement (Figs. 10B and 11B).

DISCUSSION

We have demonstrated that the expression of collecting duct water channel AQP2 and AQP3 was severely reduced after 4 wk of lithium treatment and that this was associated with an impairment of urinary concentration. In contrast to AQP2 and AQP3, no major reduction in AQP4 expression was observed by immunoelectron microscopy, and the expression of proximal nephron water channel AQP1 was not decreased but was maintained at the control levels in the lithium-induced polyuric condition. Moreover, the expression of several major renal sodium transporters (NHE3, BSC-1, Na-K-ATPase, and TSC) was increased or unchanged, suggesting that altered sodium transporter expression may not be significantly involved in the development of lithium-induced polyuria.

Water and Sodium Balance in Lithium-Treated Rats

Rats treated chronically with lithium in relatively high doses and that had free access to water (with or without access to additional NaCl) showed severe polyuria, hypotonic urine to plasma levels or below, and a very high water intake, consistent with severe NDI and as also seen in severe cases of patients treated with lithium salts. In protocol 1, lithium-treated rats had free access to a NaCl block and thus received a higher daily sodium intake compared with control rats. This supply of sodium along with high doses of lithium was necessary for maintaining these rats during the experiments and for preventing lithium intoxication and fatal outcome. This is consistent with previous observations that high lithium doses could be tolerated only by animals receiving NaCl simultaneously with lithium (50) and that lithium intoxication has been shown to be reversed by administration of sodium (57). Moreover, Shalmi et al. (53) demonstrated that lithium was reabsorbed in the distal nephron of kidney only in sodium-depleted rats and not in the sodium-replete condition.

Fig. 5. Semiquantitative immunoblotting of membrane fractions of whole kidneys. A and C: immunoblots reacted with anti-type 3 Na/H exchanger (NHE3) antibody and revealed a single ~87-kDa band. B and D: densitometric analyses revealed that whole kidney NHE3 abundance was significantly increased in high-dose-lithium-treated rats (protocol 1). In contrast, there was no significant changes in the NHE3 abundance in low-dose-lithium-treated rats (protocol 2). *P < 0.05.

Fig. 6. Semiquantitative immunoblotting of membrane fractions of whole kidneys. A and C: immunoblots reacted with anti-type 2 Na-Pi cotransporter (NaPi-2) antibody and revealed a ~85-kDa band. B and D: densitometric analyses revealed a marked decrease in whole kidney NaPi-2 abundance in high-dose-lithium-treated rats (protocol 1). In contrast, there was no significant changes in the NaPi-2 abundance in low-dose-lithium-treated rats (protocol 2). *P < 0.05.
rats. Thus, in lithium-treated rats with high sodium intake, urinary sodium excretion rates were significantly increased mainly because of the high sodium intake.

In protocol 2, rats on low-dose lithium treatment receiving the same daily amount of food also had a significantly increased urine output and decreased urinary concentration, consistent with lithium-induced NDI. However, despite the fact that these rats had the same daily sodium intake as the control rats, the lithium-treated rats also had an increased urinary sodium excretion, suggesting that chronic lithium treatment in itself causes significant natriuresis, albeit not nearly as severe as the lithium-treated rats in protocol 1.

Reduced Abundance of AQP2 and AQP3 in the Collecting Duct But Maintenance of AQP4 Abundance

We demonstrated that expression of the vasopressin-regulated collecting duct water channel AQP2 and AQP3 was severely reduced after 4 wk of high-dose or low-dose lithium therapy and that this was significantly associated with a severe impairment of urinary concentration. In contrast to AQP2 and AQP3, no major reduction in AQP4 expression was observed by immunoelectron microscopy.

The collecting duct represents the final site for the control of water excretion into the urine. Water permeability of the collecting duct is tightly regulated, under the control of vasopressin, which causes a dramatic increase in collecting duct water permeability, allowing reabsorption of water from the tubular fluid down an osmotic gradient. Vasopressin binds to the V2 receptors present in the basolateral membrane of collecting duct principal cells. Acting through the GTP-binding protein Go, the interaction of vasopressin with the V2 receptor activates adenylyl cyclase, which accelerates the production of cAMP from ATP (30). Subsequently, cAMP binds to the regulatory subunit of protein kinase A, resulting in dissociation of the regulatory subunit from the catalytic subunit. This activates the catalytic subunit, which phosphorylates various proteins (30), including AQP2 (15). AQP2 is then translocated from intracellular vesicles to the plasma membrane (6, 45), thereby increasing the water permeability of the apical plasma membrane. When vasopressin is removed, water permeability returns to basal levels, reflecting endocytic retrieval of AQP2 water channels (45), which may subsequently be available for reuse (25). Because lithium has been shown to inhibit the vasopressin-induced adenylyl cyclase activity and cAMP levels in medullary collecting duct (7), this inhibition is likely to be the cause of the reduced expression of vasopressin-regulated water channel AQP2, and the resulting decreased osmotic water permeability of collecting duct is then a key factor in the etiology of massive diuresis.

Fig. 7. Semiquantitative immunoblotting of membrane fractions of whole kidneys. A and C: immunoblots were reacted with anti-Na-K-ATPase (alpha-1 subunit) and revealed a ~96-kDa band. B and D: densitometric analyses revealed no changes in whole kidney Na-K-ATPase abundance in either high-dose (protocol 1)- or low-dose (protocol 2)-lithium-treated rats.

Fig. 8. Semiquantitative immunoblotting of membrane fractions of whole kidneys. A and C: immunoblots were reacted with anti-type 1 bumetanide-sensitive cotransporter (BSC-1) antibody and recognized a strong, broad band of molecular mass (146–176 kDa) centered at ~161 kDa. B and D: densitometric analyses revealed that whole kidney BSC-1 abundance was significantly increased in high-dose-lithium-treated rats (protocol 1). In contrast, there was no significant changes in the BSC-1 abundance in low-dose-lithium-treated rats (protocol 2). *P < 0.05.
Our data presented here also demonstrated severe downregulation of AQP3 in kidneys of rats with lithium-induced NDI. However, the mechanisms underlying regulation of AQP3 expression is presently not well established. AQP3 is localized in the basolateral plasma membrane domains of collecting duct principal cells (11, 21, 34). Immunoelectron microscopy demonstrated a predominant labeling of AQP3 in the baso-
lateral plasma membranes with little labeling of intracellular vesicles, suggesting that AQP3 is not regulated by vesicular trafficking [in contrast to the findings with AQP2 (45)]. Immunoblotting has shown that thirsting of normal rats for 48 h (11) or [deamino-Cys1-D-Arg8]-vasopressin (dDAVP) treatment of Brattleboro rats for 5 days (56) induces a marked increase in AQP3 expression. Thus there is clear evidence that AQP3 regulation is related to changes in vasopressin and water balance. However, there are several examples in which there is a decoupling of AQP2 and AQP3 expression, indicating that more needs to be learned regarding the mechanism controlling AQP3 expression (46). Moreover, the signaling mechanisms involved in the maintained expression of another collecting duct water channel, AQP4, are presently not understood, but the data presented here indicate that they are distinct from those regulating AQP2 and AQP3 expression.

It should be mentioned that several studies demonstrated significantly higher plasma concentrations of arginine vasopressin (AVP) in lithium-treated rats (2, 17, 54). Consistently, AVP gene expression in the hypothalamic paraventricular and supraoptic nuclei was demonstrated to be upregulated in rats with lithium-induced NDI (2), presumably due to the relative dehydration. It should also be emphasized that the main cause of polyuria in lithium-treated rats (or humans) is due to NDI. However, lithium treatment also induces polydipsia (38), but this is believed to be insignificant. It is highly unlikely that the severe downregulation of AQP2 and AQP3 seen in response to lithium treatment is secondary to the increased urine production. First of all, polydipsia is relatively insignificant in lithium-induced NDI (as described above). Moreover, we have previously shown that treatment with furosemide for 1 or 5 days, producing maintained polyuria, is associated
with maintained AQP2 expression (39, 41). Similarly, in a rat model with polyuria due to diabetes mellitus (glycosuria), AQP2 expression is increased (44). In both models, the maintained or increased expression is probably due to increased vasopressin levels. Thus at present there is no evidence for an effect of urine flow to modulate AQP2 or AQP3 expression.

**Increased or Maintained Abundance of BSC-1 in the TAL**

The Na-K-2Cl cotransporter BSC-1 (or NKCC2) (16, 62), which is localized at the apical plasma membrane domains of medullary and cortical TAL segments (47), mediates the apical NaCl transport in these water-impermeable segments. Several factors have been demonstrated to regulate the abundance of BSC-1 levels. An increase in the delivery of NaCl to the loop of Henle by chronic oral saline loading is known to up-regulate BSC-1 levels (12). Moreover, expression of BSC-1 in the TAL is also known to be regulated by vasopressin (shown by use of dDAVP, a V2-receptor selective agonist), and this regulation may be involved in the long-term regulation of the countercurrent multiplication system (27). Because expression of the Na-K-2Cl cotransporter is increased in response to dDAVP (27, 31) and the V2 receptor is coupled to activation of adenyl cyclase, the upregulation of BSC-1 by vasopressin may be a result of elevated levels of cAMP. Consistent with this, a cAMP-regulatory element was identified in the mouse NKCC2 gene (20).

In lithium-treated rats in which the plasma vasopressin levels are known to be increased (2, 17, 54), the expression of BSC-1 levels were significantly increased in rats on high-dose lithium treatment (with high sodium intake) or maintained in rats on low-dose lithium treatment (same sodium intake as control animals). Because the expression levels of vasopressin-regulated AQP2 and AQP3 were dramatically decreased in the same animals, this indicates that there may be other yet unknown or different molecular mechanisms involved in the regulation of vasopressin-regulated water channels and sodium (co)transporters. Since Christensen et al. (7) demonstrated that lithium affects adenyl cyclase in both the collecting duct and the thick ascending limb, our results suggest that other mechanisms inducing BSC-1 expression override the vasopressin-adenyl cyclase pathway.

**Decreased Expression of NaPi-2 in Lithium-Treated Rats**

Our results demonstrated that NaPi-2 expression in the proximal tubule was significantly decreased in rats with high-dose lithium treatment. NaPi-2 is expressed in the proximal tubule and contributes to renal proximal tubular phosphate reabsorption (5). Inhibition of proximal tubular Na-Pi cotransport by parathyroid hormone has previously been characterized by showing a significantly decreased expression of NaPi-2 in the brush border of renal proximal tubules in response to parathyroid hormone treatment (43). Lithium treatment has been reported to raise serum calcium and lower serum phosphate concentrations and to increase urinary calcium excretion in humans and rats (4, 8). Because these changes may be ascribed to the effects of lithium-induced hyperparathyroidism (4), it may be possible that lithium-induced hyperparathyroidism may decrease the NaPi-2 expression in lithium-treated rats. Moreover, because hypercalcemia is known to be associated with downregulation of AQP2 (10, 52), hyperparathyroidism-induced hypercalcemia may also contribute to lithium-induced NDI.

**Increased Expression of NHE3 in Lithium-Treated Rats**

In renal proximal tubule, the apical membrane Na+/H+ exchanger NHE3 is the predominant transporter for active secretion of H+ and reabsorption of Na+ and HCO3-. Several in vitro studies have demonstrated that the lithium inhibits Na+ influx through the inhibition of Na+/H+ exchange in rabbit brush-border membrane (22, 37). In contrast, in vivo studies with long-term lithium treatment in rats demonstrated enhanced rates of Na+/H+ antiport across the brush-border membrane in proximal tubule (63). Our results with in vivo chronic lithium treatment in rats demonstrated that the whole kidney abundance of NHE3 was significantly increased in rats treated with high doses of lithium, probably as a compensatory phenomenon. Recent studies have demonstrated that enhanced NHE3 protein abundance and activity are involved in the adaptation of bicarbonate reabsorption during chronic metabolic acidosis (1, 61). Moreover, increased NHE3 activity in the proximal tubule was observed in response to enhanced flow rates in this segment (49). Thus it may be possible that the increased expression of NHE3 in lithium-treated rats could be due to indirect effects of mild chronic metabolic acidosis, possibly related to the impairment of urinary acidification induced by lithium (3, 51) or increased flow rates in the proximal tubule. However, further studies are needed to determine the mechanisms involved in the regulation of proximal tubular sodium transporters NHE3 and NaPi-2 expression in lithium-treated rats.

**Increased Natriuresis Despite Increased or Unchanged Expression of Sodium Transporters in Lithium-Treated Rats**

Our results demonstrated that rats with lithium-induced NDI had increased natriuresis despite increased or unchanged expression of several renal sodium transporters, even with low doses of lithium treatment with same amount of daily sodium intake (protocol 2). Because lithium is known to substitute for sodium ion on several sodium transporters, which may provide a pathway for lithium entry into cells (18), uptake of lithium may be made by the apically expressed sodium transporters. Consistent with this, micropuncture studies demonstrated that high doses of lithium can inhibit sodium reabsorption in both proximal tubule and distal convoluted tubules (19). Moreover, a recent study demonstrated that chronic lithium treatment reduced aldosterone-stimulated sodium reabsorption through the amiloride-sensitive sodium...
channels in the distal nephron in rats (58). In protocol 1 the lithium-treated rats had a much higher salt intake than did control animals. Thus the maintained (Na-K-ATPase and TSC) or increased (BSC-1) expression of several sodium transporters could be viewed as an appropriate response to the increased sodium load. In protocol 2 the sodium intake is virtually identical in the two groups, which is consistent with the maintained levels of sodium transporter expression between lithium-treated and control rats. The presented data are consistent with previous evidence for regulation of these sodium transporters in physiological conditions with altered sodium and water intake [studies by Knepper and associates (27, 29, 31)]. Thus it is unlikely that these sodium transporters participate in the development of severe NDI. Downregulation of NaPi-2 may participate, whereas the role of increased expression of NHE3 is as yet difficult to interpret (it may be compensatory in response to the increased sodium load, but it may also reflect changes in the acid-base status of these animals). It should also be emphasized that other transporters may be involved in the sodium balance in lithium-induced NDI, e.g., the epithelial sodium channel (ENaC). This is a focus for further studies. In conclusion, the maintained or increased expression of some sodium transporters investigated is likely to reflect, at least partly, secondary compensatory changes to preserve water and sodium in the setting of marked polyuria and natriuresis.

Summary

In summary, markedly downregulated AQP2 and AQP3 in the collecting duct appear to play a crucial role in the development of lithium-induced polyuria. In contrast, the increased or unchanged expression of AQP1, NHE3, Na-K-ATPase, BSC-1, and TSC indicates that proximal nephron water channel AQP1 and major renal sodium transporters may not contribute to the development of lithium-induced polyuria.

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