Lithium-induced NDI in rats is associated with loss of α-ENaC regulation by aldosterone in CCD

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1The Water and Salt Research Center and 2Institute of Anatomy, University of Aarhus, Aarhus, Denmark; 3Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Taegu, Korea; 4Institute of Clinical Medicine, University of Aarhus, Aarhus, Denmark; and 5Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland

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Nielsen, Jakob, Tae-Hwan Kwon, Jørgen Frøkiær, Mark A. Knepper, and Søren Nielsen. Lithium-induced NDI in rats is associated with loss of α-ENaC regulation by aldosterone in CCD. Am J Physiol Renal Physiol 290: F1222–F1233, 2006. First published December 6, 2005; doi:10.1152/ajprenal.00321.2005.—Lithium-induced nephrogenic diabetes insipidus (Li-NDI) is associated with increased urinary sodium excretion and decreased responsiveness to aldosterone and vasopressin. Dysregulation of the epithelial sodium channel (ENaC) is thought to play an important role in renal sodium wasting. The effect of 7-day aldosterone and spironolactone treatment on regulation of ENaC in rat kidney cortex was investigated in rats with 3 wk of Li-NDI. Aldosterone treatment of rats with Li-NDI decreased fractional excretion of sodium (0.83 ± 0.02), whereas spironolactone did not change fractional excretion of sodium (1.10 ± 0.11) compared with rats treated with lithium alone (1.11 ± 0.05). Plasma lithium concentration was decreased by aldosterone (0.31 ± 0.03 mmol/l) but unchanged with spironolactone (0.84 ± 0.18 mmol/l) compared with rats treated with lithium alone (0.54 ± 0.04 mmol/l). Immunoblotting showed increased protein expression of α-ENaC, the 70-kDa form of γ-ENaC, and the Na-Cl cotransporter (NCC) in kidney cortex in aldosterone-treated rats, whereas spironolactone decreased α-ENaC and NCC compared with control rats treated with lithium alone. Immunohistochemistry confirmed increased expression of α-ENaC in the late distal convoluted tubule and connecting tubule and also revealed increased apical targeting of all three ENaC subunits (α, β, and γ) in aldosterone-treated rats compared with rats treated with lithium alone. Aldosterone did not, however, affect α-ENaC expression in the cortical collecting duct (CCD), which showed weak and dispersed labeling similar to that in rats treated with lithium alone. Spironolactone did not affect ENaC targeting compared with rats treated with lithium alone. This study shows a segment specific lack of aldosterone-mediated α-ENaC regulation in the CCD affecting both α-ENaC protein expression and trafficking, which may explain the increased sodium wasting associated with chronic lithium treatment.

epithelial sodium channel; cortical collecting duct; hypertension; nephrogenic diabetes insipidus

CHRONIC LITHIUM TREATMENT is commonly used in the management of patients with bipolar affective disorders (53). Lithium treatment is, however, often complicated by side effects including polyuria, urinary concentrating defect (3, 13, 26, 30), and increased urinary sodium excretion (4, 5, 41, 49, 51, 52).

The molecular mechanism causing lithium-induced sodium loss is not well understood. Lithium is excreted mainly by the kidney (37). In the kidney tubule, lithium is reabsorbed in competition with sodium by the type 3 Na/H exchanger (NHE3), type 1 bumetanide-sensitive Na-K-2Cl cotransporter (NKCC1), and the epithelial sodium channel (ENaC) (18, 20, 37). Approximately 60% of the filtered lithium load is reabsorbed in the proximal tubule, and 20% is reabsorbed in the thick ascending limb, connecting tubule (CNT), and cortical collecting duct (CCD) (37). During conditions with extracellular fluid volume contraction and activation of the renin-angiotensin-aldosterone system, increased sodium reabsorption also leads to increased lithium reabsorption. Thus daily sodium intake modulates renal lithium clearance (43), and insufficient dietary sodium intake can lead to fatal lithium intoxication in patients. Understanding of the cause of the sodium loss is therefore clinically important. The underlying mechanism is thought to involve dysregulation of ENaC through a decreased responsiveness to vasopressin and aldosterone in the CCD (4, 36, 50, 52).

ENaC consists of three homologous subunits, α-, β-, and γ-ENaC (9). ENaC is the main site of sodium transport across the apical plasma membrane in the CNT and CCD (19), where it reabsorbs a large fraction of the sodium delivered from the distal convoluted tubule (DCT) (1, 46). ENaC regulation is complex and includes changes in protein expression of the individual subunits (14, 31), redistribution of ENaC subunits to and from the apical plasma membrane (28, 31), and changes in channel open probability (2, 10–12, 21, 39, 40, 54, 55).

Vasopressin is known to increase protein expression of the β- and γ-ENaC subunits, whereas aldosterone increases protein expression of α-ENaC (14, 29, 31). Redistribution of ENaC-containing vesicles to the apical plasma membrane is induced by both vasopressin (7, 8, 33, 45) and aldosterone (29, 31, 38). In a previous study (36), we showed a segment-specific downregulation of the vasopressin-regulated β- and γ-ENaC subunits in the CCD and outer medulla but not in the CNT in rats with lithium-induced nephrogenic diabetes insipidus (NDI), in which plasma aldosterone and vasopressin levels are known to be increased (17). Furthermore, ENaC was only expressed in the apical plasma membrane domain in the CNT, not in the CCD, despite elevated plasma aldosterone. The decreased apical ENaC expression in the CCD is consistent with other previous studies (4, 50, 52) demonstrating decreased amiloride-sensitive sodium transport in chronically lithium-treated rats, suggesting a decreased aldosterone responsiveness.

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In the present study, we examined the direct effect of exogenous aldosterone and spironolactone (a mineralocorticoid receptor antagonist) treatment on renal sodium handling and ENaC regulation in the CNT and CCD of kidneys in rats with lithium-induced NDI. We studied rats with lithium-induced NDI on a relatively high daily sodium intake (3.4 mmol Na⁺·day⁻¹·200 g body wt⁻¹) during the experimental period to prevent extracellular volume depletion and increased endogenous plasma aldosterone secretion. We hypothesized that aldosterone responsiveness would only be observed in the CNT, whereas 7-day aldosterone infusion would be associated with unchanged α-ENaC protein abundance and no apical ENaC targeting in the CCD. The specific purposes of the present study therefore were 1) to examine the changes in subcellular ENaC immunolocalization and ENaC subunit expression in kidney cortex in response to long-term aldosterone or spironolactone treatment in rats with lithium-induced NDI and 2) to correlate these changes with the changes in renal function and handling of sodium and lithium. In addition to the changes in ENaC in the kidney cortex, we also examined the protein expression of other key sodium transporters expressed in tubule segments proximal to the CNT and CCD.

METHODS

Animal protocol: fixed lithium treatment with a fixed amount of sodium intake. Male Wistar rats (body weight 190–210 g; Møllegaard Breeding Center, L.I. Skensved, Denmark) were housed individually in normal rat cages for 18 days. During this period, we gave the rats a food ration per 200 g body wt consisting of 20 g of dry ground rat chow pellets (Altronic no. 1320; Chr. Petersen, Ringsted, Denmark), 20 ml of tap water, extra NaCl to give a total Na content of 3.4 mmol, and 0.8 mmol of LiCl. In addition, all rats had free access to water. Thus the rats had access to 20 g dry food/200 g body wt and ad libium water intake.

At day 18, the lithium-treated rats were housed in metabolic cages. At this point, an addition four untreated control rats were included in the study. In the metabolic cage, all lithium-treated rats were given (per 200 g body wt) 20 g of dry food, 3.4 mmol NaCl/20 g food, and 0.9 mmol of Li. The lithium content in the food was marginally increased to maintain the daily dose, because the food intake in the lithium-treated rats was less than expected. The untreated controls received the same food without lithium and were offered only the average food ration per 200 g body wt consisting of 20 g of dry ground rat pellets, 10 ml of tap water, extra NaCl to give a total Na content of 3.4 mmol, and 0.8 mmol of LiCl. In addition, all rats had free access to water. Thus the rats had access to 20 g dry food/200 g body wt and ad libium water intake.

After 7 days of equilibration in the metabolic cages, the lithium-treated rats were divided into three groups. In five lithium-treated rats, osmotic minipumps (model 2002; Alzet, Palo Alto, CA) were implanted subcutaneously under light halothane anesthesia (Halocarbon Laboratories, River Edge, NJ). The osmotic minipumps delivered 200 μg aldosterone/day (A6628; Sigma Chemical, St. Louis, MO) dissolved in DMSO (25% vol/vol) and sterile saline (75% vol/vol). Another six lithium-treated rats had spironolactone (3378; Sigma Chemical) added to the food in an amount of 200 mg·kg⁻¹·day⁻¹. The last eight lithium-treated rats received no additional treatment. Thus four different groups were used: 1) rats treated with lithium alone for 27 days, 2) rats treated with lithium for 27 days plus aldosterone cotreatment for the last 7 days, 3) rats treated with lithium for 27 days plus spironolactone cotreatment for the last 7 days, and 4) untreated control rats. The daily water intake and food intake was monitored throughout the study. During the entire experiment there was a 12:12-h artificial light-dark cycle, a temperature of 21 ± 2°C, and humidity of 55 ± 2%. Urine for clearance studies was collected over 24-h periods.

Immediately after removal of the right kidney, a large perfusion needle was inserted in the abdominal aorta from the aortic bifurcation. The inferior vena cava was immediately cut open to establish an outlet for the fixative. Blood was flushed from the kidneys with cold 0.01 mol/l PBS (pH 7.4) for 15 s before switching to cold 3% paraformaldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) for 3 min. The rats were euthanized during the process of fixation by rapid bleeding. The kidney was removed, and the midregion was sectioned into 2- to 3-mm transverse sections and immersion fixed for an additional 1 h, followed by 3 × 10-min washes with 0.1 mol/l cacodylate buffer (pH 7.4). Further processing is described in Immunohistochemistry.

The animal protocols were approved by the boards of the Institute of Anatomy and Institute of Clinical Medicine, University of Aarhus, according to the licenses for the use of experimental animals issued by the Danish Ministry of Justice.

Analysis of plasma and urine biochemistry. The blood samples were centrifuged for 15 min at 4,000 g in a tabletop centrifuge, and plasma was transferred to Eppendorf tubes. Measurements of plasma and urinary concentrations of sodium, potassium, creatinine, and urea were determined using a Vitros 950 analyzer (Johnson & Johnson). Measurement of plasma and urine osmolality was carried out by freezing-point depression (Advanced Osmometer, model 3900; Advanced Instruments, Norwood, MA, and Osmomat 030-D; Gonotec, Berlin, Germany). Plasma and urine concentrations of lithium were determined using a PerkinElmer Analyst 300 atomic absorption spectrometer. Plasma concentration of aldosterone was measured using a commercially available radioimmunoassay kit (Coat-A-Count; Diagnostic Products, Los Angeles, CA). Creatinine clearance was calculated as Ccr = (Ucreative × Vvolume)/(Ce × Ccreat.) × 100.

Semiquantitative immunoblotting. The procedure was similar to what has been described in detail previously (23, 48). The dissected renal cortex, inner stripe of the outer medulla, and inner medulla were homogenized (Ultra-Turrax T8 homogenizer; IKA Labortechnik, Staufen, Germany) in ice-cold isolation solution containing 0.3 mol/l sucrose, 25 mmol/l imidazole, 1 mmol/l EDTA, 8.5 μmol/l leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride, pH 7.2. To remove large cellular debris and nuclei, the homogenates were centrifuged at 4,000 g for 15 min at 4°C, and the supernatant was pipetted off and kept on ice for further processing. The total protein concentration was measured (BCA protein assay reagent kit; Pierce, Rockford, IL), and all samples were adjusted with isolation solution to reach the same final protein concentrations, solubilized at 65°C for 15 min in Laemmli sample buffer, and then stored at −20°C. To confirm equal loading of protein, an initial gel was stained with Coomassie blue dye as described previously (48). SDS-PAGE was performed on 9 or 12% polyacrylamide gels. The proteins were transferred from the gel electrophoretically (Bio-Rad Mini Protein II) to nitrocellulose membranes (Hybond ECL, RPN3032D; Amersham Pharmacia Biotech, Little Chalfont, UK). After transfer, the blots were blocked with 5% milk in PBS-T (80 mmol/l Na₂HPO₄, 20 mmol/l NaH₂PO₄, 100 mmol/l NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with primary antibodies. The sites of antibody-antigen reaction were visualized with horseradish peroxidase-conjugated secondary antibodies (P447 or P448, diluted 1:3,000; DAKO, Glostrup, Denmark) with an enhanced chemiluminescence (ECL or ECL+ plus) system and exposure to photographic film (Hyperfilm ECL, RPN3103K; Amersham Pharmacia Biotech). The band densities were quantified by scanning the films and normalizing the densitom-
etry values to facilitate comparisons. Results are listed as the relative band densities between the groups and are not absolute, hence the term semiquantitative immunoblotting.

**Immunohistochemistry.** The tissue was dehydrated in graded ethanol, and left overnight in xylene. After tissue embedding in paraffin, the sections were dewaxed with xylene and rehydrated with graded ethanol. Sections had endogenous peroxidase activity blocked with 0.5% H2O2 in absolute methanol for 10 min. With the use of a microwave oven, the sections were boiled in a target retrieval solution (1 mmol/l Tris, pH 9.0, with 0.5 mmol/l EGTA) for 10 min. After cooling, nonspecific binding was produced within 10 min by incubation with 0.05% 3,3-diaminobenzidine tetrachloride (Kem-en Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H2O2. Mayer’s hematoxylin was used for counterstaining, and after dehydration, coverslips were mounted with hydrophilic medium (Eukitt; O. Kindler, Freiburg, Germany). For analyses, sections were washed 3 times (diluted in PBS with 0.1% BSA and 0.3% Triton-X-100) overnight at 4°C. The sections were washed 3 × 10 min with PBS blocking buffer containing 1% BSA, 0.05% saponin, and 0.2% gelatin. The sections were incubated with primary antibody 10 min with PBS blocking buffer containing 1% BSA, 0.05% saponin, and 0.2% gelatin and incubated with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit immunoglobulin, DAKO P448; DAKO) for 1 h at room temperature. After 3 × 10-min rinses with PBS wash buffer, the sites of antibody-antigen reaction were visualized with a brown chromogen endogenous plasma aldosterone level in rats with lithium-induced NDI was subsequently randomized into three intervention groups: 1) oral lithium treatment for an additional 7 days, 2) aldosterone infusion for an additional 7 days, or 3) aldosterone infusion plus subcutaneous spironolactone treatment for an additional 7 days. The rats with lithium-induced NDI were subsequently randomized into three intervention groups: 1) oral lithium treatment for an additional 7 days, 2) oral lithium treatment plus subcutaneous aldosterone infusion for an additional 7 days, or 3) oral lithium and spironolactone treatment for an additional 7 days. The rats

<table>
<thead>
<tr>
<th>Table 1. Physiological data</th>
<th>Li</th>
<th>Li + Aldosterone</th>
<th>Li + Spironolactone</th>
<th>Control</th>
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<tr>
<td>Number of rats</td>
<td>8</td>
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<td>6</td>
<td>4</td>
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<td>Body wt, g</td>
<td>256 ± 7</td>
<td>256 ± 4</td>
<td>234 ± 5*</td>
<td>219 ± 11</td>
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<td>Food, g/200 g body wt</td>
<td>17.1 ± 0.4</td>
<td>15.9 ± 0.4</td>
<td>15.1 ± 0.6*</td>
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<td>Li intake, g/200 g body wt</td>
<td>0.77 ± 0.02</td>
<td>0.73 ± 0.04</td>
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<td>Urine output, ml/24 h</td>
<td>94 ± 9</td>
<td>212 ± 9*</td>
<td>63 ± 10*</td>
<td>12 ± 3</td>
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<td>Plasma</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Osmolality, mosmol/kg H2O</td>
<td>301 ± 1</td>
<td>301 ± 2</td>
<td>302 ± 2</td>
<td>298 ± 1</td>
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<td>Na+, mmol/l</td>
<td>143 ± 1</td>
<td>146 ± 1*</td>
<td>142 ± 1</td>
<td>143 ± 1</td>
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<td>K+, mmol/l</td>
<td>4.0 ± 0.1</td>
<td>3.1 ± 0.2*</td>
<td>4.7 ± 0.4</td>
<td>4.4 ± 0.1</td>
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<td>Urea, mmol/l</td>
<td>5.2 ± 0.4</td>
<td>3.5 ± 0.2*</td>
<td>7.1 ± 1.0</td>
<td>4.2 ± 0.3</td>
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<td>Creatine, mmol/l</td>
<td>25.5 ± 0.6</td>
<td>23.2 ± 0.9*</td>
<td>29.3 ± 1.6*</td>
<td>23.7 ± 0.3</td>
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<td>Li+, mmol/l</td>
<td>0.54 ± 0.04</td>
<td>0.32 ± 0.03*</td>
<td>0.84 ± 0.18</td>
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</tr>
<tr>
<td>Aldosterone, mmol/l</td>
<td>1.1 ± 0.3</td>
<td>7.5 ± 0.4*</td>
<td>4.1 ± 1.3*</td>
<td>0.6 ± 0.2</td>
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<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmolality, mosmol/kg H2O</td>
<td>294 ± 37</td>
<td>112 ± 5*</td>
<td>422 ± 80</td>
<td>1791 ± 367</td>
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<tr>
<td>Na+, mmol/l</td>
<td>31 ± 3</td>
<td>12 ± 1*</td>
<td>43 ± 7</td>
<td>250 ± 55</td>
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<tr>
<td>K+, mmol/l</td>
<td>53 ± 6</td>
<td>19 ± 1*</td>
<td>76 ± 16</td>
<td>405 ± 90</td>
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<tr>
<td>Urea, mmol/l</td>
<td>136 ± 17</td>
<td>50 ± 2*</td>
<td>204 ± 40</td>
<td>836 ± 158</td>
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<tr>
<td>Creatine, mmol/l</td>
<td>0.51 ± 0.05</td>
<td>0.22 ± 0.01*</td>
<td>0.85 ± 0.18</td>
<td>4.54 ± 1.34</td>
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<tr>
<td>Li+, mmol/l</td>
<td>6.28 ± 0.75</td>
<td>2.44 ± 0.11*</td>
<td>8.13 ± 1.68</td>
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</tr>
<tr>
<td>UUNaV, mmol/24 h</td>
<td>3.52 ± 0.14</td>
<td>3.32 ± 0.17</td>
<td>2.74 ± 0.10*</td>
<td>3.23 ± 0.09</td>
</tr>
<tr>
<td>Creatine clearance, ml/min/kg⁻¹</td>
<td>6.06 ± 0.14</td>
<td>7.41 ± 0.44*</td>
<td>5.37 ± 0.30*</td>
<td>5.84 ± 0.43</td>
</tr>
<tr>
<td>(UUNaV/PNaV)/(UCreatine/PCreatine)</td>
<td>1.11 ± 0.05</td>
<td>0.83 ± 0.02*</td>
<td>1.10 ± 0.11</td>
<td>1.06 ± 0.14</td>
</tr>
</tbody>
</table>

Values are means ± SE. UUNaV, urinary sodium excretion; (UUNaV/PNaV)/(UCreatine/PCreatine), fractional excretion of sodium. * P < 0.05, Li vs. Li + aldosterone and Li vs. Li + spironolactone.
treated with lithium alone maintained a steady polyuria and a decreased urinary concentration compared with untreated control rats [Table 1; portions of these physiological data have also been presented in a parallel study (35)]. The daily urinary sodium excretion was similar between aldosterone-treated rats and rats treated with lithium alone, whereas the spironolactone-treated rats showed a marginal decrease that, at least in part, can be explained by a small decrease in the daily food intake by spironolactone-treated rats (Table 1). The aldosterone-treated rats had a significantly decreased \( \frac{U_{Na}P_{Na}}{U_{Creatine}P_{Creatine}} \) (an index of fractional sodium excretion) compared with rats treated with lithium alone (Table 1), suggesting an increased sodium reabsorption induced by aldosterone treatment. Spironolactone did not change \( \frac{U_{Na}P_{Na}}{U_{Creatine}P_{Creatine}} \). The renal creatinine clearance \( (C_{cr}) \) was increased in aldosterone-treated rats and decreased in spironolactone-treated rats compared with rats treated with lithium alone (Table 1).

Interestingly, there was a large increase in urine production in aldosterone-treated rats with lithium-induced NDI compared with rats treated with lithium alone, whereas spironolactone treatment caused a decreased urine production compared with rats treated with lithium alone (Table 1). The increased polyuria induced by aldosterone treatment was associated with a decreased plasma lithium concentration compared with rats treated with lithium alone, whereas spironolactone treatment did not change plasma lithium concentration (Table 1).

Aldosterone significantly increased protein expression of \( \alpha \)-ENaC in renal cortex of rats with lithium-induced NDI. Under normal conditions aldosterone is an important regulator of \( \alpha \)-ENaC protein expression, but in our previous study (36), we showed that \( \alpha \)-ENaC was not increased in kidney cortex of rats with lithium-induced NDI despite significantly elevated plasma aldosterone. In this study, to determine whether this is due to decreased aldosterone responsiveness, we therefore carried out semiquantitative immunoblotting on protein samples prepared from cortex plus the outer stripe of the outer medulla. Immunoblots showed a markedly increased \( \alpha \)-ENaC protein expression in response to aldosterone treatment in rats with lithium-induced NDI compared with rats treated with lithium alone, whereas expression was decreased in response to spironolactone treatment compared with rats treated with lithium alone (Fig. 1A). A summary of densitometric analysis of immunoblots is shown in Table 2. The total protein expression of \( \gamma \)-ENaC was unchanged between groups, but aldosterone-treated rats showed a partial molecular mass shift from an 85-kDa band to a 70-kDa band (Fig. 1C). This is a known aldosterone effect thought to represent a proteolytic cleavage of the 85-kDa \( \gamma \)-ENaC form (31). The protein abundance of the \( \beta \)-ENaC subunit was unchanged in both aldosterone- and spironolactone-treated rats compared with rats treated with lithium alone (Fig. 1B).

Aldosterone did not upregulate \( \alpha \)-ENaC protein expression or induce ENaC trafficking in the CCD in rats with lithium-induced NDI. We have previously shown that lithium-induced NDI specifically affected ENaC regulation in the CCD. To investigate the direct effect of aldosterone infusion on ENaC trafficking in the kidney cortex tubule segments expressing ENaC (i.e., late DCT, CNT, and CCD) in rats with lithium-induced NDI, we examined tissue sections stained for the ENaC subunits. Immunolabeling of \( \alpha \)-ENaC in the CCD prin-

![Fig. 1. Semiquantitative immunoblots for protein prepared from cortex plus the outer stripe of the outer medulla (OSOM). Immunoblots were reacted with epithelial sodium channel (ENaC) subunits \( \alpha \)-ENaC (A), \( \beta \)-ENaC (B), and \( \gamma \)-ENaC (C). The equality of protein amount loaded was assured by Coomassie blue staining of the gel after electrophoresis (not shown). A: the \( \alpha \)-ENaC band appeared at 85 kDa and was increased in aldosterone-treated rats with lithium-induced nephrogenic diabetes insipidus (NDI; Li+Aldo rats) but decreased in spironolactone-treated rats with lithium-induced NDI (Li+Spiro rats) compared with rats treated with lithium alone (Li rats). B: the \( \beta \)-ENaC band seen at \( \sim 85 \) kDa was unchanged between groups. C: \( \gamma \)-ENaC was seen as a narrow band around 85 kDa and a broader band around 70 kDa. The 85-kDa band was decreased but the 70-kDa band was increased in the Li+Aldo rats compared with Li rats. In the Li+Spiro rats, the 85- and 70-kDa bands were unchanged compared with Li rats. *\( P < 0.05 \) compared with Li rats.]

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principal cells was very weak in all three groups of lithium-treated rats compared with the untreated control rats (Fig. 2, A–C vs. D). Moreover, the labeling intensity in aldosterone-treated rats did not appear different from that in rats treated with lithium alone, and the immunolabeling of the apical plasma membrane was not increased (Fig. 2, A and B). The labeling intensity of α-ENaC in the spironolactone-treated rats appeared marginally decreased compared with the rats treated with lithium alone (Fig. 2, A and C). Consistent with previous findings, the expression of β-ENaC (not shown) and γ-ENaC was markedly reduced in the CCD of the lithium-treated rats compared with untreated control rats, and neither aldosterone nor spironolactone affected the labeling pattern in the lithium-treated rats (Fig. 2, E–G vs. H). Thus there was no evidence of aldosterone-induced increase of α-ENaC protein expression (in CCD) in contrast to the immunoblotting results (in kidney cortex + outer stripe of outer medulla), and there was no evidence of increased apical targeting of ENaC subunits in the CCD in aldosteronetreated rats with lithium-induced NDI. 

Aldosterone caused distinct redistribution of ENaC subunits to the apical cell domain in the DCT and the CNT and increased α-ENaC expression. In contrast to the findings in the CCD, there was stronger immunolabeling of α-ENaC at the apical plasma membrane domain and weaker cytoplasmic labeling in the CNT cells in aldosterone-treated rats with lithium-induced NDI (Fig. 3F) compared with both the CCD in the same animal (Fig. 2B) and the CNT in rats treated with lithium alone (Fig. 3E). The rats treated with lithium alone showed only faint and dispersed cytoplasmic immunolabeling (Fig. 3E), and the spironolactone-treated rats (Fig. 3G) showed weak labeling, slightly fainter than that in rats treated with lithium alone. The increased labeling intensity and apical targeting of α-ENaC in the aldosterone-treated rats was not limited to the CNT but also was observed in the late DCT (Fig. 3B). The DCT showed markedly increased apical and cytoplasmic labeling compared with rats treated with lithium alone (Fig. 3A), indicating a normal response to aldosterone in OCT and CNT.

### Table 2. Densitometric analysis of ENaC immunoblots

<table>
<thead>
<tr>
<th>Number of rats</th>
<th>Li</th>
<th>Li + Aldosterone</th>
<th>Li + Spironolactone</th>
</tr>
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<tbody>
<tr>
<td>Cortex + OSOM</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>NHE3</td>
<td>1.00 ± 0.09</td>
<td>0.95 ± 0.05</td>
<td>0.71 ± 0.05*</td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.00 ± 0.04</td>
<td>0.85 ± 0.10</td>
<td>0.73 ± 0.04*</td>
</tr>
<tr>
<td>NCC</td>
<td>1.00 ± 0.09</td>
<td>0.88 ± 0.13</td>
<td>0.64 ± 0.06*</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>1.00 ± 0.13</td>
<td>3.12 ± 0.15*</td>
<td>0.57 ± 0.07*</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>1.00 ± 0.09</td>
<td>1.17 ± 0.10</td>
<td>1.01 ± 0.08</td>
</tr>
<tr>
<td>γ-ENaC total</td>
<td>1.00 ± 0.10</td>
<td>0.91 ± 0.11</td>
<td>1.03 ± 0.01</td>
</tr>
<tr>
<td>γ-ENaC 85kDa</td>
<td>1.00 ± 0.17</td>
<td>0.50 ± 0.05*</td>
<td>1.10 ± 0.09</td>
</tr>
<tr>
<td>γ-ENaC 70kDa</td>
<td>1.00 ± 0.26</td>
<td>6.29 ± 0.77*</td>
<td>1.10 ± 0.20</td>
</tr>
<tr>
<td>Na-K-ATPase α1</td>
<td>1.00 ± 0.04</td>
<td>1.18 ± 0.08</td>
<td>0.63 ± 0.06*</td>
</tr>
<tr>
<td>ISOM</td>
<td>1.00 ± 0.11</td>
<td>1.27 ± 0.12</td>
<td>0.60 ± 0.08*</td>
</tr>
<tr>
<td>NKCC2</td>
<td>1.00 ± 0.06</td>
<td>1.09 ± 0.08</td>
<td>1.00 ± 0.04</td>
</tr>
<tr>
<td>Na-K-ATPase α1</td>
<td>1.00 ± 0.03</td>
<td>0.92 ± 0.04</td>
<td>0.46 ± 0.10*</td>
</tr>
<tr>
<td>IM</td>
<td>1.00 ± 0.15</td>
<td>0.81 ± 0.14</td>
<td>0.59 ± 0.16</td>
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</table>

Values are means ± SE. NHE3, type 3 Na/H exchange transporter; NKCC2, Na-K-Cl cotransporter type 2; NCC, Na-C1 cotransporter; ENaC, epithelial sodium channel; OSOM, outer stripe of outer medulla. *P < 0.05, Li vs. Li + aldosterone and Li vs. Li + spironolactone.

The labeling intensity of α-ENaC in the late DCT of spironolactone-treated rats appeared decreased (Fig. 3C) compared with the rats treated with lithium alone and control rats (Fig. 3, A and D), suggesting a decreased mineralocorticoid receptor activation.

To confirm that the increased apical immunolabeling was restricted to the CNTs, we examined tissue sections labeled with both α-ENaC (shown in green) and the CNT/DCT marker protein calbindin-D28k (shown in red) (27, 47) by using confocal laser scanning microscopy. The tubule segments with increased apical α-ENaC in aldosterone-treated rats (Fig. 3J) were colabeled with calbindin-D28k (Fig. 3J, inset). The α-ENaC labeling in calbindin-D28k-positive tubules (Fig. 3, I, K, and L, insets) from the other experimental groups was weak and seemed dispersed in the cytoplasm without distinct apical labeling (Fig. 3, I, K, and L).

Immunolabeling with β-ENaC and γ-ENaC in the CNT cells was dispersed throughout the cytoplasm in rats treated with lithium alone (Fig. 4, A and E), whereas aldosterone-treated rats showed markedly increased labeling in the apical plasma membrane domain and decreased immunolabeling intensity in the cytoplasm (Fig. 4, B and F) consistent with redistribution of the ENaC complex induced by aldosterone (31). The spironolactone-treated rats and control rats showed dispersed labeling in the cytoplasm with no distinct apical labeling, similar to rats treated with lithium alone (Fig. 4, C, D, H, and G). The immunolabeling pattern for β-ENaC and γ-ENaC in the late DCT was not markedly different from that in the CNT (not shown). The tubule identity was confirmed by double-labeling of γ-ENaC and calbindin-D28k. The tubule segments with increased apical γ-ENaC labeling in aldosterone-treated rats (Fig. 4J) were colabeled with calbindin-D28k (Fig. 4J, inset). The other groups showed dispersed cytoplasmic labeling without distinct apical labeling calbindin-D28k-positive cells (Fig. 4, I, K, and L, insets). Thus the labeling pattern was similar to what was observed with immunoperoxidase labeling.

Interestingly, we also found increased apical labeling of γ-ENaC in some, but not all, of the initial CCDs of aldosterone-treated rats compared with rats treated with lithium alone (not shown). Consistent with previous results (36), we did not detect any γ-ENaC labeling more distal in the CNT in any of the lithium-treated rats (not shown).

Increased expression of NCC in response to aldosterone in the DCT of rats with lithium-induced NDI. In a previous study, the sodium-chloride cotransporter NCC, a known aldosterone target expressed in the DCT (25), was not increased in lithium-treated rats despite an increase in plasma aldosterone compared with untreated control rats, suggesting decreased responsiveness to aldosterone. To test this finding directly, we examined the protein expression of NCC in the kidney cortex of the aldosterone-treated rats with lithium-induced NDI. Aldosterone induced a significantly increased protein expression of NCC in aldosterone-treated rats and decreased expression in the spironolactone-treated rats compared with rats treated with lithium alone (Fig. 5C).

Spironolactone treatment decreased protein expression of NHE3, NKCC2, and Na-K-ATPase α1-subunit in rats with lithium-induced NDI. The aldosterone and spironolactone treatments were associated with significant changes in creatinine clearance and thus tubular sodium load. We therefore examined the protein expression of sodium transporters ex-
pressed in tubule segments proximal to the segments with ENaC expression by semiquantitative immunoblotting. The protein expression of NHE3, NKCC2, and Na-K-ATPase α1-subunit was markedly decreased in the spironolactone-treated rats compared with rats treated with lithium alone (Fig. 5, A, B, and D), whereas aldosterone did not affect these transporters. In the inner stripe of the outer medulla of the aldosterone-treated rats, there were no changes in NHE3, NKCC2 or Na-K-ATPase α1-subunit compared with rats treated with lithium alone, whereas spironolactone-treated rats had markedly decreased protein expression of NHE3 and Na-K-ATPase α1-subunit but unchanged NKCC2 compared with rats treated with lithium alone (Fig. 6, A–C). In the inner medulla, the Na-K-ATPase α1-subunit showed no change (Fig. 6D). The downregulation of the NHE3, NKCC2, and Na-K-ATPase α1-subunit is consistent with a decreased tubular load.

**DISCUSSION**

In this study we have investigated the effects of aldosterone and spironolactone on renal sodium and lithium handling and ENaC regulation in rats with lithium-induced NDI. The most important finding was that aldosterone did not effectively regulate α-ENaC protein expression and ENaC trafficking in the CCD of rats with lithium-induced NDI. In contrast, there was marked upregulation and apical targeting of α-ENaC in the CNT and late DCT. The lack of response to aldosterone in the CCD may explain the sodium wasting commonly observed in rats with lithium-induced NDI.

Reduced responsiveness to aldosterone in CCD but not in CNT in rats with lithium-induced NDI. The purpose of this study was to investigate whether exogenously administered aldosterone can regulate ENaC expression in rats with lithium-
induced NDI. The importance of decreased effects of aldosterone as a cause of renal sodium wasting has been described previously, although precise knowledge of the molecular mechanism is lacking (4, 5, 36, 49–52). We previously demonstrated a segment-specific downregulation of vasopressin-regulated $\alpha$-ENaC and $\gamma$-ENaC subunits observed in the CCD but not in the CNT in rats with lithium-induced NDI. These findings were compatible with the lithium-induced inhibition of vasopressin-stimulated adenylate cyclase activity that is responsible for the decreased aquaporin 2 (AQP2) and AQP3 expression (13, 26, 30). Decreased protein abundance of $\beta$-ENaC and $\gamma$-ENaC may alone explain lithium-induced urinary sodium wasting. Studies of gene knockout models and *Xenopus* oocyte expression models investigating the importance of $\beta$-ENaC and $\gamma$-ENaC have shown that an absence of either subunit results in markedly decreased ENaC activity for sodium transport (6, 9, 22, 32). Furthermore, our laboratory previously showed a decreased response to aldosterone by lack of increased $\alpha$-ENaC expression in the kidney cortex and absent ENaC trafficking in CCD, despite markedly elevated plasma aldosterone concentration in the lithium-treated rats (36). In this study, we directly tested the effect of aldosterone

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**Fig. 3.** Immunoperoxidase and confocal microscopy of $\alpha$-ENaC in the distal convoluted tubule (DCT; A–D) and connecting tubule (CNT; E–H). Labeling of $\alpha$-ENaC in the DCT and CNT cells was faint and dispersed in Li rats (A and E), Li + Sprio rats (C and G), and control rats (D and H). In contrast, the Li + Aldo rats showed an increased labeling of the apical plasma membrane domain in both the DCT and CNT (B and F) compared with Li rats (A and E). In addition, the labeling intensity in the cytoplasm of DCT cells in Li + Aldo rats was markedly increased compared with that in Li rats. (B vs. A). With double labeling, the tubule segment with strong apical $\alpha$-ENaC labeling (J, green labeling) in Li + Aldo rats was also calbindin-D28k positive (J, inset, red labeling), thus identifying the tubule as CNT or late DCT. The other groups showed only weak and dispersed labeling of $\alpha$-ENaC in calbindin-D28k-positive segments. Arrowheads indicate labeling at the apical plasma membrane domain; arrows indicate dispersed cytoplasmic labeling. Scale bar, 20 $\mu$m.
infusion on ENaC regulation. Aldosterone treatment increased α-ENaC protein expression and apical targeting in the late DCT and CNT but not in the CCD. The segment-specific lack of effect of aldosterone on α-ENaC expression and targeting in CCD provides direct evidence for decreased responsiveness to aldosterone. The lack of response to increased aldosterone may explain the sodium wasting observed in the condition of lithium-induced NDI. The CCD-specific defects on ENaC regulation should, however, be considered in relation to the recent findings from the CCD-selective α-ENaC knockout mice (42). The selective deletion of α-ENaC in CCD of mice did not result in sodium loss, and the knockout mice were resistant to sodium restriction challenge, suggesting that only the CNT and late DCT are quantitatively important in maintaining sodium balance (42). Whether these results also can be applied to rats, however, was not determined. Studies in conscious rats chronically catheterized and servo-controlled for sodium and water balance have shown that sodium delivery to the amiloride-sensitive segments is identical in untreated control and lithium-treated rats with moderate plasma lithium concentrations (~0.5 mmol/l) (50). Moreover, the natriuretic effect of chronic lithium administration was due entirely to decreased amiloride-sensitive sodium transport, i.e., ENaC-mediated sodium transport. The sodium wasting observed in
chronically lithium-treated rats therefore could be related to decreased capacity to reabsorb sodium in segments expressing ENaC. Since our results suggest that the late DCT and CNT are responding normally, the dysregulation of ENaC in the CCD therefore appears to play an important role in sodium wasting in the condition of lithium-induced NDI.

**NCC protein expression was increased with aldosterone infusion.** The Na-Cl cotransporter NCC (also known as the thiazide-sensitive Na-Cl cotransporter) is expressed in the DCT and is regulated by aldosterone (25). Previously NCC was found to be unchanged or decreased with lithium treatment compared with untreated control rats, suggesting a decreased responsiveness to aldosterone (24, 26, 36). In the present study, the NCC protein expression was increased in aldosterone-treated rats and decreased in spironolactone-treated rats compared with rats treated with lithium alone. Moreover, the marked increase in the NCC is consistent with increased α-ENaC expression observed in the late DCT, suggesting that aldosterone action is preserved in this tubular segment.

**Spironolactone treatment caused decreased Na-K-ATPase α1-subunit, NHE3, and NKCC2 protein expression.** The large decrease in Na-K-ATPase α1-subunit and NHE3 protein expression in the cortex and inner stripe of the outer medulla in the spironolactone-treated rats could be related to the decreased glomerular filtration rate observed in these rats. Particularly, the decreased protein expression of Na-K-ATPase and NKCC2 in the inner stripe of the outer medulla suggests that there is a decreased expression in the medullary thick ascending limb, compatible with a decreased delivery of tubular fluid out of the proximal tubule (15). Consistent with this finding is that NKCC2 protein expression in the cortex also decreased, compatible with a decreased NKCC2 expression in the cortical thick ascending limb. It therefore appears that spironolactone caused an extracellular fluid volume contraction, resulting in decreased glomerular filtration rate and decreased distal delivery.

**Decreased plasma lithium concentration in aldosterone-treated rats with lithium-induced NDI.** The renal handling of lithium in distal tubule segments has been extensively studied,
and one proposed hypothesis for the lithium reabsorption in sodium-depleted conditions is that the low end-tubular sodium concentration leads to reduced sodium reabsorption and hyperpolarization of the apical membrane, resulting in a condition that favors lithium reuptake (44). In our study, the increased creatinine clearance in the aldosterone-treated rats resulted in an increased tubular sodium and water load and, consequently, increased tubular sodium reabsorption to maintain steady state. This is partly accommodated by increased reabsorption in the proximal tubule through the glomerulotubular balance and load-dependent increased reabsorption in the thick ascending limb. Moreover, in the DCT, CNT, and CCD, sodium reabsorption could be enhanced by the aldosterone-regulated proteins NCC and ENaC. Therefore, the increased tubular sodium load may reduce the reabsorption of lithium and plasma lithium concentration.

In conclusion, we have shown a segment-specific lack of response to aldosterone on α-ENaC protein expression and intracellular ENaC redistribution in the CCD in rats with lithium-induced NDI, whereas aldosterone-mediated ENaC regulation in the late DCT and CNT appears normal. These results extend our previous findings of decreased expression of β-ENaC and γ-ENaC in the CCD and further support evidence that the CCD is the principal site for lithium-induced abnormalities in renal tubular sodium handling and increased urinary sodium excretion in rats with lithium-induced NDI. The underlying mechanism as to how lithium induces the altered responsiveness to aldosterone specifically in the CCD remains to be determined.

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