Aldosterone increases urine production and decreases apical AQP2 expression in rats with diabetes insipidus

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ALDOSTERONE AND VASOPRESSIN are two essential hormones in the regulation of body fluid homeostasis. Under normal conditions, renal water reabsorption is controlled through vasopressin-mediated regulation of the water permeability in the renal connecting tubule (CNT) and collecting ducts (11, 22). This occurs through a V2-receptor-mediated activation of the adenylate cyclase, leading to increased cAMP accumulation and protein kinase A (PKA) activation, promoting intracellular trafficking of the water channel aquaporin 2 (AQP2) to the apical plasma membrane and increased AQP2 protein expression (11, 14, 33). Aldosterone regulates sodium reabsorption in part through the mineralocorticoid receptor-mediated increase of the α-subunit of the epithelial sodium channel ENaC and increased ENaC trafficking (29). There is a complex interaction between vasopressin and aldosterone, and the role of aldosterone in vasopressin-mediated water permeability has been subject to several studies in isolated perfused tubule preparations and other experimental systems. Synergistic effects of mineralocorticoids on vasopressin-induced osmotic water permeability have been shown in toad urinary bladder and isolated perfused cortical collecting duct (CCD) from rabbits (7, 16, 17). In contrast, mineralocorticoids alone have not been shown to regulate osmotic water permeability in any of the rat models (7, 16, 17, 38), and there are no changes in urine production in normal rats infused with high dose aldosterone (25). In congenitally vasopressin-deficient Brattleboro (BB) rats (37) with low AQP2 expression and polyuria (12, 36, 44), experimental mineralocorticoid deficiency (adrenalectomy) or spironolactone (a mineralocorticoid receptor blocker) has been associated with decreased diluting capacity compared with BB rats with intact adrenals, whereas the urine flow was not markedly affected (15). Moreover, pharmacological interference with the renin-angiotensin system in BB rats using either captopril (an angiotensin-converting enzyme inhibitor) or spironolactone (a mineralocorticoid receptor blocker) has been shown to reduce urine production (20, 41), suggesting that aldosterone may play a role in the regulation of water excretion in vivo in the absence of vasopressin.

To better understand the interaction between aldosterone and vasopressin in settings with impaired vasopressin action, we examined the effect of aldosterone or treatment with the mineralocorticoid receptor antagonist spironolactone on the urinary water excretion and parallel changes in AQP2 trafficking and expression. Two models with impaired vasopressin signaling were applied, namely lithium-induced nephrogenic diabetes insipidus (NDI) and vasopressin-deficient BB rats with central diabetes insipidus (CDI).

In lithium-induced NDI, vasopressin action is interrupted, at least in part, through an inhibition of the adenylate cyclase activity in collecting duct, resulting in reduced accumulation of cAMP and decreased PKA activation (10). This reduces AQP2

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protein expression and decreases apical targeting, consequently causing polyuria and a urinary concentrating defect similar to what is observed in vasopressin-deficient BB rats (23, 27, 44).

METHODS

Animal protocol for Li-NDI rats: aldosterone and spironolactone treatment of rats with lithium-induced NDI. Male Wistar rats (body wt 190–210 g; Møllegaard Breeding Center, L1. Skensved, Denmark) were daily given a 40-g food ball/200 g body wt. This food ball was made by mixing 20 g of ground rat chow pellets (Altromin no. 1320; Chr. Petersen, Ringsted, Denmark) with 20 ml tap water and additional sodium chloride to give a total of sodium content of 3.4 mmol·40 g food ball−1·day−1. All lithium-treated rats received 0.8 mmol LiCl (L 4408; Sigma) added to the food ball. In addition to the water in the food mixture, the rats had free access to a water bottle. The first 18 days the rats were housed individually in normal rat cages, and each received 40 g/200 g body wt of the food mix per day. The controls were offered only the average voluntary food intake measured (out of the 40 g/200 g body wt offered) in the lithium-treated rats. During the last 10 days, the lithium-treated rats were housed in metabolic cages, and four additional untreated control rats were included in the study. In the metabolic cage, the rats were given 20 g/200 g body wt dry ground rat chow pellets with added NaCl and LiCl. After 2 days of acclimatization, the lithium-treated rats were divided into 3 groups. Five lithium-treated rats had osmotic minipumps (model 202; Alzet, Palo Alto, CA) implanted subcutaneously below the right kidney, the left kidney was perfusion fixed by insertion of a perfusion needle (21 gauge) in the abdominal aorta, and the inferior vena cava was cut to establish an outlet. During this procedure the rat was killed. Blood was flushed from the kidneys with cold 0.1 M PBS (pH 7.4) for 15 s before switching to cold 3% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 min. The kidney was removed, and the midregion was sectioned into 2- to 3-mm transverse sections and immersion fixed for an additional hour, followed by 3 × 10-min washes with 0.1 M cacodylate buffer, pH 7.4. The tissue was dehydrated in graded ethanol and left overnight in xylene. After tissue embedding in paraffin, 2-μm sections were cut on a rotary microtome (Leica Microsystems, Herlev, Denmark).

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

Analysis of plasma and urine biochemistry. The blood was centrifuged for 15 min at 4,000 g in a table-top centrifuge, and plasma was transferred to Eppendorf tubes. Measurements of plasma and urinary concentrations of Na+, K+, creatinine, and urea were determined by Vitros 950 (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 lithium concentration was determined by a Perkin-Elmer Analyst 300 atomic absorption spectrometer. Plasma aldosterone was measured using a commercially available RIA kit (Coat-A-Count; Diagnostic Products, Los Angeles, CA) and gamma counter. Creatinine clearance was calculated as 

\[ C_{cr} = \left( \frac{U_{creatinine}}{C_{creatinine}} \times \frac{V_{u}}{24 h} \right) \]

Osmolar clearance was calculated as 

\[ C_{osmol} = \left( \frac{U_{osmolality}}{C_{creatinine}} \times \frac{V_{u}}{24 h} \right) \]

Water clearance was calculated as 

\[ C_{H2O} = \left( \frac{U_{volume}}{24 h} - C_{osmol} \right) \]

Semi-quantitative immunoblotting. The procedure was similar to what has been described in detail previously (21, 44). The dissected renal cortex, inner stripe of the outer medulla (ISOM), and IM were homogenized (Ultra-Turrax T8 homogenizer; IKA Labortechnik, Staufen, Germany) in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, and 1 mM 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 (Pierce BCA protein assay reagent kit; Pierce, Rockford, IL), and all samples were adjusted with isolation solution to reach the same final protein concentrations, solubilized with SDS 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 (44). SDS-PAGE was performed on 9 or 12% polyacrylamide gels. The proteins were transferred from the gel electrophoretically (Bio-Rad Mini Protean 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 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) for 1 h and incubated overnight at 4°C with antibodies directed against AQP1 and AQP2. The AQP1 antibody has been characterized previously (44). For this study, a new AQP2 antibody was prepared using the same sequences of immunizing peptide (NH2-CEVRRRQSVELHSPQSLPRGSKA-COOH) described previously (34). The specificity was evaluated by 1) detection of the immunizing peptide on immunoblot using immune-serum and as negative control preimmune serum from the same rabbit; 2) detection of AQP2 in a protein sample prepared from whole kidney homogenates using affinity-purified anti-AQP2 antibody and showing ablation of the AQP2 detection when anti-AQP2 antibody was preincubated with surplus of the immunizing peptide; 3) detection of AQP2 immunostaining of collecting duct principal cells using sections of rat kidney, and 4) ablation of the immunocytochemical labeling using anti-AQP2 antibody preincubated with immunizing peptide. The sites of antibody-antigen reaction were visualized with horseradish perox-
idase-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 quantitated by scanning the films and normalizing the densitometry values to facilitate comparisons. Results are listed as the relative band densities between the groups and not absolute, hence the term semiquantitative immunoblotting.

Immunohistochemistry. For immunolabeling, 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 mM EGTA) for 10 min. After cooling, nonspecific binding was blocked with 50 mM NH4Cl in PBS for 30 min followed by 3 × 10 min with PBS blocking buffer containing 1% BSA, 0.05% saponin, and 0.2% gelatin. The sections were incubated with primary antibody (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 wash buffer containing 0.1% 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 produced within 10 min by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Kem-en Tek, Copenhagen, Denmark) dissolved in distilled water with 0.1% H2O2, Mayers hematoxylin was used for counterstaining, and after dehydration cover slips were mounted with hydrophobic medium (Eukitt; O. Kindler, Freiburg, Germany). For sections prepared for immunofluorescence, a secondary fluorescent antibody was used (goat anti-rabbit IgG Alexa Fluor 488 11008 and goat anti-mouse IgG Alexa Fluor, 546 11003; Molecular Probes, Eugene, OR). After 1 h incubation at room temperature and 3 × 10 min washes with PBS, cover slips were mounted with a hydrophilic mounting medium containing antifading reagent (n-propyl-gallat, P-3101; Sigma Chemical, St. Louis, MO). Light microscopy was carried out with a Leica DMRE microscope (Leica Microsystems). Laser-scanning confocal microscopy was carried out on a Leica TCS-SP2 laser confocal microscope (Heidelberg, Germany).

Semiquantitative confocal laser scanning microscopy. For the semiquantitative analysis, microscope settings (light intensity, PMT offset and gain, sampling period, and averaging) were identical for all rats, and the observer was blinded for the treatment of the individual rats. From each rat, five to eight images of tubule segments colabeled with AQP2 and 28 kDa calbindin-D and five to eight images of tubules only positive for AQP2 were obtained. Digital images were analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) to detect regions of interest and measurements of the sum of pixel intensities in the apical and basolateral cell compartments using semiautomated macros. The dynamic range was set such that the tissue with the most intense fluorescence signal only had few saturated pixels. The apical plasma membrane length of the tubules in the region of interest was measured by a calibrated manual trace of the apical plasma membrane, and the sum of pixel intensities was normalized to the length of the apical plasma membrane.

Presentation of data and statistical analyses. Quantitative data are presented as means ± SE. For blots, the results represent the mean of two blots. This was done by normalizing the absolute band densities to the mean of the controls on the same blot and then using the average normalized band density for analysis. Data were analyzed by one-way ANOVA followed by Bonferroni’s multiple-comparisons test. Multiple-comparison tests were only applied when a significant difference was determined in the ANOVA (P < 0.05). P values <0.05 were considered statistically significant.

RESULTS

Aldosterone treatment markedly increased the urine production in lithium-treated rats while the plasma lithium concentration was decreased. Consistent with previous studies (27), rats in protocol 1 treated with lithium for 20 days developed polyuria (107 ± 8 ml/day, n = 19) compared with untreated control rats (17 ± 1 ml/day, n = 4, P < 0.05). The rats with lithium-induced NDI were divided into the following three groups and continued on lithium treatment: 1) Li group receiving no further treatment, 2) Li + Aldo group receiving subcutaneous aldosterone infusion, and 3) Li + Spiro group receiving oral spironolactone for 7 days. Figure 1 shows the time course of urinary output after day 20. Interestingly, subcutaneous aldosterone infusion to the rats with lithium-induced NDI caused a marked increase in urine production compared with rats treated with lithium alone (271 ± 14 vs. 120 ± 11 ml/24 h, P < 0.05). Spironolactone treatment, on the other hand, caused a decreased urine production compared with rats treated with lithium alone (74 ± 11 vs. 120 ± 11 ml/24 h, P < 0.05). The aldosterone or spironolactone treatment was also associated with changes in urine osmolality inversely related to the changes in urine production (Table 1). There were no changes in the osmolar clearance among the lithium-treated rats, but free water clearance was markedly increased in response to aldosterone treatment compared with rats treated with lithium alone and unchanged in response to spironolactone treatment compared with rats treated with lithium alone (Table 1).

The tubular fluid load (glomerular filtration rate) as determined by creatinine clearance was increased in response to aldosterone, whereas it was decreased in rats treated with spironolactone compared with rats treated with lithium alone (Table 1). Moreover, lithium clearance was also increased in response to aldosterone but decreased in response to spironolactone treatment (Table 1). Although lithium clearance as a
measure of distal tubular delivery is debated, the increased rather than decreased lithium clearance in response to aldosterone treatment of rats with lithium-induced DI may indicate that there is an increase in the distal tubule fluid delivery in response to aldosterone treatment and a decrease in response to spironolactone treatment compared with rats treated with lithium alone.

Interestingly, the increased urine production in response to aldosterone was associated with significantly decreased plasma lithium concentration compared with rats treated with lithium alone (Table 1). In contrast, the plasma lithium concentration was unchanged in response to spironolactone treatment (Table 1). Moreover, aldosterone treatment was associated with a decreased plasma urea and potassium concentration compared with rats treated with lithium alone, although these parameters were unchanged in response to spironolactone treatment. All physiological data are summarized in Table 1.

**Aldosterone treatment did not affect AQP2 protein expression in the cortex and IM.** The large changes in urine production induced by aldosterone infusion to rats with lithium-induced DI suggested possible alterations in the expression of renal AQP2 protein. We therefore carried out immunoblot analyses using an antibody specific for the vasopressin-regulated water channel AQP2. With the use of protein prepared from the cortex plus the outer stripe of the outer medulla and protein prepared from the IM, there were no major changes in AQP2 expression in response to aldosterone or spironolactone treatment when compared with rats treated with lithium alone (Fig. 2, A and C, and Table 2), although there were statistically significant differences between groups in protein prepared from the ISOM (Fig. 2B and Table 2). The expression of AQP1 in cortex was unchanged (Table 2).

**Table 1. Functional data from rats in Li-NDI protocol**

<table>
<thead>
<tr>
<th></th>
<th>Li</th>
<th>Li + Aldosterone</th>
<th>Li + Spironolactone</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>8</td>
<td>5</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Body wt, g</td>
<td>256±2</td>
<td>256±5*</td>
<td>234±5*</td>
<td>219±11*</td>
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<tr>
<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>
<td>14.7±1.5</td>
</tr>
<tr>
<td>Plasma Osmolality, mosmol/kgH2O</td>
<td>301±1</td>
<td>301±2</td>
<td>302±2</td>
<td>298±1</td>
</tr>
<tr>
<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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<tr>
<td>K+, mmol/l</td>
<td>4.7±0.4</td>
<td>4.7±0.4</td>
<td>4.4±0.1</td>
<td>4.4±0.1</td>
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<td>Urea, mmol/l</td>
<td>7.1±0.4</td>
<td>7.1±0.4</td>
<td>4.2±0.3</td>
<td>4.2±0.3</td>
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<tr>
<td>Creatinine, μmol/l</td>
<td>29.3±1.6*</td>
<td>29.3±1.6*</td>
<td>23.7±0.3</td>
<td>23.7±0.3</td>
</tr>
<tr>
<td>Li+, mmol/l</td>
<td>0.84±0.18</td>
<td>0.84±0.18</td>
<td>0.84±0.18</td>
<td>0.84±0.18</td>
</tr>
<tr>
<td>Aldosterone, nmol/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>
</tr>
<tr>
<td>Urine Clearance</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmol, μl·min⁻¹·kg⁻¹</td>
<td>294±37</td>
<td>112±5*</td>
<td>422±80</td>
<td>1,860±367</td>
</tr>
<tr>
<td>H2O, μl·min⁻¹·kg⁻¹</td>
<td>296±14</td>
<td>271±8</td>
<td>262±7</td>
<td>215±29</td>
</tr>
<tr>
<td>Creatinine, mmol/min/kgH2O</td>
<td>30±39</td>
<td>464±29*</td>
<td>-43±35</td>
<td>-172±19</td>
</tr>
<tr>
<td>Li, mmol/min/kgH2O</td>
<td>6.06±0.14</td>
<td>7.41±0.44*</td>
<td>5.37±0.41*</td>
<td>5.84±0.43</td>
</tr>
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*Values are means ± SE. Control rats are not included in statistical analysis. *P < 0.05, Li vs. Li + aldosterone and Li vs. Li + spironolactone.
Aldosterone decreased AQP2 labeling in the apical plasma membrane domain of the CNTs from rats with lithium-induced NDI. To investigate whether there were changes in the subcellular localization and to determine if there were possible axial differences in AQP2 expression (observed as differences in labeling density) along the CNT and collecting duct in the different lithium-treated groups, we carried out an immunocytochemical analysis. AQP2 immunolocalization was investigated in the three different segments as follows: 1) CNT, 2) initial CCD, which is a transitional zone between CNT and CCD located in the outer region of the cortex representing initial CCD (iCCD), and 3) CCD located in the medullary rays (Figs. 3 and 4). Tissue sections were double labeled with AQP2 and the CNT/distal convoluted tubule (DCT) marker protein calbindin-D$_{28k}$ (43) to confirm the identity of the individual

Table 2. Densitometry analysis of AQP2 immunoblots in Li-NDI protocol

<table>
<thead>
<tr>
<th></th>
<th>Li</th>
<th>Li + Aldosterone</th>
<th>Li + Spironolactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rats</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Cortex AQP2</td>
<td>1.00±0.09</td>
<td>0.75±0.07</td>
<td>1.36±0.22</td>
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<tr>
<td>AQP1</td>
<td>1.00±0.13</td>
<td>1.08±0.10</td>
<td>1.35±0.11</td>
</tr>
<tr>
<td>ISOM AQP2</td>
<td>1.00±0.07</td>
<td>0.81±0.05*</td>
<td>1.32±0.05*</td>
</tr>
<tr>
<td>IM AQP2</td>
<td>1.00±0.06</td>
<td>0.89±0.06</td>
<td>1.09±0.09</td>
</tr>
</tbody>
</table>

Values are means ± SE. AQP, aquaporin; ISOM, inner stripe of the outer medulla; IM, inner medulla. *P < 0.05, Li vs. Li + aldosterone and Li vs. Li + spironolactone.

Fig. 3. Immunohistochemical labeling of AQP2 in kidney cortex. AQP2 labeling was seen in the apical plasma membrane domain (arrows) and at the basolateral plasma membrane domain (arrowheads) and dispersed within the cytoplasm. In the connecting tubule cells (CNT) and the initial cortical collecting duct principal cells (iCCD) there was a reduced labeling intensity in the apical plasma membrane domain in response to aldosterone treatment (Li + Aldo) compared with rats treated with lithium alone (B vs. A and F vs. E). In addition, there was increased AQP2 labeling in the basolateral plasma membrane domain in response to aldosterone (Li + Aldo) compared lithium treatment (Li) alone (F vs. E). In the CNT, spironolactone (Li + Spiro) did not induce differences in labeling pattern compared with rats treated with lithium (Li) alone (C vs. A), whereas there was an increased apical labeling in the iCCD in response to spironolactone (G vs. E). Only untreated control rats showed strong AQP2 labeling in the cortical collecting duct (J–L). Magnification: ×1,000.
Aldosterone decreased AQP2 labeling in the apical plasma membrane domain in the transitional zone between CNT and CCD. Similar to observations in the CNT segment, aldosterone-treated rats showed a markedly decreased AQP2 labeling in the apical cell domain in the iCCD (tubules not colabeled with calbindin-D28k, in the outer cortex) compared with rats treated with lithium only (Fig. 3, E vs. F, and Fig. 4, E vs. F). Moreover, the spironolactone-treated rats showed an increased AQP2 labeling in the apical cell domain compared with rats treated with lithium only (Fig. 3, G vs. E, and Fig. 4, G vs. E). The decreased apical labeling of AQP2 in aldosterone-treated rats was associated with an increased labeling in the basolateral plasma membrane domain compared with rats treated with lithium only, whereas spironolactone did not affect the AQP2 labeling in the basolateral plasma membrane domain compared with the rats treated with lithium only (Fig. 3, E–G, and Fig. 4, E–G). The untreated controls showed very strong AQP2 labeling in the apical plasma membrane domain compared with the lithium-treated groups, whereas the basolateral labeling was similar (Fig. 3, H vs. E, Fig. 4, H vs. E, and Fig. 5, B and C).

In the CCDs located in the medullary rays, there was strong AQP2 labeling in the apical plasma membrane domain of the untreated control rats (Fig. 3L). In contrast, all three groups of lithium-treated rats showed much weaker apical AQP2 labeling (Fig. 3, I–K).

Table 3. Semiquantitative analysis of AQP2 immunofluorescence in Li-NDI protocol

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Li</th>
<th>Li + Aldosterone</th>
<th>Li + Spironolactone</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apical CNT</td>
<td>37 ± 3</td>
<td>23 ± 3*</td>
<td>41 ± 6</td>
<td>82 ± 9</td>
</tr>
<tr>
<td>Basolateral</td>
<td>42 ± 4</td>
<td>21 ± 4*</td>
<td>56 ± 4*</td>
<td>150 ± 10</td>
</tr>
<tr>
<td>CNT</td>
<td>74 ± 12</td>
<td>88 ± 4</td>
<td>89 ± 10</td>
<td>93 ± 8</td>
</tr>
<tr>
<td>iCCD</td>
<td>29 ± 8</td>
<td>61 ± 6*</td>
<td>48 ± 12</td>
<td>33 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SE. CNT, connecting tubule; iCCD, initial cortical collecting duct. Control rats are not included in statistical analysis. Unit is sum of pixel intensities/apical plasma membrane length in μm. *P<0.05 Li vs. Li + aldosterone and Li vs. Li + spironolactone.
Apical AQP2 immunofluorescence intensity correlated with daily urine production. To determine whether the semiquantitative analysis of AQP2 expression in the apical cell compartment could be related to physiological parameters, we investigated the relationship between daily urine output and AQP2 immunofluorescence labeling intensity in the apical cell compartment of CNT and iCCD, respectively. This showed an inverse relationship between urine output and AQP2 labeling in the apical cell compartment (Fig. 6). The analysis was done on the AQP2 signal in the entire apical half of the cell, as shown in Fig. 5A, and thus represented both AQP2 in the apical plasma membrane and subapical vesicles and more dispersed apical cytoplasmic labeling. To determine whether the AQP2 labeling in the apical cell compartment correlates with labeling in the apical plasma membrane domain (apical membrane and the adjacent subapical region), the images used for semiquantitative analysis were further examined and scored by three blinded observers for the degree of labeling intensity in the apical plasma membrane domain on a scale from 0 to 5. These results were similar to the results obtained from the semiquantitative analysis indicating that increased signal in the apical cell compartment (in the entire apical half of the cell) is associated with increased labeling in the apical plasma membrane domain (data not shown).

AQP2 immunolabeling in the ISOM and IM showed decreased apical labeling in rats treated with aldosterone treatment. In the aldosterone-treated rats, there was a decreased labeling of the apical plasma membrane domain compared with rats treated with lithium (Li) alone (Fig. 7, B vs. A and F vs. E). The spironolactone-treated rats did not appear markedly different compared with the rats treated with lithium alone (Fig. 7, C vs. A and G vs. E). Consistent with immunoblotting results, there were no clear difference in the overall AQP2 labeling in the ISOM and IM among the lithium-treated rats.

Aldosterone treatment increased urine production in BB rats, whereas spironolactone treatment decreased urine production. To determine whether the effects of aldosterone seen in rats with lithium-induced NDI were also seen in other models of diabetes insipidus, we investigated the effect of aldosterone and spironolactone treatment in vasopressin-deficient BB rats. As shown in Fig. 8, aldosterone infusion for 7 days to BB rats produced an increase in urine production compared with untreated BB rats (323 ± 9 vs. 182 ± 7 ml/24 hours).
h, $P < 0.05$), whereas spironolactone treatment resulted in a decrease in urine production compared with untreated BB rats (127 ± 3 vs. 182 ± 7 ml/24 h, $P < 0.05$). This is consistent with the effects seen in rats with lithium-induced NDI. The changes in urine production were associated with inverse changes in urine osmolality, as shown in Table 4. Moreover, the solute free water clearance was markedly increased in aldosterone-treated BB rats compared with untreated BB rats, whereas it was markedly decreased in the spironolactone-treated BB rats compared with untreated BB rats. Additional physiological data are summarized in Table 4.

Whole kidney AQP2 protein expression was only marginally increased with aldosterone treatment. Immunoblotting using homogenate prepared from whole kidney revealed a modest increase in the protein abundance of AQP2 in the aldosterone-treated BB rats compared with untreated BB rats (132 ± 8 vs. 100 ± 3%, $P < 0.05$), whereas it was unchanged in spironolactone-treated rats compared with untreated BB rats [118 ± 8 vs. 100 ± 3%, not significant (NS)]. AQP1 protein expression was unchanged in aldosterone-treated or spironolactone-treated BB rats compared with untreated BB rats (BB: 100 ± 4%, BB + Aldo: 112 ± 6%, and BB + Spiro: 113 ± 6%, NS, blot not shown).

Aldosterone-treated BB rats exhibited markedly decreased expression of AQP2 in the apical plasma membrane domain. To determine whether aldosterone induced similar changes in subcellular distribution of AQP2 as observed in rats with lithium-induced NDI, we examined immunoperoxidase and immunofluorescence-labeled sections from the three different groups. The aldosterone-treated BB rats showed markedly decreased AQP2 labeling in the apical plasma membrane.

![Image](https://www.ajprenal.org)
domain of principal cells of the CCD (Fig. 9, B and E) compared with untreated BB rats, which showed predominantly dispersed cytoplasmic AQP2 labeling (Fig. 9, A and D). Conversely, spironolactone-treated BB rats showed increased AQP2 labeling in the apical plasma membrane domain (Fig. 9, C and F) compared with untreated BB rats (Fig. 9, A and D) or BB + Aldo rats (Fig. 9, B and E). These changes were most predominant in the iCCD and CCD of the cortex. In contrast, in the CNT, aldosterone and spironolactone caused similar changes, although less prominent (data not shown).

DISCUSSION

The present study demonstrated a marked increase in urine production in response to aldosterone treatment of rats with lithium-induced NDI and in vasopressin-deficient BB rats with CDI. Importantly, aldosterone treatment was associated with impaired apical trafficking of AQP2 in the CNT and CCD. In addition to the decreased apical AQP2 expression in both the CNT and in the iCCD, an increased basolateral AQP2 expression was seen in the iCCD. Consistent with this, aldosterone treatment was associated with increased free water clearance in both lithium-treated rats and BB rats. Treatment with the aldosterone receptor antagonist spironolactone was associated with a decreased urine production and an apparent increase in the AQP2 labeling in the apical plasma membrane domain, suggesting that the aldosterone receptor could be involved in the regulation of AQP2 trafficking and urine production in conditions of diabetes insipidus.

What is the significance of altered subcellular distribution of AQP2 in the CNT and collecting duct? The role of increased AQP2 expression in the apical plasma membrane is well documented by increased osmotic water permeability and urine concentration (35). In contrast, little is known about the role of AQP2 expression in the basolateral plasma membrane (9, 34, 35). AQP2 is regulated by intracellular trafficking for short-term regulation and by altered protein expression for long-term regulation of water balance (35). The most important mediator of both trafficking and protein expression is the peptide hormone vasopressin for regulation of renal water excretion and body water balance. Vasopressin-mediated AQP2 trafficking is mediated through the V2 receptor, resulting in Gs protein-mediated stimulation of the adenylate cyclase and resulting in increased cAMP. The increased concentration of cAMP causes activation of the cAMP-dependent protein kinase PKA, which subsequently phosphorylates AQP2 and stimulates the AQP2-bearing subapical vesicles to be fused with the apical plasma membrane, increasing the water permeability (for a review see Ref. 35). In addition to the cAMP-mediated pathway, vasopressin also causes an increase in intracellular Ca\(^{2+}\) mobilization from ryanodine-sensitive intracellular Ca\(^{2+}\) stores, which is thought to play an important role in the calmodulin-mediated exocytosis process of the AQP2-transporting vesicles (8, 35).

In the present study, we observed altered AQP2 trafficking in conditions with vasopressin resistance or lacking vasopressin. Therefore, the observed changes in intracellular AQP2 trafficking are not likely mediated by the vasopressin pathway. Indeed, previous studies have demonstrated that several different regulatory pathways could be involved in the AQP2 regulation in addition to the vasopressin-cAMP pathways. For example, 1) A recent study demonstrated a cAMP-independent and cGMP-dependent pathway for AQP2 membrane insertion in renal epithelial cells (4). In this study, exogenous cGMP or increased endogenous cGMP level induced by sodium nitroprusside, and atrial natriuretic peptide (ANP) treatment stimulated relocation of AQP2 from cytoplasmic vesicles to the plasma membrane in rat kidney collecting duct principal cells and LLC-PK1 cells stably transfected with AQP2 (4). Consistent with these findings, we recently demonstrated a strong plasma membrane staining for AQP2 in both the HEK-293 cells transiently transfected with AQP2 that were exposed to ANP and the inner medullary collecting duct (IMCD) principal

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Fig. 9. Immunoperoxidase microscopy and confocal laser scanning microscopy of AQP2 in the kidney cortex of Brattleboro rats. Brattleboro rats treated with aldosterone, or Brattleboro rats treated with spironolactone. AQP2 labeling was seen in the apical plasma membrane domain (arrows), the basolateral plasma membrane domain (arrowheads), and dispersed within the cytoplasm. Untreated Brattleboro rats (BB) showed mainly dispersed AQP2 labeling (A and D), whereas aldosterone-treated Brattleboro rats showed mainly AQP2 labeling in the basolateral cell compartment (B and E). Spironolactone-treated Brattleboro rats showed AQP2 labeling mainly in the apical cell compartment and apical plasma membrane domain (C and F). In the confocal images (D–F), there was no colabeling with 28 kDa calbindin. Magnification: ×250 in A–C and ×630 in D–F.
cells of rats systemically treated with ANP (45a). In the present study, aldosterone may have caused extracellular fluid volume expansion through sodium reabsorption, and this may have increased ANP secretion, which is known to promote diuresis and natriuresis (5). However, as mentioned above, ANP induced increased plasma membrane AQP2 expression. Moreover, the time course of ANP effects on diuresis and water excretion indicate that the effect is transient (6). Thus aldosterone-induced increased ANP secretion is not likely to be involved in the decreased apical AQP2 expression observed in the present study; 2) PGE2 has been demonstrated to play an important role in antagonizing the effect of vasopressin on osmotic water permeability in the renal collecting duct. This effect has been attributed to both inhibition of cAMP synthesis and elevation of cytosolic Ca2+ in rabbit CCDs (19, 40) and rat terminal IMCD, causing decreased trafficking of AQP2 to the apical plasma membrane and/or activating AQP2 retrieval from the apical plasma membrane (30, 32, 46). In addition, it has recently been proposed that the signaling pathway underlying the diuretic effect of PGE2 includes the cAMP and Ca2+-independent activation of the Rh-o-kinase and formation of F-actin (42); 3) we have recently demonstrated that protein expression of medullary AQP2 and p-AQP2 (AQP2 phosphorylated in the PKA-phosphorylation consensus site Ser-256) was significantly decreased in response to dDAVP and ANG II AT1 receptor antagonist cotreatment, compared with dDAVP treatment alone (24). This may suggest that ANG II AT1 receptor activation may also play a role in regulation of AQP2 in the collecting duct (24); and 4) aldosterone has been shown to cause rapid changes in second messengers, including inositol trisphosphate (IP3), diacylglycerol, cAMP, and intracellular Ca2+ (for review see Ref. 3). Aldosterone-mediated increased cAMP or Ca2+ would be expected to promote increased trafficking of AQP2, whereas the possible activation of the phosphoinositide pathway and the effect of protein kinase C and phospholipase C may both include a pre-cAMP and post-cAMP signaling to reduce the osmotic water permeability (for review see Ref. 22) by reducing apical plasma membrane AQP2. The role of protein kinase C and phospholipase C in the trafficking of AQP2 in aldosterone- or spironolactone-treated Li rats or BB rats remains to be established.

The key finding in the present study was that aldosterone treatment caused a decreased apical AQP2 expression in the CNT and in the iCCD and an increased basolateral AQP2 expression in the iCCD in rats with diabetes insipidus. In parallel to these findings, aldosterone treatment was associated with increased urine output and free water clearance in both lithium-treated rats and BB rats. In contrast, spironolactone treatment was associated with reduced urine output and in increased apical AQP2 expression. The role of basolateral AQP2 and the regulatory mechanisms involved in the targeting of AQP2 to the basolateral plasma membrane are not understood. However, it is likely that the apical and basolateral targeting processes may be independent events, possibly regulated by separate mechanisms. Initial studies investigating basolateral AQP2 expression were focused on the IMCD (34); however, basolateral AQP2 expression is also described in the cortex with considerable differences along the different tubule segments (9). It was shown that basolateral AQP2 labeling was most prominent in the CNT and IM of Sprague-Dawley rats and BB rats. In Sprague-Dawley rats, vasopressin V2-receptor antagonist treatment for 2 h was associated with markedly decreased apical AQP2 expression in the entire length of the collecting duct, whereas basolateral AQP2 was increased in the CCD but unchanged in CNT and IM. Long-term vasopressin treatment of BB rats was also associated with increased basolateral AQP2 expression in the CCD and less prominent in CNT and IMCD. Thus these data indicate that vasopressin signaling pathways are importantly involved in the changes in AQP2 redistribution to the basolateral membrane in the CCD. In the present study, we demonstrated that AQP2 is also redistributed to the basolateral plasma membrane domains in response to aldosterone in the absence of functional vasopressin signaling, e.g., in rats with lithium-induced NDI or vasopressin-deficient BB rats. This suggests that vasopressin-independent pathways are, at least in part, involved in the AQP2 targeting to the basolateral plasma membrane in conditions with no vasopressin action.

The mechanisms underlying the effect of aldosterone on altered AQP2 expression in the apical and basolateral plasma membrane are unknown. As mentioned, aldosterone has previously been shown to increase vasopressin-mediated water reabsorption whereas aldosterone alone had no intrinsic effect on water reabsorption (7, 16, 17, 38), suggesting that aldosterone only has a permissive role affecting apical AQP2. Moreover, recent studies have shown that aldosterone can increase the intracellular cAMP concentration, which may contribute to the increase in apical AQP2 trafficking (39). Similarly, aldosterone has also been shown to cause changes in intracellular Ca2+ and IP3, which could possibly be involved in increased apical expression of AQP2 (2, 18). Therefore, these results do not explain the observed paradoxical diuretic effect of aldosterone treatment with decreased apical AQP2 expression in the CNT and CCD and increased basolateral targeting in CCD occurring in the conditions exhibiting an absence of vasopressin secretion in BB rats (CDI) or a resistance of vasopressin action in lithium-induced NDI.

The mechanism for the decreased apical AQP2 expression and increased basolateral targeting in response to aldosterone treatment remains elusive. One explanation could be decreased targeting to the apical plasma membrane due to alterations in the protein-sorting process that is responsible for directing proteins to the apical plasma membrane. It has recently been shown that one form of autosomal dominant diabetes insipidus is the result of a mutation in a motif in the COOH terminus of the protein that is responsible for directing proteins to the apical plasma membrane (1). It is therefore possible that a novel aldosterone-mediated interaction or modification of sorting motifs may prevent an apical targeting of AQP2. It could be speculated that the decreased apical expression of AQP2 in response to aldosterone treatment in conditions of diabetes insipidus could be related to the increased glomerular filtration rate and/or the increased tubular flow rate in the distal nephron and collecting duct. However, previous studies on diabetes mellitus, which is known to cause distinct glomerular hyperfiltration, osmotic diuresis, and polyuria and a significant elevation of plasma vasopressin levels, exhibited a sixfold increase of urine output and a twofold increase of inner medullary AQP2 expression, including increased apical AQP2 targeting as a compensatory process (31). Thus changes in tubular flow rate per se do not decrease the AQP2 expression. Moreover, rats treated with furosemide for 5 days showed a sevenfold increase of the urine

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out but no changes of the inner medullary AQP2 expression (26). This also suggests that the changes in medullary osmolality and tubular flow may be not the major factors causing changes in AQP2 expression. However, it should be noted that, in the condition of diabetes insipidus, even small changes in glomerular filtration rate can markedly affect urine production (13), although this will not explain the changes in AQP2 trafficking.

It should also be noted that aldosterone treatment in rats with lithium-induced NDI and BB rats was associated with a low plasma potassium level, consistent with the well-known aldosterone effect seen in normal physiological conditions. Previously, it has been demonstrated that rats with hypokalemia induced by feeding rats a potassium-deficient diet have polyuria and decreased AQP2 protein expression in the IM and cortex (28). Immunocytochemistry revealed decreased AQP2 labeling in principal cells of both inner medullary and CCDs (28). However, the AQP2 labeling was mainly observed in the apical plasma membrane and subapical intracellular vesicles in hypokalemic rats (28). In the present study we demonstrate decreased apical AQP2 labeling in the CNT and CCD in response to aldosterone treatment in rats with lithium-induced NDI, which also showed hypokalemia. Thus the decreased apical AQP2 labeling is not likely to be related to the hypokalemic condition.

In conclusion, we present a study that shows a novel effect of aldosterone on urine production in two different models of diabetes insipidus. The changes in urine output were associated with a decreased apical expression of AQP2 in the CNT and CCD. Aldosterone treatment aggravated polyuria in diabetes insipidsus induced by chronic administration of lithium in rats. J Clin Invest 75: 1869–1879, 1985.


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