Hydrochlorothiazide attenuates lithium-induced nephrogenic diabetes insipidus independently of the sodium-chloride cotransporter

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†Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; ‡Department of Nephrology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; ¶Society of Experimental Laboratory Medicine, Amersfoort, the Netherlands; §Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland; and ¶Institute of Anatomy, Zurich Center for Integrative Human Physiology, University of Zurich, Zurich, Switzerland

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Sinke AP, Kortenoeven ML, de Groot T, Baumgarten R, Devuyst O, Wetzels JF, Loffing J, Deen PM. Hydrochlorothiazide attenuates lithium-induced nephrogenic diabetes insipidus independently of the sodium-chloride cotransporter. Am J Physiol Renal Physiol 306: F525–F533, 2014. First published December 19, 2013; doi:10.1152/ajprenal.00617.2013.—Lithium is the most common cause of nephrogenic diabetes insipidus (Li-NDI). Hydrochlorothiazide (HCTZ) combined with amiloride is the mainstay treatment in Li-NDI. The paradoxical antidiuretic action of HCTZ in Li-NDI is generally attributed to increased sodium and water uptake in proximal tubules as a compensation for increased volume loss due to HCTZ inhibition of the NaCl cotransporter (NCC), but alternative actions for HCTZ have been suggested. Here, we investigated whether HCTZ exerted an NCC-independent effect in Li-NDI. In polarized mouse cortical collecting duct (mMkCCD) cells, HCTZ treatment attenuated the Li-induced downregulation of aquaporin-2 (AQP2) water channel abundance. In these cells, amiloride reduces cellular Li influx through the epithelial sodium channel (ENaC). HCTZ also reduced Li influx, but to a lower extent. HCTZ increased AQP2 abundance on top of that of amiloride and did not affect the ENaC-mediated transcellular voltage. MkCCD cells did not express NCC mRNA or protein. These data indicated that in mkPCCD cells, HCTZ attenuated lithium-induced downregulation of AQP2 independently of NCC and ENaC. Treatment of Li-NDI NCC knockout mice with HCTZ revealed a significantly reduced urine volume, unchanged urine osmolality, and increased cortical AQP2 abundance compared with Li-NDI NCC knockout mice. HCTZ treatment further resulted in reduced blood Li levels, creatinine clearance, and alkalized urinary pH. Our in vitro and in vivo data indicate that part of the antidiuretic effect of HCTZ in Li-NDI is NCC independent and may involve a tubuloglomerular feedback response-mediated reduction in glomerular filtration rate due to proximal tubular carbonic anhydrase inhibition.

lithium; nephrogenic diabetes insipidus; hydrochlorothiazide; sodium-chloride cotransporter

VASOPRESSIN (AVP) REGULATES renal water uptake and urine concentration. Binding of AVP to the vasopressin type-2 receptor in the basolateral membrane of renal collecting duct principal cells results in the redistribution of aquaporin-2 (AQP2) water channels from intracellular vesicles to the apical membrane. Driven by an osmotic gradient, water will enter the principal cells through AQP2 and will exit through AQP3 or AQP4 in the basolateral membrane, resulting in concentrated urine. Besides this short-term regulation, AVP also regulates AQP2 expression in the long term (38).

Lithium, a monovalent cation, is the drug of choice for the treatment of bipolar disorders and is prescribed to 0.1% of the population (26, 40). In addition, lithium is used to treat schizoaffective disorders and depression and is under consideration as a therapeutic for alcoholism, Alzheimer’s disease, autoimmune deficiency syndrome (AIDS), and cluster headaches (11, 13, 31). Unfortunately, lithium decreases the urine concentrating ability in 50% of treated patients, while ~20% of patients develop symptomatic nephrogenic diabetes insipidus (NDI), a disorder in which the AVP-induced antidiuresis is impaired, resulting in polyuria and polydipsia (3, 36, 44). Lithium-induced NDI (Li-NDI) is the most common form of NDI, and patients are at risk for dehydration-induced lithium toxicity, cyst formation, and end-stage renal disease (40). For most Li-NDI patients, however, cessation of lithium therapy is not an option, because the bipolar disorder has a larger impact on the patient’s quality of life.

Together with the diuretic amiloride, hydrochlorothiazide (HCTZ) is the mainstay drug to treat Li-NDI. Indeed, after the initial observation that HCTZ decreased urine volume and increased urine osmolality in patients with central or nephrogenic diabetes insipids (9), it later also appeared to be effective in reducing polyuria and increasing urine osmolality in Li-NDI patients (12). Despite its use for >50 years, however, the precise mechanism by which thiazide diuretics elicit their paradoxical antidiuretic effect is still unclear. Thiazides are well known to inhibit the NaCl cotransporter (NCC) in the renal distal convoluted tubule, thereby decreasing sodium and chloride reabsorption. It is generally accepted that this thiazide-induced decrease in renal sodium uptake temporarily induces hypovolemia, which leads to a decreased glomerular filtration rate, increased renin-aldosterone-angiotensin system (RAAS) activation, and a compensatory increase in proximal tubule sodium and water reabsorption. Consequently, less sodium and water are delivered to the collecting duct and less urine produced.

This, however, does not seem to be whole story. At first, Kim et al. (17) showed that HCTZ partially increased the AQP2 abundance in Li-NDI rats. Since AVP is already increased in Li-NDI (2), it is unlikely that the increased AQP2 abundance is due to HCTZ-induced increases in AVP levels.
More importantly, however, HCTZ also increased the water permeability in isolated rat inner medullary collecting ducts from rats, suggesting that thiazides also directly act on the vasopressin-AQP2 axis of principal cells (5).

Therefore, the goal of the present study was to investigate whether HCTZ may indeed directly act on AQP2 expression and may reduce Li-NDI independently of its action of NCC.

MATERIALS AND METHODS

Cell culture. mpkCCDcl4 (clone 14) or mpkCCDcl4 cells stably transfected with a 3.0-kb AQP2 promoter luciferase construct were cultured as described (14, 20). Cells were seeded at a density of 1.5 × 10⁶ cells/cm² on semipermeable filters (Transwell, 0.4-μm pore size, Corning Costar, Cambridge, MA) and cultured for 8 days. Unless stated otherwise, the cells were exposed to 1 nM dDAVP at the basolateral side for the final 96 h to induce AQP2 expression. Lithium was administered as indicated. Amiloride (10 μM) or HCTZ (100 μM) was added to the apical and basolateral side of the filters for the final 48 h. At the end of the experiment, transcellular electrical resistance was measured using a Millicell-ERS meter (Millipore, Bedford, MA).

Lithium assays. Determination of intracellular lithium concentrations was done as described (19). Briefly, mpkCCDcl4 cells were grown as above on 4.7-cm² filters. To determine the extent of lithium contamination from the extracellular side, FITC-dextran was added to the lithium-containing medium to a final concentration of 10 μM just before harvesting, after which the medium was mixed. Then, the filters were washed three times with isosmotic sucrose (pH 7.3) at 4°C, and cells were lysed by sonication in 1 ml of Milli-Q water. For the 800-μl sample, the concentration of lithium was determined by flame photometry, from which the total amount of lithium in the sample was calculated.

For the 100-μl sample, the concentration of FITC-dextran was measured using spectrofluorophotometry (Shimadzu RF-5301) at 492 (excitation)- and 518-nm (emission) wavelengths. By comparing the obtained values with a twofold FITC-dextran dilution series, the FITC-dextran concentration in each sample was determined, from which the extent of extracellular Li⁺ contamination was calculated and subtracted from the total amount to obtain the intracellular lithium amount. With the FITC-dextran concentration used, a contamination >1:5,000 would be detected. To correct for differences in cellular yield, the intracellular lithium amounts were normalized for the protein amount in each sample, which was determined using a Bio-Rad Protein Assay (Munich, Germany).

Luciferase assay. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) following the manufacturer’s instructions. Luminescence was measured for 10 s using an EG&G Berthold Lumat LB9507 luminometer (Bad Wildbad, Germany). To verify that equal amounts of protein per sample were used for the luciferase assay, protein concentration was determined using a Bio-Rad Protein Assay as described elsewhere (4). Light absorbance at 595 nm was measured using a Helios omega spectrophotometer (Thermo Scientific, Rockford, IL).

Real-time RT-PCR. mpkCCDcl4 cells were grown on semipermeable filters for 8 days as described above, and total RNA was isolated using TRIzol extraction reagent (GIBCO, Rockville, MD), according to the manufacturer’s instructions. To remove genomic DNA, total...
RNA was treated with RNase-free DNase (Promega) for 1 h at 37°C, extracted with phenol/chloroform, and precipitated. RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase and random primers (Promega). During cDNA production, a negative control without reverse transcriptase enzyme was taken along to exclude possible genomic DNA contamination. To amplify NCC cDNA, primers binding to position 709 (GTCCA-GAAATGGCCATGAG) and 1169 (GTGGGTGTGGCCATGCAC) were used. To amplify β-actin, primers binding to position 505 (gatgcccctgtgtgaccac) and 705 (acagcttccctactgtggt) were taken along as positive controls for cDNA amplification. cDNA from total mouse kidney was taken along as a positive control sample for NCC.

Experimental animals. Male NCC knockout mice (25, 35) on C57BL/6J background (35–40 wk old, n = 24) were maintained in a temperature-controlled room with lights on between 0800 and 2000. Mice were divided into three groups (n = 8). Group 1 (controls) mice were given a normal diet (sniiff R/M-H V1534, sniiff Spezialdiatien, Soest, Germany) for 10 days. Group 2 (lithium) mice received LiCl (Merck, Darmstadt, Germany) solubilized in water and added to the chow to give a concentration of 40 mmol/kg of dry food, previously shown to give a clinically relevant serum lithium concentrations in mice (7). Group 3 (lithium-HCTZ) mice received, in addition to the diet of group 2, 350 mg HCTZ/kg dry food (Sigma-Aldrich, Steinheim, Germany) in their diet. All mice had free access to water, food, and a sodium-chloride block. For the final 48 h of the experiment, mice were housed in metabolic cages to measure water intake and urine output during the final 24 h. All animal experiments were approved by the Animal Experiments Committee of the Radboud University Medical Centre (RUNMC).

Mice were anesthetised with isoflurane, after which their blood was removed by orbital puncture. Then, mice were euthanized by cervical dislocation, and the kidneys were rapidly removed. As described below, one kidney was processed for immunohistochemistry, whereas the other kidney was used for immunoblotting. For immunoblotting, the tissue was homogenized in 1 ml of ice-cold homogenization buffer A (20 mM Tris, 5 mM MgCl2, 5 mM Na2HPO4, 1 mM EDTA, 80 mM sucrose, 1 mM PMSF, 5 μg/ml pepstatin A, 5 μg/ml leupeptin, and 5 μg/ml aprotinin) using a polytron homogenizer (VWR International, Amsterdam, The Netherlands) and cleared from nuclei and unbroken cells by centrifugation at 4,000 g for 15 min. The supernatant was then diluted in Laemmli buffer to a final protein concentration of 1 μg/μl.

Blood and urine analyses. Following orbital puncture, whole blood was analyzed immediately for sodium, potassium, hematocrit, and pH using an EGG+ cartridge and I-Stat Clinical Analyzer (Abbott, Hoofddorp, The Netherlands). The remaining blood was collected in a BD microtainer SST tube (Becton Dickinson, Breda, The Netherlands) for serum and centrifuged at 10,000 g for 3 min to sediment the red blood cells. Serum and urine samples were analyzed for osmolality using an osmometer (Fiske, Needham Heights, MA), and electrolyte concentrations were measured on a Synchron CX. Urine PGE2 levels were determined by measuring a stable PGE2 metabolite (PGEM) after chemical derivatization of PGE2 and its primary metabolites, 13,14-dihydro-15-keto PGE2 and 13,14-dihydro-15-keto PGA2, to the single PGEM compound. Urine was diluted 150 (control) or 75 (lithium-treated) times. PGEM concentrations were determined with the PGE metabolite EIA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. SDS-PAGE, blotting, and blocking of the polyvinylidene difluoride membranes were done as described (16). Membranes were incubated for 16 h at 4°C with 1:2,000-diluted affinity-purified rabbit pre-c-tail (41) or 1:3,000-diluted affinity-purified rabbit-7 AQP2 antibodies (10), all diluted in Tris-buffered saline Tween 20 (TBS-T) supplemented with 1% nonfat dry milk. After washing in TBS-T, blots were incubated for 1 h with 1:5,000-diluted goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma, St. Louis, MO). Proteins were visualized using enhanced chemiluminescence (ECL; Pierce, Rockford, IL). Densitometric analyses were performed using Bio-Rad quantification equipment (Bio-Rad 690c densitometer, Chemidoc XRS) and software (QuantityOne). Equal loading of the samples was confirmed by subsequent staining of the blots with Coomassie blue.

Immunohistochemistry. Kidneys were fixed by immersion for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer at 4°C, embedded in paraffin, and cut into 3- to 4-μm-thick sections. After deparaffinization, sections were placed in a microwave oven and heated for 10 min at 98°C in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval. Subsequently, sections were incubated overnight at 4°C with 1:2,000 affinity-purified rabbit pre-c-tail AQP2 antibody (16) and 1:2,000-diluted rabbit polyclonal H-ATPase antibody as described (42, 43). The bound primary antibodies were revealed with Cy3-coupled goat-anti rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). To check for unspecific binding of primary or secondary antibodies, incubations with nonimmune sera or without any primary antibodies were performed. All control experiments were negative. Cryosections were studied by epifluorescence using a Leica microscope (Wetzlar, Germany). Images were acquired with a charge-coupled device camera and processed electronically using Adobe Photoshop and Microsoft Powerpoint software. Adjustments for brightness and contrast were kept constant for each kidney section. The various segments of the renal collecting system were identified based on the histotopographical localization. Connecting tubules were stained with iso-osmotic

Fig. 2. HCTZ attenuates lithium entry without affecting transcellular voltage in mpkCCD cells. mpkCCD cells were grown as described in the legend for Fig. 1. During the final 24 h, the cells were incubated in the absence (C) or presence of lithium only (L) or with lithium and the addition of 10 μM amiloride (L Am), 100 μM HCTZ (L T), or 10 μM amiloride and 100 μM HCTZ (L T Am). A: intracellular lithium concentrations in pmol/μg protein ± SE were determined, corrected for contamination with extracellular lithium and normalized for the amount of protein. B: transcellular voltage of the conditions described above. All data are derived from 3 independent filters per condition. *Significant difference from control (C), P < 0.05.

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Table 1. Metabolic parameters of NCC −/− mice treated for 10 days with a standard, lithium, or lithium+HCTZ diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 8)</th>
<th>L (n = 8)</th>
<th>L+T (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum osmolality, mosmol/kg H2O</td>
<td>332 ± 2</td>
<td>328 ± 2</td>
<td>325 ± 2*</td>
</tr>
<tr>
<td>Serum sodium, mmol/l</td>
<td>153 ± 0.7</td>
<td>151 ± 0.5*</td>
<td>151 ± 0.3*</td>
</tr>
<tr>
<td>Serum potassium, mmol/l</td>
<td>4.6 ± 0.1</td>
<td>4.8 ± 0.1</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Serum lithium, mol/l</td>
<td>/</td>
<td>0.99 ± 0.02</td>
<td>0.79 ± 0.06†</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.03 ± 0.00</td>
<td>0.05 ± 0.00*</td>
<td>0.07 ± 0.00**†</td>
</tr>
<tr>
<td>Blood ionized calcium, mol/l</td>
<td>1.21 ± 0.01</td>
<td>1.26 ± 0.01*</td>
<td>1.28 ± 0.01*</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.35 ± 0.01</td>
<td>7.34 ± 0.01</td>
<td>7.36 ± 0.01</td>
</tr>
<tr>
<td>Blood hematocrit, PCV</td>
<td>0.38 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>Urine volume, μl g⁻¹·24 h⁻¹</td>
<td>39 ± 5</td>
<td>211 ± 35*</td>
<td>127 ± 17††</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kg H2O</td>
<td>3,420 ± 279</td>
<td>1,019 ± 95*</td>
<td>1,131 ± 81*</td>
</tr>
<tr>
<td>Urine sodium, mmol/l</td>
<td>121 ± 24</td>
<td>113 ± 16</td>
<td>93 ± 21</td>
</tr>
<tr>
<td>Urine potassium, mmol/l</td>
<td>540 ± 43</td>
<td>132 ± 20*</td>
<td>170 ± 16*</td>
</tr>
<tr>
<td>Urine lithium, mmol/l</td>
<td>/</td>
<td>17 ± 3</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>Urine creatinine, mg/dl</td>
<td>61 ± 6</td>
<td>15 ± 2*</td>
<td>18 ± 2*</td>
</tr>
<tr>
<td>Urine pH</td>
<td>6.11 ± 0.07</td>
<td>6.56 ± 0.27</td>
<td>7.45 ± 0.11††</td>
</tr>
<tr>
<td>Total sodium excretion, mmol</td>
<td>0.12 ± 0.02</td>
<td>0.62 ± 0.2*</td>
<td>0.31 ± 0.1</td>
</tr>
<tr>
<td>Total potassium excretion, mmol</td>
<td>0.55 ± 0.04</td>
<td>0.62 ± 0.06</td>
<td>0.54 ± 0.04</td>
</tr>
<tr>
<td>Total lithium excretion, mmol</td>
<td>/</td>
<td>79 ± 10</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>FE (Na), %</td>
<td>0.04 ± 0.01</td>
<td>0.35 ± 0.10*</td>
<td>0.24 ± 0.05*</td>
</tr>
<tr>
<td>FE (K), %</td>
<td>5.5 ± 0.8</td>
<td>9.9 ± 0.9*</td>
<td>13.2 ± 0.7††</td>
</tr>
<tr>
<td>FE (Li), %</td>
<td>5.9 ± 0.6</td>
<td>11.8 ± 1.6†</td>
<td>11.8 ± 1.6†</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>1.9 ± 0.4</td>
<td>10.1 ± 0.1*</td>
<td>6.0 ± 0.0††</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>27.8 ± 0.8</td>
<td>25.5 ± 0.7</td>
<td>26.6 ± 0.4</td>
</tr>
<tr>
<td>Water intake, μl g⁻¹·24 h⁻¹</td>
<td>93 ± 14</td>
<td>338 ± 47*</td>
<td>245 ± 24*</td>
</tr>
<tr>
<td>Food consumption, mg·g⁻¹·24 h⁻¹</td>
<td>112 ± 9</td>
<td>116 ± 13</td>
<td>128 ± 12</td>
</tr>
<tr>
<td>Feces production, mg·g⁻¹·24 h⁻¹</td>
<td>54 ± 6</td>
<td>62 ± 10</td>
<td>66 ± 7</td>
</tr>
</tbody>
</table>

Values are means ± SE. NCC, Na-Cl cotransporter; HCTZ, hydrochlorothiazide; T, HCTZ; L, lithium; PCV, packed cell volume; FE, fractional excretion; /, below detection limit. *P < 0.05 compared with control mice. †P < 0.05 compared with mice treated with lithium only.
level that was not different from controls (Fig. 1B). This indicated that HCTZ attenuates the reduced AQP2 gene transcription induced by lithium.

By blocking ENaC, amiloride strongly reduced the cellular influx and intracellular concentration of lithium in mpkCCD cells (19). To examine whether and to what extent HCTZ would affect intracellular lithium levels, mpkCCD cells were incubated for 24 h without or with lithium, the latter in the presence or absence of amiloride, HCTZ, or both. With lithium only, the intracellular lithium concentration amounted 78 ± 7 pmol/μg protein (Fig. 2A). HCTZ significantly reduced the intracellular lithium concentration by 38%. In comparison, amiloride reduced the lithium concentration by 84%, consistent with ENaC being the major influx pathway for lithium (19) (Fig. 2A). Although not significantly different from amiloride only, the intracellular lithium concentration was further reduced by >50% when amiloride was combined with HCTZ.

Due to electrogenic transcellular transport of sodium and potassium via ENaC, ROMK, and the Na-K-ATPase, a transcellular voltage (Tv) develops over mpkCCD cell monolayers. Compared with control cells, lithium slightly, but significantly, reduced the Tv of mpkCCD cells (Fig. 2B). As anticipated, amiloride strongly reduced the Tv to <6% of that of lithium-treated cells. With HCTZ, however, the Tv did not differ from that of lithium-treated cells. The Tv of cells treated with amiloride and HCTZ was not different from that of amiloride only.

Together, these data indicated that HCTZ partially protects mpkCCD cells against lithium-induced downregulation of AQP2 abundance and that the mechanism of protection differs from that of amiloride action on ENaC.

*mpkCCD cells do not express NCC.* As the HCTZ-blocked influx of lithium in mpkCCD cells could be mediated by NCC, we analyzed its expression. However, immunoblot analysis did not show any band that could be attributed to NCC (not shown). To detect NCC in the most sensitive manner, we therefore employed RT-PCR analyses. Following treatment of mpkCCD cells with or without dDAVP, the latter in the absence or presence of lithium, mRNA was isolated and reverse transcribed. Mouse kidney RNA was used as a positive control. Despite clear detection of β-actin in all RT+ samples analyzed, indicating the use of sufficient amounts of cDNA, the NCC band of 461 bp was only obtained for mouse kidney control mRNA (Fig. 3). These data revealed that NCC is not expressed in mpkCCD cells and thus that NCC was not involved in the HCTZ-induced attenuation of the reduction of AQP2 abundance with lithium in mpkCCD cells.

**Effect of HCTZ on development of Li-NDI in NCC knockout mice.** To test whether HCTZ would then also attenuate Li-NDI independently of NCC in vivo, NCC knockout mice were obtained and maintained on 40 mg/kg body wt lithium chow with or without HCTZ for 10 days. All blood and urine parameters are summarized in Table 1. In line with earlier studies (6, 19), mice treated with lithium developed severe polyuria and polydipsia combined with a significantly reduced urine osmolality. Importantly, NCC knockout mice treated with lithium and HCTZ showed a significant reduction of urinary water loss and a nearly significant reduction in drinking volume compared with lithium-treated mice. Interestingly, HCTZ treatment did not change the urine osmolality.

As observed before (7, 19), lithium-treated mice showed a tendency for a slightly lower body weight, which was partially corrected in the mice treated with HCTZ. We observed no differences in food intake, feces production, serum sodium, and potassium concentrations. However, there were differences in the urinary excretions of various solutes. Consistent with earlier reports (19, 28, 39), lithium treatment significantly increased total and fractional sodium excretion. In the HCTZ group, however, total sodium excretion did not differ from controls, while its fractional excretion was in between that of controls and lithium-treated mice. The fractional excretion of
potassium was increased in lithium-treated mice and further increased in the HCTZ group, while creatinine clearance was reduced in lithium and further reduced in the lithium-HCTZ group. Interestingly, HCTZ treatment resulted in a lower serum lithium concentration together with an increased fractional lithium excretion. Moreover, HCTZ treatment did not affect blood pH, but led to an increased urine pH compared with both control and lithium-treated mice.

Li-NDI coincides with increased urinary PGE2 levels (20). In line with these data, urinary PGE2 levels showed a significant (9-fold) increase with lithium treatment compared with controls (Fig. 4). Additional HCTZ treatment again reduced urinary PGE2 to 30% of the level obtained in lithium-treated mice.

Whole kidney immunoblotting and immunohistochemistry were used to further analyze the effect of HCTZ on relevant proteins. Immunoblots revealed that lithium significantly reduced AQP2 abundance to 9% of control kidneys, and that HCTZ slightly, but significantly, increased renal AQP2 abundance to 16% of normal values (Fig. 5). Consistent with our immunoblot data, immunohistochemistry revealed that lithium treatment nearly completely abolished AQP2 expression throughout the kidney (Fig. 6). In the HCTZ-treated mice, AQP2 abundance was partially recovered (Fig. 6C), which was mainly observed in the CNT (Fig. 6, E and F) and CCD (Fig. 6, H and I).

The proximal tubules have an important role in acid-base regulation, in which the sodium/hydrogen exchanger-3 (NHE-3) is the major hydrogen efflux pathway. Considering the increased urinary pH, we therefore tested its expression in our mice. Interestingly, immunoblot analysis revealed the NHE-3 abundance was significantly reduced in our lithium-HCTZ-treated mice compared with lithium treatment only (Fig. 7).

**DISCUSSION**

**HCTZ attenuates an NCC-independent lithium-induced downregulation of AQP2 in mpkCCD cells.** Similar but less pronounced compared with amiloride (7, 19), HCTZ reduced the intracellular lithium concentration and increased AQP2 transcription and AQP2 abundance in mpkCCD cells. However, several of our data indicate that the mechanism of action of HCTZ differs from that of amiloride: First, at an amiloride concentration that fully blocks ENaC (concentration used was ~10-fold higher than its IC50) (18) and that partially blocks
NHE-1 and -3 (30), cells treated with HCTZ on top of amiloride displayed a significantly higher abundance of AQP2 than with amiloride only. Second, in contrast to amiloride, HCTZ did not decrease the Tv, indicating that the effect of HCTZ is independent from an electrogenic transporter or channel such as ENaC. The HCTZ-blocked electroneutral influx of lithium was not conferred by NCC, as mpkCCD cells appeared not to express NCC (Fig. 3). Therefore, we conclude that in mpkCCD cells, HCTZ attenuates lithium-induced downregulation of AQP2 independently of both ENaC and NCC.

**HCTZ attenuates Li-NDI in mice lacking NCC.** In Li-NDI rodents, HCTZ treatment resulted in antidiuresis, increased urine osmolality, and increased AQP2 levels (17). Despite the absence of NCC, HCTZ treatment of our Li-NDI NCC knockout mice also showed a significantly reduced urine volume, indicating that at least part of the HCTZ-induced antidiuresis in Li-NDI is NCC independent. As HCTZ increases urine osmolality in normal rodents, the unchanged urine osmolality with HCTZ in our Li-NDI mice is likely due to the absence of NCC. In line with our in vitro data, HCTZ also significantly attenuated the lithium-induced decrease in overall AQP2 abundance (Fig. 5). This increase was rather small, which is likely explained by our observation that HCTZ increased the AQP2 abundance in the cortical collecting duct only (Fig. 6). Considering the unchanged urine osmolality and the fact that urine osmolality is mainly determined by the extent of water taken up in the inner medulla, where the abundance of AQP2 was unchanged, the contribution of the increased AQP2 levels to the observed urine volume reduction with thiazide is likely negligible. It remains to be established why HCTZ increases AQP2 abundance in the cortex only.

**HCTZ-induced, NCC-independent urine volume reduction may be due to carbonic anhydrase inhibition.** At present, it is unclear how HCTZ reduces lithium-induced polyuria in our NCC knockout mice. The reduced sodium excretion of our HCTZ-treated mice coincided with normal blood sodium concentration and hematocrit, indicating that less sodium was consumed. As it is well known and part of NDI therapy that reduced sodium intake in NDI results in reduced polyuria with unchanged urine osmolality, this could explain the reduced polyuria in our thiazide-treated mice. As food intake was indifferent between the lithium and lithium-thiazide group (Table 1), our lithium-thiazide mice have likely taken less of the salt block. The primary signal for reduced urinary sodium excretion with thiazide can be due to a reduced appetite for sodium or increased sodium reabsorption. A reduced sodium appetite for sodium, however, is unlikely for several reasons. At first, thiazides have been reported to increase sodium appetite (33). Second, the reduced urinary PGE2 with thiazides is opposite to what is expected with a reduced sodium appetite. Cyclooxygenase (COX)-2 is the major COX isoform contributing to the regulated production of prostaglandins affecting salt and water homeostasis and is localized to the macula densa/cortical thick ascending limb and to a subset of medullary interstitial cells (45). Macula densa COX-2 and its product PGE2 regulate renin production, and this enzyme and urinary release of PGE2 are stimulated with hypovolemia and suppressed under hypervolemic conditions. Similarly, in Li-NDI rats, which are hypovolemic due to the massive water loss, macula densa COX-2 abundance is increased, whereas medullary COX-1 and COX-2 are decreased (21) and urinary PGE2 is increased (e.g., Fig. 4). If HCTZ had reduced salt appetite in our Li-NDI mice, they would have become more hypovolemic, which would lead to increased urinary PGE2 levels. In contrast, our HCTZ-treated mice have reduced PGE2 levels compared with lithium-treated mice. Thus the reduced sodium intake is likely a compensatory mechanism and is therefore also not likely to constitute the primary explanation for the reduced polyuria.

Instead, our present data suggest that the observed antidiuresis is most likely mainly due to HCTZ inhibition of carbonic anhydrases (CAs) in mainly proximal tubules: In this segment, CAs facilitate conversion of water and CO2 into hydrogen and HCO3−, which is massive, as 80% of the renal HCO3− is regenerated here. Removal of the produced hydrogen occurs mainly via the apical sodium/hydrogen exchanger NHE3 (34) and is therewith the major contributor to the proximal tubule sodium uptake, which counts up to 67% of our filtered sodium and, consequently, of water. Moreover, it is well known that, like sodium, most filtered lithium is taken up by the proximal tubules and that blood lithium levels vary with proximal tubule sodium reabsorption (8, 40). Inhibition of proximal tubule CAs prevents intracellular hydrogen formation and bicarbonate re-absorption (1, 27). The reduced hydrogen production precludes NHE3 activity, resulting in alkalinated urine, and reduced proximal tubular sodium and water uptake, which, as reported
for NHE3 knockout mice (1, 35), increases delivery of fluid to the macula densa. This increased delivery of fluid reduces PGE2 release from the macula densa, resulting in a tubuloglomerular feedback (TGF) response-mediated glomerular filtration rate (GFR) reduction (23).

Thiazides are known to inhibit CAs (32, 37), and an inhibitory action of HCTZ on CAs in our mice is indicated by the alkalized urine of our HCTZ-treated Li-NDI NCC mice compared with those of the Li-NDI group (Table 1). In line with thiazide action on proximal tubule CAs, blood lithium levels were reduced in our HCTZ-treated mice compared with our Li-NDI mice (Table 1; Fig. 7). In hypovolemic states, including Li-NDI mice and mice treated with thiazides, proximal tubular NHE-3 abundance is upregulated, which is ascribed to increased RAAS activity (15, 29). In our study, NHE3 abundance was significantly downregulated in the thiazide-treated Li-NDI mice compared with the Li-NDI mice. Moreover, and in accordance with a consequent increased fluid delivery to the macula densa, our HCTZ mice had reduced urinary PGE2 levels and showed decreased creatinine clearance (15, 29).

In line with the data of Cesar et al. (5) that HCTZ increased the water permeability in isolated inner medullary collecting ducts from normal and Brattleboro rats, our in vitro data indicated that HCTZ also directly attenuates lithium-induced downregulation of AQP2 in principal cells. A possible target could be the recently identified sodium transport pathway via SLC4A8, as this Na+-driven Cl-/HCO3- exchanger mediates electroneutral, amiloride-resistant, HCTZ-sensitive, transepithelial NaCl absorption in cortical collecting ducts of mice (22).

Taken together, our in vitro and in vivo data reveal that in mice the reduction in urine volume (antidiuretic action) with HCTZ in Li-NDI is, at least partially, NCC independent and that this likely involves inhibition of CA activity. This knowledge may lead to the development of improved treatment modalities for Li-NDI.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


