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Abstract

To reduce lithium-induced nephrogenic diabetes insipidus (lithium-NDI), bipolar patients are treated with thiazide/amiloride, which are thought to induce antidiuresis by a compensatory increased pro-urine uptake in proximal tubules. However, as thiazides induced an antidiuresis in lithium-NDI mice lacking the sodium-chloride co-transporter and alkalinized their urine, this concept was challenged and suggested that inhibition of carbonic anhydrases (CA) conferred the beneficial thiazide effect. Therefore, we here tested the effect of the CA-specific blocker acetazolamide in lithium-NDI.

In collecting duct (mpkCCD) cells, acetazolamide reduced the cellular lithium content and attenuated lithium-induced downregulation of AQP2 through a mechanism different from amiloride. Treatment of lithium-NDI mice with acetazolamide or thiazide/amiloride revealed a similar antidiuresis, increased urine osmolality and AQP2 abundance. The reduced inulin clearance and cortical NHE3 expression demonstrated that the antidiuresis with acetazolamide was partially due to a tubular-glomerular feedback (TGF) response and reduced GFR. The TGF response and/or direct effect on collecting duct principal or intercalated cells may underlie the reduced urinary PGE2 levels with acetazolamide, thereby contributing to the attenuated lithium-NDI. Thiazide/amiloride-treated mice showed hyponatremia, hyperkalemia, hypercalcaemia, metabolic acidosis and increased serum lithium concentrations; side effects also observed in patients, but not seen in acetazolamide-treated mice.

In conclusion, we have shown that CAs activity contributes to lithium-NDI development, that acetazolamide attenuates lithium-induced NDI by inducing a TGF response and through a direct action on collecting duct cells, and that acetazolamide attenuates lithium-NDI development similar to thiazide/amiloride but with less side effects.

Introduction

Lithium is the drug of choice for the treatment of bipolar disorders and is also regularly used to treat schizoaffective disorders and depression. Lithium is a frequently prescribed drug as it is provided to 0.1% of the western population. Unfortunately, in 2-85% of patients, and depending on age, lithium usage leads to nephrogenic diabetes insipidus (Li-NDI), a disorder characterized by an impaired response of the kidney to vasopressin (AVP) leading to polyuria and polydipsia[1-3]. Patients with Li-NDI are at risk for dehydration-induced lithium toxicity, and prolonged lithium treatment might lead to cyst formation and end stage renal disease[4]. However, cessation of lithium therapy is not an option for most patients with NDI, because bipolar disorder symptoms have a larger impact on the patient's quality of life.

From studies in rats, it became clear that Li-NDI develops in two stages. At short term (10 days), Li-NDI coincides with downregulation of aquaporin-2 (AQP2) water channels, which is due to a reduced AQP2 transcription[5-7]. Despite an increased proliferation of the AQP2-expressing principal cells of the collecting duct, long-term lithium treatment (4 weeks) also results in a severe loss of AQP2-expressing principal cells, which might be attributed to a lithium-induced G2/M phase cell cycle arrest[8, 9]. This principal cell loss is 'compensated' by an increased number of α-intercalated cells, which are involved in acid secretion[8].

To reduce polyuria in patients receiving lithium, a low-sodium diet together with thiazide and amiloride diuretics is prescribed[10]. Amiloride acts on the principal cell epithelial sodium channel (ENaC) and we and others found that amiloride blocks principal cell lithium entry through ENaC, thereby attenuating polyuria in rodents and humans[11-13]. Thiazides are known to block sodium and chloride reabsorption via the NaCl co-transporter (NCC) in the renal distal convoluted tubule and the antidiuretic effect has been ascribed to a hypovolemia-induced activation of the renin-angiotensin-aldosterone system (RAAS) and a compensatory increased uptake of sodium and water in proximal tubules. Recently, however, we discovered

that thiazide also has an NCC-independent effect, as NCC knockout mice with Li-NDI showed a clear antidiuretic response upon treatment with thiazide[14].

As urine of our thiazide-treated mice was alkalinized and thiazides are derived from carbonic anhydrase (CA) inhibitors[15], our data indicated that the antidiuretic effect of thiazide in Li-NDI may involve CA inhibition. CAs catalyse the hydration of carbon dioxide to form carbonic acid, which then rapidly dissociates to form protons and bicarbonate, and play major roles in pH balance regulation. Here, we show that CAs are indeed involved in lithium-induced AQP2 downregulation and that, by inducing a tubular glomerular feedback response and through direct action on collecting duct cells, the CA-selective drug acetazolamide not only attenuates Li-NDI, but yields superior *in vivo* effects as compared to the presently-used treatment for Li-NDI.

Results

The clinically used drug acetazolamide attenuates lithium-induced downregulation of AQP2 in mpkCCD cells

MpkCCD cells are mouse collecting duct cells showing dDAVP-dependent expression of endogenous AQP2 and we have shown that thiazide reduces lithium-induced downregulation of AQP2 in these cells, while they lack NCC expression[7, 11, 14]. As our previous animal studies suggested that thiazides reduced polyuria in our NCC knockout mice by inhibiting CAs, we wanted to test whether acetazolamide, a stable CA inhibitor and commonly used in patients, could also rescue lithium-induced AQP2 downregulation in mpkCCD cells. Indeed, whereas lithium again reduced the AQP2 abundance in mpkCCD cells, acetazolamide significantly attenuated this downregulation (Fig. 1A, B). As our data suggest that both thiazide and acetazolamide influence lithium-reduced AQP2 abundances through CAs, we assessed whether the action mechanism of acetazolamide differs from that of amiloride. If so, we anticipated that acetazolamide and amiloride together should attenuate the lithium-induced AQP2 downregulation better than cells treated with amiloride only. Indeed, immunoblotting revealed a significantly higher AQP2 abundance in cells treated with amiloride and acetazolamide as compared to amiloride only (Fig. 1C, D). Others and we discovered that ENaC is the main cellular entry site for lithium and that amiloride strongly reduced the intracellular lithium levels in mpkCCD cells[11, 14]. Determination of the intracellular lithium concentrations revealed that amiloride indeed reduced the intracellular lithium concentration with 87%, while this was only 30% with acetazolamide (Fig. 1E). The mean intracellular lithium concentration was nominally lower with amiloride/acetazolamide; however, there was no significant difference (p=0.23). Transcellular transport of sodium and potassium via ENaC, ROMK and the Na/K-ATPase is electrogenic and therefore generates a transcellular voltage

(Tv) over mpkCCD cell monolayers. Lithium slightly reduced the Tv, which was not further decreased with acetazolamide (Fig. 1F). In contrast, amiloride completely blocked the Tv in lithium-treated mpkCCD cells. Together, these data reveal that the CA-specific inhibitor acetazolamide attenuates lithium-induced downregulation of AQP2 *in vitro* and that its mechanism of action is different from that of amiloride.

Acetazolamide attenuates development of lithium-induced NDI in mice

To investigate whether acetazolamide attenuates development of Li-NDI, mice were maintained on lithium chow only or on lithium combined with acetazolamide or thiazide/amiloride for 10 days. As reported[11, 16], mice treated with lithium developed severe polyuria and polydipsia, combined with a significantly-reduced urine osmolality (Fig. 2A-C). Interestingly, acetazolamide treatment induced a significant antidiuresis and increase in urine osmolality, which was slightly, but not significantly, better than in mice treated with thiazide/amiloride. Consistent with the induced antidiuresis, water intake was significantly reduced with the acetazolamide treatment as compared to lithium only.

As long-term lithium treatment coincides with reduced AQP2 and increased H^+-ATPase abundance in the kidney[6], we also analyzed their abundances. Immunoblot analysis revealed that lithium reduced AQP2 abundance (Fig. 3A, B), which was significantly attenuated by both acetazolamide and thiazide/amiloride (Figs. 3A-D). With acetazolamide or thiazide/amiloride, however, AQP2 levels did not return to control levels. H^+-ATPase levels were similar for all groups (Supplementary Figs. 1A-D), which is in line with the notion that lithium-induced collecting duct remodeling is not present after 10 days of lithium treatment in rodents[9, 16].

To examine segment-specific effects of the different therapies on AQP2 abundance, immunohistochemistry was performed. Consistent with our immunoblot data, lithium

treatment strongly reduced AQP2 staining, which was clearly attenuated in kidneys of mice treated with lithium and acetazolamide or thiazide/amiloride (Fig. 3E). Interestingly, while lithium abolished AQP2 expression in the entire kidney, the increased AQP2 abundance in the thiazide/amiloride treated group mainly localized to the inner medulla of the kidney, whereas in the acetazolamide treated mice, AQP2 abundance was increased along the connecting tubule and entire collecting duct. Consistent with our immunoblot data, immunohistochemistry revealed no clear changes in H^+-ATPase labeling between the different groups (Supplementary Fig. 2).

Acetazolamide shows an improved overall electrolyte balance over thiazide/amiloride.

Some patients on thiazide/amiloride therapy have been reported to develop hyponatremia, hyperkalemia, metabolic acidosis and/or hypercalcemia[17-20]. Moreover, initiating thiazide/amiloride treatment in Li-NDI patients often leads to elevated blood lithium levels, necessitating adjustment of the lithium dose[21]. Therefore, to assess and compare the effect of acetazolamide and thiazide/amiloride on these parameters, we analyzed blood and urine for lithium and electrolyte levels (Table 1). Indeed, while mice treated with thiazide/amiloride had a reduced bodyweight and developed hyponatremia, hyperkalemia, and a metabolic acidosis, these parameters were not affected in acetazolamide-treated mice. Also, thiazide/amiloride treatment induced hypercalcemia, which was significantly reduced with acetazolamide, although not to control levels. Moreover, and consistent with patients, serum lithium concentrations were significantly increased in our thiazide/amiloride mice, but were unchanged in our acetazolamide mice as compared to lithium controls.

The antidiuretic effect of acetazolamide coincides with a lowered GFR and a reduced prostaglandin E2 release

Paradoxically, the clear antidiuresis found in our acetazolamide group (Figs. 2A-C) coincided with a significantly-increased creatinine clearance (Fig. 4A). However, as the creatinine clearance in especially mice highly depends on both creatinine secretion and reabsorption in the proximal tubule, the segment mainly influenced by acetazolamide[22, 23], we hypothesized that acetazolamide may affect proximal tubular creatinine reabsorption/secretion in our mice and thus that the creatinine clearance did not properly reflect the GFR. Therefore we used FITC-inulin to determine the GFR in an identically-performed animal experiment. While acetazolamide again significantly attenuated Li-NDI (not shown), the clearance of FITC-inulin was significantly reduced with acetazolamide (Fig. 4B), indicating that acetazolamide reduced the GFR. Urinary prostaglandin E2 (PGE2), which extensively contributes to AQP2 downregulation in Li-NDI[24], was significantly increased in our Li-NDI mice, but was fully attenuated in our mice treated with lithium/acetazolamide (Fig. 4C). Moreover and consistent with its CA inhibitory action in proximal tubules, acetazolamide further increased urinary pH (Fig. 4D) and strongly reduced the abundance of NHE3 in the renal cortex as compared the cortex of mice treated with lithium only (Fig. 4E, F).

Discussion

Acetazolamide is superior to thiazide/amiloride to attenuate lithium-NDI.

Our mouse studies revealed that acetazolamide attenuates development of Li-NDI to the same extent as thiazide/amiloride, but, in contrast to acetazolamide, our thiazide/amiloride treated mice developed hyponatremia, hyperkalemia, and metabolic acidosis. In humans, hyponatremia is mostly a consequence of upregulated AQP2 expression by high circulating AVP levels. Although AVP levels are elevated in Li-NDI, this cannot explain hyponatremia in our thiazide/amiloride treated mice, because AQP2 is downregulated here. Instead, our data indicate that the hyponatremia is due to the induced natriuresis caused by thiazide and amiloride in a status of polyuria and polydipsia, because our Li-NDI mice were normonatremic and our thiazide/amiloride mice were highly natriuretic as compared to the other groups. Note, however, that part of the increased natriuresis must be due to an increased consumption of salt from the provided salt block, because food intake was not increased. The mice apparently drank water to satiety, because the hematocrit was not different between the groups (not shown). Similarly, congenital NDI patients also sometimes develop hyponatremia when treated with thiazide combinations[25, 26].

The observed hyperkalemia is likely due to inhibition of ENaC by amiloride, as renal secretion of potassium occurs only in exchange of ENaC-mediated sodium reabsorption[27]. Lithium itself can lead to metabolic acidosis[28], which would even be increased with inhibition of bicarbonate uptake by acetazolamide, but we only found metabolic acidosis in our thiazide/amiloride treated mice. Whereas metabolic acidosis in our acetazolamide group may have been compensated by increased ventilation, metabolic acidosis in the thiazide/amiloride group may be secondary to the observed hyperkalemia, as mammals attenuate hyperkalemia at the expense of development of metabolic acidosis[29].

Our Li-NDI mice developed hypercalcemia, which was sustained in our thiazide/amiloride treated mice, but not in our acetazolamide mice. Hyperparathyroidism is common with lithium-using patients and the occurrence of hypercalcemia in Li-NDI has been ascribed to inhibition of calcium-sensing receptor signaling by lithium in the parathyroid[30-32]. The corrected blood calcium levels with acetazolamide, however, may be unrelated to the parathyroid effect of lithium, because plasma calcium is increased by bone resorption, a process that involves CA2 activity in osteoclast and that is strongly inhibited by acetazolamide[33, 34].

Another important advantage of the use of acetazolamide over thiazide/amiloride is that plasma lithium concentrations remained unchanged with acetazolamide. Blood lithium is mainly set by the amount reabsorbed in proximal tubules, a process in which the apical NHE3 is highly involved and which is stimulated by thiazide[35, 36]. Considering this, the unaltered blood lithium levels with acetazolamide is best explained by combinatory effects of the reduced NHE3 abundance in proximal tubules and the reduced GFR.

Acetazolamide attenuates Li-NDI by a dual mode of action

Our data indicate that the observed antidiuresis and reduced GFR with acetazolamide is due to a tubular glomerular feedback (TGF) response caused by inhibition of CAs in the proximal tubule[37, 38]. Ninety percent of renal HCO_3^- is reabsorbed in proximal tubules, which is strongly facilitated by CA4/14 (luminal/apical), CA2 (intracellular) and CA4/12 (basolateral) hydrating CO_2 and dehydrating H_2CO_3[39]. In this process, secretion of protons by NHE3 is important. By blocking these CA, acetazolamide prevents the intracellular generation of H^+, which is needed for NHE3 to reabsorb filtered Na^+[40-43]. The consequently increased tubular salt and water load in the proximal tubule leads to an increased fluid delivery and tubular $[Cl^-]$ at the macula densa, which induces a TGF response (i.e. a reduced GFR)[38]. Indeed,

acetazolamide in our Li-NDI mice led to an increased urinary pH, reduced NHE3 abundance and reduced GFR and are in agreement with reported data on NHE3 knockout mice[38, 41, 44].

Besides intercalated cells (see below), the increased fluid delivery to the macula densa with acetazolamide may also partially explain the observed lower urinary levels of PGE2, thereby attenuating Li-NDI. By acting on EP1/3 receptors, increased urinary PGE2 levels in Li-NDI reduce principal cell AQP2 expression and thus water reabsorption[45]. In Li-NDI, a fraction of the elevated urinary PGE2 levels is thought to be derived from macula densa and surrounding cTAL cells, which produce PGE2 to increase renin synthesis and release in response to a reduced fluid delivery to the TAL/hypovolemia[43, 44]. As such, the increased fluid delivery to the TAL with acetazolamide will reduce the cortical release of PGE2 and therewith Li-NDI.

However, our *in vitro* data indicate that acetazolamide also directly protects collecting duct cells from lithium, but it is at present unclear whether *in vivo* acetazolamide acts directly on principal cells or indirectly via intercalated cells. Support for the first is that mpkCCD cells endogenously express and show proper regulation of the typical principal cell proteins, AQP2 and ENaC. Moreover, mpkCCD cells express high CA2 mRNA levels (http://esbl.nhlbi.nih.gov/mpkCCD-transcriptome/), which is also expressed in principal cells *in vivo*[39, 46-48]. Also, the *in vivo* activity of ENaC, the lithium entry site of principal cells, has been reported to be functionally paired with CA activity, as, CA inhibition by acetazolamide reduced the intracellular pH and reduced ENaC activity in sweat duct cells and colon[49, 50].

However, mpkCCD cells may not fully represent principal cells and, as intercalated cells express abundant levels of CAs 2, 4, 12, and 15[39], acetazolamide may increase principal cell AQP2 expression and water uptake indirectly by inhibiting ACs in intercalated cells. Indeed, long-term lithium treatment leads to metabolic acidosis, which underlies the increased number of α-intercalated cells[11, 51] and, as lithium inhibits intercalated cell H^+-ATPase and H^+/K^-

ATPase activity[52, 53], it has been suggested that acidosis-induced proliferation of α-intercalated cells may contribute to Li-NDI[16]. It is unlikely, however, that attenuation of Li-NDI in our mice is due to direct action of acetazolamide on α-intercalated cells or collecting duct remodeling for several reasons: At first, acetazolamide increases the number of α-intercalated cells in rodents, as it causes acidosis itself[54, 55]. Secondly and consistent with the unchanged H^+-ATPase expression in our mice, collecting duct remodeling is not observed with 10 days of lithium treatment, but only starts at about 4 weeks of treatment[9, 56].

An effect of acetazolamide on β-intercalated cells, however, may be more likely. While lithium treatment did not reduce the number of these cells[57], exciting recent studies revealed the existence of extensive cross-talk between β-intercalated and principal cells in the regulating collecting duct function[58-61]. While Eladari *et al* elegantly showed that in rodents, the sodium-dependent chloride bicarbonate exchanger (NDCBE/SLC4a8) and chloride bicarbonate exchanger protein pendrin allow for NaCl reabsorption through β-intercalated cells, chloride permeation through pendrin also appeared necessary for ENaC mediated sodium reabsorption and expression[62]. Also, in mice lacking functional intercalated cell-specific H^+-ATPase, the observed natriuresis and aquaresis was due to dysfunctional and lower abundances of ENaC and pendrin/NDCBE, and reduced AQP2 levels, respectively[60]. The authors further showed that the lack/inhibition of β-intercalated cells H^+-ATPase led, via flow-stimulated luminal ATP release, to autocrine and paracrine release of PGE2, which reduced the cortical and medullary ENaC activity and AQP2 abundance. Importantly, thiazides inhibited NCC-independent NaCl reabsorption through NDCBE/pendrin[59] and acetazolamide reduced pendrin abundance[63], drugs we showed to attenuate Li-induced downregulation of AQP2 and Li-NDI through a similar mechanism. As such, the attenuated Li-NDI with acetazolamide, which was given during the entire lithium treatment, may be due to an impaired functioning of pendrin, resulting in increased ATP/PGE2 release, reduced

ENaC activity in principal cells, and thus reduced influx of lithium from pro-urine. The finding that acetazolamide is beneficial chiefly in the cortical segments that contain intercalated cells is consistent with the possibility that intercalated cell CAs could be involved. A prime candidate here is CA12, as it is highly sensitive to acetazolamide and patients with reduced CA12 activity have a preponderance to hyponatremic dehydration[64]. Whether one of these mechanisms underlies the beneficial effect of acetazolamide in Li-NDI remains to be studied.

Taken together, we have shown that CAs activity contributes to Li-NDI development, that acetazolamide attenuates lithium-induced NDI by inducing a TGF response and through a direct action on the collecting duct, and that acetazolamide attenuates Li-NDI development similar to thiazide/amiloride but with less side effects.

Concise methods

Cell culture

MpkCCD cells were cultured as described[65]. Cells were seeded at a density of 1.5×10^5 cells/cm^2 on semi-permeable 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 of dDAVP at the basolateral side for the last 96 hr, to induce AQP2 expression. Lithium and compounds were administered as indicated. At the end of the experiment, transcellular electrical resistance and voltage were measured using a Millicell-ERS meter (Millipore corp., Bedford, MA, USA). On day 8, cells were harvested and lysed in Laemmli buffer for western blotting or stored in Trizol reagent (Invitrogen, Carlsbad, CA) at -80°C for RNA-isolation.

Lithium assays

Determination of intracellular lithium concentrations was done as described[11]. Shortly, mpkCCD$_{cl4}$ cells were grown on 4.7 cm^2 filters. To determine the extent of lithium contamination from the extracellular side, fluorescein isothiocyanate (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 iso-osmotic sucrose (pH 7.3) at 4°C and cells were lysed by sonication in 1 ml milli-Q water. Of 800 μl sample, the amount of lithium was determined by flame photometry, from which the total amount of lithium in the sample was calculated.

Of 100 μl sample, the amount of FITC-dextran was measured using spectrofluorophotometry (Shimadzu RF-5301, Japan) at 492 nM (excitation) and 518 nm (emission) wavelengths. By comparing the obtained values with a two-fold FITC-dextran dilution series, the FITC-dextran concentration in each sample was determined, from which the extent of extracellular lithium contamination was calculated. This was subtracted from the total amount to obtain the

intracellular lithium amount. With the used FITC-dextran concentration, a contamination above 1:5000 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 the Biorad Protein Assay (München, Germany).

Experimental animals

8-10 weeks old female C57Bl6/JOlaHsd mice (Harlan Laboratories, The Netherlands) were maintained in a temperature-controlled room with lights on 8.00 AM and PM. They received normal diet (ssniff R/M-H V1534, ssniff Spezialdiaten GmbH, Soest, Germany) with additions (see below) and water ad libitum for 10 days. For the experiments, mice were divided into four groups (n = 8), which were treated as follows: Group *1:* Control mice given a normal diet; Group *2:* Normal diet with 40 mmol LiCl /kg of dry food.[66] Group *3:* diet of group 2 with 200 mg amiloride[11] and 350 mg hydrochlorothiazide per kg dry food[36]. Group 4: diet of group 2 with 180 mg acetazolamide /kg dry food.[67] LiCl, amiloride, hydrochlorothiazide and acetazolamide were solubilized in water and mixed with the chow after which it was dried. All mice had free access to water, food, and a sodium-chloride block. For the last 48 hr of the experiment, mice were housed in metabolic cages to measure water intake and urine output during the last 24 hr. Mice were anesthetized with isofluorothane after which their blood was removed by orbita extraction. Then, mice were killed by cervical dislocation and the kidneys rapidly removed. One kidney was processed for immunohistochemistry, whereas the other kidney was used for immunoblotting, both as described below. For immunoblotting, the tissue was homogenized using a Polytron homogenizer (VWR international, Amsterdam, The Netherlands) in 1 ml of ice-cold homogenization buffer A (20 mM Tris, 5 mM $MgCl_2$, 5 mM Na_2HPO_4, 1 mM EDTA, 80 mM sucrose and protease inhibitors (1 mM PMSF, 5 µg/ml pepstatin A, 5 µg/ml leupeptin, and 5

μg/ml a-protinin) and cleared from nuclei and unbroken cells by centrifugation at 4000 x g for 15 min and diluted in Laemmli buffer to a final protein concentration of 1 μg/μl.

Determination of glomerular filtration rate using FITC-inulin.

To determine the glomerular filtration rate via the FITC-inulin clearance method [68, 69] we used mice as described above and these mice were also treated as before (n=8/group). Four days after the start of the diet, minipumps (Model 2001, Alzet) containing 3% FITC inulin were subcutaneously implanted in the isofluorane-anesthetized mice. At treatment days 9 and 10, mice were housed in metabolic cages and 24 hr urine was collected in amber tubes at day 10. During this 24 hr, metabolic cages and urine collection tubes were covered with aluminium foil to prevent exposure to light. Traces of left FITC-inulin urine in metabolic cages were added to the collected urine by washing the cage with 5 mL of 500 mmol HEPES buffer. On day 10, mice were anesthetized with isofluorane, blood was collected by retro-orbital bleeding and mice were killed by cervical dislocation. Urine fluorescence was determined using a Cytofluor II fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham, MA, USA) with 485 nm excitation and 538 nm emission. The excretion rate of inulin (24 hr urinary fluorescence counts/plasma fluorescence counts per ml) was taken as the GFR.

Blood and urine analysis

Whole blood was analyzed immediately for sodium, potassium, hematocrit, and pH using the EG7+ cartridge and the I-Stat Clinical Analyzer (Abbott BV, Hoofddorp, The Netherlands). The remaining blood was collected in a BD microtainer SST tube (REF #365968, Becton Dickinson BV, Breda, The Netherlands) for serum and centrifuged at 10.000 x 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 CX5 analyzer (Beckman Coulter, Brea, CA), following the manufacturer's protocols. Urine PGE2 levels were determined by measuring stable PGE2 metabolite (PGEM) after chemical derivation of PGE2 and its primary metabolites, 13,14-dihydro-15-keto PGE2 and 13,14-dihydro-15-keto PGA2, to the single PGEM compound. PGEM concentrations were determined with the prostaglandin E metabolite EIA kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions.

Immunoblotting

MpkCCD cells from 1.13 cm^2 filters were lysed in 200 µl Laemmli buffer and sonicated. MpkCCD lysate and 5-10 µg kidney material in Laemmli were denatured for 30 min at 37°C. Protein concentration was determined using the BioRad protein assay (Bio-Rad Laboratories GmbH, München, Germany), according to manufacturer's instructions. SDS-PAGE, blotting and blocking of the PVDF membranes were done as described[70]. Membranes were incubated for 16 hr at 4°C with 1:2000 affinity-purified rabbit pre-c-tail AQP2 antibody recognizing aa 236-255,[71] in Tris-Buffered Saline Tween-20 (TBS-T) supplemented with 1% non-fat dried milk. In an identical way, other blots were incubated with a rabbit CA12 antibody (kind gift from Prof. William S. Sly, Saint Louis University School of Medicine, St. Louis, MO, USA) and a rabbit CA2 antibody (Abcam, Cambridge, UK). After washing in TBS-T, all blots were incubated for 1 hr with 1:5000-diluted goat anti-rabbit IgG's (Sigma, St. Louis, MO) as secondary antibody coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Densitrometric analyses were performed using Biorad quantification equipment (Bio-Rad 690c densitometer, Chemidoc XRS) and software (QuantityOne, Bio-Rad Laboratories GmbH, München, Germany). Equal loading of the samples was confirmed by staining of the blots with Coomassie blue.

Immunohistochemistry

Kidneys were fixed by immersion for 24h in 4% paraformaldehyde in 0.1M phosphate buffer at 4°C, embedded in paraffin and cut into 3-4 μm-thick sections. After de-paraffinization, sections were placed into a microwave oven and heated for 10 minutes 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 1:80000-diluted rabbit polyclonal AQP2 antibodies or 1:2000-diluted rabbit polyclonal H^+-ATPase antibodies as described[72, 73]. The bound primary antibodies were revealed with Cy3-coupled goat-anti rabbit IgG (Jackson ImmunoResearch Laboratories, PA, USA). To check for unspecific binding of primary or secondary antibodies, incubations with non immune sera or without any primary antibodies were performed. All control experiments were negative. Cryosections were studied by epifluorescence using a Leica microscope (Wetzlar, Germany). Connecting tubules and cortical collecting ducts were distinguished based on their specific localization in the cortical labyrinth and the medullary rays, respectively. Images were acquired with a charge-coupled device camera. For overviews, single images were taken with the automated scanning mode of the microscope and afterwards stitched using the Leica Application Suite. Digital images were processed electronically with Adobe Photoshop and Microsoft Powerpoint software. Adjustments for brightness and contrast were kept constant for each kidney section.

Statistics

One-way ANOVA with Bonferroni correction was applied. A *p* value of less than 0.05 was considered significant. Data are presented as mean and standard error of the mean (SEM).

Study approval

All animal studies (DEC nr 2011-010) were approved by the Animal Ethical Committee of the Radboud University Medical Center.

Statement of competing financial interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figure legends

Figure 1. **Acetazolamide reduces lithium-induced downregulation of AQP2 abundance in mpkCCD cells.**

Native mpkCCD cells were grown to confluence for 4 days and subsequently exposed to 1 nM dDAVP for another 4 days. During the last 2 days, cells were incubated in the absence (Ctr) or presence of lithium only (Li^+) or with lithium and 100 μM acetazolamide (Acz), 10 μM amiloride (Am), 100 μM hydrochlorothiazide and amiloride (T+Am) or amiloride and acetazolamide (Am+Acz). At the basolateral and apical side, final concentrations of 1 and 10 mM lithium were used, respectively. Following measurements of transcellular voltage (**F**), cells were lysed and subjected to AQP2 immunoblotting (**A**, **C**). Molecular masses (in kDa) are indicated. The signals for non-glycosylated (29 kDa) and complex-glycosylated (40-45 kDa) AQP2 were densitometrically quantified (**B**, **D**). Mean values +/- SEM of normalized AQP2 abundance are given relative to control. (**E**) Intracellular lithium concentrations were determined, which were corrected for contamination with extracellular lithium and normalized for the amount of protein ($[Li^+]$ +/- SEM in pmol/μg protein). Data from 3 independent experiments, one way ANOVA, Bonferroni's Multiple Comparison Test, *$p < 0.05$.

Figure 2. **The antidiuretic effect of thiazide/amiloride and acetazolamide treatment on lithium-treated mice.** Urine volume (**A**), water intake (**B**) and urine osmolality (**C**) of untreated mice (CTR) or of mice treated for 10 days with lithium (Li^+) or in combination with thiazide/amiloride (T+Am) or acetazolamide (Acz). During the last 48 hr mice were housed in metabolic cages and during the last 24 hr water intake was measured and urine was collected

to determine urine volume and osmolality. n=8 mice/group, one way ANOVA, Bonferroni's Multiple Comparison Test, *p<0.05.

Figure 3. **Thiazide/amiloride and acetazolamide reduce lithium-induced downregulation of AQP2 in lithium-NDI mice.** (**A-D**) Immunoblot and corresponding densitometric analysis of AQP2 of mouse kidneys that are untreated (Ctr), treated with lithium only (Li$^+$) or with lithium together with acetazolamide (Acz) or thiazide/amiloride (T+Am). The signals for AQP2 densitometrically quantified (**B** and **D**). Mean values +/- SEM of normalized AQP2 abundance is given relative to control. Asterisks indicate significant differences (p<0.05) from controls. Equal loading of the samples was confirmed by staining of the blots with coomassie blue (Cm). One way ANOVA, Bonferroni's Multiple Comparison Test, *p<0.05. (**E**) Paraffin sections of immersion-fixed kidneys from control (Ctr; A, E, I), lithium-treated (Li$^+$; B, F, J), lithium/amiloride/thiazide-treated (Li$^+$ + T + Am; C, G, K), and lithium/acetazolamide-treated mice (Li$^+$ + Acz; D, H, L) were incubated with a rabbit polyclonal AQP2 antibody followed by a Cy3-coupled goat-anti rabbit IgG. Overviews (A-D) and high magnifications of representative connecting tubules (CNT; E-H), and cortical collecting ducts (CCD; I-L).

Figure 4. **The effect of acetazolamide on urinary pH, NHE3 abundance, glomerular filtration rate and prostaglandin-E2 release.** Mice were treated for 10 days with control diet (Ctr) or diet containing lithium (Li$^+$) only or lithium in combination with acetazolamide (Acz). During the last 48 hr, mice were housed in metabolic cages and during the last 24 hr urine was collected to determine creatinine clearance (**A**), PGE2 levels (**C**) and urinary pH (**D**). At day 10, mice were sacrificed and blood and kidneys were isolated, enabling the analysis of renal NHE3 abundance (**E, F**). In (**E**) the arrow indicates the ~85kDa band of NHE3. To measure GFR, using FITC-inulin, the above-mentioned experiment was repeated,

however at day 4 osmotic minipumps containing FITC-inulin, were implanted and at day 10 FITC-inulin levels were measured in 24 hr urine and serum (**B**). n=8 mice/group, one way ANOVA, Bonferroni's Multiple Comparison Test, *p<0.05.

Table 1. Metabolic parameters of mice treated for 10 days with standard chow only (Ctr) or together with lithium (Li⁺), lithium + thiazide + amiloride (Li⁺ + T + Am), or lithium + acetazolamide (Li⁺ + Acz).

	Ctr	Li$^+$	Li$^+$ + Am + T	Li$^+$ + Acz
Serum Osmolality (mOsm/kg)	320 ± 1	319 ± 1	311 ± 0.5**	321 ± 3 †
Serum Sodium (mmol/l)	150 ± 0.3	149 ± 0.4	139 ± 0.8**	150 ± 0.5†
Serum Potassium (mmol/l)	5.3 ± 0.1	5.6 ± 0.2	7.6 ± 0.5**	5.4 ± 0.2†
Serum Lithium (mmol/l)	/	0.63 ± 0.04*	2.11 ± 0.12**	0.69 ± 0.04†
Serum Creatinine (mg/dl)	0.08 ± 0.01	0.09 ± 0.00	0.06 ± 0.01**	0.04 ± 0.01**/†
Blood Ionized Calcium (mmol/l)	1.24 ± 0.00	1.32 ± 0.01*	1.34 ± 0.01	1.27 ± 0.01**/†
Blood pH	7.34 ± 0.01	7.32 ± 0.01	7.24 ± 0.02**	7.35 ± 0.02†
Urine sodium (mmol/l)	352 ± 40	46 ± 6*	484 ± 68 **	112 ± 12†
Urine potassium (mmol/l)	870 ± 83	131 ± 15*	154 ± 19	271 ± 31**/†
Urine lithium (mmol/l)	/	20 ± 3*	24 ± 3	38 ± 8
Urine Creatinine (mg/dl)	70 ± 7	10 ± 1*	14 ± 2	18 ± 2**
Total sodium excretion (mmol/24 hr)	0.17 ± 0.02	0.16 ± 0.01	1.26 ± 0.12**	0.22 ± 0.02†
Total potassium excretion (mmol/24 hr)	0.42 ± 0.05	0.55 ± 0.02*	0.40 ± 0.03**	0.54 ± 0.05
Total lithium excretion (mmol/24 hr)	/	84 ± 3*	64 ± 6	72 ± 11
Bodyweight (g)	18.9 ± 0.4	18.0 ± 0.2*	16.0 ± 0.5**	17.4 ± 0.4†
Food Intake (mg/g/24 hr)	214 ± 8	198 ± 10	188 ± 5	228 ± 5**/†
Feces Production (mg/g/24 hr)	111 ± 7	98 ± 8	82 ± 2	123 ± 9†

Li⁺, lithium; T, thiazide; Am, amiloride; Acz, acetazolamide.

Values are means ± s.e.m.

*   P<0.05 compared to control treatment

**   P<0.05 compared to lithium treatment

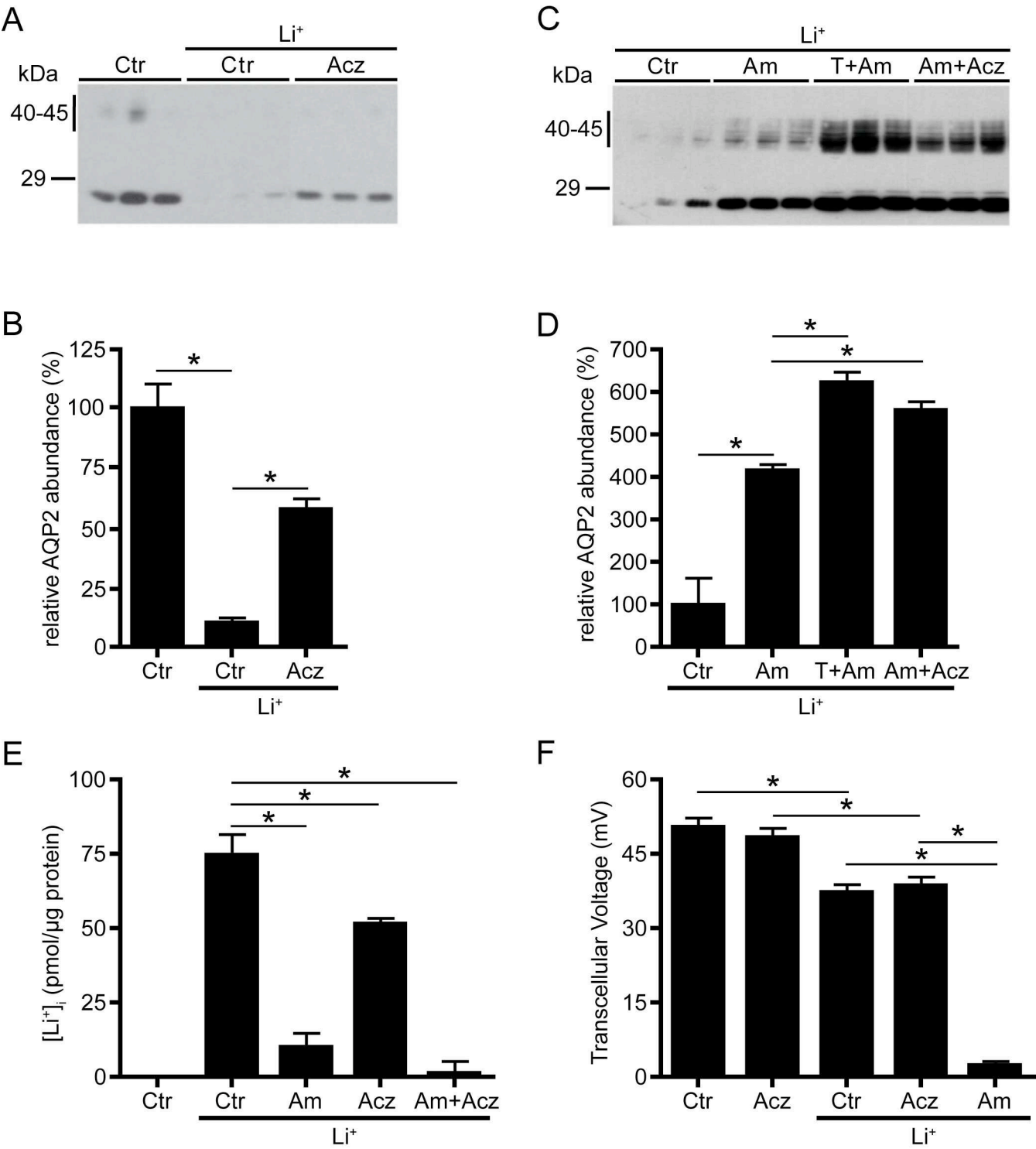† P<0.05 compared to lithium/thiazide/amiloride treatment
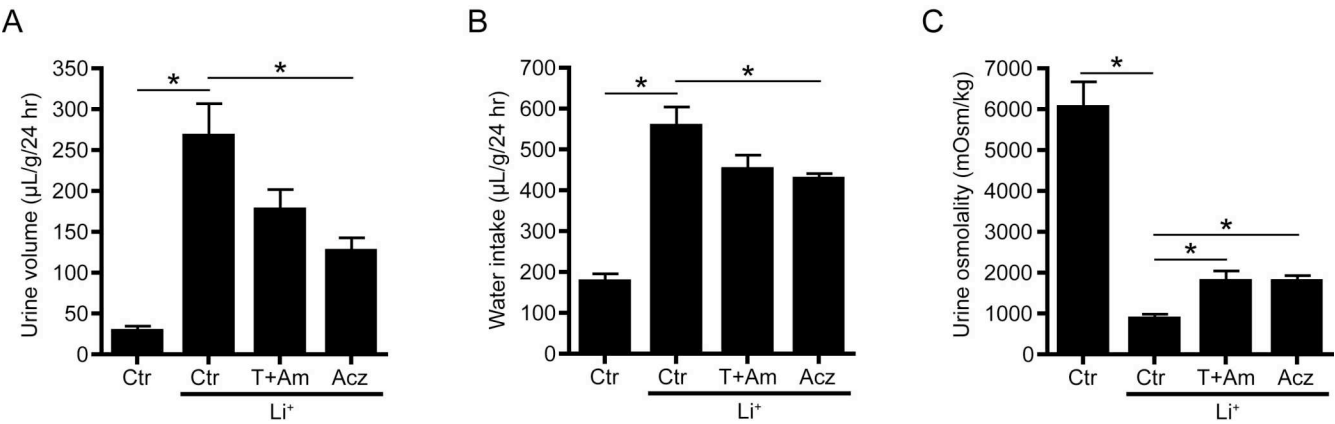
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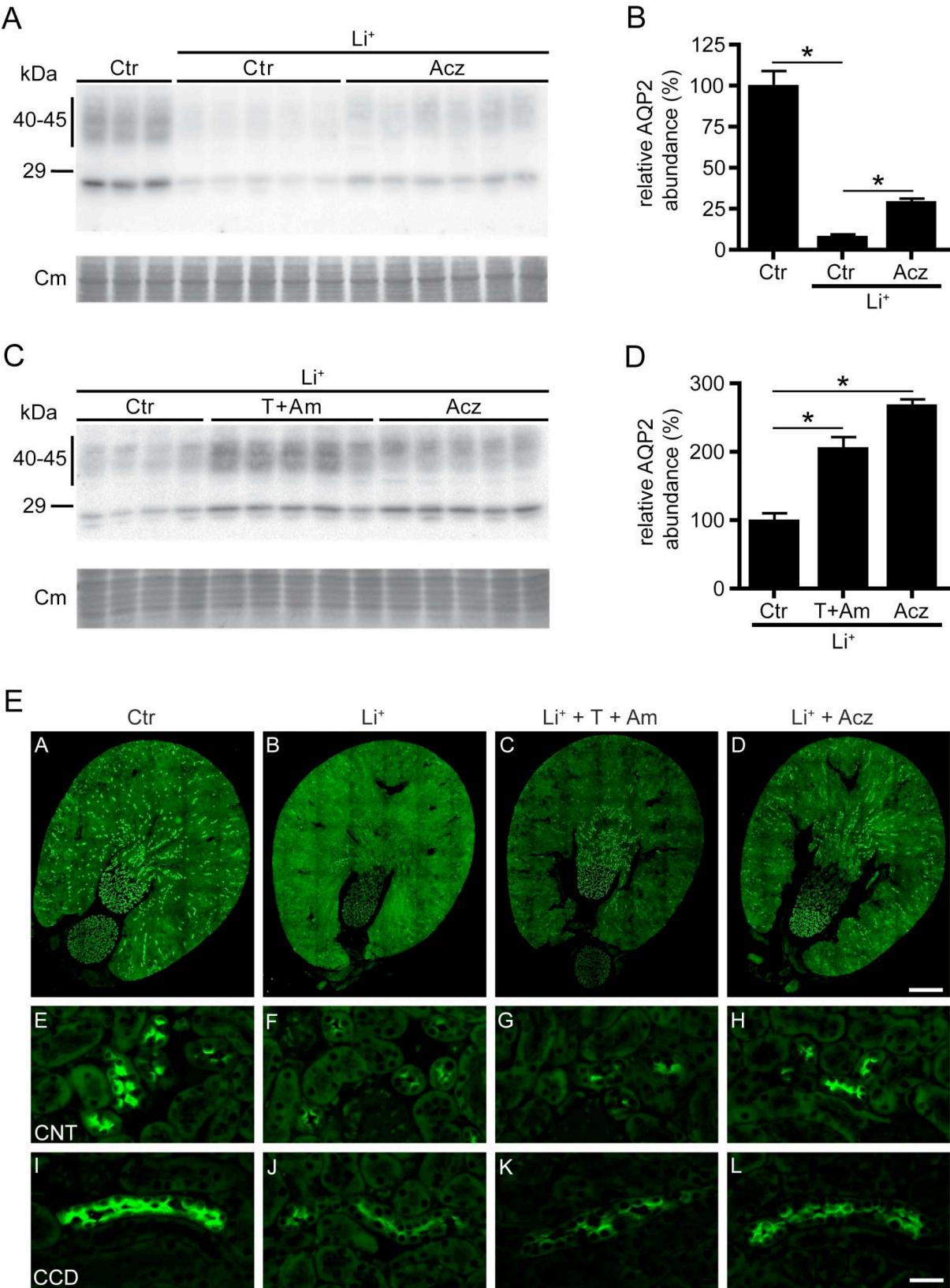
Figure 1

Figure 2

Figure 3

Figure 4