Clopidogrel attenuates lithium-induced alterations in renal water and sodium channels/transporters in mice

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Abstract Lithium (Li) administration causes deranged expression and function of renal aquaporins and sodium channels/transporters resulting in nephrogenic diabetes insipidus (NDI). Extracellular nucleotides (ATP/ADP/UTP), via P2 receptors, regulate these transport functions. We tested whether clopidogrel bisulfate (CLPD), an antagonist of ADP-activated P2Y₁₂ receptor, would affect Li-induced alterations in renal aquaporins and sodium channels/transporters. Adult mice were treated for 14 days with CLPD and/or Li and euthanized. Urine and kidneys were collected for analysis. When administered with Li, CLPD ameliorated polyuria, attenuated the rise in urine prostaglandin E₂ (PGE₂), and resulted in

significantly higher urinary arginine vasopressin (AVP) and aldosterone levels as compared to Li treatment alone. However, urine sodium excretion remained elevated. Semi-quantitative immunoblotting revealed that CLPD alone increased renal aquaporin 2 (AQP2), Na-K-2Cl cotransporter (NKCC2), Na-Cl cotransporter (NCC), and the subunits of the epithelial Na channel (ENaC) in medulla by 25–130 %. When combined with Li, CLPD prevented downregulation of AQP2, Na-K-ATPase, and NKCC2 but was less effective against downregulation of cortical α - or γ -ENaC (70 kDa band). Thus, CLPD primarily attenuated Li-induced downregulation of proteins involved in water conservation (AVP-sensitive), with modest effects on aldosterone-sensitive proteins potentially explaining sustained natriuresis. Confocal immunofluorescence microscopy revealed strong labeling for P2Y₁₂-R in proximal tubule brush border and blood vessels in the cortex and less intense labeling in medullary thick ascending limb and the collecting ducts. Therefore, there is the potential for CLPD to be directly acting at the tubule sites to mediate these effects. In conclusion, P2Y₁₂-R may represent a novel therapeutic target for Li-induced NDI.

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Introduction

Extracellular nucleotides (ATP/ADP/UTP), acting through purinergic type 2 (P2) receptors, regulate water and sodium transport in the mammalian kidney. They achieve this by virtue of the ability of purinergic signaling to oppose the actions of arginine vasopressin (AVP) and/or aldosterone in the kidney [1–8]. It is believed that the autocrine and/or paracrine

effects mediated by extracellular nucleotides exert tonic inhibitory influence on the actions of these important hormones that regulate water and sodium conservation by the kidney and thus their homeostasis [5–9]. Conversely, one can deduce that the same purinergic signaling may play a significant role in the development of clinical conditions in which the sensitivity of the kidney to AVP or aldosterone is altered, such as acquired nephrogenic diabetes insipidus (NDI) or aldosterone escape. In fact, reports from our and other laboratories support this concept [3, 9–15].

Chronic administration of lithium for the treatment of bipolar disorder continues to be the major cause of acquired NDI in clinical patients [16]. Conversely, the use of lithium has been unraveling previously unknown phenomenon in renal physiology [17]. Previously using mice lacking $P2Y_2$ receptor, we demonstrated the significant role played by this receptor in the genesis of polyuria, natriuresis, and kaliuresis associated with lithium-induced NDI [12, 13]. The $P2Y_2$ receptor is UTP/ATP-activated and a G_q/G_{11} (and possibly G_i)-coupled receptor, which enhances phosphoinositide signaling and reduces cellular cAMP levels. These signaling pathways explain the opposing action of $P2Y_2$ receptor toward AVP. While these findings offer a potential drug target for the treatment of lithium-induced NDI, drugs that antagonize $P2Y_2$ receptor in vivo are not currently available, thus limiting our ability to test this hypothesis further and the usefulness of this approach.

A different purinergic receptor, the $P2Y_{12}$ receptor, is an ADP-activated receptor predominantly expressed in blood platelets and microglia and astrocytes in the brain. It is coupled to G_i/G_o , and its activation inhibits adenylyl cyclase and thus reduces cellular cAMP levels. $P2Y_{12}$ receptor activation also enhances phosphoinositide signaling [18] and plays a critical role in platelet activation and clot formation. The anti-clotting drug or blood thinner, clopidogrel bisulfate acts by inhibiting $P2Y_{12}$ receptors on platelets. Clopidogrel (CLPD) has been in clinical use for more than 15 years, and it has been proven to be safe. CLPD is a pro-drug activated in the liver by cytochrome P450 enzymes (CYP2C19) generating its active metabolite (Act-Met), which constitutes about 15 % of the ingested drug. The Act-Met irreversibly binds to the $P2Y_{12}$ receptor by forming disulfide bridges and thus prevents its activation. Apart from platelets and microglia and astrocytes in the brain, $P2Y_{12}$ receptor messenger RNA (mRNA) expression has been reported in several organs or tissues, including the kidney [19]. Detailed studies conducted in our laboratory confirmed the expression of $P2Y_{12}$ receptor mRNA in all regions of the rodent kidney and protein in several structures of the rodent kidney [20]. Based on these findings and the ability of $P2Y_{12}$ receptor to decrease cellular cAMP levels and enhance phosphoinositide signaling, we hypothesized that administration of CLPD could have a significant ameliorating effect on lithium-induced alterations in renal

water and sodium channels/transporters in the kidneys of mice. Here, we present experimental evidence that supports our hypothesis by documenting that CLPD significantly ameliorates lithium-induced NDI in mice, apparently by normalizing the alterations in the major water and sodium channels or transporters or exchangers along the nephron and collecting duct. We also show the localization of $P2Y_{12}$ receptor protein in tubular segments and vasculature of the mouse kidney, which suggests that the observed beneficial effects of CLPD on lithium-induced NDI are potentially mediated through the $P2Y_{12}$ receptor.

Methods

Experimental animals The animal procedures described here were approved by the Institutional Animal Care and Use Committees (IACUCs) of the Veterans Affairs Salt Lake City Health Care System, Salt Lake City, Utah and the University of Southern California, Los Angeles, California. Except during urine collections, all mice were housed in standard plastic cages with bedding and regulated light–dark cycles. Throughout the experimental period, the mice had free access to food and drinking water.

Li-induced nephrogenic diabetes insipidus This was performed at the VA Salt Lake City Health Care System by the methods established in our laboratory [12, 13, 20, 21]. Specific pathogen-free B6D2 mice (males, aged 3 to 4 months) obtained from the in-house breeding colony were randomly divided into four groups (five to seven mice/group). There were no significant differences in the mean body weights among the groups. Group 1 (control mice) were fed regular rodent chow; group 2 received clopidogrel bisulfate (CLPD; 80 mg/kg bw/day) in drinking water; group 3 was fed lithium chloride-added diet (40 mmol/kg chow); and group 4 received a combination of lithium in food and CLPD in drinking water. Lithium diet was custom prepared by MP Biomedicals (Solon, OH). Clopidogrel bisulfate was administered orally by mixing finely powdered tablets (Bristol-Myers Squibb, New York, NY) in drinking water. The concentration of the drug in the drinking water was adjusted daily based on the water consumption of the animals on the previous day. Variations in water consumption between two consecutive days are very low and are unlikely to result in a significant difference in the consumption of the amount of drug. When compared with the human clinical dose of CLPD and adjusted to the K_m factor (ratio of body surface area to body weight) of the species (man vs. mouse) [22], the dose used was approximately 5-fold higher than the human effective dose (HED). Toxicological evaluation of CLPD showed that doses as high as 165 mg/kg bw/day for up to 4 weeks in rats did not produce toxicity [23]. The experimental period lasted for 14 days at which point the

mice were humanely euthanized. Twenty-four hour urine samples were collected for two consecutive days before and toward the end of the experimental period. Blood samples were collected at the time of euthanasia, and serum was separated after clotting. Kidneys were harvested at necropsy, and cortical and medullary regions of the kidneys were dissected out, flash frozen, and then processed for laboratory assays.

Effect of delayed administration of CLPD on lithium-induced polyuria This was performed at the VA Salt Lake City Health Care System. After collecting the 24-h baseline urine samples (day 0), two groups of age-matched male B6D2 mice were fed lithium-added diet for 5 days. Twenty-four hour urine samples were collected again on day 5 and analyzed for urine output and osmolality. After confirming the onset of lithium-induced polyuria, one group of mice ($N=5$) continued feeding on lithium-added diet. The other group ($N=7$) was administered CLPD (80 mg/kg bw/day) in drinking water in addition to lithium-added food. Urine output and osmolality were monitored in both groups on days 14 and 21, and then, the mice were humanely euthanized.

Urine and serum analysis Aliquots of urine samples were centrifuged at $1000\times g$ to obtain clear supernatants. Osmolality of the clear supernatants were determined by the vapor pressure method (Wescor, Logan, UT). Urinary sodium and serum lithium levels were measured with an *EasyLyte* (Medica, Bedford, MA) analyzer. Urine AVP and aldosterone concentrations were determined by ELISA kits (Enzo Life Sciences, Farmingdale, NY), and urinary PGE2 excretion was quantified by EIA (Cayman Chemical Co., Ann Arbor, MI) as described previously [12, 13, 24, 25].

Western blot analysis of kidney tissue samples Cortical and medullary tissue samples were processed separately and analyzed for protein abundances of aquaporin-2 (AQP2) and sodium transporters or channels by semi-quantitative immunoblotting as previously described [13, 25]. Briefly, samples were prepared by homogenizing the frozen tissues in a buffer containing protease inhibitors. After determining the protein concentrations, the homogenates were solubilized in Laemmli sample buffer. Quality of tissue sample preparation was assessed by staining loading gels with Coomassie blue (Gelcode Blue, Pierce Endogen, Rockford, IL), and then examining the sharpness of the bands. For immunoblotting for sodium transporter/channel proteins, aliquots of samples were shipped to Georgetown University, Washington, DC. Blots were run by loading 10–30 μg of protein from each sample into individual lanes of minigels of 7.5, 10, or 12 % polyacrylamide (precast, Bio-Rad, Hercules, CA or Novex, Life Technologies, Grand Island, NY). After electrotransfer of size-fractionated proteins to nitrocellulose membranes, blots were probed with our own peptide-derived rabbit polyclonal

antibodies against AQP2, sodium-hydrogen exchanger-type 3 (NHE3), the bumetanide-sensitive Na-K-2-CL cotransporter (NKCC2), the thiazide-sensitive Na-Cl cotransporter (NCC), the Na phosphate cotransporter-type 2 (NaPi-2), and the three subunits of the epithelial sodium channel (ENaC, α , β , and γ), as previously described [13, 25]. Our polyclonal antibodies were based on immunizing peptides originally designed, characterized, and published by Dr. Mark Knepper, a leader in the field of polyclonal antibodies directed to various channel and transporter proteins in the kidney. The production of these peptide-derived antibodies was outsourced to commercial companies. Upon characterization in immunoblots in our laboratory, these antibodies gave the same band pattern as published by Dr. Knepper. We used commercially available antibodies against the α -1 subunit of Na-K-ATPase (05–369, Millipore Corp., Temecula, CA). Loading accuracy was evaluated by probing the blots with β -actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO or Cell Signaling Technology, Inc., Danvers, MA). Band densities of transporter/channel proteins were determined and normalized to the densities of the respective β -actin bands.

Localization of P2Y₁₂-R protein by confocal immunofluorescence We designed, generated, and characterized a peptide-derived rabbit polyclonal antibody which detects both rat and mouse P2Y₁₂ receptor [20]. Confocal immunofluorescence microscopy was performed at the University of Southern California by methods described previously [20, 26, 27]. Kidneys of specific pathogen-free mice (C57BL6J) from the in-house breeding colony were fixed in situ by perfusion of 4 % paraformaldehyde, followed by overnight immersion in the same fixative at 4 °C. Kidney tissues were embedded in paraffin; 4- μm sections were cut, deparaffinized, and rehydrated. To retrieve antigens, slides were heated for 2×10 min in a microwave with medium heat in phosphate-buffered saline (PBS) and allowed to cool for 40 min. Sections were then fixed with 4 % paraformaldehyde for 10 min and permeabilized for 10 min with 0.1 % Triton X-100 in PBS. Goat serum (1:20 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS was applied to sections for 1 h to block non-specific binding. Sections were then probed with P2Y₁₂ receptor rabbit polyclonal antibody overnight followed by incubation with secondary Alexa fluor 594-conjugated goat anti-rabbit antibody (Invitrogen) for 1 h. Some sections were double labeled first by incubation with AQP2 goat polyclonal antibody (sc-9882 from Santa Cruz, CA) for 1 h at room temp (1:100 dilution), followed by incubation for 1 h with donkey anti-goat secondary Alexa fluor 488-conjugated antibody (1:500 dilution). Other sections were double labeled first by incubation with a NKCC monoclonal mouse antibody (recognizes both NKCC1 and NKCC2, kind gift from Dr. Lytle at University of California at Riverside) for 1.5 h at room temperature (1:2000 dilution), followed by

incubation for 1 h with donkey anti-mouse secondary Alexa fluor 594-conjugated antibody (1:500 dilution). 4',6-Diamino-2-phenylindole (DAPI) was used to stain the nuclei.

Statistical analysis Quantitative data are expressed as mean $\pm$ SEM. Data are comprehensively analyzed using both one- and two-way analysis of variance (ANOVA) and displayed to reveal contribution by the two experimental conditions, namely treatment with lithium or CLPD independently, and by their interactions when administered in combination. Differences between individual pairs of means were determined by a multiple comparisons test (Holm–Sidak or Kruskal–Wallis analysis of variance on ranks) following a significant ($p < 0.05$) one-way ANOVA. Kruskal–Wallis was used when data were not normally distributed or variances were not equal. Differences due to the main factors, lithium or CLPD administration, and their interactions were determined by two-way ANOVA using Sigma Stat Software (Chicago, IL). When only two groups with unequal sample size (n) were present (Fig. 7), Mann–Whitney non-parametric method was used. p Values for two-way ANOVA were shown in the table below the figures. Results of one-way ANOVA multiple comparisons testing are shown above the bars, as “A,” “AB,” “B,” “C,” etc. Means with letters in common are not different from each other. The letter A is assigned to the nominally highest means, and they are significantly different from means assigned the letter, B alone, but not AB, etc. Means assigned AB are neither different from means assigned A or B. Multiple comparisons testing are only done when the one-way ANOVA p value for all groups is less than 0.05.

Results

Effect of CLPD on lithium-induced polyuria and the decrease in AQP2 protein abundance in the kidney As shown in the Fig. 1a, b, administration of lithium induced polyuria, as assessed by marked increase in urine output associated with significant decrease in urine osmolality. Administration of CLPD significantly reversed the lithium-induced alterations in urine output and osmolality and significantly reduced lithium-induced water consumption (4.2 ± 0.6 lithium alone vs. 3.0 ± 0.4 lithium + CLPD, ml/day/20 g body weight; $p < 0.01$). Furthermore, administration of CLPD alone caused a modest decrease in urine output associated with a significant increase in urine osmolality. Thus, CLPD per se appears to increase the urinary concentrating ability of the kidney. In order to assess whether the observed effects of CLPD on the urinary concentrating ability are associated with changes in the protein abundances of collecting duct water channel AQP2, we performed semi-quantitative immunoblotting on the whole tissue homogenates of medullary and cortical regions of the kidneys of the mice subjected to treatment with CLPD and/or lithium. As shown in Fig. 1c, d, lithium

treatment resulted in a modest decrease in AQP2 protein abundance in the medulla. Administration of CLPD increased AQP2 protein to levels higher than the untreated control animals. Administration of CLPD alone resulted in over a 2-fold increase in AQP2 protein abundance in the medulla. A similar pattern of changes in AQP2 protein were seen in the cortical tissue homogenates (Fig. 1e, f). Thus, overall, the administration of CLPD prevented lithium-induced polyuria and increased AQP2 protein levels higher than the control values, especially in the medulla.

Proximal tubule sodium transporters and exchangers

Figure 2 shows representative Western blots for cortex homogenates and the densitometry summary of what would be primarily proximal tubule-expressed proteins. Lithium caused a significant downregulation of the α -1 subunit of Na-K-ATPase which was partially restored by CLPD. For the sodium hydrogen exchanger (NHE3), CLPD tend to reduce its abundance in the absence of lithium but increased it when lithium was present. The abundance of the sodium phosphate cotransporter (NaPi-2) was not significantly affected by CLPD or lithium.

TAL and DCT sodium transporters and exchangers

Figure 3 displays representative blots of medulla and cortex (as indicated) and the densitometry summary for thick ascending limb (TAL) and distal convoluted tubule (DCT) sodium transporters and exchangers. Even in the absence of lithium, CLPD led to a significant increase in the abundances of the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2) and the thiazide-sensitive Na-Cl cotransporter. In addition, in combination with lithium, CLPD prevented the decrease in the abundances of these proteins or even restored them to higher levels. There were no significant effects of treatment on medullary NHE3 or α -1 Na-K-ATPase levels (primarily TAL-associated).

The ENaC Representative blots for the ENaC subunits and densitometry summaries are shown in Fig. 4 (cortex) and Fig. 5 (medulla). In cortex (Fig. 4), lithium reduced the abundance of α -ENaC. This reduction was not restored by CLPD. Lithium or CLPD had no effect on β -ENaC, but lithium led to a reduction in both the 85 and the 70 kDa bands associated with γ -ENaC. CLPD abolished the reduction in the 85 kDa band and attenuated the reduction in the 70 kDa band. In medulla (Fig. 5), lithium did not downregulate any of the subunits of ENaC; however, CLPD led to a significant upregulation of α - and γ -ENaC (85 kDa band) in the absence of lithium. The rises were attenuated in the presence of lithium.

Urinary excretion of PGE2, AVP, aldosterone, and sodium

Lithium-induced NDI has been ascribed to resistance of the kidney to arginine vasopressin (AVP), apparently due to increased production of renal PGE2 among other factors. So, in

Fig. 1 Effect of clopidogrel on lithium-induced polyuria and decrease in AQP2 protein abundance in the kidney. Groups of mice (five to seven mice/group) were fed lithium-added diet and/or addition of clopidogrel (CLPD) to drinking water for 14 days and euthanized. Control (CNT) mice did not receive any treatment. Whole tissue homogenates of renal medulla or cortex were immunoblotted for AQP2 and β -actin proteins. AQP2 band densities were normalized by corresponding β -actin band densities. **a** Urine output, **b** urine osmolality, **c**, **d** AQP2 protein immunoblot profile for medullary tissue homogenates and the corresponding normalized band densities. **e**, **f** AQP2 protein immunoblot profile for cortical tissue homogenates and the corresponding normalized band densities. Statistical significance, determined by two-way ANOVA, is shown at the bottom of each set of data. Results of multiple comparisons testing following a significant one-way ANOVA are shown as *letters above bars* on graphs. “A” was assigned to the highest mean and is significantly higher than “B,” but not “AB,” and “C” is significantly different from “B”; CNT controls, CLPD clopidogrel

order to assess the role of these two in the amelioration of lithium-induced polyuria by CLPD, we determined the urinary excretion of PGE2 and AVP in the 24-h collections, which represent an overall production of these two during the entire day. As expected, lithium feeding caused marked (6-fold) increase in urinary PGE2, which was reduced to slightly less than 4-fold by CLPD administration (Fig. 6a). The administration of lithium did not affect urinary AVP significantly, although resulted in a modest increase (Fig. 6b). However, the combination of CLPD with lithium induced marked increase (5.3-fold) in urinary AVP levels as compared to the control group. Interestingly, the administration of CLPD per se increased the urinary AVP levels by 2-fold (Fig. 6b).

In order to explore whether a change in the production of aldosterone plays a role in the observed alterations in the ENaC subunits and NCC, we determined urinary aldosterone excretion. As shown in Fig. 6c, lithium feeding decreased urinary aldosterone levels, which were restored by the administration of CLPD. Despite elevated aldosterone and vasopressin levels, CLPD administration did not ameliorate lithium-induced natriuresis (Fig. 6d). Finally, administration of CLPD modestly but significantly increased blood lithium levels (0.38 ± 0.01 vs. 0.54 ± 0.05 mmol/l, $p < 0.02$).

Effect of delayed administration of CLPD on lithium-induced polyuria We evaluated whether delayed

Fig. 2 Effect of clopidogrel and/or lithium treatment on protein abundances of proximal tubular sodium transporters/channels in mice. Immunoblots for different proximal tubular sodium transporters/exchangers protein abundances in (upper panel). Bar graph summary of the densitometric analysis of the immunoblots shown in the upper panel (lower panel). Bars show percent changes in band densities of different proteins in treated groups relative to the corresponding values in control mice (100 %). Statistical significance, determined by two-way ANOVA, is shown at the bottom of the bar graphs. Results of multiple comparisons testing following a significant one-way ANOVA are shown as letters above bars on graphs. *N.S.* no significant differences, *CNT* controls, *CLPD* clopidogrel

administration of CLPD can reverse lithium-induced polyuria. Feeding lithium for 5 days induced significant degree of polyuria, as assessed by increase in urine output and decrease in urine osmolality, which did not change significantly by prolonging lithium feeding period for 21 days (Fig. 7). When one cohort of mice was administered CLPD starting from day 5, there was significant amelioration of lithium-induced polyuria (Fig. 7).

Confocal immunofluorescence imaging of P2Y₁₂ receptor in normal mouse kidney Using confocal immunofluorescence imaging, we evaluated whether the observed effects of CLPD on lithium-induced alterations in various membrane transporters/channels/exchangers along the nephron and collecting duct could be mediated via its known pharmacological target, the ADP-activated P2Y₁₂ receptor. Figure 8 panels a–c show predominant expression of P2Y₁₂ receptor on the brush border of proximal tubules and arterioles, in addition to weaker expression in the cortical collecting ducts (along with

Fig. 3 Effect of clopidogrel and/or lithium treatment on protein abundances of TAL and DCT sodium transporters in mice. Immunoblots for protein abundances of different sodium transporters/exchangers of thick ascending limb (TAL) and distal convoluted tubule (DCT) (upper panel). Bar graph summary of the densitometric analysis of the immunoblots shown in the upper panel (lower panel). Bars show percent change in band densities of different proteins in treated groups relative to the corresponding values in control mice (100 %). Statistical significance, determined by two-way ANOVA, is shown at the bottom of the bar graphs. Results of multiple comparisons testing following a significant one-way ANOVA are shown as letters above bars on graphs. *N.S.* no significant differences, *CNT* controls, *CLPD* clopidogrel

AQP2 protein). Panels d–f of Fig. 8 depict weaker expression (as compared to proximal tubules) of P2Y₁₂ receptor in the medullary thick ascending limb, mostly on the basolateral aspect, while the expression of NKCC2 is predominantly on the apical domain. Panels g–i of Fig. 8 show the expression of both P2Y₁₂ receptor and AQP2 in the medullary collecting duct cells, often colocalizing on the apical domain. Thus, P2Y₁₂ receptor is expressed in the renal tubular segments that are involved in the transport of water and sodium, albeit at lower levels than vasculature and proximal tubule.

Fig. 4 Effect of clopidogrel and/or lithium treatment on protein abundances of ENaC subunits in the cortical collecting duct of mice. Immunoblots for protein abundances of subunits of epithelial sodium channel (ENaC) in the cortical collecting duct (*upper panel*). Bar graph summary of the densitometric analysis of the immunoblots shown in the *upper panel* (*lower panel*). Bars show percent change in band densities of different proteins in treated groups relative to the corresponding values in control mice (100 %). Statistical significance, determined by two-way ANOVA, is shown at the bottom of the bar graphs. Results of multiple comparisons testing following a significant one-way ANOVA are shown as letters above bars on graphs. CNT controls, CLPD clopidogrel

Discussion

In this work, we demonstrated that administration of CLPD, a selective and irreversible antagonist of P2Y₁₂ receptor, ameliorated lithium-induced polyuria and decrease in AQP2 protein abundance in the kidney and significantly attenuated the rise in urine PGE₂, which is often associated with lithium treatment in humans and experimental animals [21, 28, 29]. In addition, we showed that CLPD treatment increased protein levels of the bumetanide-sensitive Na-K-2Cl cotransporter (NKCC2) and the thiazide-sensitive Na-Cl cotransporter (NCC) in the TAL and DCT, respectively, even in the absence of lithium. Furthermore, administration of CLPD was associated with higher urinary AVP and aldosterone levels as compared to lithium treatment alone. Confocal immunofluorescence microscopy using P2Y₁₂ receptor-specific antibody revealed expression of this receptor in proximal tubule brush border, blood vessels, medullary TAL, and collecting duct,

Fig. 5 Effect of clopidogrel and/or lithium treatment on protein abundances of ENaC subunits in medullary collecting duct of mice. Immunoblots for protein abundances of subunits of epithelial sodium channel (ENaC) in the medullary collecting duct (*upper panel*). Bar graph summary of the densitometric analysis of the immunoblots shown in the *upper panel* (*lower panel*). Bars show percent change in band densities of different proteins in of treated groups relative to the corresponding values in control mice (100 %). Statistical significance, determined by two-way ANOVA, is shown at the bottom of the bar graphs. Results of multiple comparisons testing following a significant one-way ANOVA are shown as letters above bars on graphs. N.S. no significant differences, CNT controls, CLPD clopidogrel

consistent with the effects of CLPD administration being mediated through P2Y₁₂ receptor in specific renal tubular segments.

A variety of reports support a role for extracellular nucleotides (ATP/ADP/UTP) in fine-tuning of salt and water reabsorption along the nephron and collecting duct [reviewed in 4–7, 11]. Most of these reports ascribed the effects of extracellular nucleotides to the UTP/ATP-activated P2Y₂ receptor, which is expressed widely and predominantly in the kidney. Previously, we documented that mice lacking P2Y₂ receptor are significantly resistant to lithium-induced polyuria, natriuresis, and kaliuresis [12, 13]. These effects seen in P2Y₂ receptor knockout mice are apparently related to the ability of this receptor to oppose the actions of both AVP and aldosterone [2, 7, 12, 13, 25, 30]. However, very little is known about the expression and function of the ADP-activated P2Y₁₂ receptor in the kidney, although its selective antagonist CLPD has been in the clinical use for more than 15 years. Taking a

Fig. 6 Effect of clopidogrel and/or lithium treatment on the urinary parameters. Groups of mice (five to seven mice/group) were fed Li-added diet and/or addition of CLPD to drinking water for 14 days and euthanized. Control mice did not receive any treatment. Twenty-four hour urine samples were collected prior to euthanasia and assayed. **a** Urinary excretion of prostaglandin E2 (PGE2), **b** urinary excretion of arginine

vasopressin (AVP), **c** urinary excretion of aldosterone, and **d** urinary excretion of sodium. Statistical significance, determined by two-way ANOVA, is shown at the bottom of the bar graphs. Results of multiple comparisons testing following a significant one-way ANOVA are shown as letters above bars on graphs. CLPD clopidogrel

Fig. 7 Delayed administration of clopidogrel reverses lithium-induced polyuria. Two groups of mice were fed lithium-added diet for 21 days. Starting from day 5, one group of mice ($N=7$) received clopidogrel in drinking water, in addition to lithium-added food (solid circles). The other group ($N=5$) received only lithium (solid squares). Urine output and osmolality were monitored periodically as shown on the x-axis. Asterisks indicate significant difference from the lithium-alone group on the corresponding day by Mann–Whitney non-parametric method

cue that signaling mediated through $P2Y_2$ and $P2Y_{12}$ receptors are somewhat similar in the sense that both decrease cellular cAMP levels, we started studying the expression of $P2Y_{12}$ receptor in the rodent kidneys and the effect of pharmacological blockade of it on lithium-induced NDI. Initially, using a peptide-derived polyclonal antibody specific for $P2Y_{12}$ receptor, we immunolocalized the receptor protein in rat kidney. We also showed that the pharmacological blockade of $P2Y_{12}$ receptor ameliorates lithium-induced polyuria in rats, as assessed by urinary parameters and AQP2 protein abundance in the kidney [20]. However, there are well-known differences between rat and mouse models of renal pathophysiology and their responses to drugs. Furthermore, lithium administration is known to affect not just renal aquaporins but also renal sodium transporters/channels as well. In this context, the novelty of the current study is that (i) it shows that the observed ameliorating effect of CLPD against lithium-induced NDI is not species-specific and can be observed in mice also and (ii) it comprehensively evaluates alterations in natriuresis and renal expression of the major sodium transporters, channels, and exchangers vis-à-vis the hormones and autocrine agents that are known to influence urinary excretion of water and sodium. Finally, the current work provides new information that may be relevant clinically, namely the ability of CLPD to reverse established lithium-induced polyuria.

Immunofluorescence labeling revealed the presence of $P2Y_{12}$ receptor protein in the major renal tubular segments involved in the absorption of water and sodium, such as proximal tubules, thick ascending limb, and collecting ducts. Interestingly, the intracellular localization of the receptor protein

Fig. 8 Confocal immunofluorescence imaging of P2Y₁₂ receptor in normal mouse kidney. **a–c** Profiles of cortical region showing P2Y₁₂ receptor labeling (red) in arterioles (white arrows) and brush border membrane (yellow arrowhead) and AQP2 labeling (green) on the apical domain of cortical collecting ducts (asterisks); merging of P2Y₁₂ and AQP2 fluorescence can be seen as yellow in **b** and **c**. **d–f** Profiles of outer medullary region showing P2Y₁₂ labeling (red) in thick ascending

limbs (TAL) (white arrows in **d**) and NKCC2 labeling (green; yellow arrowheads in **e**); overlay of P2Y₁₂ and NKCC2 protein fluorescence in TAL can be seen (white arrows in **f**). **g–i** Profiles of inner medullary region showing P2Y₁₂ labeling of collecting ducts (CD) (red; white arrows in **g**) and AQP2 protein (green; yellow arrowheads in **h**); merging of P2Y₁₂ and AQP2 fluorescence can be seen in CD (yellow; white arrows in **i**). Bar represents 20 μm

in these tubular segments is very different. Thus, in the proximal tubules, the P2Y₁₂ receptor is heavily expressed on the brush border, whereas in the medullary TAL, it is predominantly on the basal aspects. In the collecting duct cells, although distributed throughout the cells, the receptor protein clearly colocalized with AQP2 in the apical membrane. In the following paragraphs, we attempt to correlate the distribution pattern of P2Y₁₂ receptor with our findings on the protein expression of water and sodium channels and transporters in CLPD treated mice with/without lithium.

Intriguingly, administration of CLPD alone increased urinary concentrating ability, urinary AVP excretion, and protein abundances of AQP2 and NKCC2 in the cortex and medulla and NCC in the cortex, suggesting a potential role for P2Y₁₂ receptor in the regulation of urine concentration under basal conditions. One can also argue that the primary effect of CLPD could be due to increased AVP production. While that possibility cannot be ruled out, previously we showed that blockade of P2Y₁₂ receptor in primary cultures of rat inner medullary collecting duct (IMCD) cells by PSB-0739, a selective, potent, and reversible antagonist markedly potentiated the effect of desmopressin (dDAVP) on the expression of

AQP2 and production of cAMP [20]. Based on these findings, we postulated that blockade of P2Y₁₂ receptor relieves the tonic inhibitory effect of this receptor on cAMP production and thus enhances the effect of AVP/dDAVP [20]. These in vitro studies also provided direct experimental evidence functionally linking blockade of P2Y₁₂ receptor to augmented cAMP production and AQP2 expression in IMCD cells exposed to dDAVP. Hence, we speculate that blockade of P2Y₁₂ receptor may relieve the AVP-resistant state in lithium-induced NDI. The decreased production of PGE₂ as observed in the current study might have also lessened the development of AVP resistance induced by lithium.

Another important observation in this study is that CLPD effectively reversed polyuria even when administered after the onset of lithium-induced polyuria. Although this is presented in a shorter period study, if established in a longer period study, this finding illustrates a potential therapeutic use of CLPD for amelioration of polyuria in the clinic in patients that present with lithium-induced NDI.

With respect to the major sodium channels, exchangers, and transporters, we found that CLPD administration had a predominant effect on the protein abundances in TAL and

DCT with much more modest effects on collecting duct. It is surprising that despite its heavy expression in the brush border of proximal tubules, CLPD did not alter the protein abundance of NHE3 or NaPi-2; however, both of these proteins have also been demonstrated to be regulated by phosphorylation and/or changes in subcellular localization [31], which was not evaluated in this study. On the other hand, CLPD alone markedly increased the protein abundance of NKCC2 in both medulla and cortex and also attenuated the marginal lithium-induced decrease in this protein at both sites. Since AVP is known to increase NKCC2 protein abundance [32–34], the CLPD-induced increase in NKCC2 protein may be due to increased AVP levels. Maintenance or enhanced expression of NKCC2 would be expected to attenuate lithium-induced concentrating deficits, as observed here.

Similarly, the thiazide-sensitive NCC protein was markedly increased by CLPD alone and remained high despite lithium treatment. Furthermore, lithium had little effect on the abundance of this protein, in agreement with our previous work [13]. NCC protein levels are highly sensitive to increases in circulating aldosterone levels [35, 36]; therefore, it is possible that the trend for an increase in aldosterone with CLPD ($p=0.088$, two-way ANOVA) may have contributed to upregulation of NCC. It is not known whether the observed increase in NCC protein is due to increased AVP levels, although the latter is known to regulate NCC function [37].

In general, the protein levels of the ENaC subunits have not been shown to be as consistently sensitive to lithium, as, for example, AQP2, although all are collecting duct principal cell-expressed proteins [13, 38]. In fact, lithium is thought to mediate many of its effects on the urinary concentrating mechanism via its entry into the collecting duct principal cell through ENaC [39, 40]. This may be because one of the primary effects of lithium is to desensitize the collecting duct to AVP, and AQP2 expression is markedly affected by this signaling pathway [41]. In contrast, while the β - and γ -ENaC subunits have also been shown to be upregulated by AVP, the changes in expression (either mRNA or protein) with dDAVP, AVP administration, or thirsting, however, are modest [32, 42, 43]. Therefore, restoration of AVP sensitivity of the collecting duct and/or the rise in circulating AVP levels would be expected to have less effect on ENaC subunits than on AQP2 or NKCC2 proteins.

In contrast, lithium had a dramatic effect to downregulate the 70 kDa band of γ -ENaC (in cortex). This is potentially the cleaved or “activated” form of γ -ENaC [44, 45]. CLPD was able to partially, but not fully, restore this downregulation. Similarly, α -ENaC in the cortex was modestly but significantly reduced (~20 %) by lithium and not restored by CLPD. An increase in α -ENaC or the 70 kDa band of γ -ENaC has been demonstrated consistently on Western blots with low-sodium diet or aldosterone infusion in rodents [46–48]. In this regard, lithium treatment has also been associated with aldosterone

insensitivity of the renal tubule [38] although less is understood regarding the mechanism(s) underlying this effect. Nonetheless, the failure of CLPD to reverse these changes in ENaC subunits may suggest aldosterone insensitivity, which was not reversed by CLPD. This may have played a role in the fact that lithium-induced natriuresis was not attenuated. In fact, CLPD significantly increased urine sodium (two-way ANOVA).

Finally, the amelioration of lithium-induced alterations in mice observed here was not due to reduced intake of lithium resulting in lower blood lithium levels. On the other hand, similar to our previous report in rat model [20], administration of CLPD resulted in modest but significantly higher blood lithium levels in mice. In our previous report in the rat model, we also showed that CLPD did not alter lithium levels in inner medulla. However, for want of sufficient tissue, we could not assay inner medullary tissue lithium levels in the mice. But, the finding that despite higher levels of blood lithium levels, when administered with CLPD, the effects of lithium on the kidney are significantly ameliorated in the mice makes that assay unnecessary. We believe that these observations may find application in the clinic by reducing the lithium dose when combined with CLPD without compromising the therapeutic blood levels of lithium.

In summary, our study identified a significant role for the ADP-activated P2Y₁₂ receptor in renal transport functions in health and in lithium-induced nephrogenic diabetes insipidus and thus expanded the scope of purinergic regulation of renal transport functions.

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References

1. Rieg T, Bunday RA, Chen Y, Deschens G, Junger W, Insel PA, Vallon V (2007) Mice lacking P2Y₂ receptors have salt-resistant hypertension and facilitated renal Na⁺ and water reabsorption. *FASEB J* 21:3717–3726. doi:10.1096/fj.07-8807com
2. Zhang Y, Sands JM, Kohan DE, Nelson RD, Martin CF, Carlson NG, Kamerath CD, Ge Y, Klein JD, Kishore BK (2008) Potential role of purinergic signaling in urinary concentration in inner medulla: insights from P2Y₂ receptor gene knockout mice. *Am J*

- Physiol Renal Physiol 295:F1715–F1724. doi:[10.1152/ajprenal.90311.2008](https://doi.org/10.1152/ajprenal.90311.2008)
3. Wildman SS, Marks J, Turner CM, Yew-booth L, Peppiat-Wildman CM, King BF, Shirley DG, Wang W, Unwin RJ (2008) Sodium-dependent regulation of renal amiloride-sensitive currents by apical P2 receptors. *J Am Soc Nephrol* 19:731–742. doi:[10.1681/ASN.2007040443](https://doi.org/10.1681/ASN.2007040443)
 4. Vallon V (2008) P2 receptors in the regulation of renal transport mechanisms. *Am J Physiol Ren Physiol* 294:F10–F27. doi:[10.1152/ajprenal.00432.2007](https://doi.org/10.1152/ajprenal.00432.2007)
 5. Kishore BK, Nelson RD, Miller RL, Carlson NG, Kohan DE (2009) P2Y₂ receptors and water transport in the kidney. *Purin Signal* 5:491–499. doi:[10.1007/s11302-009-9151-5](https://doi.org/10.1007/s11302-009-9151-5)
 6. Prætorius H, Leipziger J (2010) Intrarenal purinergic signaling in the control of renal tubular transport. *Annu Rev Physiol* 72:377–393. doi:[10.1146/annurev-physiol-021909-135825](https://doi.org/10.1146/annurev-physiol-021909-135825)
 7. Vallon V, Rieg T (2011) Regulation of renal transport mechanisms. *Am J Physiol Ren Physiol* 301:F463–F475
 8. Leipziger J (2011) Luminal nucleotides are tonic inhibitors of renal tubular transport. *Curr Opin Nephrol Hypertens* 20:518–522. doi:[10.1097/MNH.0b013e3283487393](https://doi.org/10.1097/MNH.0b013e3283487393)
 9. Toney GM, Vallon V, Stockand JD (2012) Intrinsic control of sodium excretion in the distal nephron by inhibitory purinergic regulation of the epithelial Na⁺ channel. *Curr Opin Nephrol Hypertens* 21: 52–60. doi:[10.1097/MNH.0b013e32834db4a0](https://doi.org/10.1097/MNH.0b013e32834db4a0)
 10. Wildman SSP, Kang ESK, King BF (2009) ENaC, renal sodium excretion and extracellular ATP. *Purinergic Signal* 5:481–489. doi:[10.1007/s11302-009-9150-6](https://doi.org/10.1007/s11302-009-9150-6)
 11. Stockand JD, Mironova E, Bugaj V, Rieg T, Insel PA, Vallon V, Peti-Peterdi J, Pochynyuk O (2010) Purinergic inhibition of ENaC produces aldosterone escape. *J Am Soc Nephrol* 21:1903–1911. doi:[10.1681/ASN.2010040377](https://doi.org/10.1681/ASN.2010040377)
 12. Zhang Y, Pop IL, Carlson NG, Kishore BK (2012) Genetic deletion of the P2Y₂ receptor offers significant resistance to development of lithium-induced polyuria accompanied by alterations in PGE₂ signaling. *Am J Physiol Ren Physiol* 302:F70–F77. doi:[10.1152/ajprenal.00444.2011](https://doi.org/10.1152/ajprenal.00444.2011)
 13. Zhang Y, Li L, Kohan DE, Ecelbarger CM, Kishore BK (2013) Attenuation of lithium-induced natriuresis and kaliuresis in P2Y₂ receptor knockout mice. *Am J Physiol Ren Physiol* 305:F407–F416. doi:[10.1152/ajprenal.00464.2012](https://doi.org/10.1152/ajprenal.00464.2012)
 14. Zhang Y, Morris KL, Sparrow SK, Dwyer KM, Enjyoji K, Robson SC, Kishore BK (2012) Defective renal water handling in transgenic mice over-expressing human CD30/NTPDase1. *Am J Physiol Ren Physiol* 303:F420–F430. doi:[10.1152/ajprenal.00060.2012](https://doi.org/10.1152/ajprenal.00060.2012)
 15. Zhang Y, Robson SC, Morris KL, Heiney KM, Dwyer KM, Kishore BK, Ecelbarger CM (2015) Impaired natriuretic response to high-NaCl diet plus aldosterone infusion in mice over-expressing human CD39, an ectonucleotidase (NTPDase1). *Am J Physiol Renal Physiol* doi: [10.1152/ajprenal.00125.2014](https://doi.org/10.1152/ajprenal.00125.2014) [Epub ahead of print]
 16. Grünfeld JP, Rossier BC (2009) Lithium nephrotoxicity revisited. *Nat Rev Nephrol* 5:270–276. doi:[10.1038/nrneph.2009.43](https://doi.org/10.1038/nrneph.2009.43)
 17. Kishore BK, Ecelbarger CM (2013) Lithium: a versatile tool for understanding renal physiology. *Am J Physiol Ren Physiol* 304: F1139–F1149. doi:[10.1152/ajprenal.00718.2012](https://doi.org/10.1152/ajprenal.00718.2012)
 18. Dorsam R, Kunapalli SP (2004) Central role of the P2Y₁₂ receptor in platelet activation. *J Clin Invest* 113:340–345. doi:[10.1172/JCI200420986](https://doi.org/10.1172/JCI200420986)
 19. Pausch MH, Lai M, Tseng E, Paulsen J, Bates N, Kwak S (2004) Functional expression of human and mouse P2Y₁₂ receptors in *Saccharomyces cerevisiae*. *Biochim Biophys Res Commun* 324: 171–177. doi:[10.1016/j.bbrc.2004.09.034](https://doi.org/10.1016/j.bbrc.2004.09.034)
 20. Zhang Y, Peti-Peterdi J, Müller CE, Carlson NG, Baqi Y, Strasburg DL, Heiney KM, Villanueva K, Kohan DE, Kishore BK (2015) P2Y₁₂ receptor localizes in the renal collecting duct and its blockade augments arginine vasopressin action and alleviates nephrogenic diabetes insipidus. *J Am Soc Nephrol* pii: ASN.2014010118. [Epub ahead of print]
 21. Zhang Y, Nelson RD, Carlson NG, Kamerath CD, Kohan DE, Kishore BK (2009) Potential role of purinergic signaling in lithium-induced nephrogenic diabetes insipidus. *Am J Physiol Ren Physiol* 296:F1194–F1201. doi:[10.1152/ajprenal.90774.2008](https://doi.org/10.1152/ajprenal.90774.2008)
 22. Reagan-Shaw S, Nihal M, Ahmad N (2007) Dose translation from animal to human studies revisited. *FASEB J* 22:659–661. doi:[10.1096/fj.07-9574LSF](https://doi.org/10.1096/fj.07-9574LSF)
 23. Reist M, Rou-de Vos M, Montseny JP, Mayer KM, Carrupt PA, Berger Y, Testa B (2000) Very slow chiral inversion of clopidogrel in rats: a pharmacokinetic and mechanistic investigation. *Drug Metab Disp* 28:1405–1410
 24. Zhang Y, Kohan DE, Nelson RD, Carlson NG, Kishore BK (2010) Potential involvement of P2Y₂ receptor in diuresis of postobstructive uropathy in rats. *Am J Physiol Ren Physiol* 298: F634–F642. doi:[10.1152/ajprenal.00382.2009](https://doi.org/10.1152/ajprenal.00382.2009)
 25. Zhang Y, Lishrop R, Ecelbarger CM, Kishore BK (2011) Renal sodium transporter/channel expression and sodium excretion in P2Y₂ receptor knockout mice fed high-NaCl diet with/without aldosterone infusion. *Am J Physiol Ren Physiol* 300:F567–F668. doi:[10.1152/ajprenal.00549.2010](https://doi.org/10.1152/ajprenal.00549.2010)
 26. Hanner F, Lan L, Nguyen TX, Yu A, Peti-Peterdi J (2012) Intrarenal localization of the plasma membrane channel pannexin 1. *Am J Physiol Ren Physiol* 303:F1454–F1459. doi:[10.1152/ajprenal.00206.2011](https://doi.org/10.1152/ajprenal.00206.2011)
 27. Kishore BK, Zhang Y, Gevorgyan H, Kohan DE, Schiedel AC, Müller CE, Peti-Peterdi J (2013) Cellular localization of adenine receptors in the rat kidney and their functional significance in the inner medullary collecting duct. *Am J Physiol Ren Physiol* 305: F1298–F1305. doi:[10.1152/ajprenal.00254.2013](https://doi.org/10.1152/ajprenal.00254.2013)
 28. Allen HM, Jackson RL, Winchester MD, Deck LV, Allon M (1989) Indomethacin in the treatment of lithium-induced nephrogenic diabetes insipidus. *Arch Intern Med* 149:1123–1126. doi:[10.1001/archinte.1989.00390050095019](https://doi.org/10.1001/archinte.1989.00390050095019)
 29. Sugawara M, Hashimoto K, Ota Z (1988) Involvement of prostaglandin E₂, cAMP, and vasopressin in lithium-induced polyuria. *Am J Physiol Regul Integr Comp Physiol* 254:R863–R869
 30. Mironova E, Boiko N, Bugaj V, Kucher V, Stockand JD (2015) Regulation of Na⁺ excretion and arterial blood pressure by purinergic signalling intrinsic to the distal nephron: consequences and mechanisms. *Acta Physiol* 213:213–221. doi:[10.1111/apha.12372](https://doi.org/10.1111/apha.12372)
 31. McDonough AA (2010) Mechanisms of proximal tubule sodium transport regulation that link extracellular fluid volume and blood pressure. *Am J Physiol Regul Integr Comp Physiol* 298:R851–R861. doi:[10.1152/ajpregu.00002.2010](https://doi.org/10.1152/ajpregu.00002.2010)
 32. Knepper MA, Kim G-H, Fernández-Llama ECA (1999) Regulation of thick ascending limb transport by vasopressin. *J Am Soc Nephrol* 10:628–634
 33. Ecelbarger CA, Kim GH, Wade JB, Knepper MA (2001) Regulation of the abundance of renal sodium transporters and channels by vasopressin. *Exp Nephrol* 171:227–234. doi:[10.1006/exnr.2001.7775](https://doi.org/10.1006/exnr.2001.7775)
 34. Ares GR, Caceres PS, Ortiz PA (2011) Molecular regulation of NKCC2 in the thick ascending limb. *Am J Physiol Ren Physiol* 301:F1143–F1159. doi:[10.1152/ajprenal.00396.2011](https://doi.org/10.1152/ajprenal.00396.2011)
 35. Kim GH, Masilamani S, Turner R, Mitchell C, Wade JB, Knepper MA (1998) The thiazide-sensitive Na-Cl cotransporter is an aldosterone-induced protein. *Proc Natl Acad Sci U S A* 95: 14552–14555. doi:[10.1073/pnas.95.24.14552](https://doi.org/10.1073/pnas.95.24.14552)
 36. Eladiri D, Chambrey R, Picard N, Hadchouel J (2014) Electrochemical absorption of NaCl by the aldosterone-sensitive distal nephron: implications for normal electrolytes homeostasis and blood pressure regulation. *Cell Mol Life Sci* 71:2879–2895

37. Mutig K, Saritas T, Uchida S, Kahl T, Borowski T, Paliege A, Böhlick A, Bleich M, Shan Q, Bachmann S (2010) Short-term stimulation of the thiazide-sensitive $\text{Na}^+\text{-Cl}^-$ cotransporter by vasopressin involves phosphorylation and membrane translocation. *Am J Physiol Ren Physiol* 298:F502–F509. doi:[10.1152/ajprenal.00476.2009](https://doi.org/10.1152/ajprenal.00476.2009)
38. Nielsen J, Kwon TH, Frøkiær J, Knepper MA, Nielsen S (2006) Lithium-induced NDI in rats is associated with loss of α -ENaC regulation by aldosterone in CCD. *Am J Physiol Ren Physiol* 290:F1222–F1233. doi:[10.1152/ajprenal.00321.2005](https://doi.org/10.1152/ajprenal.00321.2005)
39. Kortenoeven ML, Li Y, Shaw S, Gaeggeler HP, Rossier BC, Wetzels JF, Deen PM (2009) Amiloride blocks lithium entry through sodium channel thereby attenuating the resultant nephrogenic diabetes insipidus. *Kidney Int* 76:44–53. doi:[10.1038/ki.2009.91](https://doi.org/10.1038/ki.2009.91)
40. Christensen BM, Zuber AM, Loffing J, Stehle JC, Deen PM, Rossier BC, Hummler E (2011) α -ENaC-mediated lithium absorption promotes nephrogenic diabetes insipidus. *J Am Soc Nephrol* 22:253–261. doi:[10.1681/ASN.2010070734](https://doi.org/10.1681/ASN.2010070734)
41. Kortenoeven ML, Schweer H, Cox R, Wetzels JF, Deen PM (2012) Lithium reduces aquaporin-2 transcription independent of prostaglandins. *Am J Physiol Cell Physiol* 302:C131–C140. doi:[10.1152/ajpcell.00197.2011](https://doi.org/10.1152/ajpcell.00197.2011)
42. Nicco C, Wittner M, DiStefano A, Jounier S, Bankir S, Bouby N (2001) Chronic exposure to vasopressin upregulates ENaC and sodium transport in the rat renal collecting duct and lung. *Hypertens* 38:1143–1149. doi:[10.1161/hy1001.092641](https://doi.org/10.1161/hy1001.092641)
43. Sauter D, Fernandes S, Goncalves-Mendes N, Boulkroun S, Bankir L, Loffing J, Bouby N (2006) Long-term effects of vasopressin on the subcellular localization of ENaC in the renal collecting system. *Kidney Int* 69:1024–1032. doi:[10.1038/sj.ki.5000211](https://doi.org/10.1038/sj.ki.5000211)
44. Bhalla V, Hallows KR (2008) Mechanisms of ENaC regulation and clinical implications. *J Am Soc Nephrol* 19:1845–1854. doi:[10.1681/ASN.2008020225](https://doi.org/10.1681/ASN.2008020225)
45. Svenningsen P, Friis UG, Bistrup C, Buhl KB, Jensen BL, Skøtt O (2011) Physiological regulation of epithelial sodium channel by proteolysis. *Curr Opin Nephrol Hypertens* 20:529–533. doi:[10.1097/MNH.0b013e328348bcc7](https://doi.org/10.1097/MNH.0b013e328348bcc7)
46. Masilamani S, Kim GH, Mitchell C, Wade JB, Knepper MA (1999) Aldosterone-mediated regulation of ENaC α , β , and γ subunit proteins in rat kidney. *J Clin Invest* 104:R19–R23
47. Masilamani S, Wang X, Kim GH, Brooks H, Nielsen J, Nielsen S, Nakamura K, Stoke JB, Knepper MA (2002) Time course of renal Na-K-ATPase, NHE3, NKCC2, NCC and ENaC abundance changes with dietary NaCl restriction. *Am J Physiol Ren Physiol* 283:F648–F657
48. Riazi S, Khan O, Tiwari S, Hu X, Ecelbarger C (2006) Rosiglitazone regulates ENaC and Na-K-2Cl cotransporter (NKCC2) in the obese Zucker rat. *Am J Nephrol* 26:245–257
49. Zhang Y, Carlson NG, Kishore BK, Ecelbarger CM (2013) Pharmacological blockade of P2Y₂ receptor reverses lithium-induced downregulation of NKCC2, NCC, and α -ENaC protein abundances in mice (Abstract). *J Am Soc Nephrol* 24:537A