Lithium reduces aquaporin-2 transcription independent of prostaglandins

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Kortenoeven ML, Schweer H, Cox R, Wetzels JF, Deen PM. Lithium reduces aquaporin-2 transcription independent of prostaglandins. Am J Physiol Cell Physiol 302: C131–C140, 2012. First published August 31, 2011; doi:10.1152/ajpcell.00197.2011.—Vasopressin (AVP)-stimulated translocation and transcription of aquaporin-2 (AQP2) water channels in renal principal cells is essential for urine concentration. Twenty percent of patients treated with lithium develop nephrogenic diabetes insipidus (NDI), a disorder in which the kidney is unable to concentrate urine. In vivo and in mouse collecting duct (mpkCCD) cells, lithium treatment coincides with decreased AQP2 abundance and inactivation of glycogen synthase kinase (Gsk) 3β. This is paralleled in vivo by an increased renal cyclooxygenase 2 (COX-2) expression and urinary prostaglandin PGE2 excretion. PGE2 reduces AVP-stimulated water reabsorption, but its precise role in lithium-induced downregulation of AQP2 is unclear. Using mpkCCD cells, we here investigated whether prostaglandins contribute to lithium-induced downregulation of AQP2. In these cells, lithium application reduced AQP2 abundance, which coincided with Gsk3β inactivation and increased COX-2 expression. Inhibition of COX by indomethacin, leading to reduced PGE2 and PGF2α levels, or dexamethasone-induced downregulation of COX-2 both increased AQP2 abundance, while PGE2 addition reduced AQP2 abundance. However, lithium did not change the prostaglandin levels, and indomethacin and dexamethasone did not prevent lithium-induced AQP2 downregulation. Further analysis revealed that lithium decreased AQP2 protein abundance, mRNA levels and transcription, while PGE2 reduced AQP2 abundance by increasing its lysosomal degradation, but not by reducing AQP2 gene transcription. In conclusion, our data reveal that in mpkCCD cells, prostaglandins decrease AQP2 protein stability by increasing its lysosomal degradation, indicating that in vivo paracrine-produced prostaglandins might have a role in lithium-induced NDI via this mechanism. However, lithium affects also AQP2 gene transcription, which is prostaglandin independent.

Lithium is the drug of choice for the treatment of bipolar disorders. It is also used regularly to treat schizoaffective disorders as well as depression. In addition, lithium is also under consideration as a therapeutic for many diseases including Alzheimer’s disease, acquired immunodeficiency syndrome, and amyotrophic lateral sclerosis (20, 26, 51). Lithium is a frequently prescribed drug and is used by 1 in 1,000 of the population (59). Approximately 20% of patients undergoing lithium treatment develop symptomatic nephrogenic diabetes insipidus (NDI), a disorder characterized by polyuria and polydipsia due to a urinary concentrating defect (9). This makes lithium-induced NDI the most common form of NDI.

Studies in rats have shown that lithium-induced NDI occurs in conjunction with AQP2 downregulation (41, 43), and in line with this, lithium treatment reduces urinary AQP2 excretion in humans, indicating a decreased renal AQP2 expression (61). Lithium mainly enters the cell via the epithelial sodium channel ENaC (16, 36), and inactivates glycogen synthase kinase (Gsk) 3β. The development of NDI and inactivation of Gsk3β are temporally related to an increased cyclooxygenase-2 (COX-2) expression in the kidney in rats, leading to an increased urinary prostaglandin E2 (PGE2) excretion (53). The fact that PGE2 reduces AVP-stimulated water reabsorption in the collecting duct (30, 46) could suggest an important role for PGE2 in lithium-NDI development. This is also suggested by studies showing that blocking prostaglandin production by indomethacin reduces the urine volume of lithium-treated rats (34) as well as of lithium-induced NDI patients (2, 62).

In vitro, mouse collecting duct (mpkCCD) cells are a good model system to study the effects of lithium on AVP-induced AQP2 expression. Following induction of endogenous AQP2 expression, addition of clinically relevant concentrations of lithium to the apical side caused a reduction in AQP2 abundance and resulted in Gsk3β inactivation (36, 42). Lithium mainly entered the mpkCCD cells via ENaC, as amiloride partially blocked cellular lithium entry, Gsk3β inactivation, and AQP2 downregulation (36). In the present study, we used the mpkCCD model system to gain additional insights into the mechanism whereby lithium reduces AQP2 abundance and the potential role of prostaglandins in this process.

MATERIALS AND METHODS

Cell culture. Mouse mpkCCD-c14 cells were essentially grown as described (27). Cells were seeded at a density of 1.5 × 105 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 treated for the last 96 h with 1 mM 1-desamino-8-D-arginine vasopressin (dDAVP) to the basolateral side, to maximally induce AQP2 expression (42). Cells were incubated with 1 mM lithium chloride at the basolateral side and 10 mM lithium chloride at the apical side or 20 μM zinc chloride at both sides of the cells for the last 24 or 48 h. Dexamethasone (1 μM), indomethacin (10 μM), PGE2 (1 μM) (all from Sigma, St. Louis, MO), or PGF2α (1

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µM; Calbiochem, San Diego, CA) was administered at both sides of the cells during the last 48 h.

Transfection and generation of a stable mpkCCD cell line with a 3.0 AQP2 promoter-luciferase reporter construct was previously described (37).

Immunoblotting. mpkCCD cells, from a 1.13-cm² filter were lysed in 200 µl Laemmlı buffer, and 15-µl samples were analyzed. PAGE, blotting, and blocking of the polyvinylidene difluoride membranes were done as described (33). Membranes were incubated for 16 h with 1:3,000 diluted affinity-purified rabbit 7 R AQP2 antibodies (18), 1:1,000 diluted rabbit anti-Ser9-Gsk3β (Cell Signaling Technology, Beverly, MA), 1:5,000 diluted mouse anti-Gsk3β (BD Transduction Laboratories, Lexington, KY), 1:1,000 diluted mouse anti-COX-2 (Cayman Chemicals, Ann Arbor, MI) in Tris-buffered saline Tween-20 (TBS-T) supplemented with 1% wt/vol nonfat dried milk. Next, blots were incubated for 1 h with 1:5,000 diluted goat anti-rabbit IgGs or 1:2,000 goat anti-mouse IgGs (Sigma) coupled to horseradish peroxidase. Proteins were visualized using enhanced chemiluminescence (ECL, Pierce, Rockford, IL). Films were scanned with a Bio-Rad 690c densitometer, and signals were analyzed using Bio-Rad software. Twofold dilution series of a control sample were blotted in parallel to allow semi-quantification. Equal loading of the samples was confirmed by subsequent staining of the blots with Coomassie Brilliant Blue G250 (Serva, Heidelberg, 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.

RT-PCR. mpkCCD cells were grown on semipermeable filters for 8 days as described above, and total RNA was treated with DNase (Promega) in DNase buffer, incubated for 1 h at 37°C, extracted with phenol/chloroform, and precipitation. RNA was reverse-transcribed into cDNA using Moloney murine leukemia virus (M-MLV) Reverse Transcriptase and random primers (Promega). During cDNA production, a control reaction without the reverse transcriptase enzyme was conducted to exclude amplification of genomic DNA.

SYBR Green real-time quantitative PCR was performed on an iQ5 Real-Time PCR Detection System from Bio-Rad by utilizing the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and primers for the prostanoid receptors or the mouse AQP2 gene. To amplify AQP2 mRNA, primers binding to exon 2 (CTCCCAA-CAATGCACACGC) and exon 3 (GAGCAGCCGGTGAAATAGAT) were used. To amplify AQP2 pre-mRNA, the exon 3 primer was used together with an intron 2 primer (GGCACGCTCAAGCTGCTCTG). Primers for prostanoid receptors were designed to be intron overlapping (see Table 1). Signals for the housekeeping gene, which was amplified in parallel, were used to normalize for differences in the amount of starting cDNA, using either primers for β-actinor ribosomal 18S (Table 1).

Prostanoid analysis. Samples were prepared as described (56) with minor modifications. Briefly, cell culture supernatants were spiked with ~1 ng of deuterated internal standards, and the methoximes were obtained through reaction with an O-methylhydroxylamine hydrochloride-acetate buffer. After acidification to pH 3.5, prostanoid derivatives were extracted, and the pentfluorobenzylesters were formed. Samples were purified by thin layer chromatography, and a broad zone with Rf 0.03–0.4 was eluted. After withdrawal of the organic layer, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to GC/MS/MS analysis on a Finnigan MAT TSQ700 GC/MS/MS (Thermo Electron, Dreieich, Germany) equipped with a Varian 3400 gas chromatograph (Palo Alto, CA) and a CTC A200S autosampler (CTC Analytics, Zwingen, Switzerland).

RESULTS

Effect of lithium on Gsk3 and COX-2. In vivo, lithium treatment has been reported to lead to the inhibition of Gsk3β, which is suggested to lead to increased COX-2 abundance and prostaglandin release (52, 53). COX-1 and COX-2 catalyze the production of prostaglandins (13), and the released prostaglandins activate prostanoid receptors in a paracrine fashion, resulting in reduced AVP-induced water permeability in the collecting duct (29, 30, 46, 67).

To test whether a similar response to lithium is seen in mpkCCD cells, these cells were grown for 8 days, the last 4 days in the presence of dDAVP to induce AQP2 expression, and treated with lithium for the last 48 h. Because in patients on lithium therapy, the concentration of lithium in serum is around 1 mM and around 10 mM in urine (59), cells were treated with 10 mM at the apical and 1 mM at the basolateral side. While AQP2 levels were decreased with lithium, Gsk3β activity was reduced as shown by the increased Ser9-phosphorylation of an unchanged total amount of Gsk3β (Fig. 1A). Zinc also inhibits Gsk3β activity (32). Therefore we incubated mpkCCD cells with zinc to determine whether inhibition of Gsk3β by another metal ion also affects AQP2 abundance. Administration of 20 µM zinc reduced AQP2 protein levels and increased phosphorylation of Gsk3β in mpkCCD cells, again without changing total Gsk3β expression levels (Fig. 1A). Both lithium and zinc significantly increased COX-2 abundance (Fig. 1B).

Effect of COX-2 on AQP2 expression in mpkCCD cells. Dexamethasone decreases COX-2 mRNA and protein abundance in various cell types (40, 63). Because previous studies have shown that prostaglandins inhibit AVP-induced water reabsorption in vivo (30, 46), we investigated whether dexamethasone-decreased COX-2 expression inversely correlates with AQP2 abundance in mpkCCD cells. Incubation with 1 µM dexamethasone caused a significant downregulation of COX-2 and increase of AQP2 abundance (Fig. 2A). However, dexamethasone did not affect lithium-induced upregulation of COX-2 nor downregulation of AQP2 (Fig. 2A).

To test whether indomethacin, which inhibits both COX-1 and COX-2 activity, increases AQP2 abundance, cells were grown as described above, the last 48 h in the presence of 10 µM indomethacin. Even though COX-2 abundance was increased, immunoblotting revealed increased AQP2-abundance with indomethacin (Fig. 2B), underscoring the correlation between COX activity, prostaglandin release, and AQP2 abundance. Interestingly, even in the presence of lithium, lithium decreased AQP2 abundance and increased abundance of COX-2 (Fig. 2B), suggesting that the effect of lithium on

Table 1. Primer sequences for prostanoid receptors and housekeeping genes

<table>
<thead>
<tr>
<th>Protein</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<tbody>
<tr>
<td>EP1</td>
<td>GAGGCAGCCGGCGTCATATGCTGCGG</td>
<td>GAGGCAGCCGGCGTCATATGCTGCGG</td>
</tr>
<tr>
<td>EP4</td>
<td>TACACCCCTTCTCTTACAT</td>
<td>TTGACCCGGTTGCTTGCTGA</td>
</tr>
<tr>
<td>FP</td>
<td>GCTCAGGCGGTTGCACTCAC</td>
<td>TTGACCACTGCTTTGGAAT</td>
</tr>
<tr>
<td>TP</td>
<td>GTGGGCATCATGGGCTGGTTG</td>
<td>CACAGGGCAGTAGATTGACAGCG</td>
</tr>
<tr>
<td>h-Actin</td>
<td>GTATGCGCTCTGCTGCTCAGAC</td>
<td>AGATTTTCTCCCTTGACAGCTG</td>
</tr>
<tr>
<td>18S</td>
<td>GATCCGCTTGGACCCACATT</td>
<td>GATCCGCTTGGACCCACATT</td>
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EP, PGE2 receptor; FP, PGF2α receptor; TP, thromboxane A2 receptor.
AQP2 abundance may not be solely generated through prostaglandins, or that indomethacin did not completely block the effect of lithium on prostaglandin production.

Involvement of prostaglandins and their receptors in lithium-induced downregulation of AQP2 in mpkCCD cells. COX-1 and -2 catalyze the conversion of arachidonic acid to prostaglandin H$_2$ (PGH$_2$). PGH$_2$ serves as a substrate for various prostaglandins and thromboxanes. These newly generated products bind their respective G protein-coupled receptors from the extracellular side and activate their respective intracellular signaling pathways (Fig. 3A). Since lithium treatment increased COX-2 abundance, we questioned which prostaglandin could mediate the lithium-induced downregulation of AQP2.

The prostaglandin receptors EP1, EP4, FP, and TP could be detected by RT-PCR in mpkCCD cells, while results for the IP receptor were inconclusive. The same receptors were found after lithium treatment (data not shown). The release of the agonists of these receptors was investigated in cells treated with or without lithium, to determine the effect of lithium on prostanoid production. mpkCCD cells were grown as above and incubated with lithium for the last 24 h. The medium was collected, and the amounts of prostaglandins were determined. Prostaglandin concentrations from fresh medium (before addition to cells) were subtracted. In dDAVP-treated cells, the amounts of PGE$_2$ and PGF$_{2\alpha}$ produced were clearly above the detection limit, while levels of 6-keto-PGF$_{1\alpha}$ (a stable metabolite of PGF$_2\alpha$) and thromboxane B$_2$ (a stable metabolite of TxA$_2$) were low and close to or similar to the levels detected in fresh medium (Fig. 3B). There was no significant difference in the concentration of prostaglandins released into the apical or basolateral compartment (not shown). The concentrations of PGE$_2$, 6-keto-PGF$_{1\alpha}$, PGF$_{2\alpha}$, and TxB$_2$ were not significantly changed with lithium.

Because coincubation with indomethacin increased AQP2 abundance compared with lithium alone (Fig. 2B), we also analyzed prostaglandin levels from cells treated with lithium and indomethacin. Compared with lithium-treated cells, indomethacin significantly decreased PGE$_2$ and PGF$_{2\alpha}$ levels (Fig. 3B), suggesting that a reduction of these prostaglandins is involved in the attenuating effect of indomethacin on lithium-induced AQP2 downregulation.

Because lithium did not affect prostanoid production in mpkCCD cells, we investigated whether a change in prostanoid receptor expression could explain the AQP2 downregulation. The effect of lithium on the relative expression of the different prostanoid receptors was analyzed by quantitative RT-PCR (Q-RT-PCR) and normalized for the amount of ribosomal 18S. As shown in Fig. 3C, the expression of FP and EP1 receptors was significantly decreased with lithium, while the expression of the TP or EP4 receptors was unchanged. Because signaling via the FP and EP1 receptors is expected to inhibit water reabsorption (11, 29), a downregulation of these receptors cannot explain the decrease in AQP2 expression conferred by lithium.

Effect of lithium on AQP2 transcription. Previously, we showed that lithium decreases AQP2 mRNA levels (42), suggesting an effect of lithium on AQP2 transcription and/or mRNA stability. Gene transcription results in the formation of a pre-mRNA transcript consisting of introns and exons, which is spliced to mRNA within minutes. While pre-mRNA levels are mainly determined by transcriptional rates, mRNA levels are regulated by both transcription/splicing and degradation (17). Therefore, when lithium affects transcription only, the AQP2 mRNA/pre-mRNA ratio should not decrease, but if lithium has an effect on mRNA stability, the relative amount of AQP2 mRNA would be decreased compared with AQP2 pre-mRNA levels. So, to investigate this, Q-RT-PCR was performed on mpkCCD cDNA using primers amplifying AQP2...
with 10 B: mpkCCD cells were grown as in exon 3 primer). Following lithium treatment, AQP2 mRNA (exon 2 and 3 primer) and pre-mRNA (intron 2 and 3 primer) were subjected to immunoblotting for AQP2 and COX-2. C, control; Li, lithium; D, dexamethasone; In, indomethacin. *Significant differences (P < 0.05).

Fig. 2. Effect of COX-2 on AQP2 expression. A: mpkCCD cells were grown to confluence and treated with 1 nM dDAVP for 4 days. For the last 48 h the cells were treated with 1 μM dexamethasone (Dex) with or without lithium. B: mpkCCD cells were grown as in A. For the last 48 h the cells were treated with 10 μM indomethacin with or without lithium. In A and B, cells were lysed and subjected to immunoblotting for AQP2 and COX-2. C, control; Li, lithium; D, dexamethasone; In, indomethacin. *Significant differences (P < 0.05).

mRNA (exon 2 and 3 primer) and pre-mRNA (introns 2 and 3 primer). Following 4 h of lithium treatment, AQP2 pre-mRNA was decreased to 40% of control levels, whereas a nonsignificant decrease in AQP2 mRNA levels was observed (Fig. 4A). After 6 h, both AQP2 mRNA and pre-mRNA were decreased to 30–40%, which was sustained until at least 24 h. Together, these data reveal that lithium reduces AQP2 mRNA levels by reducing AQP2 gene transcription and not by reducing AQP2 mRNA stability.

If lithium affects AQP2 transcription, one would also expect that the production of an AQP2 irrelevant transcript driven by the AQP2 promoter would show reduced expression after lithium treatment. To test this hypothesis, mpkCCD cells were stably transfected with pGL3-AQP2-3.0-luc, a construct containing luciferase cDNA under control of a 3-kb fragment of the mouse AQP2 promoter, generating mpkCCD-AQP2-luc cells. Moreover, mpkCCD-luc cells were generated as a negative control, by stably transfecting the luciferase construct lacking the AQP2 promoter (pGL3-luc). Pooled colonies were seeded on filters and grown with or without dDAVP for the last 4 days and treated with or without lithium during the last 48 h. Basal luciferase activity was around 100 times lower in mpkCCD-luc cells compared with mpkCCD-AQP2-luc cells (Fig. 4B). Moreover, dDAVP application significantly increased luciferase activity in mpkCCD-AQP2-luc, but not mpkCCD-luc cells, revealing that dDAVP efficiently stimulates transcription from the 3.0-kb AQP2 promoter. Importantly, lithium significantly decreased the dDAVP-induced luciferase activity in mpkCCD-AQP2-luc cells to ~60%, which underscores our data above that lithium reduces AQP2 gene transcription.

Effect of prostaglandins on AQP2 transcription. Because lithium reduces AQP2 transcription of the 30-kb fragment of the promoter, this establishes a model system to further study the transcriptional mechanisms leading to AQP2 downregulation by lithium. To test whether lithium-induced reductions in AQP2 transcription result from the release of prostaglandins, mpkCCD-AQP2-luc cells were grown without or with dDAVP for 4 days and incubated for the last 48 h with or without indomethacin, PGE2, or PGF2α, dDAVP application again significantly increased luciferase activity (Fig. 5A). Indomethacin, however, did not further increase the luciferase activity, and PGE2 or PGF2α application did not reduce luciferase activity, which suggests that prostaglandins do not affect AQP2 transcription. In addition to these prostaglandins, incubation with PGD2, the PGI2 analog carbacyclin, or the thromboxane analog U-4661 did not reduce luciferase activity (data not shown).

To establish whether the effect on luciferase expression mimics the effect on AQP2 mRNA levels, Q-RT-PCR assays were done. Consistent with the data above, AQP2 mRNA levels were not changed with PGE2 or PGF2α, or indomethacin, while the lithium control showed a clear reduction in mRNA (Fig. 5B).

The absence of any effect of prostaglandins on AQP2 mRNA levels indicated that they reduce AQP2 abundance by affecting AQP2 protein stability. In mpkCCD cells, chemical messengers like ATP and dopamine counteract vasopressin-induced AQP2 abundance by targeting it for lysosomal degradation, which can be blocked with the inhibitor chloroquine(8). Therefore, to test the effect of prostanoids on AQP2 stability directly, cells were incubated with dDAVP for 4 days of which the last day with or without lithium or PGE2, all in the presence or absence of the lysosome inhibitor chloroquine. Immunoblotting revealed that, in contrast to lithium, PGE2 addition did not decrease AQP2 abundance in the presence of chloroquine (Fig. 6). Both lithium and PGE2 reduced AQP2 abundance in the absence of chloroquine.

Together, these data reveal that prostaglandins reduce the dDAVP-induced AQP2 abundance solely by increasing lysosomal degradation of AQP2. This indicates that the effect of lithium on AQP2 transcription is mediated independently of prostaglandins.

DISCUSSION

mpkCCD cells as a model system to study lithium-induced regulation of AQP2 expression. Our data reveal that mpkCCD cells are a proper model to study lithium-induced changes in AQP2 expression. The 1 mM lithium used at the basolateral side in our experiments is similar to serum lithium levels found
in patients, which are generally between 0.6 and 1.5 mM (59).
From the cortex to the medulla of the collecting duct, lithium
is concentrated as micropuncture studies in rats showed an
increased lithium concentration in the early distal nephron and
a more severe increase in the final urine (28). Also, in lithium-
treated patients, lithium concentrations of 10 –15 mM are
found in their urine (3, 22). Collecting duct concentrations of
lithium may thus vary from 1 mM in the cortex to 10 –15 mM
in the medulla. On the basis of these in vivo concentrations, we
chose to use 10 mM lithium at the apical side in our experi-
ments.

First, we show that incubation of the cells with lithium at
these clinically relevant concentrations did lead to a downregu-
lation of AQP2. This is in agreement with our previous studies
(36, 42) and consistent with AQP2 downregulation observed in
vivo in lithium-NDI rats (36, 41, 43). In this study, we further
show that the AQP2 downregulation coincides with inactiva-
tion of Gsk3β/H9252, in agreement with previous studies in lithium-
NDI mice (53) and mpkCCD cells (36). We furthermore show
that Gsk3 inactivation coincides with an increased abundance
of COX-2, which is consistent with previous in vivo effects of
Gsk3 on COX-2 (52) as well as the observed increase in
COX-2 abundance observed in lithium-NDI mice (53). In
addition, we show that changes in COX-2 expression or activ-
ity influence AQP2 abundance in mpkCCD cells, as shown by
the administration of both dexamethasone, leading to specific

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downregulation of COX-2 (6), and indomethacin, blocking both COX-1 and COX-2 (60). Similarly, treatment with a COX-2 inhibitor increases AQP2 abundance in rats with lithium-induced NDI or bilateral ureteral obstruction (34, 50).

Although COX-2 is highly expressed in renal interstitial cells (23, 53), it is at present controversial whether COX-2 is also expressed in collecting duct cells. While immunohistochemistry reveals strong COX-2 expression in the collecting duct in three studies (1, 55, 64), absence of it was suggested in three other studies (12, 24, 25). Using collecting ducts isolated from AQP2-green fluorescent protein transgenic mice, Ye et al. (66) recently showed a 10 times lower expression of COX-2 mRNA in collecting duct cells compared with kidney cells not originating from collecting ducts, suggesting that constitutive COX-2 expression is low. Increased COX-2 levels, however, have been detected in collecting ducts of animals that were dehydrated or subjected to a chronic NaCl load (55, 64) indicating that COX-2 expression in collecting duct cells is increased under stress conditions. This suggests that COX-2 expression may also be induced in collecting duct cells during lithium treatment. Besides COX-2, COX-1 is highly expressed in the collecting duct (23).

The major prostaglandins produced in mpkCCD cells were PGE2 and PGF2α. In line with PGE2 and PGF2α being produced in mpkCCD cells, PGE2 and PGF2α have been shown to be mainly produced in the collecting ducts, being highest in the medulla (19).

**Lithium treatment does not increase prostaglandin production in mpkCCD cells.** Since the abundance of COX-2 increased after treatment with lithium, we investigated which of the COX-derived compounds could mediate the lithium-induced downregulation of AQP2. However, the lithium-induced increase in COX-2 abundance did not result in elevated prostaglandin levels in media of mpkCCD cells. This absence of increase in prostaglandins may be due to a lack of free arachidonic acid, which is rate-limiting in the production of prostaglandins (7, 31, 45, 49).

Blocking COX by indomethacin increased AQP2 abundance in cells treated with or without lithium, suggesting that also in cells without lithium, AQP2 abundance is decreased by the action of endogenously produced prostaglandins. Indomethacin significantly reduced released PGE2 and PGF2α levels but did not affect 6-keto-PGF1α (a stable metabolite of PGI2) or TxB2 (a stable metabolite of TxA2) levels, suggesting that a reduction of PGE2 and/or PGF2α is involved in the attenuating effect of indomethacin on lithium-induced AQP2 downregulation.
However, no effect of lithium on prostaglandin production was found in mpkCCD cells, which suggested that the effect of lithium on AQP2 expression in these cells occurs independently of prostaglandins. This is also suggested by the observation that, although indomethacin blocked COX activity, leading to reduced prostaglandin production, it did not prevent the lithium-induced downregulation of AQP2.

Dexamethasone reduces COX-2 abundance by decreasing COX-2 mRNA stability by inhibiting p38 (40). Interestingly, lithium did completely block the effect of dexamethasone on COX-2 expression, which suggests cross talk between the p38 pathway and the Gsk3β-NF-κB-mediated effect on COX-2 transcription, or a direct effect of lithium on p38 activity.

The absence of a lithium-induced increase in PGE2 production in our cells illuminates a difference from in vivo, because in vivo lithium leads to a large increase in PGE2 production (53). This difference is likely due to the fact that in vivo, the lithium-induced increase in prostaglandins is thought to be derived from the interstitial instead of principal cells (53). In line with the beneficial effects of COX-2 inhibition on AQP2 abundance in lithium-NDI rats (34), however, addition of PGE2 to mpkCCD cells leads to AQP2 downregulation.

**Lithium decreases expression of the EP1 and FP receptors in mpkCCD cells.** Expression of EP1, EP4, FP, IP, and TP receptors was detected in mpkCCD cells, while no expression was seen of the DP, EP2, and EP3 receptors. Of the prostaglandin receptors found to be expressed, the EP1, FP, and TP receptors couple to pathways counteracting the vasopressin-induced Gs/cAMP pathway. TP receptors are unlikely to be involved in AQP2 downregulation under normal circumstances, because TxA2 levels produced are low and indomethacin, leading to increased AQP2 abundance, does not reduce TxA2 production.

Application of lithium decreased the expression of FP and EP1 receptors. However, because signaling via these receptors...
is expected to inhibit water reabsorption (11, 29), a downregulation of these receptors cannot explain the decrease in AQP2 expression conferred by lithium and may instead be a compensatory mechanism.

In agreement with the expression in mpkCCD cells, collecting duct cells express EP1, EP4, FP, and TP receptors and lack DP and EP2 receptors (10, 11, 54, 57). While expression of the IP receptor in mpkCCD cells is inconclusive, also this receptor is found in the collecting duct (35). In contrast to the mpkCCD cells, however, the EP3 receptor, which couples to the AVP-counteracting G_{i} pathway, is also expressed in collecting ducts, and may thus, besides EP1 and FP receptors, be involved in prostaglandin-dependent downregulation of AQP2 in vivo. Conclusive evidence for the roles of particular prostaglandin receptors in mediating a prostaglandin-induced downregulation of AQP2 in lithium-induced NDI awaits studies using collecting duct specific knockout of these receptors.

**Lithium decreases AQP2 transcription independently of prostaglandins.** 
PGE$_2$ application to mpkCCD cells reduced AQP2 abundance, in line with the inhibitory effect of PGE$_2$ on AVP-stimulated water absorption in the collecting duct (30, 46). This prostaglandin-induced AQP2 downregulation could be prevented by coincubation with the lysosome inhibitor chloroquine, showing that the effect of prostaglandins on AQP2 abundance is mediated by increasing AQP2 degradation. Interestingly, and in contrast to lithium, addition of PGE$_2$ or PGF$_{2\alpha}$ did not decrease AQP2 transcription or mRNA abundance. In line with this, indomethacin, blocking prostaglandin production, increased dDAVP-stimulated AQP2 protein abundance but did not increase AQP2 transcription or mRNA levels. In agreement with previous results (42), we here found that lithium does not affect AQP2 degradation but decreases AQP2 mRNA by decreasing AQP2 gene transcription. Together, these data indicate that in vivo the reduction of AQP2 abundance by lithium consists of at least two parts, being an increased AQP2 degradation due to increased prostaglandin levels and a reduction of AQP2 gene transcription due to a prostaglandin-independent effect of lithium.

A limitation of our cell model is the absence of the EP3 receptor, which is found in vivo in the collecting duct. EP3 activation inhibits cAMP generation via G$_i$ (11) and might therefore inhibit AVP-stimulated AQP2 transcription by decreasing CRE-mediated transcription. Although this might contribute to the AQP2 downregulation, earlier studies show no decrease in cAMP after lithium treatment in vivo (42), suggesting that an in vivo effect of lithium on AQP2 transcription is EP3 independent.

**Model of lithium-induced NDI.** On the basis of the present and earlier data, we propose the following model (Fig. 7): lithium leads to AQP2 downregulation in the principal cells, and, in the long term, to an increase in apoptosis as well as proliferation, resulting in a decreased fraction of principal cells (14, 15, 47).

Lithium reduces AQP2 abundance through two mechanisms: at first, lithium enters renal medullary interstitial cells and, possibly, renal principal cells, resulting in increased production of PGE$_2$ (39, 53). Lithium entry in principal cells occurs through the epithelial sodium channel ENaC (16, 36), but the entry pathway of interstitial cells is unknown. In either cell type, this leads to inactivation of Gsk3β, which increases the abundance of COX-2 (52, 53) and release of prostaglandins. The increase in COX-2 expression has been reported to occur in the interstitium in vivo (53), suggesting that the increased urinary PGE$_2$ excretion is predominantly due to the medullary interstitial cells. The released prostaglandins are detected by prostaglandin receptors on principal cells, leading to lysosomal degradation of AQP2 and a decline in urine concentrating ability.

Secondly, lithium decreases AQP2 abundance by reducing AQP2 gene transcription. The ENaC-mediated entry of lithium in principal cells leads to inactivation of Gsk3β (36, 53). In line with an important role of Gsk3β in AQP2 expression, other Gsk3β inhibitors also lead to a downregulation of AQP2 expression in mpkCCD cells (36). The effect on transcription is prostaglandin independent and is likely a consequence of inhibition of one of multiple pathways steered by Gsk3β, like signaling involving the transcription factors nuclear factor of activated T-cells (NFAT), β-catenin, or hypoxia-inducible factor (HIF). This decreased AQP2 transcription will result in a further decline of the AQP2 abundance and of the urine concentrating ability.

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**Fig. 7.** Model of the lithium-induced inhibition of AQP2-mediated water reabsorption. AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; ENaC, epithelial sodium channel; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PR, prostaglandin receptor; V2R, vasopressin type 2 receptor; CREB, cAMP responsive element-binding protein. For details, see text.
In conclusion, our data show that in mpkCCD cells, lithium decreases AQ2 protein abundance as well as AQ2 gene transcription. Lithium decreases AQ2 protein independently of the endogenous prostaglandin production in mpkCCD cells. Our data furthermore show that in mpkCCD cells, released prostaglandins decrease AQ2 protein stability by increasing its lysosomal degradation. On the basis of these observations, in vivo paracrine produced prostaglandins might have an additional role in lithium-induced NDI by decreasing AQ2 protein stability, without affecting AQ2 transcription. Such an effect would currently be difficult to adequately separate from the “direct” effect of lithium on AQ2 transcription and abundance in the intact animal. The two identified pathways leading to a decrease in AQ2 abundance might also have consequences for the clinical treatment of lithium-NDI. Both COX inhibitors and the ENaC blocker amiloride have been shown to individually reduce the NDI phenotype (2, 4, 5, 38, 62). However, since the effect of lithium on prostaglandin production in interstitial cells could be blocked by COX inhibitors, and the entry of lithium into the principal cells could be blocked by amiloride treatment, a beneficial effect of combining these two therapies for the treatment of lithium-NDI could potentially improve the treatment of lithium-NDI in the clinic. Future studies in humans addressing such therapeutic approaches are needed to definitively clarify this matter.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are disclosed by the author(s).

REFERENCES


