TRANSLATIONAL PHYSIOLOGY

Treating lithium-induced nephrogenic diabetes insipidus with a COX-2 inhibitor improves polyuria via upregulation of AQP2 and NKCC2

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[1]*Department of Internal Medicine, College of Medicine, and* [2]*Institute of Biomedical Sciences, Hanyang University, Seoul;* [3]*Department of Internal Medicine, Konyang University College of Medicine, Nonsan;* [4]*Department of Anatomy, Chungnam National University College of Veterinary Medicine, Daejeon, Korea; and* [5]*Laboratory of Kidney and Electrolyte Metabolism, National Institutes of Health, Bethesda, Maryland*

Kim G-H, Choi NW, Jung J-Y, Song J-H, Lee CH, Kang CM, Knepper MA. Treating lithium-induced nephrogenic diabetes insipidus with a COX-2 inhibitor improves polyuria via upregulation of AQP2 and NKCC2. *Am J Physiol Renal Physiol* 294: F702–F709, 2008. First published January 23, 2008; doi:10.1152/ajprenal.00366.2007.—Prostaglandin E_2 may antagonize vasopressin-stimulated salt absorption in the thick ascending limb and water absorption in the collecting duct. Blockade of prostaglandin E_2 synthesis by nonsteroidal anti-inflammatory drugs (NSAIDs) enhances urinary concentration, and these agents have antidiuretic effects in patients with nephrogenic diabetes insipidus (NDI) of different etiologies. Because renal prostaglandins are derived largely from cyclooxygenase-2 (COX-2), we hypothesized that treatment of NDI with a COX-2 inhibitor may relieve polyuria through increased expression of Na-K-2Cl cotransporter type 2 (NKCC2) in the thick ascending limb and aquaporin-2 (AQP2) in the collecting duct. To test this hypothesis, semiquantitative immunoblotting and immunohistochemistry were carried out from the kidneys of lithium-induced NDI rats with and without COX-2 inhibition. After male Sprague-Dawley rats were fed an LiCl-containing rat diet for 3 wk, the rats were randomly divided into control and experimental groups. The COX-2 inhibitor DFU (40 mg·kg^{-1}·day^{-1}) was orally administered to the experimental rats for an additional week. Treatment with the COX-2 inhibitor significantly relieved polyuria and raised urine osmolality. Semiquantitative immunoblotting using whole-kidney homogenates revealed that COX-2 inhibition caused significant increases in the abundance of AQP2 and NKCC2. Immunohistochemistry for AQP2 and NKCC2 confirmed the effects of COX-2 inhibition in lithium-induced NDI rats. The upregulation of AQP2 and NKCC2 in response to the COX-2 inhibitor may underlie the therapeutic mechanisms by which NSAIDs enhance antidiuresis in patients with NDI.

cyclooxygenase-2; lithium; aquaporin-2; Na-K-2Cl cotransporter; DFU

NEPHROGENIC DIABETES INSIPIDUS (NDI) is a clinical syndrome in which the kidney is unable to concentrate urine despite normal or elevated concentrations of the antidiuretic hormone arginine vasopressin. It can be divided into congenital and acquired forms, and lithium treatment is one of the major causes of the acquired form of NDI (5). In lithium-induced NDI rat models, downregulation of aquaporin-2 (AQP2) has been demon-strated (29, 32) to be the underlying molecular basis of acquired NDI (22).

For the treatment of NDI, nonsteroidal anti-inflammatory drugs (NSAIDs) or cyclooxygenase-2 (COX-2) inhibitors have been useful (2, 39, 44), but their therapeutic mechanisms were not clearly defined. Previously, we showed that in a lithium-induced NDI rat model hydrochlorothiazide produced the paradoxical antidiuresis via upregulation of AQP2 (25), suggesting that AQP2 may be involved in the therapeutic mechanisms as well as in the pathophysiology of lithium-induced NDI.

The antidiuretic effect of NSAIDs in NDI can be explained by the role of prostaglandins in the regulation of renal water excretion. NSAIDs inhibit the enzyme cyclooxygenase and thereby block prostaglandin synthesis in the kidneys. Urinary prostaglandin E_2 (PGE_2) increased in congenital NDI due to either V2 receptor (33) or AQP2 (21) mutation as well as in acquired NDI due to lithium treatment (20). Renal PGE_2 plays an important role in modulating the effect of vasopressin on osmotic water permeability in the renal collecting duct, where it attenuates antidiuretic action. Studies in animals have shown that PGE_2 inhibits vasopressin-stimulated water permeability (19, 34, 42).

In addition, PGE_2 may also inhibit sodium and chloride absorption in the thick ascending limb of Henle's loop and increase medullary blood flow, thus lowering medullary interstitial osmotic driving forces (27). Active sodium chloride transport in the thick ascending limb of Henle's loop occurs mainly through the Na-K-2Cl cotransporter type 2 (NKCC2), setting up a corticomedullary concentration gradient. This medullary interstitial hypertonicity drives water absorption through the AQP2 water channel in the collecting duct in the presence of vasopressin (24).

Thus it is conceivable that when NSAIDs are used in NDI, amelioration of polyuria can be achieved by enhancing absorption of sodium chloride in the thick ascending limb and absorption of water in the collecting duct, respectively. Because the renal effects of prostaglandins are derived from COX-2, we hypothesized that treatment of NDI with the COX-2 inhibitor may relieve polyuria through upregulation of NKCC2 in the thick ascending limb and/or AQP2 in the

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collecting duct. To test this hypothesis, semiquantitative immunoblotting and immunohistochemistry were carried out in the kidneys of lithium-induced NDI rats with and without COX-2 inhibition.

METHODS

Animals and experimental protocols. Specific pathogen-free male Sprague-Dawley rats (Orient Bio, Seongnam, Korea), weighing 180–220 g, were used for induction of NDI and treatment with the COX-2 inhibitor. The experimental protocol was approved by the institutional Animal Care and Use Committee of Hanyang University. Throughout the study, a fixed amount of regular rat chow (15 g·180 g body wt^{-1}·day^{-1}) was given, and drinking water was freely accessible. After NDI was induced by offering lithium chloride (40 mmol lithium/kg dry food for 3 wk) (25), lithium-induced NDI rats were randomly divided into DFU-treated rats and control rats. For the selective COX-2 inhibition, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methanesulfo-nylphenyl)-2(5H)-furanone 1 (DFU; 40 mg·kg^{-1}·day^{-1}) was orally administered in food to the treated group for an additional week. For preparation of the lithium and DFU mixture in the diet, we first dissolved LiCl 170 mg in water (100 ml) and then added DFU at a concentration of 0.53 mg/ml. DFU, a related analog of rofecoxib (16), was kindly donated by Merck. Lithium-containing food was continuously offered to both groups throughout the study period.

Semiquantitative immunoblotting. After the whole-animal experiments, the rats were killed by decapitation, and kidneys were rapidly removed and placed in chilled isolation solution containing 250 mM sucrose, 10 mM triethanolamine (Sigma, St. Louis, MO), 1 µg/ml leupeptin (Sigma), and 0.1 mg/ml phenylmethylsulfonyl fluoride (Sigma) titrated to pH 7.6. Next, the whole kidneys were homogenized in 10 ml of ice-cold isolation solution at 15,000 rpm with three strokes for 15 s using a tissue homogenizer (PowerGun 125, Fisher Scientific, Pittsburgh, PA). After homogenization, total protein concentration was measured using the bicinchoninic acid protein assay reagent kit (Sigma) and adjusted to 2 µg/µl with isolation solution. The samples were then solubilized by adding 1 vol 5× Laemmli sample buffer/4-vol sample and heating to 60°C for 15 min.

Initially, "loading gels" were done on each sample set to allow fine adjustment of loading amount to guarantee equal loading on subsequent immunoblots. Five micrograms of protein from each sample were loaded into each individual lane and electrophoresed on 12% polyacrylamide-SDS minigels using a Mini-PROTEAN III electrophoresis apparatus (Bio-Rad, Hercules, CA), and then stained with Coomassie blue dye (G-250, Bio-Rad; 0.025% solution made in 4.5% methanol and 1% acetic acid). Selected bands from these gels were scanned (GS-700 Imaging Densitometry, Bio-Rad) to determine density (Molecular Analyst version 1.5, Bio-Rad) and relative amounts of protein loaded in each lane. Finally, protein concentrations were "corrected" to reflect these measurements.

For immunoblotting, the proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes (Bio-Rad). After being blocked with 5% skim milk in PBS-T (80 mM Na$_2$HPO$_4$, 20 mM NaH$_2$PO$_4$, 100 mM NaCl, 0.1% Tween 20, pH 7.5) for 30 min, membranes were probed overnight at 4°C with the respective primary antibodies. For probing blots, all antibodies were diluted into a solution containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/dl sodium azide, 50 mg/dl Tween 20, and 0.1 g/dl bovine serum albumin (pH 7.5). The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (31458, Pierce, Rockford, IL) diluted to 1:3,000. Sites of antibody-antigen reaction were viewed using enhanced chemiluminescence substrate (ECL RPN 2106, Amersham Pharmacia Biotech, Buckinghamshire, UK) before exposure to X-ray film (Hyperfilm, Amersham Pharmacia Biotech). Relative quantitation of the band densities from immunoblots was carried out by densitometry using a laser densitometer (GS-700 Imaging Densitometry, Bio-Rad).

Immunohistochemistry. The kidneys were perfused by retrograde perfusion via the abdominal aorta with 1% PBS to remove blood and then with periodate-lysine-paraformaldehyde (PLP; 0.01 M NaIO$_4$, 0.075 M lysine, 2% paraformaldehyde, in 0.0375 M Na$_2$HPO$_4$ buffer, pH 6.2) for kidney fixation for 3 min. After completion of perfusion, each kidney was sliced into 5-mm-thick pieces and immersed in 2% PLP solution overnight at 4°C. Sections of PLP-fixed tissue were cut transversely through the kidney using a vibratome to a thickness of 50 µm and were processed for immunohistochemistry using an indirect immunoperoxidase method. For higher magnification, slices of kidney tissue were dehydrated and embedded in polyester wax, and 5-µm sections were cut and mounted on gelatin-coated glass slides.

The sections were dewaxed with a graded series of ethanol and treated with 3% H$_2$O$_2$ for 30 min to eliminate endogenous peroxidase activity. They were blocked with 6% normal goat serum (S-1,000, Vector Laboratories, Burlington, CA) for 15 min and then incubated overnight at 4°C with the respective primary antibodies diluted in PBS. After incubation, they were washed with PBS and incubated for 30 min in biotinylated goat anti-rabbit IgG (BA-1000, Vector Laboratories) at room temperature. Next, streptavidin-peroxidase from a Vectastatin ABC kit (PK-4000m Vector Laboratories) was added for 30–60 min at room temperature. The sections were washed with PBS and incubated in a 3,3′-diaminobenzidine substrate kit (SK-4100, Vector Laboratories). The slides were mounted with Canadian balsam.

Primary antibodies. For semiquantitative immunoblotting and immunohistochemistry, we used previously characterized polyclonal antibodies against AQP2 (35) and NKCC2 (23). Commercially available antibodies against COX-2 (Cayman Chemical, Ann Arbor, MI) and β-actin (Sigma) were purchased.

Measurement of urinary PGE$_2$. A commercial enzyme immunoassay kit (Assay Designs, Ann Arbor, MI) was used to determine PGE$_2$ in the urine of experimental animals. The assay was performed according to the manufacturer's instructions. One hundred microliters of a urine sample or standard solution was mixed with 50 µl of assay buffer (Tris-buffered saline containing proteins and sodium azide as a preservative), PGE$_2$ antibodies, and PGE$_2$-alkaline phosphatase conjugate in the microplate provided. The mixture was incubated overnight at 4°C. After a series of washes, the substrate (*p*-nitrophenyl phosphate) was applied to the microplate for 1 h at 37°C. After the reaction was stopped with a solution of trisodium phosphate in water, optical density was measured at 450 nm. A standard curve was obtained using optical densities of standard samples and used to determine the PGE$_2$ concentrations in urinary samples.

Determination of medullary osmolality. The kidneys were rapidly removed when the rats were killed. The medulla was cut out and weighed. After being weighed, the medullary tissue samples were dried over a desiccant at 60°C for 8 h. After drying, the samples were reweighed, and fractional water content was calculated by (wet weight − dry weight) ÷ wet weight. The dried tissue samples were then reconstituted to measure solute concentrations following the

Table 1. *Physiological parameters in NDI rats with and without DFU treatment*

	NDI Control ($n = 5$)	DFU Treatment ($n = 5$)
Body weight, g	301±8	294±9
Volume, ml/day	134±15	68±10*
Osmolality, mosmol/kgH$_2$O	247±40	402±51*
Na$^+$, µmol/day	2,255±151	2,255±401
K$^+$, µmol/day	4,168±218	3,674±448
Cl$^-$, µmol/day	3,619±275	3,312±413
Creatinine clearance, ml/min	1.32±0.12	1.03±0.04

Values are means ± SE. NDI, nephrogenic diabetes insipidus; DFU, 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methanesulfonylphenyl)-2(5H)-furanone 1. *$P < 0.005$ by Mann-Whitney *U*-test.
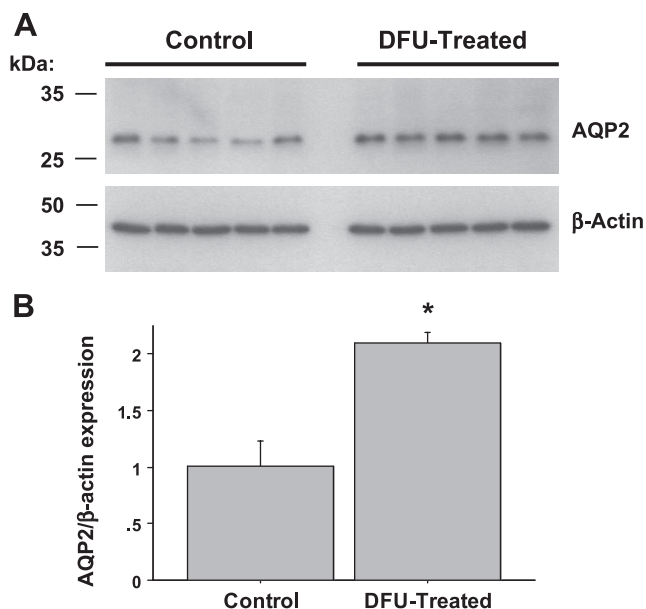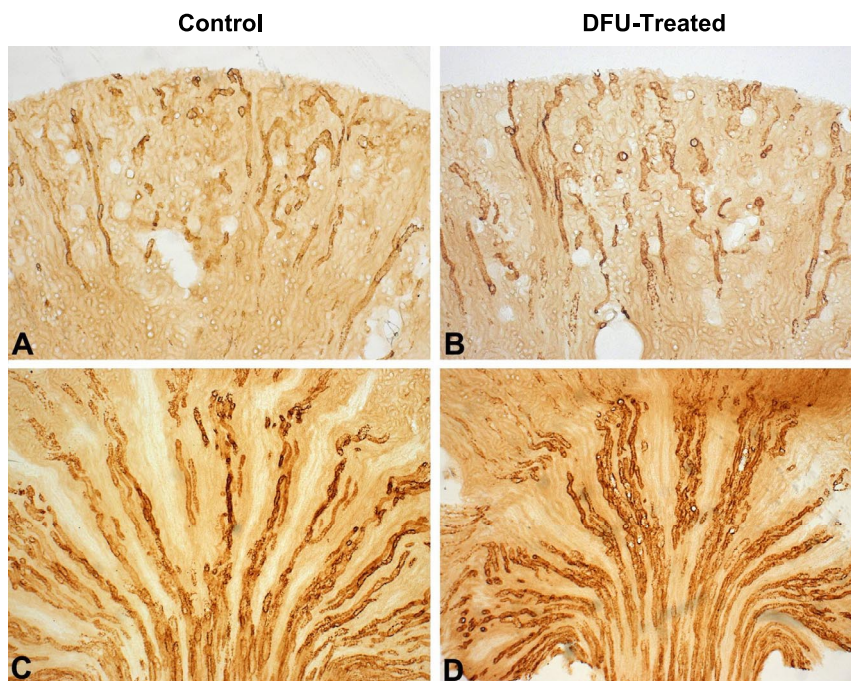
Fig. 1. Effect of cyclooxygenase-2 (COX-2) inhibition on aquaporin-2 (AQP2) abundance in lithium-induced nephrogenic diabetes insipidus (NDI) rat kidneys. *A*: immunoblot reacted with anti-AQP2 antibody shows an increase in AQP2 abundance in lithium-induced NDI rats with COX-2 inhibition (DFU-treated) vs. without COX-2 inhibition (control). Each lane was loaded with a protein sample from a different rat. *B*: densitometric analysis reveals a significant (*$P < 0.01$) increase in whole-kidney AQP2 abundance in lithium-induced NDI rats with COX-2 inhibition (DFU-treated) compared with those without COX-2 inhibition (control).

work of Schmidt-Nielsen et al. (43). Osmolality from the tissue water was determined by freezing-point depression osmometry (Fiske, Burlington, MA). Total tissue osmolality was calculated as follows (28): total osmolality [mosmol/kgH$_2$O] = [urea] + 2[Na$^+$] + 2[K$^+$]. Na$^+$ and K$^+$ were determined by ion-selective electrodes and urea by the colorimetric method using a Bayer autoanalyzer (model ADVIA 1650).

Statistics. Values are presented as means ± SE. Quantitative comparisons between the groups were made by the Mann-Whitney *U*-test (Statview software; Abacus Concepts, Berkeley, CA). To facilitate comparisons in the semiquantitative immunoblotting, we normalized the band density values by dividing by the mean value for the normal control group. Thus the mean for the normal control group is defined as 100%. $P < 0.05$ was considered as indicative of statistical significance.

RESULTS

NDI was successfully induced by lithium administration for 3 wk, and urine volume was remarkably increased to 101 ± 14 ml/day ($n = 10$) compared with the baseline value of 12 ± 2 ml/day ($n = 10$). This degree of polyuria was comparable to that of our previous study (25), in which downregulation of AQP2 was demonstrated. The rats ate all of the offered food during the course of the day, and body weight steadily increased during the study period in both control and DFU-treated groups.

Table 1 summarizes changes in physiological parameters in response to DFU treatment in lithium-induced NDI rats. After the treatment with DFU for 7 days, urine volume decreased significantly, but there was no significant change in control NDI rats. Consistent with the change in urine volume, urine osmolality was higher in DFU-treated rats than in control rats. However, urinary excretion of sodium, potassium, and chloride was not affected by the DFU treatment. Creatinine clearance showed a somewhat decreasing tendency in DFU-treated group but was not statistically changed (Table 1). The 24-h urinary PGE$_2$ excretion was significantly decreased in DFU-treated rats compared with the lithium-induced NDI controls (81 ± 20 vs. 236 ± 49 ng/day, $P < 0.05$, $n = 5$/group).

Figure 1 shows the effect of DFU treatment on AQP2 protein abundance in lithium-induced NDI rats. Semiquantitative immunoblotting from whole-kidney homogenates revealed that DFU treatment for 7 days caused a significant

Fig. 2. Light micrographs of 50-μm-thick vibratome sections of kidney from lithium-induced NDI rat without (*A* and *C*) and with COX-2 inhibition (*B* and *D*) illustrating AQP2 immunoreactivity in cortex (*A* and *B*) and outer medulla (*C* and *D*). In COX-2 inhibitor (DFU)-treated rats, AQP2 immunoreactivity was increased in the cortical collecting duct (*B*) and outer medullary collecting duct (*D*) compared with control rats. Magnification: ×200 (*A*–*D*).

increase in the abundance of AQP2 protein (Fig. 1*A*). When densitometry was adjusted using β-actin band density, DFU treatment markedly increased the expression of AQP2 in the whole kidney to 209 ± 10% of the controls ($P < 0.01$, Fig. 1*B*).

Figure 2 demonstrates AQP2 immunohistochemistry from NDI control and DFU-treated rats, confirming the effects of DFU treatment. Compatible with the immunoblotting results, it revealed that AQP2 expression was increased by DFU treatment in both the cortex and medulla. At high magnification, DFU-treated rats showed a stronger apical AQP2 labeling along the collecting duct compared with NDI control rats (Fig. 3).

In addition, semiquantitative immunoblotting from whole-kidney homogenates revealed that DFU treatment for 7 days caused a significant increase in the abundance of NKCC2 protein (Fig. 4*A*). When densitometry was adjusted using β-actin band density, DFU treatment significantly increased the expression of NKCC2 in the whole kidney to 176 ± 29% of the controls ($P < 0.05$, Fig. 4*B*).

Figure 5 shows NKCC2 immunohistochemistry from NDI control and DFU-treated rats, confirming the effects of DFU treatment. Compatible with the immunoblot results, it revealed that NKCC2 expression was increased by DFU treatment in both cortex and outer medulla (Fig. 5, *A–D*). At high magnification, results from DFU-treated rats showed stronger apical NKCC2 labeling along the thick ascending limb compared with NDI control rats (Fig. 5, *E* and *F*).

We also measured medullary osmolality from the excised tissue (43) to see whether this increase in NKCC2 expression had an effect on the corticomedullary osmotic gradient. There was no significant difference in measured medullary osmolality between DFU-treated rats and NDI controls (517 ± 42 vs. 468 ± 39 mosmol/kgH₂O, $n = 5$/group). Total tissue osmolality estimated by an alternative method (2[Na$^+$] + 2 [K$^+$] +

[urea]) (28) also was not different between the two conditions (403 ± 25 mmol/kgH₂O in DFU-treated rats vs. 376 ± 28 mmol/kgH₂O in NDI controls).

Finally, we tested whether the expression of COX-2 protein was affected by our DFU treatment. The abundance of
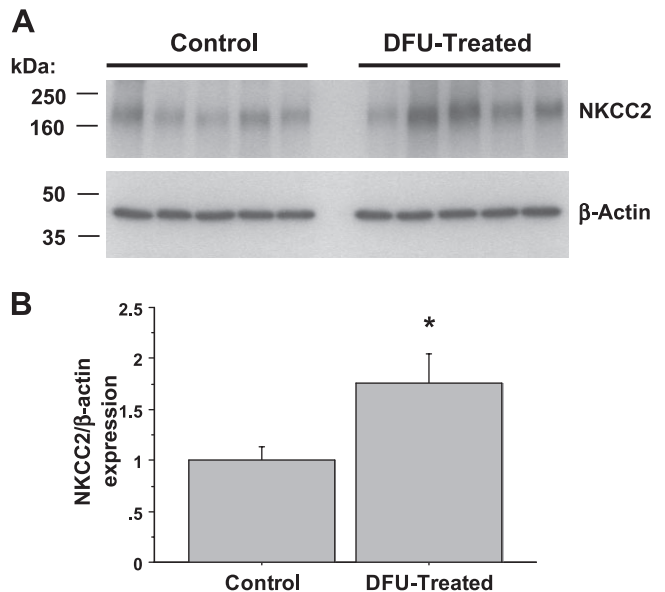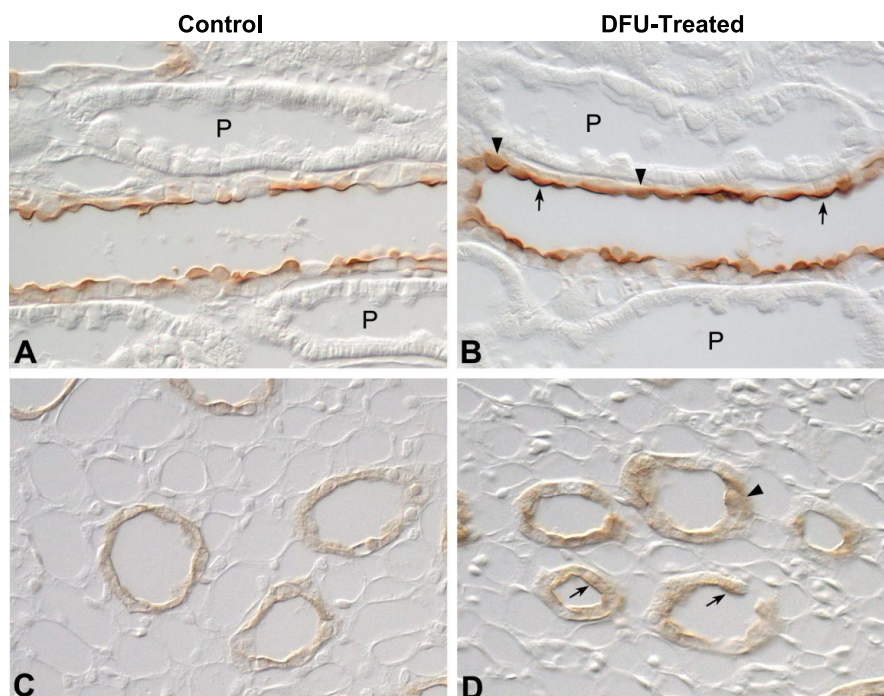

Fig. 4. Effect of COX-2 inhibition on Na-K-2Cl cotransporter type 2 (NKCC2) abundance in lithium-induced NDI rat kidneys. *A*: immunoblot reacted with anti-NKCC2 antibody shows an increase in NKCC2 abundance in lithium-induced NDI rats with COX-2 inhibition (DFU-treated) vs. without COX-2 inhibition (control). Each lane was loaded with a protein sample from a different rat. *B*: densitometric analysis reveals a significant ($P < 0.05$) increase in whole kidney NKCC2 abundance in lithium-induced NDI rats with COX-2 inhibition (DFU-treated) compared with those without COX-2 inhibition (control).

Fig. 3. Light micrographs of kidney from lithium-induced NDI rat without (*A* and *C*) and with COX-2 inhibition (*B* and *D*) illustrating AQP2 immunoreactivity in cortical collecting duct (*A* and *B*) and outer medullary collecting duct (*C* and *D*). In COX-2 inhibitor-treated rats, AQP2 immunoreactivity was increased in the apical plasma membrane (arrows) and distributed in the cytoplasm (arrowheads) of the cortical collecting duct (*B*) and outer medullary collecting duct (*D*). P, proximal tubule. Magnification: ×620 (*A–D*).
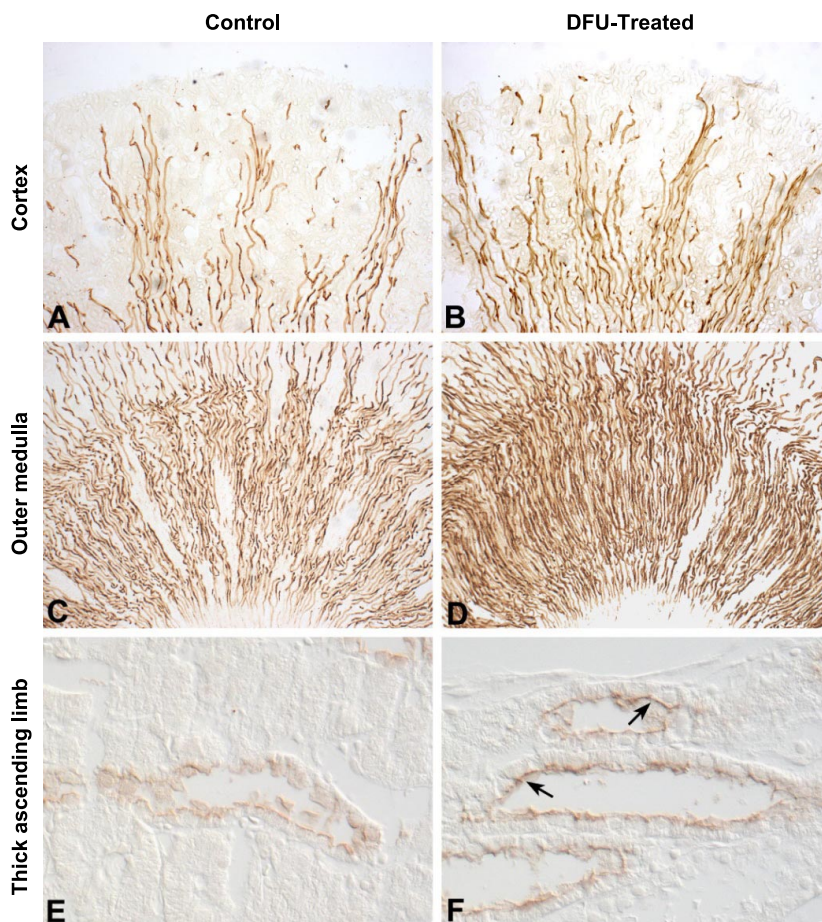
Control   **DFU-Treated**

Fig. 5. Light micrographs of immunostaining for NKCC2 in kidneys from lithium-induced NDI rat without (*A*, *C*, and *E*) and with COX-2 inhibition (*B*, *D*, and *F*) in the cortex (*A* and *B*), outer medulla (*C* and *D*) and outer medullary thick ascending limb (TAL; *E* and *F*). In COX-2 inhibitor-treated rats, NKCC2 immunoreactivity was increased in cortical TAL (*B*) and outer medullary TAL (*D*). With higher magnification of outer medullary TAL (*F*), NKCC2 immunoreactivity was markedly increased in the apical plasma membrane compared with control rats (arrows). Magnification: ×200 (*A*–*D*); ×620 (*E* and *F*).

COX-2 protein was increased in both cortex ($207 \pm 11\%$ vs. control, $P < 0.05$) and medulla ($177 \pm 20\%$ vs. control, $P < 0.01$) of DFU-treated rat kidneys (Fig. 6), suggesting a compensatory response as previously demonstrated (11, 18, 36), and providing further confirmation that our DFU treatment was successful in the inhibition of COX-2 enzyme activity.

DISCUSSION

Lithium treatment is associated with marked downregulation of AQP2 expression (29, 32), consistent with clinical observation of impaired urinary concentration among patients receiving long-term lithium therapy (6). Lithium-induced NDI, therefore, offers a model system with which to develop a therapeutic strategy for this pathophysiological AQP2 downregulation. Analogous to the long-term regulation of AQP2 by vasopressin, the expression level of the thick ascending limb NKCC2 is important for maintaining urinary concentration (24). In this study, we demonstrate that in lithium-induced NDI rats, treatment with a COX-2 inhibitor improves the urinary concentration defect via upregulation of the expression of AQP2 and NKCC2 proteins in the kidney.

AQP2 is the apical water channel of the principal cells and is the chief target for regulation of collecting duct water permeability by vasopressin (35). Vasopressin-regulated water transport in the collecting duct is markedly influenced by PGE_2, probably via the EP1 and EP3 receptors (7), and

increased renal PGE_2 production has been suggested to play an important role in promoting lithium-induced polyuria. Previous studies in rats indicate that urinary PGE_2 is increased by lithium treatment and that indomethacin, a nonselective COX inhibitor, reduced both urine PGE_2 production and urine volume in lithium-induced NDI (47). Recently, lithium-induced NDI in mice was shown to be accompanied by increases in both renal COX-2 expression and urine PGE_2 excretion (40). Thus COX-2 inhibition could be a therapeutic option to relieve polyuria in lithium-induced NDI.

Our results showed that the expression level of AQP2 can be modified by COX-2 inhibition in lithium-induced NDI rat kidneys. Consistent with the inhibitory action on prostaglandin synthesis, urinary PGE_2 excretion was markedly reduced in response to the COX-2 inhibitor we used. PGE_2 may therefore have impaired the vasopressin-regulated AQP2 response in the collecting duct in lithium-induced NDI rat kidneys. Recently, Norregaard et al. (36) reported that selective COX-2 inhibition prevented downregulation of renal inner medullary AQP2 expression in rats with bilateral ureteral obstruction, another animal model of NDI.

As a result of their frequent hyperosmolar state, patients with X-linked NDI, as well as those on lithium therapy, exhibit increased serum vasopressin levels (38, 41). Increased vasopressin levels would enhance vasopressin-stimulated synthesis of renal PGE_2 by the collecting duct epithelial cells (17). Infusion of vasopressin caused antidiuresis in water-loaded
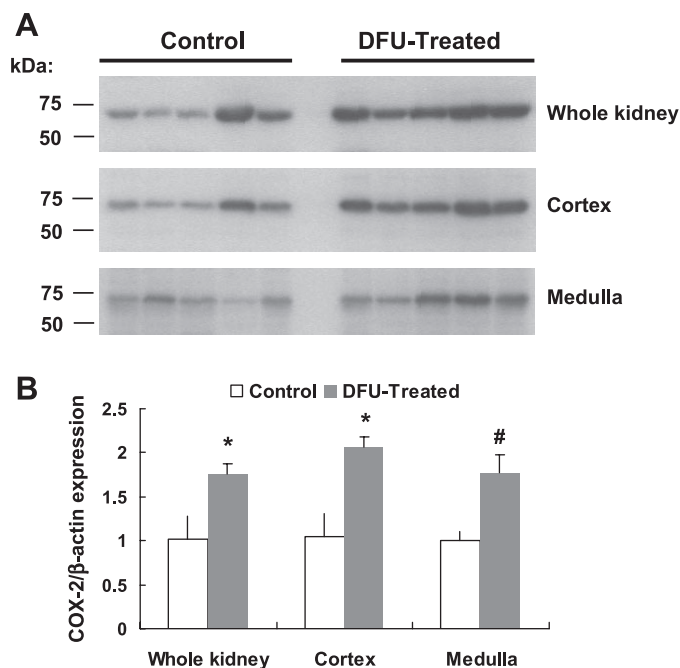
A

B

Fig. 6. Effect of COX-2 inhibition on COX-2 protein abundance in lithium-induced NDI rat kidneys. *A*: immunoblots reacted with anti-COX-2 antibody show increases in COX-2 protein abundance in lithium-induced NDI rats with COX-2 inhibition (DFU-treated) vs. without COX-2 inhibition (control). Each lane was loaded with a protein sample from a different rat. *B*: densitometric analyses reveal that the abundance of COX-2 protein was significantly increased in both renal cortex and medulla of lithium-induced NDI rats with COX-2 inhibition (DFU-treated) compared with those without COX-2 inhibition (control). *$P < 0.05$, #$P < 0.01$ by Mann-Whitney *U*-test.

dogs, and the antidiuretic activity of vasopressin was greatly enhanced by the administration of prostaglandin synthesis inhibitors (10). Besides, inhibition of renal prostaglandin synthesis is associated with an enhanced response to vasopressin (1, 3) via an increase in cAMP synthesis (13, 31). Among several types of PGE$_2$ receptors, EP3 receptors are coupled to adenylyl cyclase through the inhibitory G protein G$_i$ to inhibit cAMP production (7, 19).

Thus it is conceivable that the increased AQP2 protein abundance by COX-2 inhibition in our NDI model might be cAMP dependent. Li et al. (30), however, recently demonstrated that both in mpkCCD cells and in vivo the lithium-induced downregulation of AQP2 occurred independently of adenylate cyclase activity and vasopressin-induced cAMP levels. Consistent with this, previous studies showed in lithium-induced NDI rats that administration of cAMP did not interrupt lithium-induced polyuria (14) and that treatment with indomethacin had no effect on vasopressin-stimulated cAMP production (48). Alternatively, whether pathways of glycogen synthase kinase 3 and phospho-inositol kinases (30) or steps distal to the generation of cAMP such as AKAP18δ (4) are involved in lithium-induced NDI need to be identified.

Other factors independent of the presence of vasopressin may contribute to the increase in the reabsorption of water by the collecting duct during diuresis as well as antidiuresis when the synthesis of renal prostaglandins is inhibited. The role of the increase in osmolality of the medullary interstitium was suggested in the antidiuretic effect of indomethacin in Brattleboro rats (46). Previously, an increased concentration of

NaCl in the renal papilla has been reported when renal prostaglandin synthesis is inhibited (15). This increase in the renal medullary sodium chloride concentration would be expected to increase the osmotic gradient during water reabsorption across the terminal collecting duct. Thus nonspecific prostaglandin synthesis inhibitors as well as selective COX-2 inhibitors can enhance the hydrosmotic response to vasopressin to increase the permeability of the cells to water. Given these facts, the role of NKCC2 in the medullary thick ascending limb was addressed.

In the medullary thick ascending limb, NKCC2 plays an important role in long-term regulation of the countercurrent multiplication system, enhancing urinary concentration (24). In this study, we showed that COX-2 inhibition increased NKCC2 expression in lithium-induced NDI rat kidneys. Previously, administration of NSAIDs indomethacin and diclofenac to normal rats substantially increased the NKCC2 protein abundance (12). Thus PGE$_2$ seems to have a long-term inhibitory effect on NKCC2 protein expression. It is known that PGE$_2$ binds to the EP3 receptor in the thick ascending limb, which couples to adenylyl cyclase in an inhibitory manner via the heterotrimeric G protein G$_i$ (7).

The expression of NKCC2 may also be dependent on the level of intracellular cAMP (26) because Ecelbarger et al. (8) found that, in mice with heterozygous disruption of the G$_s$α gene, both cAMP production and NKCC2 expression from the thick ascending limb were reduced by half. In addition, the abundance of NKCC2 protein is chronically regulated by vasopressin (24). These previous studies suggest an association between V2-mediated signaling and the level of NKCC2 expression in lithium-induced NDI.

As mentioned above, however, COX-2 inhibition may ameliorate polyuria in NDI by a mechanism independent of the presence of vasopressin. Although plasma vasopressin was raised in lithium-induced NDI rats, it was not affected by the chronic administration of indomethacin (47). In patients with congenital NDI, plasma vasopressin concentrations were not further increased by the treatment with indomethacin (41). Instead, Stoff et al. (46) found in both water-loaded Sprague-Dawley rats and Brattleboro rats that treatment with indomethacin or meclofenamate slowed water diuresis and increased the osmolality of the renal papilla by raising sodium and urea content. Thus endogenous prostaglandins seem to have a direct effect on solute transport by renal tubules.

With COX-2 inhibition in lithium-induced NDI, however, increasing NaCl absorption by the NKCC2 may not have affected the corticomedullary osmotic gradient because neither measured nor calculated renal medullary osmolality was significantly changed by our DFU treatment in lithium-induced NDI rats. According to Christensen et al. (8), both cortical and medullary osmolalities were not lowered by lithium treatment in rats. It was only in the renal papilla of lithium-induced NDI rats (8) and indomethacin-treated Brattleboro rats (46) that the osmolality was significantly affected.

As an indirect determinant of the osmotic interstitial gradient in the renal medulla, the role of prostaglandins in maintaining renal medullary blood flow may be important. PGE$_2$ directly dilates descending vasa recta, and increased medullary blood flow may contribute to the increased interstitial pressure that occurs as renal perfusion pressure increases, leading to enhanced salt excretion (44). 20-Hydroxyeicosatetraenoic acid

(20-HETE), the major eicosanoid in the kidney, was recently reported to produce differential effects in the kidney, i.e., vasoconstriction in the cortex and vasodilation in the medulla (37). Thus medullary blood flow may be affected by COX-2 inhibition, resulting in an alteration of the medullary interstitial osmotic gradient.

In conclusion, treatment of lithium-induced NDI by COX-2 inhibition improves polyuria via upregulation of AQP2 and NKCC2 in the kidney. The upregulation of AQP2 and NKCC2 in response to the COX-2 inhibition may underlie the therapeutic mechanisms by which NSAIDs enhance antidiuresis in patients with NDI. Further investigations are necessary to elucidate the intracellular signaling pathways involved in the action of COX-2 inhibitors.

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