Antidiuretic Effect of Hydrochlorothiazide in Lithium-Induced Nephrogenic Diabetes Insipidus Is Associated with Upregulation of Aquaporin-2, Na-Cl Co-transporter, and Epithelial Sodium Channel

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Abstract. Thiazides have been used in patients with nephrogenic diabetes insipidus (NDI) to decrease urine volume, but the mechanism by which it produces the paradoxical antidiuretic effect remains unclear. Previous studies have reported that downregulation of aquaporin-2 (AQP2) is important for the development of lithium-induced (Li-induced) polyuria and that hydrochlorothiazide (HCTZ) increases renal papillary osmolality and Na+ concentration in Brattleboro rats. For elucidating the molecular basis of the antidiuretic action of HCTZ in diabetes insipidus, whether administration of HCTZ may affect the expression of AQP2 and major renal Na+/Cl− transporters in Li-induced NDI rats was investigated, using semiquantitative immunoblotting and immunohistochemistry. After feeding male Sprague-Dawley rats Li chloride–containing rat diet for 4 wk, HCTZ or vehicle was infused subcutaneously via osmotic minipump. Urine output was significantly decreased by HCTZ treatment, whereas it was not changed in vehicle-treated rats. Urine osmolality was also higher in HCTZ-treated rats than in vehicle-treated rats. Semiquantitative immunoblotting using whole-kidney homogenates revealed that HCTZ treatment caused a significant partial recovery in AQP2 abundance from Li-induced downregulation. AQP2 immunohistochemistry showed compatible findings with the immunoblot results in both cortex and medulla. The abundances of thiazide-sensitive NaCl co-transporter and α-epithelial sodium channel were increased by HCTZ treatment. Notably, HCTZ treatment induced a shift in molecular weight of α-epithelial sodium channel from 85 to 70 kD, consistent with previously demonstrated aldosterone stimulation. The upregulation of AQP2 and distal renal Na+/Cl− transporters in response to HCTZ treatment may account for the antidiuretic action of HCTZ in NDI.

In nephrogenic diabetes insipidus (NDI), the kidney is unable to concentrate urine despite normal or elevated concentrations of the antidiuretic hormone arginine vasopressin. The acquired form of NDI is much more common than the congenital form, which has mutations either in the vasopressin V2 receptor gene or in the aquaporin-2 (AQP2) gene (1). Lithium (Li) treatment (2), hypokalemia (3), and ureteral obstruction (4) are three common causes of acquired NDI, and all have been associated with downregulation of AQP2 (2–4).

One of the recommended regimens for the treatment of NDI is low-sodium diet coupled with thiazides, although amiloride is preferably used to mitigate Li-induced polyuria in humans because of its blunting the inhibitory effect of Li on water transport in the collecting duct (5). Thiazide diuretics paradoxically decrease urine volume and increase urine osmolality presumably by producing a mild sodium depletion (6). The widely accepted hypothesis suggests that thiazide-induced sodium depletion causes a reduction in distal delivery associated with enhanced fractional water reabsorption in the collecting duct (6,7). However, the mechanism by which thiazide diuretics produce their paradoxical antidiuretic effect in patients or animals with diabetes insipidus is poorly understood. Inhibition of NaCl reabsorption in the distal convoluted tubule alone does not account for the antidiuretic effect of the thiazide diuretics (6).

According to studies by Walter et al. (8) and Shirley et al. (9) on Brattleboro rats, the antidiuretic effect of hydrochlorothiazide (HCTZ) is secondary not only to the extracellular
volume contraction but also to redistribution of body sodium and increase in papillary osmolality. In addition, the possibility that thiazide diuretics can directly affect water permeability in the collecting duct epithelium was suggested (10), and a recent in vitro microperfusion study reported that HCTZ enhanced water absorption in inner medullary collecting duct from normal rats and from Brattleboro rats (11).

These previous notions led us to hypothesize that to produce the antidiuretic effect in diabetes insipidus, HCTZ may directly or indirectly act in the distal nephron segments and collecting duct to affect expression of water channels and sodium transporters. To test this hypothesis, we used semiquantitative immunoblotting and immunohistochemistry to investigate the effects of HCTZ on the expression of AQP2, Na,H,exchanger type 3 (NHE3), bumetanide-sensitive Na-K-2Cl co-transporter type 2 (NKCC2), thiazide-sensitive NaCl co-transporter (NCC), and epithelial sodium channel (ENaC) in Li-induced NDI rat kidneys.

**Materials and Methods**

**Animals and Experimental Protocols**

Specific pathogen-free male Sprague-Dawley rats (SLC Inc., Shizuoka, Japan) that weighed 180 to 220 g were used to produce Li-induced NDI animals and to treat with HCTZ. Initially, the rats were randomly allocated to normal controls (n = 4) and Li-induced NDI rats (n = 7). For Li administration, Li chloride was added to the rat diet to give a concentration of 40 mmol Li/kg dry food as described previously (2,12). Normal controls were given the same amount of food without Li, and all rats were allowed free access to water. After 3 wk of Li administration, NDI induction was confirmed by measurements of urine output and urine osmolality.

Then, after an additional week of Li administration to ensure the induction of NDI, Li-induced NDI rats were randomly divided into vehicle-treated rats (n = 4) and HCTZ-treated rats (n = 3). For chronic HCTZ treatment for 7 d, osmotic minipumps (model 2ML1; Alzet, Palo Alto, CA) were implanted subcutaneously to deliver 3.75 mg/d HCTZ (YuHan Corp., Seoul, Korea) as described previously (13). HCTZ was dissolved in a 1.7% ethanolamine solution, and the minipumps that contained vehicle (ethanolamine) alone were implanted in vehicle-treated rats. Li-containing food was offered continuously to both groups throughout the study period. Considering the role of sodium depletion in the antidiuretic effect of thiazides in NDI (14,15), no salt was added to the drinking fluid. Serum samples were collected at the time each rat was killed for determination of the serum Li concentration by a colorimetric method (VITROS 250 Chemistry System; Ortho-Clinical Diagnostics, Raritan, NJ). The kidneys were perfused by retrograde perfusion via the abdominal aorta with 1% PBS to remove blood and then with periodate-lysine-paraformaldehyde (0.01 M NaO, 0.075 M lysine, 2% paraformaldehyde, in 0.0375 M Na2HPO4 buffer [pH 6.2]) for the kidney fixation for 3 min. After completion of perfusion, each kidney was sliced into 5-mm-thick pieces and immersed in 2% periodate-lysine-paraformaldehyde solution overnight at 4°C. Each slice was dehydrated with a graded series of ethanol and embedded in polyester wax. The embedded pieces of kidney were sectioned at 5-μm thickness on a microtome (RM 2145; Leica Instruments GmbH, Nussloch, Germany).

**Semiquantitative Immunoblotting**

After the whole-animal experiments, the rats were killed by decapitation, and kidneys were rapidly removed and placed in chilled isolation solution that contained 250 mM sucrose, 10 mM triethanolamine (Sigma, St. Louis, MO), 1 μg/ml leupeptin (Sigma), and 0.1 mg/ml PMSF (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/ml with isolectric focusing (ITF). Five micrograms of protein from each sample was loaded into each individual lane and electrophoresed on 12% polyacrylamide-SDS minigels using 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.

**Immunohistochemistry**

The kidneys were perfused by retrograde perfusion via the abdominal aorta with 1% PBS to remove blood and then with periodate-lysine-paraformaldehyde (0.01 M NaO, 0.075 M lysine, 2% paraformaldehyde, in 0.0375 M Na2HPO4 buffer [pH 6.2]) for the kidney fixation for 3 min. After completion of perfusion, each kidney was sliced into 5-mm-thick pieces and immersed in 2% periodate-lysine-paraformaldehyde solution overnight at 4°C. Each slice was dehydrated with a graded series of ethanol and embedded in polyester wax. The embedded pieces of kidney were sectioned at 5-μm thickness on a microtome (RM 2145; Leica Instruments GmbH, Nussloch, Germany).

The sections were dewaxed with a graded series of ethanol and treated with 3% H2O2 for 30 min to eliminate endogenous peroxidase activity. They were blocked with 6% normal goat serum (S-100; Vector Laboratories, Burlingame, CA) for 15 min and then incubated overnight at room temperature. Next, peroxidase standard Vectastain ABC kit (PK-4000; Vector Laboratory) were added for 30 min at room temperature. The sections were washed with PBS and incubated in 3,3’-diaminobenzidine substrate kit (SK-4100; Vector Laboratories, Burlingame, CA) for 15 min with 0.05% H2O2. 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 quantification of the band densities from immunoblots was carried out by densitometry using a laser densitometer (GS-700 Imaging Densitometry; Bio-Rad).

**Primary Antibodies**

For semiquantitative immunoblotting and immunohistochemistry, we used previously characterized polyclonal antibodies. Affinity-
purified polyclonal antibodies against AQP2 (17), NHE3 (18), NKCC2 (19), NCC (20), and ENaC (21) were used.

Statistical Analyses

Values are presented as mean ± SEM. Quantitative comparisons between the groups were made by Mann-Whitney U test (Statview software; Abacus Concepts Inc., 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

Induction of NDI by Li Administration

Diabetes insipidus (DI) was successfully induced by Li administration for several weeks. Urine output measured at the end of the third week of the experiment was remarkably high in Li-administered rats (n = 7; 110 ± 7 ml/d) compared with normal controls (n = 4; 10 ± 3 ml/d). Urine osmolality was also remarkably lower in Li-administered rats (89 ± 10 mOsm/kgH₂O) than in normal controls (1670 ± 287 mOsm/kgH₂O).

Effects of Chronic HCTZ Treatment

In NDI (Li-administered) rats, the effects of chronic HCTZ treatment were tested after 7 d of continuous infusion. After HCTZ treatment, urine output was significantly decreased (n = 3; 46 ± 11 ml/d), whereas it did not show a significant change in vehicle-infused rats (n = 4; 127 ± 1 ml/d). Urine osmolality was also higher in HCTZ-treated rats (557 ± 139 mOsm/kgH₂O) than in vehicle-treated rats (207 ± 9 mOsm/kgH₂O). Considering the influence of HCTZ treatment on renal Li clearance (22–25), we measured serum Li levels. The individual values (mEq/L) were 0.3, 0.3, 0.4, and 0.5 in Li + vehicle-treated rats; 0.4, 0.5, and 0.5 in Li + HCTZ-treated rats; and <0.2 in all normal controls. Thus, the serum Li level was not significantly different between vehicle and HCTZ treatment in Li-NDI rats. Figure 1 summarizes serial changes of daily urine output and urine osmolality in response to Li with and without HCTZ treatment.

Figure 2 shows an immunoblot of AQP2 from whole-kidney homogenates. The abundance of NHE3 was not significantly altered by chronic Li administration but increased by HCTZ treatment in Li-induced NDI rats (292 ± 127 mOsm/kgH₂O). Conversely, the abundance of NCC was not significantly affected by Li administration but increased by HCTZ treatment in Li-induced NDI rats (271 ± 270 mOsm/kgH₂O).

Figure 5 shows NCC immunohistochemistry from the renal cortex of normal control, Li + vehicle-treated, and Li + hydrochlorothiazide-treated rats (n = 3).

Figure 1. Urine output (A) and urine osmolality (B) at basal state (D0), after lithium (Li) administration (D21), and after hydrochlorothiazide treatment (D35). Data represent mean ± SEM. Control, normal control rats (n = 4); Li, Li + vehicle-treated rats (n = 4); Li + TZ, Li + hydrochlorothiazide-treated rats (n = 3).
HCTZ-treated rats. Compatible with the immunoblot result, it demonstrates a marked increase in immunostaining in Li + HCTZ-treated versus Li + vehicle-treated rat.

**Discussion**

We demonstrate that, in Li-induced NDI rats, upregulation of AQP2, NCC, and ENaC is induced by chronic HCTZ treatment. A partial recovery from Li-induced AQP2 downregulation and aldosterone-activated upregulation of distal renal Na⁺ transporters may account for the antidiuretic action of HCTZ in NDI. These results provide new information on the renal mechanisms of the antidiuretic effect of thiazide diuretics in DI.

**HCTZ Treatment Partially Reverses Li-Induced AQP2 Downregulation**

Chronic Li therapy is known to be a major cause of NDI (26), and downregulation of AQP2 has been shown to be associated with the development of severe polyuria in both central and nephrogenic DI (27–30). Consistent with previous studies (2,12), we showed that the abundance of AQP2 in rat kidney was markedly decreased (~80%) after several weeks of Li administration. This was associated with an increase (~11-fold) in urine output and a decrease in urine osmolality (~95%) in the Li-induced NDI rats. With HCTZ treatment for 1 wk, polyuria was significantly reduced (~65%) and urine osmolality was also markedly enhanced (~170%). Thiazide diuretics may affect renal Li clearance and hence alter Li toxicity. When chlorothiazide was administered to Na⁺-restricted healthy humans, it acutely increased Li clearance by ~25% (22). Conversely, when using thiazides chronically with Li, these diuretics decrease renal clearance of Li (23). Specifically, thiazide diuretics have demonstrated the greatest potential to increase Li concentrations, with a 25 to 40% increase in concentrations often evident after initiation of therapy (24,25). According to the results of the serum Li levels from our animals, HCTZ treatment does not seem to have affected Li toxicity in our animal model of NDI.

When thiazide diuretics are administered to patients with DI, the effect of reduction in urine volume and elevation of urine osmolality was first observed by Crawford and Kennedy (31) and later confirmed by others (32,33). However, this apparent clinical pharmacologic paradox has not been clearly understood, and the following mechanism has been proposed to account for the antidiuretic effect of thiazides (6,7). An initial reduction of sodium reabsorption in the distal convoluted tu-
bule increases sodium excretion and causes extracellular fluid volume contraction. As a result, the GFR decreases and the proximal tubular sodium and water reabsorption increases. Consequently, less water and sodium are delivered to the collecting ducts, and less water is excreted.

Consistent with this notion, in a study on Brattleboro rats with central DI, Walter et al. (34) showed that the antidiuresis that follows acute administration of HCTZ is entirely secondary to the natriuresis and consequent sodium depletion induced by the drug. However, Shirley et al. (9) showed that the mechanism of sustained antidiuresis during chronic HCTZ administration in DI differs from that of the acute response. When they recorded distal delivery and urine flow simultaneously during chronic thiazide antidiuresis, the allover change in distal delivery (−15%) or the enhanced proximal solute and water reabsorption was considered to be too small to account for the profound antidiuresis. Thus, an additional antidiuretic effect on distal nephron segments can be postulated, and our study was undertaken to address this issue.

AQP2 is exclusively expressed in the principal cells of the connecting tubule and collecting duct and is the predominant vasopressin-regulated water channel (29). In this study, we...
demonstrated that the abundance of AQP2 was almost doubled by chronic HCTZ treatment in Li-induced NDI rats. Therefore, the Li-induced AQP2 downregulation was partially (from 20 to 40% of normal controls) reversed by chronic HCTZ treatment. Recently, an in vitro microperfusion study performed by Cesar and Magaldi (11) showed that HCTZ increased osmotic and diffusional water permeabilities in inner medullary collecting duct from normal rats (in the absence of vasopressin) and from Brattleboro rats. Our results suggest that the increase in water permeability induced by HCTZ in NDI should not be limited to the inner medullary collecting duct because our AQP2 immunohistochemistry in cortex showed the similar pattern to that in inner medulla (Figure 3). These data support the hypothesis that thiazides may act in segments beyond the distal convoluted tubule. However, we cannot exclude the possibility that the various transporters are regulated by secondary effects of thiazides including alterations in total body salt and water; changes in aldosterone or angiotensin II; and changes in total body potassium, magnesium, or calcium.

Previously, Gronbeck et al. (28) investigated whether the abundance of the subcellular distribution of AQP2 protein is changed in Brattleboro rats by 5 d of treatment with bendroflumethiazide. In contrast with our result, they reported no change in the expression of AQP2 associated with bendroflumethiazide-induced antidiuresis. Vasopressin may be required for the thiazide-induced increase in AQP2. In the aspect of pharmacologic properties, HCTZ significantly inhibits carbonic anhydrase activity, whereas bendroflumethiazide lacks the carbonic anhydrase inhibiting activity (35).

HCTZ Treatment Upregulates NCC and ENaC in Li-Induced NDI

We also tested whether the abundances of major renal Na\(^+\) transporters are affected by HCTZ treatment in Li-induced NDI. According to studies by Kwon et al. (12) and Nielsen et al. (36), urinary sodium excretion is markedly increased in rats with Li-induced NDI. This wasting of sodium was explained by a marked decrease in the abundance of $\beta$-ENaC and $\gamma$-ENaC in the cortex and outer medulla (36). In addition, HCTZ inhibits sodium chloride transport in the distal convoluted tubule (37). Thus, sodium depletion is expected in Li-induced NDI and HCTZ-treated NDI rats.

In our results from Li-induced NDI rats, the changes in the abundance of major Na\(^+\) transporters in proximal tubule, loop of Henle, and distal convoluted tubule are consistent with a previous study (12). The abundance of NHE3, the major apical Na\(^+\) transporter in proximal tubule, was not affected by Li with and without HCTZ treatment. The abundance of NKCC2, the major apical Na\(^+\) transporter in thick ascending limb of Henle’s loop, was increased in both Li + vehicle-treated and Li + HCTZ-treated groups. This result seems to be compatible with upregulation of NKCC2 by vasopressin (19).

Our interest was to determine whether any changes occur in the expression of distal Na\(^+\) transporters in response to chronic HCTZ treatment in Li-induced NDI. Previously, the osmolality of renal papillary interstitial fluid from HCTZ-treated Brattleboro rats was shown to be considerably greater than that from untreated animals, accompanied by a significant increase in sodium concentration (9). In this study, the abundance of NCC in distal convoluted tubule was not altered by Li administration but increased by HCTZ treatment in Li-induced NDI rats. This result can be interpreted in light of the notion from our previous studies: (1) aldosterone upregulates the expression of NCC (20), and (2) chronic HCTZ treatment induces a compensatory increase in the abundance of NCC (13).

ENaC is the major apical Na\(^+\) pathway in connecting tubule and collecting duct and is activated by aldosterone. We showed a characteristic change in the ENaC subunit proteins, which is consistent with the response to aldosterone stimulation as previously demonstrated (21). With chronic HCTZ treatment, Li-induced NDI rat kidneys revealed an increase in the abundance of $\alpha$-ENaC and a shift in molecular weight of $\gamma$-ENaC from 85 to 70 kD. This finding was shown to be accompanied by a striking redistribution of ENaC from the cytoplasmic to the apical region of the collecting duct principal cells (21). Taken together with the result of the NCC expression, aldosterone release must have been stimulated by HCTZ treatment in our Li-induced NDI rats.

Potential mechanisms of the increase in $\alpha$-ENaC protein abundance include increased transcription of the $\alpha$-ENaC gene (38–41) and alterations in protein synthesis (42) or degradation (43). A likely explanation for a shift in the molecular weight of the $\gamma$ subunit would be a physiologic proteolytic cleavage in response to aldosterone stimulation (21). Studies of ENaC expressed in Xenopus oocytes have demonstrated that serine proteases applied from the extracellular side activate the channel by increasing the open probability (44). Vallet et al. (45) identified an epithelial serine protease, CAP1, that activates ENaC and is induced by aldosterone, providing a possible explanation for the observed molecular weight shift of the $\gamma$ subunit.

Upregulation of NCC and ENaC induced by HCTZ treatment would enhance sodium reabsorption along the distal segments of nephron. The upregulation of NCC tends to mitigate the primary effect of the thiazide diuretic. Besides, the increased ENaC-mediated transport in response to HCTZ decreases luminal osmolality in the cortical connecting tubule and collecting duct and increases the driving force for water reabsorption.

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References


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