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Reverse pharmacological effect of loop diuretics and altered rBSC1 expression in rats with lithium nephropathy

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Background. Renal urinary concentration is associated with enhanced expression of rBSC1, a rat sodium cotransporter, in the thick ascending limb of Henle. Increased expression of rBSC1 was reported recently in nephrogenic diabetes insipidus induced by lithium chloride (Li nephropathy). However, the pathophysiological implication of altered rBSC1 expression has not yet been investigated.

Methods. Li nephropathy was induced in rats by an oral administration of 40 mmol lithium/kg dry food. In rats with reduced urinary osmolality to less than 300 mOsm/kg H_2O, we examined the expression of rBSC1 mRNA and protein, plasma arginine vasopressin (AVP) and RNA expression of kidney-specific water channel, aquaporin-2 (AQP2), of collecting ducts. Rats with Li nephropathy were treated with furosemide (3 mg/kg body weight), which blocks the activity of rBSC1, and changes in urine concentration, plasma AVP, medullary accumulation of Li ions, and apical AQP2 expression were determined.

Results. Rats with Li nephropathy showed increased rBSC1 RNA and protein expression and reduced AQP2 RNA. In these rats, furosemide, which induces dilution of urine and polyuria in normal rats, resulted in a progressive and significant rise in urine osmolality from 167 ± 11 (mean ± SD) at baseline to 450 ± 45 mOsm/kg H_2O at three hours after administration, and significant oliguria. In the same rats, plasma AVP decreased significantly from 5.7 to 3.0 pg/mL. In addition, recovery of apical AQP2 expression was noted in a proportion of epithelial cells of the collecting ducts. Although Li^+ in the renal medulla was slightly lower in rats with Li nephropathy treated with furosemide, statistical significance was not achieved.

Conclusions. Our results suggest that dehydration or high plasma AVP results in an enhanced rBSC1 expression in Li nephropathy, and that rBSC1 expression is closely associated with the adverse effects of Li ions on collecting duct function.

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One of the major toxic effects of lithium ions is nephrogenic diabetes insipidus (Li nephropathy) [1]. The reversibility of the disease suggests that lithium ions have certain physiological effects on tubular function [2]. The recent identification of cell surface membrane proteins that are involved in renal urinary concentration has shed novel insights into the pathophysiology of Li nephropathy [3, 4]. Enhanced expression of a kidney-specific apical water channel in the collecting duct (AQP2) is associated with renal urinary concentration [5–8]; however, Li nephropathy causes specific down-regulation of AQP2 in collecting ducts, directly leading to defective urinary concentration [3].

A kidney-specific sodium cotransporter (BSC1; bumetanide-sensitive cotransporter) in the thick ascending limb of Henle supplies NaCl for countercurrent multiplier to concentrate urine [9, 10]. Our previous studies [11, 12] and those of Kim et al [13] have demonstrated a high expression of rat BSC1 (rBSC1) in dehydrated animals or following arginine vasopressin (AVP) stimulation, indicating that rBSC1 up-regulation also is associated closely with renal urinary concentration. Kwon et al, however, reported the presence of enhanced rBSC1 expression in rats with Li nephropathy and highly defective urinary concentration mechanisms [4]. Thus, the present study was undertaken to assess the paradox of rBSC1 expression in Li nephropathy.

Lithium ion (Li^+) entry into the renal tubular cell and its accumulation in renal medulla occurs through sodium transporters, which exhibit a high selectivity for Li ions as well as Na ions [14]. rBSC1 is a major sodium supplier for countercurrent multiplier and, thus, increased rBSC1 may promote transcellular Li transport and its accumulation in interstitial space of renal medulla [15], possibly influencing renal tubular function. Since loop-diuretics, such as furosemide, inhibit the functional ability of rBSC1,

Li^+ uptake though rBSC1 can be transiently blocked by furosemide administration [16]. Therefore, in the present study, we investigated various physiological parameters in a rat model of Li nephropathy before and after administration of furosemide, and examined rBSC1 expression. Our results demonstrated that altered rBSC1 expression is involved in the pathophysiology of Li nephropathy.

METHODS

Experimental protocol

Male Sprague-Dawley rats weighing around 250 g were treated orally with lithium chloride as described previously [3]. Briefly, 32 rats received 40 mmol/L lithium chloride (LiCl)/kg of dry food. During the subsequent 11 to 13 days, rats had free access to water and were maintained in a humidity- and temperature-controlled room with a 12/12 hour light/dark cycle. Seven days after the start of LiCl administration, each rat was moved to an individual metabolic cage for urine collection. The baseline urinary volume and osmolality measurements used for further examinations are summarized in Table 1. When osmolality decreased to $<$300 mOsm/kg H_2O, kidneys from six rats were removed for RNA (right kidneys) and protein extraction (left kidneys from 3 rats) and for immunohistochemistry (left kidneys from 3 rats), and 14 rats were catheterized as described previously [5, 12]. Briefly, catheters filled with heparinized (100 U/mL) 0.9% NaCl were placed in the femoral artery and vein. Each rat was housed in a separate cage and provided with water containing 5% dextrose to ensure adequate postoperative hydration. On the following day, after urine collection for one hour, a 3-mL blood sample was withdrawn that was replaced with a simultaneous injection of an equal volume of donor rat blood. Then, each rat was injected intraperitoneally with furosemide (3 mg/kg body weight) or saline, and returned to its individual metabolic cage. Urine was collected every one hour for three hours in awakened animals. Another 3-mL blood sample was withdrawn from each treated rat, and then each rat was deeply anesthetized and the kidneys removed. Each kidney was divided into three parts: the cortex, outer medulla, and inner medulla. Samples from the right kidney were homogenized in 4 mol/L guanidine with 25 mmol/L sodium citrate and 0.7% mercaptoethanol for RNA extraction, and those from the left kidney were homogenized in 2 mL of phosphate-buffered saline (PBS), 1% triton, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) for protein extraction. The left kidneys of six rats (3 treated with furosemide and 3 with saline) were sliced and fixed in 95% ethanol and used for immunohistochemistry. The resting 12 rats with Li nephropathy were treated with furosemide or saline as mentioned above, and the renal medulla was separated and used to measure Li ion in the tissue.

Six control rats without LiCl treatment were used for RNA and protein extraction and for immunohistochemistry. The experimental protocol was approved by the Ethics Committee for Animal Experimentation at Tohoku University.

Competitive PCR for rBSC1

Competitive polymerase chain reaction (PCR) analysis for rBSC1 was performed as described previously [11, 17]. Briefly, a point mutation for the formation of *EcoR*I site was induced in the middle of a 352 bp of a partial fragment for the kidney-specific sodium cotransporter (rBSC1) cDNA. A series of diluted mimic cDNA was mixed with a constant amount of sample template cDNA of the renal outer medulla and co-amplified using rBSC1 primers. PCR proceeded for 25 cycles, and then the products were co-incubated with *EcoR*I for three hours at 37°C. The sample and mimic cDNA were designed to produce 352 and 180 bp fragments, respectively. After agarose gel electrophoresis, the intensity of the band in each sample and mimic cDNA was measured using a densitometer. The amount of rBSC1 mRNA in the sample was calculated from the equivalent point (40 $\times$ mimic for rBSC1 at the equivalent point/1 μg RNA).

Competitive PCR for AQP2

To construct a mimic cDNA, as described previously [5, 12], a pair of PCR primers was designed to frame the major part of AQP2 cDNA (760 bp), which contained *Sph*I and *Sac*I restriction enzyme sites in the middle of the product. By deleting 180 bp between these sites, the final PCR product was 580 bp, which was obtained and used to mimic cDNA for competitive PCR. A series of diluted mimic cDNA was mixed with the same amount of sample template cDNA (from 0.05 μg RNA) from each part of the kidney and co-amplified using the AQP2 primer set. PCR was performed using 25 cycles. Following agarose gel electrophoresis, the intensity of the bands in each sample and those of the mimic cDNA was measured using a densitometer. The amount of AQP2 mRNA in the sample was calculated from the equivalent point (20 $\times$ amount of mimic cDNA at the equivalent point/ 1 μg RNA).

Western blots

We prepared peptide-directed rabbit polyclonal antibodies that specifically recognize amino acid 33-55 of rBSC1 according to Kim et al's study [13]. The antisera to rBSC1 was affinity-purified using a column on which 1 mg of the appropriate synthetic peptides were immobilized via covalent linkage to Sepharose beads (Sepharose 4B; Amersham Bioscience, Little Chalfont, Buckinghamshire, UK). Immunoblotting using the specific antibody

Table 1. Effects of LiCl treatment

	Lithium treatment	
	Before	After
N	20	20
Body weight *g*	254 ± 9	270 ± 9[a]
Water intake *mL/24 h*	43 ± 11	136 ± 37[a]
Urine output *mL/6 h*	4.2 ± 1.7	20.7 ± 9.2[a]
Urine osmolality *mOsm/kg H_2O*	899 ± 285	198 ± 69[a]
Lithium intake *mmol/day*	0	0.65 ± 0.14

Data are means ± SD.
[a] $P < 0.05$ vs. before lithium treatment

against rBSC1 was performed as previously described [12, 17]. Briefly, the renal outer medulla was homogenized in 2 mL of PBS, 1% triton, 1% deoxycholate, 0.1% SDS, and 0.1 mmol/L PMSF. One hundred micrograms of protein in each lane were loaded in Laemmli SDS-polyacrylamide (8%) gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane was blocked for one hour and exposed to antibody diluted in 2.5% milk powder/TBST (10 mmol/L Tris-HCl, pH 8.5, 150 mmol/L NaCl, and 0.1% Tween 20) overnight at 4°C and then to a second antibody (peroxidase-linked anti-rabbit Ig) for one hour at room temperature. After washing, antigen-antibody complexes were visualized with a chemiluminescence system.

Immunohistochemistry

Ethanol-fixed specimens were stained as described previously [11]. Briefly, 4-μm thick sections were reacted for one hour with immune serum diluted 1:100 anti-AQP2 antibodies in 1% bovine serum albumin (BSA) in PBS. The antibody against AQP2 protein was a kind gift from Dr. Sei Sasaki (Second Department of Internal Medicine, Tokyo Medical and Dental School). Then, sections were incubated for 30 minutes with a horseradish peroxidase (HRP)-labeled polymer conjugated to anti-rabbit immunoglobulin (Envision; Dako Corporation, Carpinteria, CA, USA), and the sites of HRP were visualized using diaminobenzidine (DAB), H_2O_2, and were analyzed with a photomicroscope.

Measurements of AVP

Arginine vasopressin was measured by radioimmunoassay (RIA) as described previously [18]. Briefly, AVP was extracted using octacasyl-silane packed in a cartridge (Sep-Pak C18 cartridge; Waters Associates, Milford, MA, USA) and assayed using specific antibodies to AVP (Mitsubishi Petrochemical, Tokyo, Japan). The recovery rate was 72.4 ± 6.8% ($N = 30$).

Measurement of Li^+ in renal medulla

Lithium ions in renal medulla were determined according to a previous study in which solute accumulation

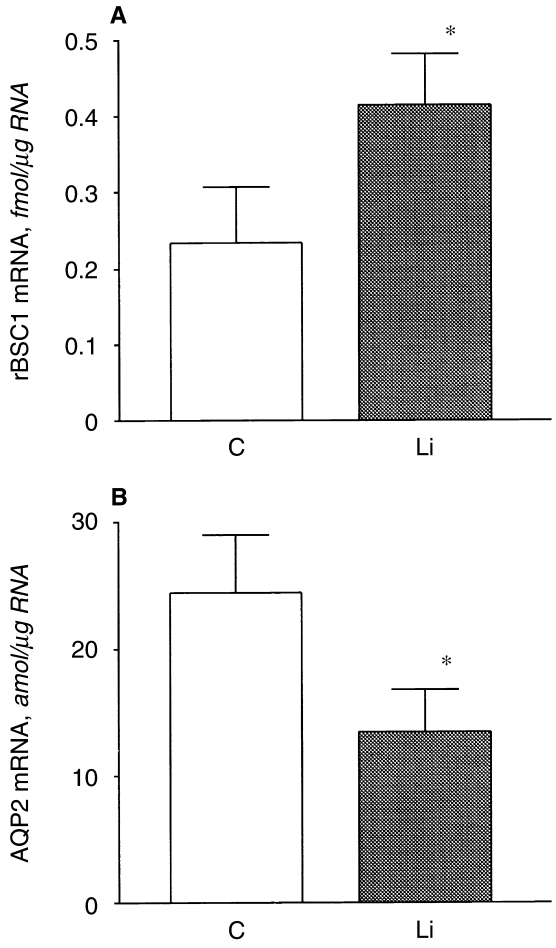

Fig. 1. Competitive polymerase chain reaction (PCR) of rBSC1 (*A*) and AQP2 (*B*) in controls (C) and lithium nephropathy (Li) rats. Data represent the mean ± SD of 6 determinations in each condition (*$P < 0.05$).

in renal papilla was measured [19]. Briefly, the renal medullas were separated, put in microcentrifuge tubes, and quickly weighed. The tubes were then plunged into an 80°C water bath for 60 minutes to inactivate the enzymes. The tubes were dried in an oven at 80°C for 48 hours and reweighed after the tissue had attained complete dryness. Distilled water was then added and the bottles were plunged into a water bath at 80°C for two hours to facilitate extraction of the Li ions into water. The tubes were cooled to room temperature and reweighed to determine the volume of the added water. After extraction the supernatant was analyzed by an electrolyte analyzer (NOVA11$^+$; NOVA Biochemical, Waltham, MA, USA). The concentration of Li ions was expressed per kilogram of wet tissue weight.

Other measurements and statistical analysis

Plasma and urinary osmolality was determined using an osmometer (model 3D2; Advanced Instruments, Needham Heights, MA, USA). Creatinine and urea concentrations

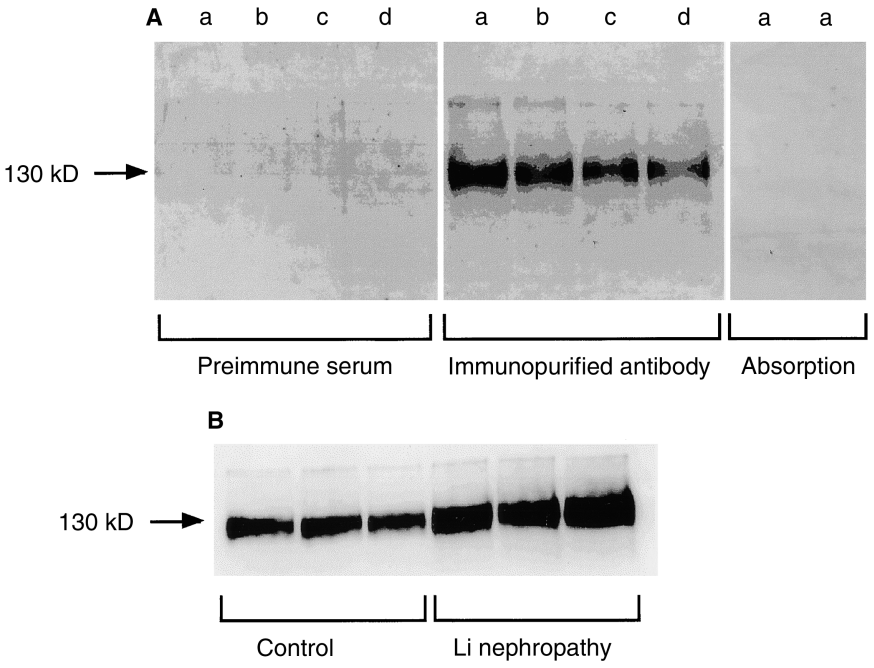

Fig. 2. Immunoblotting of rBSC1. Characteristics of peptide-directed rabbit polyclonal antibodies that recognize amino acid 33-55 of rBSC1 (*A*) and rBSC1 protein expression in Li nephropathy (*B*). (A) Comparison among pre-immunized serum, immunopurified antibody, and antibody absorption demonstrates mature bands at approximately 130 kD only in the lanes hybridized with immunopurified antibody. The amounts of protein loaded are: a, 150 μg; b,125 μg; c, 100 μg; and d,75 μg. The intensity of the band increases proportionately with the amount of protein loaded in the lane. (B) Total protein (100 μg) from control rats or rats with Li nephropathy is loaded in each lane.

in plasma and urine were measured by an autoanalyzer. Differences between physiological and laboratory data were examined for statistical significance using paired and unpaired *t* tests followed by the Student *t* test. The results are expressed as mean ± SD. A P value <0.05 was considered significant.

RESULTS

Changes in urinary output and osmolality

Changes in body weight, urinary output and osmolality in rats with Li nephropathy are depicted in Table 1. There were significant increases in body weight, water intake urinary output and a significant decrease in urinary osmolality in rats with Li nephropathy, compared to baseline.

Changes in expression levels of rBSC1 and AQP2 mRNAs

The results of competitive PCR analyses of rBSC1 in the outer medulla and AQP2 in the inner medulla are demonstrated in Figure 1. rBSC1 transcripts in 1 μg of total RNA was significantly higher in rats with Li nephropathy than in control rats. In contrast, AQP2 mRNA was significantly lower in rats with Li nephropathy.

Changes in expression level of rBSC1 protein

Characteristics of peptide-directed rabbit polyclonal antibodies that recognized amino acid 33-55 of rBSC1 are shown in Figure 2. When hybridized with immunopurified antibody, mature bands of approximately 130 kD appeared; the intensity of which changed according to the amount of protein loaded in the lane (Fig. 2A). Hybridization with pre-immune serum (left lanes) or absorption (right lanes) of anti-rBSC1 antibody by immunized peptide (incubation of 100 μg antibody with 30 μg peptide for 24 hours at 4°C) showed no labeling. Figure 2B represents immunoblots of rBSC1 in rats with Li nephropathy; demonstrating that the intensity of the signal in rats treated with LiCl was stronger than those of controls.

Effects of furosemide on rats with Li nephropathy

In rats with Li nephropathy treated with saline, hypotonic urine was persistent and large changes in body weight were noted even within the first three hours (Fig. 3 and Table 2). Furosemide treatment resulted in a marked decrease in urinary volume, which was noted at two hours, and a significant rise in urinary osmolality (Fig. 3). As expected, changes in body weight were less than those in saline-treated rats. Furthermore, furosemide induced a significant reduction in plasma AVP concentration but did not alter plasma osmolality and sodium levels (Table 2). These results indicate that the reduction of urinary volume induced by furosemide ameliorated (though not completely) the dehydrated condition during the three-hour observation period. A longer follow-up of six hours revealed the reappearance of less concentrated urine (350 ± 119 mOsm/kg H_2O) even in rats treated with furosemide.

To assess the effect of furosemide on the absorption of Li^+ through rBSC1, Li ions in the renal medulla were

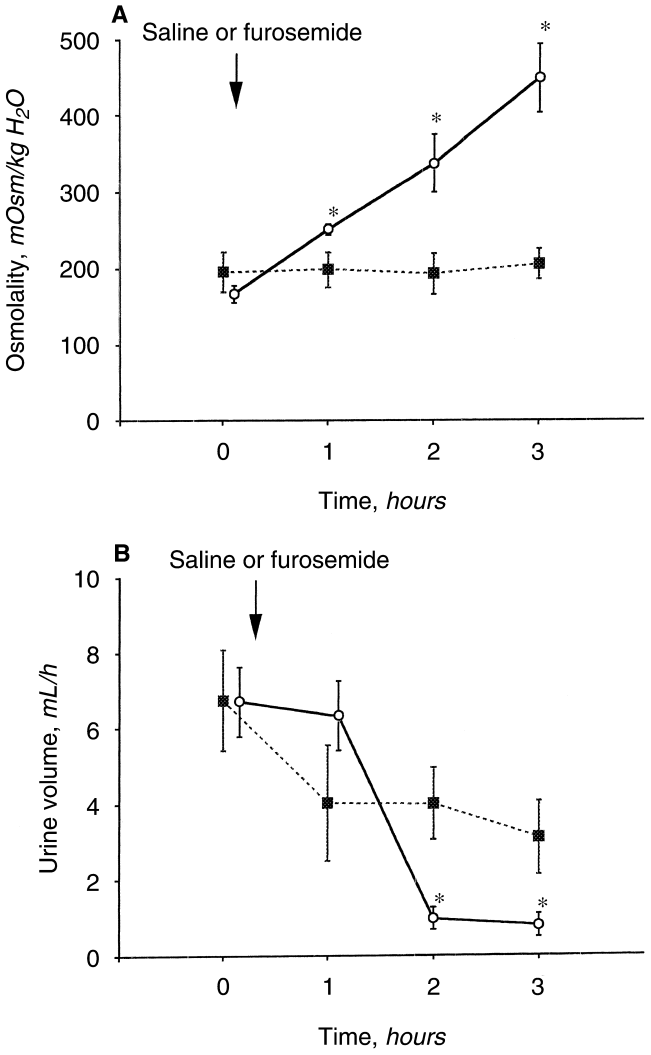

Fig. 3. Effects of furosemide treatment in rats with Li nephropathy. (*A*) Saline treatment did not change the urinary hypotonicity in rats with Li nephropathy (■), while furosemide treatment resulted in a significant rise in urinary osmolality (○). (*B*) Saline treatment of rats with Li nephropathy resulted in oliguria (■), while furosemide treatment resulted in a significant fall in urine volume (○). Data are mean ± SD. *$P < 0.05$ vs. before treatment.

measured as shown in Table 2. Although Li^+ levels in the renal medulla were slightly lower in rats treated with furosemide, this was not statistically significant (Table 2).

Apical AQP2 expression

To determine the mechanism(s) of altered urinary osmolality and of oliguria induced by furosemide in rats with Li nephropathy, we examined AQP2 expression in collecting duct by immunohistochemistry. In kidneys of control rats, which developed dehydration in response to 24-hour water deprivation, a strong intensity signal was noted especially along the apical membrane (Fig. 4A). In kidneys of rats with Li nephropathy, the signal intensity was lower in the cytoplasmic area of collecting duct cells

Table 2. Effects of furosemide on rats with Li nephropathy

	Furosemide		Saline
	Before	After	After
N	7	7	7
Change in body weight %		4.3 ± 0.9	5.9 ± 3.0
Plasma			
Osmolality *mOsm/kg H_2O*	285 ± 1	288 ± 2	291 ± 9
Sodium *mEq/L*	139 ± 1	140 ± 1	140 ± 2
Urea *mg/dL*	9.3 ± 0.9	11.6 ± 1.4	11.7 ± 2.6
Creatinine *mg/dL*	0.5 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
AVP pg/mL	5.7 ± 1.5	3.0 ± 1.2[a]	
Li+ in renal medulla *nmol/g*	2.58 ± 0.67	2.29 ± 0.86	

Data are means ± SD.
[a] $P < 0.05$ vs. before

(Fig. 4B). In rats with Li nephropathy treated with furosemide, the staining intensity also was limited but the signal was noted in the apical area of the cells (Fig. 4C).

DISCUSSION

Our present study confirms the previous findings of elevated plasma AVP [20] and enhanced rBSC1 expression [4] in a rat model of Li nephropathy. We have previously shown an up-regulation of rBSC1 under dehydration conditions associated with elevation of plasma AVP levels [11]. In this regard, Kim and coworkers demonstrated that AVP directly induced an up-regulation of this cotransporter [13]. Anai et al, who identified elevated plasma AVP levels in Li nephropathy, speculated that decreased plasma volume due to increased urinary volume probably simulated AVP synthesis [20]. Thus, dehydration or elevated AVP due to reduced plasma volume is thought to physiologically promote rBSC1expression in Li nephropathy.

In contrast to rBSC1, AQP2 RNA expression was reduced in our rat model of Li nephropathy. This finding is similar to that reported previously by Marples et al in this disease [3]. Our present study also demonstrates that AQP2 expression is probably impaired at a transcriptional level. Since the expression levels of both AQP2 RNA and protein are reported to be associated with dehydration or elevated AVP [5–8, 21, 22], these abnormalities in AQP2 expressions are considered to be associated directly with urinary concentrating defect in Li nephropathy. Together with the observation of rBSC1 expression, our finings indicate that tubular injury in Li nephropathy most likely is located only in the collecting duct until dehydration induces a reduction in the renal plasma flow.

Sodium transporters, including rBSC1, possess a high selectivity for Li ions as well as Na ions, and thereby allow the entry of Li^+ into the renal medulla. Dousa and Barnes found a close association between plasma AVP level and medullary Li^+ accumulation in rats with Li ne-

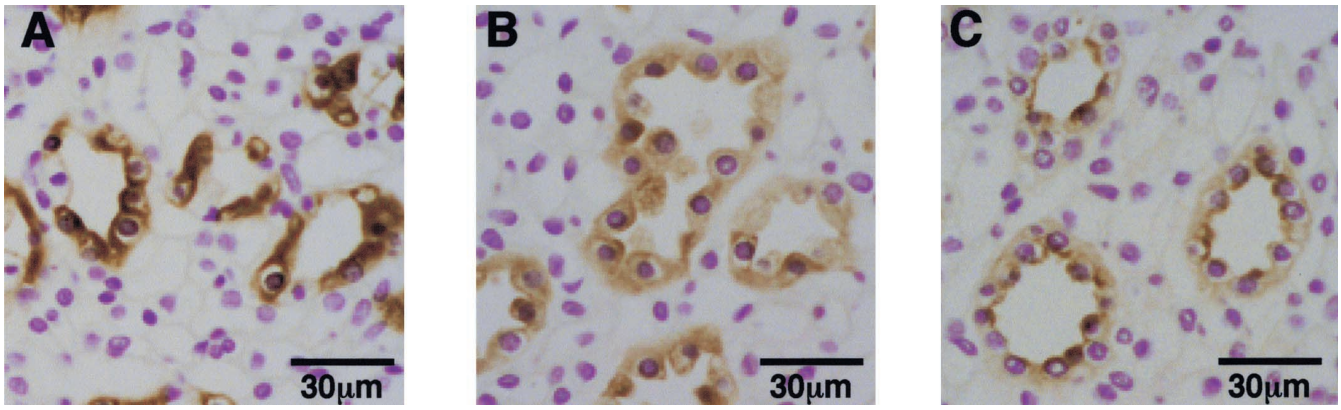

Fig. 4. Immunohistochemistry of AQP2. (*A*) A representative example of a kidney from normal but dehydrated rat. (*B*) A representative example of a kidney from a rat with Li nephropathy. (*C*) A representative example of a kidney from a rat with Li nephropathy treated with furosemide.

phropathy [15]. Therefore, increased rBSC1, even though this increase might be a physiological reaction to dehydration or to elevated plasma AVP, may promote Li^+ accumulation in renal medulla in Li nephropathy. We asked whether this rBSC1-enhanced Li^+ accumulation plays any pathophysiological role in Li nephropathy. To this end, we blocked the rBSC1 function by loop diuretics, using furosemide in rats with Li nephropathy. Interestingly, furosemide induced urinary concentration and a reduction of urine volume in rats with Li nephropathy. In addition, immunohistochemical analysis showed that the furosemide treatment resulted in a slight recovery of apical AQP2 expression. These present findings strongly suggest the rBSC1 expression influences collecting duct function, at least apical AQP2 expression in the renal medulla.

To assess the mechanism of the association between rBSC1 expression and AQP2 expression in Li nephropathy, Li^+ accumulation in the renal medulla was examined. Li^+ in the renal medulla, however, was not significantly lower in rats treated with furosemide in the present study and, thus, it is difficult to conclude that Li^+ transport by rBSC1 induced the toxicity. Li^+ entry into renal medulla occurs through other sodium transporters [15, 23], and a part of the absorbed Li^+ may quickly return to systemic circulation. These conditions may mask the difference induced by furosemide.

In the present study furosemide significantly reduced the plasma AVP concentration in rats with Li nephropathy. Thus, furosemide, which induces urinary excretion in normal rats, reduces urinary volume and ameliorates the dehydration state in rats with Li nephropathy. However, the longer follow-up period revealed the reappearance of less concentrated urine even in rats treated with furosemide. Furosemide blocks sodium entry into the renal medulla and reduces the osmotic gradient in renal medulla in a longer follow-up period. Therefore, based on the results of the present study, the effects of furosemide on Li nephropathy seem to be limited and transient.

Apical AQP2 expression involves AQP2 synthesis and its translocation to the membrane, and both of these processes are cAMP-dependent [6, 7, 24]. Impairment of AVP-dependent cAMP formation by LiCl has been reported in human renal medulla [25] in addition to collecting duct of the rat model of Li nephropathy [26, 27]. Since apical staining for AQP2 protein was noted in the kidney of rats treated with furosemide, even a slight reduction of medullary Li^+ may result in the recovery of AQP2 translocation. We measured the cAMP concentration both in urine and whole kidney before and after the furosemide treatment, and no difference was observed (data not shown). These findings suggest that it is difficult to detect a limited alteration of cAMP formation, which induced a slight enhancement of apical AQP2 expression and a moderate urinary concentration. Therefore, the association between the Li^+ accumulation in renal medulla and cAMP formation in collecting ducts remains to be investigated using other experimental techniques, such as in vitro microperfusion.

In conclusion, our present study demonstrates that the dehydrated state or elevated plasma AVP results in a physiological increase in rBSC1 expression in a rat model of Li nephropathy. Increased rBSC1, in turn, is associated with a disturbed collecting duct function.

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