Increases in the Expression Levels of Aquaporin-2 and Aquaporin-3 in the Renal Collecting Tubules Alleviate Dehydration Associated with Polyuria in Diabetes Mellitus

Masako SATAKE,[#] Nobutomo IKARASHI,[#] Mai KAGAMI, Naoki OGIUE, Takahiro TODA, Yasushi KOBAYASHI, Wataru OCHIAI, and Kiyoshi SUGIYAMA*

Department of Clinical Pharmacokinetics, Hoshi University; 2–4–41 Ebara, Shinagawa-ku, Tokyo 142–8501, Japan.
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Enhanced expression of renal aquaporin-2 (AQP2) has been reported when polyuria occurs in diabetic animal models. The purpose of this study was to clarify the possibility that increased AQP2 expression in the kidneys play a role as a compensatory mechanism to alleviate diabetic dehydration. Lithium carbonate (Li_2CO_3), which decreases the renal expression of AQPs, was administered to streptozotocin (STZ)-induced model mice of type I diabetes mellitus (STZ mice), to investigate the relationship between urine volume and renal AQP expression. Plasma glucose and urine glucose levels were similar between STZ mice given feed containing Li_2CO_3 for 10 d and un-treated STZ mice. Urine volume increased to 70 ml/d for the Li_2CO_3-treated STZ mice, compared to 36 ml/d for un-treated STZ mice. No changes were observed in creatinine clearance or the mRNA expression levels of sodium myo-inositol transporter and taurine transporter, which are genes associated with the regulation of osmotic pressure in the kidney, in the Li_2CO_3-treated STZ mice relative to un-treated STZ mice. Protein expression levels of AQP2 and aquaporin-3 (AQP3) of the renal inner medulla were significantly decreased in the Li_2CO_3-treated STZ mice, compared to levels in the STZ group. This study revealed that the decreased expression levels of AQP2 and AQP3 in the kidney increased the urine volume in mice without a change in urinary osmotic pressure. The results of this study suggest that the increased renal AQP2 and AQP3 expression, in the setting of polyuria, physiologically serves as a compensatory mechanism to alleviate dehydration in diabetes mellitus.

Key words aquaporin-2; lithium carbonate; diabetes mellitus; streptozotocin; sodium myo-inositol transporter; taurine transporter

Aquaporins (AQPs) are water channels found widely in bacteria, plants, animals, and humans. There are presently 13 known types of aquaporins in humans (AQP0 to AQP12), which are expressed in various organs.[1)] In the kidney, several members of the AQP family are expressed, which are responsible for regulating water volume in the body. Especially in the renal collecting duct, AQP2, which is expressed on the luminal membranes of principal cells, and AQP3 and AQP4, which are expressed on the basolateral membranes of principal cells, are involved in urine concentration by inducing the re-absorption of water from the luminal side to the basolateral side.[2)] The capacity for water re-absorption in the renal collecting duct is thought to determine urine volume. Because either protein mutations or a decreased expression of AQP2 is found in patients with nephrogenic diabetes insipidus who present with marked polyuria, AQP2 is considered to play the most important role in water re-absorption in the renal collecting duct.[3,4)]

Enhanced expression of renal AQP2 has been reported when polyuria occurs in streptozotocin (STZ)-induced type I diabetes model mice.[5,6)] In addition, we previously found that the level of renal AQP2 expression increases along with the pathological progression of type II diabetes mellitus using KKAy mice, the animal model of type II diabetes.[7)] Although this increased AQP2 expression in the kidneys has been regarded as a possible compensatory mechanism to alleviate dehydration in polyuria, the physiological significance of the increase in renal AQP2 expression in diabetes remains unknown. Recently, a method to generate a mouse model of nephrogenic diabetes insipidus by administering lithium carbonate (Li_2CO_3) was reported.[8,9)] Almost all of the Li_2CO_3 absorbed into the body is eliminated unchanged *via* glomerular filtration. Lithium is incorporated into principal cells of the collecting tubules *via* epithelial sodium channels that are dominantly expressed on the collecting tubules.[10)] The incorporated lithium decreases cAMP by suppressing the activity of adenylate cyclase (AC), resulting in the inhibition of pathways for both AQP2 and AQP3 transcription and membrane transfer.[8,9,11)] Using Li_2CO_3, we constructed an experimental system to decrease only the expression levels of AQPs, with no change in urinary osmotic pressure, to investigate the relationship between urine volume and the expression levels of AQPs. In this study, we developed this experimental system by administering Li_2CO_3 to STZ-induced type I diabetes model mice.

MATERIALS AND METHODS

Materials 2-Mercaptoethanol, bromophenol blue, Folin and Ciocalteu's phenol reagent, polyoxyethylene (20) sorbitan monolaurate (Tween 20), sodium dodecyl sulphate (SDS), and 2-amino-2-hydroxymethyl-1,3-propanediol (Tris) were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Anti-rat aquaporin 1, anti-rat aquaporin 2, anti-rat aquaporin 3, and anti-rat aquaporin 4 antibodies were purchased from Alomone Labs, Ltd. (Jerusalem, Israel). Streptozotocin, anti-rabbit antibody coupled to horseradish peroxidase, bovine serum albumin (BSA), ethylenediamine tetraacetic acid (EDTA), leupeptin trifluoroacetate salt, and phenylmethanesulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). Enhanced chemiluminescence system (ECL) plus Western blotting detection reagents were purchased from GE Healthcare (Chalfont St. Giles, U.K.). Skim milk powder was purchased from

* To whom correspondence should be addressed. e-mail: sugiyama@hoshi.ac.jp
[#] These authors contributed equally to this work.

Table 1. Primer Sequences of Mouse mRNA

Target	Accession number	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
SMIT	NM_53715	AGGAGTCCTTGGGTTGGAAC	ACTGCAACAAGGCCTCCAG
TauT	NM_17208	GTTCTGGGAGCGCAACGT	ACCGAACACCCTTCCAGATG
NKCC2	U20974	GTCTCGGTGTGATTATCATCGG	ATCCGTTTGTGGCGATAGCAG
UT-A1	AF366052	AAGGAGATGTCTGACAGCAACA	GGGCTGGGTGTGTATCCTG
GLUT2	NM_031197	CTGGAGCCCTCTTGATGGGA	CCAGTCCTGAAATTAGCCCACA
SGLT2	NM_133254	TGAGTGGAATGCGCTCTTCG	CTTGCGGAGGTACTGAGGC
18S rRNA	X00686	GTCTGTGATGCCCTTAGATG	AGCTTATGACCCGCACTTAC

SMIT, sodium myo-inositol transporter; TauT, taurine transporter; NKCC2, Na–K–Cl cotransporter 2; UT-A1, urea transporter A1; GLUT2, glucose transporter 2; SGLT2, sodium/glucose cotransporter 2; 18S rRNA, 18S ribosomal RNA.

Snow Brand Milk Products Co., Ltd. (Tokyo, Japan). An RNeasy mini kit was purchased from Qiagen Inc. (Valencia, CA, U.S.A.). A high capacity cDNA reverse transcription kit was purchased from Applied Biosystems (Foster City, CA, U.S.A.). iQ SYBR green supermix and RNase-free water were purchased from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Tris EDTA buffer solution (TE buffer; pH 8.0) was purchased from Nacalai Tesque (Kyoto, Japan). Primers were purchased from Invitrogen (Tokyo, Japan). All other reagents were of the highest commercially available grade.

Development of STZ Model Mice ICR mice (six weeks old) were purchased from Sankyo Labo Service (Tokyo, Japan). Each mouse was kept at room temperature (24±1 °C) and 55±10% humidity in a 12-h light cycle (artificial illumination: 08:00—20:00). The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University.

After a 12-h fast, mice were given a single intravenous administration of 150 mg/kg STZ dissolved in 0.05 M citrate buffer (pH 4.5). After two weeks of administration, blood samples were taken from the eyeground. Mice with greater than 500 mg/dl of plasma glucose were used in this study. Normal mice were given a single intravenous injection of citrate buffer.

Li_2CO_3 Treatment Normal mice and STZ treated mice were provided with control chow, chow containing 0.1% or 0.2% Li_2CO_3 for 10 d, *ad libitum*. After 7 d of administration, water intake and 24-h urine volume were measured. Twenty-four-hour urine volume was measured from 11:00 to the same time on the following day using a metabolic cage (Natsume Seisakusho Co., Ltd., Tokyo, Japan). After 10 d, animals were anesthetised under diethyl ether and a blood sample was collected from the abdominal vena cava using heparin. The kidney was removed, frozen in liquid nitrogen, and stored at −80 °C.

Blood and Urine Analysis The blood samples were centrifuged (1000×***g*** for 15 min at 4 °C), and plasma was stored at −80 °C until assays were performed. The urine samples were centrifuged (1000×***g*** for 15 min at 4 °C). The plasma glucose concentration and urine glucose concentration were enzymatically quantified using a Glucose CII-Test Wako (Wako Pure Chemical Industries, Ltd.). The plasma creatinine and urine creatinine concentrations were determined using a Creatinine assay kit (BioVision, Inc., Mountain View, CA, U.S.A.). Creatinine clearance (C_{cr}), as an indicator of glomerular filtration rate, was calculated *via* $C_{cr}=(C_u/C_p)\times V$, where C_u is the concentration of creatinine in the urine, C_p is the concentration of creatinine in plasma, and V is the urine flow rate in millilitres per minute.[12)]

RNA Preparation from Tissue Samples RNA was extracted from the outer medulla and inner medulla isolated from the kidney using the RNeasy mini kit, respectively. RNA extraction was performed according to the kit protocol. The resulting solution was diluted 25-fold with TE buffer solution, and the RNA purity and concentration (μg/ml) were calculated by measuring the absorbance at 260 and 280 nm using a spectrophotometer (U-2800; Hitachi High Technologies, Tokyo, Japan).

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) A high-capacity cDNA synthesis kit was used to synthesise cDNA from 1 μg of RNA. TE buffer solution was used to dilute the cDNA 20-fold to prepare a cDNA TE buffer solution. The expression of target genes was detected by preparing the primers listed in Table 1 and performing real-time RT-PCR. To each well of a 96-well PCR plate, 25 μl of iQ SYBR green supermix, 3 μl of forward primer for the target gene (5 pmol/μl), 3 μl of reverse primer (5 pmol/μl), 4 μl of cDNA TE buffer solution, and 15 μl of RNase-free water were added. The denaturation temperature was set at 95 °C for 15 s, the annealing temperature was 56 °C for 30 s, and the elongation temperature was 72 °C for 30 s. The fluorescence intensity of the amplification process was monitored using the My iQ™ single-color, real-time RT-PCR detection system (Bio-Rad Laboratories). The mRNA levels were normalised against 18S rRNA.

Preparation of the Membrane Fraction for Immunoblotting Fractionation of the kidney tissue was performed using the method of Marples *et al.*[13)] with slight modification. The inner medulla and cortex were separated from the kidney and homogenised (1250 rpm, 5 strokes) in dissecting buffer (0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, 1 μM PMSF; pH 7.2) on ice. The homogenate was centrifuged (4000×***g*** for 15 min at 4 °C). The resulting supernatant was centrifuged (17000×***g*** for 30 min at 4 °C) and the supernatant was removed. Dissecting buffer was added to the precipitate and homogenised using an ultrasonic homogeniser (UH-50, SMT Co., Ltd., Tokyo, Japan) to yield the plasma membrane-enriched fraction.

Electrophoresis and Immunoblotting The protein concentration was measured by the method of Lowry *et al.*,[14)] and BSA was used to generate a standard curve.

Electrophoresis was performed using the Laemmli method.[15)] Four micrograms of protein were diluted 2-fold in loading buffer (84 mM Tris, 20% glycerol, 0.004% bromophenol blue, 4.6% SDS, 10% 2-mercaptoethanol; pH 6.3), boiled for 5 min and separated on a 7.5% polyacrylamide gel. After electrophoresis, proteins were transferred to a PVDF

membrane using the CompactBLOT system (AE-7500, Atto Corp., Tokyo, Japan). Membranes were blocked for 1 h in 1.0% skim milk and incubated with rabbit anti-AQP1 (1 : 500), rabbit anti-AQP2 (1 : 1000), rabbit anti-AQP3 (1 : 500), or rabbit anti-AQP4 (1 : 500) for 1 h at room temperature. Membranes were then washed in TBS-Tween (20 mM Tris–HCl, 137 mM NaCl, 0.1% Tween 20; pH 7.6) and incubated with donkey anti-rabbit immunoglobulin G (IgG) (whole molecule) horse radish peroxidase conjugate (AQP1 and AQP3; 1 : 2000, AQP2 and AQP4; 1 : 5000) for 1 h at room temperature. Membranes were then washed and developed using the ECL plus detection reagents. Bands were visualised using a Lumino-image analyzer (LAS-3000 mini; Fuji Film, Tokyo, Japan).

Statistical Analysis Numerical data were expressed as means±standard deviation. The significance of differences was examined by the ANOVA method, followed by the Tukey test. Results with p values <0.05 were considered significant.

RESULTS

Effects of Li_2CO_3 on Plasma Glucose Level, Urine Volume, Water Intake, and Creatinine Clearance The plasma glucose level in the normal mice was approximately 200 mg/dl, which did not change after Li_2CO_3 treatment. The plasma glucose and urine glucose levels in the STZ mice were approximately 800 mg/dl and 3 g/d, respectively. The plasma glucose and urine glucose levels of STZ mice receiving feed containing 0.1% or 0.2% Li_2CO_3 were nearly the same as those of the STZ mice (Figs. 1A, B).

The urine volume of the normal mice was approximately 1 ml/d, which increased to 7 ml/d after Li_2CO_3 treatment. The urine volume of the STZ mice was approximately 36 ml/d. In the Li_2CO_3-treated STZ mice, the urine volume increased significantly compared to that in the STZ mice. Specifically, the urine volume in the 0.2% Li_2CO_3-treated STZ group was 70 ml/d, which was approximately twice the volume in the STZ group (Fig. 1C).

Water intake in the normal mice was approximately 2 ml/d, which increased approximately six fold after Li_2CO_3 treatment. Water intake in the STZ mice was 50 ml/d. Water intake in the Li_2CO_3-treated STZ mice increased significantly relative to that in the STZ group. In particular, water intake in the 0.2% Li_2CO_3-treated STZ group was 80 ml/d, which was approximately 1.6 times higher than that in the STZ group (Fig. 1D).

Creatinine clearance (C_{cr}) in the normal mice was approximately 0.3 ml/min, which did not change after Li_2CO_3 treatment. C_{cr} in either the STZ mice or the Li_2CO_3-treated STZ mice was nearly the same as that in the normal mice (Fig. 1E).

Effects of Li_2CO_3 on Osmoregulatory Genes To investigate the osmotic pressure of urine in the collecting tubules, the expression levels of the osmoregulatory genes were measured.[16—19] The mRNA expression level of sodium myo-inositol transporter (SMIT) in STZ mice was twice as high as that of normal mice. Li_2CO_3 did not exert any effect on the mRNA expression levels of SMIT in either STZ mice or normal mice (Fig. 2A). The mRNA expression levels of taurine transporter (TauT) showed the same tendency as that of SMIT (Fig. 2B).

Effects of Li_2CO_3 on the Expression Levels of Sodium Transporter, Urea Transporter, and Glucose Transporter in the Kidneys The mRNA expression levels of sodium transporter (Na–K–Cl cotransporter 2; NKCC2), urea transporter (urea transporter A1; UT-A1), and glucose transporters (glucose transporter 2; GLUT2, sodium/glucose co-

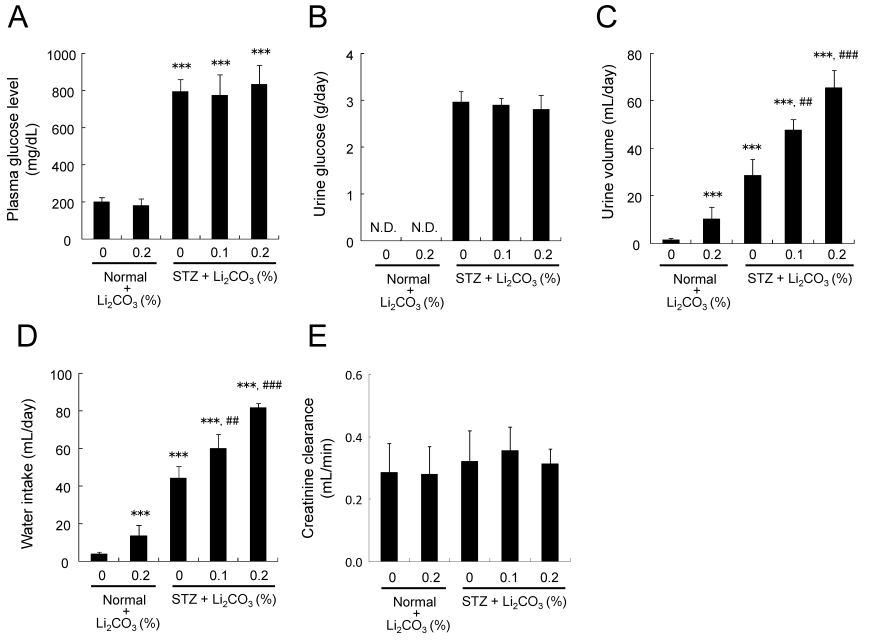

Fig. 1. Changes in Plasma Glucose Level (A), Urine Glucose Level (B), Urine Volume (C), Water Intake (D), and Creatinine Clearance (E) Following Treatment of Normal Mice and STZ Mice with Lithium Carbonate

Normal mice and STZ treated mice were provided with control chow, chow containing 0.1% or 0.2% Li_2CO_3 for 10 d, *ad libitum*. Seven days after administration, water intake and 24-h urine volume were measured. After 10 d, plasma glucose and urine glucose level were measured. Plasma and urinary creatinine concentrations were determined to calculate creatinine clearance. Data show the mean±S.D. of six mice per group. Tukey's test significances; *** $p<0.001$, compared to normal mice. ## $p<0.01$, ### $p<0.001$, compared to STZ mice.

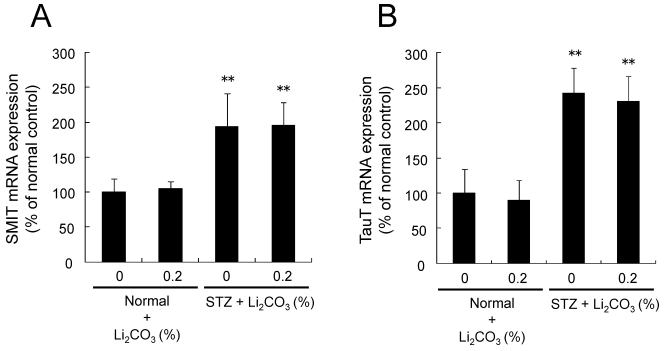

Fig. 2. Changes in the mRNA Expression Levels of Sodium Myo-inositol Transporter (SMIT, A) and Taurine Transporter (TauT, B) in the Renal Outer Medulla Following Treatment of Normal Mice and STZ Mice with Lithium Carbonate

Normal mice and STZ treated mice were provided with control chow, chow containing 0.2% Li_2CO_3 for 10 d, *ad libitum*. The kidneys of the mice were removed, and the mRNA expression levels of SMIT and TauT in the renal outer medulla were measured by real-time RT-PCR. 18S rRNA was used as a house-keeping gene, and the averages of values derived from the un-treated normal mice were expressed as 100%. Data show the mean±S.D. of six mice per group. Tukey's test significances; $^{**}p<0.01$, compared to normal mice.

transporter 2; SGLT2) in the renal inner medulla, where the collecting tubules were dominantly present, were measured (Fig. 3).

The mRNA expression level of NKCC2 in STZ mice was nearly equal to that of the normal mice. Li_2CO_3 treatment did not exert any changes in the mRNA expression levels of NKCC2 in both normal and STZ mice.

The mRNA expression levels of UT-A1, GLUT2, and SGLT2 of STZ mice were significantly higher than those of normal mice. Li_2CO_3 treatment significantly decreased the expression levels of these mRNAs in both normal and STZ mice.

Effect of Li_2CO_3 on the Expression Levels of AQP1, AQP2, AQP3, and AQP4 in the Kidneys The expression level of AQP1 in the plasma membrane fraction of the renal cortex, where the proximal tubules were dominantly present, was determined by the Western blotting method. AQP1 was identified as two bands (Fig. 4A). One of these appeared at around 25 kDa and represented the deglycosylated form of AQP1, while the other appeared at around 35—45 kDa and represented a glycosylated form of AQP1. This glycosylation is associated with the stability and intracellular translocation of AQP,[20,21] but has no effect on water permeability.[22] Therefore, in the present study, the AQP1 protein expression level was analysed using the sum of the intensities of these two bands. The expression level of AQP1 in STZ mice was nearly equal to that of normal mice. Li_2CO_3 treatment did not exert any changes on the expression level of AQP1 in either normal or STZ mice.

AQP2 was identified as two bands in the Western blot of the plasma membrane fraction of the renal inner medulla, where the collecting tubules were dominantly present. One of these bands appeared at around 29 kDa, while the other appeared at around 40—50 kDa (Fig. 4B). The sum of the intensities of these two bands in STZ mice was approximately 2.7 times higher than that of normal mice. Li_2CO_3 treatment significantly decreased the expression levels of AQP2 in both normal and STZ mice.

AQP3 was identified as two bands in the Western blot of the plasma membrane fraction of the renal inner medulla,

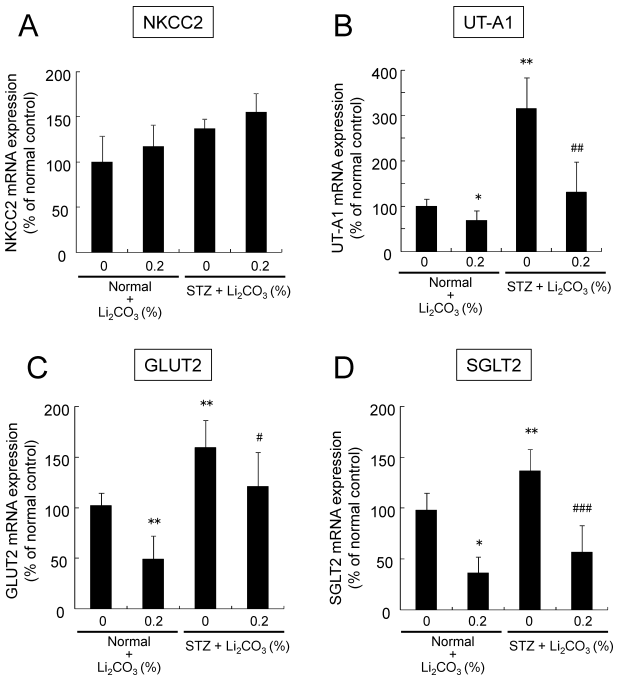

Fig. 3. mRNA Expression Levels of Sodium, Urea, and Glucose Transporter in the Kidneys

Normal mice and STZ treated mice were provided with control chow, chow containing 0.2% Li_2CO_3 for 10 d, *ad libitum*. The kidneys of the mice were removed, and the mRNA expression levels of NKCC2 (A), UT-A1 (B), GLUT2 (C) and SGLT2 (D) in the inner medulla were measured by real-time RT-PCR. 18S rRNA was used as a house-keeping gene, and the averages of values derived from the un-treated normal mice were expressed as 100%. Data show the mean±S.D. of six mice per group. Tukey's test significances; $^{*}p<0.05$, $^{**}p<0.01$, compared to normal mice. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, compared to STZ mice.

where the collecting tubules were dominantly present. One of these bands appeared at around 27 kDa and represented a deglycosylated form of AQP3, while the other band appeared at around 33—40 kDa and represented a glycosylated form of APQ3 (Fig. 4C). The expression level of AQP3 in STZ mice was significantly higher than that of normal mice. Li_2CO_3 treatment significantly decreased the expression levels of AQP3 in both normal and STZ mice.

AQP4 was identified as a single band, which appeared at around 33 kDa, in the Western blot of the plasma membrane fraction of the renal inner medulla, where the collecting tubules were dominantly present (Fig. 4D). The expression level of AQP4 in STZ mice was nearly equal to that of normal mice. Li_2CO_3 treatment did not exert any changes on the expression level of AQP4 in either normal or STZ mice.

DISCUSSION

The administration of STZ to mice causes an insulin deficiency by destroying β cells in the pancreas, leading to an increase in plasma glucose level and the presence of urine glucose. In addition, it has been reported that the presence of urine glucose causes polyuria, and subsequently, an increase in water intake.[23] Mice with severe diabetes were used in this study (Fig. 1). The levels of plasma glucose and urine glucose in the Li_2CO_3 treated STZ group were nearly the same as those in the STZ group (Figs. 1A, B), but both urine volume and water intake increased significantly in the Li_2CO_3 treated STZ group (Figs. 1C, D). These findings indicated that Li_2CO_3 was capable of increasing urine volume

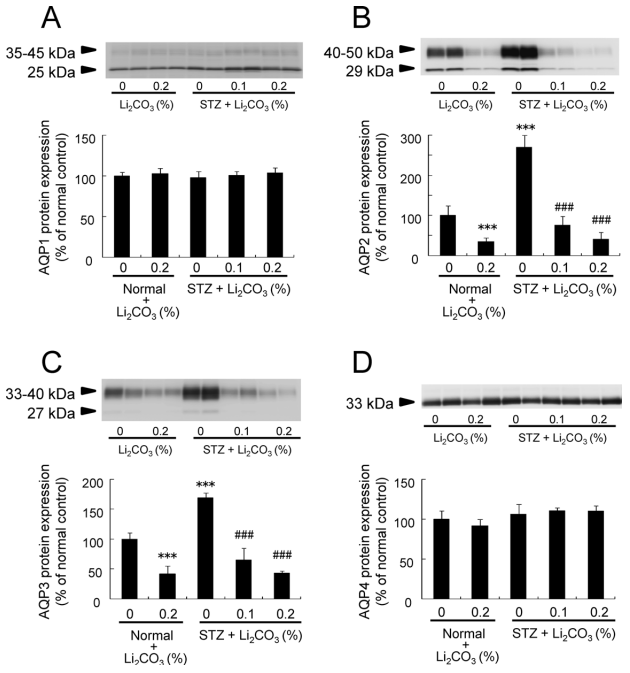

Fig. 4. Protein Expression Levels of AQP1 (A), AQP2 (B), AQP3 (C), and AQP4 (D) in the Kidneys

Normal mice and STZ treated mice were provided with control chow, chow containing 0.1% or 0.2% Li_2CO_3 for 10 d, *ad libitum*. After completion of administration, the kidneys were removed and the renal cortex and inner medulla were separated. Using the plasma membrane fraction separated by centrifugation, protein expression levels of AQP1 in the renal cortex and of AQP2, AQP3, and AQP4 in the renal medulla were determined by Western blotting. The averages of values derived from the normal mice were expressed as 100%. Data show the mean±S.D. of six mice per group. Tukey's test significances; *** $p<0.001$, compared to normal mice. ### $p<0.001$, compared to STZ mice.

without exerting an effect on plasma glucose level.

Approximately 150 l of primary urine is generated per day by the human kidneys. This primary urine is re-absorbed at the proximal tubules, the descending limb of Henle's loop, the distal tubules, and the collecting tubules at levels of 65%, 15%, 15%, and 4%, respectively. In total, this system generates approximately 1.5 l of urine, which is excreted each day. The capacity for water re-absorption in the renal collecting duct is thought to determine urinary volume.[4] Thus, the urine volume depends on both the amount of primary urine generated and the volume of water re-absorption at the renal uriniferous tubules and collecting tubules. In this study, we calculated C_{cr} values from the plasma and urinary creatinine concentrations to examine the generated volume of primary urine.[11] Based on these calculations, no change in C_{cr} was observed after Li_2CO_3 was administered to STZ mice (Fig. 1E). This finding suggested that the volumes of primary urine were similar between Li_2CO_3-treated STZ mice and untreated STZ mice.

Recently, it has been revealed that AQP plays an important role in regulating urine volume. AQP1 is dominantly expressed in the renal cortex, including the proximal tubules, while AQP2, AQP3, and AQP4 are mainly expressed in the renal inner medulla, where the collecting tubules are dominantly present.[2] Vasopressin is known to regulate urinary volume *via* AQP2 and AQP3, expressed in the renal collecting duct. Vasopressin binds to the V_2 receptor expressed on the basolateral membranes of the renal collecting duct principal cells, which in turn activates AC. The cAMP produced by the activation of AC activates protein kinase A (PKA).[24,25] The cAMP response element-binding protein (CREB) phosphorylation is mediated *via* the activation of PKA.[26,27] The phosphorylation of CREB regulates the transcription and the expression level of AQP2 and AQP3.[28,29] It is also known that the expression levels of AQP1 and AQP4 are not regulated by vasopressin.[30,31] Increased secretion of vasopressin in diabetic patients and diabetic animals has also been reported.[32,33] In this study, it was shown that the AQP2 and AQP3 levels of STZ mice were higher than those of normal mice, while the AQP1 and AQP4 levels of STZ mice were nearly equal to those of normal mice. These results were consistent with other reports,[30,31] suggesting that urine concentration was controlled by vasopressin in the collecting tubules, not in the proximal tubules. In addition, the possibility was suggested that the increase of the urine volume by Li_2CO_3 treatment was caused by reduced water re-absorption in the collecting tubules, not in the proximal tubules.

Water re-absorption in the collecting tubules depends on urinary osmotic pressure and AQP expression.[34,35] To evaluate the urinary osmotic pressure in the collecting tubules, where final urine volume was determined, the mRNA expression levels of the osmoregulatory genes (SMIT and TauT) and transporters related to osmotic pressure were measured.

The levels of SMIT and TauT expression were enhanced along with the increase in osmotic pressure.[16—19] The mRNA expressions of SMIT and TauT in the renal outer medulla were nearly the same between the STZ mice treated with Li_2CO_3 and un-treated mice (Fig. 2). This suggested that Li_2CO_3 administration caused no change in the osmotic pressure in the renal collecting tubules.

The osmotic pressure in the collecting tubules is determined by sodium, urea, and glucose levels. The effects of Li_2CO_3 treatment on sodium, urea, and glucose transporters were investigated. Li_2CO_3 treatment caused no changes in the mRNA expression levels of sodium transporter (NKCC2), but significantly reduced the mRNA expression levels of urea transporter (UT-A1) and glucose transporters (GLUT2, SGLT2) (Fig. 3). These results suggested the possibility that Li_2CO_3 treatment did not change sodium re-absorption, but reduced urea and glucose re-absorption. Under the assumption that the volume of primary urine that reached the collecting tubules both in normal and STZ mice are the same with or without Li_2CO_3 treatment, it was deduced that the urinary osmotic pressure of Li_2CO_3 treated mice will decrease due to increased urine volume. However, in this study, SMIT and TauT did not change. These results suggested that one of the reasons why the increased urine volume that was induced by Li_2CO_3 treatment did not affect the osmotic pressure in the collecting tubules was decreased urea and glucose re-absorption combined with unchanged sodium re-absorption. These results suggested that Li_2CO_3 treatment did not change the osmotic pressure in the collecting tubules.

The expression levels of AQPs in the renal inner medulla, where the collecting tubules were dominantly present, were measured by the Western blotting method.[4] The expression levels of AQP2 and AQP3 in the plasma membrane fraction of the inner medulla in STZ mice were significantly higher than those of normal mice (Figs. 4B, C). The expression levels of AQP2 and AQP3 in the plasma membrane fraction of the inner medulla were significantly decreased by Li_2CO_3 treatment (Figs. 4B, C). These results indicated that a

decrease in the expression levels of AQP2 and AQP3 in the kidneys resulted in an increase in urine volume (Figs. 1C, 4B, C). It is known that AQPs transport water according to an osmotic gradient.[34,35] In the kidneys, water is transported from the lumen to the blood vessels, because the osmotic pressure on the blood vessel side is always higher than the pressure inside the collecting tubules.[36] Therefore, this finding strongly suggests the possibility that increased expression levels of AQP2 and AQP3 in the renal collecting tubules play a role in promoting water re-absorption in the kidneys.

The urine volumes attributed to diabetic osmotic diuresis and to AQP2 and AQP3 were estimated as follows. The urine volume attributed to diabetic osmotic diuresis was calculated by determining the urine volume difference between two groups of mice that had different osmotic pressures but the same expression levels of AQP2 and AQP3 (difference between 0.2% Li_2CO_3-treated STZ mice and 0.2% Li_2CO_3-treated normal mice; Figs. 1C, 2, 4B, C) The urine volume attributed to diabetic osmotic diuresis deduced from the above calculation was roughly 63 ml. The urine volume attributed to AQP2 and AQP3 was calculated by determining the urine volume difference between two groups of mice that had the same osmotic pressures but different AQP2 and AQP3 levels (difference between 0.2% LI_2CO_3-treated STZ mice and un-treated STZ mice; Figs. 1C, 2, 4B, C) The urine volume attributed to AQP2 and AQP3 deduced from the above calculation was roughly 34 ml. From these results, the theoretical urine volume of STZ mice was calculated to be 30 ml [normal mice urine volume (1 ml)+63 ml−34 ml], which closely approximated the observed value (36 ml). This result revealed that the increased levels of AQP2 and AQP3 in diabetics can play a role in alleviating dehydration.

Li_2CO_3 has been used to treat manic-depression. It has been reported that hydrochlorothiazide, which is a diuretic, suppresses the AQP reduction in the kidneys of lithium-induced diabetes insipidus rats, and results in the improvement of diabetes insipidus.[37] However, in this study, the urine volume of Li_2CO_3-treated STZ mice was significantly higher than that of Li_2CO_3-treated normal mice (Fig. 1C). This result indicates the possibility that Li_2CO_3 may exacerbate polyuria in diabetic patients. Therefore, it is suggested that extra care should be taken when Li_2CO_3 is used for diabetic patients.

A positive correlation between renal AQP2 expression and urine volume has been reported in polyuria due to diabetes, regardless of type (type I or type II).[5—7] The results of this study strongly suggest that the increased expression levels of AQP2 and AQP3 can promote the re-absorption of water and play a role as a compensatory mechanism to complement the reduction of water content in the body. In diabetes patients, although a significant decrease in body water due to polyuria has been a concern, there have been few actual cases of dehydration. We believe that this compensatory mechanism may play a role in preventing dehydration in polyuria associated with diabetes by increasing the expression levels of AQP2 and AQP3 in the kidneys.

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