Original Article

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Urinary Concentration Defect and Renal Glycosuria in Cyclosporine-treated Rats

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Background: Urinary concentration impairment is a major feature of cyclosporine nephrotoxicity.
Methods: We explored two possible mechanisms that may underlie cyclosporine-induced polyuria; water, and/or osmotic diuresis. Cyclosporine was subcutaneously injected to normal salt-fed Sprague-Dawley rats at a daily dose of 25 mg/kg for 2 weeks (Experiment I) and 7.5 mg/kg for 6 weeks (Experiment II).
Results: In Experiment I, cyclosporine treatment caused an increase in urine volume (2.7±0.5 vs. 10.3±1.13 mL/d/100 g BW, p<0.001) and a decrease in urine osmolality (2,831±554 vs. 1,379±478 mOsm/kg H_2O, p<0.05). Aquaporin-2 (AQP2) protein expression decreased in cyclosporine-treated rat kidneys (cortex, 78±8%, p<0.05; medulla, 80±1%, p<0.05). Experiment II also showed that urine volume was increased by cyclosporine treatment (4.97±0.66 vs. 9.65±1.76 mL/d/100 g BW, p<0.05). Whereas urine osmolality was not affected, urinary excretion of osmoles was increased (7.5±0.4 vs. 14.9±1.4 mosmoles/d/100 g BW, p<0.005). Notably, urinary excretion of glucose increased in cyclosporine-treated rats (7±1 vs. 10,932±2,462 mg/d/100 g BW, p<0.005) without a significant elevation in plasma glucose. In both Experiment I and II, GLUT2 protein expression in the renal cortex was decreased by cyclosporine treatment (Experiment I, 55±6%, p<0.005; Experiment II, 88 ±3%, p<0.05).
Conclusion: Both water diuresis and osmotic diuresis are induced by cyclosporine nephrotoxicity. AQP2 and GLUT2 downregulation may underlie water and osmotic diuresis, respectively.

Key Words: Aquaporin-2, Cyclosporine, GLUT2, Osmotic diuresis, Water diuresis

INTRODUCTION

Tubulointerstitial injury is a characteristic feature of cyclosporine nephrotoxicity and may be associated with tubular dysfunction, such as urinary concentration defect. Long-term administration of cyclosporine to rats induces proximal and distal tubular damage, associated with polyuria, natriuresis, and glycosuria[1]. Previous animal studies reported that the expression of both water channels and sodium transporters were downregulated by cyclosporine administration, consistent with renal water and sodium loss[2,3]. Interestingly, these animal experiments were performed using a salt-depleted rat model which causes severe striped interstitial fibrosis[4].

Based on the pathophysiology of renal water excretion, polyuria can be classified into water diuresis and osmotic diuresis. The main causes of water diuresis are primary polydipsia and diabetes insipidus, and cyclosporine nephropathy may induce nephrogenic diabetes insipidus due to tubulointerstitial lesions. The Henle's loop and collecting duct are the two essential segments in the kidney for urine concentration as aquaporin-1 (AQP1) and the Na-K-2Cl cotransporter type 2 (NKCC2) contribute to countercurrent ex-

change and multiplication in the thin descending limb and the thick ascending limb of the Henle's loop, respectively. In the collecting duct, aquaporin-2 (AQP2) is the major molecule exerting water reabsorption to produce the final concentrated urine[5]. Thus, it is conceivable that AQP1, NKCC2 and/or AQP2 may be disturbed in nephrogenic diabetes insipidus[6].

The other category of polyuria, osmotic diuresis, is characterized by increased excretion of urinary osmoles. Major endogenous urinary osmoles are sodium, potassium, chloride, urea, and ammonium. However, glucose is not detected in normal urine[7] because of effective proximal tubular reabsorption. Glucose is reabsorbed in the proximal tubule by the sodium-glucose cotransporters (mainly SGLT2) and is released into circulation *via* GLUT2 in the basolateral membrane[8]. *SGLT2* was reported to be the gene responsible for congenital renal glycosuria[9], and mutations in the *GLUT2* gene were demonstrated in Fanconi-Bickel syndrome[10].

Glycosuria has been reported as an index of cyclosporine nephrotoxicity in humans[11] and rats[12]. However, whether cyclosporine-induced glycosuria is nephrogenic (renal glycosuria) or secondary to hyperglycemia remains unclear becaus e cyclosporine is also implicated as a diabetogenic drug[13,14]. Because proximal tubular dysfunction is an early sign of cyclosporine nephrotoxicity[15], abnormal glucose reabsorption in the proximal tubule may lead to renal glycosuria and consequent osmotic diuresis. In this study, we asked if water and/or osmotic diuresis underlie cyclosporine-induced polyuria and investigated the molecular bases for the urinary concentration defect.

MATERIAL AND METHODS

1. Animal experiments

Specific pathogen-free male Sprague-Dawley rats (Orient Bio Inc., Seongnam, Korea) weighing 180-220 g, were used for two animal experiment protocols of subcutaneous cyclosporine administration. In Experiment I, a daily large dose (25 mg/kg/d) was given for two weeks[16]. In Experiment II, a daily small dose (7.5 mg/kg/d) was used for six weeks[17]. Both animal protocols were adopted to induce tubular defect without remarkable structural damages. Control rats received a daily subcutaneous injection of the vehicle solution only, and 6 rats were assigned to each group. All rats were placed on regular (not low-sodium) rat chow (Lab Diet 5053, Orient Bio Inc.), and they were fed and watered ad lib. Urine and plasma samples were obtained at the end of each animal experiment. Urine samples were collected from metabolic cages for measurement of sodium, potassium, chloride, glucose, urea nitrogen, creatinine, and osmolality. Sodium, potassium, and chloride were measured with ion-selective electrodes, and creatinine was measured using Jaffe method with an automated analyzer (AU680, Beckman Coulter, Brea, CA). Urine osmolality was measured with an osmometer (ADVIA 2430, Precision Systems, Basking Ridge, NJ). Our experimental protocols were approved by the institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-10-005).

2. Immunoblot analysis

Manually dissected slices of kidney cortex and medulla were homogenized in a buffer containing 250 mM sucrose, 10 mM triethanolamine, 1 μg/mL leupeptin, and 0.1 mg/mL phenylmethylsulfonyl fluoride titrated to pH 7.6. Coomassie-stained "loading gels" were done to assess the quality of the protein by sharpness of the bands and to adjust protein concentrations before immunoblotting. For immunoblotting, the proteins were transferred electrophoretically from unstained gels to nitrocellulose membranes (Bio-Rad, Hercules, CA). After being blocked with 5% skim milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, 0.1 percent Tween-20, pH 7.5) for 1 h, membranes were probed overnight at 4℃ with the respective primary antibodies: rabbit polyoclonal anti-AQP1 and anti-AQP2 (Alomone Labs, Jerusalem, Israel), rabbit polyclonal anti-Na-K-2Cl cotransporter type 2 (NKCC2)[18] (kindly donated by Dr. Mark Knepper at the National Institutes of Health, Bethesda, MD), rabbit polyclonal anti-GLUT2 (Chemicon International, Temecula, CA), and mouse monoclonal anti-β-actin (Sigma, St. Louis, MO). The secondary antibody was goat anti-rabbit or goat anti-mouse IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA). Sites of antibody-antigen reaction were viewed using enhanced chemiluminescence substrate (GenDEPOT, Barker, TX) before exposure to X-ray film (Agfa-Gevaert, Mortsel, Belgium).

Relative quantitation of the band densities from immunoblots was carried out by densitometry using a laser scanner and Quantity One software (Basic version 4.6.9, Bio-Rad).

3. Immunohistochemistry

Periodate-lysine-paraformaldehyde-fixed, paraffin-embedded 4-μm sections of the left kidneys were used for immunohistochemical examination of the vehicle- and cyclos- porine-treated rats. Sections were deparaffinized with a graded series of ethanol. Endogenous peroxidase activity was removed by incubation with 3% H_2O_2 for 30 min, and heat-induced epitope retrieval was performed using 0.01 mM sodium citrate (pH 6.0) at 2,450 MHz and 800 W for 14 min in a microwave oven. Tissues were blocked with 10% normal donkey serum for 30 min and then incubated overnight at 4°C with the rabbit polyclonal anti-GLUT2 (Chemicon International). The DAKO Envision kit (Dako, Glostrup, Denmark) was used to conduct peroxidase labeling at room temperature, and the sections were washed with Tris buffer and incubated in 0.05% 3,3'-diaminobenzidine (DAB) and 0.033% H_2O_2. The tissue was counterstained with hematoxylin, and slides were mounted with Canadian balsam (Sigma, Saint Louis, MO).

4. Quantitative polymerase chain reaction (qPCR) for SGLT2 mRNA

Total RNA was isolated from the rat whole kidneys with the TRIzol® Reagent (Life Technologies, Carlsbad, CA). RNA was quantified by spectrophotometry, and cDNA synthesis was performed on 3 μg of RNA with SuperScript® III Reverse Transcriptase (Life Technologies, Carlsbad, CA). For quantitative polymerase chain reaction (PCR), 45 ng of cDNA served as a template for PCR amplification using Brilliant SYBR green QPCR master mix according to the manufacturer's instructions (FastStart DNA Master SYBR Green I, Roche Molecular Biochemicals, Mannheim, Germany). The SGLT2 gene-specific sequences used were the forward primer 5′-CATTGTCTCAGGCTGGCACTGG-3′ and the reverse primer 5′-GGACACTGCCACAATGAACACC-3′, respectively[19]. The thermal profile of the LightCycler® Instrument (Roche Molecular Biochemicals, Mannheim, Germany) was optimized with an initial denaturation of 10 minutes at 95℃ and 45 amplification cycles with each 10 seconds at 95℃, 10 seconds at 58℃ and 10 seconds at 72℃. The comparative Ct method was used to determine the relative amounts of target-mRNA levels calculated for each sample by expressing the target-mRNA level as a percentage of GAPDH mRNA levels. Ct ratios were analyzed using the LightCycler® Software (Version 4.05). Specificity was ensured by postrun melting curve analysis.

5. Statistics

Values are presented as means±SE. Comparisons between cyclosporine-treated and control rats were performed by the Mann-Whitney U-test. P values of less than 0.05 were considered as indicative of statistical significance.

RESULTS

1. Urine parameters and plasma glucose

In Experiment I, urine volume measured during the final day was larger in the cyclosporine-treated rats compared with controls (27.2±3.1 vs. 8.9±1.7 mL, $p<0.001$). As expected, urine osmolality in cyclosporine-treated rats was lower than that in controls (1,379±478 vs. 2,831±554 mOsm/kg H_2O, $p<0.05$). Although both groups of animals steadily achieved weight gain during the study period, the control rats were heavier than cyclosporine-treated rats at the end of the experiment (333±9 vs. 265±6 g, $p<0.001$). Thus we presented urine data of solute excretion, adjusting values per 100 g body weight (BW) (Table 1).

As shown in Table 1, daily urine excretion of osmoles was not significantly different between the control and cyclosporine-treated rats. Urine excretion of sodium and chloride were not significantly affected by cyclosporine treatment. However, urine excretion of potassium, glucose, and urea was significantly elevated in cyclosporine-treated rats. In particular, glycosuria was remarkable in cyclosporine-treated rats (control, 2±1 vs. cyclosporine-treated, 4,865±2,932 mg/d/100 g BW; $p<0.005$). Figure 1 shows the results of the dipstick test. The difference in specific gravity between the groups was clear. In contrast to the controls, the urine glucose from the cyclosporine-treated rats was strongly positive (Fig. 1). Although plasma glucose seemed to be elevated in cyclosporine-treated rats, the difference

did not reach statistical significance (control, 150±39 vs. cyclosporine-treated, 215±53 mg/dL; p=0.11).

Cyclosporine treatment also caused an increase in urine volume in Experiment II (Table 2). Inconsistent with Experiment I, urine osmolality was not significantly different between the two groups. Instead, daily urine excretion of osmoles was significantly increased in the cyclosporine-treated rats compared with controls (14.9±1.4 vs. 7.5±0.4 mosmoles/d/100 g BW, p<0.005). Whereas urine excretion of sodium, potassium, chloride, and urea were not significantly increased by cyclosporine treatment, considerable glycosuria was noted in cyclosporine-treated rats (10,932± 2,462 vs. 7±1 mg/d/100 g BW; p<0.005). Notably, plasma glucose was not significantly different between the groups (control, 102±8 vs. cyclosporine-treated, 127±14 mg/dL).

Figure 2 shows a significant association between glycosuria and urine output. Although urine volume was positively correlated with urine excretion of glucose in both animal experiments, the correlation was stronger in Experiment II (r=0.90, p<0.0001) than in Experiment I (r=0.81, p<0.005).

2. AQP1, AQP2, and NKCC2 protein expression in the kidney

In Experiment I, immunoblot analysis revealed that AQP2

Table 1. Animal data from Experiment I[a]

	Vehicle Control (n=6)	CsA Treatment (n=6)	p-value[b]
Body weight (g)	333±9	265±6	<0.001
Urine			
Volume (mL/d/100 g BW)	2.67±0.47	10.25±1.13	<0.005
Osmolity (mOsm/kg H_2O)	2,831±554	1,379±478	<0.05
Osmoles (mosmoles/d/100 g BW)	7.92±2.12	11.8±1.8	NS
Na^+ (μmol/d/100 g BW)	463±64	502±62	NS
K^+ (μmol/d/100 g BW)	711±96	1,073±85	<0.05
Cl^- (μmol/d/100 g BW)	505±70	668±50	NS
Glucose (mg/d/100 g BW)	2±1	4,865±2,932	<0.005
Urea nitrogen (μg/d/100 g BW)	786±138	1,269±94	<0.05
Serum creatinine (mg/dL)	0.60±0.00	0.62±0.04	NS
Cr clearance Creatinine clearance (uL/min100 g BW)	205±25	343±18	<0.05

Data are mean±SE.
[a]Cyclosporine (CsA) was subcutaneously injected with a daily dose of 25 mg/kg BW for 2 weeks in Sprague-Dawley rats.
[b]Comparison by Mann-Whitney U-test; NS, not significant; Cr, creatinine.

Table 2. Animal data from Experiment II[a]

	Vehicle Control (n=6)	CsA Treatment (n=6)	p-value[b]
Body weight (g)	309±7	275±5	<0.01
Urine			
Volume (mL/d/100 g BW)	4.97±0.66	9.65±1.76	<0.05
Osmolity (mOsm/kg H_2O)	1,591±157	1,953±593	NS
Osmoles (mosmoles/d/100 g BW)	7.5±0.4	14.9±1.4	<0.005
Na^+ (μmol/d/100 g BW)	664±26	535±48	<0.05
K^+ (μmol/d/100 g BW)	901±69	934±47	NS
Cl^- (μmol/d/100 g BW)	692±32	664±43	NS
Glucose (mg/d/100 g BW)	7±1	10,932±2,462	<0.005
Urea nitrogen (μg/d/100 g BW)	1,457±141	1,701±134	NS

Data are mean SE.
[a]Cyclosporine (CsA) was subcutaneously injected with a daily dose of 7.5 mg/kg BW for 6 weeks in Sprague-Dawley rats.
[b]Comparison by Mann-Whitney U-test; NS, not significant.

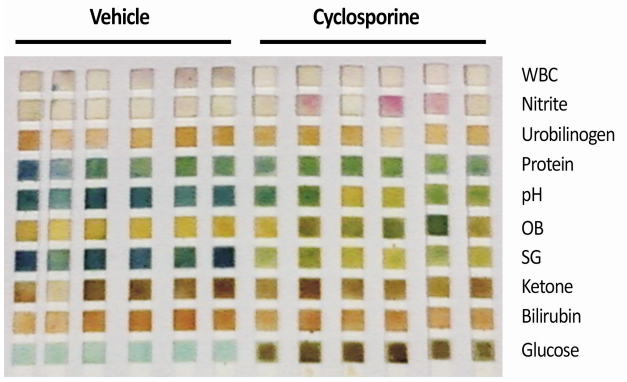

Fig. 1. Urine dipstick test results from cyclosporine-treated versus vehicle-treated control rats. Cyclosporine (25 mg/kg BW/d) was administered for 2 weeks, and each dipstick was tested from a different rat. In contrast to controls, all cyclosporine-treated rats demonstrate strong positive responses for glycosuria (bottom). Consistently, urine specific gravity (SG) was remarkably increased in cyclosporine-treated rats. OB, occult blood; WBC, white blood cell.

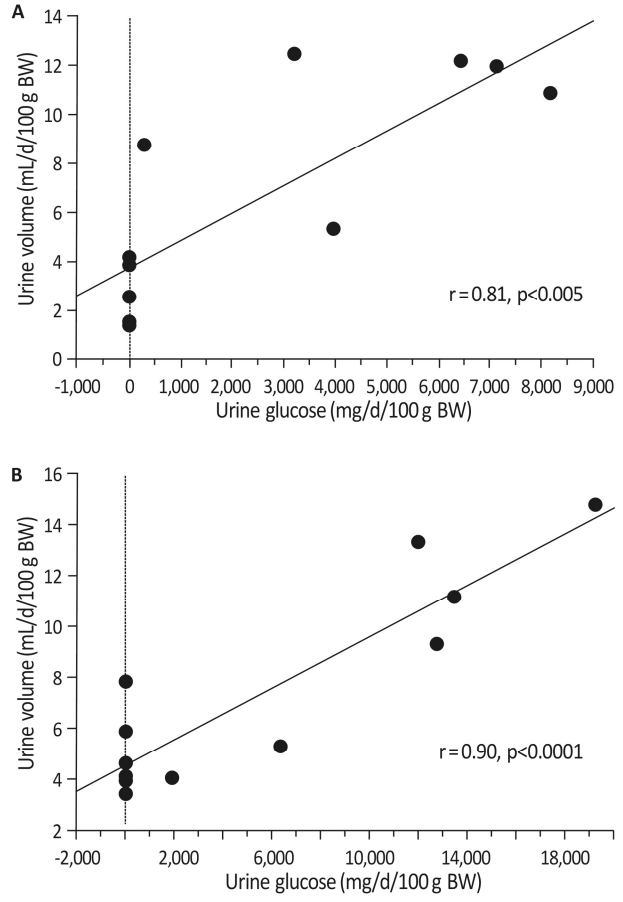

Fig. 2. Relationship between glycosuria and urine output. Urine volume was positively correlated with urine excretion of glucose in Experiment I (**A**, cyclosporine 25 mg/kg BW/d for 2 weeks) and Experiment II (**B**, cyclosporine 7.5 mg/kg BW for 6 weeks).

protein expression decreased in cyclosporine-treated rat kidneys compared with that of controls (cortex, 78±8 vs. 100±4%, p<0.05; medulla, 80±1 vs. 100±9%, p<0.05). However, AQP1 protein expression was not significantly different between the groups (cortex, 110±4 vs. 100±4%; medulla, 102±3 vs. 100±6%). Although NKCC2 protein abundance appeared to decrease in the cyclosporine-treated rats, the difference did not reach statistical significance (Fig. 3).

In Experiment II, AQP1 and AQP2 protein expression were not significantly affected by cyclosporine treatment (Fig. 4). On the other hand, the expression of NKCC2 protein in the renal medulla was increased in cyclosporine-treated rats compared with that of controls (361±60 vs. 100±42%, p<0.05).

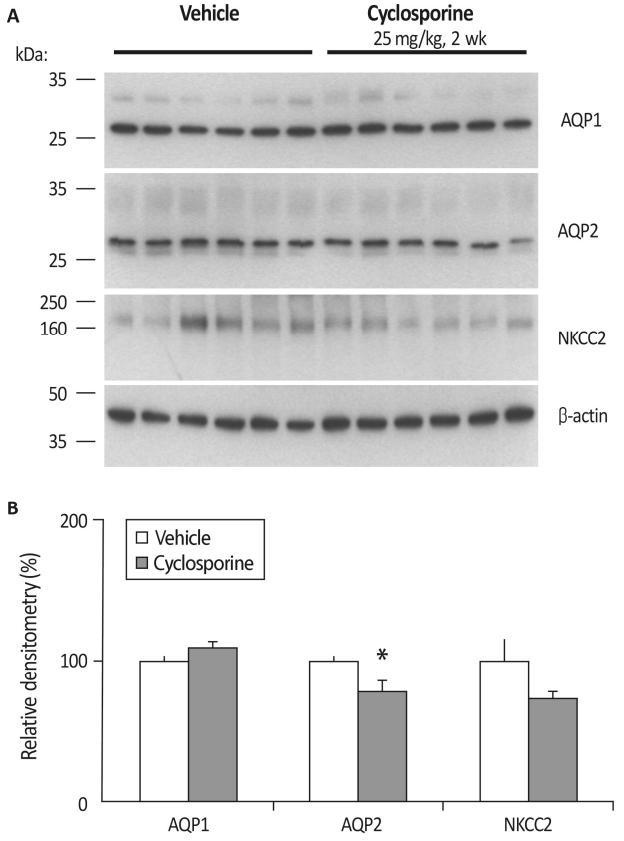

Fig. 3. Effects of high-dose (25 mg/kg BW/d) short-term (2 weeks) cyclosporine treatment on protein abundances of aquaporin-1 (AQP1), aquaporin-2 (AQP2), and Na-K-2Cl cotransporter type 2 (NKCC2) in rat renal cortex. Immunoblots, in which each lane was loaded with a protein sample from a different rat, reacted with respective specific antibodies are shown (**A**). Densitometric analyses reveal a significant decrease in AQP2 abundance in cyclosporine-treated rats (**B**). *=p<0.05.

3. Renal expression of GLUT2 protein and SGLT2 mRNA

We investigated whether the expressions of major glucose transporters are altered by cyclosporine treatment. Figure 5 shows the results of immunoblot analyses for GLUT2 in renal cortical homogenates. Notably, cyclosporine treatment decreased the expression of GLUT2 protein in both Experiment I (100±3 vs. 55±6%, $p<0.005$) and Experiment II (100±3 vs.88±3%, $p<0.05$). This altered expression of GLUT2 protein was confirmed by immunohistochemistry. Cyclosporine treatment caused a considerable decrease in GLUT2 expression in the basolateral membrane of the proximal tubule (Fig. 6). However, glomerular morphology and tubulointerstitial lesions were unremarkable.

Next, we examined SGLT2 mRNA expression from renal cortices using quantitative real-time PCR. No significant difference in the expression of SGLT2 mRNA was found between the groups (control, 100±5 vs. cyclosporine-treated, 96±8%). We did not obtain SGLT2 immunoblot data because specific antibodies reactive to rat renal SGLT2 were not available.

DISCUSSION

We used two different methods of chronic cyclosporine administration to rats. These were successful in inducing urinary concentration defect without overt tubulointerstitial injury. In contrast with previous studies[2,3], the effects of cyclosporine nephrotoxicity on the renal tubular transporters or channels were selective. Our novel finding was that GLUT2 protein was downregulated by cyclosporine treatment, leading to renal glycosuria.

When a larger dose of cyclosporine was administered for two weeks, the main mechanism of polyuria appeared to be water diuresis. This is due to both the decrease in urine osmolality and the unchanged excretory rate of urine osmoles (Table 1). Compatible with this finding, AQP2 protein was downregulated by cyclosporine treatment (Fig. 3). However, the levels of AQP1 and NKCC2 protein were not significantly altered. Therefore, the collecting duct, and not Henle's loop, should be the locus of cyclosporine nephrotoxicity presenting with nephrogenic diabetes insipidus[5].

On the other hand, osmotic diuresis was prominent when a smaller dose of cyclosporine was administered for six weeks. The excretion of total urinary osmoles was elevated, but urine osmolality was unaffected (Table 2). Consistent with this finding, the expression of AQP1 and AQP2 protein was not altered. Interestingly, NKCC2 protein was upregulated by cyclosporine treatment (Fig. 4). This result is compatible with the result from Lim et al. They showed that in the outer medulla, NKCC2 protein expression was increased by cyclosporine (15 mg/kg/d) treatment for 7 days[3]. This upregulation of NKCC2 may partially explain the reason why urine sodium excretion was reduced in cyclosporine-treated rats (Table 2). Because cyclosporine decreases the prostaglandin E2 synthesis in the kidney, the EP3-receptor-

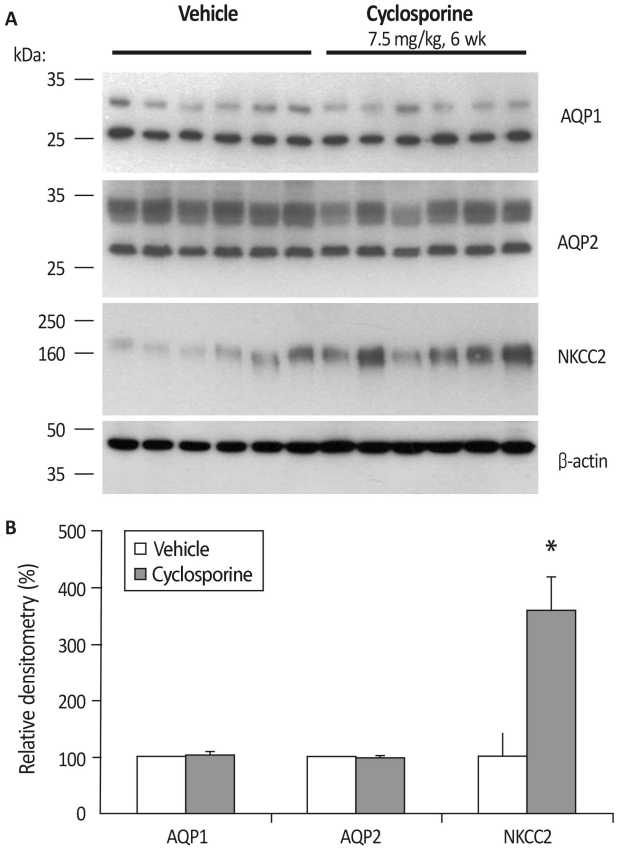

Fig. 4. Effects of low-dose (7.5 mg/kg BW/d) long-term (6 weeks) cyclosporine treatment on protein abundances of aquaporin-1 (AQP1), aquaporin-2 (AQP2), and Na-K-2Cl cotransporter type 2 (NKCC2) in rat renal medulla. Immunoblots, in which each lane was loaded with a protein sample from a different rat, reacted with respective specific antibodies are shown (**A**). Densitometric analyses reveal a significant increase in NKCC2 abundance in cyclosporine-treated rats (**B**). *=$p<0.05$.

mediated inhibition of cAMP production may increase the NKCC2 expression[20].

Despite different mechanisms underlying polyuria between Experiment I and II, cyclosporine administration consistently reduced GLUT2 protein expression in the proximal tubule. This GLUT2 downregulation was compatible with glycosuria in cyclosporine-treated rats. SGLT2 was also expected to be downregulated by cyclosporine nephrotoxicity. However, we could not examine the expression of SGLT2 protein because of a lack of specific antibodies. According to our qPCR results for SGLT2 mRNA, transcriptional downregulation of SGLT2 is unlikely in cyclosporine nephrotoxicity.

Previous experimental evidences support the notion that calcineurin inhibitors, including tacrolimus and cyclosporine, are diabetogenic[14,21]. When cyclosporine (50 mg/kg) was orally administered to rats for 7 days, morphological and functional damages to the pancreas were substantial, including severe degranulation and hydropic degeneration of islet β-cells, hyperglycemia and hypoinsulinemia[22]. In addition, cyclosporine impaired insulin-stimulated glucose uptake by adipocytes[23]. Finally, cyclosporine can induce insulin resistance via metabolic alterations in liver, muscle and adipose tissue[24].

Compatible with these previous studies, blood glucose levels tended to increase when a high dose of cyclosporine was used in Experiment I. In Experiment II, however, the blood glucose level was not affected by the smaller dose of cyclosporine. Glycosuria was consistently remarkable in both experiments, and it clearly contributed to osmotic diuresis (Fig. 2). Furthermore, the expression of GLUT2 in the proximal tubule was decreased by cyclosporine administration in both experiments (Figs. 5 & 6). Taken altogether, glycosuria was thought to be caused by impaired glucose reabsorption in the proximal tubule. In contrast, GLUT2 expression is enhanced in diabetes mellitus due to compensatory upregulation[25].

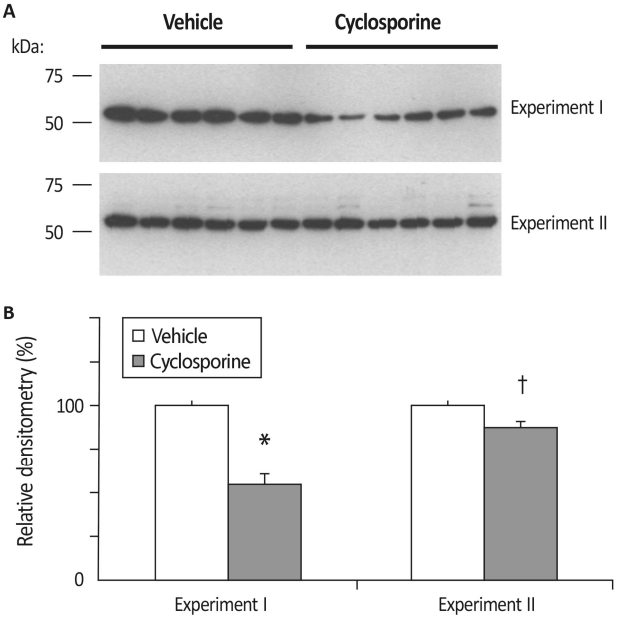

Fig. 5. Effects of cyclosporine treatment on GLUT2 protein abundance in rat renal cortex. Immunoblots reacted with an anti-GLUT2 antibody are shown from two different animal experiments using cyclosporine. Experiment I used a high-dose (25 mg/kg BW) short-term (2 weeks) protocol, and Experiment II used a low-dose (7.5 mg/kg BW) long-term (6 weeks) protocol (**A**). Densitometric analyses reveal a significant decrease in renal cortical GLUT2 protein abundance in cyclosporine-treated rats in Experiment I and II (**B**). *=p<0.005; †=p<0.05.

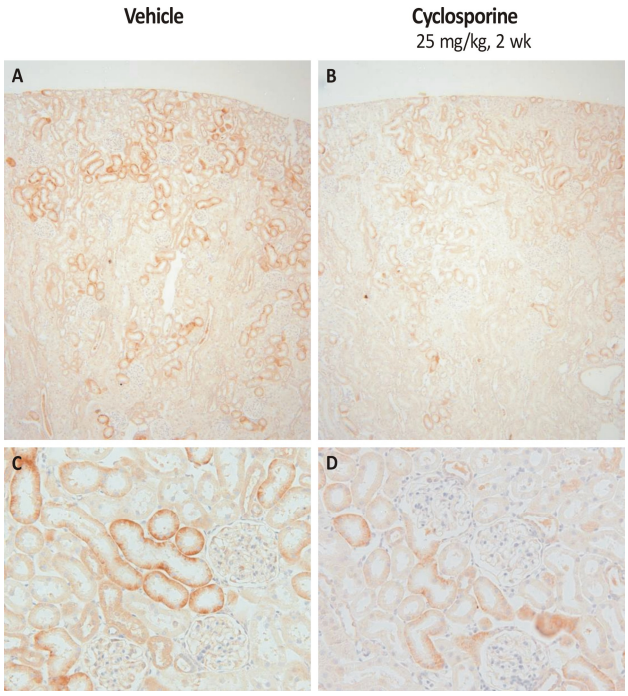

Fig. 6. Immunohistochemistry of GLUT2 in rat renal cortex. Sprague-Dawley rats were treated with vehicle (**A**, **C**) or cyclosporine 25 mg/kg BW/d for 2 weeks (**B**, **D**). In cyclosporine-treated rats, GLUT2 immunoreactivity was decreased in basolateral membranes in proximal tubule. Magnification: **A** and **B**, ×100; **C** and **D**, ×400.

CONCLUSION

In summary, a urinary concentration defect was induced by administering cyclosporine to rats. At a larger dose of cyclosporine, water diuresis was primarily associated with AQP2 downregulation. However, osmotic diuresis was steadily produced by different protocols of cyclosporine administration. Renal glycosuria, without overt hyperglycemia, contributed to osmotic diuresis, and GLUT2 downregulation in the proximal tubule may underlie renal glycosuria in cyclosporine nephrotoxicity.

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Disclosure

The authors declare no conflict of interest.

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