Cyclophosphamide-induced vasopressin-independent activation of aquaporin-2 in the rat kidney

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Cyclophosphamide is an alkylating agent used extensively to treat malignancy and rheumatological disease. It can produce an antidiuretic effect when a high dose is injected intravenously into water-loaded patients (35). We recently reported that the occurrence of hyponatremia was not rare when low-dose pulse cyclophosphamide was given to patients with lupus nephritis and lymphoma (23). However, the mechanisms by which cyclophosphamide induces water retention in the kidney have not been defined.

Although many drugs can cause water retention, mechanistic explanations as to how they decrease water excretion from the kidneys are lacking. In contrast to drugs that enhance vasopressin release, such as vincristine, cyclophosphamide is classified as a drug that potentiates the renal action of vasopressin (34). More specifically, water retention may result from the direct effects of cyclophosphamide on the collecting duct epithelium, because plasma vasopressin levels are not elevated in patients following the administration of intravenous cyclophosphamide (2, 5, 22). Furthermore, antidiuresis was reported to occur in response to intravenous cyclophosphamide in an 8-yr-old girl with central diabetes insipidus (6), excluding the possibility of the syndrome of inappropriate antidiuretic hormone secretion (SIADH).

These previous studies led us to hypothesize that cyclophosphamide or its active metabolites may directly dysregulate the expression of water channels and/or sodium transporters in the kidney. According to DeFronzo et al. (13), the effect of cyclophosphamide-induced water retention was related temporally with the urinary excretion of active metabolites of the drug, and 4-hydroperoxycyclophosphamide (4-HC) is the main hepatic active metabolite of cyclophosphamide (36).

This study was undertaken to investigate whether intrarenal mechanisms of urinary concentration are activated by treatment with cyclophosphamide or 4-HC. In particular, we explored the roles of the V2 vasopressin receptor (V2R), aquaporin-2 water channel (AQP2), and Na-K-2Cl cotransporter type 2 (NKCC2) in cyclophosphamide-induced water retention.

**MATERIALS AND METHODS**

*Animal experiments.* Specific pathogen-free male Sprague-Dawley rats weighing 210–230 g (Orient Bio, Seongnam, Korea) were used. Based on the results of our preliminary study (20), we performed two different animal experiments. In each experiment, the rats were randomly divided into vehicle-treated controls and cyclophosphamide-treated rats. In animal experiment 1, kidneys were harvested 12 h after a single intraperitoneal administration of cyclophosphamide [25 mg/kg body wt (BW)], whereas in animal experiment 2, kidneys were harvested 72 h after a single intraperitoneal administration of cyclophosphamide (50 mg/kg BW) (Fig. 1). Before cyclophosphamide administration, rats were water-loaded with a gelled-agar diet for 3 days. The diet comprised 1% agar, 72% water, and 27% powdered rat chow (15), and the rats received 70 g of this preparation per 200 g BW each day. Plasma and urine were obtained at the end of
each animal experiment, and vasopressin levels were determined from EDTA plasma by RIA (Vasopressin Direct RIA, Buhlmann Laboratories). This experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University.

Table 1. Primer sequences for qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (F) and reverse (R) Primer Sequences</th>
<th>PCR Product, bp</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
</table>
| AQP1 | F 5'-CTGCTGCCCATTGACTACACTG-3’  
R 5'-GGTTTGAAGTTGGCCGGTAG-3’ | 89 | NM_012778.1 |
| AQP2 | F 5’-GGCTTCTCATTTGGCTGAGGT-3’  
R 5’-CCTGACGGAGTTCTACTGGAG-3’ | 125 | NM_012909.2 |
| AQP3 | F 5’-AGCAAGATCTGAGCTGGGAGT-3’  
R 5’-CTGGGCTTTAAAAGGGGAC-3’ | 214 | NM_031703.1 |
| NKCC2 | F 5’-CGGCTGCTGCTAGATGCCAAA-3’  
R 5’-ATGACTGGGGAGAACTGG-3’ | 189 | NM_001270617 |
| V2R | F 5’-GCTCTGTATTTGGCTGAGGT-3’  
R 5’-TCAAGATTGATGAGGAGGA-3’ | 110 | NM_019136 |

qPCR, quantitative PCR; AQP1, aquaporin-1 water channel; AQP2, aquaporin-2 water channel; AQP3, aquaporin-3 water channel; AQP7, aquaporin-7 water channel; NKCC2, Na-K-2Cl cotransporter type 2; V2R, V2 vasopressin receptor.
the experiment at day 5. Culture medium was Dulbecco’s modified Eagle’s medium/F12 without phenol red, containing 80 mM urea, 130 mM NaCl, 10 mM HEPES, 1 mM sodium selenate, 5 nM hydrocortisone, 5 nM 3,5-diiodo-thyronine, 1 mM sodium selenate, 5 nM transferrin, and 10% fetal bovine serum (pH 7.4, 640 mmol/kg H2O). Previously, V2R-mediated small interfering (si)RNA delivery against AQP2 was demonstrated in these cells (18).

To study whether 4-HC treatment induces AQP2 upregulation, IMCD cells were treated for 24 h with vehicle (PBS-free culture medium) or two different doses of 4-HC at both the apical and basolateral sides of the cells. 4-HC was purchased from Niomec (Bielefeld, Germany), and therapeutic (10 µM) or toxic (30 µM) concentrations of 4-HC were chosen based on clinical (1, 8) and preclinical (3, 7) study findings. Next, to examine whether 4-HC-induced AQP2 upregulation was mediated by V2R stimulation, primary cultured IMCD cells were pretreated with a V2R antagonist, tolvaptan (10 or 50 nM, basolateral side only), for 3 h, followed by cotreatment with dDAVP (1 nM, basolateral side only) or 4-HC (10 µM, basolateral side only) for an additional 24 h. For semiquantitative immunoblotting, cell lysate was obtained in RIPA buffer (10 mM Tris-HCl, 0.15 M NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.02% sodium azide, 1 mM EDTA, pH 7.4) containing proteinase and phosphatase inhibitors (0.4 µg/ml leupeptin, 0.1 mg/ml pepstatin, 0.1 mM Na3VO4, 25 mM NaF, and 0.1 µM okadaic acid). For immunocytochemistry, cells were fixed with 3% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature.

**Immunofluorescence microscopy of primary cultured IMCD cells.** IMCD cells were grown to confluence in a Transwell chamber (0.4-µm pore size, Transwell Permeable Supports, catalog no. 3460, Corning) for 4 days and treated on day 5 after seeding according to the experimental protocols (9, 23). On day 5, IMCD cells were subjected to treatment with vehicle or 4-HC (10 or 30 µM, both apical and basolateral sides of the cells) for 1 day and then fixed with 3% paraformaldehyde in PBS, pH 7.4, for 20 min at room temperature. After fixation, cells were washed twice in PBS and permeabilized with 0.3% Triton X-100 in PBS at room temperature for 15 min. Cells were washed and incubated with anti-AQP2 antibody (1:400, AB3274, Millipore) in PBS overnight at 4°C, and nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI; D1306, Molecular Probes). After incubation, cells were washed in PBS and incubated with goat-anti-rabbit IgG Alexa Fluor 488 secondary antibody (A11008, Molecular Probes) for 2 h at room temperature. Cells were washed in a

**Immunohistochemistry for AQP2 in rat kidneys from animal experiment 1**. Kidneys were harvested 12 h after a single intraperitoneal administration of cyclophosphamide (25 mg/kg BW) in Sprague-Dawley rats. The immunohistochemistry revealed significant increases in AQP2 and NKCC2 in cyclophosphamide-treated rats vs. control rats. *P < 0.05.
hydrophilic mounting medium containing antifading reagent (P36930, Molecular Probes). AQP2 immunolocalization was observed using a laser-scanning confocal microscope (Zeiss LSM 5 EXCITER, Jena, Germany).

The immunofluorescence of AQP2 was detected at 530 nm with an excitation wavelength of 490 nm. During evaluating of 4-HC-induced subcellular redistribution of AQP2, identical microscope settings (light intensity, gain value, sampling period, and averaging) were used between groups, and the observer was blinded to treatment data. Digital images were collected and analyzed using the Zeiss Aim Image Examiner program.

X-Z images of AQP2-labeled cells were randomly selected from each group, and immunofluorescence intensity per distance (60 pixels) from the apical pole to the base of the cells (vertical lines drawn just next to the nuclei stained with DAPI) was acquired. The total number of cells examined was 150 (50 for vehicle-treated controls, 50 for the 10 μM 4-HC-treated group, and 50 for the 30 μM 4-HC-treated group).

Quantitative PCR analysis. Total RNA was isolated from rat kidneys and primary cultured IMCD cells with TRIzol Reagent (Life Technologies, Carlsbad, CA). cDNA synthesis was performed on 3 μg of RNA with SuperScript III Reverse Transcriptase (Life Technologies). For quantitative (q) PCR, 100 ng of cDNA served as a template for PCR amplification using the Brilliant SYBR green QPCR master mix, according to the manufacturer’s instructions (FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). A serial dilution (1 ng-μg/μl) of cDNA was used as a template to generate a standard curve. Nested primers were used to amplify the standard and kidney cDNA samples (Table 1). Standard and unknown samples were amplified in duplicate in 96-well plates. The thermal profile of the LightCycler Instrument (Roche Molecular Biochemicals) was optimized as follows: an initial denaturation for 5 min at 95°C followed by 45 amplification cycles, each consisting of 10 s at 95°C and 45 s at 60°C. Specificity was verified by melting-curve analysis. Relative mRNA expression was determined by normalizing the expression of each target to GAPDH mRNA levels. Results were analyzed by the 2^(-ΔΔCt) method (25).

cAMP measurement in IMCD suspensions. Intracellular cAMP levels were measured in IMCD suspensions prepared from normal kidneys of Sprague-Dawley rats (200–250 g, Orient Bio), as previously described (10, 14). Acutely isolated IMCD suspensions were incubated with 1 mM IBMX for 30 min to inhibit cyclic nucleotide phosphodiesterases. In the presence of IBMX, 10 nM dDAVP, 10 μM 4-HC, and 10 μM 4-HC + 100 nM tolvaptan (S2593, Selleckchem) suspensions were treated for an additional 30 min. The reaction was terminated by adding 10% trichloroacetic acid and placing the sample tube on ice for 10 min. The cAMP levels in the supernatant were determined using a competitive enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). The protein content of each suspension aliquot was measured, and the results were expressed in picomoles per milligram protein.

Statistics. Values are presented as means ± SE. Quantitative comparisons between groups were performed using the Mann-Whitney U-test (Statview software; Abacus Concepts, Berkeley, CA). To
facilitate immunoblot and qPCR comparisons, we normalized the band density or the relative mRNA values by dividing them by the mean value for the vehicle-treated control group. Thus the mean for the control group was defined as 100%.

**RESULTS**

**Renal response to cyclophosphamide administration in Sprague-Dawley rats.** In animal experiment 1, kidneys were harvested 12 h after a single intraperitoneal administration of cyclophosphamide (25 mg/kg BW) in Sprague-Dawley rats. Urine output measured over the experimental period decreased significantly in cyclophosphamide-administered rats ($n=6$, 6.63 ± 1.8 ml) compared with vehicle-treated controls ($n=6$, 22.5 ± 2.4 ml; $P<0.05$). However, neither urine osmolality (824 ± 73 vs. 763 ± 58 mosmol/kgH$_2$O) nor plasma sodium concentration (143 ± 2 vs. 143 ± 1 mmol/l) differed significantly between groups.

In the kidney, expression of AQP2, NKCC2, and V2R was upregulated by cyclophosphamide administration. Immuno-blotting results for AQP1, AQP2, AQP3, AQP7, and NKCC2 from whole kidney homogenates and densitometric values are shown in Fig. 2. Whereas alterations in levels of AQP1 and AQP7 were not significant, expression of AQP2 (137 ± 10 vs. 100 ± 6%; $P=0.01$), AQP3 (123 ± 7 vs. 100 ± 10%; $P<0.05$), and NKCC2 (205 ± 32 vs. 100 ± 11%; $P<0.05$) was significantly increased by cyclophosphamide administration. These immunoblot findings were confirmed by immunohistochemistry. Figure 3 shows that cyclophosphamide-treated rats had stronger apical AQP2 labeling along the collecting duct than controls. This change in AQP2 subcellular localization was quantitatively evaluated based on immunofluorescence intensity using confocal laser-scanning microscopy. Figure 4 demonstrates that cyclophosphamide treatment induced marked increases in apical-to-cytoplasmic MPI ratios for AQP2 immunofluorescence labeling in the cortex (6.21 ± 0.83 vs. 1.69 ± 0.26; $P<0.05$) and medulla (7.90 ± 0.40 vs. 2.80 ± 0.50; $P<0.05$).

In Fig. 5, NKCC2 immunostaining in the thick ascending limb appears to be increased in cyclophosphamide-treated rat kidneys compared with controls. When mRNA expression levels were examined by qPCR, a significant increase in V2R expression was observed in cyclophosphamide-treated rats compared with vehicle-treated controls.

**Fig. 6.** Real-time quantitative PCR for AQP1, AQP2, AQP3, NKCC2, and vasopressin-2 receptor (V2R) in rat kidneys from animal experiment 1. Kidneys were harvested 12 h after a single intraperitoneal administration of cyclophosphamide (25 mg/kg BW) in Sprague-Dawley rats. A: V2R graphs of fluorescence against cycle number were discriminated between vehicle-treated controls (blue) and cyclophosphamide-treated rats (red). B: the level of V2R mRNA expression was significantly increased by cyclophosphamide administration vs. vehicle administration. Horizontal bar represents the threshold cycle (Ct). *$P<0.05$.

**Fig. 7.** A: immunoblots of AQP1, AQP2, AQP3, and NKCC2 in rat kidneys from animal experiment 2. Kidneys were harvested 72 h after a single intraperitoneal administration of cyclophosphamide (50 mg/kg BW) in Sprague-Dawley rats. The immunoblots were reacted with anti-AQP1 (28-kDa nonglycosylated and 35-kDa glycosylated bands), anti-AQP2 (29-kDa nonglycosylated and 35-kDa glycosylated bands), anti-AQP3 (27-kDa nonglycosylated and 40-kDa glycosylated bands), anti-AQP7 (28 kDa), anti-NKCC2 (170 kDa), and anti-GAPDH antibody (37 kDa). Each lane was loaded with a protein sample from a different rat. B: densitometric analyses revealed significant increases in AQP1, AQP2, and NKCC2 expression in cyclophosphamide-treated rats vs. control rats. *$P<0.05$. †$P<0.01$. 

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was observed in cyclophosphamide-treated rats compared with controls (169 ± 18 vs. 100 ± 13%; \( P < 0.05 \)). Although AQP1, AQP2, and NKCC2 mRNA levels tended to be higher in cyclophosphamide-treated rats than control rats, these changes were not statistically significant (Fig. 6).

In animal experiment 2, kidneys were harvested 72 h after single administration of a large dose (50 mg/kg BW) of cyclophosphamide (Fig. 1). Urine and plasma data from vehicle-treated controls (\( n = 7 \)) and cyclophosphamide-treated rats (\( n = 7 \)) revealed no significant differences (data not shown). Plasma levels of arginine vasopressin were not significantly different between vehicle-treated controls (experiment 1, 48.9 ± 4.1 pg/ml; experiment 2, 30.2 ± 10.6 pg/ml) and cyclophosphamide-administered rats (experiment 1, 58.1 ± 11.0 pg/ml; experiment 2, 54.3 ± 29.5 pg/ml). However, patterns of alterations in AQP2, NKCC2, and V2R expression in the kidney were consistent with those observed in animal experiment 1. As shown in Fig. 7, cyclophosphamide-treated rat kidneys were characterized by significant increases in expression of AQP1 (115 ± 4 vs. 100 ± 6%; \( P < 0.05 \)), AQP2 (201 ± 26 vs. 100 ± 13%; \( P < 0.01 \)), and NKCC2 (149 ± 12 vs. 100 ± 19%; \( P < 0.05 \)) proteins vs. vehicle-treated control kidneys. These immunoblot findings were confirmed by immunohistochemistry, as shown in Fig. 8. In qPCR analysis, both AQP2 (135 ± 5 vs. 100 ± 10%; \( P < 0.05 \)) and V2R (127 ± 8 vs. 100 ± 9%; \( P < 0.05 \)) mRNA levels were significantly increased above control levels by cyclophosphamide administration (Fig. 9).

Effects of 4-HC treatment on AQP2 and V2R expression in primary cultured IMCD cells. We tested whether the active cyclophosphamide metabolite 4-HC can directly regulate AQP2 and/or V2R expression in the absence of vasopressin. Immunoblot analysis of primary cultured IMCD cells revealed that treatment of these cells with a therapeutic concentration (10 μM) of 4-HC for 1 day induced an increase in AQP2

![Fig. 8. Immunohistochemistry for AQP1, AQP2, and NKCC2 in rat kidneys from animal experiment 2. Kidneys were harvested 72 h after a single intraperitoneal administration of cyclophosphamide (50 mg/kg BW) in Sprague-Dawley rats. In cyclophosphamide-treated rats, immunostaining for AQP1, AQP2, and NKCC2 appears to be increased in the proximal convoluted tubule (PCT), cortical collecting duct (CCD), and thick ascending limb (TAL) compared with control rats.](image-url)

![Fig. 9. Real-time quantitative PCR for AQP1, AQP2, AQP3, NKCC2, and V2R in rat kidneys from animal experiment 2. Kidneys were harvested 72 h after a single intraperitoneal administration of cyclophosphamide (50 mg/kg BW) in Sprague-Dawley rats. AQP2 (A) and V2R (B) graphs of fluorescence against cycle number were discriminated between vehicle-treated controls (blue) and cyclophosphamide-treated rats (red). Cyclophosphamide administration induced significant increases in AQP2 and V2R mRNA expression relative to control levels (C). Horizontal bar represents the Ct.* \( P < 0.05 \).](image-url)
protein abundance (118 ± 8 vs. 100 ± 10%, \(P < 0.05\)), whereas a higher toxic concentration (30 \(\mu M\)) of 4-HC was associated with decreased AQP2 protein abundance (76 ± 12 vs. 100 ± 10%, \(P < 0.05\), Fig. 10).

Laser-scanning confocal microscopy demonstrated that apical and lateral translocation of AQP2 was increased when IMCD cells were treated with 4-HC (10 \(\mu M\)) compared with vehicle-treated cells (X-Z images in Fig. 11, A and B). In contrast, intracellular labeling intensity of AQP2 decreased after 4-HC treatment (10 \(\mu M\), Fig. 11, A and B). A toxic concentration of 4-HC (30 \(\mu M\)), however, was associated with markedly decreased AQP2 labeling intensity in the cells, and nuclei stained with DAPI were shrunken, possibly due to cell toxicity (Fig. 11C). To quantify 4-HC-induced subcellular redistribution of AQP2, pixel densities were measured as a function of apical-to-basal position. The X-Z images of AQP2-labeled IMCD cells were randomly selected, and the fluorescence intensities along the selected distance [60 pixels, from the apical pole (position 0) to the basal part (position 60) of the cells] were acquired in each group. The results showed that, compared with vehicle-treated cells, both 4-HC (10 and 30 \(\mu M\), 24 h) treatments induced a shift in AQP2 immunolabeling (490-nm excitation and 530-nm emission wavelengths) to the apical portion of the cells (Fig. 11, D–G).

To examine the changes in the mRNA levels of AQP2 and V2R in 4-HC-treated IMCD cells, qPCR was performed. Transcript levels of AQP2 (185 ± 27 vs. 100 ± 16%, \(P < 0.05\)) and V2R (221 ± 28 vs. 100 ± 4%, \(P < 0.05\)) were significantly higher in IMCD cells treated with 4-HC (10 \(\mu M\)) for 1 day than...
vehicle-treated cells. Graphs of SYBR green fluorescence from AQP2 and V2R as a function of number of cycles for control and experimental observations are presented in Fig. 12. Effects of V2R antagonist cotreatment on 4-HC-induced AQP2 expression in primary cultured IMCD cells. To examine whether 4-HC-induced AQP2 expression was mediated by V2R stimulation, tolvaptan was cotreated in primary cultured IMCD cells. Immunoblotting of AQP2 was initially performed to evaluate the effects of tolvaptan cotreatment on dDAVP- or 4-HC-induced AQP2 expression and to determine the optimal dose of tolvaptan for inhibiting the effects of vasopressin stimulation on AQP2 expression (Fig. 13A). The results demonstrated that dDAVP (1 nM, basolateral, 24 h)-induced AQP2 expression was significantly increased by 4-HC treatment relative to vehicle- (blue) and cyclophosphamide-treated rats (red). Both AQP2 and V2R mRNA against cycle number were discriminated between vehicle-treated controls onstrated that dDAVP (1 nM, basolateral, 24 h)-induced AQP2 stimulation on AQP2 expression (Fig. 13A). This was also seen in 4-HC (10 µM)-treated IMCD cells, where 4-HC-induced AQP2 expression was attenuated by tolvaptan cotreatment (50 nM, basolateral, 27 h, Fig. 13A).

Based on these results, 50 nM tolvaptan was used to evaluate whether 4-HC-induced AQP2 expression is blocked by a V2R antagonist. Figure 13, B and C, shows that AQP2 protein expression was significantly increased in 4-HC (10 µM)-treated cells (614 ± 149% of vehicle-treated control, P < 0.05) and that the 4-HC-induced increase in AQP2 expression was significantly attenuated when the cells were cotreated with tolvaptan (50 nM, basolateral, 27 h: 187 ± 22% of vehicle-treated control, P < 0.05 vs. 4-HC-treated cells, Fig. 13, B and C). The latter finding indicates that 4-HC may induce an increase in AQP2 protein via V2R stimulation.

Fig. 12. Real-time quantitative PCR analysis of AQP2 and V2R levels in primary cultured IMCD cells. Cells were treated with vehicle (n = 3) and 4-HC (10 µM, n = 3) for 24 h. AQP2 (A) and V2R (B) graphs of fluorescence against cycle number were discriminated between vehicle-treated controls (blue) and cyclophosphamide-treated rats (red). Both AQP2 and V2R mRNA expression were significantly increased by 4-HC treatment relative to vehicle-only treatment (C). Quantitative real-time RT-PCR experiments for AQP2, V2R, and β-actin were performed 3 times independently. Horizontal bar represents the C. *P < 0.05.

DISCUSSION

The antidiuretic hormone (AVP)-V2R-cAMP-AQP2 water channel axis is so crucial that dysregulation of AVP-induced AQP2 is implicated in many clinical disorders of water homeostasis (21). In this study, we demonstrated that cyclophosphamide induces short-term upregulation of V2R and AQP2 expression in the kidney. Importantly, these results were confirmed in vitro in the absence of vasopressin stimulation. This novel finding may underlie the renal water retention induced by intravenous cyclophosphamide administration during management of rheumatological and malignant disorders.

Secretion of the antidiuretic hormone AVP is enhanced in most settings of hyponatremia. Impaired urinary dilution leading to water retention and hyponatremia is predominantly derived from nonsomitic stimulation of vasopressin release with upregulation of V2R and AQP2 expression in the kidney. Importantly, these results were confirmed in vitro in the absence of vasopressin stimulation. This novel finding may underlie the renal water retention induced by intravenous cyclophosphamide administration during management of rheumatological and malignant disorders.

The term “syndrome of inappropriate antiidiuresis (SIAD)” was proposed as an alternative to SIADH because a subgroup of patients with features of SIADH does not have elevated plasma vasopressin levels (32). Thus dilutional hyponatremia may be produced either by excessive AVP release (central) or by constitutive activation of V2R (nephrogenic). The latter type of SIAD is referred to as nephrogenic syndrome of inappropriate antidiuresis (NSIAD), and was originally de-
scribed from gain-of-function mutations of the V2 vasopressin receptor in two infants (16).

We also obtained results dissociating the upregulation of V2R from AVP release. In the absence of AVP stimulation, apical translocation of AQP2 was remarkably increased when primary cultured IMCD cells were treated with 4-HC, a major metabolite of cyclophosphamide. This effect was induced by a dose of 4-HC consistent with serum levels of toxic cyclophosphamide metabolites in patients that receive the prodrug (1, 8), although higher dosages produced cytotoxic effects. In addition, significant increases in AQP2 and V2R mRNA expression were demonstrated in both in vivo rat kidney and in vitro primary cultured IMCD cells. Moreover, AQP2 upregulation and cAMP accumulation in response to 4-HC were significantly reduced by tolvaptan cotreatment in primary cultured IMCD cells and IMCD suspensions, respectively. Thus, taken together, these results suggest the possibility of drug-induced NSIAD. A recent clinical study postulated NSIAD to be an underlying mechanism of hyponatremia in elderly patients treated with antidepressants (26). Interestingly, carbamazepine was reported to increase AQP2 expression in the rat IMCD (11). However, whether the expression of V2R is altered by drugs has not been explored.

In this study, cyclophosphamide-induced upregulation of V2R was accompanied by increased expression of both AQP2 and NKCC2 in the apical membranes of the collecting duct and thick ascending limb, respectively. These results are consistent with the notion that V2R is located mainly in the collecting duct and thick ascending limb for the action of AVP (27). Increased expression of NKCC2 is likely to play an important role in enhancing the countercurrent multiplication system in the thick ascending limb (19) and would promote water retention from the collecting duct.

We also found that the expression of AQP1 was increased by cyclophosphamide, confirming our preliminary study findings (20). AQP1 is constitutively expressed in the apical and basolateral membranes of renal proximal tubules and descending thin limbs and does not participate in the regulation by vasopressin of renal water excretion (28). Thus its regulation remains elusive; our experiments showed that expression of AQP1 was altered by drug administration. Further studies are required to elucidate the intracellular pathways that regulate the expression of AQP1 in the renal proximal tubule. Few studies have investigated the regulation of AQP1, but angiotensin II may increase AQP1 expression in the kidney (4).

We conclude that in rat kidneys, cyclophosphamide may activate V2R and induce upregulation of AQP2 and NKCC2 or directly increase AQP2 trafficking in the absence of vasopressin stimulation. These intrarenal mechanisms suggest the possibility of drug-induced nephrogenic syndrome of inappropriate antidiuresis.

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REFERENCES


