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

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¹Institute of Biomedical Sciences, Hanyang University College of Medicine, Seoul, Korea; ²Department of Biochemistry and Cell Biology, Kyungpook National University School of Medicine, Taegu, Korea; and ³Division of Nephrology, Department of Internal Medicine, Hanyang University College of Medicine, Seoul, Korea

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Cyclophosphamide-induced vasopressin-independent activation of aquaporin-2 in the rat kidney. Am J Physiol Renal Physiol 309: F474–F483, 2015. First published June 24, 2015; doi:10.1152/ajprenal.00477.2014. —Because cyclophosphamide-induced hyponatremia was reported to occur without changes in plasma vasopressin in a patient with central diabetes insipidus, we hypothesized that cyclophosphamide or its active metabolite, 4-hydroperoxycyclophosphamide (4-HC), may directly dysregulate the expression of water channels or sodium transporters in the kidney. To investigate whether intrarenal mechanisms for urinary concentration are activated in vivo and in vitro by treatment with cyclophosphamide and 4-HC, respectively, we used water-loaded male Sprague-Dawley rats, primary cultured inner medullary collecting duct (IMCD) cells, and IMCD suspensions prepared from male Sprague-Dawley rats. In cyclophosphamide-treated rats, significant increases in renal expression of aquaporin-2 (AQP2) and Na-K-2Cl cotransporter type 2 (NKCC2) were shown by immunoblot analysis and immunohistochemistry. Apical translocation of AQP2 was also demonstrated by quantitative immunocytochemistry. In both rat kidney and primary cultured IMCD cells, significant increases in AQP2 and vasopressin receptor type 2 (V2R) mRNA expression were demonstrated by real-time quantitative PCR analysis. Confocal laser-scanning microscopy revealed that apical translocation of AQP2 was remarkably increased when primary cultured IMCD cells were treated with 4-HC in the absence of vasopressin stimulation. 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. We demonstrated that, in the rat kidney, cyclophosphamide may activate V2R and induce upregulation of AQP2 in the absence of vasopressin stimulation, suggesting the possibility of drug-induced nephrogenic syndrome of inappropriate antidiuresis (SIADH).

cyclophosphamide; aquaporin-2; Na-K-2Cl cotransporter type 2; vasopressin receptor type 2; hyponatremia

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

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Primer sequences for qPCR

<table>
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<th>Gene</th>
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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, 10% fetal bovine serum (pH 7.4, 640 mosmol/kgH₂O). 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 (FBS-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 protease and phosphatase inhibitors (0.4 μg/ml leupeptin, 0.1 mg/ml pefabloc, 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.

Fig. 2. A: immunoblots of aquaporin-1 (AQP1), aquaporin-2 (AQP2), aquaporin-3 (AQP3), and Na-K-2Cl cotransporter type 2 (NKCC2) 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 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 AQP2, AQP3, and NKCC2 in cyclophosphamide-treated rats vs. control rats. *P < 0.05.

Fig. 3. 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. In cyclophosphamide-treated rats, immunostaining for AQP2 appears to be increased along the collecting duct. The arrow in the inset (high magnification) of the inner medullary collecting duct (IMCD) indicates that apical AQP2 labeling was remarkably enhanced by cyclophosphamide administration. CCD, cortical collecting duct; OMCD, outer medullary collecting duct.
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). RNA was quantified by spectrophotometry, and 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-fg/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. 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.

In the kidney, expression of AQP2, NKCC2, and V2R was upregulated by cyclophosphamide administration. Immunoblotting 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 remarkable 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...
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. in 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 \( \mu \)M) of 4-HC for 1 day induced an increase in AQP2
protein abundance (118 ± 8 vs. 100 ± 10%, P < 0.05), whereas a higher toxic concentration (30 μM) 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 μM) 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 μM, Fig. 11, A and B). A toxic concentration of 4-HC (30 μM), 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 though the vertical lines drawn just next to the nuclei stained by DAPI in the cells) were acquired in each group. The results showed that, compared with vehicle-treated cells, both 4-HC (10 and 30 μM, 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 μM) 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-treated controls (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.

**Effects of V2R antagonist cotreatment on 4-HC-induced cAMP production in IMCD suspensions.** To confirm the effects of tolvaptan cotreatment on 4-HC-induced upregulation of AQP2 and V2R in IMCD cells, we measured cAMP levels in response to 4-HC with and without tolvaptan cotreatment in rat IMCD suspensions. After incubation for 30 min, cAMP levels were significantly increased by 10 μM 4-HC (17.3 ± 0.6 pmol/mg protein) compared with vehicle-treated controls (8.5 ± 0.6 pmol/mg protein; P < 0.01). The 4-HC-induced increase in cAMP was comparable to that of 10 nM dDAVP treatment (20.5 ± 1.3 pmol/mg protein). Notably, it was significantly reduced by cotreatment with 100 nM tolvaptan (12.4 ± 1.1 pmol/mg protein, P < 0.01, Fig. 14).

**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 nonosmotic 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 antidiuresis (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. 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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