Excretion of urinary exosomal AQP2 in rats is regulated by vasopressin and urinary pH

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Higashijima Y, Sonoda H, Takahashi S, Kondo H, Shigemura K, Ikeda M. Excretion of urinary exosomal AQP2 in rats is regulated by vasopressin and urinary pH. Am J Physiol Renal Physiol 305: F1412–F1421, 2013. First published August 28, 2103; doi:10.1152/ajprenal.00249.2013.—Urinary exosomes are small vesicles secreted into urine from all renal epithelial cell types and known to contain proteins that are involved in renal secretion and reabsorption. Among these proteins, urinary exosomal aquaporin-2 (AQP2) has been suggested to be useful for diagnosis of renal disease. However, the mechanisms underlying the excretion of urinary exosomal AQP2 are largely unknown. In this study, we examined the mechanisms of urinary exosomal AQP2 excretion in vivo, using diuretics including furosemide (FS), an inhibitor of the sodium-potassium-chloride symporter; acetazolamide (ACTZ), an inhibitor of carbonic anhydrase; OPC-31260 (OPC), a vasopressin type 2 receptor antagonist; and NaHCO3, a urinary alkalinizing agent. Samples of urine from rats were collected for 2 h just after treatment with each diuretic, and urinary exosomes were isolated by ultracentrifugation. Urinary exosomal AQP2 excretion was dramatically increased by treatment with FS accompanied by urine acidification or with ACTZ accompanied by urine alkalinization. Immunohistochemistry showed that apical exosomal AQP2 (35, 41). Although both studies suggested that vasopressin enhanced the excretion of exosomal AQP2 through its short-term effect is related to renal AQP2 protein level. The short-term effect of vasopressin is mediated by trafficking of AQP2 from intracellular vesicles to the apical plasma membrane, resulting in a rapid increase of water reabsorption (17, 19, 30, 42). Furthermore, vasopressin also increases the expression of AQP2 protein in renal collecting duct cells by increasing transcription, contributing to the late phase of increased water reabsorption induced by vasopressin (6, 11, 12, 20, 31).

Since the discovery of AQP2 in human urine by Kanno et al. (14) in 1995, many studies have explored the biological significance of urinary AQP2 (5, 7, 28, 32). As a result, it is now thought that the urinary excretion of AQP2 reflects the action of vasopressin on renal cells through both short-term and long-term effects (7, 14, 28, 32). The short-term effect of vasopressin is mediated by trafficking of AQP2 from intracellular vesicles to the apical plasma membrane, and the long-term effect is related to renal AQP2 protein level.

Despite the extensive work that has been done on urinary AQP2, to our knowledge, only two studies have directly examined the mechanisms underlying the excretion of urinary exosomal AQP2 (35, 41). Although both studies suggested that vasopressin enhanced the excretion of exosomal AQP2 through the long-term effect, the involvement of the short-term effect of vasopressin in the excretion of urinary exosomal AQP2 has remained unclear. The reason for this is that a certain volume of urine is required to isolate urinary exosomes and that little...
urine is produced during the short-term phase of the vasopressin effect. Besides the regulatory effect of vasopressin, other factors that can affect the urinary excretion of exosomal AQP2 have yet to be elucidated.

In the present study, we examined whether the short-term effect of vasopressin was involved in the excretion of urinary exosomal AQP2 in vivo. To overcome the paucity of urine production during the short-term effect of vasopressin, in this study using rats we used diuretics, including furosemide (FS), an inhibitor of the sodium-potassium-chloride symporter (29); acetazolamide (ACTZ), an inhibitor of carbonic anhydrase (29); OPC-31260 (OPC), a V2 receptor antagonist (43); and NaHCO₃, a urinary alkalinizing agent, as diuretic treatment was expected to facilitate production of a sufficient urine volume, accompanied by an increased blood level of vasopressin through systemic volume depletion. As a result, we found that the short-term effect of vasopressin was to increase the abundance of urinary exosomal AQP2 and that urine alkalinization also exerted a similar effect independently of vasopressin.

**MATERIALS AND METHODS**

**Animals and drug administration.** All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals at the University of Miyazaki and conducted in compliance with the Law Concerning the Protection and Control of Animals (Japan Law No. 105, October 1, revised on June 22, 2005). Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88 of the Ministry of the Environment, Japan, April 28, 2006), and the Guidelines for Animal Experimentation (the Japanese Association for Laboratory Animal Science, May 22, 1987).

Male Sprague-Dawley (SD) rats aged 9 wk were purchased from Kyudo (Saga, Japan) or Charles River Japan (Kanagawa Japan). OPC was generously donated by Otsuka Pharmaceutical (Tokyo, Japan). FS (20 mg/kg; Sanofi Aventis, Tokyo, Japan), ACTZ (50 mg/kg; Sanwa Kagaku Kenkyusho, Aichi, Japan), OPC (10 mg/kg), or vehicle (saline) was given via subcutaneous injection. NaHCO₃ (3 mmol/l; Otsuka Pharmaceutical) was administered orally by gavage. When OPC was coadministered with another agent, each drug was administered 15 min after OPC. All animals were kept in metabolic cages and given free access to water during the study period.

**Analyses of blood and urine parameters.** Urinary concentrations of electrolytes, plasma creatinine, and plasma urea nitrogen were analyzed using an autoanalyzer (Fuji Film Medical, Tokyo, Japan). Urine pH was measured using litmus paper (GE Healthcare Japan, Tokyo, Japan), and analyzed using an autoanalyzer (Fuji Film Medical, Tokyo, Japan). Urine and plasma vasopressin concentration, collected blood was drawn into chilled plastic tubes containing Aprotinin (500 KIU/mL) and 4 mM EDTA, 70 mM p-amidinophenyl methanesulfonyl fluoride hydrochloride, and a complete protease inhibitor cocktail (Roche) on ice. Immediately after collection, urine was centrifuged at 1,000 g for 15 min, and the supernatant was centrifuged at 17,000 g for 15 min to remove the urinary sediment. The resulting supernatant was centrifuged at 200,000 g for 1 h at 4°C (Beckman Instruments, Brea, CA). The resulting pellet was suspended in a solution containing a protease inhibitor mixture (10-fold dilution) and the suspension was mixed with 4x sample buffer (8% SDS, 50% glycerol, 250 mM Tris·HCl, 0.05% bromphenol blue, and 200 mM DTT), followed by incubation for 30 min at 37°C. Each urinary exosomal protein sample was loaded in each lane with the same amount of total protein.

Proteins were separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. After blocking with Tris-buffered saline including 5% skim milk and 0.05% Tween 20, the membrane was incubated with Tris-buffered saline including 1.7% skim milk, 0.05% Tween 20, and a primary antibody, which was a rabbit polyclonal antibody against either AQP2 (cat no. AQP-002; Alomone Laboratories, Jerusalem, Israel) or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), at 4°C for 1 h. The blots were then incubated with Tris-buffered saline including 1.7% skim milk, 0.05% Tween 20, and a peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Danvers, MA) at 37°C for 45 min. The antibody-antigen reaction was detected using a Super Signal chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA). The resulting band was quantified using the WinRoof software package version 5.7 (Mitani, Tokyo, Japan).

In each series of experiments, we always included a control group composed of multiple numbers of animals. The protein samples from the corresponding control animals were always loaded in each gel for normalized quantification.

**Immunohistochemistry.** The paraffin-embedded kidney blocks were cut into sections 2-μm thick, placed on slides, and then deparaffinized and rehydrated. The antigen was retrieved by heating the slide in 10 mM EDTA, pH 9.0, for 15 min at 95°C. The resulting pellet was dissolved in an isolation solution, followed by mixing in 4x sample buffer. This solution was incubated for 30 min at 37°C. Each renal protein sample was loaded in each lane with the same amount of total protein.

Real-time PCR. Total RNA was isolated from each part of the kidney using a RNeasy Protect Minikit (Qiagen, Tokyo, Japan) with DNase digestion. The isolated RNA was reverse-transcribed to cDNA using an 5 Script cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). Rat GAPDH and AQP2 were amplified using a Power SYBR Green RT-PCR Reagents Kit (Applied Biosystems, Carlsbad, CA) employing the following primers: forward (5’-atcccttacctcaggcttt-3’) and reverse (5’-ctccgttagcttgatc-3’) for GAPDH and forward (5’-aaggggacagaggtttg-3’) and reverse (5’-aggagcaagcagagagt-3’) for AQP2. GAPDH was used for normalization of cDNA input. The expression of each gene was detected and analyzed using a 7900HT system (Applied Biosystems).

**Statistical analysis.** All data are expressed as means ± SE. Differences from the control group were analyzed by one-way ANOVA followed by Dunnet’s test (a parametric test) or by Steel’s test (a nonparametric test) for multiple comparisons. Differences between
### Table 1. Changes in body weight and urinary parameters after treatment with diuretics

<table>
<thead>
<tr>
<th></th>
<th>Control (Saline)</th>
<th>FS</th>
<th>ACTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change of body weight, g/h</td>
<td>2.9 ± 0.3 (n = 13)</td>
<td>11.0 ± 0.5* (n = 13)</td>
<td>6.8 ± 1.2* (n = 12)</td>
</tr>
<tr>
<td>Urine volume, ml/h</td>
<td>1.3 ± 0.2 (n = 13)</td>
<td>9.0 ± 0.3* (n = 13)</td>
<td>4.3 ± 0.3* (n = 12)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mEq/h</td>
<td>0.10 ± 0.01 (n = 13)</td>
<td>0.80 ± 0.03* (n = 13)</td>
<td>0.45 ± 0.03* (n = 12)</td>
</tr>
<tr>
<td>Urinary potassium excretion, mEq/h</td>
<td>0.11 ± 0.02 (n = 13)</td>
<td>0.26 ± 0.01* (n = 13)</td>
<td>0.28 ± 0.02* (n = 12)</td>
</tr>
<tr>
<td>Urinary chloride excretion, mEq/h</td>
<td>0.09 ± 0.01 (n = 13)</td>
<td>0.81 ± 0.04* (n = 13)</td>
<td>0.15 ± 0.01 (n = 12)</td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH2O</td>
<td>687.8 ± 60.7 (n = 13)</td>
<td>320.5 ± 5.7* (n = 13)</td>
<td>446.1 ± 21.4 (n = 12)</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.89 ± 0.10 (n = 13)</td>
<td>7.25 ± 0.07* (n = 13)</td>
<td>8.73 ± 0.07* (n = 12)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. FS, furosemide; ACTZ, acetazolamide. *P < 0.01 vs. control.

### RESULTS

#### Changes in urine and blood parameters after treatment with diuretics

The parameters of urine collected for 2 h just after treatment with diuretic (0- to 2-h urine) are summarized in Table 1. Compared with the control group, urine volume was significantly increased after treatment with FS or ACTZ, accompanied by a decrease in urinary osmolality. The degree of the diuretic effect was more pronounced in the FS group than in the ACTZ group. Urinary sodium and potassium excretion were significantly increased in both groups, but urinary chloride excretion was increased only in the FS group. Compared with the control group, urinary pH was significantly low in the FS group and significantly high in the ACTZ group.

We also measured blood parameters (Table 2) after each treatment. The plasma creatinine concentration was not altered in either of the groups, but the plasma urea nitrogen concentration was significantly increased only in the FS group relative to the control. The plasma vasopressin level was significantly increased in the FS group and tended to be increased in the ACTZ group. FS significantly increased the blood HCO₃⁻ concentration, and ACTZ significantly lowered both the blood pH and HCO₃⁻ concentration.

These urinary and blood data indicated that FS induced rapid dramatic systemic dehydration and acidification of the urine and that ACTZ caused moderate systemic dehydration and acidosis, with urinary alkalization. These changes well reflect the pharmacological actions of FS and ACTZ, which are attributable to inhibition of the sodium-potassium-chloride symporter and inhibition of carbonic anhydrase, respectively (29).

#### Urinary exosomal AQP2 protein excretion after treatment with diuretics

Next, we examined whether diuretics affected urinary excretion of exosomal AQP2 protein in 0- to 2-h urine. As shown in Fig. 1, immunoblot analysis revealed that urinary exosomal AQP2 protein excretion was dramatically increased in the FS and ACTZ groups compared with the control. Interestingly, the degree of exosomal AQP2 protein excretion was significantly higher in the ACTZ group than in the FS group. When we compared total creatinine excretion into 0- to 2-h urine, there was no significant difference between the three groups (data not shown).

It is known that FS has a shorter half-life than ACTZ (FS: 0.3–3.4 h; ACTZ: 6–9 h; ref. 29), so this difference may explain the greater urinary exosomal AQP2 protein excretion in the ACTZ group. We therefore compared the two groups in terms of urinary volume and urinary exosomal AQP2 protein excretion into 0- to 1-h urine after treatment with the diuretics. In this case it was difficult to include control data because sometimes an insufficient amount of urine was collected within such a short period, and therefore, we directly compared the two groups. Although the 0- to 1-h urine volume was still larger in the FS group than in the ACTZ group (FS: 13.1 ± 0.7 ml, n = 7; ACTZ: 4.9 ± 0.6 ml, n = 8), the degree of urinary exosomal AQP2 protein excretion in the ACTZ group still tended to be higher than that in the FS group (FS: 100 ± 76.5%; ACTZ: 269.8 ± 309.8%). These results suggested that the greater increase of urinary exosomal AQP2 excretion in the ACTZ group may not have been attributable to the difference in the half-life of the diuretics.

#### Analysis of the renal AQP2 protein level after treatment with diuretics

As the degree of exosomal AQP2 protein excretion was significantly increased by treatment with diuretics, we examined the abundance of renal AQP2 protein 2 h after treatment with FS or ACTZ. Figure 2A shows a representative immunoblot, and Fig. 2B summarizes quantification across multiple experiments for renal AQP2 protein. Although in the OM and IM, the levels of AQP2 protein did not differ among the control, FS, and ACTZ groups, FS and ACTZ significantly reduced the abundance of renal AQP2 protein in the cortex relative to the control. This suggested that renal cortical AQP2 protein was the source of the increased amount of urinary exosomal AQP2 induced by the diuretics.

We also examined the renal expression of AQP2 protein using an immunohistochemical technique (Fig. 2, C–H). Along

### Table 2. Changes in blood parameters after treatment with diuretics

<table>
<thead>
<tr>
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<th>Control (Saline)</th>
<th>FS</th>
<th>ACTZ</th>
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<tbody>
<tr>
<td>Plasma creatinine concentration, mg/dl</td>
<td>0.20 ± 0.02 (n = 7)</td>
<td>0.25 ± 0.07 (n = 12)</td>
<td>0.22 ± 0.04 (n = 12)</td>
</tr>
<tr>
<td>Plasma urea nitrogen concentration, mg/dl</td>
<td>14.56 ± 0.67 (n = 7)</td>
<td>21.91 ± 0.53† (n = 12)</td>
<td>14.01 ± 0.58 (n = 12)</td>
</tr>
<tr>
<td>Plasma vasopressin level, %control</td>
<td>100.0 ± 14.2 (n = 7)</td>
<td>291.3 ± 60.9* (n = 12)</td>
<td>234.5 ± 51.4 (n = 12)</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.31 ± 0.02 (n = 5)</td>
<td>7.35 ± 0.01 (n = 3)</td>
<td>7.17 ± 0.01† (n = 6)</td>
</tr>
<tr>
<td>PCO₂, mmHg</td>
<td>63.02 ± 2.38 (n = 5)</td>
<td>65.40 ± 1.82 (n = 3)</td>
<td>68.70 ± 2.29 (n = 6)</td>
</tr>
<tr>
<td>HCO₃⁻, mmol/l</td>
<td>31.32 ± 0.32 (n = 5)</td>
<td>36.13 ± 0.55† (n = 3)</td>
<td>25.02 ± 0.65† (n = 6)</td>
</tr>
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</table>

Data are expressed as means ± SE. *P < 0.05 and †P < 0.01 vs. control.
with the data from immunoblot analysis, immunohistology at low magnification (Fig. 2, C, E, and G) demonstrated a reduced level of AQ2 protein in the cortex after treatment with FS or ACTZ. At high magnification (Fig. 2, D, F, and H), the apical expression level of AQ2 protein was markedly increased in the FS and ACTZ groups (Fig. 2, F and H), indicating that the short-term renal effect of vasopressin occurred during the actions of the diuretics.

Analysis of renal AQ2 mRNA after treatment with diuretics. Protein analysis indicated that the diuretics selectively affected the expression of AQ2 in the cortex. Therefore, we investigated the levels of renal AQ2 mRNA using a real-time PCR technique. As shown in Fig. 3, although we found no significant changes in AQ2 mRNA levels in both the OM and IM, the level of AQ2 mRNA in the renal cortex was significantly increased after treatment with FS or ACTZ. These PCR results supported the selective actions of FS and ACTZ indicated by the protein analyses, as shown in Fig. 2.

Urinary exosomal AQ2 protein excretion after coadministration of OPC with FS or ACTZ. The above data suggested that the increase of urinary exosomal AQ2 protein induced by treatment with FS or ACTZ was mediated by the short-term effect of vasopressin on the renal cortex. We then examined the effect of a V2 receptor antagonist, OPC, on the increased urinary level of exosomal AQ2 induced by FS or ACTZ. The OPC was preinjected before the diuretic treatment. The 0- to 2-h urinary parameters are summarized in Table 3. Compared with the control (saline + saline), urine volume was significantly increased after treatment with saline + FS, OPC + FS, saline + ACTZ, and OPC + ACTZ, along with decreases in urinary osmolality. Also, the urine volume tended to increase and the urinary osmolality was significantly decreased in the OPC + saline group.

Figure 4A shows a representative immunoblot, and Fig. 4B summarizes quantification across multiple experiments for urinary exosomal AQ2 levels in 0- to 2-h urine. When we compared the control group (saline + saline) with the OPC group (OPC + saline), there was no significant difference between them. Coadministration of OPC with FS completely inhibited the FS-induced increase in the excretion of urinary exosomal AQ2. On the other hand, coadministration of OPC with ACTZ partially inhibited the ACTZ-induced increase in excretion of urinary exosomal AQ2.

Urinary excretion of exosomal AQ2 protein after treatment with NaHCO3. In comparison with the FS group, the ACTZ group produced less urine, but the level of urinary excretion of exosomal AQ2 was higher and the inhibitory effect of OPC was partial. Because ACTZ is known to alkalize urine (29) and this action was also confirmed by the present study (Tables 1 and 3), we next examined the effect of urine alkalization on the urinary excretion of exosomal AQ2. For this experiment, we employed NaHCO3, which is well known to cause urinary alkalization and diuresis. Tables 4 and 5 summarize the urine and blood parameters, respectively. As shown in Table 4, NaHCO3 increased the urinary pH, along with osmotic diuresis. The degree of urine alkalization was comparable to that of ACTZ (Tables 1 and 3). The blood parameters indicated that treatment with NaHCO3 resulted in alkalosis with an increased concentration of HCO3-.

Figure 5A shows a representative immunoblot, and Fig. 5B summarizes quantification across multiple experiments for 0- to 2-h urine after treatment with NaHCO3. The amount of urinary exosomal AQ2 was significantly increased in the NaHCO3 group relative to the control.

DISCUSSION

It is well known that the renal expression and localization of AQ2 are regulated by the vasopressin/V2 receptor pathway (21, 22). After activation of the V2 receptor by vasopressin, AQ2 is rapidly translocated from intracellular vesicles to the apical plasma membrane in the renal connecting tubule and collecting duct cells, and AQ2 then accumulates in the apical membrane, resulting in an acute increase of water reabsorption (the short-term effect of vasopressin; Refs. 17, 19, 30, 42). This short-term effect is known to occur within 1 h (17, 30, 42) after
activation of the V2 receptor. On the other hand, treatment with vasopressin increases the expression of AQP2 protein in the renal cells mainly through the enhancement of transcription, contributing to the late phase of water reabsorption induced by vasopressin (the long-term effect of vasopressin). Because previous in vitro and in vivo studies have demonstrated a marginal increase of AQP2 protein expression 3 h after stimulation of the V2 receptor (11, 31), and a gradual increase of its expression thereafter, the long-term effect is thought to occur 3 h or more after activation of the V2 receptor. In this study, urine volume was increased and osmolality was reduced within 2 h after treatment with FS and ACTZ. Each treatment also resulted in an increase of the plasma vasopressin level. Among the three renal regions, the level of AQP2 protein in the OM
Changes in body weight and urinary parameters after coadministration of OPC-31260 with FS or ACTZ

and IM remained unchanged, whereas the cortical AQP2 protein level decreased with enhancement of the apical expression of AQP2 after each treatment. Under these conditions, both diuretics dramatically increased the urinary excretion of exosomal AQP2. When the V2 receptor antagonist OPC was coadministered, the FS- and ACTZ-induced increases were diminished and reduced, respectively. These data strongly indicated that the urinary excretion of exosomal AQP2 increased in response to the diuretics used in the present study during the short-term phase of the vasopressin effect.

In terms of diuretic action, the effect of FS was more pronounced than that of ACTZ (Table 1). Also, the blood level of vasopressin was higher in the FS group than in the ACTZ group (Table 1). However, the effect of ACTZ on urinary exosomal AQP2 excretion was more pronounced than that of FS. When we compared the two groups in terms of urinary volume and urinary exosomal AQP2 excretion into 0- to 1-h urine after treatment with the diuretics, the results indicated that the difference in the half-life of the diuretics was unlikely to explain the greater increase of urinary exosomal AQP2 excretion in the ACTZ group than that in the FS group. On the other hand, as shown in both Tables 1 and 2, ACTZ alkalized the urine and caused systemic acidosis. Therefore, it was probable that alkalinized urine and/or systemic acidosis increased the urinary excretion of exosomal AQP2. When we examined the effect of NaHCO3, it was found to significantly increase the urinary excretion of exosomal AQP2 (Fig. 5), accompanied by urinary alkalinization and systemic alkalosis (Tables 4 and 5). Furthermore, in a preliminary experiment, when rats were treated with ammonium chloride (1 mmol/rat po), an agent for acidifying urine, the pH of 0- to 2-h urine was 5.7 ± 0.1 (n = 4). In this case, the urinary excretion level of exosomal AQP2 was not significantly different from that of control animals treated with saline (control: 100 ± 17%, n = 7; ammonium chloride: 167 ± 59%, n = 3). Moreover, in the present study, OPC abolished the FS-induced excretion of urinary exosomal AQP2, while the increased excretion of urinary exosomal AQP2 induced by treatment with ACTZ or NaHCO3 was only partially sensitive to treatment with the V2 receptor antagonist. These data strongly suggest that urine alkalinization increases, and independently regulates, the urinary excretion of exosomal AQP2.

The plasma vasopressin level was not altered by treatment with NaHCO3 relative to the control (Table 5). Despite this, coadministration of OPC partially inhibited the NaHCO3-induced increase in the urinary excretion of exosomal AQP2. Using LLC-PK1 cells, Zalyapin et al. (44) demonstrated that the affinity of vasopressin for the V2 receptor was higher at pH 7.4 than at pH 5.5. Since we observed alkalosis in rats after treatment with NaHCO3, one possible reason for the partial sensitivity of the V2 receptor antagonist to NaHCO3-induced urinary excretion of exosomal AQP2 is increased activation of the V2 receptor under conditions of alkalosis without any increase in the plasma vasopressin level.

In the present study, treatment with OPC alone significantly decreased urinary osmolality, but urinary excretion of exosomal AQP2 was not reduced. The reason for this is currently unclear. We observed that OPC caused slightly urinary alkalinization (Table 3), and therefore, this might have masked any reduction in the urinary excretion of exosomal AQP2 in response to OPC treatment. Alternatively, the basal urinary exosomal excretion of AQP2 might be maintained by factors other than vasopressin. It has been reported that calcitonin induces cAMP-dependent AQP2 trafficking in connecting tu-

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**Table 3. Changes in body weight and urinary parameters after coadministration of OPC-31260 with FS or ACTZ**

<table>
<thead>
<tr>
<th></th>
<th>Control (Saline + Saline)</th>
<th>OPC + Saline</th>
<th>Saline + FS</th>
<th>OPC + FS</th>
<th>Saline + ACTZ</th>
<th>OPC + ACTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change of body weight, g/h</td>
<td>( \Delta 3.7 \pm 0.7 ) (n = 14)</td>
<td>( \Delta 5.4 \pm 0.4 ) (n = 8)</td>
<td>( \Delta 12.5 \pm 0.7 \dagger ) (n = 5)</td>
<td>( \Delta 13.9 \pm 0.7 \dagger ) (n = 7)</td>
<td>( \Delta 8.2 \pm 1.1 \dagger ) (n = 10)</td>
<td>( \Delta 10.3 \pm 1.1 \dagger ) (n = 11)</td>
</tr>
<tr>
<td>Urine volume, ml/h</td>
<td>1.7 ± 0.2 (n = 14)</td>
<td>3.0 ± 0.6 (n = 8)</td>
<td>10.6 ± 0.4† (n = 5)</td>
<td>10.9 ± 0.6† (n = 7)</td>
<td>5.1 ± 0.7† (n = 10)</td>
<td>7.7 ± 0.8† (n = 11)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mEq/h</td>
<td>0.11 ± 0.01 (n = 14)</td>
<td>0.11 ± 0.02 (n = 8)</td>
<td>1.00 ± 0.04† (n = 5)</td>
<td>0.86 ± 0.06† (n = 7)</td>
<td>0.48 ± 0.04† (n = 10)</td>
<td>0.52 ± 0.03† (n = 11)</td>
</tr>
<tr>
<td>Urinary potassium excretion, mEq/h</td>
<td>0.13 ± 0.02 (n = 14)</td>
<td>0.13 ± 0.03 (n = 8)</td>
<td>0.32 ± 0.02† (n = 5)</td>
<td>0.27 ± 0.02† (n = 7)</td>
<td>0.26 ± 0.03† (n = 10)</td>
<td>0.30 ± 0.02† (n = 11)</td>
</tr>
<tr>
<td>Urinary chloride excretion, mEq/h</td>
<td>0.09 ± 0.01 (n = 14)</td>
<td>0.10 ± 0.03 (n = 8)</td>
<td>0.95 ± 0.03† (n = 5)</td>
<td>0.85 ± 0.06† (n = 7)</td>
<td>0.14 ± 0.01 (n = 10)</td>
<td>0.17 ± 0.01 (n = 11)</td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH2O</td>
<td>640.5 ± 50.5 (n = 14)</td>
<td>341.4 ± 48.2† (n = 8)</td>
<td>327.8 ± 9.8† (n = 5)</td>
<td>279.6 ± 7.7† (n = 7)</td>
<td>434.1 ± 13.2† (n = 10)</td>
<td>337.1 ± 8.2† (n = 11)</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.33 ± 0.13 (n = 14)</td>
<td>7.56 ± 0.15 (n = 8)</td>
<td>7.05 ± 0.12 (n = 5)</td>
<td>6.86 ± 0.07* (n = 7)</td>
<td>8.94 ± 0.11† (n = 10)</td>
<td>8.62 ± 0.05† (n = 11)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. OPC, OPC-31260. *P < 0.05 and †P < 0.01 vs. control.

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bule and cortical collecting duct cells in parallel with an increase in urine concentration (3). Also, Wang et al. (40) have observed that continuous infusion of atrial natriuretic factor evoked transient diuresis (peak at 10 min) followed by reduced production of urine to control levels (30–90 min after infusion); later, in the reduced urine production phase, a marked increase in apical labeling of AQP2 was evident in the collecting duct cells. Furthermore, the renin-angiotensin-aldosterone axis (16, 18) and prostanoids (E-prostanoid receptors 2 and 4; Ref. 23) have been reported to provide an additional mechanism for regulating the trafficking of AQP2. These data indicate that in addition to vasopressin, apical expression of AQP2 is regulated by other factors, suggesting that such factors might contribute to maintaining the basal urinary exosomal excretion of AQP2, leading to insensitivity to V2 receptor antagonists under basal conditions. It is noteworthy that, recently, it has been reported that in response to saline, urinary excretion of AQP2 (not in the exosomal fraction) is abnormally increased in patients with essential hypertension showing a lack of vasopressin dependency (10).

Our present study showed that treatment with FS or ACTZ decreased the expression of AQP2 protein and increased expression of the mRNA only in the renal cortex. These data suggest that the increased excretion of urinary exosomal AQP2 in response to diuretics originated mainly from the renal cortex and that the decreased level of AQP2 protein in renal cortical cells activated a compensatory mechanism, such as enhancement of AQP2 gene transcription. So far, region-specific regulation of AQP2 has been reported in various models such as food-deprived rats (1), mice lacking the apical Na\(^+\)/H\(^+\) ex-

### Table 4. Changes in body weight and urinary parameters after NaHCO\(_3\) treatment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (Saline)</th>
<th>NaHCO(_3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change of body weight, g/h</td>
<td>Δ3.6 ± 0.6 (n = 7)</td>
<td>Δ5.0 ± 0.6 (n = 7)</td>
</tr>
<tr>
<td>Urine volume, ml/h</td>
<td>1.1 ± 0.1 (n = 7)</td>
<td>2.7 ± 0.3* (n = 7)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mEq/h</td>
<td>0.11 ± 0.01 (n = 7)</td>
<td>0.70 ± 0.06* (n = 7)</td>
</tr>
<tr>
<td>Urinary potassium excretion, mEq/h</td>
<td>0.13 ± 0.03 (n = 7)</td>
<td>0.29 ± 0.03* (n = 7)</td>
</tr>
<tr>
<td>Urinary chloride excretion, mEq/h</td>
<td>0.09 ± 0.01 (n = 7)</td>
<td>0.29 ± 0.03* (n = 7)</td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH(_2)O</td>
<td>786.0 ± 112.1 (n = 7)</td>
<td>906.9 ± 34.3* (n = 7)</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.53 ± 0.20 (n = 7)</td>
<td>8.55 ± 0.04* (n = 7)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P < 0.01 vs. control.
chancer NHE3 (Nhe3−/− mice) (2), and rats with lithium-induced nephrogenic diabetes insipidus treated with aldosterone (18). Among them, there are some similarities between our data obtained with diuretics and those in Nhe3−/− mice. Nhe3−/− mice exhibited polyuria with lower osmolality, a reduced level of AQP2 protein particularly in the renal cortex, and an increased circulating plasma level of vasopressin, compared with wild-type controls. The lack of apical NHE3 is thought to decrease reabsorption of fluid and HCO3− in the proximal tubule. These decreases caused by NHE3 deficiency resemble those resulting from inhibition of carbonic anhydrase by ACTZ. Interestingly, Nhe3−/− mice showed reduced expression of sodium-potassium-chloride symporter (NKCC2) protein in the thick ascending limb, which is a primary target of FS. Although we did not investigate renal expression level of NKCC2 protein after treatment with ACTZ, the inhibition of NKCC2 activity might be associated with the downregulation of renal cortical AQP2 protein.

A number of previous studies of urinary exosomes in humans and experimental animals have suggested that some exosomal proteins may be potential biomarkers for several renal-related diseases. These include fetuin-A (46), activating transcription factor 3 (45), and AQP1 (34) for acute kidney injury, Wilms tumor 1 (15, 45) for early podocyte injury, NKCC2 and sodium-chloride transporter (8, 27) for hypertension, the phosphorylated form of the sodium chloride cotransporter and prostatin for aldosteronism (25, 37), and AQP2 (24).

**Table 5. Changes in blood parameters after NaHCO3 treatment**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (Saline)</th>
<th>NaHCO3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma creatinine concentration, mg/dl</td>
<td>0.19 ± 0.01 (n = 7)</td>
<td>0.20 ± 0.00 (n = 7)</td>
</tr>
<tr>
<td>Plasma urea nitrogen concentration, mg/dl</td>
<td>15.54 ± 0.70 (n = 7)</td>
<td>13.61 ± 0.63 (n = 7)</td>
</tr>
<tr>
<td>Plasma vasopressin level, %control</td>
<td>100.0 ± 18.7 (n = 6)</td>
<td>92.1 ± 32.1 (n = 6)</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.26 ± 0.02 (n = 5)</td>
<td>7.34 ± 0.01* (n = 6)</td>
</tr>
<tr>
<td>PCO2, mmHg</td>
<td>68.0 ± 2.61 (n = 5)</td>
<td>66.3 ± 2.61 (n = 6)</td>
</tr>
<tr>
<td>HCO3−, mmol/l</td>
<td>30.6 ± 0.23 (n = 5)</td>
<td>36.3 ± 0.81* (n = 6)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P < 0.01 vs. control.

![Fig. 5. Levels of urinary exosomal AQP2 after NaHCO3 treatment. A: 0- to 2-h urine was collected from rats treated with saline or NaHCO3. A typical immunoblot with urinary exosomal samples is shown. Immunoblot image separately into groups is shown because the original image included data for the other samples in the middle 4 lanes between the control and NaHCO3 groups. Separate images originated from the same blot, while retaining the original quality. Each lane contained the same amount of creatinine. B: quantitative data obtained from immunoblot analyses of urinary exosomal samples are summarized. Each value is expressed as a percentage of the mean value of the urinary exosomal AQP2 level in the control group. Data are expressed as means ± SE; n = 7 in each group. **P < 0.01, compared with the control group.](http://ajprenal.physiology.org/)

![Fig. 6. Levels of urinary exosomal AQP2 after coadministration of OPC with NaHCO3. A: 0- to 2-h urine was collected from rats with saline or NaHCO3 15 min after preinjection of saline or OPC. A typical immunoblot with urinary exosomal samples is shown. Each lane contained the same amount of creatinine. B: quantitative data obtained from immunoblot analyses of urinary exosomal samples are summarized. Each value is expressed as a percentage of the mean value of the urinary exosomal AQP2 level in the control group. Data are expressed as means ± SE; n = 6 for the control, n = 6 for the saline + NaHCO3, n = 7 for the OPC + NaHCO3 groups. **P < 0.01, compared with the control group. A significant difference was not seen between the control and the OPC + NaHCO3 groups. #P < 0.05, compared between the saline + NaHCO3 and the OPC + NaHCO3 groups.](http://ajprenal.physiology.org/)
for the urinary concentrating defect in patients with American cutaneous leishmaniasis. Studies of urinary exosomal biomarker proteins are still in the early stages, since scarcely 10 yr have passed since urinary exosomes were first reported by Knepper and colleagues (27). Therefore, any candidate proteins will require validation in large patient cohorts. Furthermore, various important questions about how urinary exosomal excretion of such candidate proteins is regulated remain unanswered. In the present study to examine the mechanism responsible for regulation of urinary exosomal AQP2 excretion in vivo, we found two contributory factors: plasma vasopressin and urinary alkalization. We anticipate that these findings will contribute to future clinical application of urinary exosomal AQP2 as a diagnostic marker for patients with kidney diseases.

REFERENCES


