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 localization of AQP2 was clearly evident and the plasma vasopressin level was increased after each treatment. Although treatment with OPC alone had no significant effect, coadministration of OPC completely inhibited the FS-induced and partially reduced the ACTZ-induced responses, respectively. Treatment with NaHCO3 increased the excretion of urinary exosomal AQP2 accompanied by urine alkalinization. This increased response was partially inhibited by coadministration of OPC. These data suggest that an increased plasma level of vasopressin promoted the excretion of urinary exosomal AQP2 and that urine alkalinization also increased it independently of vasopressin.

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EXOSOMES ARE SMALL VESICLES <100 nm in diameter secreted from various cell types such as dendritic cells, B lymphocytes, endothelial cells, and epithelial cells (13, 26, 38). Exosomes are released into the extracellular space by fusion of the outer membrane of multivesicular bodies with the cell surface (13, 26, 38). Interestingly, the contents of exosomes, such as proteins, mRNAs, and microRNAs, can be transferred to the target cells by fusion of the exosome membrane with the target cell plasma membrane, and therefore, exosomes are thought to mediate communication between cells (4, 33, 36).

Urinary exosomes are released into urine from all nephron segments. So far, Pisitkun and colleagues (9, 27) have identified 1,132 exosomal proteins, of which at least 34 are related to kidney diseases such as autosomal-dominant or autosomal-recessive nephrogenic diabetes insipidus [aquaporin-2 (AQP2)], Bartter syndrome (sodium-potassium-chloride symporter 2), and autosomal recessive renal tubule acidosis (carbonic anhydrase 2). Based on the characteristics of exosomes, several groups have tried to identify a novel biomarker among exosomal proteins. Zhou et al. (45, 46) reported that urinary exosomal fetuin-A and activating transcription factor 3 were increased in a rat model of acute kidney injury. Our group has demonstrated that urinary exosomal AQP1 is decreased in a similar model (34). More recently, Oliviera et al. (24) reported that urinary exosomal AQP2 might be a potential biomarker for the urinary concentration defect in patients with American cutaneous leishmaniasis. These findings indicate that examination of urinary exosomes could lead to the discovery of new noninvasive biomarkers of kidney disease.

Fluid balance is regulated by water reabsorption in the kidney, and a certain part of this renal function is mediated by a water channel protein, AQP2, expressed mainly in the renal connecting tubule and collecting duct cells (21, 22). In response to volume depletion or an increase in plasma osmolality, vasopressin is released from the posterior pituitary, and then vasopressin binds to a specific vasopressin type 2 receptor, the V2 receptor, in the basolateral membrane of renal cells. Upon stimulation of the V2 receptor by vasopressin, AQP2 is translocated 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 Low 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 (Sagai, 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/kg; Otsuka Pharmaceutical) was administered orally by gavage. When we measured the plasma vasopressin concentration, collected blood was drawn into chilled tubes containing EDTA (1 mg/ml) and aprotinin (500 KIU/ml), and the plasma fraction was isolated by usual centrifugation (1,600 g for 15 min at 4°C). Plasma vasopressin concentration was determined using an EIA kit (cat no. 900-017; Enzo Life Sciences, Farmingdale, NY), which is a competitive immunoassay (39).

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) or a pH meter (ISPTCOM, Saitama, Japan). Blood pH, PCO₂, and HCO₃⁻ were analyzed using a blood gas analyzer (Fuso Pharmaceutical Industries, Osaka, Japan). When we measured the plasma vasopressin concentration, collected blood was drawn into chilled tubes containing EDTA (1 mg/ml) and aprotinin (500 KIU/ml), and then a plasma fraction was isolated by usual centrifugation (1,600 g for 15 min at 4°C). Plasma vasopressin concentration was determined using an EIA kit (cat no. 900-017; Enzo Life Sciences, Farmingdale, NY), which is a competitive immunoassay (39).

Isolation of urinary exosomes, and protein analyses of exosomes and kidney. The procedure for isolation of urinary exosomes was performed as described previously (34). Briefly, urine was collected from rats for an appropriate period of time (2 h for most experiments) with a collection tube containing a protease inhibitor mixture (60 μl of 130 mM EDTA, 70 mM p-amidinophenyl methanesulfonfyl fluoride hydrochloride, and a complete protease inhibitor cocktail tablet) 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 (Optima TL Ultracentrifuge; Beckman Instruments, Brea, CA). The resulting pellet was suspended in a solution containing 10 mM HEPES (pH 7.4)-150 mM NaCl, and the suspension was mixed with 4× sample buffer (8% SDS, 50% glycerol, 250 mM Tris-HCl, 0.05% bromophenol 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 1% 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 GPDH (Santa Cruz Biotechnology, Santa Cruz, CA), at 37°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 distilled water at 121°C for 5 min, and then the endogenous peroxidase was quenched using a 3% H₂O₂ solution. The slide was then incubated with anti-AQP2 antibody diluted to 1:200 for 1 h at room temperature, followed by incubation with Envision System labelled Polymer reagent (Dako Japan, Tokyo, Japan) for 45 min at room temperature. The reaction product was visualized by treatment with 3,3'-diaminobenzidine tetrahydrochloride, and the slide was counterstained with hematoxylin.

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 iScript 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'-atccctgccacccatattg-3') and reverse (5'-ttactccttgagccttg-3') for GAPDH and forward (5'-ctctcgagcctcttg-3') and reverse (5'-aggggacagccagttg-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>$\Delta 2.9 \pm 0.3$ ($n = 13$)</td>
<td>$\Delta 11.0 \pm 0.5$* ($n = 13$)</td>
<td>$\Delta 6.8 \pm 1.2$* ($n = 12$)</td>
</tr>
<tr>
<td>Urine volume, mEq/h</td>
<td>$1.3 \pm 0.2$ ($n = 13$)</td>
<td>$9.0 \pm 0.3$* ($n = 13$)</td>
<td>$4.3 \pm 0.3$* ($n = 12$)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mEq/h</td>
<td>$0.10 \pm 0.01$ ($n = 13$)</td>
<td>$0.80 \pm 0.03$* ($n = 13$)</td>
<td>$0.45 \pm 0.03$* ($n = 12$)</td>
</tr>
<tr>
<td>Urinary potassium excretion, mEq/h</td>
<td>$0.11 \pm 0.02$ ($n = 13$)</td>
<td>$0.26 \pm 0.01$* ($n = 13$)</td>
<td>$0.28 \pm 0.02$* ($n = 12$)</td>
</tr>
<tr>
<td>Urinary chloride excretion, mEq/h</td>
<td>$0.09 \pm 0.01$ ($n = 13$)</td>
<td>$0.81 \pm 0.04$* ($n = 13$)</td>
<td>$0.15 \pm 0.01$ ($n = 12$)</td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH₂O</td>
<td>$687.8 \pm 60.7$ ($n = 13$)</td>
<td>$320.5 \pm 57.9$* ($n = 13$)</td>
<td>$446.1 \pm 21.4$ ($n = 12$)</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>$7.89 \pm 0.10$ ($n = 13$)</td>
<td>$7.25 \pm 0.07$* ($n = 13$)</td>
<td>$8.73 \pm 0.07$* ($n = 12$)</td>
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</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 the 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 excretion in the ACTZ group. We therefore 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. 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 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 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
OPC was preinjected before the diuretic treatment. The effect of a V2 receptor antagonist, OPC, on the increased effect of vasopressin on the renal cortex. We then examined the treatment with FS or ACTZ was mediated by the short-term that the increase of urinary exosomal AQP2 protein induced by concentration of OPC with FS or ACTZ.

Protein analysis indicated that the diuretics selectively affected the expression of AQP2 in the cortex. There-fore, we investigated the levels of renal AQP2 mRNA using a real-time PCR technique. As shown in Fig. 3, although we expressed as means ± SE; n = 13 for the control, n = 13 for the FS, and n = 12 for the ACTZ groups. *P < 0.05 and **P < 0.01, compared with the control group. ##P < 0.01, compared between the FS and the FS groups.

The above data suggested that the increase of urinary exosomal AQP2 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 AQP2 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 AQP2 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 AQP2. On the other hand, coadministration of OPC with ACTZ partially inhibited the ACTZ-induced increase in excretion of urinary exosomal AQP2.

Urinary excretion of exosomal AQP2 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 AQP2 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 AQP2. For this experiment, we employed NaHCO3, which is well known to cause urinary alkalization and 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 AQP2 was significantly increased in the NaHCO3 group relative to the control.

Urinary exosomal AQP2 protein level after coadministration of OPC with NaHCO3. Although the blood vasopressin level was not affected by treatment with NaHCO3 (Table 5), NaHCO3 increased the volume of urine, suggesting that vasopressin potentially affects the kidney. Therefore, we performed an experiment in which OPC was coadministered with NaHCO3. Figure 6A shows a representative immunoblot and Fig. 6B summarizes quantification across multiple experiments for exosomal AQP2 excretion in 0- to 2-h urine. Coadministration of OPC with NaHCO3 partially inhibited the NaHCO3-induced increase in the excretion of urinary exosomal AQP2, similar to the results obtained when OPC was coadministered with ACTZ (Fig. 4).

DISCUSSION

It is well known that the renal expression and localization of AQP2 are regulated by the vasopressin/V2 receptor pathway (21, 22). After activation of the V2 receptor by vasopressin, AQP2 is rapidly translocated from intracellular vesicles to the apical plasma membrane in the renal connecting tubule and collecting duct cells, and AQP2 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

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**Fig. 2. Levels of renal AQP2 protein expression after treatment with diuretics. A: kidney samples were collected from rats treated with saline (control), FS, or ACTZ. Typical immunoblots for renal AQP2 and GAPDH in the renal cortex are shown. Each lane contained the same amount of protein (30 μg/lane). B: Quantitative data were obtained from immunoblot analyses of renal AQP2 in the cortex, outer medulla (OM), and inner medulla (IM). After normalization to the corresponding level of GAPDH protein, each normalized value is expressed as a percentage of the mean value of the renal AQP2 protein level in the control group. Data are expressed as means ± SE; n = 6 in each group. *P < 0.05, compared with the control group. Other groups did not differ significantly from the corresponding control group. C–H: immunohistochemistry of AQP2 in the renal cortex from rats treated with saline (control; C and D), FS (E and F), or ACTZ (G and H). Representative examples of staining at low (C, E, and G) and high (D, F, and H) magnification are shown. Black boxes in C, E, and G indicate the regions of high-magnification in D, F, and H, respectively. Brown staining indicates the presence of AQP2. Bars = 100 μm (low magnification) and 20 μm (high magnification).**
and IM remained unchanged, whereas the cortical AQ2 protein level decreased with enhancement of the apical expression of AQ2 after each treatment. Under these conditions, both diuretics dramatically increased the urinary excretion of exosomal AQ2. When the V2 receptor antagonist OPC was coadministered, the FS- and ACTZ-induced increases were diminished and reduced, respectively. These data strongly suggest that urine alkalization increases, and independently regulates, the urinary excretion of exosomal AQ2.

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 AQ2. Using LLC-PK1 cells, Zalypin 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 AQ2 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 AQ2 was not reduced. The reason for this is currently unclear. We observed that OPC caused slightly urinary alkalization (Table 3), and therefore, this might have masked any reduction in the urinary excretion of exosomal AQ2 in response to OPC treatment. Alternatively, the basal urinary exosomal excretion of AQ2 might be maintained by factors other than vasopressin. It has been reported that calcitonin induces cAMP-dependent AQ2 trafficking in connecting tu-

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

<table>
<thead>
<tr>
<th>Parameter</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>Δ3.7 ± 0.7</td>
<td>Δ5.4 ± 0.4</td>
<td>Δ12.5 ± 0.7†</td>
<td>Δ13.9 ± 0.7†</td>
<td>Δ8.2 ± 1.1†</td>
<td>Δ10.3 ± 1.1†</td>
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<tr>
<td>(n = 14)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td>(n = 10)</td>
<td>(n = 11)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>Urine volume, ml/h</td>
<td>1.7 ± 0.2</td>
<td>3.0 ± 0.6</td>
<td>10.6 ± 0.4†</td>
<td>10.9 ± 0.6†</td>
<td>5.1 ± 0.7†</td>
<td>7.7 ± 0.8†</td>
</tr>
<tr>
<td>(n = 14)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 11)</td>
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</tr>
<tr>
<td>Urinary sodium excretion, mEq/h</td>
<td>0.11 ± 0.01</td>
<td>0.11 ± 0.02</td>
<td>1.00 ± 0.04†</td>
<td>0.86 ± 0.06†</td>
<td>0.48 ± 0.04†</td>
<td>0.52 ± 0.03†</td>
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<td>(n = 14)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 11)</td>
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<tr>
<td>Urinary potassium excretion, mEq/h</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.03</td>
<td>0.32 ± 0.02†</td>
<td>0.27 ± 0.02†</td>
<td>0.26 ± 0.03†</td>
<td>0.30 ± 0.02†</td>
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<tr>
<td>(n = 14)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 11)</td>
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<tr>
<td>Urinary chloride excretion, mEq/h</td>
<td>0.09 ± 0.01</td>
<td>0.10 ± 0.03</td>
<td>0.95 ± 0.03†</td>
<td>0.85 ± 0.06†</td>
<td>0.14 ± 0.01</td>
<td>0.17 ± 0.01</td>
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<td>(n = 14)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH2O</td>
<td>640.5 ± 50.5</td>
<td>341.4 ± 48.2†</td>
<td>327.8 ± 9.8†</td>
<td>279.6 ± 7.7†</td>
<td>434.1 ± 13.2†</td>
<td>337.1 ± 8.2†</td>
</tr>
<tr>
<td>(n = 14)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 11)</td>
<td></td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.33 ± 0.13</td>
<td>7.56 ± 0.15</td>
<td>7.05 ± 0.12</td>
<td>6.86 ± 0.07*</td>
<td>8.94 ± 0.11†</td>
<td>8.62 ± 0.05†</td>
</tr>
<tr>
<td>(n = 14)</td>
<td>(n = 8)</td>
<td>(n = 5)</td>
<td>(n = 7)</td>
<td>(n = 10)</td>
<td>(n = 11)</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. OPC, OPC-31260. *P < 0.05 and †P < 0.01 vs. control.
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 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 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 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 urine concentration (3).

Table 4. Changes in body weight and urinary parameters after NaHCO₃ treatment

<table>
<thead>
<tr>
<th>Control (Saline)</th>
<th>NaHCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Change of body weight, g/h</td>
<td>Δ3.6 ± 0.6 (n = 7)</td>
</tr>
<tr>
<td>Urine volume, ml/h</td>
<td>1.1 ± 0.1 (n = 7)</td>
</tr>
<tr>
<td>Urinary sodium excretion, mEq/h</td>
<td>0.11 ± 0.01 (n = 7)</td>
</tr>
<tr>
<td>Urinary potassium excretion, mEq/h</td>
<td>0.13 ± 0.03 (n = 7)</td>
</tr>
<tr>
<td>Urinary chloride excretion, mEq/h</td>
<td>0.09 ± 0.01 (n = 7)</td>
</tr>
<tr>
<td>Urinary osmolality, mosmol/kgH₂O</td>
<td>786.0 ± 112.1 (n = 7)</td>
</tr>
<tr>
<td>Urinary pH</td>
<td>7.53 ± 0.20 (n = 7)</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P < 0.01 vs. control.
changen 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 AQPI (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 prostanin for aldosteronism (25, 37), and AQP2 (24).
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.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: Y.H. and M.I. conception and design of research; Y.H., H.S., S.T., H.K., and M.I. performed experiments; Y.H., H.S., S.T., H.K., K.S., and M.I. analyzed data; Y.H. and M.I. interpreted results of experiments; Y.H., H.S., S.T., H.K., K.S., and M.I. performed experiments; Y.H., H.S., S.T., H.K., K.S., and M.I. drafted manuscript; M.I. edited and revised manuscript; M.I. approved final version of manuscript.

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