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Original Article

Acetazolamide enhances the release of urinary exosomal aquaporin-1

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ABSTRACT

Background. Renal aquaporin-1 (AQP1), a water channel protein, is known to be secreted into urine, conveyed by nano-sized extracellular vesicles called exosomes. A previous study has demonstrated that acetazolamide (AZ), a diuretic that inhibits carbonic anhydrases, alters the expression level of AQP1 in cultured cells. Here we investigated whether AZ alters the release of urinary exosomal AQP1 *in vivo*.

Methods. The effect of AZ on urinary exosomal AQP1 secretion was examined in rats and compared with furosemide (another diuretic), $NaHCO_3$ (an alkalizing agent) and NH_4Cl (an acidifying agent). Urine, blood and kidney samples were obtained 2 h after each treatment. Urinary exosomes were isolated by a differential centrifugation technique and urinary exosomal proteins were analyzed by immunoblotting.

Results. The release of exosomal AQP1 into urine was markedly increased after treatment with AZ, accompanied by alkaluria and metabolic acidosis. Immunohistochemistry clearly demonstrated that AZ increased the apical membrane expression of AQP1 in the proximal tubules. AZ did not affect the release of exosomal marker proteins (tumor susceptibility gene 101 protein and apoptosis-linked gene 2 interacting protein X). Treatment with furosemide did not change, whereas $NaHCO_3$ and NH_4Cl decreased the exosomal release of AQP1.

Conclusion. The present findings indicate that AZ increases the release of exosomal AQP1 into urine in association with enhanced apical membrane expression of AQP1.

Keywords: acetazolamide, aquaporin-1, exosomes, extracellular vesicles

INTRODUCTION

Aquaporins (AQPs) are water channel proteins; 13 AQP isoforms (AQP0–AQP12) having been identified and characterized in mammals to date. Among them, AQP1 was the first to be isolated by Agre's group, and is known to be expressed at both the apical and basolateral membrane in proximal tubule cells in the kidney, contributing to transcellular water reabsorption in this segment. It has been shown that AQP1 is also localized in both the thin descending loop of Henle and the descending vasa recta in the kidney, where it plays important roles in countercurrent multiplication and countercurrent exchange to maintain the corticomedullary osmotic gradient [1, 2].

Exosomes, a type of extracellular vesicle (<100 nm), are known to be released into extracellular fluid from various cell types upon fusion of the outer membrane of late endosomes—the multivesicular bodies—with the cell membrane [3–5]. Exosomes that are derived from all nephron segments are known to be released into the urinary space, and these urinary exosomes contain many types of renal functional proteins that are specifically expressed in each segment, including transporters, ion channels and AQPs. These functional proteins are involved in reabsorption and secretion of solutes and water molecules in the kidney. Therefore, it is considered that any alteration in the abundance of these proteins in exosomes might reflect dysfunction of a specific renal segment, a concept that has led to the discovery of a novel biomarker for kidney diseases [6, 7]. In fact, it has been reported that some renal functional proteins in urinary exosomes have potential as biomarkers of renal-related diseases, including AQP1 [8] and organic anion transporters [9] for acute kidney injury, AQP2 for gentamicin-induced nephrotoxicity [10] and the urine concentrating defect in American

cutaneous leishmaniasis [11], Na^+-K^+-Cl^- cotransporter 2 for Bartter syndrome [12], and Na^+-Cl^- cotransporter for Gitelman's syndrome [13, 14], pseudohypoaldosteronism type II [14, 15] and primary aldosteronism [16]. Although evidence for the biomarker value of urinary exosomal protein has accumulated, the mechanisms underlying alterations of exosomal release from renal epithelial cells into urine are still largely unknown, especially under *in vivo* conditions [2].

Acetazolamide (AZ), a carbonic anhydrase (CA) inhibitor, is used for treatment of glaucoma and edema [17]. AZ is able to potently inhibit both the membrane-bound (CAIV) and cytoplasmic forms of CA (CAII) in renal proximal tubules, thus almost completely abolishing $NaHCO_3$ reabsorption in this segment, causing polyuria with alkaluria and occasional metabolic acidosis. Recently, it has been reported that AZ lowers the level of AQP1 expression in cultured cells, suggesting a novel mechanism of diuresis [18]. This observation also suggests that AZ affects the urinary exosomal release of AQP1. To our knowledge, however, this hypothesis has not been examined.

If AZ has an ability to modulate the release of urinary exosomal AQP1, AZ might become a powerful tool for investigating the mechanisms underlying alterations of exosomal AQP1 release from renal epithelial cells under *in vivo* conditions. Therefore, in this study, we examined the effect of AZ on exosomal release of AQP1 into rat urine compared with furosemide (FS; a diuretic that inhibits the Na^+-K^+-Cl^- cotransporter), $NaHCO_3$ (an alkalizing agent) and NH_4Cl (an acidifying agent).

MATERIALS AND METHODS

Animal studies

All animal studies were performed in accordance with the Guidelines for Institutional Care and Use of Laboratory Animals at the University of Miyazaki. Ten-week old male Sprague Dawley (SD) rats (Kyudo, Saga, Japan) were used. AZ (50 mg/kg; Sanwa Kagaku Kenkyusho, Aichi, Japan), FS (20 mg/kg; Sanofi Aventis, Tokyo, Japan) or vehicle (0.9% NaCl solution) was given via subcutaneous injection. In another series of experiments, $NaHCO_3$ (3 mmol/rat; Otsuka Pharmaceutical), NH_4Cl (1.5 mmol NH_4Cl + 1.5 mmol NaCl/rat; Otsuka Pharmaceutical) or vehicle (3 mmol NaCl/rat) was administered orally by gavage. All animals were kept in metabolic cages and given free access to water. Urine, blood and kidney samples were obtained 2 h after injection.

Analyses of blood and urine parameters

Measurements of the urinary concentrations of electrolytes, plasma creatinine and plasma urea nitrogen were performed using an autoanalyzer (Fuji Film Medical, Tokyo, Japan). For measurement of urine pH, a pH meter (ISFTCOM, Saitama, Japan) was used. Blood concentrations of electrolytes, pH and pCO_2 were analyzed using an i-STAT system (Abbott Japan, Tokyo, Japan). Also, an osmometer (Osmostation om-6060; Arkray, Kyoto, Japan) was used for measurement of urinary osmolality.

Isolation of urinary exosomes

Exosomes were isolated from urine as described previously [10]. Briefly, urine was gathered from rats for 2 h at room temperature. After collection, the urinary exosomal fraction was obtained using a differential centrifugation technique (1000*g* for 15 min, 17 000*g* for 15 min, 200 000*g* for 1 h). The resulting pellet was diluted in a solution containing a protease inhibitor mixture and then the solution was mixed with 4× sample buffer (8% SDS, 50% glycerol, 250 mM Tris–HCl, 0.05% bromophenol blue, 200 mM DTT). Thereafter, the mixture was incubated for 30 min at 37°C.

For immunoblot analysis, the loading volume of each urinary exosomal sample was adjusted so that the loaded amount of creatinine was constant [18].

Kidney protein sample

The kidney was separated into the cortex and medulla and each part was homogenized for 5 min at 4°C using a homogenizer (BioMedical Science Inc.). The homogenate was sequentially centrifuged at 1000*g* for 10 min and at 200 000*g* for 1 h at 4°C. The resulting pellet was dissolved in homogenization buffer (300 mM sucrose, 1.3 mM EDTA, 25 mM imidazole, complete protease inhibitor cocktail) and mixed with 4× sample buffer. The mixture was then incubated for 30 min at 37°C. The total protein concentration in each sample before addition of 4× sample buffer was measured using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). For immunoblot analysis, the loading volume was adjusted so that the loaded amount of total protein was constant.

Immunoblot analyses

Immunoblot analysis was performed as described previously [10]. The primary antibodies used in this study were anti-AQP1 antibody (cat. no. sc-20810; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-tumor susceptibility gene 101 protein antibody (TSG101, cat. no. ab-125011; Abcam, Cambridge, MA, USA), anti-apoptosis-linked gene 2 interacting protein X antibody (Alix, cat. no. sc-49268; Santa Cruz Biotechnology) and anti-β-actin antibody (cat. no. sc-4778; Santa Cruz Biotechnology). The secondary antibodies were peroxidase-conjugated anti-rabbit IgG antibody (cat. no. cs-7074; Cell Signaling Technology, Danvers, MA, USA), anti-goat IgG antibody (cat. no. P0449; Dako Japan, Tokyo, Japan) and anti-mouse IgG antibody (cat. no. 1858413; Thermo Fisher Scientific, Rockford, IL, USA). Bands were detected using a chemiluminescence detection system (Thermo Fisher Scientific) and quantified using the ImageQuant TL software package (GE Healthcare, Uppsala, Sweden).

Immunohistochemistry

Sections 2 μm thick from paraffin-embedded kidney blocks were placed on slides and then deparaffinized and rehydrated. After retrieval of the antigen and inactivation of endogenous peroxidase, the slides were reacted with anti-AQP1 antibody, followed by incubation with the Envision System Labelled Polymer Reagent (Dako, Japan). The antigen was visualized by

treatment with 3,3′-diaminobenzidine tetrahydrochloride. Nuclei were stained with hematoxylin.

For quantification of anti-AQP1 antibody positivity, line expression profiles were generated using blue-colored images extracted from full-color images and analyzed using the WinROOF image system (Mitani, Tokyo, Japan). More than 20 proximal tubules for each animal were quantified.

Statistical analyses

All data are expressed as mean ± SE. Differences from the control group for multiple comparisons were analyzed by Steel's test. Differences between two groups were examined by *t*-test. In all tests, differences at $P < 0.05$ were accepted as statistically significant.

RESULTS

The urinary parameters after treatment with AZ (AZ group) or FS (FS group) are summarized in Table 1. In comparison with the control group, urine volume was significantly increased after treatment with AZ or FS. The diuretic effect of FS was more pronounced than that of AZ and, concomitantly, urinary osmolality was significantly decreased by FS. Urinary excretion of Na^+ and K^+ was significantly increased in both groups. In contrast, although urinary Cl^- excretion was markedly increased by FS, the effect of AZ was modest. Urinary pH was significantly higher in the AZ group and significantly lower in the FS group than in the control group.

Data for blood parameters are summarized in Table 2. The plasma urea nitrogen concentration was significantly increased only in the FS group relative to the control, probably due to prerenal azotemia caused by rapid and dramatic dehydration. Blood Na^+ and K^+ concentrations were not altered in either of the groups. The blood Cl^- concentration was significantly increased in the AZ group and decreased in the FS group. AZ significantly lowered the blood HCO_3^- concentration accompanied by a decrease in blood pH, and FS significantly increased the blood HCO_3^- concentration. These changes in urinary and blood parameters well reflect the pharmacological actions of AZ and FS in terms of inhibitions of carbonic anhydrase and Na^+-K^+-Cl^- cotransporter, respectively [17].

Next, we tested whether AZ or FS affected the exosomal release of AQP1 into urine. In this examination, in accordance with previous studies, each sample of urinary exosomal protein was loaded in each lane with the same amount of urinary creatinine [10, 19]. As shown in Figure 1, immunoblot analysis demonstrated a significant increase in the level of urinary exosomal AQP1 in the AZ group and a decreasing tendency in the FS group compared with the control group. When we examined the glycosylated and nonglycosylated forms of AQP1 separately (Figure 1), we found that AZ increased both forms to a similar extent.

Since AZ significantly increased the level of urinary exosomal AQP1, we next examined the effect of each diuretic on the renal abundance of AQP1. As shown in Figure 2, immunoblot analyses revealed no significant changes in the level of renal AQP1 expression in either the cortex or the medulla in both groups relative to the control group. We then examined the renal expression of AQP1 using immunohistochemistry. As shown in Figure 3, the apical expression level of AQP1 in proximal tubule cells of the cortex and outer medulla was increased by AZ but not by FS. Therefore, quantification of apical and basolateral AQP1 abundance in the proximal tubules was performed using immunohistochemistry images captured by a digital camera attached to a microscope. Figure 4 summarizes

Table 1. Changes in body weight and urinary parameters after treatment with AZ or FS

	Control ($n = 8$)	AZ ($n = 10$)	FS ($n = 10$)
Change in body weight (g/h)	2.75 ± 0.37	7.80 ± 0.47*	12.90 ± 0.41*
Urine volume (mL/h)	1.20 ± 0.17	4.98 ± 0.25*	11.74 ± 0.30*
Urinary Na^+ excretion (mEq/h)	0.09 ± 0.01	0.76 ± 0.03*	1.39 ± 0.04*
Urinary K^+ excretion (mEq/h)	0.08 ± 0.02	0.34 ± 0.02*	0.38 ± 0.02*
Urinary Cl^- excretion (mEq/h)	0.07 ± 0.01	0.16 ± 0.01*	1.43 ± 0.06*
Urine osmolality (mOsm/kg)	469.75 ± 51.69	509.80 ± 18.39	317.50 ± 3.94*
Urinary pH	7.52 ± 0.21	8.76 ± 0.04*	6.95 ± 0.12

Data are expressed as mean ± SE.
*$P < 0.05$ versus control.

Table 2. Changes in blood parameters after treatment with AZ or FS

	Control ($n = 8$)	AZ ($n = 10$)	FS ($n = 9$–10)
Creatinine concentration (mg/dL)	0.16 ± 0.02	0.16 ± 0.02	0.27 ± 0.03
Urea nitrogen concentration (mg/dL)	15.15 ± 0.61	16.86 ± 1.04	28.47 ± 0.96*
Na^+ concentration (mmol/L)	138.38 ± 1.87	139.60 ± 1.01	137.33 ± 0.88
K^+ concentration (mmol/L)	3.53 ± 0.18	3.78 ± 0.15	3.41 ± 0.05
Cl^- concentration (mmol/L)	104.00 ± 1.20	109.20 ± 0.90*	94.44 ± 1.03*
HCO_3^- concentration (mmol/L)	25.56 ± 1.11	20.77 ± 0.77*	31.24 ± 1.13*
pCO_2 (mmHg)	48.08 ± 1.87	55.07 ± 2.28	55.08 ± 1.78*
pH	7.33 ± 0.01	7.19 ± 0.01*	7.36 ± 0.02

Data are expressed as mean ± SE.
For the FS group, urinary Na^+, K^+ and Cl^- excretion was tested in 9 animals, whereas other parameters were tested in 10 animals, due to the paucity of samples.
*$P < 0.05$ versus control.

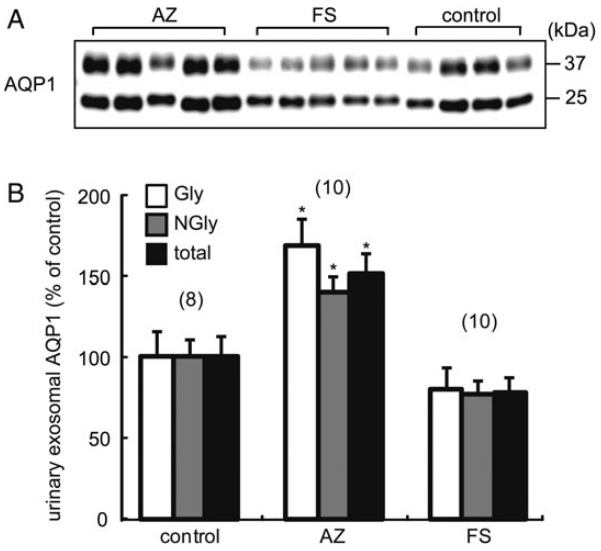

FIGURE 1: Urinary exosomal AQP1 levels after treatment with AZ or FS. (**A**) Typical immunoblot for urinary exosomal AQP1 after treatment with AZ, FS or saline (control). Two-hour urine samples were collected. Each loaded sample contained 10 μg/lane creatinine. Two bands are seen: the upper band is glycosylated and the lower band is nonglycosylated. (**B**) Results of immunoblotting were analyzed quantitatively. The data are expressed as a percentage of the mean value for the control group. Gly, Ngly and total indicate the glycosylated, nonglycosylated and glycosylated plus nonglycosylated bands, respectively. Data are expressed as mean ± SE. Parenthetic numbers indicate the number of animals tested. *$P < 0.05$ compared with the corresponding control group.

the results. AZ significantly increased the ratio of the pixel intensity at the apical membrane relative to that in the perinuclear region. Interestingly, in contrast to the level of apical expression, AZ significantly decreased the ratio of the pixel intensity at the basolateral membrane relative to that in the perinuclear region.

Since treatment with AZ is known to cause metabolic acidosis with alkaluria, due to inhibition of carbonic anhydrase (as also shown in Table 1), we examined the effect of $NaHCO_3$ (an alkalizing agent) or NH_4Cl (an acidifying agent) on exosomal release of AQP1 into urine. In this experiment, the osmolality of the solution administered in each group was evenly adjusted (see Materials and Methods). The urinary and blood parameters are summarized in Tables 3 and 4. In comparison with the control group, $NaHCO_3$ caused significant alkalosis with alkaluria, whereas NH_4Cl induced significant acidosis with aciduria. The urinary osmolalities did not differ significantly among the three groups (Table 3). In the $NaHCO_3$ group, a significant increase in blood HCO_3^- and a decrease in Cl^- concentrations were observed. On the other hand, NH_4Cl significantly decreased the blood HCO_3^- and increased the Cl^- concentrations.

Next, we examined the effect of $NaHCO_3$ or NH_4Cl on the urinary level of exosomal AQP1. As shown in Figure 5, decreases in the levels of urinary exosomal AQP1 in both the $NaHCO_3$ and the NH_4Cl groups were observed in comparison with the control group.

Since many previous studies have indicated that TSG101 and Alix play important roles in the biogenesis of multivesicular bodies, which are organelles that include vesicles of preformed exosomes, the levels of TSG101 and Alix have been considered

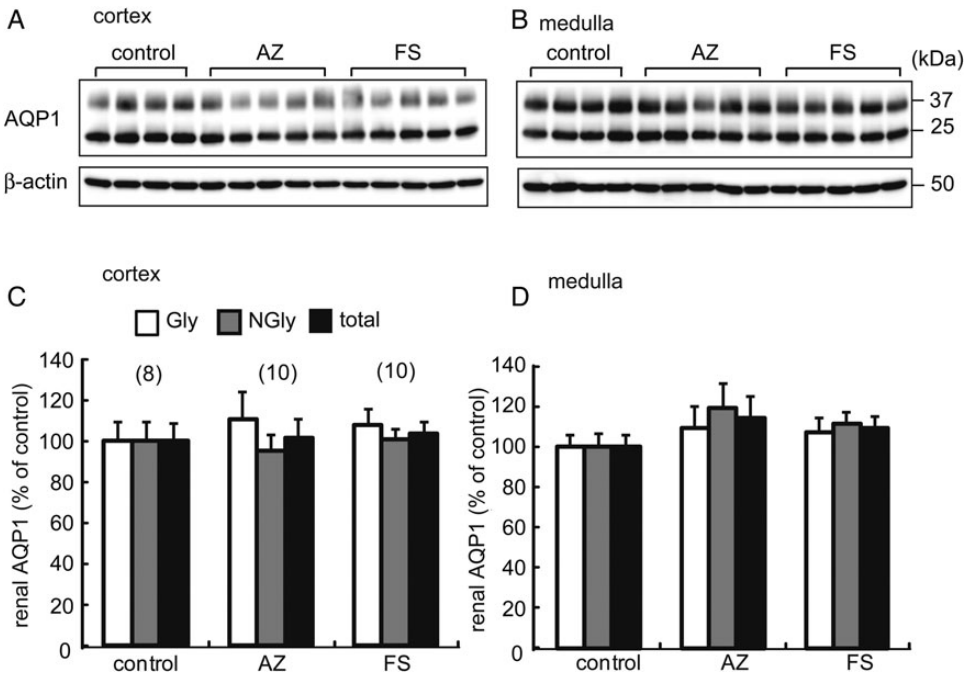

FIGURE 2: Renal expression of AQP1 after treatment with AZ or FS. Typical photographs of immunoblots for renal AQP1 and β-actin in the (**A**) renal cortex and (**B**) medulla. Kidney samples were obtained 2 h after each treatment. Each loaded sample contained 1 μg total protein/lane. Immunoblotting results for the (**C**) renal cortex and (**D**) medulla were analyzed quantitatively. After normalization relative to the corresponding level of β-actin, each value was expressed as a percentage of the mean value for the control group.

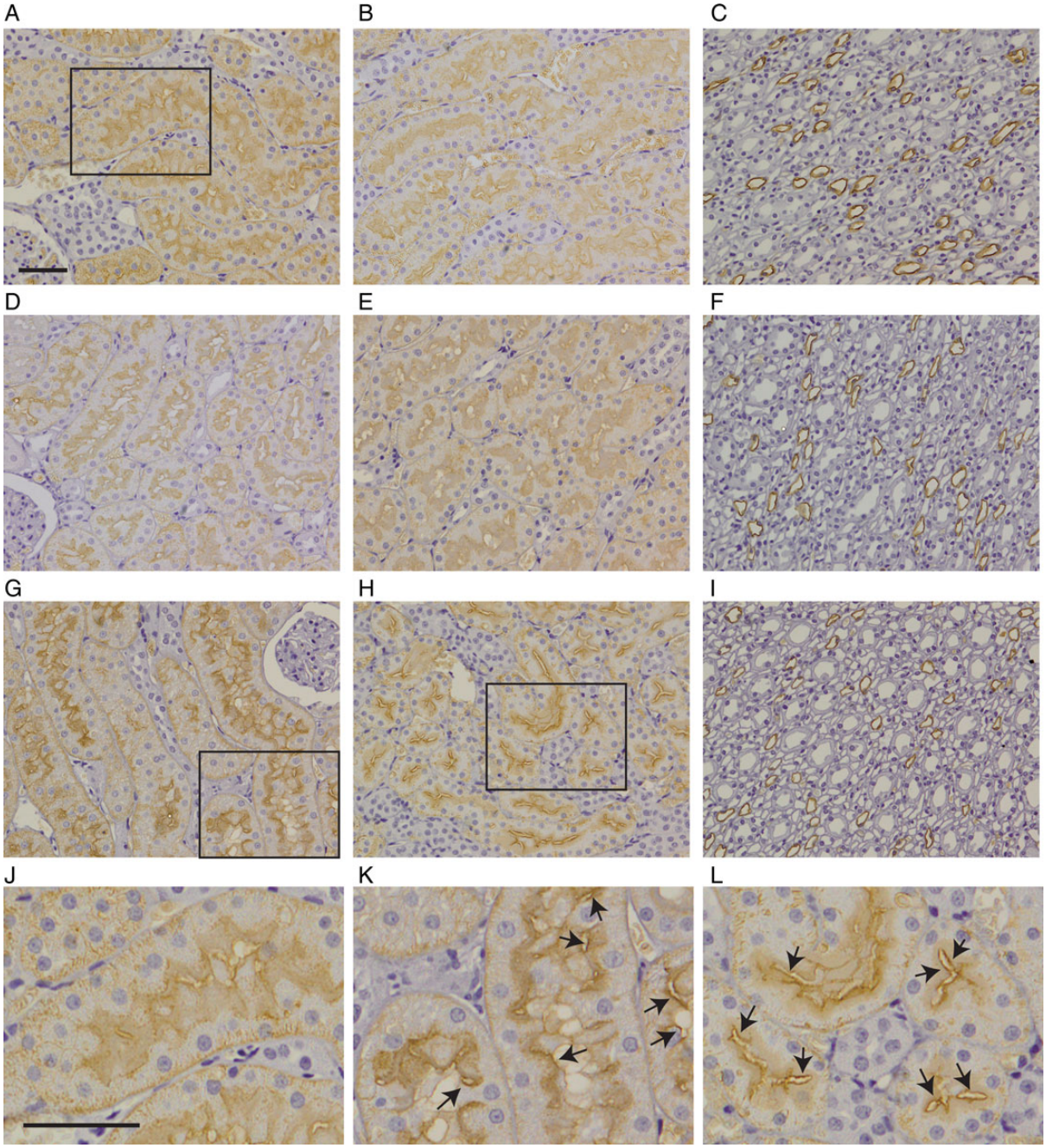

FIGURE 3: Immunohistochemistry of renal AQP1 after treatment with AZ or FS. Immunohistochemistry for renal AQP1 in the control group obtained from the (**A**) cortex, (**B**) outer medulla and (**C**) inner medulla. (**D**–**F**) Immunohistochemistry for renal AQP1 in the FS group. (**G**–**I**) Immunohistochemistry for renal AQP1 in the AZ group. (**J**–**L**) Black boxes in (A), (G) and (H) correspond to the region of high magnification in (J), (K) and (L), respectively. Brown color indicates AQP1. Bars = 50 μm.

to be proportional to the number of exosomes [20, 21]. Therefore, we examined the urinary exosomal levels of TSG101 and Alix after treatment with AZ. Immunoblot analyses showed that the level of TSG 101 or Alix (Figure 6) was not affected by AZ treatment. These data indicated that the total number of exosomes released into the urine was not affected by treatment with AZ.

Finally, we also checked the effect of $NaHCO_3$ or NH_4Cl on exosomal release of TSG 101 and Alix into urine. As shown in Figure 7, treatment with either $NaHCO_3$ or NH_4Cl decreased the exosomal release of both proteins. This suggests that the decrease in the release of AQP1 by $NaHCO_3$ and NH_4Cl (Figure 5) is probably attributable to a lower total number of exosomes released into urine.

DISCUSSION

In this study, we found that urine volume and pH were increased and that the blood pH was decreased within 2 h after

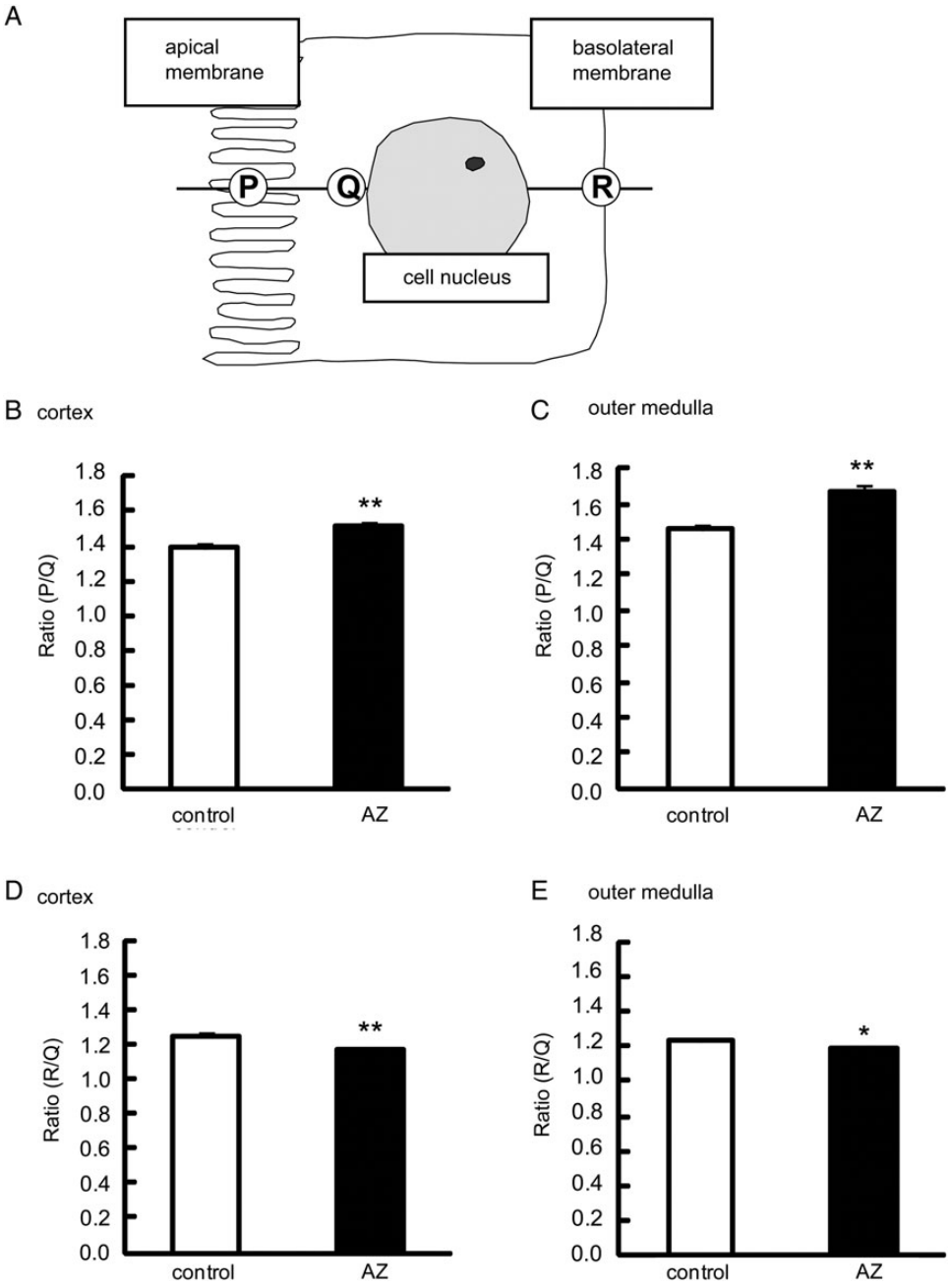

FIGURE 4: Quantification of immunohistochemistry for renal AQP1 after treatment with AZ. (**A**) Illustration showing points for quantification. Line expression profiles at a line including P (apical membrane), Q (perinucleus) and R (basolateral membrane) were generated and then pixel intensities at the respective points were quantified. (**B**–**E**) More than 20 proximal tubules in the cortex (B and D) or outer medulla (C and E) for each animal (total 3 animals) were quantified, and then the ratios of P/Q (B and C) and R/Q (D and E) were calculated. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the control group.

treatment with AZ, a diuretic that inhibits carbonic anhydrases. Under these conditions, AZ dramatically increased the level of urinary exosomal AQP1. Also, AZ enhanced the apical expression level of AQP1 in proximal tubules. On the other hand, AZ had no effect on the levels of urinary exosomal TSG101 and Alix, known to be markers of exosomes [20, 21]. FS, another diuretic that inhibits Na^+-K^+-Cl^- cotransporter, induced marked diuresis but did not increase the level of urinary exosomal AQP1. These data strongly indicate that exosomal release of AQP1 into urine was specifically increased by AZ, accompanied by translocation of AQP1 in the proximal tubules. This increase appeared to be independent of either the diuretic effect or the increase in the number of exosomes released into urine.

AZ clearly caused alkaluria and acidosis. Therefore, these deviations from physiological conditions might be possible factors involved in the increase of urinary exosomal AQP1 by AZ. In order to examine this possibility, we investigated the effect of $NaHCO_3$ or NH_4Cl on the urinary level of exosomal AQP1. Although $NaHCO_3$ increased the blood and urine pH, the level of

Table 3. Changes in body weight and urinary parameters after treatment with $NaHCO_3$ or NH_4Cl

	Control ($n = 8$)	$NaHCO_3$ ($n = 9$)	NH_4Cl ($n = 9$)
Change of body weight (g/h)	3.75 ± 0.31	3.67 ± 0.62	2.78 ± 0.32
Urine volume (mL/h)	3.52 ± 0.27	3.61 ± 0.40	2.53 ± 0.27*
Urinary Na^+ excretion (mEq/h)	0.93 ± 0.08	0.94 ± 0.10	0.55 ± 0.06*
Urinary K^+ excretion (mEq/h)	0.40 ± 0.02	0.37 ± 0.04	0.34 ± 0.03
Urinary Cl^- excretion (mEq/h)	0.90 ± 0.08	0.51 ± 0.05*	0.60 ± 0.07*
Urine osmolality (mOsm/kg)	894.63 ± 37.42	860.67 ± 34.11	985.56 ± 49.90
Urinary pH	6.61 ± 0.17	8.22 ± 0.15*	6.03 ± 0.17*

Data are expressed as mean ± SE.
*P < 0.05 versus control.

Table 4. Changes in blood parameters after treatment with $NaHCO_3$ or NH_4Cl

	Control ($n = 8$)	$NaHCO_3$ ($n = 9$)	NH_4Cl ($n = 9$)
Creatinine concentration (mg/dL)	0.19 ± 0.01	0.22 ± 0.01	0.20 ± 0.02
Urea nitrogen concentration (mg/dL)	14.96 ± 0.57	17.33 ± 0.47*	18.68 ± 0.70*
Na^+ concentration (mmol/L)	142.38 ± 0.80	140.44 ± 0.58	141.00 ± 0.31
K^+ concentration (mmol/L)	3.59 ± 0.08	3.49 ± 0.05	3.78 ± 0.12
Cl^- concentration (mmol/L)	103.00 ± 0.27	97.33 ± 0.52*	106.13 ± 0.54*
HCO_3^- concentration (mmol/L)	26.04 ± 0.31	30.52 ± 0.45*	23.42 ± 0.67*
pCO_2 (mmHg)	53.25 ± 1.07	54.83 ± 0.94	52.96 ± 2.02
pH	7.30 ± 0.01	7.35 ± 0.01*	7.26 ± 0.01*

Data are expressed as mean ± SE.
*P < 0.05 versus control.

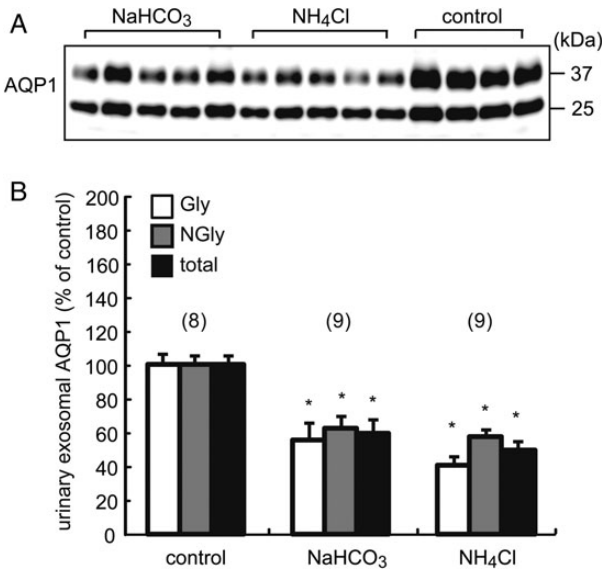

FIGURE 5: Urinary exosomal AQP1 levels after treatment with $NaHCO_3$ or NH_4Cl. (**A**) Typical immunoblot for urinary exosomal AQP1 after treatment with $NaHCO_3$, NH_4Cl or NaCl (3 mmol/rat, control). (**B**) Immunoblotting results were analyzed quantitatively. Each value is expressed as a percentage of the mean value for the control group. *P < 0.05 compared with the corresponding control group.

exosomal AQP1 was reduced. Treatment of rats with NH_4Cl gave rise to metabolic acidosis and aciduria, accompanied by a reduction in the level of urinary exosomal AQP1. Taken together, these results suggest that the effect of AZ on the exosomal release of AQP1 is unlikely to be mediated through a secondary action such as alkaluria and acidosis, but reflects a direct action of AZ on the cells expressing AQP1. Since AZ increased the level of urinary exosomal AQP1 within 2 h after treatment, the rapidity of this effect lends support to the above notion.

So far, a number of reports have indicated that AZ is able to act on AQP1 at the cellular level [18, 22–24]. Although it has been thought that detection of the inhibitory effect of AZ on the water permeability of AQP1 is dependent on its level of expression in the cell, assay methods employed and period of treatment with AZ [24–27], several groups have independently observed that AZ reduces the osmotic water permeability of AQP1 in *Xenopus* oocytes expressing AQP1 [22–24]. Furthermore, it has been reported that the level of AQP1 expression in cultured mammalian cells is altered by treatment with AZ. These observations favor a direct action of AZ at the cellular level [18].

In the present study, AZ increased the exosomal release of AQP1 without any significant decrease in its renal expression level. Previously we observed that treatment of rats with AZ increased the exosomal release of AQP2 by >1000% relative to control animals and that this increase was accompanied by a 50% decrease of renal AQP2 abundance [19]. In the present study, we observed only a 50% increase in the exosomal release of AQP1 by AZ, and therefore the apparent lack of any reduction in the renal AQP1 level by AZ appeared to be due to the modest effect of AZ on the release of exosomal AQP1.

Since AQP1 has been shown to be abundantly expressed in the apical membrane of proximal tubules under basal conditions in adult animals and to be insensitive to vasopressin [1, 2], AQP1 was originally considered to be constitutively expressed in the proximal tubules. In the present study, however, we clearly showed that the level of apical AQP1 expression was increased within 2 h after treatment of rats with AZ. Similarly,

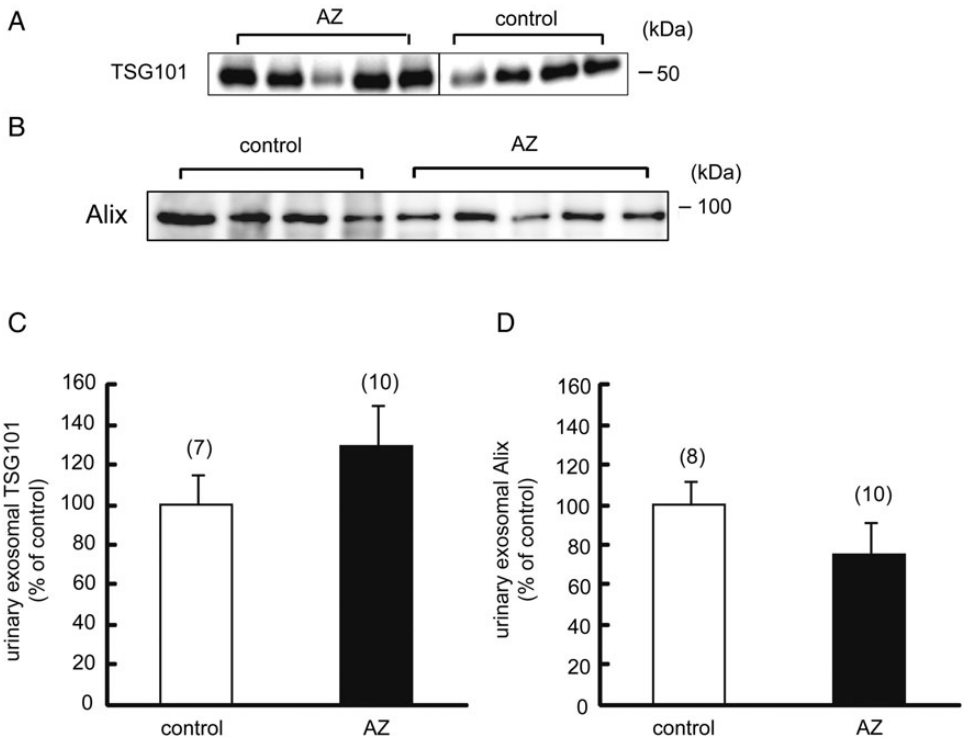

FIGURE 6: Urinary exosomal TSG101 and Alix levels after treatment with AZ. (**A**) Typical immunoblot for urinary exosomal TSG101 after treatment with AZ or saline (control). The original blot for the image shown here had 14 lanes, including the left 5 lanes for the AZ group, the middle 5 lanes for a group that was not directly related to this result and the right 4 lanes for the control group. Therefore, the part for the middle 5 lanes was removed and then the image was built while maintaining the original quality. The dividing line indicates the splice junction. (**B**) Typical immunoblot for urinary exosomal Alix after treatment with AZ or saline (control). (**C**) Immunoblotting results for (A) were analyzed quantitatively. Each value is represented as a percentage of the mean value for the control group. (**D**) Immunoblotting results for (B) were analyzed quantitatively. Each value is represented as a percentage of the mean value for the control group.

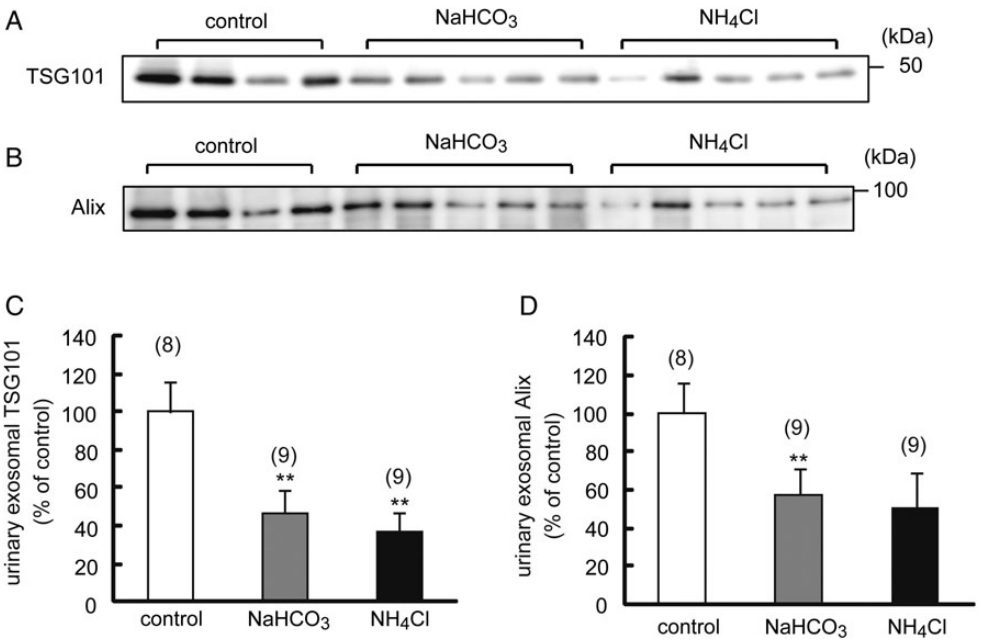

FIGURE 7: Urinary exosomal TSG101 and Alix levels after treatment with $NaHCO_3$ or NH_4Cl. (**A**) Typical immunoblot for urinary exosomal TSG101 after treatment with $NaHCO_3$, NH_4Cl or NaCl (3 mmol/rat, control). (**B**) Typical immunoblot for urinary exosomal Alix after treatment with $NaHCO_3$, NH_4Cl or NaCl (3 mmol/rat, control). (**C**) Immunoblotting results for (A) were analyzed quantitatively. Each value is represented as a percentage of the mean value for the control group. (**D**) Immunoblotting results for (B) were analyzed quantitatively. Each value is represented as a percentage of the mean value for the control group. **$P < 0.01$ and compared with the control group.

Zhang *et al.* [18] recently observed that AZ increased the plasma membrane expression of AQP1 in HK-2 cells, which are immortalized proximal tubule epithelial cells, within 6 h after administration. They also investigated the mechanisms responsible in detail and found that the increased plasma membrane expression was associated with binding of AQP1 to the heavy chain of myosin, a member of the motor protein family. These data suggest that AQP1 can be rapidly translocated to the plasma membrane in response to AZ and that this translocation is possibly mediated by motor proteins. Further work to examine the involvement of motor proteins in the trafficking of AQP1 to the apical membrane upon AZ stimulation under *in vivo* conditions will be needed.

In contrast to apical membrane expression, basolateral expression of AQP1 was decreased after AZ treatment. To our knowledge, this is the first evidence for an association between the apical and basolateral membrane expression of AQP1. Yui *et al.* [28] proposed a model for the transcytotic pathway of AQP2 trafficking involving initial basolateral insertion of AQP2, followed by retrieval and transcytosis into an apical delivery pathway. They have also hypothesized that several molecules such as Rab5, EEA1 and clathrin are involved in this pathway. A further study will be needed to clarify whether or not this model is applicable to AZ-induced AQP1 trafficking.

We observed that increased exosomal release of AQP1 was not dependent on the number of exosomes released into urine. Although currently the reason for this is unclear, the following hypothesis can be considered. When endosomes containing nano-sized intraluminal vesicles, known as multivesicular bodies, are trafficked to and fused with the plasma membrane, intraluminal vesicles are released into the extracellular space as exosomes. The biogenesis of multivesicular bodies is known to be mediated by the endosomal sorting complex required for transport (ESCRT) machinery [29–31]. The biogenesis is initiated by capturing of ubiquitinated cargo proteins by a component of the ESCRT machinery. Blanc *et al.* [32] observed that the abundance of AQP1 in mouse reticulocytes decreases during their *in vitro* maturation and that this reduction is associated with the release of exosomes. Furthermore, they observed that the release of AQP1 is suppressed by a proteasome inhibitor, suggesting that ubiquitination is involved in the exosomal release of AQP1. Ubiquitination of AQP1 has also been reported to occur within 6 h after treatment of HK cells with AZ [18], suggesting that the possible mechanisms for the increased exosomal release of AQP1 in response to AZ under *in vivo* conditions could include (i) ubiquitination of AQP1 in response to AZ, (ii) selective incorporation of AQP1 into intraluminal vesicles, (iii) formation of AQP1-rich intraluminal vesicles in multivesicular bodies and (iv) release of the intraluminal vesicles into urine, leading to increased exosomal release of AQP1 without any alteration in the number of exosomes released into urine. This hypothesis will need to be tested in future studies.

We have reported that renal ischemia/reperfusion specifically reduces exosomal release of AQP1 into the urine of rats and human patients, suggesting the usefulness of urinary exosomal AQP1 for detection of ischemia/reperfusion injury [8]. Besides renal ischemia/reperfusion injury, although AQP1 has been detected in whole urine and not exclusively in the exosomal fraction, urinary AQP1 has been reported to be a sensitive and specific biomarker of kidney cancers, such as clear cell carcinoma, derived from proximal tubule cells [33–35]. Since urinary AQP1 is reportedly associated with urinary small vesicles [36], it is likely that urinary exosomal AQP1 is increased in patients with clear cell carcinoma. Together, these reported data suggest that urinary exosomal AQP1 could be developed as a diagnostic tool for certain types of renal disease. We anticipate that our present findings will contribute to future work aimed at elucidating the mechanisms underlying the release of urinary exosomal AQP1 *in vivo*.

AUTHORS' CONTRIBUTIONS

H.K. and M.I. designed the research; A.A., H.S., S.O., Y.H., H.K. and M.I. performed the research; A.A., H.S., S.O. and M.I. analyzed the data; A.A., H.S. and M.I. interpreted the results of experiments; A.A. and M.I. wrote the manuscript.

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CONFLICT OF INTEREST STATEMENT

None declared.

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