

URINARY EXCRETION OF AQUAPORIN-2 IN PATIENTS WITH DIABETES INSIPIDUS

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Abstract Background. Urine-concentrating ability is regulated by vasopressin. Recently, the specific water-channel protein of the renal collecting duct, known as aquaporin-2, was cloned. However, it is not certain whether this molecule is responsive to vasopressin.

Methods. We measured the urinary excretion of aquaporin-2 and its response to vasopressin in 11 normal subjects and 9 patients with central or nephrogenic diabetes insipidus. The urine samples were collected during periods of dehydration and hydration and after the administration of vasopressin. Urine samples were analyzed for aquaporin-2 by the Western blot assay and immunogold labeling, and the amount of aquaporin-2 was determined by radioimmunoassay.

Results. Aquaporin-2 was detectable in the urine in both soluble and membrane-bound forms. In the five normal subjects tested, the mean (\pm SE) urinary excretion of

aquaporin-2 was 11.2 ± 2.2 pmol per milligram of creatinine after a period of dehydration, and it decreased to 3.9 ± 1.9 pmol per milligram of creatinine ($P = 0.03$) during the second hour after a period of hydration. In the six other normal subjects, an infusion of desmopressin (1-desamino-8-D-arginine vasopressin) increased the urinary excretion of aquaporin-2 from 0.8 ± 0.3 to 11.2 ± 1.6 pmol per milligram of creatinine ($P < 0.001$). The five patients with central diabetes insipidus also had increases in urinary excretion of aquaporin-2 in response to the administration of vasopressin, but the four patients with X-linked or non-X-linked nephrogenic diabetes insipidus did not.

Conclusions. Aquaporin-2 is detectable in the urine, and changes in the urinary excretion of this protein can be used as an index of the action of vasopressin on the kidney. (N Engl J Med 1995;332:1540-5.)

THE permeability of the collecting ducts of the kidney to water increases rapidly in response to vasopressin. In what is called the shuttle hypothesis, this increase is believed to be mediated by the movement of a vasopressin-regulated water-channel protein to the apical membranes of the cells of the collecting ducts.¹⁻⁴ The aquaporins are a family of membrane proteins that function as water-selective channels in many water-transporting tissues.⁵ The first aquaporin to be identified, aquaporin-1 (initially termed an aquaporin of channel-forming integral protein), is present in red cells, proximal and descending tubules of the kidney, and other tissues.^{6,7}

We recently cloned a water-channel protein specific to the collecting duct of the kidney, aquaporin-2, first termed aquaporin-CD, from rats and humans. It is a 271-amino-acid protein with a molecular weight of 29,000. This aquaporin is localized to the apical region of collecting-duct cells, which suggests that it is the vasopressin-regulated water channel.^{8,9} To determine the responsiveness of this protein to vasopressin, we measured the urinary excretion of aquaporin-2 and its response to vasopressin in normal subjects and patients with central and nephrogenic diabetes insipidus.

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METHODS

Normal Subjects and Patients

We studied four groups of subjects. The protocol was approved by the appropriate institutional review boards, and all subjects gave informed consent.

The first group consisted of five normal men ranging in age from 31 to 46 years. At 9 a.m., after an overnight period of dehydration during which they were not allowed to drink water, the subjects were asked to urinate and then to drink 1 liter of water in a 20-minute period. Starting at 10 a.m., urine samples were collected every 60 minutes for 3 hours. The samples obtained in this and the three subsequent studies were analyzed immediately or stored at -80°C until their use.

The second group consisted of five patients (three women and two men, ranging in age from 40 to 72 years) who had idiopathic central diabetes insipidus and regularly took desmopressin (1-desamino-8-D-arginine vasopressin, or DDAVP) intranasally twice daily. The patients discontinued desmopressin therapy 24 hours before the study. After an overnight fast (during which free intake of water was allowed), the patients were asked to urinate, after which they were given 10 U of arginine vasopressin (Sankyo Pharmaceutical, Tokyo, Japan) subcutaneously. Urine samples were collected at one-hour intervals for three hours after the injection.

The third study group consisted of four male patients with nephrogenic diabetes insipidus — three with the X-linked form and one with the non-X-linked form. All three patients with X-linked nephrogenic diabetes insipidus (who ranged in age from 37 to 50 years) had mutations in the gene for the vasopressin V_2 receptor.¹⁰ The patient with non-X-linked nephrogenic diabetes insipidus was hospitalized at the age of five months with fever and dehydration, at which time his plasma sodium concentration was 161 mmol per liter. He had normal fibrinolytic and vasodilatory responses to desmopressin, but his urinary osmolality did not increase, indicating a functional abnormality distal to the vasopressin V_2 receptor.¹¹ We have sequenced the coding regions of both the aquaporin-2 gene and the vasopressin V_2 receptor gene in this patient and have found no mutations (unpublished data). The three patients with X-linked nephrogenic diabetes insipidus were not taking any medications. The patient with non-X-linked nephrogenic diabetes insipidus discontinued his regular medication (hydrochlorothiazide) 36 hours before the desmopressin-infusion test.

The fourth group consisted of six normal subjects (two women and four men, ranging in age from 23 to 35 years). All the patients in the third and fourth groups received intravenous infusions of desmopressin (0.3 μg per kilogram of body weight, up to a maximal dose of 24 μg ; Ferring Pharmaceuticals, Malmö, Sweden) in 100 ml of saline over a 20-minute period.¹² Urine samples were collected at 30-minute intervals before the start of infusion and for 3 hours afterward.

Western Blot Analysis

A polyclonal antibody against a synthetic portion of the C-terminal of human aquaporin-2 raised in rabbits⁹ was used in the Western blot analysis. This portion of the aquaporin-2 molecule has no sequence homology with other aquaporins. Samples of urine or suspensions of normal human renal medullary membranes⁹ were diluted in a half volume of a loading buffer containing 9 percent sodium dodecyl sulfate (SDS), 195 mM TRIS-hydrochloric acid, 30 percent glycerol, and 15 percent 2-mercaptoethanol and heated at 70°C for 10 minutes. The samples were subjected to SDS-polyacrylamide-gel electrophoresis (SDS-PAGE) with 10 to 20 percent gradient gels and were then transferred to polyvinyl membranes (Immobilon, Millipore, Bedford, Mass.). The membranes were incubated with antibody (dilution, 1:500), and the reaction products were visualized with protein A labeled with iodine-125 (ICN Biochemicals, Irvine, Calif.). Some urine samples were concentrated by ultrafiltration (cut-off, a molecular weight of 3000; Microcon-3, Amicon, Beverly, Mass.) before SDS-PAGE. The renal medullary tissue used was tumor-free tissue from kidneys removed during surgery to treat renal cell carcinoma.

Radioimmunoassay

Urinary aquaporin-2-like immunoreactivity was measured by a specific radioimmunoassay that used the same antibody that was employed in the Western blot analysis. A synthetic peptide (Tyr⁹-aquaporin-2[V257-A271]) corresponding to the 15-amino-acid sequence of the C-terminal of aquaporin-2 was radioiodinated with iodine-125 (New England Nuclear, Boston) by the lactoperoxidase method, as described elsewhere.¹³ For the assay, 0.1 ml of the urine sample or of a standard, 0.1 ml of assay buffer (0.05 M sodium phosphate [pH 7.4], 0.08 M sodium chloride, 0.01 M EDTA, 0.1 percent bovine serum albumin, 0.1 percent Triton X-100, and 0.01 percent sodium azide), and 0.1 ml of the antibody (final dilution, 1:12,000) were incubated at 4°C for 24 hours, followed by the addition of 0.1 ml of the radiolabeled synthetic peptide (approximately 10,000 cpm) and further incubation at 4°C for 48 hours. Bound and free quantities of radiolabeled ligand were separated by the double-antibody method. The serial-dilution curve of the urine samples was parallel to that of the standard (data not shown). Each sample was analyzed in duplicate, and the intra- and interassay coefficients of variation were less than 10 percent. The minimal detectable quantity of aquaporin-2 was 25 pg per tube, and an amount equivalent to 600 pg per tube caused 50 percent inhibition of binding of the radiolabeled ligand. Urinary creatinine concentrations were measured with an AutoAnalyzer.

Immunolabeling of Urinary Aquaporin-2

Fresh samples of urine were first centrifuged at low speed (2000 rpm) for 15 minutes to remove cellular elements, then the supernatants were centrifuged at high speed (120,000 $\times g$) for 30 minutes. The resulting sediments were suspended in 7.5 mM phosphate buffer (pH 7.4) containing 20 μg of phenylmethylsulfonyl fluoride per milliliter of solution, 1 μg of pepstatin A per milliliter, and 1 μg of leupeptin per milliliter. An equal volume of 0.5 M sucrose was added to each suspension, and the resulting mixture was then mixed with an equal volume of a twofold concentration of periodate-lysine-paraformaldehyde fixative at 4°C for 40 minutes. After three washings, the sediment was suspended in an equal volume of a solution containing 20 percent polyvinylpyrrolidone and 1.84 M sucrose in phosphate-buffered saline. The sediment was frozen in an immersion cryofixation system (KF-80, Reichert-Jung, Vienna, Austria), and ul-

trathin cryosections were cut at -100°C and collected on copper grids. The grids were placed on droplets of the antibody solution (dilution, 1:200) at room temperature for one hour. After being rinsed, the grids were incubated for 30 minutes with a 1:100 dilution of a F(ab')₂ fragment of a goat antirabbit IgG antiserum to which 10-nm particles of gold had been conjugated. The grids were rinsed with phosphate-buffered saline, fixed with 2 percent glutaraldehyde, and stained with 2 percent uranyl acetate after adsorption staining with a mixture of polyvinyl alcohol and uranyl acetate. The sections were examined at 100 kV in an electron microscope (H 7100, Hitachi, Ibaraki, Japan).

Statistical Analysis

The results are expressed as means \pm SE. Statistical data were calculated by analysis of variance. All P values are two-sided.

RESULTS

Western Blot Analysis of Urine

Immunoblots of urine samples obtained after a 12-hour overnight dehydration showed aquaporin-2 with molecular sizes of 29 kd and 40 to 50 kd (Fig. 1, lane 2), as did the samples of membranes from renal medullary tissue (Fig. 1, lane 1). The broad band at 40 to 50 kd represents a glycosylated form of the 29-kd pro-

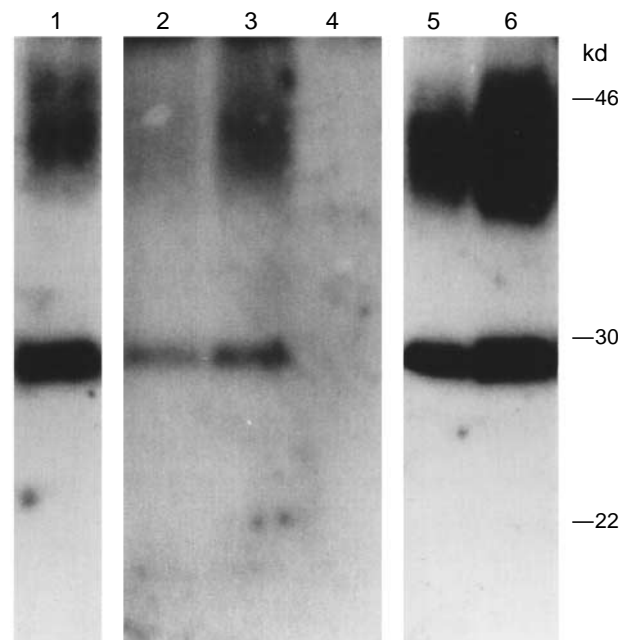


Figure 1. Western Blot Analysis of Urine Samples from Normal Subjects and Normal Renal Medullary Tissue.

Lane 1 shows a suspension of renal medullary tissue (10 μg); lane 2, a urine sample obtained after the subject underwent a 12-hour period of dehydration (10 μl); lane 3, a urine sample concentrated threefold (10 μl); lane 4, cellular sediment obtained after low-speed centrifugation of a urine sample (10 μg); and lanes 5 and 6, membrane fractions of urine after ultracentrifugation (lane 5, 0.2 μg ; lane 6, 0.5 μg). The membranes were incubated with anti-aquaporin-2 antibody and visualized with ¹²⁵I-labeled protein A. Markers of molecular weight were subjected to electrophoresis simultaneously and detected by staining with Coomassie blue (not shown).

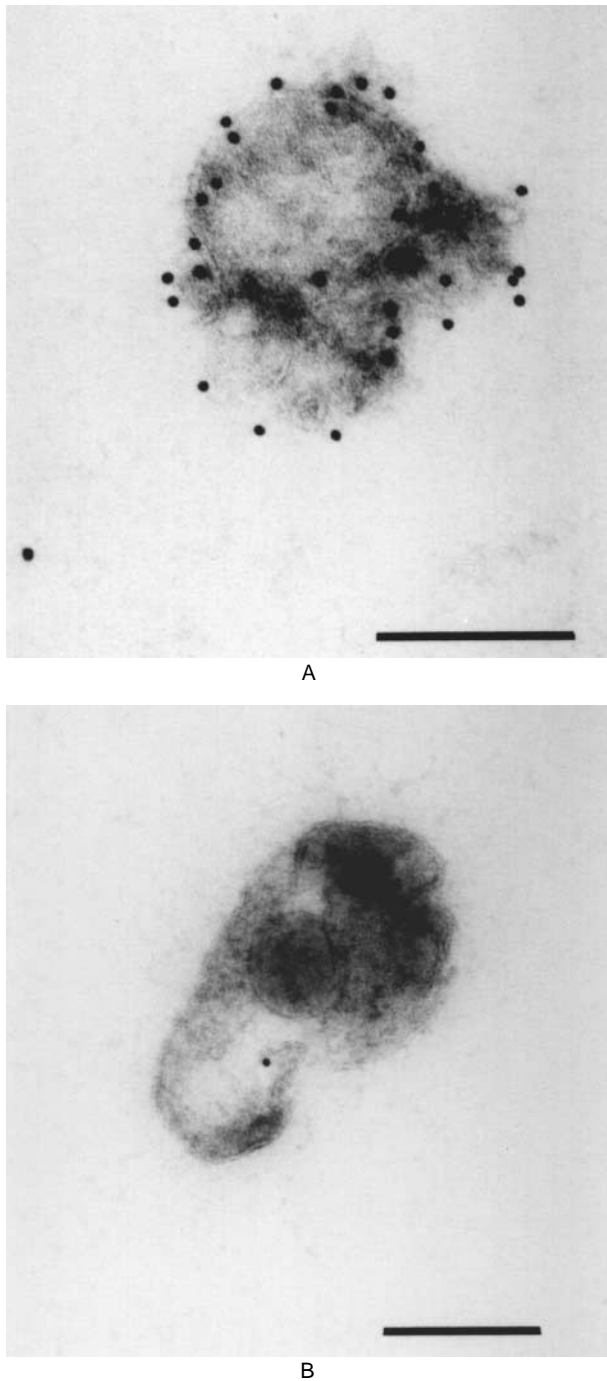


Figure 2. Immunogold Labeling of Urinary Sediment Obtained by Ultracentrifugation.

The urine sample was obtained from a normal subject after a 12-hour period of dehydration. The sediment was immunostained with antibody against aquaporin-2 (Panel A) and nonimmune serum (Panel B). After being rinsed, the sediment samples were incubated with a 1:100 dilution of a $F(ab')_2$ fragment of goat anti-rabbit IgG antiserum to which 10-nm particles of gold had been conjugated. After staining with 2 percent uranyl acetate, the sections were examined at 100 kV in an electron microscope. The particles of gold appeared as black dots. The bars represent 200 nm.

tein.⁹ The amount of aquaporin-2 detected increased when the urine was concentrated threefold by ultrafiltration (Fig. 1, lane 3). These results suggest that intact aquaporin-2 is excreted in the urine. Aquaporin-2 was not detected in the cellular urinary sediment obtained by low-speed centrifugation (Fig. 1, lane 4), ruling out the possibility that collecting-duct cells in the urine are the source of urinary aquaporin-2. The sediments obtained by high-speed ultracentrifugation of urine (at $120,000\times g$) contained abundant amounts of aquaporin-2 protein (Fig. 1, lanes 5 and 6), indicating that some of the aquaporin-2 in urine is bound to membrane structures. Quantification by radioimmunoassay indicated that 42.3 ± 3.8 percent of the aquaporin-2 in the urine of the nine normal subjects studied was deposited as sediment by this ultracentrifugation procedure. Recently, Preston et al. reported finding aquaporin-1 in cellular urinary sediment obtained by low-speed centrifugation.¹⁴ The lack of staining in the sediment obtained at low speed in our Western blot analysis (Fig. 1, lane 4) indicated that our antibody did not cross-react with aquaporin-1.

Immunoelectron-Microscopical Analysis of the Sediment of Urine Centrifuged at High Speed

Immunoelectron-microscopical analysis of the urinary sediment obtained by ultracentrifugation revealed membrane structures forming vesicle-like shapes. Immunogold labeling with specific antibody against aquaporin-2 indicated the localization of aquaporin-2 along these membranes (Fig. 2A); staining with nonimmune serum was negative (Fig. 2B).

Urinary Excretion of Aquaporin-2 in Normal Subjects and Patients with Diabetes Insipidus

Effect of Dehydration and Water Loading in Normal Subjects

After overnight dehydration, the mean (\pm SE) urinary excretion of aquaporin-2 by five normal subjects was 11.2 ± 2.2 pmol per milligram of creatinine. The mean values decreased to 3.9 ± 1.9 and 3.5 ± 1.5 pmol per milligram of creatinine two and three hours, respectively, after water loading ($P=0.03$ and $P=0.02$) (Fig. 3). The mean urinary osmolality decreased from 933 ± 55 mOsm per kilogram of water to 436 ± 272 and 627 ± 246 mOsm per kilogram of water after two and three hours, respectively.

Effect of Arginine Vasopressin in Normal Subjects and Patients with Diabetes Insipidus

The mean basal excretion of aquaporin-2 by the five patients with central diabetes insipidus was 0.4 ± 0.05 pmol per milligram of creatinine (Fig. 4). A subcutaneous injection of arginine vasopressin increased urinary excretion of aquaporin-2 by four to six times ($P<0.001$) during the first hour after the injection. The values decreased thereafter in most patients. The mean urinary osmolality increased from 142 ± 16 to 334 ± 65 mOsm per kilogram of water after one hour,

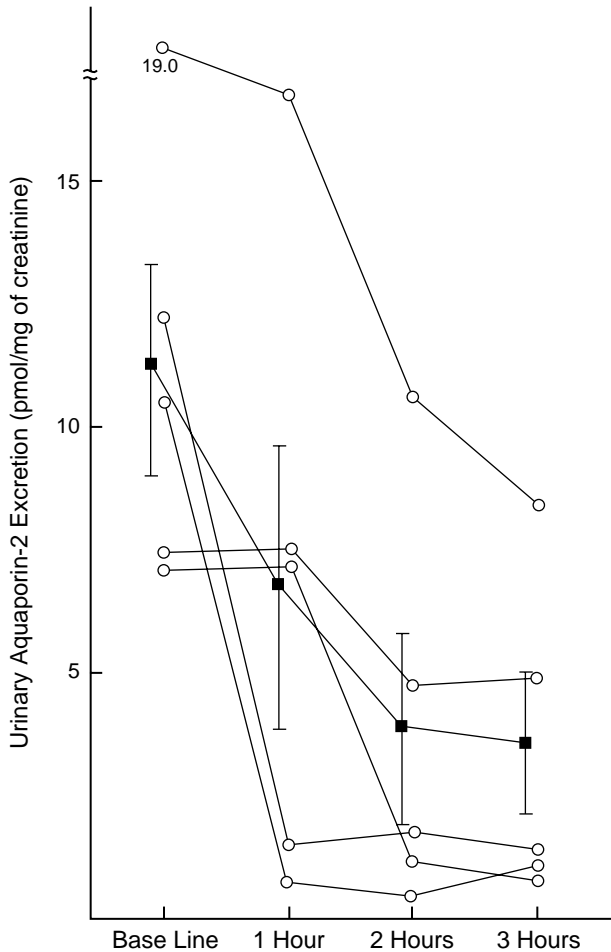


Figure 3. Effect of Dehydration and Water Loading on Urinary Excretion of Aquaporin-2 in Normal Subjects.

Urine samples were collected after the subjects had undergone dehydration overnight (base line) and were then collected hourly for 3 hours after the subjects had drunk 1 liter of water in a 20-minute period. The open circles represent the values for each subject, and the solid squares and bars the means (\pm SE). $P=0.03$ and $P=0.02$ for the comparisons of the mean base-line value with the mean values after two and three hours, respectively.

to 520 ± 83 mOsm after two hours, and to 548 ± 57 mOsm after three hours.

The six normal subjects who received 20-minute infusions of desmopressin had a rapid 14-fold increase in mean urinary excretion of aquaporin-2, from 0.8 ± 0.3 to 11.2 ± 1.6 pmol per milligram of creatinine ($P < 0.001$). Although the values decreased subsequently, they remained elevated for three hours after the start of the infusion (Fig. 5A). The mean urinary osmolality increased from 592 ± 113 mOsm per kilogram of water to 762 ± 87 , 803 ± 94 , and 875 ± 121 mOsm per kilogram of water in these subjects after one, two, and three hours, respectively. In contrast, neither urinary excretion of aquaporin-2 nor osmolality (approximately 130 mOsm per kilogram of water) increased during or after

the infusion of desmopressin in any of the four patients with nephrogenic diabetes insipidus (Fig. 5B).

DISCUSSION

According to the shuttle hypothesis,¹⁻⁴ the vasopressin-regulated water channel is localized in the apical plasma membrane and the membranes of the cytoplasmic vesicles of collecting-duct cells in the kidney. Cytoplasmic vesicles carrying the water-channel proteins fuse to the apical membrane in response to vasopressin, thereby increasing the permeability of the membrane to water. Immunohistochemical studies using specific antibodies against aquaporin-2 showed that the localization of aquaporin-2 in tissue is consistent with that predicted by the shuttle hypothesis.^{5,6,15,16} Endocytosis of apical membranes containing water-channel proteins is known to be stimulated by vasopressin in collecting-duct tissue and toad bladders. This retrieval of membranes is accelerated by the influx of water into

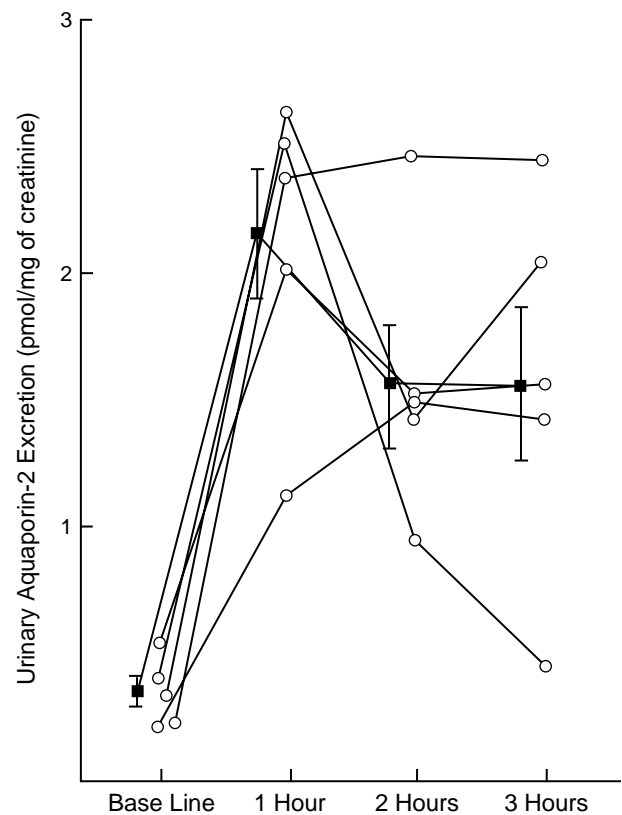


Figure 4. Effect of an Injection of Arginine Vasopressin on Urinary Excretion of Aquaporin-2 in Patients with Central Diabetes Insipidus.

Urine samples were collected after the patients had undergone fasting overnight (base line) and were then collected hourly for three hours after a subcutaneous injection of 10 U of arginine vasopressin. The open circles represent the values for each patient, and the solid squares and bars the means (\pm SE). $P < 0.001$, $P = 0.002$, and $P = 0.003$ for the comparisons of the mean base-line value with the mean values after one, two, and three hours, respectively.

the cell, and it may be involved in readjustment of the permeability of vasopressin-responsive tissues to water.¹⁷⁻¹⁹ Thus, vasopressin appears to stimulate the turnover of membranes in two directions; one is the fusion of vesicles carrying water-channel proteins to the apical membrane, and the other is the endocytotic retrieval of apical membrane containing the water-channel proteins. In this study we demonstrated that aquaporin-2 is present in the urine of humans and that it is present on the membranes of vesicle-like structures in urinary sediments obtained by high-speed centrifugation. The latter findings indicate that some particulate aquaporin-2 is shed into the luminal fluid. The possibility of the disposal of water-channel proteins has not been considered in the framework of the shuttle hypothesis.

The urinary excretion of aquaporin-2 increased during antidiuresis in normal subjects, whether it was induced by a period of dehydration or by the infusion of vasopressin. The best explanations for this increase are that vasopressin increases the movement of aquaporin-2-carrying vesicles to the apical membrane and that a fraction of the apical membrane reaches the urine by exocytosis. Alternatively, the endocytotic vesicles that carry the water-channel proteins may be released di-

rectly into the urine. Whatever the mechanism or mechanisms, the increased urinary excretion of aquaporin-2 induced by vasopressin probably results from the increased movement of aquaporin-2 to the apical membrane.

The patients with central diabetes insipidus, like the normal subjects, had increased urinary excretion of aquaporin-2 in response to vasopressin, suggesting that the urine-concentrating mechanisms in collecting-duct cells in the kidney were intact, but this increase was less than that in normal subjects. This finding may have occurred because the patients lacked endogenous vasopressin, which is important for the expression of aquaporin-2 messenger RNA and protein. The 5' flanking region of the aquaporin-2 gene contains an element that is responsive to cyclic AMP²⁰ (vasopressin increases the intracellular concentration of cyclic AMP in collecting-duct cells), and long-term administration of vasopressin increases the content of aquaporin-2 protein in the kidney medulla.^{21,22}

Congenital nephrogenic diabetes insipidus is a rare, inherited disorder characterized by renal unresponsiveness to vasopressin. In most families it is transmitted as an X-linked recessive disorder caused by mutations in the vasopressin V₂-receptor gene, which is located

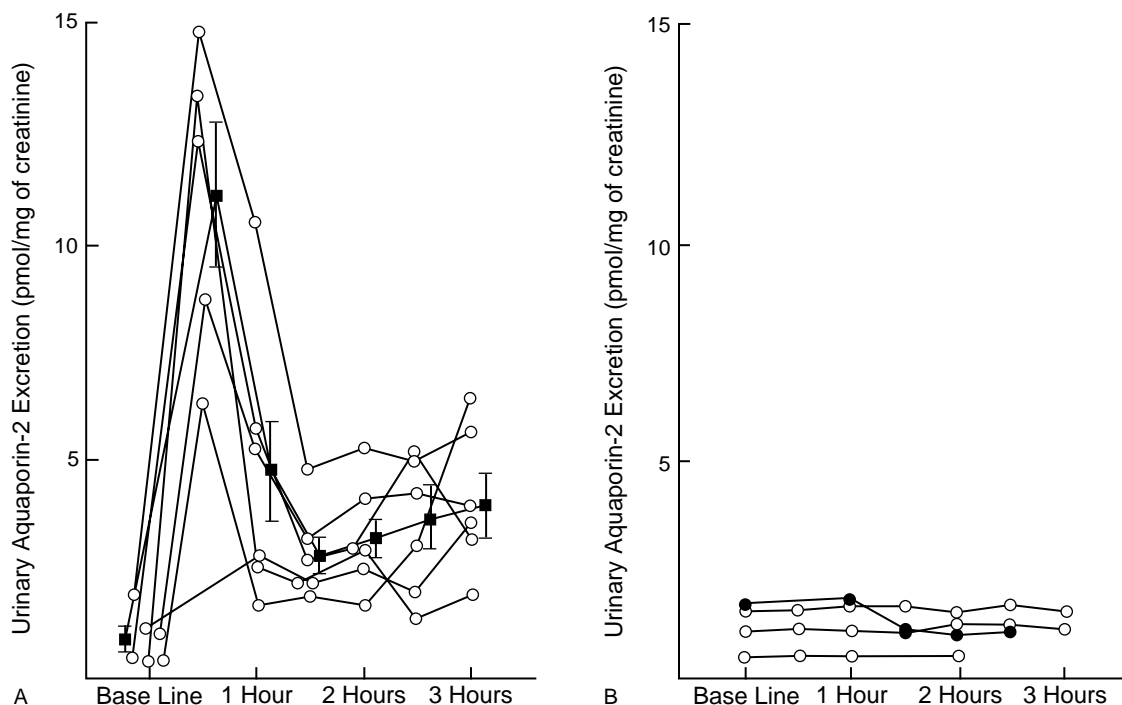


Figure 5. Effect of an Infusion of Desmopressin on Urinary Excretion of Aquaporin-2 in Normal Subjects (Panel A) and Patients with Nephrogenic Diabetes Insipidus (Panel B).

All the subjects received intravenous infusions of desmopressin (0.3 µg per kilogram) for 20 minutes. The urine samples were collected at the start of the infusion (base line) and at 30-minute intervals for 3 hours. In Panel A, the open circles represent the values for each subject, and the solid squares and bars the means (±SE). ($P < 0.001$, $P = 0.003$, $P = 0.03$, and $P = 0.01$ for the comparisons of the mean base-line value with the mean values after 1/2 hour, 1 hour, 2 1/2 hours, and 3 hours, respectively.) In Panel B, the open circles represent the values in the three patients with X-linked nephrogenic diabetes insipidus, and the solid circles represent the values in a patient with non-X-linked nephrogenic diabetes insipidus.

on the X chromosome.^{10,23-25} In patients with non-X-linked nephrogenic diabetes insipidus, who have normal V₂ receptors,^{11,24,26} the disorder may be caused by abnormalities in the aquaporin-2 protein.^{27,28} The patients we studied with either form of the disorder had no increases in urinary excretion of aquaporin-2 in response to vasopressin. The absence of such an increase in patients with nephrogenic diabetes insipidus may help differentiate this disorder from central diabetes insipidus.

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