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Urinary Content of Aquaporin 1 and 2 in Nephrogenic Diabetes Insipidus

Peter M.T. Deen, Rémon A.M.H. van Aubel, Angenita F. van Lieburg, and Carel H. van Os

ABSTRACT

Hereditary nephrogenic diabetes insipidus (NDI) is caused by mutations in either the X-chromosomal gene encoding the vasopressin V2-receptor or in the autosomal gene encoding aquaporin-2. Expressed in Xenopus oocytes, the AQP2 gene mutations found in NDI have been shown to reduce the stability of the encoded protein. This study investigated the in vivo stability of mutant and wild-type aquaporin-2 proteins by measuring their excretion in urine of NDI patients and healthy individuals. On immunoblots, the urine samples from healthy volunteers revealed clear aquaporin-1 and aquaporin-2 signals in antidiuretic but not diuretic states. In the urine of a female patient, whose NDI is explained by low expression of the wild-type V2-receptor gene, aquaporin-2 excretion was high and comparable with that in a healthy individual during antidiuresis. In the urine of a male patient with a non-sense mutation in the V2-receptor gene, a weak aquaporin-2 signal was detected. In NDI patients with mutations in the aquaporin-2 gene, aquaporin-2 could not be detected in urine, suggesting a low stability of mutant aquaporin-2 proteins. In four out of seven NDI patients, aquaporin-1 excretion was relatively high, which suggests a compensatory increase in proximal reabsorption in NDI.

Key Words: (Anti)diuresis, collecting duct, water channel, vasopressin V2-receptor, urine concentration

In humans, the kidney produces 180 L of glomerular filtrate daily, of which normally more than 99% is reabsorbed. In this process, molecular water channels, aquaporins, have been shown to play an important role (1). In the proximal tubules and descending limbs of Henle, the constitutive reabsorption of 165 L is presumably mediated by aquaporin-1 (AQP1) (1). Depending on the need of the body, the remaining 15 L can either be reabsorbed in the collecting duct in response to the antidiuretic hormone, arginine vasopressin (AVP), or be expelled. Upon the binding of AVP to its V2-receptor (V2R) in the basolateral membrane of collecting duct cells, a chain of signaling events is initiated, resulting in the insertion of vesicles containing aquaporin-2 (AQP2) into the apical membrane and increased reabsorption of water (2).

In patients suffering from nephrogenic diabetes insipidus (NDI), the kidney fails to concentrate urine in response to AVP. In the majority of these patients, the disease is caused by a mutation in the X-chromosomal V2R gene (3-5). Mutations in the autosomal AQP2 gene, which has been assigned to chromosome 12 (6,7), have been shown to be responsible for NDI in families that show an autosomal mode of inheritance. Until now, a one-nucleotide deletion and three missense mutations in the AQP2 gene of NDI patients have been described (8,9). From expression studies in oocytes, it appeared that these missense AQP2 proteins were nonfunctional and were equally or less stable than wild-type AQP2 (10). On immunoblots, both AQP1 and AQP2 have been detected in urine (7,11), thereby enabling the assessment of the in vivo stability of mutant AQP2 proteins. Because the urine osmolality of NDI patients is usually below 200 mosmol/kg, we first tested whether AQP1 and AQP2 can be detected in diuretic states in healthy individuals. We subsequently analyzed the urine from seven NDI patients, four of whom have a mutation in the AQP2 gene and three who have a mutation in the V2R gene, to address the stability of mutant AQP2 proteins in vivo and the presence of wild-type AQP2 in NDI patients with mutations in the V2R. In addition, we assessed whether urinary AQP1 expression levels are different in NDI patients.

METHODS

Urine Samples

Healthy Persons. Urine samples from four healthy volunteers (aged 24, 25, 33, and 51 yr; 1 woman, 3 men) were collected according to the following protocol. After 3 h of drinking at least 0.75 L water per h, a diuretic (D) urine sample was collected. The next morning, after emptying the bladder before going to sleep and fluid restriction from 8 p.m. to 7 a.m., an antidiuretic (AD) urine sample was obtained. The osmolality of the urine samples was determined by standard procedures. To determine the (sub)cellular frac-
tions containing AQP1, AQP2, and Tamm-Horsfall proteins (THP), a fractionation protocol was adopted that efficiently separates intracellular vesicles from plasma membranes as tested for AQP1 and AQP2 on rat kidney tissue (12). In brief, an antidiuretic urine sample was divided into two portions and subsequently concentrated or consecutively centrifuged for 15 min at 4000 g, 30 min at 17,000 g, and 1 h at 200,000 g. The supernatant of the 200,000-g fraction was centrifuged. Cells, nuclei, and large fragments are sedimented at 4000 g, plasma membranes are enriched in the 17,000-g pellet, and intracellular vesicles are found in the 200,000-g pellet (12). The pellets of the different fractions were resuspended in homogenization buffer A (20 mM Tris [pH = 7.4], 5 mM MgCl₂, 5 mM NaHPO₄, 1 mM EDTA, 1 mM dithiothreitol, 100 μM ouabain, 1 mM phenylmethylsulphonyl fluoride, 5 μg/mL leupeptin and pepstatin, and 80 mM sucrose). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), relative volume-equivalents were loaded (i.e., the amount of AQP1, AQP2, or THP in the concentrated urine is identical to the total amount contained in the fractions).

**NDI Patients.** Urine samples were collected from four NDI patients with mutations in the AQP2 gene and from three patients with mutations in the V₂R gene (Table 1). The patients carrying AQP2 gene mutations are homozygous for a deleted nucleotide at Position 463 (originally annotated as Position 369) or homozygous for Gly64Arg, Arg187Cys (9), or Ala147Thr (not published) substitutions. The V₂R mutations found in the male patients consist of a small deletion (528del7; originally annotated as 457del7) and a T204N substitution (13), respectively, whereas the female patient is heterozygous for a two-nucleotide deletion in the Region 337-340 (14). All nucleotide deletions result in frameshifts.

Urine samples were concentrated by ultrafiltration using Amicon stirred cells with YM10 ultrafiltration membranes (Amicon Inc., Beverly, MA) according to a protocol provided by the manufacturer. Protein concentrations of urine samples were determined using the BioRad protein assay (Bio-Rad, München, Germany).

**Immunoblotting**

From erythrocyte ghost membranes, prepared according to Benneth (15), and urine samples of healthy persons and NDI patients, protein equivalents of 18 μg were denatured and separated by SDS-PAGE as described (10). From the patient with the T204N mutation in the V₂R, only 10 μg was available. After electrophoresis, the gel was stained with Coomassie brilliant blue or used for immunoblotting essentially as described (16). In brief, proteins were transferred at 4°C for 105 min at 100 V to 0.45-μm nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using Towbin buffer (17). The efficiency of protein transfer was checked by staining membranes with ponceau red. The Western blot was subsequently analyzed with enhanced chemiluminescence (ECL, Amersham, England), to visualize sites of antigen-antibody reactions, according to the manufacturer and as modified by Sheng and Schuster (18). As primary antibodies we used: a 1:50 dilution of a mouse monoclonal antibody raised against dog AQP1 (19) (gift from M.L. Jennings, Galveston, TX); a 1:3000 dilution of affinity-purified rabbit anti-AQP2 immunoglobulins (10); a 1:10000 dilution of goat antiserum directed against human THP (Organon Teknika Co., West Chester, PA); or a 1:1000 dilution of affinity-purified rabbit anti-spectrin antibodies (20) (gift from P. Agre, Baltimore, MD). When indicated, a 1:300 dilution of affinity-purified rabbit antibodies directed against the N-terminus of human AQP1 was used (20) (gift from P. Agre, Baltimore, MD). As secondary antibodies, we used a 1:2000 dilution of affinity-purified sheep anti-mouse IgG antiserum (whole molecule; Sigma Chemical Co., St. Louis, MO), a 1:5000 diluted affinity-purified goat anti-rabbit IgG antiserum (whole molecule; Dakopatts Corp., Glostrup, Denmark). All three secondary antibodies were coupled to horseradish peroxidase. When indicated, luminographic signals were scanned with an LKB Ultrascan XL laser densitometer (Pharmacia LKB, Sweden).

**RESULTS**

**Healthy Individuals**

To investigate AQP2 excretion in urine, D and AD urine samples from four healthy volunteers were collected and concentrated. The osmolality of D urine samples was 100 mosmol/kg or less, whereas the osmolality of AD urine samples was ≥ 700 mosmol/kg. Coomassie staining of a gel loaded with 18-μg protein equivalents of the urine samples revealed a striking difference in protein pattern between D and AD urine samples, including considerably more low-molecular-mass proteins (LMMP) (i.e., < 66 kd) in D urine samples (Figure 1). These differences were also apparent when the samples were analyzed by immunoblotting (Figure 2). AQP2 antibodies demonstrated a 29-kd AQP2 band in all four AD urine samples. No signal was detectable in D urine samples, except in the urine sample of Volunteer 2, in which a weak 29-kd signal was obtained. To analyze the influence of diuresis or anti-diuresis on the excretion of other proteins from tubular cells, Western blots were also analyzed for AQP1 and THP. In the kidney, AQP1 is exclusively expressed in the proximal tubules and descending limbs of Henlé's loop (21), whereas THP is

**Table 1. AQP2/V₂R mutation, sex, age, and medication of NDI patients**

<table>
<thead>
<tr>
<th>Annotation</th>
<th>AQP2/V₂R mutation</th>
<th>Sex</th>
<th>Age (yr)</th>
<th>Medication</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AQP2-Δ</td>
<td>Homozygous for 463del in AQP2</td>
<td>M</td>
<td>18</td>
<td>No</td>
<td>(9)</td>
</tr>
<tr>
<td>AQP2-GR</td>
<td>Homozygous for G64R in AQP2</td>
<td>F</td>
<td>30</td>
<td>Thiazide/amiloride</td>
<td>(9)</td>
</tr>
<tr>
<td>AQP2-RC</td>
<td>Homozygous for R187C in AQP2</td>
<td>M</td>
<td>30</td>
<td>No</td>
<td>(9)</td>
</tr>
<tr>
<td>AQP2-AT</td>
<td>Homozygous for A147T in AQP2</td>
<td>M</td>
<td>4</td>
<td>Thiazide/Indomethacin</td>
<td>To be published</td>
</tr>
<tr>
<td>V₂R-TN</td>
<td>T204N in V₂R</td>
<td>M</td>
<td>6</td>
<td>Thiazide/amiloride</td>
<td>(13)</td>
</tr>
<tr>
<td>V₂R-Δ+</td>
<td>Heterozygous for 337del/21 in V₂R</td>
<td>F</td>
<td>12</td>
<td>Thiazide</td>
<td>(14)</td>
</tr>
<tr>
<td>V₂R-Δ-</td>
<td>528del7 in V₂R</td>
<td>M</td>
<td>13</td>
<td>Thiazide/amiloride</td>
<td>(13)</td>
</tr>
</tbody>
</table>
Urinary Content of Aquaporin 1 and 2 in NDI

Figure 1. Coomassie blue-stained polyacrylamide gel of diuretic (D) and antidiuretic (AD) urine samples from four healthy volunteers (1-4). Eighteen-μg protein-equivalents were loaded. The molecular mass of marker proteins (in kd) is indicated.

Figure 2. Immunoblot analysis of AQP1, AQP2, and THP in diuretic (D) and antidiuretic (AD) urine samples from four healthy volunteers (1-4). Eighteen-μg protein-equivalents of urine samples were separated by SDS-PAGE and blotted. AQP1, AQP2, and THP proteins were visualized by chemiluminescence autoradiography.

localized to the thick ascending limbs of Henle's loop and early distal convoluted tubules (22). With AQP1 antibodies, the 28-kd AQP1 band was detected in some D urine samples, but considerably more AQP1 was observed in all AD urine samples (Figure 2). For THP, both the nonglycosylated band of 87 kd and the glycosylated 97-kd band were detected in D and AD urine, and again, larger amounts were present in AD urine (Figure 2). Immunoblot analysis of fractionated antidiuretic urine and subsequent densitometric scanning revealed that 40% of urinary AQP1 and AQP2 is contained in the 200,000-g pellet, whereas the remaining 60% is found in the 200,000-g supernatant (Figure 3). Urinary THP was totally sedimented in the 4000-g pellet. With the AQP1, AQP2, and THP antibodies, no other signals were detected.

Figure 3. Immunoblot analysis of AQP1, AQP2, and THP in fractions of antidiuretic urine. Urine was concentrated (conc.) or consecutively centrifuged for 15 min at 4000 g (15'-4k), 30 min at 17,000 g (30'-17k), and 1 h at 200,000 g (1h-200k-p). Relative volume-equivalents of the concentrated urine, of the resuspended pellets of the different centrifugation steps, and of the remaining supernatant (1h-200k-s) were separated by SDS-PAGE and blotted. AQP1, AQP2, and THP proteins were visualized by chemiluminescence autoradiography.

NDI Patients

To determine whether AQP2 proteins are present in the urine of NDI patients carrying mutations in the AQP2 or V2R genes (Table 1), the urine samples of seven patients were concentrated and subjected to immunoblotting (Figure 4). D and AD urine samples of Volunteer 4 (Figures 1, 2) were included as controls (Lanes 4-D and 4-AD, respectively). A strong signal for the expected 29-kd AQP2 band was obtained in AD urine of the healthy volunteer and in the urine sample of the female NDI patient who was heterozygous for a V2R mutation (Lane V2R-A/+). Furthermore, the male patient with a deletion in the V2R gene (Lane V2R-A) showed a weak AQP2 signal compared with the AD urine of the healthy volunteer. No AQP2 signal was detected in the urine of any of the four patients with

Figure 4. Immunoblot analysis of AQP1 and AQP2 in urine samples of NDI patients. The annotations and mutation In the AQP2 gene (AQP2) or V2R gene (V2R) are given in Table 1. For comparison, diuretic (D) and antidiuretic (AD) urine samples of healthy individual 4 were loaded. AQP1 and AQP2 were visualized by chemiluminescence autoradiography. Fluid intake was ad libitum.
mutations in the AQP2 gene nor in urine of the male patient whose V2R gene encodes a T204N substitution. Staining of an identical blot with AQP1 antibodies revealed AQP1 signals at the expected 28 kd in urine samples of all NDI patients (Figure 4). In the urine of three patients with mutations in the AQP2 gene (AQP2-At, AQP2-GR, and AQP2-RC), the AQP1 signals were weak even when compared with D urine of the healthy volunteer. However, the intensity of the AQP1 signals in urine of one patient with a mutation in AQP2 (Lane AQP2-AT), and all patients with V2R mutations (V2R-TN, V2R-Δ/+, and V2R-Δ) was similar to the intensity of the AQP1 signal in AD urine of the healthy volunteer.

Because AQP1 is also present in erythrocytes, detected AQP1 proteins in urine might derive from erythrocyte membranes. To test this possibility, the urine samples were analyzed for the presence of spectrin, a 200- to 240-kd protein abundantly and specifically expressed in erythrocytes (20). Immunoblotting of different amounts of isolated erythrocyte ghost membranes, together with urine proteins from five NDI patients and Volunteer 4, revealed that urine AQP1 signals were similar to AQP1 levels between 0.18 and 1.8 µg of red blood cell membrane proteins (Figure 5). Identical bands and relative intensities were obtained with rabbit antibodies directed against the N-terminal of AQP1 (not shown). Analysis of the same blot with erythrocyte spectrin-specific antibodies revealed a clear band of > 200 kd in a 0.018-µg protein equivalent of erythrocyte ghost membranes (Figure 5). No spectrin signal was detected in lanes containing urine samples, even after longer exposure times.

DISCUSSION

In urine, a wide variety of proteins is present, including approximately 40% albumin, 15% immunoglobulins, and 40% tissue proteins originating from renal and other urogenital tissues (23). However, the relative abundance of nephron constituents in urine is not constant because it shows intra- and interindividual variability and is also influenced by short-term physiological factors, such as urinary flow rate, age, sex, and circadian rhythm (24). The clear differences in Coomassie-stained protein profiles (Figure 1) and detected levels of AQP1, AQP2, or THP excretion (Figure 2) in D urine or AD urine samples of different healthy individuals underscore the interindividual variability. Furthermore, the intrindividual differences between D and AD urine samples corroborate the influence of urinary flow rate and circadian rhythm on urinary protein content. In this respect, the shift to more LMMP in D urine samples, compared with corresponding AD urine samples, is remarkable (Figure 1). Usually, LMMP, which pass through the glomerular filter, are almost completely reabsorbed and catabolized by the proximal tubule (25). However, in diuresis, the observed high excretion of LMMP has been ascribed to a decreased reabsorption in proximal tubules (23,24,26). The relatively high content of blood LMMP in diuretic urine explains the relatively low levels of AQP1, AQP2, and THP in these urine samples, compared with AD urine. Induction of AQP2 expression in anti-diuresis, however, also appears to contribute to elevated AQP2 levels in AD urine. In dehydrated rats, renal AQP2 mRNA and protein levels are increased upon activation of the V2R (27–30). In NDI patients with V2R mutations, a similar mechanism appears to occur: in the urine of the patients without a functional V2R, AQP2 is not, or is hardly, detectable (Figure 4: Lanes V2R-TN and V2R-Δ), whereas in the urine of the patient encoding one mutant and one functional V2R, strongly elevated AQP2 levels are detected (Figure 4: Lane V2R-Δ/+). Low AQP2 levels in urine of NDI patients with V2R mutations and increased levels of urinary AQP2 in healthy humans after dehydration (or AVP injection) have also been found by others (31). Interestingly, fractionation of AD urine revealed that 40% of all urinary AQP1 and AQP2 is contained in the 200,000-g pellet, whereas the remaining 60% is found in the supernatant. A similar amount of AQP2 in the 200,000-g pellet has been found by others (31). Application of the used fractionation protocol to homogenized kidneys of rats treated with 1-desamino-8-arginine-vasopression revealed that AQP1 was enriched in a plasma membrane fraction (17,000 g), whereas AQP2 was found in plasma membranes and in intracellular vesicles (200,000-g pellet) (12). Apparently, urinary AQP1 and AQP2 are present in small vesicles only. Whether urinary AQP1 and AQP2 stem from intracellular vesicles released from renal cells or from shedded cells remains to be established. The solubilized form of AQP1 and AQP2 could well be a consequence of high urea concentrations in AD urine.

Mutant proteins that fold incorrectly or that are not assembled properly in vitro are mostly retarded in the endoplasmic reticulum and are typically degraded in a pre-Golgi compartment (32,33). Until now, missense AQP2 proteins in NDI patients have been shown to be nonfunctional and impaired in their targeting to the plasma membrane of Xenopus oocytes. The mutant Arg187Cys AQP2, however, was more stable, whereas Gly64Arg and Ser216Pro AQP2 were less stable than wild-type AQP2 (10). Because the osmolality of the urine of NDI patients (e.g., see References 9,13) is in
patients could be anticipated. However, NDI patients with AQP2 proteins, which will not be recognized by our AQP1 signal in the AD control subject, the excreted mutants in the AD control. This indicates that in an NDI patient expressing only a limited amount of functional V_2R, enough wild-type AQP2 is synthesized and excreted to be clearly detectable in urine. Because in our NDI patients carrying AQP2 gene mutations, a mutation in the V_2R gene is unlikely or has been excluded, the absence of AQP2 in their urine must be attributed to reduced stability of the mutant AQP2 proteins. The urine from one patient (AQP2-Δ) served as a negative control, because his AQP2 gene encodes truncated AQP2 proteins, which will not be recognized by our antibody. Consequently, no conclusion can be drawn concerning the stability of these mutant AQP2 proteins.

The differences in stability between missense AQP2 proteins and wild-type AQP2 in humans, as concluded from the study presented here, seem more pronounced than previously concluded from expression studies in Xenopus oocytes. This is most likely the result of a smaller amount of AQP2 mRNA present in collecting tubule cells compared with the amount injected into oocytes. Furthermore, as has been shown for the insulin receptor, mutant AQP2 proteins in collecting tubule cells might downregulate their own mRNA expression, a phenomenon that does not occur in oocytes. A similar situation has been described in a person whose AQP1 gene encoded a P368L mutation. In this person, AQP1 could not be detected, whereas after 2 days of expression in Xenopus oocytes, this mutant AQP1 protein had a stability similar to wild-type AQP1.

No spectrin could be detected in the urine samples, whereas in red blood cell membranes containing similar amounts of AQP1 as the urine samples, the spectrin signal was strong. Therefore, urinary AQP1 must be of renal origin. Compared with the AQP1 signal in the AD control subject, the excreted amounts of AQP1 were similar in all NDI patients with a V_2R mutation and in one patient with an AQP2 mutation. In the urine of the other three NDI patients with AQP2 mutations, AQP1 signals were weak.


