Urinary Aquaporin 2 and Calciuria Correlate with the Severity of Enuresis in Children

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Abstract. This study examined the hypothesis that nocturnal enuresis might be paralleled by aquaporin 2 (AQP2) urinary excretion. Eighty children who experienced nocturnal enuresis were studied and compared with 9 healthy children. The 24-h urine samples were divided into two portions: night collections and day collections. Creatinine equivalents of urine samples from each patient were analyzed by Western blotting. AQP2 levels were semiquantified by densitometric scanning and reported as a ratio between the intensity of the signal in the day urine sample versus the night urine sample (D/N AQP2 ratio). The D/N AQP2 ratio was 0.59 ± 0.11 (n = 9) in healthy children and increased to 1.27 ± 0.24 (n = 10) in a subpopulation of enuretic children who had low nocturnal vasopressin levels. In enuretic children who displayed hypercalciuria and had normal vasopressin levels, the D/N AQP2 ratio was 1.05 ± 0.27 (n = 8). These data indicate that reduced secretion of vasopressin and absorptive hypercalciuria are independently associated with an approximately twofold increase in the urinary D/N AQP2 ratio. When low nocturnal vasopressin levels were associated with hypercalciuria, a nearly threefold increase in the D/N AQP2 ratio was observed (1.67 ± 0.41, n = 11). In addition, in all enuretic patients tested, the urinary D/N AQP2 ratio correlates perfectly with the severity of the disorder (nocturnal polyuria). The findings reported in this article indicate that urinary AQP2 correlates with the severity of enuresis in children.

Nocturnal enuresis (NE) is a pathologic state that is more frequent in 5- to 10-yr-old children and is characterized by urine loss during the night in children over the age at which bladder control is supposed to be present. Its frequency is 15 to 20% in 5-yr-old children, decreasing to 7% by the age of 10 and to 1 to 2% in adults, particularly males (1,2). Urinary bladder dysfunction and reduction in the nocturnal vasopressin levels that lead to a high nighttime urine output have been indicated as the two major causes of NE (3,4).

In normal subjects, the decreased urine production observed at night, about half that produced during the day, is associated with a nocturnal increase in vasopressin secretion. However, recent data obtained by treating primary NE using desmopressin acetate (dDAVP; a selective V2 receptor agonist) suggest that dDAVP may be useful in some but not in all patients with NE. The authors suggested that in some patients there might be a defect at the vasopressin receptor level or in the signal transduction pathway associated with receptor stimulation by vasopressin (4). The findings thus suggest various causes of NE related to different patterns of vasopressin action.

Kuznetsova et al. (5) suggested that a main role in inducing NE is played by a decrease in the reabsorption of osmotically active solutes, particularly sodium ions. The authors concluded that the beneficial effect of dDAVP is most likely due to a decrease in natriuresis. A recent study suggested that NE can be caused by absorptive hypercalciuria and can be treated with a combination of diet and dDAVP (6). Despite the diversity of possible causes of NE, studies have shown that in a high proportion of the patients examined, the night urine output is relatively increased (7–10). Moreover, it has been hypothesized that excessive bladder filling causes enuresis when the amount of urine exceeds functional bladder capacity (7).

Renal water excretion is regulated by the peptide hormone vasopressin in two separate ways: (1) the short-term regulation resulting from shuttling of the vasopressin-sensitive water channel aquaporin 2 (AQP2) from intracellular vesicles into the apical membrane of collecting-duct principal cells via regulated exocytosis (11,12) and (2) a long-term regulation that is seen after a prolonged (>24 h) increase in circulating vasopressin, which in turn results in an increase in AQP2 (13) and AQP3 renal expression (14,15) in collecting-duct principal cells. AQP2 is downregulated in multiple forms of acquired nephrogenic diabetes insipidus characterized by severe polyuria (16–19). Recent findings from Wen et al. (20) demonstrated that in rats, AQP2 is excreted in the urine and that excretion is proportionally increased in response to vasopressin or thirsting, via a selective apical pathway.

The aim of this work was to analyze whether the vasopres-
sin-sensitive water channel AQP2 might play a role in the osmoregulatory function of the kidney in children who experience primary NE. To explore this hypothesis, 80 children who were experiencing primary NE were examined between January 1999 and June 1999 and compared with 9 healthy children as a control group. AQP2 urine excretion was evaluated in all patients. This article reports the first detailed analysis of alterations of AQP2 excretion associated with NE in humans.

Materials and Methods

Patients and Study Design

From January 1999 to June 1999, a total of 89 children with a mean age of 9.7 yr were admitted to the hospital ( Policlinico, University of Bari) for a 48-h investigation. Among these children, 80 had significant primary NE (three or more wet nights weekly) and 9 were healthy controls, hospitalized for acute pharyngotonsillitis. For the latter group, we obtained a special informed consent from their parents on the day before their discharge, authorizing us to perform our routine investigations.

Routine evaluation for all patients included detailed medical history, physical examination, micturition list, blood count, serum creatinine and electrolytes, urinalysis and culture, vestibular swab in females, urinary electrolytes and urinary creatinine levels, vasopressin, renin and aldosterone measurements, kidney and bladder ultrasound, and uroflowmetry. For each patient, the 24-h urine samples were collected and divided into two portions: night collections (8:00 p.m. to 8:00 a.m.) and day collections (8:00 a.m. to 8:00 p.m.). All urine samples were collected for 24 h with a natural urination. In children with NE, urine was collected during the night with a plastic bag adapted to external genitalia. This kind of bag is commonly used in patients who have cutaneous urinary diversion, such as ureterocutaneostomy. Children with clinically psychiatric, neurologic, musculoskeletal, cardiovascular, or genitourinary disease were excluded from the study. On the day when urine samples were collected, blood samples were taken from the decubital vein at 4:00 a.m. in supine position before the patient got up, to measure renin levels and also to exclude the last meal. Samples were taken at 8:00 a.m., 2:00 p.m. and 8:00 p.m. to 8:00 a.m. (8:00 p.m. to 8:00 a.m.) and day collections (8:00 a.m. to 8:00 p.m.).

Urine Processing

AQP2 excretion in the urine was semiquantified in a total of 80 enuretic children and in 9 healthy children. To semiquantify the amount of AQP2 excreted in the urine, the day and night urine samples from each patient were spun down at 3000 \( \times g \) for 10 min at 4°C to remove cellular debris in the presence of the following protease inhibitors: 2 mM phenylmethylsulphonyl fluoride, 1 \( \mu g/\)ml leupeptin, and 1 \( \mu g/\)ml pepstatin. A total of 150 \( \mu g \) of creatinine equivalent of each sample were then concentrated by ultrafiltration using Centricon tubes (Millipore, Bedford, MA) with 10,000 D cutoff according to the protocol provided by the manufacturer. Concentrated proteins were subjected to immunoblot analysis to semiquantify the amount of AQP2 in the sample.

Immunoblotting

Concentrated urine samples were mixed with 1 vol of Laemmli sample buffer containing 1 mM dithiothreitol and denatured for 1 min at 60°C and loaded on 13% acrylamide gels. After sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the proteins were immunooblotted on Immobilon-P (Millipore). The efficiency of the transfer was checked by staining the membranes with Coomassie Blue. Subsequently, the blots were blocked in blotting buffer (150 mM NaCl, 20 mM Tris-HCl [pH 7.4], and 1% Triton X-100) containing 5% nonfat dry milk, for 1 h. Membranes were washed and incubated with 1:300 dilution of rabbit anti-human AQP2 serum (generous gift from Dr. Walter Rosenthal, FMP, Berlin). Membranes were washed and incubated with anti-rabbit alkaline-phosphatase 1:5000 dilution (Sigma, St. Louis, MO). Antigen-antibody reactions were visualized using the substrates 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate, 0.48 mM nitro blue tetrazolium in 10m Tris-HCl, [pH 9.5]. Controls using preimmune serum or omission of primary or secondary antibody revealed no labeling.

The density of the 29 kD band of AQP2 that showed up was measured by densitometry and quantified using the NIH software. For each sample, a twofold dilution series was performed to ensure that the densitometric units were linear over a wide range. Finally, the ratio between the densitometric units of the daytime 29 kD band of the AQP2 versus the nighttime 29 kD band were calculated for each patient.

Statistical Analysis

Values are presented in the text as mean ± SEM. Comparisons between groups were made by unpaired \( t \) test.

Results

The enuretic children were divided into three subgroups: G1 with low nocturnal vasopressin levels and a higher nighttime than daytime diuresis, G2 with low nocturnal vasopressin levels and a balanced nighttime and daytime diuresis, and G3 with normal vasopressin levels and a lower nighttime than daytime diuresis. These three subgroups were compared with healthy children (G4). Table 1 summarizes the principal parameters evaluated in the 80 enuretic children and in the 9 healthy children (G4). It has recently been established that AQP2 is excreted in the urine and that its excretion increases in response to acute vasopressin treatment or thirsting, indicating that conditions characterized by increased vasopressin levels and altered AQP2 expression in the apical membrane are associated with increased AQP2 excretion (20). On the basis of these observations, the 24-h urine samples were collected for each patient and divided into two portions: night collections (8:00 p.m. to 8:00 a.m.) and day collections (8:00 a.m. to 8:00 p.m.).

The urine samples were analyzed for a semiquantitative expression of AQP2. Routinely, 150 \( \mu g \) of creatinine equivalents of concentrated urine samples were loaded on the gels, and AQP2 was semiquantified by Western blot. The densitometric units obtained for the 29 kD band of AQP2 were found to be linear over a wide range at this creatinine concentration (Figure 1). Figure 2A reports a representative Western blot of urine samples from healthy children and from enuretic children probed with specific anti-human AQP2 antibodies. In healthy children, the AQP2 level detected in a daytime urine sample was less than half that detected in the nighttime urine sample. In contrast, the situation was reversed in most of the enuretic
Table 1. Principal parameters evaluated in enuretic children and in healthy children

<table>
<thead>
<tr>
<th></th>
<th>G1 (n = 21)</th>
<th>G2 (n = 39)</th>
<th>G3 (n = 20)</th>
<th>Control (n = 9)</th>
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<tbody>
<tr>
<td>Daytime diuresis (ml)</td>
<td>409.5 ± 44.0***</td>
<td>481.5 ± 40.0**</td>
<td>487.5 ± 40.0***</td>
<td>733.7 ± 42.0</td>
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<tr>
<td>Nighttime diuresis (ml)</td>
<td>661.4 ± 60.0**</td>
<td>442.0 ± 30.0*</td>
<td>395.0 ± 25.0</td>
<td>357.5 ± 18.0</td>
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<td>24-h diuresis (ml)</td>
<td>1071.0 ± 102.0</td>
<td>923.0 ± 54.0</td>
<td>882.0 ± 55.0</td>
<td>1051.3 ± 59.0</td>
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<tr>
<td>N/D diuresis</td>
<td>1.64 ± 0.08***</td>
<td>0.94 ± 0.03***</td>
<td>0.87 ± 0.06***</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>Urine osmolality (mOsm/kg water)</td>
<td>719.0 ± 37.0***</td>
<td>783.0 ± 24.0*</td>
<td>746.0 ± 51.0*</td>
<td>931.0 ± 22.0</td>
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<tr>
<td>Vasopressin (pg/ml)</td>
<td>1.06 ± 0.12***</td>
<td>1.22 ± 0.11***</td>
<td>4.0 ± 0.15</td>
<td>4.08 ± 0.04</td>
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<tr>
<td>CaU/CrU D</td>
<td>0.13 ± 0.02*</td>
<td>0.12 ± 0.01**</td>
<td>0.18 ± 0.04*</td>
<td>0.046 ± 0.004</td>
</tr>
<tr>
<td>CaU/CrU N</td>
<td>0.22 ± 0.02***</td>
<td>0.20 ± 0.01***</td>
<td>0.22 ± 0.03***</td>
<td>0.048 ± 0.004</td>
</tr>
<tr>
<td>FE Na D</td>
<td>0.66 ± 0.06</td>
<td>0.76 ± 0.04</td>
<td>0.83 ± 0.08</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>FE Na N</td>
<td>0.95 ± 0.06***</td>
<td>0.91 ± 0.06*</td>
<td>0.98 ± 0.09*</td>
<td>0.66 ± 0.04</td>
</tr>
<tr>
<td>FE K D</td>
<td>11.7 ± 0.99*</td>
<td>10.8 ± 0.46***</td>
<td>11.2 ± 1.06*</td>
<td>15.5 ± 0.4</td>
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<tr>
<td>FE K N</td>
<td>8.11 ± 0.76***</td>
<td>7.74 ± 0.78**</td>
<td>8.41 ± 0.87**</td>
<td>13.80 ± 1.40</td>
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<td>Renin (ug/ml per h)</td>
<td>2.43 ± 0.31***</td>
<td>2.47 ± 0.21***</td>
<td>3.18 ± 0.4*</td>
<td>4.60 ± 0.36</td>
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<td>Aldosterone (ug/ml per h)</td>
<td>110.2 ± 18.0**</td>
<td>87.8 ± 9.6**</td>
<td>89.0 ± 11.6**</td>
<td>344.0 ± 16.5</td>
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<td>Plasma calcium (mg/dl)</td>
<td>9.9 ± 0.34</td>
<td>10.0 ± 0.4</td>
<td>9.9 ± 0.37</td>
<td>9.9 ± 0.4</td>
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* CaU/CrU D, daytime urinary calcium/creatinine ratio; CaU/CrU N, nighttime urinary calcium/creatinine ratio; FE Na D, daytime fractional excretion of sodium (%); FE Na N, nighttime fractional excretion of sodium (%); FE K D, daytime fractional excretion of potassium (%); FE K N, nighttime fractional excretion of potassium (%); G1, group 1, low nocturnal vasopressin levels and a higher nighttime than daytime diuresis; G2, group 2, low nocturnal vasopressin levels and a balanced nighttime and daytime diuresis; G3, group 3, normal vasopressin levels and a lower nighttime than daytime diuresis; G4, group 4, normal vasopressin levels and nighttime diuresis half that of daytime diuresis; * P < 0.05; ** P < 0.005; *** P < 0.0001 (compared with corresponding values in the control group).

Figure 1. Correlation between creatinine urine sample concentration and the aquaporin 2 (AQP2) 29 kD scanned band density. Serial dilutions of a concentrated urine sample were run on a gel, and AQP2 signals were semiquantified by densitometry (see the Materials and Methods section). The linear regression line was calculated for sample in the range 50 to 300 µg creatinine, and the linear correlation was highly significant.

Results from the three groups (Figure 2A). When the urine samples were probed with anti-AQP3 antibodies (the aquaporin expressed in the basolateral membrane of collecting-duct principal cells, Figure 2B) no proteins were detected, confirming the finding of Wen et al. (20) indicating that AQP2 excretion in the urine does not result from collecting-duct epithelial cell shedding. By semiquantitative densitometry, we evaluated the ratio of the AQP2 signal detected in the daytime versus the nighttime urine sample for each patient from each group. The results are reported in Figure 3A. In healthy children, this ratio (D/N AQP2 ratio, G4) was 0.59 ± 0.16 (n = 9), indicating that AQP2 excretion in nighttime urine was approximately twice that in daytime urine. This result was paralleled by the night/day diuresis ratio (N/D diuresis ratio), which was found to be 0.49 ± 0.01 in healthy children (Figure 3B, G4). In contrast, in all enuretic patients tested (80 children), the D/N AQP2 ratio was significantly higher compared with that found in healthy children, displaying a modulation that perfectly correlated with the N/D diuresis ratio. The highest value for D/N AQP2 ratio in the three subgroups of enuretic children was found in G1 (1.48 ± 0.25, Figure 3A), corresponding to enuretic children who had low nocturnal vasopressin levels and a higher nighttime than daytime diuresis. Again, this situation paralleled the N/D diuresis ratio (1.64 ± 0.08, Figure 3B, G1). G2 and G3 displayed a nearly twofold increase in the D/N AQP2 ratio (0.97 ± 0.11, Figure 3A [G2]; 0.92 ± 0.16, Figure 3A, G3). G2 included children who had a balanced daytime/nighttime diuresis, whereas G3 included children who had lower nighttime than daytime diuresis. Also for G2 and G3 this situation paralleled the N/D diuresis ratio (0.94 ± 0.03, Figure 3B, G2; 0.87 ± 0.06, Figure 3B, G3).

Altogether, these results have two implications: (1) NE can be linked directly to the modulation of AQP2 urinary levels, which in turn are dependent on the AQP2 present in the apical
membrane of collecting-duct principal cells; and (2) the N/D diuresis ratio, a value that matches the severity of the disorder (nocturnal urine output), parallels the D/N AQP2 ratio.

The strict correlation between the N/D diuresis ratio and the D/N AQP2 ratio is shown in Figure 4. These two parameters seem to be perfectly superimposed with a correlation coefficient of 0.999. This finding further suggests that the N/D diuresis ratio is proportionally linked to the D/N AQP2 ratio.

It has been suggested that besides low vasopressin levels, absorptive hypercalciuria can cause NE. To test this hypothesis, we focused our attention on G1 patients. These children had low nocturnal vasopressin levels and a significantly high D/N AQP2 ratio when compared with healthy children. Figure 5A shows the creatinine equivalent calcium levels (UCa/Cr) in the daytime/nighttime urine samples for all patients from this group (Figure 5A). The estimated normal value Ca/creatinine ratio (UCa/Cr) is less than 0.2, which corresponds to a normal calcium excretion in the urine (<4mg/kg per day). Values above 0.2 UCa/Cr characterized hypercalciuria (21). On the basis of these parameters, if we consider a subpopulation of patients from G1 who displayed hypercalciuria (Figure 5A), then the corresponding D/N AQP2 ratio increased approximately threefold compared with healthy children (1.67 ± 0.41 versus 0.59 ± 0.11; Figure 5B, P < 0.001). The remaining normocalciuric patients (NC) still had approximately twice the D/N AQP2 ratio (1.27 ± 0.24 versus 0.59 ± 0.11; Figure 5B, P < 0.05), and they had low vasopressin levels only nocturnally but were not exposed to high calcium, either during the night or during the day. This indicates that low vasopressin levels in patients who display normal calciuria are associated both with a decrease in the nocturnal expression of AQP2 in the apical membrane and, consequently, with a drop in the amount of AQP2 excreted in the urine during the night, thus leading to a nearly twofold increase in the D/N AQP2 ratio. Moreover, in this group, the combination of low nocturnal vasopressin levels and hypercalciuria resulted in a more severe NE, which is paralleled by AQP2 excretion in the urine. In fact, compared with NC children, the D/N AQP2 ratio in hypercalciuric (HC) children was significantly higher (1.67 ± 0.41 versus 1.27 ± 0.24; P < 0.05). Figure 6 shows the analysis of patients from G2. The total population of this group displayed a significantly high D/N AQP2 ratio (0.97 ± 0.11 versus 0.59 ± 0.11, P < 0.01). As for the G1, HC patients from G2 had a significantly higher D/N AQP2 ratio compared with NC children (1.01 ± 0.22 versus 0.88 ± 0.10, P < 0.01). Figure 7 shows the examination of G3 patients characterized by having normal vasopressin levels. On average, the total population displayed a D/N AQP2 ratio approximately twice that of normal values (0.92 ± 0.16 versus 0.59 ± 0.11, P < 0.05). The subpopulation of HC children from G3 (who were exposed to high urinary calcium levels both during the day and during the night, Figure 7A) had a D/N AQP2 ratio of 1.05 ± 0.27, significantly higher (P < 0.05) than in the NC children from the same group (0.79 ± 0.12, Figure 7B), indicating that absorptive hypercalciuria might be associated with a significant increase in the D/N AQP2 ratio even in the presence of normal vasopressin levels. It is therefore remarkable that in all three groups of enuretic children tested, hypercalciuria significantly enhanced the D/N AQP2 ratio compared with the NC children from the same group. It is stressed that approximately 40% of enuretic children enrolled in this study displayed hypercalciuria, suggesting that hypercalciuria might play a crucial role in inducing NE.

Discussion

Urinary excretion of AQP2 in humans has been proposed to be a potential marker of collecting-duct responsiveness to vasopressin (22,23). This article is the first detailed analysis of the modulation of urinary AQP2 excretion in humans who experience NE, one of the most common pathologic states, especially in children. Although aquaporins were predicted to be involved in NE (24), a clear demonstration for such a role was lacking.

In mammalian kidney and urine, two forms of AQP2 are detected: a nonglycosylated form of 29 kD and a glycosylated form of 40 to 45 kD (22,23). To overcome the problems related
to the variability of AQP2 excretion among individuals, in this work we took advantage of the immunodetection of AQP2 in the urine samples collected during the day (8:00 a.m. to 8:00 p.m.) and during the night (8:00 p.m. to 8:00 a.m.) from the same patient; consequent calculation of the daytime/nighttime AQP2 signal was obtained by densitometry. This study demonstrates that compared with healthy subjects, in enuretic children, low nocturnal vasopressin levels are associated with an approximately twofold increase in the daytime/nighttime AQP2 ratio detectable in the urine, indicating that the amount of AQP2 present in the apical membrane of collecting-duct principal cells during the day and during the night. This probably reflects the amount of AQP2 present in the apical membrane of collecting-duct principal cells during the day and during the night. In the enuretic children examined, this situation is concurrent with a more or less severe nocturnal polyuria. A reversed situation was found in healthy children: the daytime/nighttime AQP2 ratio was found to be $0.59 \pm 0.11$, which is associated with a nighttime diuresis approximately half that of the daytime diuresis ($N/D$ diuresis ratio, $0.48 \pm 0.01$).

Moreover, our findings show that absorptive hypercalciuria in all enuretic children is associated with a significant increase in the daytime/nighttime AQP2 ratio, even in the presence of normal vasopressin levels. The modulation of AQP2 excretion in the urine raises the possibility that NE in HC children might be at least in part due to a reduction in the availability of the AQP2 water channel in the apical membrane of principal cells.

**Low Vasopressin Levels and Reduced AQP2 Excretion**

It is well established that downregulation of AQP2 occurs in multiple forms of nephrogenic diabetes insipidus (NDI) (22). The Brattleboro rats are an animal model for central NDI. This rat strain is unable to produce vasopressin and is extremely polyuric. Compared with the parent strain, the inner medulla of Brattleboro rats expressed one third of the AQP2. Vasopressin administration for 5 d increased AQP2 expression and corrected urinary concentration defects (25). This demonstrates that vasopressin modulates the AQP2 expression in the kidney and that patients with low vasopressin levels are likely to have reduced expression of AQP2. However, the short-term regulation of AQP2 trafficking at the apical membrane of collecting-duct principal cells regulated by vasopressin is probably a more relevant mechanism. AQP2 excretion in the urine depends on vasopressin action on principal cells rather than on reflecting AQP2 expression in the kidney (22). This study is in agreement with the observation that vasopressin levels modulate AQP2 excretion in the urine. Children from both G1 and G2 analyzed in this study were characterized by low nocturnal vasopressin levels in the blood. On average, the 24-h diuresis in those subjects was comparable to that found in normal subjects, whereas all of them experienced nocturnal polyuria. The AQP2
detection in the daytime and nighttime urine samples correlates perfectly with the daytime and nighttime diuresis, which indicates that the nocturnal urine output and, therefore, the severity of the nocturnal polyuria were inversely proportional to the amount of AQP2 excreted in the urine (Figure 3). These important findings suggest that changes in the urinary excretion of this protein can be used in part as an index of short-term vasopressin action. It is likely that enuretic children who have low nocturnal vasopressin levels experience bed-wetting episodes because of a decrease in AQP2 during the night. Preliminary observations made in our laboratory further support this hypothesis; in some of these patients treated with dDAVP, the urinary daytime/nighttime AQP2 ratio returned to the value observed in healthy children (0.59 ± 0.11) with concomitant interruption of enuretic episodes (not shown). It has been shown that in rats, dDAVP treatment increases urine osmolality and causes a relocation of AQP2 to the apical plasma membrane but produces only a small increase in AQP2 expression in the kidney (18). We therefore conclude that nocturnal diuresis in enuretic children from G1 and G2 might be a direct consequence of circadian variation of vasopressin levels, which in turn acutely modulates the AQP2 trafficking toward the apical membrane of collecting-duct principal cells. We cannot exclude, however, that other mechanisms might contribute to the development of nocturnal polyuria. In fact, in normal subjects, urine is concentrated as a result of the combined action of the loop of Henle and collecting duct. The loop of Henle generates high osmolality in the renal medulla by the countercurrent multiplication process while the collecting duct, in the presence of vasopressin, allows osmotic equilibration between the urine and hypertonic interstitium. The generation of high osmolality in the medulla depends mainly on NaCl absorption because of the activity of the Na+ transporters

**Figure 5.** (A) Creatinine equivalent calcium levels (UCa/Cr) in the daytime/nighttime urine samples for all patients from G1. The estimated normal value Ca/creatinine ratio (UCa/Cr) is less than 0.2, corresponding to a normal calcium excretion in the urine (<4mg/kg per day). Values above of 0.2 UCa/Cr characterized hypercalciuria. (B) Corresponding D/N AQP2 ratio. TP, total population; HC, subpopulation of hypercalciuric children; NC, subpopulation of normocalciuric children. Mean ± SEM. *P < 0.05.

**Figure 6.** (A) UCa/Cr in the daytime/nighttime urine samples for all patients from G2. (B) Corresponding D/N AQP2 ratio. Mean ± SEM. *P < 0.01.
located in the thick ascending limb. Sodium absorption is crucial for the urinary concentrating capacity. Compared with healthy children (G4), enuretic children from the three groups examined had a significantly higher nocturnal sodium excretion (G1 versus G4, P < 0.005; G2 versus G4, P < 0.05; G3 versus G4, P < 0.05). Conversely, the 24-h urinary volume was not significantly different from healthy children but had a reduced osmolality compared with controls (G1 = 719 ± 37, P < 0.005; G2 = 783 ± 24 P < 0.05; G3 = 746 ± 51, P < 0.05 compared with control G4 = 931 ± 22). This situation was accompanied by reduced renin levels in enuretic patients, although the values observed remained within the lowest limits of normality and with significantly reduced levels of aldosterone. Thus, we conclude that the concentrating defect in those enuretic children might be a consequence of multiple factors, including an impairment of AQP2 trafficking in the collecting duct, which would predict low water permeability.

Hypercalciuria and Reduced AQP2 Excretion

In a previous study, we suggested that absorptive hypercalciuria may be responsible for NE (6). In this article, we extended our previous observations that showed that high urine levels of calcium seem to decrease the amount of AQP2 detectable in the urine. Examination of a subpopulation of children from G1 who displayed hypercalciuria (Figure 5A) demonstrated that the daytime/nighttime AQP2 ratio increased by approximately threefold compared with healthy children (1.67 ± 0.41 versus 0.59 ± 0.11; Figure 4B). That was the highest value for the D/N AQP2 ratio found in the enuretic children tested. Probably, in this group, the combination of low nocturnal vasopressin levels and hypercalciuria resulted in a more severe nocturnal polyuria and is paralleled by AQP2 levels in the urine. A similar situation, although to a lesser extent, was found in children from G2. In fact, compared with NC children, low vasopressin levels associated with hypercalciuria in G2 induced a significant increase in the daytime/nighttime AQP2 ratio. Notably, our data indicate that absorptive hypercalciuria is associated with a significant increase in the daytime/nighttime AQP2 ratio even in the presence of normal vasopressin levels. In fact, on average, the whole population of enuretic children from G3, characterized by normal vasopressin levels, displayed a D/N AQP2 ratio approximately twice as high as normal values (0.92 ± 0.16 versus 0.59 ± 0.11). When we examined a subpopulation of HC children in this group (who were exposed to high urinary calcium levels both during the day and during the night), the daytime/nighttime AQP2 ratio was 1.05 ± 0.27, a value that is significantly higher than in NC children from the same group (0.79 ± 0.12). These data suggest that hypercalciuria per se might cause enuresis in some patients, probably by altering AQP2 trafficking and thus decreasing aquaporin excretion in the urine. Although the effect of hypercalciuria on the modulation of the D/N AQP2 ratio was much clearer in G1 patients, G2 and G3 patients showed a significant but modest increase in the D/N AQP2 ratio. Other factors might mask the effect of urinary calcium levels on AQP2 excretion in these groups of enuretic children.

HC enuretic children are exposed to high urinary calcium levels, which results in a parallel modulation of AQP2 excretion in the urine. Therefore, a signal transduction pathway must link urinary calcium levels to AQP2 expression in the collecting duct. Sands et al. (26) reported that in rats, hypercalcemia induces alterations in vasopressin-regulated water permeability. Moreover, it has been demonstrated (27) that a calcium-sensing signal transduction complex is present in the apical membrane of rat kidney inner medullary collecting duct that could possibly integrate calcium and water homeostasis. As the physiologically relevant calcium concentrations are in the millimolar range, this sensing mechanism functions like a low-affinity receptor. This low-affinity, calcium-sensing mechanism, however, detects small changes in the activity of calcium ions. Increases in external calcium activity modulate the production of two major intracellular second messenger systems in bovine parathyroid cells (28). First, raising external calcium activates phospholipase C, which leads to an accumulation of
in adenosine 3'-5'-cyclic monophosphate. These changes in the second messenger system will modulate the activity of a series of kinases, such as protein kinase C. Data from many laboratories have demonstrated that vasopressin-elicited apical membrane events that lead to increases in the osmotic permeability coefficient are antagonized by activation of protein kinase C (29,30), possibly through endocytotic activation of AQP2-bearing vesicles (31). We speculate that this short-term regulation induced by external calcium might occur in HC enuretic children from G1 who are exposed to high urinary calcium levels only during the night and not during the day (see Figure 3). This situation is associated with a nearly threefold increase in the D/N AQP2 ratio, which indicates that during the night, very low levels of AQP2 might be present on the apical membrane of collecting-duct principal cells in those patients, thus inducing nocturnal polyuria. Conversely, besides the mechanisms proposed here, in HC children from G3, who are exposed to high urinary calcium levels both during the day and during the night (see Figure 3), a more complex regulation of AQP2 expression in the kidney might occur. Additional studies addressing this and other potential mechanisms will lead to a better understanding of the pathophysiology of hypercalciuria-dependent NE. In this respect, it is noteworthy that more than 40% of the enuretic children analyzed in this study displayed hypercalciuria. This suggests that hypercalciuria might play a crucial role in inducing NE.

Conclusion

Our results suggest that (1) NE in children is accompanied by a two- to threefold increase in urinary daytime/nighttime AQP2 ratio, a value that correlates perfectly with the severity of the disorder (nocturnal polyuria); (2) NE in patients who have low nocturnal vasopressin levels is probably a consequence of impaired AQP2 trafficking in collecting-duct principal cells; (3) high levels of calcium in the urine seem to decrease the amount of AQP2 detectable in the urine. These data are the first detailed analyses of hypercalciuria-induced alterations of AQP2 levels in humans that might prevent formation of calcium-containing renal stones. In addition, on the basis of our observations that offer a rationale correlating NE and AQP2 urinary excretion, this work suggests that the clinical evaluation of enuretic children is enhanced by measuring simple parameters such as nocturnal and diurnal diuresis, nighttime and daytime calciuria, and vasopressin levels.

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References

19. Marples D, Frokiaer J, Dorup J, Knepper MA, Nielsen S: Hy-


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