Low-calcium diet in hypercalciuric enuretic children restores AQP2 excretion and improves clinical symptoms

GIOVANNA VALENTI,1 ANTONIA LAERA,1 SABINE GOURAUD,1 GIUSEPPE PACE,2† GABRIELLA ACETO,3 ROSA PENZA,3 FRANCESCO P. SELVAGGI,2 AND MARIA SVELTO1

Departments of 1General and Environmental Physiology, 2Emergency and Transplantation, and 3Developmental Age Biomedicine, University of Bari, 70100 Bari, Italy

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Valenti, Giovanna, Antonia Laera, Sabine Gouraud, Giuseppe Pace, Gabriella Aceto, Rosa Penza, Francesco P. Selvaggi, and Maria Svelto. Low-calcium diet in hypercalciuric enuretic children restores AQP2 excretion and improves clinical symptoms. Am J Physiol Renal Physiol 283: F895–F903, 2002. First published June 18, 2002; 10.1152/ajprenal.00354.2001.—In this study, we analyzed the effect of a therapeutic intervention in 46 enuretic children, 26 (57%) of whom were hypercalciuric. All the patients (n = 46) were treated with DDAVP for 3–6 mo. The hypercalciuric patients (n = 26) received a low-calcium diet (~500 mg/day) for the same period. After the therapy, the bed-wetting episodes stopped in 80% of the 46 patients tested. In those patients having low-AVP levels before the therapy, circulating AVP concentration returned to normal (>4 pg/ml), and the hypercalciuria was resolved in the hypercalciuric patients (calcium/creatinine ratio <0.2). Urinary aquaporin-2 (AQP2) levels were semiquantified by densitometric scanning and reported as a ratio between the intensity of the signal in the day vs. the night urine samples (day/night AQP2 ratio). In the hypercalciuric patients, the day/night AQP2 ratio returned to values close to those found in the healthy children (from 1.19 ± 0.20 before to 0.69 ± 0.10 after the treatment, n = 26, P = 0.03). In contrast, in the normocalciuric children we saw no significant modulation of AQP2 excretion (from 1.07 ± 0.14 before to 0.99 ± 0.14 after the treatment, n = 20). This study clearly demonstrates that urinary calcium levels modulate AQP2 excretion and is likely to be useful for treatment of children with enuresis.

aquaporin; hypercalciuria; enuresis; calcium-sensing receptors; vasopressin

Nocturnal enuresis is a very frequent chronic disorder in childhood that is characterized by high urine output during the night (11). In most children with primary monosymptomatic nocturnal enuresis, plasma AVP does not increase during the night as it does in the healthy children (2, 13). A first-line therapy for nocturnal enuresis is long-term treatment with nasal DDAVP, which was found useful in improving clinical symptoms, but there was some evidence that this was not sustained after treatment stopped (4, 9). Kuznetssova et al. (9) suggested that a decrease in reabsorption of osmotically active solutes plays a main role in inducing nocturnal enuresis and the beneficial effect of DDAVP would be due to a decrease in natriuresis. Disturbances in nocturnal urine production, bladder function, and arousal mechanisms have also been proposed as pathogenic factors in nocturnal enuresis (12). Present evidence suggests that childhood nocturnal enuresis must be regarded as a multifaceted problem with a variety of treatment interventions.

More recently, our laboratory proposed that absorptive hypercalciuria plays a crucial role in inducing nocturnal enuresis (15). Regulation of water excretion by the kidney predominantly occurs in the collecting duct and is regulated by the antidiuretic hormone AVP. In response to an increase in serum osmolality, AVP is released and binds to its V2 receptor on the basolateral membrane of collecting duct principal cells, with subsequent insertion of aquaporin-2 (AQP2) water channels in the apical membrane, rendering these cells water permeable (7, 8). AQP2 is also detectable in urine, and this excretion is proportionally increased, in response to AVP or thirsting, via a selective apical pathway (23). In an earlier report (21), our laboratory semiquantified AQP2 levels in enuretic children by using quantitative Western blotting. Our laboratory showed that the AQP2 levels correlated with the severity of enuresis in children and that absorptive hypercalciuria and reduced secretion of AVP are independently associated with a decrease in AQP2 urinary excretion.

In this study, we propose a therapeutic intervention that has a high cure rate and a relatively short treatment period. Comparison of urinary AQP2 levels in patients before and after the therapeutic intervention highlighted the close relationship among AVP secretion, hypercalciuria, nocturnal polyuria, and AQP2 expression/trafficking.

Methods

Patients and therapy. From April 1999 to February 2001, a total of 46 children with significant primary nocturnal enuresis histories (3 or more wet nights/wk) were admitted to the hospital (Policlinico, University of Bari) for a 48-h investiga-
tion after a therapeutic intervention (see below). During the same period, 11 healthy controls hospitalized for acute pharyngotonsillitis were also examined. For the control group, all the clinical parameters were evaluated the day before their discharge, when the children were completely healthy, with absolutely normal hydration status.

For all the children, we obtained special informed consent from their parents authorizing us to perform our routine investigations. All the nocturnal enuresis patients were treated with nasal DDAVP spray (10–30 mg/day) starting with a 3-mo titration period, followed by 15 days off to evaluate the response.

In the case of hypercalciuria, the patients received a continuous low-sodium (~5 g/day) and low-calcium diet (~500 mg/day). Follow-up visits were scheduled every 3 mo during the first year. Routine evaluation for all the patients included a detailed medical history, physical examination, micturition pattern, nocturnal enuresis, urine samples collected in two portions, night (8 PM–8 AM) and day (8 AM–8 PM). All urine samples were collected for 24 h with natural urination. In the children with nocturnal enuresis, urine was collected during the night with a plastic bag adapted to external genitalia. On the day when urine samples were collected, blood samples were taken from the decubital vein at 4 AM, the time at which maximal peak of AVP excretion is expected. Renin, aldosterone, and AVP levels were measured by radioimmunoassay (aldosterone bridge kit, Biochem Immunodies, Italia, Buhlmann Laboratories). Quantitative determination of parathyroid hormone (PTH) and 25-hydroxyvitamin D in human serum was performed with chemiluminescence assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) following the manufacturer’s instructions. Osteocalcin and calcitonin were measured by immunoenzymetric assay (DRG Diagnostic) following the manufacturer’s instructions. The concentration of creatinine in the urine samples was determined by standard automated techniques.

Urine processing. The AQP2 excretion in the urine was semiquantified in the 46 enuretic and 11 healthy children as described previously (21). Briefly, the day and night urine samples from each patient were spun down at 3,000 g for 10 min at 4°C to remove cellular debris in the presence of the following protease inhibitors: 2 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml pestatin. One hundred fifty micrograms of creatinine equivalents of each sample were then concentrated by ultrafiltration using Centricon tubes (Millipore, Bedford, MA) with 10,000-Da cutoff according to the manufacturer’s protocol. Concentrated proteins were subjected to immunoblot analysis to semiquantify the amount of AQP2 in the samples.

Immunoblotting. The immunoblotting of the urine samples with anti-human AQP2 antibody was performed as previously described (21). Briefly, concentrated urine samples were mixed with 1 vol of Laemmli sample, loaded on 13% acrylamide gels, and subsequently electroblotted on Immobilon-P (Millipore). The membranes were incubated with 1:300 dilution of rabbit anti-human AQP2 serum (a generous gift from Dr. Walter Rosenthal, Forschungsinstitut für Molekulare Pharmakologie, Berlin, Germany). The membranes were washed and incubated with anti-rabbit alkaline phosphatase 1:5,000 dilution (Sigma). Antigen-antibody reactions were visualized by using the substrates 0.56 mM 5-bromo-4-chloro-3-indolyl phosphate and 0.48 mM nitro blue tetrazenium in 10 mM Tris·HCl (pH 9.5). Controls using preimmune serum or omission of the primary or secondary antibody revealed no labeling.

Density of the 29-kDa band of AQP2 was measured by densitometry and semiquantified by using Image software (11), an image processing and analysis program for Macintosh computers developed at the National Institutes of Health. The ratio between the densitometric units of the daytime vs. the nighttime 29-kDa band of AQP2 was calculated for each patient.

Statistical analysis. Values are presented in the text as means ± SE. Comparisons between groups were made by Student’s unpaired t-test. A P value <0.05 was considered significant.

RESULTS

In this study, we analyzed the effect of a therapeutic intervention in a population of 46 enuretic children with a mean age of 9.7 yr who were previously analyzed for urinary AQP2 excretion (21). A group of 11 healthy children (G4) of the same mean age was enrolled as the control. The enuretic children were divided into three subgroups: G1, low nocturnal AVP levels and higher nighttime than daytime diuresis; G2, low nocturnal AVP levels and balanced nighttime and daytime diuresis; and G3, normal AVP levels and lower nighttime than daytime diuresis. Among them, nearly 57% were hypercalciuric, suggesting that hypercalciuria might play a significant role in inducing nocturnal enuresis. A specific purpose of this study was to investigate whether lowering urinary calcium (UCa) concentration would result in an amelioration of the clinical symptoms and analyze whether this might have any effect on the modulation of urinary AQP2 excretion. The therapeutic intervention consisted of the hypercalciuric children following a low-calcium diet (~500 mg/day) for 3–6 mo. All the patients were treated with DDAVP during the same period. After this period of strictly controlled diet, the hypercalciuric children were asked to maintain their daily calcium intake with a diet slightly below average normal needs, corresponding to ~1,000 mg/day.

To have more information on the safety of the therapeutic approach, in a subgroup of the treated children, we compared bone mineral density, PTH, 25-hydroxyvitamin D, phosphate, plasma calcium, osteocalcin, and calcitonin levels measured before and after the treatment (Table 1). The bone mineral densitometry (spinal vertebrae) was measured after 6 mo of the low-calcium diet. As shown in Table 1, despite the fact that the low-calcium intake followed by the patients enrolled in this study is somewhat below the Recommended Dietary Allowances (800–1,200 mg/day), no significant modifications of all the parameters were observed.

Table 2 summarizes the principal parameters evaluated in enuretic children from the three groups before and at least 6 mo after the therapy was stopped. The parameters were also compared with a control group of 11 healthy children (G4). On average, very similar results were obtained in patients from G1 and G2. In both groups, the therapy was 90% effective, with the
Table 1. Evaluation of the low-Ca diet effects on some clinical parameters related to calcium handling

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Enuretic Children (n = 7)</th>
<th>Normal Values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Bone mineral densitometry, z score</td>
<td>+0.90 ± 0.01</td>
<td>+0.87 ± 0.02</td>
</tr>
<tr>
<td>Phosphate, mg/ml</td>
<td>4.1 ± 1.0</td>
<td>4.1 ± 1.2</td>
</tr>
<tr>
<td>Parathyroid hormone, pg/ml</td>
<td>22.8 ± 4.0</td>
<td>28.4 ± 3.6</td>
</tr>
<tr>
<td>25 Hydroxyvitamin D, ng/ml</td>
<td>27.3 ± 10.7</td>
<td>34.0 ± 12.0</td>
</tr>
<tr>
<td>Osteocalcin, pg/ml</td>
<td>24.8 ± 2.9</td>
<td>19.2 ± 4.0</td>
</tr>
<tr>
<td>Calcitonin, pg/ml</td>
<td>5.8 ± 0.77</td>
<td>5.0 ± 0.89</td>
</tr>
<tr>
<td>Plasma calcium, mg/dl</td>
<td>9.93 ± 0.37</td>
<td>9.90 ± 0.40</td>
</tr>
</tbody>
</table>

Values are means ± SE. Statistical analysis was made by Student’s t-test for paired data comparing corresponding values before and after the treatment. All the clinical parameters evaluated were not significantly different after the treatment.

enuretic episodes coming to a halt in 3–4 mo. After the therapy, the night/day (N/D) diuresis ratio returned close to values found in the healthy children (from 1.63 ± 0.05 to 0.71 ± 0.05 for G1, P < 0.001; from 1.00 ± 0.04 to 0.66 ± 0.05 for G2, P < 0.0001; control 0.49 ± 0.01). AVP levels, which were low in both G1 and G2, returned to normal (from 0.97 ± 0.13 to 3.97 ± 0.10 for G1, P < 0.0001; from 1.19 ± 0.13 to 4.30 ± 0.21 for G2, P < 0.0001; control 4.32 ± 0.13), and this was associated with a significant increase in urine osmolality for both groups (from 895.7 ± 40.7 to 922.5 ± 24.5 for G1; from 816.3 ± 32.8 to 915.0 ± 25.0 for G2; control 941.7 ± 19.5 mosmol/kg H2O). In addition to the normalization of AVP levels, renin and aldosterone levels significantly increased after the therapy (Table 2). This is probably due to a feedback mechanism reflecting an overall amelioration of renal function.

No modifications in fractional urinary sodium or potassium excretion were observed (FENa and FEK, respectively). Interestingly, in both groups, nocturnal hypercalciuria was resolved in the hypercalciuric children, reaching values of UCa/UCr < 0.2. The hypercalciuric patients had normal plasma calcium (~10 mg/dl; not shown) and normal PTH levels (~24 ng/ml; not shown).

A very different picture emerged from the analysis of G3. Six of fourteen patients (43%) tested continued to experience nocturnal enuresis with no apparent amelioration of the clinical symptoms. On average, the efficacy of the therapy was very low in this group, and only 28% of the patients recovered completely.

To semiquantify AQP2 excreted in the urine after the therapy, the 24-h urine samples were collected for each patient in two portions, night (8 PM–8 AM) and day (8 AM–8 PM). Routinely, 150 μg of creatinine equivalents of the concentrated urine samples were loaded onto the gels, and AQP2 was semiquantified by Western blot analysis as previously described (21). By semiquantitative densitometry, we evaluated the ratio of the AQP2 signal detected in the daytime vs. the

Table 2. Principal parameters evaluated in the enuretic children before and after the therapy

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Enuretic Children</th>
<th>Control G4 (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Daytime diuresis, ml</td>
<td>379.23 ± 29.5</td>
<td>566.9 ± 30.6†</td>
</tr>
<tr>
<td>Nighttime diuresis, ml</td>
<td>630.9 ± 44.5</td>
<td>395.4 ± 24.3†</td>
</tr>
<tr>
<td>24-h diuresis, ml</td>
<td>990 ± 68.2*</td>
<td>926.3 ± 23.9</td>
</tr>
<tr>
<td>N/D ri</td>
<td>1.63 ± 0.05</td>
<td>0.71 ± 0.05†</td>
</tr>
<tr>
<td>Urine osmolality, mosm/kg H2O</td>
<td>695.7 ± 40.7</td>
<td>922.46 ± 24.5†</td>
</tr>
<tr>
<td>AVP, pg/ml</td>
<td>0.97 ± 0.13</td>
<td>3.97 ± 0.10†</td>
</tr>
<tr>
<td>UCa/UCr, D</td>
<td>0.16 ± 0.02</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>UCa/UCr, N</td>
<td>0.27 ± 0.03*</td>
<td>0.18 ± 0.01*</td>
</tr>
<tr>
<td>FEK, D</td>
<td>0.67 ± 0.06</td>
<td>0.63 ± 0.06</td>
</tr>
<tr>
<td>FEK, N</td>
<td>0.92 ± 0.10</td>
<td>0.80 ± 0.06</td>
</tr>
<tr>
<td>FEna, D</td>
<td>11.54 ± 15.1</td>
<td>12 ± 0.9</td>
</tr>
<tr>
<td>FEna, N</td>
<td>9.07 ± 0.88</td>
<td>9.23 ± 0.87</td>
</tr>
<tr>
<td>Renin, μg·mL·h⁻¹</td>
<td>2.18 ± 0.36</td>
<td>3.26 ± 0.16*</td>
</tr>
<tr>
<td>Aldosterone, μg·mL·h⁻¹</td>
<td>101.05 ± 19.5</td>
<td>311.19 ± 12.7†</td>
</tr>
</tbody>
</table>

Values are means ± SE. UCa, D/UCr, D, daytime urinary calcium/vitamin creatinine ratio; UCa, N/UCr, N, nighttime urinary calcium/vitamin creatinine ratio; FEK, D, daytime fractional excretion of sodium (%); FEK, N, nighttime fractional excretion of sodium (%); FEna, D, daytime fractional excretion of potassium (%); FEna, N, nighttime fractional excretion of potassium (%); G1, group 1, low nocturnal AVP levels and a higher nighttime than daytime diuresis; G2, group 2, low nocturnal AVP levels and a balanced nighttime and daytime diuresis; G3, group 3, normal AVP levels and a lower nighttime than daytime diuresis; G4, group 4, normal AVP levels and nighttime diuresis one-half that of daytime diuresis; †P < 0.05; *P < 0.0001 (compared with corresponding values before the treatment); nm, not measured; N/D, nighttime/daytime.

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nighttime urine sample for each patient from each group before and after the therapeutic intervention. The day/night (D/N) AQP2 ratio is a good index of the modulation of AQP2 excretion in the daytime and nighttime urine samples, because AQP2 excretion is evaluated related to its internal control, thus overcoming the high variability of the parameter among different subjects.

Figure 1 shows a representative Western blotting of the urine samples taken from a healthy child and from a hypercalciuric patient before and after the therapy. The urine samples were probed with specific anti-human AQP2 antibodies. As previously shown in the healthy children, the AQP2 levels detected in the daytime urine samples were nearly one-half those detected in the nighttime urine sample (21). In contrast, the situation is reversed in most of the enuretic children (Fig. 1, before therapy). Of note, the therapy restored urinary AQP2 excretion to levels similar to those found in control children (Fig. 1, after therapy).

Figure 2 compares the N/D diuresis ratio and the D/N AQP2 ratio obtained in the three groups of enuretic children before and after the therapeutic intervention.

In both $G_1$ and $G_2$, the N/D diuresis ratio decreased significantly, with the strongest remodulation in $G_1$, consistent with an end to enuretic episodes in nearly 90% of those children. Quite interestingly, these results were very well paralleled by a significant proportional decrease in the D/N AQP2 ratio ($G_1$, from $1.41 \pm 0.26$ before to $0.69 \pm 0.13$ after the therapy, $P < 0.05$; $G_2$, from $1.11 \pm 0.18$ before to $0.70 \pm 0.10$ after the therapy, $P < 0.05$), and those ratios were close to values obtained in the healthy children ($0.59 \pm 0.11$). Again, these results confirmed the close relationship between diuresis and urinary AQP2 levels and underscored the efficacy of the therapy. As mentioned, the therapy also restored the circulating AVP levels in both $G_1$ and $G_2$ (Table 2).

In contrast, in the enuretic patients from $G_3$ (14 children), the D/N AQP2 ratio was not significantly modified after the therapeutic intervention (Fig. 2, $G_3$), and, consistently, no significant amelioration of the N/D diuresis ratio was observed. As mentioned, the efficacy of the therapy was very low in this group, and this is in agreement with the mean data obtained.

To evaluate the role of hypercalciuria in determining nocturnal enuresis, we divided the entire population into hypercalciuric ($U_{Ca}/U_{Cr} > 0.21$) and normocalciuric ($U_{Ca}/U_{Cr} < 0.21$) children in an attempt to determine whether lowering the external $U_{Ca}$ concentration with a low-calcium diet had any significant specific effect on the D/N AQP2 ratio and, in turn, on the nocturnal polyuria. Figure 3 summarizes the results obtained before and after the therapy, considering all the enuretic children tested ($G_1$–$G_3$ together), for which the data were calculated by distinguishing the hypercalciuric and the normocalciuric children in the total population of the three groups.

Clearly, on average, the hypercalciuria was resolved in the hypercalciuric children ($U_{Ca}/U_{Cr} > 0.21$), and this resulted in a significant decrease of both the N/D diuresis ratio and the D/N AQP2 ratio close to the normal values, respectively (Fig. 3). In contrast, for the normocalciuric children, despite a clear significant reduction in the N/D diuresis ratio, this result was not paralleled by a concomitant reduction in the D/N AQP2 ratio (Fig. 3). This indicates that hypercalciuria plays a major role in remodulating urinary AQP2 excretion.

As mentioned above, the therapy had a similar cure rate (90%) in the patients from $G_1$ and $G_2$, all the members of which were characterized by low AVP levels before the therapy, whereas a much lower percentage of recovery was seen in the patients from $G_3$ (<50%). To investigate the effect of the therapy in greater depth, the parameters evaluated in Fig. 3 were analyzed by grouping the two more homogeneous $G_1$ and $G_2$ patients together and analyzing $G_3$ separately. Figure 4 reports the analysis of the same parameters evaluated in Fig. 3, calculated in the $G_1$ and $G_2$ patients before and after the therapy.

Both the hypercalciuric and normocalciuric children showed a clear striking reduction in the N/D diuresis ratio, but only in the hypercalciuric children was this accompanied by a parallel restoration of the D/N AQP2 ratio; no statistically significant modification of this ratio was found in the normocalciuric children.

However, in the normocalciuric children from $G_1$ and $G_2$, it has to be emphasized that a clear tendency to a reduction in the D/N AQP2 ratio was observed. Overall, these data point to a crucial role of hypercalciuria in modulating AQP2 excretion in urine.

![Fig. 1. Representative Western blotting of the daytime and nighttime urine samples probed with anti-human aquaporin-2 (AQP2) antibody. Urine samples from hypercalciuric children ($G_1$) were tested before and after the therapeutic intervention. Note the inversion of the relative amounts of AQP2 in the urine samples probed after the therapy, which matches the distribution observed in the healthy children (Control).](http://ajprenal.org)
As mentioned, the therapy in G3 was, on average, much less efficient at stopping the bed-wetting episodes. Most of the patients still experienced nocturnal enuresis, and interestingly, also the AQP2 excretion was not normalized in either the hypercalciuric or the normocalciuric patients (Fig. 5). Rather unexpectedly, in some cases we even observed a marked increase in the D/N AQP2 ratio. It is, however, possible that the low number of patients who recovered after the treatment (4 of 14) makes it difficult to draw any conclusions on the correlation between hypercalciuria and modulation of urinary AQP2 excretion in this group.

Taking into account all the enuretic children tested after the treatment (G1–G3), we calculated that the efficacy of the therapy was ~81% in the hypercalciuric children and 80% in the normocalciuric children. Interestingly, the effect of the therapy on the modulation of urinary AQP2 excretion was clear only in the hypercalciuric children; whereas, in the normocalciuric children who recover after the treatment, we saw no statistically significant modulation of the AQP2 excretion.

Fig. 2. A: nighttime/daytime (N/D) diuresis ratio for G1–G3 measured before and after the therapy. B: creatinine equivalents of the daytime and nighttime urine samples were analyzed by Western blot for expression of AQP2 before and after the therapy in the enuretic children from G1 to G3. The densitometry of the 29-kDa band of AQP2 was calculated for each patient from each group, and the ratio between the signal in the daytime and the nighttime urine sample (D/N AQP2 ratio) was calculated. Values are mean ± SE of the obtained values for each group. n.s., Not significant. *P < 0.05, ***P < 0.0001 compared with the ratio calculated before the therapy.

Fig. 3. A: creatinine equivalent calcium levels (U_{Ca}/U_{Cr}) in the day/night (D/N) urine samples for all the patients from the three groups before and after the therapy (G1–G3). The estimated normal value of U_{Ca}/U_{Cr} is <0.2, corresponding to a normal calcium excretion in the urine (<4 mg-kr⁻¹-day⁻¹). U_{Ca}/U_{Cr} > 0.2 characterized the hypercalciuria. B: N/D diuresis ratio for each group measured before and after the therapy. C: D/N AQP2 ratio for each group measured before and after the therapy. TP, total population; HC, hypercalciuric children; NC, normocalciuric children. Values are mean ± SE. *P < 0.05, ***P < 0.0001, compared with the ratio calculated before the therapy.
To further confirm that lowering extracellular calcium has a major role in modulating AQP2 excretion, we stopped the low-calcium diet in four patients randomly chosen from G1 and G2. The combination of DDAVP and low-calcium diet was successful in lowering the nighttime UCa (Fig. 7, Cured children, night-Uca) and in lowering the D/N AQP2 close to control levels (Fig. 7, Cured children, D/N AQP2). Under those conditions, the patients were dry. Of note, when the low-calcium diet was stopped in those patients for 10 days, the night UCa increased with a concomitant significant increase in the D/N AQP2 ratio despite the fact that they were still under the DDAVP treatment (Fig. 7, Diet suspension + DDAVP). These conditions were associated with restarting of the bed-wetting episodes (N/D diuresis ratio from 0.6 ± 0.1 in cured children to 0.9 ± 0.1 in the same children after the diet suspension). These data support the view of the direct effect of extracellular calcium on urinary AQP2 excretion.

**Fig. 6.** To further confirm that lowering extracellular calcium has a major role in modulating AQP2 excretion, we stopped the low-calcium diet in four patients randomly chosen from G1 and G2. The combination of DDAVP and low-calcium diet was successful in lowering the nighttime UCa (Fig. 7, Cured children, night-Uca) and in lowering the D/N AQP2 close to control levels (Fig. 7, Cured children, D/N AQP2). Under those conditions, the patients were dry. Of note, when the low-calcium diet was stopped in those patients for 10 days, the night UCa increased with a concomitant significant increase in the D/N AQP2 ratio despite the fact that they were still under the DDAVP treatment (Fig. 7, Diet suspension + DDAVP). These conditions were associated with restarting of the bed-wetting episodes (N/D diuresis ratio from 0.6 ± 0.1 in cured children to 0.9 ± 0.1 in the same children after the diet suspension). These data support the view of the direct effect of extracellular calcium on urinary AQP2 excretion.
**DISCUSSION**

Hypercalciuria is a common problem consistently associated with nocturnal enuresis, which leads to risk of renal stone formation. It results from a renal tubular calcium leak or intestinal hyperreabsorption of calcium. On the basis of our previous findings (21) demonstrating that hypercalciuria is associated with a decrease in urinary AQP2 excretion, this study was undertaken to investigate the determinants that may correlate urinary AQP2 excretion and absorptive hypercalciuria.

A quantitative Western blot analysis of urinary AQP2 was performed to determine the renal responsiveness to DDAVP administration associated with a low-calcium diet in the case of hypercalciuria in the enuretic children. Specific treatments were also applied in the case of altered urodynamic patterns. The study group comprised 46 children who were screened for hypercalciuria by means of the UCa/UCr ratio, as well as for urinary AQP2 excretion before and after the therapeutic intervention. Besides those parameters, a complete clinical evaluation (plasma AVP, renin, aldosterone, urine osmolality, diuresis, FEK, FEna) was performed for each patient.

The major effects observed after the therapeutic intervention can be summarized as follows. First, the therapy rectified both AVP levels and hypercalciuria. Second, in both the normocalciuric and the hypercalciuric children, urine osmolality returned to normal, with complete dryness in 81% of the hypercalciuric and 80% of the normocalciuric children. Third, lowering the UCa/UCr ratio resulted in a parallel decrease in the D/N AQP2 ratio in the hypercalciuric children from G1 and G2, whereas no such obvious correlation was found in the normocalciuric subjects from the same groups. Finally, the therapeutic intervention failed in nearly 50% of the subjects from G3.

**AVP levels and urinary AQP2 excretion.** As mentioned, the modulation of urinary AQP2 excretion was clear only in the hypercalciuric children, whereas, in the normocalciuric children who recovered after the treatment, we saw no significant modulation of the urinary D/N AQP2 ratio, although we can observe a clear tendency to a decrease in this value if our analysis is restricted to G1 and G2. It is known that ~3% of AQP2 in the collecting duct is excreted into urine, and in normal subjects, there is a positive correlation with plasma AVP levels (6, 17, 18). This AVP-dependent AQP2 excretion is a result of stimulation of AQP2 mRNA expression in the kidney (3, 10). Conversely, it is well established that downregulation of AQP2 occurs in multiple forms of nephrogenic diabetes insipidus (1, 5, 6, 14, 22). The detailed analysis of the normocalciuric patients from G1 and G2 (Fig. 4), all characterized by low-circulating AVP levels, shows that after the therapy, the D/N AQP2 ratio did not return to values...
found in the control children, even though 90% of those patients recovered from bed-wetting episodes. The following considerations may give some hints in explaining this effect.

First, in this study we evaluated the D/N AQP2 ratio for each patient, which is a good index of the modulation of the AQP2 excretion in the daytime and nighttime urine samples but gives no information on the absolute amount of the excreted AQP2. It is therefore possible that the increase in the AQP2 expression/trafficking (matched by an increase in the AQP2 excretion) that might occur during the night in the patients after the treatment as a result of normalization of circulating AVP levels and that may account for the observed tendency to a decrease in the D/N AQP2 ratio is per se sufficient for the reestablishment of urinary concentrating capabilities, especially during the night, thus avoiding the nocturnal enuretic episodes. However, this decrease might not be sufficient to make the D/N AQP2 ratio low enough to reach significance after the therapy, compared with values before the therapy. It is therefore likely that even in those patients, the urinary AQP2 reflects the action of AVP on the collecting duct, as reported in several studies (16).

**Hypercalciuria and AQP2 excretion.** In our previous study, we showed that high levels of calcium are associated with an increase in the D/N AQP2 ratio. Here, we show that after the therapy in the children from G1 and G2 displaying hypercalciuria (U_Ca/U_Cr > 0.21) and exposed to high-calcium levels during the night, the nighttime hypercalciuria returned to normal levels. In those patients, the most striking observation was the strict positive correlation between the urinary D/N AQP2 ratio and the U_Ca concentration. A clear remodulation of the D/N AQP2 ratio to normal values, very well paralleled by the normalization of the U_Ca concentration, was found in those patients after the treatment with DDAVP associated with the low-calcium diet. Although the normocalciuric patients from G1 and G2 were treated with DDAVP and this resulted, as previously mentioned, in a sensitive but not statistically significant decrease in the D/N AQP2 ratio, the hypercalciuric patients were treated with both the DDAVP and the low-calcium diet, and this had a clear effect in rectifying the D/N AQP2 ratio to normal values. This result validates the hypothesis that luminal calcium modulates AVP-elicited hydromosic response as a principal direct consequence of modulation of AQP2 expression/trafficking. Together, those findings provide a link between calcium and water homeostasis, implying that an apical membrane signaling mechanism links calcium and water permeability in the renal collecting duct.

Before the therapeutic intervention, the hypercalciuric patients from G1 and G2 were exposed to high-calcium levels during the night but not during the day (Fig. 4). The resulting hypercalciuria contributed to the production of a renal concentrating defect manifested as nocturnal polyuria. To explain this effect, a calcium-sensing signal transduction complex must link the U_Ca levels to the AQP2 expression in the collecting duct. The presence of an extracellular calcium-sensing receptor protein has been reported in the apical membrane of the rat collecting duct (19). In isolated perfused rat inner medullary collecting duct, acute increases in the luminal calcium from 1 to 5 mM caused a rapid 30% reduction in AVP-elicited plasma flow (20). In addition, during sustained hypercalcemia in chronically hypercalcemic rats, the plasma flow in the inner medullary collecting duct did not increase significantly after AVP and was accompanied by ~87% reduction in AQP2 protein but not mRNA (19), which may contribute to the lack of AVP responsiveness. Moreover, chronic hypercalcemia is associated with reduced thick ascending limb NaCl reabsorption, which also contributes to the presence of a renal concentrating defect. Indeed, compared with controls, the FE Na during the night in all the patients was significantly higher before the treatment and remained unchanged after the therapy (Table 2). It is therefore likely that the improvement in the kidney concentrating ability obtained in those children after the therapeutic intervention, and leading to the normal U_Ca/U_Cr values, is a main consequence of the restoration of AQP2 trafficking/expression. In fact, in contrast with a previous finding by Kuznetsova et al. (9), we did not observe a decrease in natriuresis in the patients treated with DDAVP. A key finding of this study was the observation that the bed-wetting episodes restarted shortly after suspension of the low-calcium diet in the children despite the treatment with DDAVP (Fig. 7). This condition was accompanied with a simultaneously significant increase of the nighttime U_Ca and the D/N AQP2 ratio. Although the number of examined patients was low (n = 4), this result highlights the strict correlation between the hypercalciuria and AQP2 excretion in the urine.

The group displaying the lower rate of patient recovery was G3 (nearly 50% recovery). Those patients were treated with DDAVP, despite the normal plasma values observed. In the subpopulation of hypercalciuric children from this group, the association of DDAVP treatment with the low-calcium diet improved, but on average did not resolve, the hypercalciuria. The N/D diuresis ratio decreased but did not return to normal levels, and no significant modification of the D/N AQP2 was seen. Conversely, in the normocalciuric children from this group, the N/D diuresis ratio remained unchanged after the treatment, and the D/N AQP2 ratio paradoxically had a tendency to increase after the treatment. However, a more complex etiology of the nocturnal enuresis might be found in those patients whose treatment times were longer.

**Conclusions.** The results in the present study provide new insights into the renal mechanisms involved in nocturnal enuresis. We propose here a therapeutic intervention that allows, overall, a high cure rate over a relatively short treatment time. Although it is clear that the concentration defect observed in the enuretic children is a consequence of multiple factors that may alter both the generation of medullary interstitial hypertonicity and osmotic equilibration in the collecting ducts, further studies are required to investigate this hypothesis.
duct, quantitative analysis of AQP2 in the urine highlighted the close relationship among AVP secretion, hypercalciuria, nocturnal polyuria, and AQP2 expression/trafficking. This study clearly demonstrated that the amelioration of clinical symptoms of nocturnal enuresis after the therapy is accompanied by the regulation of urine output through the remodulation of AQP2 expression/trafficking. Another key point addressed in our study was the strict positive correlation between the hypercalciuria and AQP2 excretion in the urine, which might prevent formation of calcium-containing renal stones. The signals involved in such changes remain to be discovered but may represent a means by which long-term modulation of urinary concentrating ability can be controlled.

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