Hyperosmolality In Vivo Upregulates Aquaporin 2 Water Channel and Na-K-2Cl Co-Transporter in Brattleboro Rats

Chunling Li,* Weidong Wang,* Sandra N. Summer,* Melissa A. Cadnapaphornchai,*† Sandor Falk,* Fuminori Umenishi,* and Robert W. Schrier*

Departments of *Medicine and †Pediatrics, University of Colorado School of Medicine, Denver, Colorado

There are considerable experimental results that indicate that arginine vasopressin (AVP)-independent factors are involved in urinary concentration. This study examined the role of hyperosmolality in vivo to modulate aquaporin 2 (AQ2) and Na-K-2Cl co-transporter (NKCC2), pivotal factors in urinary concentration, in AVP-deficient Brattleboro (BB) rats. Hyperglycemia with associated hyperosmolality occurred in diabetic BB rats (BBDM). Protein abundance of AQ2 increased and was reversed by insulin in the inner medulla (IM; control 100 ± 5%; BBDM 146 ± 8%; BBDM+Ins 122 ± 9%; P < 0.001) and inner stripe of outer medulla (ISOM; control 100 ± 4%; BBDM 123 ± 8%; BBDM+Ins 93 ± 6%; P < 0.05). These results were confirmed by immunohistochemistry studies. NKCC2 rose in the ISOM but was not reversed with insulin treatment. For investigation of the role of hyperosmolality in the absence of hyperglycemia on the regulation of the expression of renal AQ and NKCC2, studies were performed with hyperosmolality that was induced by 0.5% NaCl in drinking water in BB rats. Hyperosmolality that was induced by NaCl increased significantly the protein abundance of IM AQ2 (121 ± 2 versus 100 ± 5%; P < 0.01), ISOM AQ2 (135 ± 6 versus 100 ± 5%; P < 0.001), cortex plus outer stripe of outer medulla AQ2 (121 ± 4 versus 100 ± 1%; P < 0.001), ISOM NKCC2 (133 ± 1 versus 100 ± 4%; P < 0.05), and cortex plus outer stripe of outer medulla NKCC2 (142 ± 16 versus 100 ± 9%; P < 0.05). In conclusion, hyperosmolality, secondary to either glucose or NaCl, upregulated renal AQ2 and NKCC2 in vivo in BB rats.

Renal water excretion is regulated by arginine vasopressin (AVP), which increases the water permeability in the collecting duct (CD) (1,2). Recently, the water channel aquaporin 2 (AQ2) that mediates the water transport in the CD in response to vasopressin was identified (3). It is widely known that AQ2 is the main target for the action of vasopressin to regulate CD water reabsorption and hence body water balance via the V2 receptor and cAMP pathway (4).

There is, however, some in vitro evidence for vasopressin-independent effects on the regulation of water channels. For example, it has been demonstrated that hypertonicity enhances the expression and the stability of AQ1 in the absence of AVP in cultured murine renal medullary cells (5). Hyperosmolality also increased the expression of AQ3 in vitro (6). In recent in vitro studies, a long-term (24 h) increase of extracellular tonicity by addition of NaCl or sucrose increased AQ2 expression via stimulation of AQ2 gene transcription (7). In mouse inner medullary CD mIMCD3 cell lines that were transfected with AQ2 cDNA, hyperosmolality with supplemented NaCl increased the expression of nonglycosylated AQ2 and enhanced the apical membrane insertion of nonglycosylated AQ2 in the absence of AVP. These results indicate that hyperosmolality may play a critical role in the regulation of AQ2 in the CD (8). Taken together, these in vitro studies suggest that hyperosmolality could be an important vasopressin-independent regulator of AQ2 in vivo.

In addition, Na-K-2Cl co-transporter (NKCC2) is known to play a very important role in initiating the countercurrent mechanism for urinary concentration. The transport activity of NKCC2, exogenously expressed in Xenopus laevis oocytes, has been shown to be stimulated by hypertonicity (9). Therefore, these in vitro studies indicate that hyperosmolality could be an important factor in vivo in regulating not only water channels but also NKCC2 expression. The in vivo effect of hyperosmolality independent of AVP on renal AQ2 and NKCC2, however, is not known. The purpose of our study, therefore, was to examine the effect of hyperosmolality, with either glucose or NaCl, to upregulate AQ2 and NKCC2 in Brattleboro (BB) rats, which have no detectable endogenous AVP.

Materials and Methods
Animal Model

The study protocol was approved by the University of Colorado Institutional Animal Care and Use Committee. Male BB rats that weighed between 150 and 175 g were allowed to acclimate to Denver’s altitude (1500 m) for 1 wk before any experimental protocols. The rats were housed individually in metabolic cages, exposed to a 12-h light-dark cycle and constant ambient temperature, and maintained on a standard rodent diet (Harlan Teklad Bioproducts, Indianapolis, IN) with free access to water.
Rats were allocated to the protocols indicated next. Age- and time-matched controls were prepared and observed in parallel with the untreated diabetic group, diabetic group treated with insulin, and the NaCl drinking water group in the following two protocols.

**Protocol 1 (Diabetic BB Rats)**

**Group 1.** Vehicle (0.2 ml of 0.1 M citrate buffer [pH 4.0]; Sigma, St. Louis, MO) was injected into a femoral vein of 18 control BB rats (BBCTR). The day of injection was designated as day 0.

**Group 2.** Streptozotocin (STZ; Roche Diagnostics Corporation, Indianapolis, IN; 40 mg/kg body wt prepared fresh in 0.1 M citrate buffer [pH 4.0]; Sigma) was injected into a femoral vein of 18 BB rats (BBDM) at 7 a.m. (10). Diabetes was confirmed by measuring spot urine glucose at 24 and 48 h after STZ injection. Glucose levels in blood that was obtained from the tails of the BB rats were monitored daily with an Accu-Chek glucometer (Boehringer Mannheim Corp., Indianapolis, IN). At days 12 to 14, nine rats were given 5 mg of V2-receptor antagonist OPC-31260 (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) mixed with food, twice a day.

**Group 3.** STZ was injected into nine BB rats. Glucose levels in blood were monitored while daily with a glucometer. At day 3, rats were given 5 mg of V2-receptor antagonist OPC-31260 (Otsuka Pharmaceutical Co., Ltd., Tokushima, Japan) twice a day.

**Protocol 2 (Nondiabetic BB Rats)**

**Group 1.** Nine control BB rats (BBCTR) were allowed free access to water and food.

**Group 2.** Nine BB rats were given 0.5% NaCl in drinking water (BBNaCl), with limited water intake to 200 ml/d per rat for 5 d. Rats were allowed free access to food.

Urines were collected and urine osmolality, creatinine, and glucose were measured. Rats were killed by decapitation. Trunk blood was collected for plasma AVP concentrations, serum osmolality, serum sodium, and serum creatinine concentrations. Serum and urine osmolality was measured by freezing-point depression (Advanced Instruments, Inc., Norwood, MA). Serum and urine creatinine were measured by an autoanalyzer (Beckman Instruments, Inc., Fullerton, CA). Serum sodium concentrations were measured by flame photometry. Blood glucose level was measured using a single-touch glucometer as described above. Plasma AVP concentration was assessed by RIA as described previously (11).

### Protein Isolation

After decapitation, kidneys were placed in ice-cold isolation solution that contained 250 mM sucrose, 25 mM imidazole, 1 mM EDTA (pH 7.2) with 0.1 vol% protease inhibitors (0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, and 1 μg/ml aprotinin), and 200 μM PMSF. Kidneys were dissected on ice into cortex plus outer stripe of outer medulla (OSOM+C), inner stripe of outer medulla (ISOM), and inner medulla (IM) regions. Tissue samples were homogenized immediately in a glass homogenizer at 4°C. After homogenization, protein concentration was determined for each sample by the Bradford method (Bio-Rad, Richmond, CA). Tissue protein was used for immunoblotting for AQP water channels and sodium and urea transporters.

### Electrophoresis and Immunoblotting

Samples of membrane fractions were run on 10 or 12% acrylamide gels. For each gel, an identical gel was run in parallel and subjected to Coomassie staining for confirming equal loading of protein. The other gel was subjected to Western blotting analysis. After transfer by electrotelution to nitrocellulose membranes, blots were blocked with 5% milk in PBS-T (80 mM Na2HPO4, 20 mM NaH2PO4, 100 mM NaCl, and 0.1% Tween 20 [pH 7.5]) for 1 h and incubated with primary antibodies (see next section) overnight at 4°C. After washing with PBS-T, the blots were incubated with horseradish peroxidase–conjugated secondary antibody (P448; Dako A/S, Glostrup Denmark; diluted 1:3000). After final washing as above, antibody binding was visualized using the ECL system (Amersham International, Little Chalfont, UK).

### Primary Antibodies

For semiquantitative immunoblotting and immunocytochemistry, antibodies to AQP2, AQP3, sodium-hydrogen exchanger (NHE3), and urea transporter A1 (UT-A1) have been characterized previously (12–15). Anti–Na-K-ATPase α1 antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-AQP1 was obtained from Chemicon International, Inc. (Temecula, CA). Anti–Na-K-2Cl antibody was provided by Dr. Mark A. Knepper (National Institutes of Health, Bethesda, MD).

### Table 1. Characteristics of BBDM rats with and without insulin treatment on last day of study

<table>
<thead>
<tr>
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<th>BBCTR</th>
<th>BBDM</th>
<th>BBDM+Ins</th>
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<tr>
<td>n</td>
<td>9</td>
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<tr>
<td>BGlucose (mg/dl)</td>
<td>103 ± 2</td>
<td>463 ± 29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>201 ± 41&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
<td>Sosm (mOsm/kg·H2O)</td>
<td>302 ± 3</td>
<td>317 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>291 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>SNa (mmol/L)</td>
<td>145 ± 3</td>
<td>138 ± 2</td>
<td>142 ± 3</td>
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<tr>
<td>Screa (mg/dl)</td>
<td>0.36 ± 0.02</td>
<td>0.33 ± 0.03</td>
<td>0.45 ± 0.07</td>
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<tr>
<td>UVol (μl/min per kg body wt)</td>
<td>298 ± 19</td>
<td>763 ± 51&lt;sup&gt;b&lt;/sup&gt;</td>
<td>547 ± 33&lt;sup&gt;b,c&lt;/sup&gt;</td>
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<tr>
<td>Usom (mOsm/kg·H2O)</td>
<td>165 ± 17</td>
<td>260 ± 26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>144 ± 11&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td>UGlucose (mg/dl)</td>
<td>0</td>
<td>500/1000</td>
<td>0</td>
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<sup>a</sup>Values are means ± SEM. BBCTR, control Brattleboro rats; BBDM, BB rats with diabetes induced by streptozotocin (STZ); BBDM+Ins, BB rats with diabetes induced by STZ with administration of insulin; BGlucose, blood glucose; Sosm, serum osmolality; SNa, serum sodium; Screa, serum creatinine and sodium; UVol, urine volume. Statistical analysis of results was performed using ANOVA with Student-Newman-Keuls.

<sup>b</sup><sup>P</sup> < 0.05 versus BBCTR rats.

<sup>c</sup><sup>P</sup> < 0.05 versus BBDM rats.
Immunocytochemistry

The kidneys were fixed by retrograde perfusion via the abdominal aorta with 3% paraformaldehyde, in 0.1 M cacodylate buffer (pH 7.4). Immunolabeling was performed on sections from paraffin-embedded preparation (2 μm thickness) using methods described previously in detail (16). The microscopy was carried out using a Leica DMRE light microscope.

Statistical Analyses

Values are presented as mean ± SE. Multiple group comparisons were performed using a one-way ANOVA with posttest according to Student-Newman-Keuls. Because unidirectional changes were expected with the intervention, i.e., improvement of the parameter examined, a one-way ANOVA was used. Comparisons between two groups were made by unpaired t test. P < 0.05 was considered statistically significant.

Results

Undetectable AVP in BB Rats

None of the BB rats had detectable plasma AVP concentration by RIA.

Figure 1. Immunoblot of membrane fractions of inner medulla (IM) in kidneys from Brattleboro control (BBCTR; n = 9), BB diabetic (BBDM; n = 9), and BBDM insulin-treated (BBDM + Ins; n = 9) rats. Immunoblots were reacted with affinity-purified anti-AQP2 (A), anti-AQP3 (B), and anti-Na-K-ATPase (C) antibodies. Densitometric analysis of all samples revealed that in the BBDM rats, expression of AQP2, AQP3, and Na-K-ATPase in the IM increased significantly compared with BBCTR rats. Insulin treatment significantly attenuated the rise in expression of AQP2, AQP3, and Na-K-ATPase in the IM that was seen in the nontreated BBDM rats.

Figure 2. Immunoblot of membrane fractions of inner stripe of outer medulla (ISOM) in the kidneys from BBCTR (n = 9), BBDM (n = 9), and BBDM + Ins (n = 9) rats. Immunoblots were reacted with affinity-purified anti-AQP2 (A) and anti-Na-K-2Cl co-transporter (anti-NKCC2; B) antibodies. Densitometric analysis of all samples revealed that in the BBDM rats, the expression of AQP2 and NKCC2 in the ISOM increased significantly compared with BBCTR rats. Insulin treatment attenuated the rise in the expression of AQP2 in the ISOM but not the increased expression of NKCC2 in the ISOM that was seen in the nontreated BBDM rats.
Diabetes that was induced by STZ treatment in vasopressin-deficient BB rats was associated with hyperglycemia and polyuria (Table 1). After STZ injection, from days 1 to 14, urine volume increased dramatically in BBDM rats compared with BBCTR. At days 4 to 14 after STZ injection, BBDM rats maintained elevated blood glucose levels. Serum osmolality in BBDM rats was increased in association with the hyperglycemia. With continuous long-acting insulin treatment, there was partial restoration of the blood glucose and urine flow toward control values (Table 1).

Reversal of Upregulation of Renal AQP2 in the IM and ISOM in BBDM-Ins Rats

Densitometric analysis from immunoblots revealed that the protein abundance of AQP2 in the IM and ISOM increased significantly in BBDM rats (Figures 1A and 2A). Insulin treatment reversed the upregulation of AQP2 in both IM and ISOM. Immunohistochemistry with AQP2 antibody confirmed these findings (Figure 3). In kidneys from BBCTR rats, immunohistochemistry demonstrated that AQP2 antibody labeled the apical plasma membrane domains and subapical vesicles in the CD principal cells in the IM and ISOM. In the BBDM kidneys, AQP2 labeling was much stronger in the CD principal cells in the IM and ISOM. In the BBDM+Ins kidneys, labeling density of AQP2 was no different compared with BBCTR rats. Furthermore, AQP3 and Na-K-ATPase expressions in the IM were upregulated in BBDM rats, and insulin treatment attenuated the increased expression levels of AQP3 and Na-K-ATPase in the IM (Figure 1, B and C), respectively.

In BBDM rats, the expression levels of NKCC2 increased significantly in the ISOM compared with BBCTR rats (Figure 2B). However, insulin treatment did not reverse increased expression levels of NKCC2 in the ISOM. These results were confirmed by immunohistochemistry (Figure 4). Immunohistochemistry showed that labeling of NKCC2 was seen in the apical plasma membrane domains of the medullary thick ascending limb cells in BBCTR rats (Figure 4A). In the BBDM rats, AQP3 and Na-K-ATPase expressions in the IM were upregulated in BBDM rats, and insulin treatment attenuated the increased expression levels of AQP3 and Na-K-ATPase in the IM (Figure 1, B and C), respectively.
the labeling of NKCC2 in the ISOM thick ascending limb cells was much stronger compared with that in BBCTR rats (Figure 4B). Insulin treatment did not reverse the strong labeling of NKCC2 that was seen in the BBDM kidneys (Figure 4C). Vasopressin V₂ receptor antagonist (OPC 31260) treatment increased urine flow (1005 ± 93 versus 774 ± 77 μl/min per kg; P < 0.05) in the BBDM rats.

### Nonsignificant Changes in Water Channels and Ion Transporters in BBCTR, BBDM, and BBDM+Ins Rats

The expression levels of AQP1 in the IM and OSOM+C were not different among BBCTR, BBDM, and BBDM+Ins groups. There was also no difference in UT-A1 expression between insulin-treated and nontreated BBDM rats. The protein expression levels of NHE3 in the ISOM and OSOM+C were no different. AQP2, NKCC2, and NHE3 in the OSOM+C were not different in BBCTR, BBDM, BBDM+Ins rats (Table 2).

### Hyperosmolality Induced by NaCl Loading Increased the Expression of AQP2 in the IM, ISOM, and OSOM+C as well as NKCC2 in the ISOM and OSOM+C Compared with BBCTR Rats

In Table 3 are shown the effects of NaCl drinking in BB rats (BBNaCl) on serum osmolality and sodium concentration, as well as urine volume, urine osmolality, and fractional excretion of sodium. The expression of AQP2 in the IM (Figure 5A), ISOM (Figure 5B), and OSOM+C (Figure 5C) increased significantly in BB rats with NaCl-induced hyperosmolality. The abundance of NKCC2 also was increased in the ISOM (Figure 6A) and OSOM+C (Figure 6B). There were no significantly changes in the expression of AQP3 in the IM; AQP1 in the IM; Na-K-ATPase in the IM, ISOM, and OSOM+C; UT-A1 in the IM; NHE3 in the ISOM and OSOM+C; and type 2 sodium-phosphate co-transporter in the OSOM+C in the BB rats with hyperosmolality (Table 4). Immunohistochemistry studies demonstrated an increase in AQP2 in the IM and NKCC2 in the ISOM with BBNaCl rats (Figure 7).

### Discussion

The effect of AVP to increase CD AQP2 water channel expression and trafficking to the apical membrane has been demonstrated to be pivotal in urinary concentration (4). AVP synthesis and release have been shown to be regulated by alterations in plasma osmolality and nonosmotic, baroreceptor-mediated pathways (17). Maximal urinary concentration, however, seems to be mediated by factors in addition to plasma AVP. For example, 24 h of fluid deprivation has been shown to increase urinary osmolality to greater levels than what is achieved with exogenous administration of supraphysiologic concentrations of AVP (18). Fluid deprivation in BB rats with...
out detectable plasma or hypothalamic AVP also has been shown to increase urinary osmolality (19). Similarly, V$_2$ AVP receptor antagonists can cause maximal urinary dilution, yet, in their presence, fluid deprivation increases urinary osmolality (20).

Mutation of V$_2$ vasopressin receptors is known to cause hereditary nephrogenic diabetes insipidus even though the AQP2 water channels are intact (21). Therefore, defining vasopressin-independent factors that increase AQP2 protein expression and trafficking to the apical membrane of the CD has potential clinical implications. In addition to AVP-independent effects on AQP2, an AVP-independent effect to enhance NKCC2, the initiator of the countercurrent concentrating mechanism, would have important implications.

Hyperosmolality has been shown to upregulate AQP2 in several in vitro cell culture systems (7,8), but, to date, the effect of increased osmolality in vivo to enhance AQP2 protein expression and trafficking remains to be documented clearly. In this regard, there have been conflicting results about the effects of fluid deprivation in BB rats to alter AQP2 expression (19,22). This may be due, however, to differences in the duration of fluid deprivation. Similarly, NKCC2 has been shown to be increased by vasopressin (23), but hypertonicity also has been demonstrated to increase NKCC2 expression in Xenopus oocytes in vitro in the absence of AVP (9).

On this background, the purpose of our study was to investigate whether hyperosmolality can increase AQP2 expression and trafficking as well as enhance NKCC2 activity in vivo in the absence of AVP. BB rats are an established species in which no detectable AVP is present and daily urine volume that is comparable to the rat’s body weight is excreted (24). In this study,

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**Figure 5.** Immunoblot of membrane fractions of AQP2 in the IM, ISOM, and cortex plus outer stripe of outer medulla (OSOM+C) from BBCTR (n = 9) and BB rats with hyperosmolality induced by 0.5% NaCl in drinking water (BBNaCl, n = 9). Immunoblots were reacted with affinity-purified anti-AQP2 antibodies. Densitometric analysis of all samples revealed that in the BBNaCl rats, AQP2 expression in the IM, ISOM, and OSOM+C increased significantly compared with BBCTR rats.

**Figure 6.** Immunoblot of membrane fractions of NKCC2 in the ISOM and OSOM+C in the kidneys from BBCTR (n = 9) and BBNaCl rats. Immunoblots were reacted with affinity-purified and anti-NKCC2 antibodies. Densitometric analysis of all samples revealed that in the BBNaCl rats, the expression of NKCC2 in the ISOM and OSOM+C increased significantly compared with BBCTR rats.
Two approaches were used to cause hyperosmolality, namely hyperglycemia in BBDM rats or by NaCl drinking water in BB rats.

STZ is known to cause insulin-deficient (type 1) diabetes. Moreover, in the presence of insulin deficiency, the resultant hyperglycemia in type 1 diabetes is known to increase effective extracellular fluid osmolality. In the presence of hyperosmolality secondary to hyperglycemia, AQP2 protein expression was increased in the ISOM and IM; AQP3 expression was increased in the IM. These effects on AQP2 and AQP3 were reversed by the effect of insulin administration to decrease blood glucose and serum osmolality. A previous study in BBDM rats also showed an increase in AQP2 and NKCC2, but reversibility with insulin was not undertaken (10). The effect of hyperglycemia and hyperosmolality on AQP2 protein expression was confirmed in the immunohistochemistry studies, which also demonstrated a reversible effect on the apical membrane domains of AQP2. Although NKCC2 protein expression was increased in the ISOM, an effect that also was demonstrated by immunohistochemistry, this effect was not reversible with insulin treatment. This result indicates that hyperosmolality or hyperglycemia was not the cause of the NKCC2 upregulation. Alternatively, another factor, such as a persistent negative sodium balance secondary to the initial 3 d of glucosuria, was maintaining NKCC2 upregulation despite that the later insulin treatment reversed the hyperglycemia and hyperosmolality. Other factors that are known to affect urinary concentration, namely AQP1 and UT-A1, were not altered in these studies in BBDM rats.

The foregoing studies in BBDM rats did not distinguish between whether the reversible upregulation of AQP2 was due to the hyperosmolality or hyperglycemia. Further studies were undertaken, therefore, to examine the hypothesis that in vivo hyperosmolality, independent of AVP, upregulates AQP2 and NKCC2, both pivotal factors in urinary concentration. We therefore developed an experimental model in which 0.5% NaCl drinking water in BB rats was associated with hyperosmolality in the absence of hyperglycemia. The results of these studies demonstrated that hyperosmolality in BBNaCl rats upregulated AQP2 throughout the cortical and medullary CD. NKCC2 also was upregulated by NaCl-induced hyperosmolality in vivo in both the inner stripe of the medulla as well as the outer stripe of outer medulla and cortex. The higher plasma osmolality in the BBNaCl, as compared with the hyperglycemic BB rats, may explain why the OSOM + C AQP2 water channel and NKCC2 were upregulated only in the BBNaCl rats. This observation also might suggest that medullary nephron sites are more sensitive in vivo to the effect of hyperosmolality than nephron segments in the cortex. The results in the NaCl drinking model excluded an effect of sodium depletion on NKCC2, which must have occurred early in the BBDM rats secondary to untreated glucosuria. There was no effect of hyperosmolality on the NHE3 and type 2 sodium-phosphate co-transporter in any of these studies. There was, however, a reversible effect on Na-K-ATPase in the IM in the BBDM studies, but this effect was not confirmed in the NaCl drinking studies.

Of interest is the effect of a V2 receptor antagonist to increase urine flow further in the BBDM rats, which supports an earlier observation by Serradeil-Le Gal et al. (25). In this regard, plasma oxytocin has been shown to be increased in BB rats, and oxytocin exerts its antidiuretic action via the vasopressin V2 receptor (26). Therefore, the further increase in urine flow in BB rats with a V2 receptor antagonist seems most likely to be due to inhibition of the antidiuretic effect of oxytocin.
Conclusion

Hyperosmolality in vivo is known to stimulate AVP and increase urinary concentration. These in vivo results indicate that hyperosmolality, independent of AVP, also increases AQP2 expression. This AVP-independent factor in urinary concentration of AQP2 upregulation may be complemented by an effect of hyperosmolality to upregulate NKCC2, the initiator of the countercurrent concentrating mechanism in the water-impermeable thick ascending limb. Although these results support previous in vitro observations of hyperosmolality's up-regulating AQP2 in cell culture systems in the absence of AVP, an effect of hyperosmolality on AQP1 and UT-A1 was not demonstrated.

Acknowledgments

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References