Role of vasopressin in diabetes mellitus-induced changes in medullary transport proteins involved in urine concentration in Brattleboro rats

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Kim, Dongun, Jeff M. Sands, and Janet D. Klein. Role of vasopressin in diabetes mellitus-induced changes in medullary transport proteins involved in urine concentration in Brattleboro rats. Am J Physiol Renal Physiol 286: F760–F766, 2004.—In rats with streptozotocin-induced diabetes mellitus for 10–20 days, we showed that the abundance of the major medullary transport proteins involved in the urinary concentrating mechanism, urea transporter (UT-A1), aquaporin-2 (AQP2), and the Na/H/K-2Cl cotransporter (NKCC2/BSC1), is increased, despite the ongoing osmotic diuresis. To test whether vasopressin is necessary for these diabetes mellitus-induced changes in UT-A1, AQP2, or NKCC2/BSC1, we studied Brattleboro rats because they lack vasopressin. Brattleboro rats were given vasopressin (2.4 μg/day via osmotic minipump) for 5 or 12 days. At 5 days, vasopressin increased AQP2 protein abundance but decreased UT-A1 abundance compared with untreated Brattleboro rats. At 12 days, vasopressin increased the abundance of both UT-A1 and AQP2 proteins but did not alter NKCC2/BSC1. Next, untreated Brattleboro rats were made diabetic for 10 days by injecting them with streptozotocin (40 mg/kg). Diabetes mellitus increased the abundance of AQP2 and NKCC2/BSC1 proteins, but UT-A1 protein abundance did not increase. Third, vasopressin-treated Brattleboro rats were made diabetic with streptozotocin for 10 days. In vasopressin-treated Brattleboro rats, diabetes mellitus increased UT-A1, AQP2, and NKCC2/BSC1 protein abundances. Vasopressin significantly increased UT-A1 phosphorylation in vasopressin-treated diabetic Brattleboro rats but not in the other groups of Brattleboro rats. We conclude that 1) vasopressin is necessary for UT-A1 and AQP2 in diabetic Brattleboro rats but is not necessary for the increase in UT-A1 protein in diabetic rats and is not necessary for the increase in AQP2 or NKCC2 proteins.

METHODS

Animal preparation. All animal protocols were approved by the Emory Institutional Animal Care and Use Committee. Male and female Brattleboro rats (bred in our animal facility) weighing 150–350 g received free access to 23% protein rat chow and water. To administer vasopressin, minipumps (Alzet model 2002, Durect, Cupertino, CA) delivering 2.4 μg/day of arginine vasopressin (Sigma, St. Louis, MO) were implanted subcutaneously under light ketamine anesthesia. Vasopressin was diluted with normal saline and infused at a rate of 12 μl/day for 12 days. A spot urine osmolality was measured every 2–3 days to confirm that the minipumps were working properly (model 5500 vapor pressure osmometer, Wescor, Logan, UT).

To induce diabetes mellitus, Brattleboro rats were injected with STZ (40 mg/kg body wt prepared fresh in 0.1 M citrate buffer, pH 4.0; Sigma) into a tail vein at 7 AM (10, 13, 21). Vasopressin-treated Brattleboro rats received STZ 2 days after the vasopressin-containing minipumps were placed. Diabetes mellitus was confirmed by measuring spot urine glucose at 24 and 48 h after STZ injection ( Ames N-Multi 3 i, Miles, Elkhart, IN). Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 125–200 g received free access to 23% protein rat chow and water for at least 3 days after delivery. To induce diabetes, rats were given sugar water containing 10% glucose to drink for 14 days (24). The sugar water-fed rats do not develop glucosuria (24).

Pati EnTs with uncontrolled diabetes mellitus (type I) often have osmotic diuresis, due to unabsorbed glucose in the tubule lumen, and this can result in volume depletion. In rats, we showed that at 3–5 days of diabetes mellitus induced by streptozotocin (STZ), the urea transporter (UT-A1) protein abundance is decreased and aquaporin-2 (AQP2) protein abundance is unchanged in the inner medulla tip compared with control rats (10, 13). However, from 10–20 days, the abundance of the major medullary transport proteins involved in the urinary concentrating mechanism, UT-A1, AQP2, and the Na/H/K-2Cl cotransporter (NKCC2/BSC1), is increased despite the ongoing osmotic diuresis (1, 10, 18, 26). Glucocorticoids mediate the early (3–5 days) decrease in UT-A1 protein abundance in rats with uncontrolled diabetes mellitus (13, 14, 17). However, the hormonal mediator of the subsequent increase in UT-A1, AQP2, and NKCC2/BSC1 is unknown.

One candidate mediator is vasopressin. Diabetic rats have elevated plasma vasopressin levels in some studies (4, 29) but not in another (10). However, measuring vasopressin levels in rats can be difficult because any factor that causes the rat to become anxious can result in a large and rapid release of vasopressin from the pituitary gland. The Brattleboro rat has central diabetes insipidus due to a congenital lack of vasopressin and is an excellent animal model for studying the effects of vasopressin (8). Previous studies of medullary transport proteins have generally administered vasopressin (or dDAVP, a V2-selective agonist) to Brattleboro rats for 5–7 days (5, 6, 11, 12, 27). Vasopressin increases AQP2 protein abundance in the inner medulla (5, 6, 12, 27). dDAVP increases NKCC2/BSC1 protein abundance in the outer medulla (11), although vasopressin does not (6). In contrast, vasopressin decreases UT-A1 protein abundance in the inner medulla (28). The goal of this study was to determine the effect of diabetes mellitus on UT-A1, AQP2, and NKCC2/BSC1 protein abundances in Brattleboro rats so that we could study the effect of diabetes in the absence of vasopressin.

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Table 1. Blood and urine parameters in Brattleboro rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=6)</th>
<th>Vasopressin for 12 Days (n=6)</th>
<th>Diabetes Mellitus for 10 Days (n=6)</th>
<th>Vasopressin + Diabetes Mellitus (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood glucose, mg/dl</td>
<td>118±18</td>
<td>98±12</td>
<td>332±27*</td>
<td>376±28*</td>
</tr>
<tr>
<td>BUN, mg/dl</td>
<td>25±6</td>
<td>23±1</td>
<td>19±4</td>
<td>20±1</td>
</tr>
<tr>
<td>Urine volume, ml/day</td>
<td>40±10</td>
<td>5±1*</td>
<td>110±20*</td>
<td>60±10†</td>
</tr>
<tr>
<td>Urine osmolality, mosmol/kgH2O</td>
<td>240±30</td>
<td>1,940±340*</td>
<td>320±10</td>
<td>910±50†</td>
</tr>
<tr>
<td>Urine urea conc., mmol/l</td>
<td>100±20</td>
<td>570±90*</td>
<td>80±10</td>
<td>170±10†</td>
</tr>
<tr>
<td>Glucose excretion, mmol/day</td>
<td>3±1</td>
<td>3±1</td>
<td>9±2*</td>
<td>10±1*</td>
</tr>
<tr>
<td>BW, body weight</td>
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</tbody>
</table>

Values are means ± SE. BUN, blood urea nitrogen; conc., concentration; BW, body weight. *P < 0.05 vs. control. †P < 0.05 for vasopressin vs. vasopressin and diabetes mellitus.

All rats were fed 23% protein chow and water (or sugar water) ad libitum throughout the study. Two days before death, rats were put into metabolic cages and 24-h urine was collected to measure urine volume, osmolality, and glucose and urea concentrations. Rats were killed by decapitation, and trunk blood was collected to measure glucose (One Touch Pro Diabetes Tracking Kit, Lifescan, Milpitas, CA), blood urea nitrogen (BUN; Infinity BUN reagent, Sigma), and osmolality.

Western blot analysis. Kidneys were removed and the medulla was dissected into outer medulla, inner medullary base, and inner medullary tip as previously described (10, 13). In some studies, one inner medulla was not subdivided, whereas the other kidney from the same rat was dissected into base and tip. Kidney tissue from a single rat was placed into ice-cold isolation buffer (10 mM triethanolamine, 250 mM sucrose, pH 7.6, 1 µg/ml leupeptin, and 2 mg/ml PMSF), homogenized, and SDS was added to a final concentration of 1% SDS for Western blot analysis of total cell lysate (10, 13). Total protein in each sample was measured by a modified Lowry method (Bio-Rad DC protein assay reagent, Bio-Rad, Richmond, CA). Proteins (10 µg/lane) were size separated by SDS-PAGE using 7.5, 10, or 15% polyacrylamide gels, blotted to polyvinylidene difluoride membranes (PVDF; Gelman Scientific, Ann Arbor, MI), and Western blotting was performed as described previously (10, 13). Western blots were probed with antibodies (diluted in TBS/Tween) to the following proteins: 1) UT-A1 (17), 2) AQP2 (10, 19), and 3) NKCC2/BSC1 (10, 11). Blots were quantified using an Imaging Densitometer GS670 and Molecular Analyst software (Bio-Rad). When reporting on total UT-A1 protein abundance differences, we make the assumption that the antibody recognizes both glycoproteins equally. The functional significance of these differences, however, is unclear.

Phosphorylation of UT-A1. Inner medullary collecting duct (IMCD) suspensions were incubated with 32P, and then UT-A1 protein was immunoprecipitated from equal amounts of the whole cell lysates as previously described (31). Proteins were size separated on two identical SDS-polyacrylamide gels containing an equal portion of the total immunoprecipitated protein per lane. The proteins on one gel were transferred to a PVDF membrane and the amount of immunoprecipitated UT-A1 was assayed by Western blotting. The other gel was dried, and 32P incorporation into UT-A1 was analyzed by autoradiography.

**RESULTS**

Effect of administering vasopressin to Brattleboro rats on UT-A1, AQP2, and NKCC2/BSC1. Administering vasopressin to Brattleboro rats for 12 days significantly increased urine osmolality and reduced urine volume. To test for statistically significant differences between these two groups, a paired Student’s t-test was used. To test for statistically significant differences between three or more groups, an ANOVA was used followed by a multiple comparison, protected t-test (25).

Because the increase in UT-A1 at 12 days is opposite to previous results following 5 days of vasopressin (28), we performed two additional experiments. First, we measured UT-A1 abundance in the inner medullary tip and base from Brattleboro rats that received vasopressin for 5 days. We also measured UT-A1 in whole inner medulla to provide a direct comparison to the previous study (28). UT-A1 protein abundance was decreased in the inner medullary tip and in whole inner medulla of 5-day vasopressin-treated Brattleboro rats, compared with untreated Brattleboro rats, consistent with previous studies (5, 6, 12, 27). The Brattleboro rats treated with vasopressin for 5 days had comparable increases in urine osmolality and reductions in urine volume to rats treated for 12 days (data not shown).

Second, we measured UT-A1, AQP2, and NKCC2/BSC1 protein abundances in Sprague-Dawley rats undergoing water deprivation.
diuresis for 14 days to reduce endogenous vasopressin levels. The water diuretic rats had significantly reduced urine osmolality and increased urine volume compared with control rats (Table 2). All three protein abundances were significantly decreased in the inner medullary tip (UT-A1, AQP2) or outer medulla (NKCC2/BSC1) of water diuretic rats compared with untreated Sprague-Dawley rats (Fig. 5).

**Effect of diabetes mellitus on UT-A1, AQP2, and NKCC2/BSC1 in Brattleboro rats.** Blood glucose was markedly increased in diabetic Brattleboro rats, both with and without vasopressin (Table 1). Neither vasopressin nor STZ changed BUN. Diabetic Brattleboro rats had significantly higher urine volume, urea excretion, and glucose excretion than control Brattleboro rats. Diabetes mellitus did not increase UT-A1 protein abundance in the inner medullary tip of Brattleboro rats (Fig. 6) but did increase AQP2 to 170% over control (Fig. 7). Diabetes mellitus also increased NKCC2/BSC1 protein abundance to 280% over control in the outer medulla (Fig. 8).

In Brattleboro rats that received vasopressin, diabetes mellitus again increased urine volume, urea excretion, and glucose excretion. In addition, diabetes mellitus decreased urine osmolality in vasopressin-treated Brattleboro rats compared with nondiabetic vasopressin-treated Brattleboro rats. In vasopressin-treated Brattleboro rats, diabetes mellitus increased the protein abundance of the 117-kDa UT-A1 glycoprotein to 69% over vasopressin alone but did not change the abundance of the 97-kDa UT-A1 glycoprotein (Fig. 9). UT-A1 typically runs as two glycoprotein bands (97 and 117 kDa) on Western blot (3). However, in Fig. 9, the 117-kDa band appears to have expanded to include some less glycosylated species between the 97- and 117-kDa bands. The Western blot was scanned as the discreet 97-kDa UT-A1 isoform, which did not change with

### Table 2. Blood and urine parameters in Sprague-Dawley rats

<table>
<thead>
<tr>
<th></th>
<th>Control Rats (n=6)</th>
<th>Hydrated Rats (n=6)</th>
</tr>
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<tbody>
<tr>
<td><strong>Urine volume, ml/day</strong></td>
<td>5 ± 1</td>
<td>26 ± 2*</td>
</tr>
<tr>
<td><strong>Urine osmolality, mosmol/kgH2O</strong></td>
<td>1,980 ± 80</td>
<td>270 ± 40*</td>
</tr>
<tr>
<td><strong>Urine urea concentration, mmol/l</strong></td>
<td>920 ± 30</td>
<td>90 ± 20*</td>
</tr>
<tr>
<td><strong>Urea excretion, mmol/day</strong></td>
<td>4.3 ± 0.2</td>
<td>2.3 ± 0.3*</td>
</tr>
<tr>
<td><strong>Blood urea nitrogen, mg/dl</strong></td>
<td>19 ± 1</td>
<td>12 ± 2*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. control.
diabetes (10), and the larger bands centering ~117 kDa were grouped for densitometric analysis. In Sprague-Dawley rats, it is the upper glycosylated species that are changed with diabetes (10). Diabetes mellitus also increased the protein abundances of AQP2 to 71% over vasopressin alone (Fig. 10) in the inner medullary tip and NKCC2/BSC1 to 37% over vasopressin alone in the outer medulla of vasopressin-treated Brattleboro rats (Fig. 11).

**Phosphorylation of UT-A1 protein.** The relative abundance of immunoprecipitated UT-A1 protein from IMCD suspensions was not different from the relative abundance of UT-A1 protein in whole cell lysate by Western blot analysis, i.e., control \( \approx \) diabetes mellitus alone < vasopressin (12 days) alone < vasopressin and diabetes mellitus (Fig. 12). Only IMCD suspensions from the vasopressin-treated diabetic Brattleboro rats had a significant increase in UT-A1 phosphorylation to 190% over control Brattleboro rats. IMCD suspensions from the vasopressin-treated Brattleboro rats did not show any increase in UT-A1 phosphorylation despite a significant increase in UT-A1 protein abundance.

**DISCUSSION**

The major findings in this study are that 1) administering vasopressin to Brattleboro rats for 12 days, but not for 5 days, increases UT-A1 protein abundance and 2) vasopressin is necessary for the increase in UT-A1 protein abundance in diabetic rats but is not necessary for the increase in AQP2 or

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Fig. 5. UT-A1 and AQP2 in IM tip and NKCC2/BSC1 in the outer medulla (OM) of Sprague-Dawley control (SD Ctr) and water-diuretic rats (SD Hyd). Water-diuretic rats were given 10% sucrose in water for 14 days. Right: densitometric analyses of those results. UT-A1, AQP2, and NKCC2 protein abundances were significantly decreased in the water-diuretic rat, compared with control rats. Values are means ± SE. \( *P < 0.05 \).

Fig. 6. UT-A1 in IM tip of control (BB CTR) and diabetic (BB DM) Brattleboro rats. A: Western blot of representative samples of IM tip lysates probed with anti-UT-A1 antibody. B: densitometry of protein bands in arbitrary units. There was no significant difference in the abundance of UT-A1 in diabetic Brattleboro rats compared with control Brattleboro rats. Values are means ± SE.

Fig. 7. AQP2 in IM tip of BB CTR and BB DM rats. A: Western blot of representative samples of IM tip lysates probed with anti-AQP2 antibody. B: densitometry of protein bands in arbitrary units. There was a 170% increase in the abundance of AQP2 in the diabetic brattleboro above control levels. Values are means ± SE. \( *P < 0.05 \).
NKCC2/BSC1 protein abundances. These findings suggest that the increase in the major medullary transport proteins involved in the urinary concentrating mechanism cannot be ascribed solely to vasopressin.

**Effects of vasopressin in Brattleboro rats.** Vasopressin gradually restores urinary concentrating ability in Brattleboro rats (8, 12). Correction of the urinary concentrating defect is associated with a progressive increase in inner medullary osmolality, primarily due to an increase in inner medullary urea content (8). After 3 days of vasopressin, inner medullary osmolality is only 50–75% of normal, but there is osmotic equilibration between urine and the inner medullary interstitium (8, 12). This finding suggests that the collecting duct is able to osmotically reabsorb water after 3 days of vasopressin, even though AQP2 protein abundance is subnormal (12). After 5 days of vasopressin, AQP2 protein abundance returns to normal levels (5, 6, 12, 27, and present study). After 14–28 days of vasopressin, urine and inner medullary osmolality are normal (8, 12), suggesting that urea content does not become normal until sometime between 3 and 14–28 days. After 5 days of vasopressin, UT-A1 protein abundance is decreased (28, present study) but is increased after 12 days (present study). This time course is consistent with Harrington and Valtin’s and Kishore and colleagues’ time courses (8, 12) and may help to explain the gradual correction of concentrating ability in Brattleboro rats.

Why the delayed increase in UT-A1 protein? The promoter for rat UT-A1 does not contain a consensus cAMP-response element (CRE) but does contain a tonicity enhancer element (15, 16). Thus it is possible that vasopressin may not directly
increase UT-A1 transcription but may do so indirectly by increasing the transcription of other genes, such as AQP2, NKCC2/BSC1, that begin to increase inner medullary osmolality (9, 30). Consistent with this hypothesis is that UT-A1 protein abundance increases following 2 days of water diuresis (28) but decreases following 14 days of water diuresis (present study). Vasopressin does increase AQP2 transcript (30), which could account for the more rapid increase in AQP2 protein following vasopressin administration to Brattleboro rats.

The effect of vasopressin on NKCC2/BSC1 abundance has differed in different studies (2, 6, 11). Water restriction (which raises endogenous vasopressin levels) increases NKCC2/BSC1 protein abundance and a selective V$_2$-receptor antagonist decreases it (2, 11), consistent with the presence of a CRE in the NKCC2/BSC1 promoter (9). In Brattleboro rats, dDAVP increases NKCC2/BSC1 abundance (11) but vasopressin does not (6). Knepper and colleagues (6) proposed that this difference may result from higher vasopressin levels following exogenous vasopressin administration resulting in V$_1$-receptor effects on the thick ascending limb that counteracts its V$_2$-receptor effect to increase NKCC2/BSC1. The present study is consistent with this hypothesis, because exogenous vasopressin did not increase NKCC2/BSC1, but water diuresis (which reduces endogenous vasopressin levels) did decrease it. Other potential explanations for the difference between exogenous and endogenous vasopressin include a difference in sensitivity of the thick ascending limb vs. collecting duct to vasopressin (7) or the difference in half-life and duration of action of dDAVP vs. vasopressin (23).

Effect of diabetes mellitus in Brattleboro rats. Previous studies show that UT-A1, AQP2, and NKCC2/BSC1 protein abundances increase after 10 days of diabetes mellitus in Sprague-Dawley rats (1, 10, 18). In the present study, AQP2 and NKCC2/BSC1 protein abundances increased in diabetic Brattleboro rats, indicating that vasopressin is not required for the increase in these proteins in diabetes mellitus. AQP2 is known to be regulated by both vasopressin-dependent and -independent pathways (reviewed in Ref. 20). Promeneur and colleagues (22) showed that dehydration increases AQP2 protein in Brattleboro rats, even in the absence of vasopressin, and suggested that oxytocin is acting as an antidiuretic hormone through the V$_2$ receptor. Thus it is possible that oxytocin is responsible for the increase in AQP2 protein in diabetic Brattleboro rats in the present study.

In contrast, UT-A1 protein abundance did not increase in diabetic Brattleboro rats but did increase in vasopressin-treated Brattleboro rats. This finding suggests that vasopressin is necessary for the increase in UT-A1 protein abundance during diabetes. It also suggests that a factor other than vasopressin is responsible for the increase in UT-A1 protein because vasopressin levels were not different between the two vasopressin-treated groups of Brattleboro rats. One potential mechanism for the upregulation of UT-A1 in the vasopressin-treated Brattleboro rats is an increase in luminal and/or interstitial osmolality or urea concentration, because diabetes does not change urine osmolality or urea concentration in untreated Brattleboro rats but does in the vasopressin-treated Brattleboro rats (Table 1).

Vasopressin also increased UT-A1 phosphorylation, but only in vasopressin-treated diabetic Brattleboro rats. We previously showed that an increase in UT-A1 phosphorylation by vasopressin correlates with an increase in vasopressin-stimulated urea transport in the rat IMCD (31). Thus, in the absence of vasopressin, diabetes mellitus does not increase UT-A1 protein abundance or its phosphorylation by vasopressin.

Summary. The abundances of the major medullary transport proteins involved in the urinary concentrating mechanism vary with time after the onset of diabetes mellitus in rats. At 3–5 days of diabetes mellitus, UT-A1 protein abundance is decreased in the inner medullary tip and AQP2 protein abundance is unchanged, compared with control rats, and this effect is mediated by glucocorticoids (10, 13). At 10–20 days of diabetes mellitus, UT-A1, AQP2, and NKCC2/BSC1 protein abundances are increased despite the ongoing osmotic diuresis (1, 10, 18, 26). The increases in AQP2 and NKCC2/BSC1 protein abundances are not mediated by vasopressin because these proteins increase in diabetic Brattleboro rats, but vasopressin is necessary for the increase in UT-A1 protein abundance.

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GRANTS

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