Long-term regulation of urea transporter expression by vasopressin in Brattleboro rats

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Regulation of urea concentration in the renal medullary interstitium is therefore important to promote water reabsorption in the collecting duct and to balance higher urinary osmolality. The accepted model of urea accumulation in the kidney medullary interstitium is that urea is reabsorbed in the terminal inner medullary collecting duct (IMCD) via facilitated urea transporters and is preserved by recycling pathways involving transfer of urea into the descending limb of the loop of Henle from the ascending limbs of the loop of Henle and ascending vasa recta, and countercurrent exchange pathways between the ascending and descending vasa recta (12). Presently, the cDNAs encoding two different UTs have been identified in the renal tubules. The 4.0-kb UT1 transcript encodes for a 929-amino acid residue urea transporter protein located in the apical membrane of the IMCD (18, 26, 27) and the 2.9-kb UT2 transcript is in the descending thin limbs of the loop of Henle (26, 28, 34). Physiological changes that result in modulation of UT1 and UT2 expression include water restriction and protein content in the diet.

Isolated tubule perfusion studies have demonstrated acute regulation of urea permeability in IMCD by the antidiuretic hormone arginine vasopressin (AVP). In the acute phase, AVP-stimulated urea reabsorption in the terminal IMCD begins within 5–10 min and is complete within 30–60 min (23, 33). AVP has been shown to act through at least two different G protein-coupled receptors in the kidney: V1a receptor, involving phosphatidylinositol hydrolysis and rise in intracellular Ca2⁺, and V2 receptor, which is coupled to a Gs/adenyl cyclase system. Modulation of urea permeability in the IMCD is mediated through V2 receptors followed by activation of adenylyl cyclase and increased intracellular cAMP (29).

In contrast, the presently available data suggest that high accumulation of urea in the medullary interstitium during long-term water restriction is not primarily due to the acute effect of AVP to stimulate urea permeability in the IMCD. A study by Lankford et al. (14) showed that Sprague-Dawley rats when thirsted for 24 h did not show an elevation of basal urea permeability in isolated perfused IMCD and that cAMP...
concentration in these cells was not increased. This suggests that separate mechanisms exist for urea accumulation in the medullary interstitium during acute vs. long-term water restriction. In support of this physiological finding, our previous studies demonstrated that UT1 and UT2 transcripts are differentially regulated during antiidiuresis (28). Restriction of water intake in Sprague-Dawley rats for 3 days results in upregulation only of the UT2 but not UT1 transcript in the kidney medulla. However, it is unknown whether this process is mediated by a long-term increase in the concentration of circulating antidiuretic hormone AVP, other hormones, or by secondary effects such as changes in renal hemodynamics. If AVP is indeed a main factor responsible for modulation of UT gene expression during long-term dehydration, it is unknown whether this is mediated by V₂ receptors as in acute phase or others.

In the present study, we conducted a comprehensive analysis to clarify the role of vasopressin in long-term regulation of UTs by using Brattleboro rats that congenitally lack circulating vasopressin and its potential targeted site of activation. The objectives of this study were to 1) determine the effect of thirsting on UT expression in Brattleboro rats; 2) determine the effect of chronic infusion of vasopressin on expression of both UT1 and UT2; and 3) compare the effect of AVP and the specific V₂ vasopressin-receptor agonist [deamino-Cys², D-Arg⁸]-vasopressin (dDAVP). The results show that vasopressin is essential for long-term regulation of UT1 and UT2 expression during water restriction and this hormone likely acts through activation of V₂ receptors.

METHODS

Animals. The animals used for these studies were pathogen-free male Brattleboro homozygous (di/di) rats weighing 250–300 g. All rats were equilibrated in individual cages on standard rat chow and ad libitum water intake for 1 wk after delivery. To study the effects of continuous vasopressin infusion, the rats were divided into four groups (3–4 rats/group) and were implanted with osmotic minipumps (model 2002; Alza, Palo Alto, CA) under light phenobarbital anesthesia as previously described (9). The minipumps administered vehicle (group I and II), AVP solution (group III) or dDAVP solution (group IV) at a rate of 0.5 µl/h. AVP and dDAVP were each diluted with 5% dextrose and 0.05% acetic acid in water to deliver 1.2 µg/day. This dose rate of AVP has been shown to produce comparable plasma vasopressin levels to those in normal rats during water restriction (9), and the equivalent dose rate of dDAVP was administered. After 7 days, the rats were separated and maintained in individual metabolic cages in the same room to allow 24-h urine collections. All rats had free access to food and water, except group II, which were thirsted for 24 h.

At day 8 after infusion, the rats were anesthetized with pentobarbital sodium. One kidney was rapidly removed and frozen in liquid nitrogen. The other kidney was perfused with 4% paraformaldehyde in PBS, postfixed in the same solution at 4°C, immersed in 30% ice-cold sucrose for 24 h, and stored at −80°C.

Northern analysis. Total RNA was isolated from whole kidneys by the guanidine isothiocyanate method by using cesium-trifluoroacetic acid. Total RNA (12 µg/lane) was separated in a formaldehyde/agarose gel and capillary blotted onto a nylon filter. Equal loading of each sample was determined by the intensity of the 28S and 18S ribosomal RNA bands when stained with ethidium bromide and visualized under ultraviolet light. The specific probes for UT1 and UT2 transcripts were UT1 specific, the first 652 nucleotides of UT1 cDNA, and UT2 specific, 706 nucleotides at the 5′ terminus of UT2 cDNA (27). The filter was hybridized at 42°C in 50% formamide and washed in buffer containing 0.1× SSC, 0.1% SDS at 65°C. After autoradiography, the blots were quantified by densitometry [National Institutes of Health (NIH) Image Analysis].

In situ hybridization. These studies were performed as previously described (10). Briefly, 7-µm-thick tissue sections were hybridized with 35S-labeled sense and antisense cRNA probes synthesized from the full-length rat UT2 cDNA. This probe was degraded by partial hydrolysis to ~100 nucleotides in length. This probe has been reported by our group to recognize both UT1 and UT2 transcripts in rat kidneys (27, 28). After washing at 50°C, the air-dried tissue sections were exposed overnight to Biomax MR film (Eastman Kodak, Rochester, NY). Tissue slides were coated with Kodak NTB2 emulsion and stored for 7 days before being developed and counterstained with hematoxylin and eosin.

Immunocytochemistry. Polyclonal anti-rat renal urea transport (RUT) antibodies, raised against a peptide corresponding to the NH₂-terminal 19 amino acids of UT2 (18), were used in the immunocytochemical studies (a generous gift from Dr. Mark A. Knepper, NIH). For immunostaining, the kidney tissue sections were treated with 1% SDS for 5 min to expose antigenic sites (3), rinsed three times with PBS, and preincubated with 5% normal goat serum for 15 min. Sections were then incubated for 1 h with the affinity-purified RUT antibody, washed three times with PBS, and then incubated for 1 h with goat anti-rabbit indocarbocyanine (1:100, Jackson ImmunoResearch, West Grove, PA). Specificity of the staining was tested by using anti-RUT antibody preadsorbed with immunizing peptide at 50 µg/ml.

Statistics. The data are reported as means ± SE. Statistical comparisons were made by use of an analysis of variance followed by Fisher's protected least significant difference test with statistical significance considered at P < 0.05.

RESULTS

The effect of water restriction and continuous infusion of AVP or dDAVP on urine volume and osmolality of Brattleboro rats is shown in Table 1. As has been

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<th>Table 1. Body weight, urine flow rate, and urine osmolality in control, 24-h water restriction, continuously AVP-treated, and dDAVP-treated Brattleboro rats</th>
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Values are means ± SE. n, No. of rats; AVP, [Arg⁸]-vasopressin; dDAVP, [deamino-Cys²-D-Arg⁸]-vasopressin. *P < 0.05 compared with control. †P < 0.001 compared with control. ‡P < 0.05 compared with AVP-treated group.
previously reported, Brattleboro rats, despite the lack of endogenous AVP, are still able to concentrate their urine when thirsted (2, 8). In our study 24-h water restriction resulted in a doubling of urine osmolality and a halving of urine volume with ~20% loss in their body weight. Rats treated with continuous AVP infusion at a rate of 50 ng/h for 7 days showed a significant decrease in urine volume and increased urine osmolality indicating urinary concentration. Infusion of molar equivalent amounts of dDAVP resulted in a large increase (~2 times) in urine osmolality, significantly greater than that observed for AVP treatment. This discrepancy might be a consequence of difference in steadiness of plasma vasopressin-receptor agonist (biological half-life for dDAVP and AVP is 40 and 3 min, respectively), thus resulting in different levels of V2 receptor activation (see DISCUSSION) and/or counter-effect of V1a on V2 receptor activation in modulation of the antidiuretic actions of vasopressin (15).

The effects of various treatments on the expression level of UT1 and UT2 transcripts in total kidney RNA were examined by Northern analysis (Fig. 1), and the percent change in expression levels was determined (Fig. 2). The corresponding pattern of mRNA expression was further studied by in situ hybridization (Figs. 3 and 4).

Effect of thirsting on UT mRNA expression in Brattleboro rat kidneys. Thirsting caused no detectable changes in the amount of UT2 mRNA, but a modest decrease in the level of UT1 mRNA expression (Figs. 1 and 2) was observed. This finding is consistent with studies from in situ hybridization (thirsting in Fig. 3B vs. control in Fig. 3A).

Effect of chronic infusion of vasopressin on UT mRNA expression in Brattleboro rat kidneys. In analogy to the previous observations of water-restricted normal rats (20, 28), Northern analysis revealed a substantial increase in UT2 mRNA and a decrease in UT1 mRNA in Brattleboro rats treated with dDAVP for 8 days to maximize urinary osmolality (Figs. 1 and 2). These changes, however, were not observed in the AVP-treated group, which might be due to a smaller effect of AVP at the dose used (as also reflected by smaller increase of urinary osmolality) in conjunction with the limitation of Northern analysis to detect small changes of RNA levels. We used in situ hybridization to further examine alterations in abundance and distribution of UT transcripts in the kidney in response to AVP and dDAVP administration. These studies demonstrated a progressive increase in the UT2 signal in the outer medullary regions when control (Fig. 3A), water-restricted (Fig. 3B), AVP-treated (Fig. 3C) and dDAVP-treated groups (Fig. 3D) were compared. At higher magnification (Fig. 4, A and C) it can be seen that the UT2 signals in kidneys from dDAVP-treated animals are extended along a greater length of the descending
thin limbs of the loop of Henle. Increased UT2 signals were observed in this structure both in the inner stripe of the outer medulla (Fig. 4, A and B) and in the upper portion of the inner medulla (Fig. 4, C and D). These findings suggest a much greater range of descending thin limbs involved in countercurrent exchange to preserve high urea accumulation in the medullary interstitium after long-term treatment with dDAVP (see DISCUSSION).

The changes in hybridization signals in the innermost part of the inner medulla, where UT1 is expressed, were only clearly observed in the dDAVP-treated rats. Figures 3D and 4E show weaker hybridization signals in the terminal portion of IMCD in dDAVP-treated rats compared with control (Figs. 3A and 4F). This finding is consistent with a decrease in UT1 mRNA expression levels seen by Northern analysis in the rats treated with dDAVP (Figs. 1 and 2).

Effect of vasopressin infusion on UT protein expression. To test whether UT1 and UT2 protein expression levels change in response to chronic vasopressin infusion and to determine whether these changes correlate with the mRNA expression levels, we performed immunocytochemistry using a previously characterized anti-rat UT antibody (18) that recognizes both UT1 proteins in IMCD and UT2 in descending thin limbs (Fig. 5). In agreement with a spreading of UT2 mRNA expression along descending thin limbs (Fig. 5), labeling in descending thin limbs in the outer medulla in dDAVP-treated Brattleboro rats (Fig. 5, A and C) compared with control (Fig. 5, B and D). Labeling in this segment of the dDAVP-treated group extended to the upper part of the inner stripe of the outer medulla and the basal part of the inner medulla. In the inner medulla, immunocytochemistry revealed that labeling of IMCD in the dDAVP-treated Brattleboro rats (Fig. 5E) was considerably decreased compared with control (Fig. 5F). Interestingly, only the lower part of the IMCD exhibited a strong decrease. Unlike the marked increase in aquaporin-2 labeling in the apical membrane of the IMCD in vasopressin-treated BB rats (11, 17, 22), no evidence of increased labeling of the UT1 urea transporter in the IMCD was observed in any dDAVP-treated rats.

DISCUSSION

The present study was designed to investigate the role of vasopressin in modulation of two urea transporters expressed in the kidney tubule, UT1 and UT2, in response to dehydration. Brattleboro rats, which lack circulating vasopressin, were continuously infused for 8 days with either AVP, which is a known stimulator of both V1 and V2 receptors, or dDAVP, which is a specific V2-receptor agonist. The findings that both UT1 and UT2 were not significantly altered after 24-h water restriction in Brattleboro rats highlight the importance of vasopressin in the regulation of UT1 and UT2. Long-term administration of vasopressin, in particular dDAVP, was required to upregulate UT2 in descending thin limbs of the loops of Henle and to downregulate UT1 in the terminal IMCD in a manner analogous to Sprague-Dawley rats that underwent water restriction (28). Furthermore, although UT2 expression was mainly restricted to the deep portion of the inner stripe of the outer medulla, treatment with dDAVP resulted in an extension of UT2 signals throughout the inner stripe of the outer medulla and the upper part of the inner medulla. Expression levels of the UT1 and UT2 proteins, as determined by immunocytochemistry, were altered in parallel with mRNA changes. Thus the present studies confirm the hypothesis that sustained increase in vasopressin is critical for urea transporter regulation as part of the adaptation to water restriction and to maintain appropriate hypertonicity in the kidney inner medullary interstitium.
Early studies by Valtin (32) showed that urea concentration (but not sodium) was markedly diminished in medullary and papillary tissue in parallel with lower urine osmolality in Brattleboro rats compared with control. These abnormalities could be corrected by large doses of exogenous vasopressin. Subsequently, it was shown that increased urea accumulation in the medullary interstitium during water restriction does not correlate with increased urea reabsorption in the IMCD because urea permeability in this segment was unchanged (5, 14). The findings in our present studies that expression of UT1, which mediates urea reabsorption in the apical membrane of the IMCD, is not increased after treatment with vasopressin are in agreement with previous findings.
agreement with these previous studies and our earlier findings in Sprague-Dawley rats (28). Thus upregulation of UT2 expression in thin descending limbs of the inner stripe of the outer medulla and the upper part of the inner medulla likely accounts for urea accumulation in the medullary interstitium in response to long-term infusion of vasopressin. It is also interesting that UT2 mRNA expression in Brattleboro rats is restricted to a shorter portion of descending thin limbs of the loops of Henle compared with untreated normal rats (Fig. 3 and Ref. 28). Taken together, these data show that long-term regulation of UT2 expression in the descending thin limbs of the loops of Henle is the main factor that modulates urea accumulation in the medullary interstitium in response to water restriction and that this effect is under control of the antidiuretic hormone vasopressin. Upregulation of UT2 expression should result in more efficient urea recycling that limits urea escape from the inner medulla and increases its concentration in the medullary interstitium.

Although sustained increase in vasopressin levels leads to increased expression of aquaporin-2 and aquaporin-3 mRNAs and proteins in the collecting duct with increased water reabsorption (7, 16), no such long-term increase in UT expression in the IMCD is necessary. The increased accumulation of urea is due to the higher concentration of urea entering the terminal IMCD as a result of water extraction along the collecting duct, and to increased urea recycling by UT2 in descending thin limbs. This mechanism is advantageous because it allows the maintenance of the corticomedullary urea gradient without decreasing the capacity of the
kidney to excrete urea as the daily nitrogenous waste product.

Water deprivation of Brattleboro rats for 24 h did not result in significant alteration in the transcription levels of either UT1 or UT2, as demonstrated by Northern analysis and in situ hybridization. It was demonstrated in previous studies that thirsting of Brattleboro rats causes a progressive decline in glomerular filtration rate (GFR) in parallel with decreased urine flow and increased urinary osmolality (8). It is plausible that decreases in GFR and medullary blood flow are primarily accounted for increased inner medullary urea levels and osmolality (1, 32). In addition, dehydration results in increased plasma oxytocin levels (8) that are known to stimulate urea permeability in the IMCD by binding to V₂ receptors and increasing cAMP during the acute phase (5). Increased osmolality, even in the absence of vasopressin, has been shown to increase urea permeability in the terminal IMCD by changes in intracellular calcium (13, 24). A consensus sequence for osmotic effects of gene transcription has been discovered in the promoter of several genes (4). Studies designed to induce long-term but subtle water restriction might be beneficial to demonstrate this effect.

The different expression levels of UT1 and UT2 between AVP- and dDAVP-treated animals in this study are interesting. The dose of AVP used in the present study has been shown to increase urinary osmolality to $1,000–1,200$ mosmol/kgH₂O after 5 days (9) and was similar to that used by DiGiovanni et al. (7) to demonstrate alteration of water channel expression. However, this dose rate is about two times less than that used later by Terris et al. (30, 31) to study long-term regulation of water channels and urea transporters in rats that induced maximum urinary osmolality to above $2,000$ mosmol/kgH₂O. These observations indicate that maximum V₂ receptor activation was not reached in the AVP-treated group in our present study as determined by the mean urinary osmolality of $1,200$ mosmol/kgH₂O. This explains a less dramatic increase in UT2 expression observed in these animals. Treatment with dDAVP, which is known to be more specific for V₂ receptor activation, even with the same dose, results in greater urinary osmolality ($2,400$ mosmol/kgH₂O) and therefore in a remarkable change in UT2 expression. Our data, however, cannot rule out the possibility that concurrent V₁ₐ receptor activation as a result of AVP treatment counteracts the regulatory effect of V₂ receptor stimulation. Future studies using V₁ₐ-receptor-specific antagonists in conjunction with the high dose of AVP treatment will be required to address this question.

In a recent study, UT expression was examined in Brattleboro rats by using different doses of AVP and dDAVP (5 and 0.25 µg/day, respectively) to receive a similarly high level of urine osmolality (19). After 5 days, a higher change in UT2 transcripts in the inner stripe and the basal part of the inner medulla was demonstrated in dDAVP-treated Brattleboro rats compared with AVP-treated animals, findings that are analogous to the present study. However, Northern analysis in that study revealed an increase in UT1 message in the inner medullary base of dDAVP-treated rats, in contrary to our observation that UT1 message is downregulated in the kidney inner medulla. The most plausible explanation for this discrepancy is the difference in the amount of protein intake by the animals in the two studies. It has been documented that varying protein diet can effect the abundance of UT1 mRNA (Fig. 3). Recently, Knepper and colleagues (31) showed that at least two forms of UT1 polypeptide are present in the IMCD with an apparent molecular mass of 97 and 117 kDa. Immunoblot analysis of continuous vasopressin-treated Brattleboro rats revealed a marked decrease of the 117-kDa protein, which is mostly abundant in the membrane vesicles of the IMCD, whereas the 97-kDa protein was not changed. Regulation of the 117-kDa protein may involve post-translational modification, as suggested by the authors (31). Whether the decrease of UT1 protein observed in the terminal portion of the IMCD corresponds to the changes of the 117-kDa protein remains to be determined.

In conclusion, our results demonstrate that vasopressin is responsible for increased UT2 expression in the descending thin limbs and modestly decreased UT1 expression in the IMCDs during long-term water restriction. Our data indicate that enhancement of the urea recycling pathway plays an important role to maintain medullary hypertonicity and maximize urinary concentration in this condition.

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