Urinary Concentrating Defect in Hypothyroid Rats: Role of Sodium, Potassium, 2-Chloride Co-Transporter, and Aquaporins

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Abstract. Hypothyroidism is associated with impaired urinary concentrating ability in humans and animals. The purpose of this study was to examine protein expression of renal sodium chloride and urea transporters and aquaporins in hypothyroid rats (HT) with diminished urinary concentration as compared with euthyroid controls (CTL) and hypothyroid rats replaced with L-thyroxine (HT+T). Hypothyroidism was induced by aminotriazole administration. Body weight, water intake, urine output, solute and urea excretion, serum and urine osmolality, serum creatinine, 24-h creatinine clearance, and fractional excretion of sodium were comparable among the three groups. However, with 36 h of water deprivation, HT rats demonstrated significantly greater urine flow rates and decreased urine and medullary osmolality as compared with CTL and HT+T rats at comparable plasma vasopressin concentrations. Western blot analyses revealed decreased renal protein abundance of transporters, including Na-K-2Cl, Na-K-ATPase, and NHE3, in HT rats as compared with CTL and HT+T rats. Protein abundance of renal AQP1 and urea transporters UTA1 and UTA2 did not differ significantly among study groups. There was however a significant decrease in protein abundance of AQP2, AQP3, and AQP4 in HT rats as compared with CTL and HT+T rats. These findings demonstrate a decrease in the medullary osmotic gradient secondary to impaired countercurrent multiplication and downregulation of aquaporins 2, 3, and 4 as contributors to the urinary concentrating defect in the hypothyroid rat.

Hypothyroidism is a very common clinical disorder that is associated with abnormalities in many organs, including the kidney. The ability to conserve water during periods of fluid deprivation is an important function of the kidney. Hypothyroidism has been associated with an impaired urinary concentrating capacity (1,2). However, the mechanisms of this defect at the cellular and molecular level have not been defined.

In the present study, the effect of hypothyroidism in rats on the pivotal components of the urinary concentrating mechanism have been examined and compared with euthyroid animals. These components during fluid deprivation include release of the antidiuretic hormone, arginine vasopressin (AVP), and upregulation of the abundance of aquaporin-2 (AQP2) water channels in the principal cells of the collecting duct. Activation of the countercurrent concentrating mechanism, which is initiated by increased sodium-potassium-2 chloride (Na-K-2Cl) co-transporter in the water impermeable ascending limb, creates the osmotic driving force for passive water reab-sorption across the collecting duct. There are also roles for other water channels, including aquaporins 1, 3, and 4, and for urea transporters in urinary concentration. The present study was undertaken to define the effect of hypothyroidism on these various molecular events during fluid deprivation in the rat.

Materials and Methods

Animal Model

The study protocol was approved by the University of Colorado Institutional Animal Care and Use Committee. Male Sprague-Dawley rats weighing 180 to 220 g were used for the experiments. Balance studies were conducted in metabolic cages. All animals underwent acclimation to metabolic cages for a continuous 5-d period before initiation of study. Thirty rats were divided equally into three study groups: hypothyroid (HT), hypothyroid with thyroxine replacement (HT+T), and control (CTL). All rats were subjected to the study protocol as outlined below. Seven rats in each study group were utilized for immunoblotting studies, and three in each group were utilized for immunofluorescence studies.

Hypothyroidism was induced by administration of aminotriazole (Sigma, St. Louis, MO) for 4 wk (0.5 g/kg powdered rat chow) (3) and was confirmed by tail vein serum free thyroxine measurement. HT+T rats received the same aminotriazole treatment for 6 wk. However, in the last 2 wk of study, HT+T rats also received daily intraperitoneal injection of L-thyroxine (Sigma) 50 μg/kg. Control rats received plain powdered rat chow. Food intake was controlled through matched feeding, (i.e., one CTL rat and one HT+T rat received the same daily food intake as one HT rat) to control solute, protein, and caloric intake. Powdered rat chow was obtained from Harlan Teklad Bio-
products (Indianapolis, IN) and contained 0.4% sodium. Drinking water was provided ad libitum. All animals were maintained in metabolic cages for the duration of the study to accurately assess daily food and water intake and urine output. Body weight was measured weekly.

Toward the conclusion of the aminotriazole treatment period, echocardiography was performed using a GE Vingmed System Five imaging tool for small rodents, with a 10 MHz probe. The animals were anesthetized with ketamine (40 mg/kg body wt, intraperitoneal) and xylazine (5 mg/kg body wt, intraperitoneal). Left ventricular fractional shortening was calculated from left ventricle systolic (LVIDs) and diastolic (LVIDd) diameter as ([LVIDd-LVIDs]/LVIDd × 100%). Cardiac output was calculated via measurement of the diameter of the outflow tract (LVOT), the flow through the outflow tract (VTI), and the heart rate (HR) by the formula, 0.785 × LVOT² × VTI × HR.

Two to three days after echocardiography, a 24-h water balance study was performed. Urine was collected under oil and frozen for later analysis of osmolality and creatinine. Tail vein blood sampling was performed at the conclusion of the balance study to measure serum osmolality, creatinine, and free thyroxine concentration.

Subsequently, all animals were subjected to a 36-h period of water deprivation, during which time food intake and urine output were recorded. Urine was collected under oil, and urine volume was measured every 12 h. In the final 12 h of the water deprivation period, urine was collected for osmolality and creatinine. Animals were then sacrificed by decapitation to avoid any influence of anesthesia on plasma AVP concentration (4). Trunk blood was collected for plasma AVP concentration, serum osmolality, and serum creatinine concentration.

In seven rats in each of the three study groups, kidneys were rapidly removed after decapitation and dissected on ice into cortex, outer medulla, and inner medulla regions. Kidney regions were immediately placed in chilled isolation solution containing 250 mM sucrose, 10 mM triethanolamine, pH 7.6, with 0.1% vol protease inhibitors (0.7 µg/ml pepstatin, 0.5 µg/ml leupeptin, 1 µg/ml aprotinin) and 200 µM phenylmethylsulfonyl fluoride. Tissue samples were immediately homogenized in a glass homogenizer at 4°C. After homogenization, protein concentration was determined for each sample by the Bradford method (Bio-Rad, Richmond, CA). Immunoblotting was performed on cortex samples for AQP1, Na-K-2Cl, Na-K-ATPase, and NHE3. Immunoblotting was performed on outer medulla samples for Na-K-2Cl, urea transporters, and AQP1. Inner medulla samples were utilized for immunoblotting for urea transporters and AQP2, AQP3, and AQP4.

Western Blot Analyses

SDS-PAGE was performed on 8% acrylamide gels for Na-K-2Cl, Na-K-ATPase, and NHE3, on 10% acrylamide gels for UTA1 and UTA2, and on 12% acrylamide gels for AQP. After transfer by electroelution to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blots were blocked overnight with 5% nonfat dry milk in phosphate-buffered saline (PBS(−)) and then probed with the respective antibodies for 24 h at 4°C. After washing with buffer containing PBS(−) with 0.1% Tween-20 (J. T. Baker Inc., Phillipsburg, NJ), the membranes were exposed to secondary antibody for 1.5-h at room temperature. Subsequent detection of the specific proteins was carried out by enhanced chemiluminescence (Amersham, Arlington Heights, IL) according to manufacturer’s instructions. Prestained protein markers were used for molecular mass determinations. Densitometric results were reported as integrated values (area × density of band) and expressed as a percentage compared with the mean value in controls (100%). Membranes were stained with Coomassie blue to ensure equal loading. Western blots as shown in the results section are representative of results obtained from all samples (n = 7 rats per study group; total n = 21). Densitometry results shown are obtained from all samples.

Antibodies

Antibodies to AQP2, AQP3, AQP4, Na-K-2Cl, NHE3, and UT-A1 were utilized in this study. Specificity of these antibodies has been previously documented (5–10). Anti-Na-K-ATPase α-1 antibody was obtained from Upstate Biotechnology (Lake Placid, NY). Anti-AQP1 antibody was obtained from Chemicon International, Inc. (Temecula, CA).

Immunofluorescence Studies

Sagittal sections of the kidneys from three rats in each study group were embedded in OCT compound (Sakura Finetek, U.S.A., Inc., Torrance, CA) and snap frozen in liquid nitrogen. Five micrometer sections were cut with a cryostat and collected on charged slides. The slides were stored at −80°C. The slides were then fixed overnight at 4°C with antibodies for AQP2 and Na-K-2Cl, both antibodies diluted 1:100 in PBS. AQP2 antibody has been previously characterized (5). Na-K-2Cl antibody was a kind gift from Dr. M.A. Knepper (National Institutes of Health, Bethesda, MD). Next, the slides were washed three times in PBS and incubated for 1 h at room temperature with fluorescein conjugated F(ab’)2 to rabbit IgG (Cappel) diluted 1:200 in PBS. The slides were then washed three times with PBS and viewed with a Zeiss microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY). Immunofluorescence studies were performed and viewed in one experiment to minimize variation in technique.

Biochemical Analyses

After acetone-ether extraction, measurement of AVP concentration was assessed in plasma by RIA as described previously (11). Serum and urine osmolality were measured by freezing point depression (Advanced Instruments, Inc., Norwood, MA). Serum and urine creatinine were measured (Beckman Instruments, Inc.). Twenty-four-hour creatinine clearance was used as an estimate of GFR.

Medullary Tonicity

Samples of inner medulla were placed in a preweighed Eppendorf tube containing 200 µl of deionized distilled water. The tissue was homogenized in a glass homogenizer at 4°C. Tissue osmolality was measured by freezing point depression (Advanced Instruments, Inc.). The original tissue osmolality was estimated based on the nominal dilution factor and the assumption that 80% of the wet weight was water (12).

Statistical Analyses

Statistical analysis of results was performed using ANOVA with Tukey test post-hoc. Results were expressed as mean ± SEM with P < 0.05 considered significant.

Results

Characteristics of Study Groups Just before Water Deprivation

Study group characteristics are reported in Table 1. Body weight was similar at the start of the study and after the
aminotriazole treatment period, before water deprivation. This was expected as food intake was matched among the three study groups. No significant differences were found in water intake, sodium intake, urine output, urine osmolality, serum osmolality, serum creatinine, 24-h creatinine clearance, fractional excretion of sodium, or urea excretion. Solute excretion was similar among study groups. Serum free thyroxine concentration was significantly lower in HT rats as compared with CTL and HT+T rats.

Echocardiography was performed on all rats (Figure 1). Heart rate was significantly lower in HT rats as compared with CTL and HT+T rats (Figure 1A). Cardiac output (Figure 1B) and left ventricular fraction of shortening (Figure 1C) were also significantly decreased in HT rats as compared with the other two study groups.

Water Deprivation Studies
All rats were then subjected to a 36-h period of water deprivation. During this period, HT rats demonstrated significantly higher urine flow rate (Figure 2A) than either CTL or HT+T rats. In addition, maximal urine osmolality was significantly decreased in HT rats in response to water deprivation as compared to CTL and HT+T rats (Figure 2B). Serum osmolality (CTL 303 ± 5 versus HT 314 ± 8 versus HT+T 302 ± 3 mOsm/kg H2O, P = NS) was similar among study groups. No significant differences in plasma AVP concentrations (CTL 5.8 ± 0.7 versus HT 5.4 ± 1.3 versus HT+T 6.1 ± 1.0 pg/ml, P = NS) were found in response to water deprivation.

Countercurrent Multiplication
Medullary osmolality was significantly decreased in HT rats as compared with CTL and HT+T rats in response to water deprivation (Figure 2C). Hypothyroidism was associated with significant decreases in renal protein abundance of Na-K-2Cl (Figure 3A), Na-K-ATPase (Figure 3B), and NHE3 (Figure 3C) as compared with CTL and HT+T rats. AQP1 was unchanged in renal cortex (CTL 100 ± 3 versus HT 100 ± 14 versus HT+T 117 ± 9%, P = NS), outer medulla (CTL 100 ± 20 versus HT 100 ± 28 versus HT+T 100 ± 18%, P = NS), and inner medulla (CTL 100 ± 25 versus HT 112 ± 26 versus HT+T 100 ± 19%, P = NS) in hypothyroidism in response to water deprivation. Immunofluorescence studies demonstrated decreased expression of Na-K-2Cl in kidneys of hypothyroid rats as compared with CTL and HT+T rats (Figure 4).

Urea Transporter Abundance
No statistically significant differences in inner medulla protein abundance of UTA1 (Figure 5A) or UTA3 (Figure 5B) or in outer medulla UTA2 (Figure 5C) were found among the study groups.

Aquaporin Water Channels
Hypothyroidism was associated with significant decreases in inner medulla protein abundance of AQP2 (Figure 6A), AQP3 (Figure 6B), and AQP4 (Figure 6C) as compared with CTL and HT+T rats. Hypothyroid rats also demonstrated decreased AQP2 by immunofluorescence as compared with CTL and HT+T rats (Figure 7).

Discussion
Advanced hypothyroidism to the clinical stage of myxedema is associated with hyponatremia involving both the nonosmotic release of AVP and diminished GFR (1,13–15). These events, which impair urinary dilution in advanced hypothyroidism, override the defect in urinary concentrating capacity, which also has been observed with hypothyroidism (1,2). In the present study, a model of moderate hypothyroidism was investigated in which neither hyponatremia nor a diminished GFR

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a HT, hypothyroid rats; CTL, euthyroid rats; HT + T, hypothyroid rats replaced with L-thyroxine.  

b P < 0.001 HT versus CTL; P < 0.001 HT versus HT + T.
occurred, but a reversible decrease in maximal urine concentration was observed. With the use of molecular probes, it was possible to examine the perturbations in transporters and aquaporins that are involved in this hypothyroid-related defect in urinary concentration.

In the present study, the cardiac hemodynamics characteristic of hypothyroidism were demonstrated, including a decrease in cardiac output, heart rate, and left ventricular fraction of shortening as examined by echocardiography. Perturbations in these hemodynamic measurements were completely reversed by thyroid hormone replacement. The observed maintenance of GFR in these animals indicated intact GFR autoregulation with this degree and duration of hypothyroidism. The defect in maximal urinary concentration could be related to diminished AVP release in the hypothyroid animals. However, this was not the case, because plasma AVP concentrations as measured by RIA were no different between the hypothyroid and euthyroid animals. Thus, the impaired urinary concentration constituted an AVP-resistant nephrogenic defect.

It is known that the rate of sodium chloride delivery to the thick ascending limb of Henle’s loop is a determinant of maximal urinary concentration, because sodium chloride reab-

Figure 1. Hypothyroid rats (HT) demonstrated significant decreases in heart rate (A), cardiac output (B), and left ventricular fraction of shortening (C) as compared with control (CTL) and L-thyroxine-treated hypothyroid rats (HT+T).

Figure 2. HT rats demonstrated increased urine flow rate (A), decreased maximal urinary osmolality (B), and decreased medullary osmolality (C) in response to 36-h water deprivation as compared with CTL and HT+T rats.
sorption in this water impermeable segment of the nephron is the initiating event of the countercurrent concentrating mechanism. Either a decrease in GFR or an increase in sodium chloride reabsorption in the proximal tubule could diminish delivery of sodium chloride to the thick ascending limb. Micropuncture studies, however, have demonstrated a decrease in sodium chloride reabsorption in the proximal tubule in a similar rat model of hypothyroidism (15). The present results provide a molecular basis for this observed diminution in sodium chloride reabsorption in the proximal nephron. Specifically, the renal cortical transporter proteins, NHE-3 and Na-K-ATPase, both factors involved in enhancing proximal tubule sodium reabsorption, were decreased in the hypothyroid rats. These defects were reversed with thyroid hormone replacement.

Diminished urinary urea excretion has been reported in hypothyroidism (1). Urea, along with sodium chloride, constitutes a large portion of the medullary hyperosmolar driving force for water transport; therefore, diminished protein intake as reflected by decreased urinary urea excretion could contribute to the concentrating defect in hypothyroidism. In this regard, protein restriction has been shown to decrease maximal urinary concentration (16). In preliminary studies, we observed a decreased intake of protein and calories in the hypothyroid rats. Thus, the experiments reported in the present study involved paired feeding of the three groups of rats. As a result, the urinary urea excretion rates were comparable in all three groups of rats. The solute excretion and urine flow rates were also not different among the three groups. However, even though urea excretion rates were comparable, it was possible that diminished expression of UTA1 or UTA2 protein in the collecting ducts could still contribute to the urinary concentrating defect in the hypothyroid rat. The protein abundance of these urea transporters was, however, not different between the hypothyroid and euthyroid animals.

The Na-K-2Cl co-transporter in the thick ascending limb is known to be primarily responsible for initiating the countercurrent concentrating mechanism. Thus, a diminution in this transporter protein could diminish medullary osmolality and thereby impair maximal urinary concentration. In the rat, AVP is known to upregulate the Na-K-2Cl co-transporter expression in the thick ascending limb (17). However, as already noted, in the present study plasma AVP concentrations were comparable in the hypothyroid and euthyroid animals. Moreover, in the same hypothyroid AVP rat model, the mRNA in the hypothalamus was actually found to be upregulated (4).

In the present study, the Na-K-2Cl protein was significantly decreased in the cortex and outer medulla, an observation confirmed by immunofluorescence studies. Support for the functional importance of this diminution in the thick ascending limb co-transporter in the impaired maximal urinary concentrating capacity was the significant decrease in medullary osmolality observed in the hypothyroid as compared to the euthyroid rats.

Further studies were undertaken to examine the collecting duct water channels in the hypothyroid and euthyroid state. There was a significant decrease in protein expression in AQP2, 3, and 4 in the hypothyroid rats, which was reversed...
with thyroid hormone replacement. The AQP1 protein was also studied since a decrease in this aquaporin has been shown to cause a urinary concentrating defect in both knockout mice and humans without the Colton antigen blood type (18,19). However, in the current hypothyroid rats, there was no evidence for a downregulation of AQP1.

Figure 4. Immunofluorescence studies demonstrated decreased Na-K-2Cl expression in hypothyroid rats as compared with CTL and HT+T rats. Arrows denote Na-K-2Cl expression. Magnification, ×200.
protein abundance in renal cortex, outer medulla, or inner medulla.

The relative importance of the decrease in these collecting ducts

Figure 5. No significant differences in inner medulla abundance of the urea transporters UTA1 (A) or UTA2 (B) or in outer medulla abundance of UTA2 (C) were noted among the study groups.

Figure 6. Hypothyroidism (HT) was associated with significant decreases in the inner medulla protein abundance of the aquaporin water channels, AQP2 (A), AQP3 (B), and AQP4 (C), as compared with CTL and HT+T rats.
duct water channels, AQP2, 3, and 4, is somewhat difficult to establish. Specifically, because the decreased medullary and urine osmolalities in the hypothyroid rats were not significantly different, it was not possible to establish a failure for water to equilibrate osmotically across the collecting duct. In the absence of the defect in the Na-K-2Cl and the diminished generation of the medullary osmotic gradient, however, this degree of downregulation of AQP2 protein abundance would be expected to contribute to a decrease in maximal urinary concentration. It is difficult to assess whether the observed decrease in the exit water channels on the basolateral collecting duct membrane, i.e., AQP3 and 4, would be sufficient to alter maximal urinary concentration.

In summary, the impaired maximal urinary concentrating capacity and perturbations in systemic hemodynamics in rats with moderate hypothyroidism are readily reversible with thyroid hormone replacement. The hypothyroid-related urinary concentrating defect was dissociated from a decrease in GFR, urea, and solute excretion; water, caloric, and protein intake; or a downregulation of collecting duct urea transporters. However, a diminished medullary osmotic driving force for passive water movement across the collecting duct was associated with a significant decrease in the medullary Na-K-2Cl co-transporter of the thick ascending limb. Any functional importance of the downregulation of AQP2, 3, and 4 in the collecting duct was superseded by the role of the downregulated Na-K-2Cl co-transporter in association with an impaired countercurrent concentrating mechanism.

Acknowledgments

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References


