

Renal Phenotype of UT-A Urea Transporter Knockout Mice

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The urea transporters UT-A1 and UT-A3 mediate rapid transepithelial urea transport across the inner medullary collecting duct (IMCD). In a previous study, using a new mouse model in which both UT-A1 and UT-A3 were genetically deleted from the IMCD (*UT-A1/3*^{-/-} mice), we investigated the role of these transporters in the function of the renal inner medulla. Here the authors report a new series of studies investigating more generally the renal phenotype of *UT-A1/3*^{-/-} mice. Pathologic screening of 33 tissues revealed abnormalities in both the testis (increased size) and kidney (decreased size and vascular congestion) of *UT-A1/3*^{-/-} mice. Total urinary nitrate and nitrite (NOx) excretion rates in *UT-A1/3*^{-/-} mice were more than double those in wild-type mice. Total renal blood flow was not different between *UT-A1/3*^{-/-} and wild-type mice but underwent a greater percentage decrease in response to NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME) infusion. Whole kidney GFR (FITC-inulin clearance) was not different in *UT-A1/3*^{-/-} mice compared with controls and underwent a similar increase in response to a greater dietary protein intake. Fractional urea excretion was markedly elevated in *UT-A1/3*^{-/-} mice on a 40% protein diet, reaching $102.4 \pm 8.8\%$ of the filtered load, suggesting that there may be active urea secretion somewhere along the renal tubule. Although there was a marked urinary concentrating defect in *UT-A1/3*^{-/-} mice, there was no decrease in aquaporin 2 or aquaporin 3 expression. Furthermore, although urea accumulation in the inner medulla was markedly attenuated, there was no decrease in sodium ion concentration in tissue from outer medulla or two levels of the inner medulla. These results support our conclusion that the urinary concentrating defect in *UT-A1/3*^{-/-} mice is caused by a failure of urea transport from the IMCD lumen to the inner medullary interstitium, resulting in osmotic diuresis.

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In mammals, nitrogen from dietary protein in excess of that required to satisfy the body's needs is converted to urea in the liver as part of the urea/ornithine cycle. The kidneys excrete this urea. In humans, urea production and excretion can amount to several hundred millimoles per day. Such large amounts of any other solute, for example mannitol (1,2), would obligate large amounts of water excretion by causing an osmotic diuresis. A basic model of urea handling was proposed by Berliner *et al.* (3), hypothesizing that an osmotic diuresis does not occur with urea because of the accumulation of urea in the inner medullary interstitium, which osmotically balances the high level of urea in the inner medullary collecting duct (IMCD) and urine. This urea accumulation is thought to be largely a consequence of facilitated urea transport across the IMCD epithelium mediated by phloretin-sensitive urea transporters.

In a recent study (4), we demonstrated that genetic deletion of the urea transporters UT-A1 and UT-A3 (generating of so-called *UT-A1/3*^{-/-} mice) completely abolished phloretin-sensitive, vasopressin-regulated urea transport across the IMCD.

The deletion of these urea transporters resulted in a marked defect in inner medullary urea accumulation and a marked limitation in the ability of the kidneys to conserve water caused by a urea-dependent osmotic diuresis. Therefore, the *UT-A1/3*^{-/-} line represents one of several mouse models with defective urinary concentrating mechanisms, including those with genetic disruption of the urea transporter UT-B, the CLC-K1 chloride channel, and the aquaporin 2 water channel (5–7).

In our previous study, *UT-A1/3*^{-/-} mice were used to investigate the role of UT-A1 and UT-A3 in the function of the renal inner medulla. In the new series of experiments reported in this article, we investigate more generally the renal function of *UT-A1/3*^{-/-} mice. We address whether there are changes in renal structure, renal blood flow, GFR, corticomedullary solute gradients, and Na transporter or aquaporin expression consequent to the deletion of the urea transporters. Furthermore, we have performed careful measurements of fractional urea excretion in *UT-A1/3*^{-/-} mice to address overall urea handling in kidneys in which the main urea reabsorptive mechanism beyond the proximal tubule has been deleted. We have also investigated the renal effects of changes in dietary protein intake in the *UT-A1/3*^{-/-} mice to determine whether these effects are dependent on IMCD urea reabsorption and the associated medullary urea recycling process. Finally, because UT-A urea transporters are expressed in numerous extra-renal

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tissues (8), we have begun to examine whether a nonrenal phenotype exists in *UT-A1/3*^{-/-} mice.

Materials and Methods

Whole Animal Pathology

UT-A urea transporters are expressed in numerous extrarenal tissues (8). Therefore, we performed whole animal necropsy and pathologic analysis of 33 different organs and tissues to determine if deletion of urea transporters resulted in a nonrenal phenotype. Tissues analyzed were kidneys, spleen, skeletal muscle, liver, testis, reproductive tract, brain, gastrointestinal tract (all levels), lungs, heart, thymus, pancreas, sciatic nerve, trachea, spinal cord, esophagus, femur, teeth, nasal sinuses, gall bladder, ears, urinary bladder, bone marrow, eyes, tongue, skin, parathyroid glands, Harderian glands, pituitary gland, thyroid glands, salivary glands, adrenal glands, and lymph nodes. For analysis, three male wild-type mice and three male knockout mice were compared. Animals were euthanized, and for some organs wet weight was measured. All other tissues were immersion-fixed in formaldehyde, embedded in paraffin, and histopathologic diagnosis was performed. Kidney images were captured using a Leica MZ FL III Fluorescence Stereomicroscope.

Renal Blood Flow

Measurements of renal blood flow (RBF) were performed in male *UT-A1/3*^{-/-} mice as described previously (9). After baseline measurements, the response of RBF to intravenous bolus injections of L-NAME (1 $\mu\text{g/g}$ body weight [BW]) was assessed.

Nitrate and Nitrite Measurements

As an index of total kidney nitric oxide production, we determined the levels of both nitrate and nitrite in urine using a colorimetric assay (Cayman Chemical).

Vasopressin Measurements

Serum vasopressin levels were measured by RIA (Alpco Diagnostics). Mice were euthanized by decapitation; the blood was collected and serum was separated. Because the assay required 1 ml of serum, and the amount of serum collected from each mouse by decapitation was approximately 250 μl , four mice were used for one basal measurement (12 total animals per group, $n = 3$).

Metabolic Cage Studies

Animals were maintained in mouse metabolic cages (Hatteras Instruments) for the duration of the study under controlled temperature (62°C) and light conditions (12-h light and dark cycles). Several experimental manipulations were performed.

Effect of Dietary Protein Content on Urinary Concentrating Ability and Solute Accumulation. Mice received free access to pelleted diet containing 4%, 20%, or 40% protein by weight (as casein) for 7 d before metabolic cage studies. Subsequently, mice received a fixed daily ration of 5g of gelled diet per 20 g of BW per day, also with either 4%, 20%, or 40% protein. The gelled diet was made up of 1 ml of deionized water, 4 g of special low-NaCl synthetic food (0.001% Na w/w; Research Diets), 0.2 mmol NaCl, and 25 mg agar. Prewedged drinking water was provided *ad libitum* during the initial period of the study. After 3 days of adaptation to the cages, urine was collected under mineral oil in preweighed collection vials for successive 24-h periods. After the initial collection period, each mouse received a fixed daily ration of 5.7 g of gelled diet per 20 g BW per day for 24 h; gelled diet contained 1.7 ml of deionized water. Mice did not have access to

supplemental drinking water during this period. Blood was collected and kidneys were processed as detailed.

Serum and Urine Collection. Mice were anesthetized with isoflurane and blood was collected using retro-orbital eye bleeding. Serum was separated from whole blood using centrifugation at 2000 $\times g$ and StatSampler collection tubes (StatSpin Inc). Sodium, potassium, chloride, creatinine, urea, glucose, calcium, albumin, magnesium, phosphorus, total protein, and uric acid levels in serum were determined using an autoanalyzer. Urine samples were centrifuged at 14,000 $\times g$ for 5 min and sodium, potassium, chloride, creatinine, and urea levels were determined using an autoanalyzer. Serum and urine osmolalities were determined using a vapor pressure osmometer (Wescor).

Solute Content of Kidney. Kidneys were removed and rapidly dissected into cortex, outer medulla, inner medulla base (IM1), and papilla (IM2/3). Solute content of the individual segments was performed as described previously (4). To determine initial solute concentrations, calculations were performed as detailed in the work of Schmidt-Nielsen *et al.* (10).

Determination of Whole Kidney GFR: FITC–Inulin Clearance Method. GFR measurements were made in conscious mice using FITC–inulin clearance and a modified protocol based on the work of Qi *et al.* (11). Mice received free access to pelleted diet (Research diets) containing either 4 or 40% protein by weight for 7 d before metabolic cage studies. Mice were housed individually in metabolic cages for 3 days (as detailed) before surgery and received gelled food. On the fourth day, mice were anesthetized with isoflurane and an osmotic minipump (Model 2001; Alzet) containing approximately 3% FITC–inulin was implanted subcutaneously. Inulin solution was prepared exactly as detailed (11). Mice were returned to metabolic cages and continued to receive free access to water and gelled food. On day 6 after surgery, urine was collected under mineral oil for 24 h. During urine collection, the collection vessel and metabolic cage base were covered with aluminum foil to minimize exposure of the urine to light. Cages were washed twice with 5 ml of 500 mmol HEPES (pH 7.4) to collect residual fluorescence. Serum was collected by retro-orbital eye bleeds as described. Fluorescence was measured in 10- μl samples as described previously (11) using a Victor-3 1420 Multi-label Counter (Wallac) and the GFR was estimated by the 24-h urinary FITC–inulin excretion rate, *i.e.*, urinary fluorescence counts per 24 h divided by the concentration of plasma FITC–inulin.

Adaptive Changes in Renal Sodium and Water Channels. Animals were housed in metabolic cages as detailed and received a 20% protein diet throughout the study. After 3 d of adaptation, half the animals studied were switched for 36 h to a water-restricted diet containing 1.7 ml water per day, and they did not have access to supplemental drinking water during this period. Mice were killed by decapitation and the kidneys processed for immunoblotting.

Immunoblotting. Immunoblotting was performed as described previously (12) using affinity-purified polyclonal antibodies. Quantification of the band densities from immunoblots was performed by laser densitometry (Molecular Dynamics). To facilitate comparisons, we normalized the densitometry values such that the mean for the wild-type control mice is defined as 1 (arbitrary units). Quantitative data are presented as mean \pm SEM.

Statistical Analyses

All values are quoted as means \pm SEM. Analysis was performed by *t* test or one-way ANOVA as appropriate, with significance assumed at the 5% level. If ANOVA indicated a significant difference, comparison between groups was performed with the Student-Newman-Keuls method.

Results

Survey of Tissues

Because urea transporters are expressed in numerous extra-renal tissues, a comprehensive pathologic survey of numerous tissues from *UT-A1/3*^{-/-} mice was performed to determine if any abnormalities were present. Except for the kidney and testis, no abnormalities were apparent. When normalized by brain weight, the kidneys were significantly smaller and the testis significantly larger in knockout animals compared with controls (Table 1). However, microscopic analysis could not detect any differences in cell number, cell type, or morphology. Resected kidneys of *UT-A1/3*^{-/-} mice also had greater blood congestion than kidneys from wild-type animals (representative images are shown in Figure 1A and 1B), especially in the renal medulla (Figure 1C and 1D).

Urinary Nitric Oxide Excretion

Measurements of total urinary nitrate and nitrite (NO_x) excretion rates under basal conditions on a 20% protein diet were performed to determine if the blood congestion observed in *UT-A1/3*^{-/-} mice kidneys may be related to abnormal nitric oxide (NO) production by the kidney. In knockout mice, NO_x excretion (127.0 ± 7.8 nmol/g BW per d) was more than double that in wild-type controls (52.5 ± 9.5 nmol/g BW per d).

Circulating Vasopressin Levels

Another factor that could alter renal blood flow dynamics is vasopressin. Consequently, we measured plasma vasopressin levels in *UT-A1/3*^{-/-} and wild-type mice. Under basal conditions (free access to water) on a 20% protein diet, we observed no difference in plasma vasopressin levels between *UT-A1/3*^{-/-} mice (5.9 ± 1.5 pg/ml) and wild-type controls (4.5 ± 0.76).

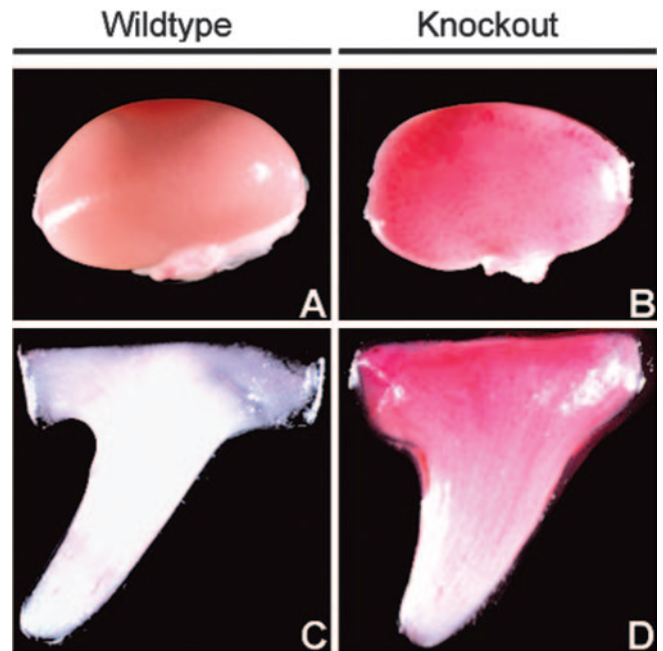


Figure 1. Blood congestion in kidneys of *UT-A1/3*^{-/-} mice. Representative images of whole kidney and inner medulla from wild-type (A and C) or *UT-A1/3*^{-/-} mice (B and D) show greater blood congestion in knockout mice.

Total RBF

Because greater blood congestion was observed in the kidneys of *UT-A1/3*^{-/-} mice, we performed measurements of total RBF in anesthetized mice (Figure 2). Total RBF in the *UT-A1/3*^{-/-} mice was not significantly different from that observed in age-matched wild-type control mice. Inhibition

Table 1. Mouse necropsy

	Wild-Type	Knockout
Body weight, g	22.4 ± 1.4	21.7 ± 1.0
Brain, g	0.443 ± 0.015	0.420 ± 0.003
Kidneys, g	0.308 ± 0.024	0.245 ± 0.012*
Kidney weight/brain weight	0.686 ± 0.043	0.546 ± 0.015*
Liver, g	1.363 ± 0.028	1.116 ± 0.072*
Liver weight/brain weight	3.078 ± 0.044	2.658 ± 0.1583
Spleen, g	0.0797 ± 0.0055	0.0527 ± 0.0068*
Spleen weight/brain weight	0.180 ± 0.016	0.125 ± 0.016
Left testis, g	0.0607 ± 0.0075	0.0803 ± 0.0033
Right testis, g	0.0603 ± 0.0064	0.0780 ± 0.0057
Combined	0.0605 ± 0.0044	0.07917 ± 0.0029*
Testes weight/brain weight	0.136 ± 0.010	0.186 ± 0.014*
Heart, g	0.126 ± 0.008	0.109 ± 0.005
Heart weight/brain weight	0.283 ± 0.01	0.258 ± 0.01
Thymus, g	0.0683 ± 0.007	0.0563 ± 0.002
Thymus weight/brain weight	0.155 ± 0.02	0.134 ± 0.01

Wet tissue weights for male mice. $n \geq 4$.

* represents a significant difference in values from knockout animals compared to corresponding wild-type control group.

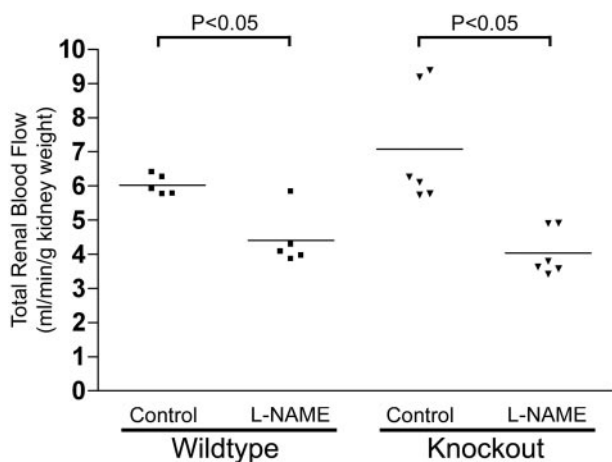


Figure 2. Total renal blood flow (RBF) in wild-type (squares) and *UT-A1/3*^{-/-} mice (triangles). Each point represents data from an individual mouse and means are shown. Administration of NG-Nitro-L-arginine methyl ester hydrochloride (L-NAME, 1 μ g/g body weight) statistically (ANOVA) decreased RBF in both groups of animals. However, no significant difference was observed in RBF between wild-type and *UT-A1/3*^{-/-} mice.

of NO production by infusion of L-NAME resulted in a significant decrease in total RBF in knockout mice and controls. However, the percentage decrease caused by L-NAME was significantly greater in *UT-A1/3*^{-/-} mice ($39.2 \pm 2.4\%$ versus $27.1 \pm 4.9\%$), suggesting a greater role for NO in the maintenance of blood flow.

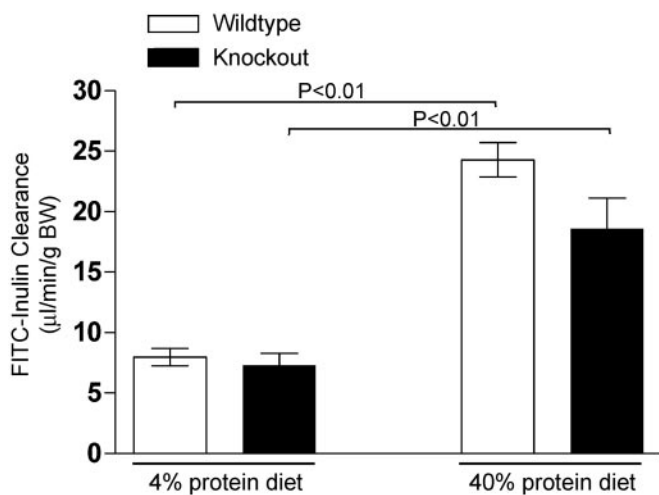


Figure 3. GFR in conscious male mice. FITC-inulin clearance for wild-type (white bars) and *UT-A1/3*^{-/-} mice (black bars). Representative values are mean \pm SEM and significant differences (ANOVA) are indicated. Administration of a high-protein diet (40%) for 7 d dramatically increased GFR in both groups of animals ($n = 5$). However, no significant difference was observed in GFR between wild-type and *UT-A1/3*^{-/-} mice.

GFR

We determined GFR in conscious *UT-A1/3*^{-/-} and wild-type mice on two levels of protein intake, 4 and 40%, using FITC-inulin clearance. Increasing the protein content of the diet more than doubled the FITC-inulin clearance in both *UT-A1/3*^{-/-} mice and wild-type controls (Figure 3 and Table 2). However, no significant differences were observed in inulin clearance between *UT-A1/3*^{-/-} and wild-type mice under either dietary condition, even when corrected for BW.

Urinary Excretion Values

A summary of the excretion of water, monovalent cations, and urea in wild-type and *UT-A1/3*^{-/-} mice on a low-protein (4%) or high-protein (40%) diet is shown in Table 2. Urine volume was greater in *UT-A1/3*^{-/-} mice only on the high-protein diet, resulting in a marked decrease in the urine/plasma (U/P) inulin ratio and a corresponding reduction in urinary osmolality. However, there were no differences in osmolar excretion in *UT-A1/3*^{-/-} mice versus wild-type controls.

On a high-protein diet, plasma urea concentration was significantly lower in *UT-A1/3*^{-/-} mice than in wild-type controls (Table 2). Plasma concentrations of Na, K, and Cl were not different between *UT-A1/3*^{-/-} mice and controls.

Fractional urea excretion (FE_{urea}) was markedly elevated in *UT-A1/3*^{-/-} versus wild-type controls on both the 4% and 40% protein diets. In fact, FE_{urea} reached $102.4 \pm 8.8\%$ of the filtered urea in the *UT-A1/3*^{-/-} mice on the 40% protein diet, a level that may be indicative of net active urea secretion along the renal tubule. Fractional excretion rates of sodium and potassium were not significantly different in *UT-A1/3*^{-/-} mice versus wild-type controls.

Urinary Concentrating Ability of *UT-A1/3*^{-/-} Mice

UT-A1/3^{-/-} mice have a urinary concentrating defect that is dependent on the level of urea excretion (4). Because the main determinant of urea excretion rate is protein intake, in the present study, the effects of three different dietary protein intakes on urinary concentrating ability were determined (Figure 4). On low protein intake (4% protein diet), there were no significant differences in fluid consumption, urine flow, or urine osmolality between wild-type and *UT-A1/3*^{-/-} mice. However, on normal protein intake (20% protein diet), *UT-A1/3*^{-/-} mice exhibited significantly greater fluid consumption and urine flow than wild-type mice, resulting in a decreased urine osmolality. This decrease in urinary concentrating ability was even greater on a 40% protein diet. Furthermore, after an 18-h water restriction (2 ml of water per day per 20 g of BW), *UT-A1/3*^{-/-} mice on either 20% or 40% protein diet (but not a 4% protein diet) were unable to reduce their urine flow and could not raise their maximal urinary osmolality above that observed under basal conditions. In the same time period, wild-type mice on either 20% or 40% protein diet reduced their urine output to 0.9 ± 0.2 and 0.8 ± 0.3 ml, respectively. During this 18-h water restriction, the BW of *UT-A1/3*^{-/-} mice on a 20% or 40% protein diet decreased by $18.2 \pm 0.4\%$ and $24.6 \pm 0.3\%$, respectively. In contrast, *UT-A1/3*^{-/-} mice on a 4% protein diet

Table 2. Urinary concentrating ability, plasma electrolytes, and urinary excretion rates

	4% Protein		40% Protein	
	Wild-Type	Knockout	Wild-Type	Knockout
Body weight, g	22.6 ± 1.3	20.9 ± 0.3	20.4 ± 0.3	20.0 ± 0.6
Urine volume, ml/24 h	2.05 ± 0.27	2.44 ± 0.30	2.12 ± 0.18	7.80 ± 0.50*
Urine osmolality, mosm/kg H ₂ O	646 ± 24	571 ± 51	2613 ± 141	788 ± 13*
Osmolar excretion, mosm/24 h	1392 ± 76	1350 ± 57	5377 ± 422	6151 ± 404
U/P Inulin	112 ± 9	85 ± 6*	262 ± 28	70 ± 9*
Inulin clearance, ml/d	290.8 ± 17.3	219.1 ± 26.5	712.6 ± 42.4	535.7 ± 77.1
Inulin clearance, μl/min per g BW	8.0 ± 0.7	7.3 ± 1.0	23.9 ± 1.42	18.6 ± 2.5
Plasma Na, mmol	150 ± 1	150 ± 0	151 ± 1	152 ± 1
Plasma Cl, mmol	123 ± 1	126 ± 1	122 ± 2	123 ± 1
Plasma K, mmol	5.1 ± 0.2	5.0 ± 0.1	5.4 ± 0.3	5.2 ± 0.3
Plasma urea nitrogen, mmol	2.1 ± 0.1	2.1 ± 0.1	8.0 ± 0.2	6.4 ± 0.4*
Urine Na, mmol	93 ± 12	87 ± 7	87 ± 3	43 ± 9*
Urine K, mmol	79 ± 6	75 ± 15	66 ± 4	24 ± 4*
Urine urea, mmol	123 ± 8	131 ± 23	1464 ± 178	462 ± 27*
Na excretion, mmol/d	239 ± 38	232 ± 32	237 ± 20	302 ± 35
K excretion, mmol/d	181 ± 26	189 ± 36	178 ± 13	188 ± 40
Urea excretion, mmol/d	317 ± 39	350 ± 71	3908 ± 372	3516 ± 485
Na clearance, ml/d	1.23 ± 0.20	1.47 ± 0.20	1.56 ± 0.13	1.97 ± 0.22
K clearance, ml/d	33.6 ± 3.8	37.1 ± 6.9	30.5 ± 2.0	33.9 ± 8.9
Urea clearance, ml/d	117.5 ± 18.3	156.6 ± 24.3	486.6 ± 46.1	556.7 ± 94.1
FE Na, %	0.41 ± 0.05	0.73 ± 0.16	0.25 ± 0.05	0.39 ± 0.12
FE K, %	11.5 ± 0.9	15.9 ± 5.6	4.3 ± 0.3	6.5 ± 1.7
FE urea, %	39.8 ± 4.6	77.1 ± 17.7	68.2 ± 5.2	102.0 ± 8.8*
U/P Na	0.61 ± 0.07	0.58 ± 0.05	0.57 ± 0.02	0.28 ± 0.06*
U/P K	16.6 ± 0.5	15.1 ± 3.5	11.2 ± 0.8	4.4 ± 0.8*
U/P Urea	57.3 ± 3.6	61.7 ± 7.1	182.5 ± 23.1	72.7 ± 6.1*

n = 5 for each group.

* represents a significant difference in values from knockout animals compared to corresponding wild-type control group.

were able to maintain fluid balance without a marked loss of body weight (3.0 ± 0.2%).

Expression of Sodium Transporters and Aquaporins

The greater urine flow in *UT-A1/3*^{-/-} mice can be explained by a urea-dependent osmotic diuresis (4). We hypothesized that changes in the expression of other channels and transporters may, in part, compensate for the observed polyuria. Therefore, we examined the abundance of the major Na transporters and aquaporins in kidneys from wild-type and *UT-A1/3*^{-/-} mice either under normal conditions, in which animals had free-access to water, or after water restriction.

Under basal conditions without water restriction, apart from a significant reduction in the expression of the type 2 Na-dependent phosphate transporter, no other major differences in Na transporter abundances were observed between the groups (Figure 5). However, after 36 h of water restriction, the abundances of both the thiazide-sensitive Na-Cl cotransporter and all three subunits of epithelial sodium channel (ENaC) were significantly greater in knockout animals compared with con-

trols. In contrast, the expression of the type 2 Na-dependent phosphate transporter was further reduced in knockout animals to a level that was virtually undetectable by immunoblotting.

Under basal conditions without water restriction (Figure 6), *UT-A1/3*^{-/-} mice had greater aquaporin 3 expression levels than wild-type controls. After water restriction, the abundances of both aquaporin 2 and aquaporin 3 increased in both *UT-A1/3*^{-/-} and wild-type mice; however, the increase in abundances of aquaporins 2 and 3 were greater in knockout mice. Thus, the concentrating defect in *UT-A1/3*^{-/-} mice cannot be attributed to a defect in long-term regulation of aquaporin 2 or 3.

Medullary Solute Gradients

In knockout mice on a high-protein diet, there was a marked depletion of osmolality and urea in the inner medulla compared with controls (Figure 7). However, there were no differences between *UT-A1/3*^{-/-} mice and wild-type control mice in Na concentrations measured in cortex, outer medulla, or inner medulla. In addition, tissue K concentration was not different

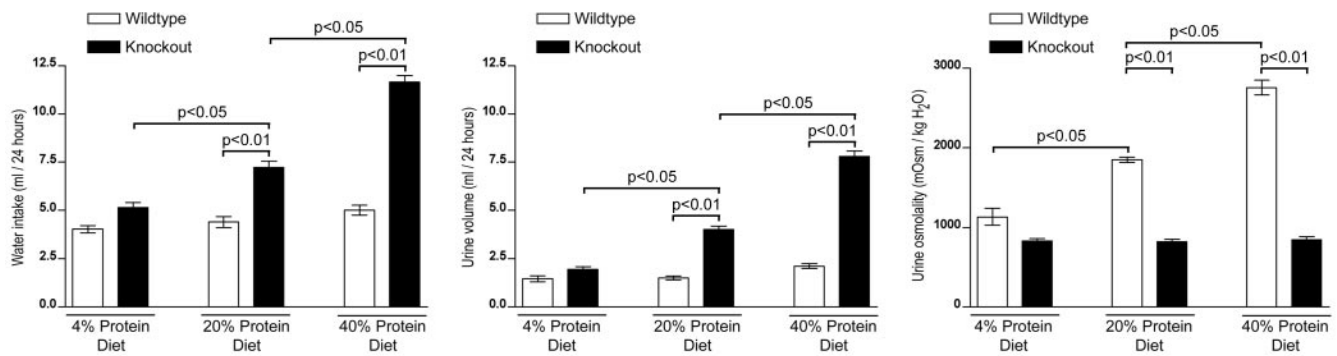


Figure 4. Water conservation and urinary concentrating ability of *UT-A1/3*^{-/-} mice. For all graphs, values are mean \pm SEM and a significant difference (ANOVA) between wild-type mice (clear bars) and *UT-A1/3*^{-/-} mice (solid bars) is indicated. Mice received 4, 20, or 40% protein intake for 7 d before and throughout the duration of the study. (A) 24-h water consumption. (B) Urine output under basal conditions (free access to drinking water). (C) Urine osmolality under basal conditions.

between *UT-A1/3*^{-/-} and wild-type mice (not shown). Furthermore, the Na concentration profiles were virtually identical when mice on a 4% diet are compared with mice on a 40% protein diet. Thus, sodium accumulation in the outer and inner

zones of the kidney medulla appears to occur largely independently of tissue urea concentrations.

Discussion

In the kidney, the transepithelial movement of urea across the IMCD is mediated by two members of the UT-A-facilitated urea transporter family: UT-A1 and UT-A3. Recently, we developed a mouse model in which both UT-A1 and UT-A3 were deleted from the IMCD (*UT-A1/3*^{-/-} mice) to investigate the role of these transporters in the function of the renal inner medulla (4). In this article, we address several

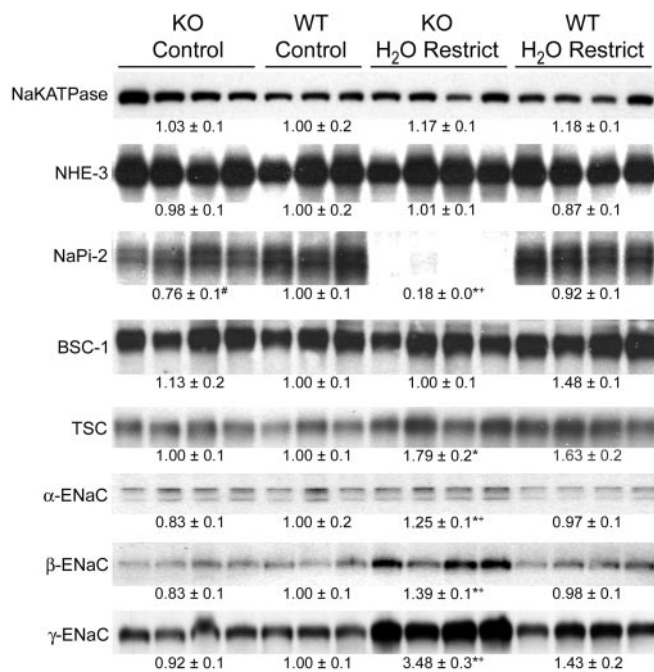


Figure 5. Immunoblots assessing sodium transporter and sodium channel abundances in whole kidney homogenates from wild-type and *UT-A1/3*^{-/-} mice. Mice received free access to water (control) or were water-restricted for 36 h. Each lane was loaded with a sample from a different animal. Values are mean band densities \pm SE normalized such that the mean for the wild-type mice under control conditions is defined as 1. *Significant change in mean band densities within a group under the two experimental conditions. #Significant change in mean band densities between the wild-type and *UT-A1/3*^{-/-} groups under control conditions. +Significant change in mean band densities between the wild-type and *UT-A1/3*^{-/-} groups under water-restricted conditions.

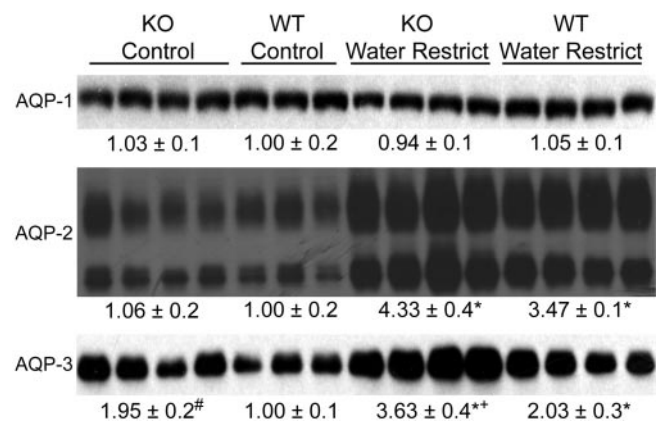


Figure 6. Immunoblots assessing aquaporin water channel abundances in whole kidney homogenates from wild-type and *UT-A1/3*^{-/-} mice. Mice received free access to water (control) or were water-restricted for 36 h. Each lane was loaded with a sample from a different animal. Values are mean band densities \pm SE normalized such that the mean for the wild-type mice under control conditions is defined as 1. *Significant change in mean band densities within a group under the two experimental conditions. #Significant change in mean band densities between the wild-type and *UT-A1/3*^{-/-} groups under control conditions. +Significant change in mean band densities between the wild-type and *UT-A1/3*^{-/-} groups under water-restricted conditions.

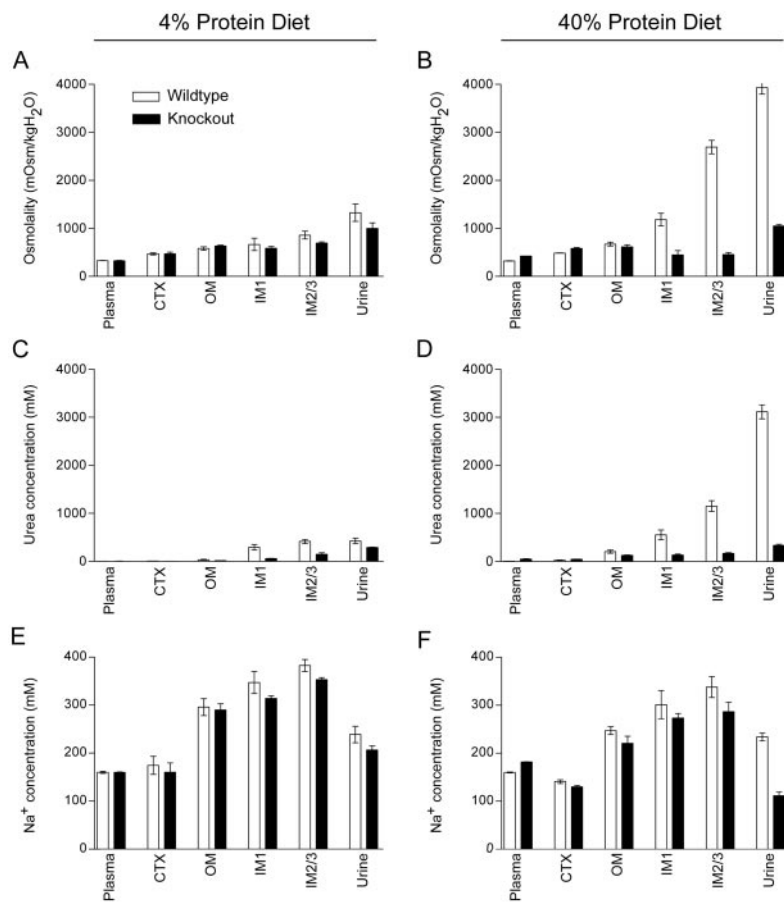


Figure 7. Comparison of kidney solute composition of wild-type and *UT-A1/3*^{-/-} mice. For all graphs, values are mean \pm SEM and a significant difference (ANOVA) between wild-type mice (white bars) and *UT-A1/3*^{-/-} mice (black bars) are indicated. Mice received either a 4 or 40% protein intake for 7 d before and throughout the duration of the study. Osmolality on either a 4% protein intake (A) or 40% protein intake (B). Urea concentration on either a 4% protein intake (C) or 40% protein intake (D). Na⁺ concentration on either a 4% protein intake (E) or 40% protein intake (F).

long-standing hypotheses about the role of urea in renal physiology.

Role of NO in Renal Water Balance

It is well recognized the renal NO production plays an important role in the regulation of water transport in the renal collecting duct, although it appears to have both inhibitory (13) and stimulatory (14) effects on collecting duct water permeability. Furthermore, NO plays an important role in regulation of thick ascending limb NaCl transport, a process critical to the urinary concentrating mechanism. Hence, it should not seem surprising that changes in NO excretion were found in the *UT-A1/3*^{-/-} mice in association with the markedly abnormal water conservation capacity of these animals. However, these studies do not directly determine the cause or the consequences of the increased NO excretion in these mice, or of the associated medullary vascular congestion.

Effect of High Protein Intake on GFR: Possible Role of Urea

It has been known for several years that diets rich in protein increase whole kidney GFR (15,16). Studies by Seney and Wright determined that this increase in GFR results from changes in the tubuloglomerular feedback (TGF) system (17,18). Furthermore, they determined that it is not an actual change in the sensing mechanism of the TGF response that is affected by high-protein diet, but rather it is an effect on the

signal causing the TGF response. They concluded that this diminished TGF response is caused, at least in part, by a reduced early distal NaCl concentration, without a change in early distal tubule osmolality (18). Bankir *et al.* (19,20) have proposed that the reduction in early distal NaCl concentration is caused by increased concentrations of urea consequent to the high protein intake. The urea concentrations in the late thick ascending limb and early distal tubule are dependent on the urea concentration of the glomerular filtrate and the extent of urea recycling, a result of passive urea secretion into the loop of Henle from urea reabsorption in the IMCD (21–23). Because urea recycling is likely to be virtually eliminated in the *UT-A1/3*^{-/-} mice, it would be predicted that the increase in GFR in response to high protein feeding would be markedly attenuated. However, in our studies, a large protein-dependent increase in GFR was observed in both *UT-A1/3*^{-/-} and wild-type mice, and we observed no significant difference in inulin clearance between the groups under either dietary condition examined. Therefore, these data suggest that urea reabsorption from the IMCD and the process of urea recycling is not an important determinant of the protein-induced increases in GFR observed.

Possibility of Active Urea Secretion along the Renal Tubule

The excretion of urea, in classical thinking, is thought to depend on two factors: the filtered load of urea and the amount of urea reabsorption that occurs along the nephron. Although

secretion of urea into the loop of Henle occurs as part of the urea recycling process discussed in the previous paragraph, this secretion is thought to occur passively. However, several pieces of evidence support the notion that *active* urea secretion may occur at some point along the renal tubule (24). Evidence for active urea secretion in rodents was initially provided by Bodil Schmidt-Nielsen (25), and microperfusion studies by Kawamura and Kokko indicated that a low rate of active urea secretion could occur in the rabbit proximal tubule (26), although net urea secretion was not detectable in another study of the rabbit proximal straight tubule (27). More recently, Kato and Sands have shown that in rats, urea can be actively secreted in the terminal IMCD (28). In our studies, the FE_{urea} in *UT-A1/3*^{-/-} mice on a high-protein diet was virtually 100% (Table 2). Conclusive evidence for active urea secretion from clearance studies would require a net fractional urea excretion significantly >100%. Although this benchmark was not achieved, given that at least 30 to 40% of the filtered load of urea is normally reabsorbed in the proximal tubule (22,29), the finding of a net fractional excretion of $102.0 \pm 8.8\%$ (Table 2) suggests the presence of active urea secretion in the mouse renal tubule. However, to examine whether there are changes in proximal tubule urea reabsorption in the *UT-A1/3*^{-/-} mice and to determine where the postulated active urea secretion occurs in the kidney nephron will require micropuncture studies.

Role of Medullary Urea Accumulation in the Renal Concentrating Mechanism

Berliner *et al.* proposed that medullary urea accumulation serves to prevent urea (present at high levels in the collecting duct lumens) from causing an osmotic diuresis (3). Our previous study with the *UT-A1/3*^{-/-} mice demonstrated that in the absence of facilitated urea transport across the IMCD, urea accumulation in the renal inner medulla is markedly attenuated (4). Consistent with the Berliner model, *UT-A1/3*^{-/-} mice fed either a normal or high-protein diet had a significantly greater fluid intake and urine flow than wild-type animals, whereas *UT-A1/3*^{-/-} mice on a low-protein diet did not show a substantial degree of polyuria. In the latter condition, hepatic urea production is low and urea delivery to the IMCD is predicted to be low, thus rendering the absence or presence of collecting duct urea transport immaterial with regard to water balance. Furthermore, when “challenged” by an 18-h water restriction, *UT-A1/3*^{-/-} mice on a 20 or 40% protein intake were unable to reduce their urine flow to levels below those observed under basal conditions, resulting in volume depletion and loss of body weight. We can conclude from these findings that the concentrating defect in *UT-A1/3*^{-/-} mice is caused by a urea-dependent osmotic diuresis; greater urea delivery to the IMCD results in greater levels of water excretion. Overall, the results are consistent with a role for urea transporters in the maintenance of water balance through their ability to prevent a urea-induced osmotic diuresis.

Potentially, regulation of urea transporters in the IMCD could play a direct role in regulation of water and NaCl excretion by modulating the extent of urea-induced osmotic diuresis. For example, the downregulation of collecting duct urea trans-

porters seen in extracellular fluid volume expanded states (30) could be viewed as a homeostatic response that could increase water and salt excretion. Furthermore, some of the effects of glucocorticoids on water balance could be a consequence of the effect of glucocorticoids to down-regulate urea transporter expression in the IMCD (31,32).

Role of Urea in the Concentration of Na and Cl in the Renal Medulla

In this study, we performed an analysis of solute concentrations in cortex, outer medulla, and two levels of the inner medulla to assess the separate effects of changes in dietary protein intake and/or deletion of the collecting duct urea transporters on corticomedullary solute gradients. Several striking observations were apparent. First, in wild-type mice, a change in dietary protein intake from 4 to 40% resulted in increased tissue osmolality that was caused solely, of the solutes measured, by a greater urea accumulation in the inner medulla. Sodium concentrations at all levels of the corticomedullary axis were unaffected by the change in dietary protein intake. Second, in agreement with studies in rats by Schmidt-Nielsen *et al.* (33), the concentration of urea in the papillary tip of wild-type mice fed a low-protein diet was equivalent to the concentration of urea in the urine, whereas in mice fed a high-protein diet, urea concentration in the urine is greater than in the papillary tip, consistent with a failure of urea to completely equilibrate between lumen and interstitium. Third, in contrast to wild-type mice, in knockout mice there was a substantially attenuated corticomedullary osmolality gradient and there was no urea gradient on either diet. However, the corticomedullary sodium gradients were virtually equivalent in wild-type and knockout mice on either level of dietary protein intake. Thus, neither marked medullary urea depletion caused by dietary protein restriction nor marked medullary urea depletion caused by deletion of collecting duct urea transporters affected the ability of the kidney to form a corticomedullary sodium gradient.

Our measurements of tissue osmolality, urea concentration, and sodium concentration suggest that there is an “osmotic gap” (approximately 500 mmol) between osmolality and $[urea] + 2x[Na]$, indicating that there are substantial amounts of unmeasured solutes. Others have reported that trimethylamines (*e.g.*, glycerophosphorylcholine and betaine), polyhydric alcohols (*e.g.*, inositol and sorbitol), amino acids, lactate, ammonium, and potassium are all accumulated in the inner medulla (34–38). These previous reports use different species and different sections of the kidney, making it difficult to directly compare the values quoted to those reported in ours. However, it is likely that these unmeasured solutes contribute to the osmotic gap that is evident in the renal inner medulla and could play a role in the urinary concentrating mechanism. Despite these differences, our data suggest that it is unlikely that NaCl accumulation in the inner medulla is dependent on either IMCD urea transport or the accumulation of urea in the IMCD interstitium as proposed in the passive concentrating mechanism (39,40).

Expression of Aquaporins and NaCl Transporters in UT-A1/3^{-/-} Mice

In general, aquaporin-2 and -3 expression levels were intact in UT-A1/3^{-/-} mice, ruling out a role for dysregulation of these transporters in the demonstrated concentrating defect. Also, in response to restriction of water intake, aquaporin-2 and aquaporin-3 abundances increased in UT-A1/3^{-/-} mice more than in control mice, reflecting a greater degree of water depletion. Furthermore, the levels of sodium transporter expression were not impaired in UT-A1/3^{-/-} mice and appeared to respond appropriately to volume depletion after water restriction. An unexplained finding was that the type 2 Na-dependent phosphate transporter manifested a marked decrease in expression level in the knockout mice *versus* controls. We have no explanation for this finding, because phosphate intake was identical in the two groups of mice.

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