Urea selective Concentrating Defect in Transgenic Mice Lacking Urea Transporter UT-B

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Urea transporter UT-B has been proposed to be the major urea transporter in erythrocytes and kidney-descending vasa recta. The mouse UT-B cDNA was isolated and encodes a 384-amino acid urea-transporting glycoprotein expressed in kidney, spleen, brain, ureter, and urinary bladder. The mouse UT-B gene was analyzed, and UT-B knockout mice were generated by targeted gene deletion of exons 3–6. The survival and growth of UT-B knockout mice were not different from wild-type litters. Urea permeability was 45-fold lower in erythrocytes from knockout mice than from those in wild-type mice. Daily urine output was 1.5-fold greater in UT-B deficient mouse (p < 0.01), and urine osmolality (Uosm) was lower (1532 ± 71 versus 2056 ± 83 mosm/kgH2O, mean ± S.E., p < 0.001). After 24 h of water deprivation, Uosm (in mosm/kgH2O) was 2403 ± 38 in UT-B null mice and 3438 ± 98 in wild-type mice (p < 0.001). Plasma urea concentration (Purea) was 30% higher, and urea urea concentration (Uurea) was 35% lower in knockout mice than in wild-type mice, resulting in a much lower Uurea/Purea ratio (61 ± 5 versus 124 ± 9, p < 0.001). Thus, the capacity to concentrate urea in the urine is more severely impaired than the capacity to concentrate other solutes. Together with data showing a disproportionate reduction in the concentration of urea compared with salt in homogenized renal inner medullas of UT-B null mice, these data define a novel “urea-selective” urinary concentrating defect in UT-B null mice. The UT-B null mice generated for these studies should also be useful in establishing the role of facilitated urea transport in extrarenal organs expressing UT-B.

Urea is the major end product of nitrogen metabolism in mammals. Urea is synthesized by the liver and excreted by the kidney. In omnivores such as humans and laboratory rodents, urea represents up to 40–50% of all urinary solutes and is markedly concentrated in urine with respect to plasma (up to 100-fold in humans and 250-fold in rodents) (1). Because of a unique intrarenal recycling process enabling urea to accumulate late in the medulla, urea contributes to the urinary concentrating mechanism and, thus, to water conservation (1, 2). Abnormally low blood urea concentration in malnutrition results in a urinary concentrating defect because of impaired intrarenal urea accumulation (3).

Physiological studies over 30 years provided evidence for the existence of facilitated urea transporters in erythrocytes and certain segments of the nephron (4). Two major subfamilies of mammalian urea transporters have been identified, the “renal tubular-type” urea transporter (UT)1-A and the “erythrocyte-vascular type” urea transporter UT-B (4–6). Five UT-A isoforms have been identified that are produced by alternative splicing of a single gene (7–9). UT-A1 (original name UT1) is expressed in the apical membrane of terminal inner medullary collecting duct cells and is thought to be involved in the vasopressin-regulated increase in urea permeability (10, 11). UT-A2 (UT2) is located in the late part of descending thin limbs of short loops of Henle and in the intermediate part of descending thin limbs of long loops, and it may facilitate urea recycling (1, 12, 13). UT-A3 is most abundant in intracellular membranes and in the apical region of inner medullary collecting duct cells (14). The localization of UT-A4 in the kidney is unclear (15). UT-A5 mRNA was localized to the peritubular myoid cells of the testis (16). A UT-B isoform (original name UT11, HUT11, and UT3) was cloned from human bone marrow (17) and rat kidney (18). In rat, UT-B is expressed in the kidney outer and inner medulla, testis, brain, bone marrow, and spleen (18–20). UT-B is a 384-amino acid protein having 62% identity to rat UT-A2. UT-B has similar membrane topology to UT-A2 based on hydropathy analysis and has functional characteristics similar to UT-A1 and UT-A2. We reported that rat UT-B heterologously expressed at a relatively high level in Xenopus oocytes functioned as a urea/water channel utilizing a common aqueous pathway (21), however, the relevance of water transport by UT-B in mammalian physiology is unresolved (22).

We report here the generation of the first transgenic mouse model of facilitated urea transporter deletion. The erythrocyte/vasa recta transporter UT-B was deleted by targeted gene disruption, and the phenotype of the null mice was studied with focus on urinary concentrating ability. Erythrocyte urea permeability in UT-B null mice was remarkably reduced, and the mice manifested a unique type of urea-selective urinary concentrating defect. Our data provide evidence that UT-B-dependent countercurrent exchange of urea in the renal medulla contributes to approximately one-third of the total capacity of the kidney to concentrate urine but contributes even more greatly to the ability of the kidney to concentrate urea itself.

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† The abbreviations used are: UT, urea transporter; RACE, rapid amplification of cDNA ends; ANOVA, analysis of variance; mRNA, messenger RNA.
Materials and Methods

CDNA Cloning and Genomic Analysis of Mouse UT-B

Kidney mRNA was isolated (Oligotex direct mRNA kit, Qiagen) from C57B16 mice. CDNA was reverse-transcribed from the mRNA using oligo(dT) (SuperScript II preamplification kit, Invitrogen). Polymerase chain reaction amplification was performed with CDNA template and primers designed from the rat UT-B sequence (sense, 5'-AGTGTGCGCTCAAGAGCTCTGCTGAGAGCAAGTACTTGGCTA-3'; antisense, 5'-CATGAGT-3'). The PCR product (~0.7 kb) was purified (Qiagex II gel extraction kit, Qiagen) and subcloned into PCRII TA cloning vector (Invitrogen). The full-length insert was sequenced. 5' and 3' end cDNA sequences were obtained by 5'- and 3'-RACE (Clontech). For functional measurements of urea and water permeability, the mouse UT-B CDNA (full-length coding sequence) was subcloned into oocyte expression vector pSP64T, and in vitro transcribed CDNA (5 ng) was expressed in Xenopus laevis oocytes as described previously (21). The structure of the mouse UT-B gene was analyzed by PCR amplification of exon-intron-exon fragments by using C57B16 mouse liver genomic DNA as template. All exons and exon-intron boundaries were sequenced.

Generation of UT-B Null Mice—A targeting vector for homologous recombination was constructed with a 1.7-kb genomic UT-B DNA fragment containing intron 2 and part of exon 3 (left arm) and a 5.5-kb fragment containing part of exon 6 and intron 7 (right arm) (see Fig. 2A). The left and right arm genomic fragments (surrounding a 1.8-kb PpolI neobpA cassette) were PCR-amplified, and a PGK-tk cassette was inserted upstream for negative selection. The targeting vector was linearized at a unique downstream NotI site and electroporated into CBl8 embryonic stem cells. Transfected embryonic stem cells were selected with G418 and FIAU for 7 days, yielding two targeted clones from 120 doubly resistant colonies on PCR screening with a neo-specific sense primer and a UT-B gene-specific antisense primer located 50 bp upstream of the targeted region. Homologous recombination was confirmed by Southern hybridization in which 10 μg of genomic DNA was digested with SpeI, electrophoresed, transferred to a Nylon + membrane (Zetabonder Biosciences, Inc.), and hybridized with a 1.1-kb CXTAGAGTACTTGGCTA-3' antisense, and 5'-CATGAGT-3' sense probe corresponding to the mouse UT-B cDNA coding sequence. The targeting strategy shown in Fig. 2A (top) separating eight exons with boundaries at residues 46, 109, 152, 217, 266, 311, and 332 in the deduced mouse UT-B amino acid sequence, identical to the boundary residues of the human UT-B gene (25). All boundaries correspond to the GT-AG rule. Genomic Southern blot analysis with EcoRI, SpeI, and Kpn1-digested mouse genomic DNA indicated a single copy UT-B gene/haploid mouse genome (data not shown).

UT-B knockout mice were generated by the gene-targeting strategy shown in Fig. 2A. Heterozygous founder mice containing the disrupted UT-B gene were bred to produce homozygous UT-B knockout mice.

Northern Blot Analysis—Total RNA from mouse tissues was isolated using TRIzol reagent (Invitrogen). mRNAs purified by Oligotex direct mRNA kit (Qiagen) were resolved on a 1% formaldehyde-agarose denaturing gel (1.5 μg/lane), transferred to a Nylon + membrane (Amersham Biosciences, Inc.), and hybridized at high stringency with a 32P-labeled cDNA (full-length coding sequence) was subcloned into oocyte expression vector pSP64T, and in vitro transcribed CDNA (5 ng) was expressed in Xenopus laevis oocytes as described previously (21). The structure of the mouse UT-B gene was analyzed by PCR amplification of exon-intron-exon fragments by using C57B16 mouse liver genomic DNA as template. All exons and exon-intron boundaries were sequenced.

RESULTS

A cDNA clone with homology to rat UT-B was isolated from C57B16 mouse kidney. The open reading frame of 1152 bp (GenBank™ accession number AF444879) encodes a 384-amino acid protein with 10 putative hydrophilic transmembrane domains. The deduced amino acid sequence is 85 and 94% identical to human and rat UT-B, respectively (Fig. 1A). Northern blot analysis revealed transcripts of 3.8 and 2.0 kb in brain, spleen, kidney, ureter, and urinary bladder (Fig. 1B). Functional analysis in Xenopus oocytes expressing mouse UT-B cDNA indicated efficient urea-transport and weak water-transport activity (Fig. 1C) in agreement with results for rat UT-B (21). Also, as found previously, urea and water transport in oocytes expressing mouse UT-B was inhibited by phloretin.

Sequence comparison of mouse UT-B CDNA with a cloned 17-kb genomic DNA indicated 7 introns (lengths 0.4, 1.8, 2.3, 1.5, 0.4, 5.5, and 2.5 kb) (Fig. 2A, top) separating eight exons with boundaries at residues 46, 109, 152, 217, 266, 311, and 332 in the deduced mouse UT-B amino acid sequence, identical to the boundary residues of the human UT-B gene (25). All boundaries correspond to the GT-AG rule. Genomic Southern blot analysis with EcoRI, SpeI, and Kpn1-digested mouse genomic DNA indicated a single copy UT-B gene/haploid mouse genome (data not shown).

UT-B knockout mice were generated by the gene-targeting strategy shown in Fig. 2A. Heterozygous founder mice containing the disrupted UT-B gene were bred to produce wild-type, heterozygous, and UT-B null mice. Genomic Southern blot analysis of mouse liver genomic DNA digested with SpeI (probed as indicated in Fig. 2A) showed the predicted 4.3-kb fragment in wild-type mice, 3.7-kb fragment in UT-B knockout mice, and both fragments in heterozygous mice (Fig. 2B). Fig. 2C shows only truncated UT-B transcripts in the kidney of knockout mice. Immunoblot analysis revealed UT-B protein in erythrocytes of wild-type but not in the UT-B null mice (Fig. 2D). Immunohistochemistry of kidney showed UT-B protein expression in medullary vasa recta of wild-type mice with no staining in UT-B null mice (Fig. 2E).

Genotype analysis of offspring from breeding of UT-B heterozygous mice indicated a nearly 1:2:1 Mendelian distribution (54 wild-type, 113 heterozygous, and 41 null mice). An analysis of growth by mouse weight (age 1–12 weeks) showed no differences among the genotypes. The UT-B null mice had grossly normal appearance, activity, and behavior. Plasma sodium, potassium, chloride, bicarbonate, and creatinine concentrations were similar in both groups as was hematocrit (48.3 ± 0.4% in wild-type and 49.2 ± 0.5% in UT-B null mice).

Urea and water permeabilities in erythrocytes were measured by stopped-flow light scattering. Urea permeability was measured from the time course of cell swelling in response to a 250 mM inwardly directed urea gradient. There was an initial rapid decrease in cell volume because of osmotically induced water efflux followed by slower cell swelling, which was the result of urea and secondary water influx. At 10 °C, urea equil.
ibrated across erythrocytes from wild-type mice with a half-time of \( \frac{1}{27} \text{s} \), giving a permeability coefficient \( (P_{\text{urea}}) \) of \( 1.1 \times 10^{-6} \text{ cm/s} \) (Fig. 3A, top curve). Urea permeability was inhibited by 96% by 0.7 mM phloretin (Fig. 3A, bottom curve). Fig. 3A, middle curves, shows urea permeability in erythrocytes from heterozygous and UT-B null mice. Urea permeability in the erythrocytes from null mice was 45-fold lower than that from wild-type mice. Fig. 3B shows the time course of osmotic cell shrinking in response to a 250 mM inwardly directed osmotic gradient of sucrose. The data from a series of mice showed that UT-B deletion did not significantly reduce erythrocyte osmotic water permeability \( (P_f) \) (0.018 ± 0.002 cm/s for wild-type mice; 0.017 ± 0.002 cm/s for UT-B null mice).

Urinary concentrating ability was measured in response to a 24 h water deprivation. Urine osmolality in the UT-B null mice increased significantly, although to a significantly lesser extent than in wild-type mice \( (p < 0.02) \). Body weight loss during this test was similar in both groups \((20.1 \pm 1.2\% \text{ wild-type mice; 21.7} \pm 0.8\% \text{ null mice}) \).

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were moderately polyuric, consuming and excreting approximately 50% more fluid than litter-matched heterozygous and wild-type mice. The difference in fluid intake and urinary output, primarily representing insensible respiratory losses, was similar in all groups. The average urine osmolality in UT-B null mice \((1532 \pm 71 \text{ mOsm/kg H}_2\text{O}) \) was significantly lower than that in wild-type mice \((2056 \pm 83 \text{ mOsm/kg H}_2\text{O}) \).

Urinary concentrating ability was measured in response to a 24 h water deprivation. Urine osmolality in the UT-B null mice increased significantly, although to a significantly lesser extent than in wild-type mice \( (p < 0.02) \). Body weight loss during this test was similar in both groups \((20.1 \pm 1.2\% \text{ wild-type mice; 21.7} \pm 0.8\% \text{ null mice}) \).

Osmolality and urea concentration were measured in plasma and urine of six wild-type and six UT-B null mice under basal conditions (food intake was equal in the two groups). Plasma osmolality was slightly but not significantly higher in UT-B null mice than in wild-type mice \((330 \pm 4 \text{ versus } 324 \pm 4 \text{ mOsm/kgH}_2\text{O}) \). The urine-to-plasma ratio for osmolality, an index of overall concentrating capacity of the kidney, was lower by one-third in UT-B null mice compared with wild-type mice \((4.0 \pm 0.2 \text{ versus } 6.1 \pm 0.4, p < 0.001) \). As shown in Fig. 5A, plasma urea concentration was significantly higher, and uri-
ponent of the inner medulla as measured on the supernatants (Fig. 5). Water permeability measured from the time course of erythrocyte volume as determined by light scattering in response to a 250 ml inwardly directed urea gradient. A, urea permeability measured in erythrocytes from wild-type mice in the absence (top) and presence (bottom) of 0.7 mM phloretin and from mice of indicated genotypes (middle) at 10 °C. B, water permeability measured from the time course of erythrocyte volume as determined by light scattering in response to a 250 ml inwardly directed sucrose gradient (n = 3).

FIG. 3. Erythrocyte urea and water permeability. Urea permeability was measured from the time course of erythrocyte volume as determined by light scattering in response to a 250 ml inwardly directed urea gradient. A, urea permeability measured in erythrocytes from wild-type mice in the absence (top) and presence (bottom) of 0.7 mM phloretin and from mice of indicated genotypes (middle) at 10 °C. B, water permeability measured from the time course of erythrocyte volume as determined by light scattering in response to a 250 ml inwardly directed sucrose gradient (n = 3).

The cDNA encoding mouse UT-B had 94% amino acid identity to a rat homolog (rUT-B) cloned previously (18). Mouse UT-B and rUT-B transcript expression differed in a few respects. Northern blot analysis revealed mouse UT-B transcripts with two sizes (3.8 and 2.0 kb) in brain, spleen, kidney, ureter, and urinary bladder, whereas a single mRNA band was found for rUT-B. The UT-B transcript was identified in rat testis (18), but it was not seen in mouse testis.

Transgenic null mice deficient in the UT-B were generated and characterized. The UT-B knockout mice expressed truncated UT-B transcript but lacked detectable UT-B protein by C-terminal antibody. Urea permeability in erythrocytes from null mice was 45-fold lower than that from wild-type mice, indicating that UT-B was functionally deleted. We found previously that rat UT-B transported some water along with urea when heterologously expressed in Xenopus oocytes (21). Mouse UT-B also functioned as a weak water channel and an efficient urea transporter in Xenopus oocytes. The osmotic water permeability in UT-B-deficient erythrocytes was not reduced by UT-B deletion, however, the high AQP1-dependent water permeability in mouse erythrocyte precluded the detection of effects of UT-B deletion. The measurement of water permeability in erythrocytes lacking UT-B and AQP1 together, when available, should be informative in this regard.

UT-B null mice exhibited normal growth and no overt abnormalities in their main biological functions, behavior, and sensory activity. The sex and genotype ratios were normally distributed, suggesting no influence of UT-B gene deletion on survival or sexual differentiation. As expected, the major phenotypic abnormality was in renal water and urea handling. Fluid intake and urine output were approximately 50% higher, and urine osmolality was approximately one-third lower in null mice than in wild-type mice. Despite this difference in fluid turnover, the concentrations of the major plasma solutes were unaltered. The very low urea permeability in red cells did not influence the hematocrit.

Urea is an important contributor to the urinary concentrating mechanism (1, 2, 26–29). Interestingly, urea contributes mostly to its own concentration, because it is the most abundant solute in the urine, except in herbivores, and because its concentration in blood is much lower than that of the other main urinary constituents. In normal human urine, urea is concentrated approximately 45-fold above plasma, K⁺ (12-fold), and Na⁺ (0.65-fold). The kidney transforms large amounts of urea-poor plasma into a small volume of urea-rich urine. Thus, besides the overall urinary concentrating defect in UT-B null mice, we investigated the possibility of a selective defect in urea concentrating capacity by measurement of the urine-to-plasma urea concentration ratio and inner medulla composition. UT-B deficiency in kidney resulted in decreased urinary urea and increased plasma urea concentrations, producing a 2-fold decrease in the urine-to-plasma urea concentration ratio. UT-B deficiency also produced a >2-fold reduction in inner medullary urea concentration with little effect on inner medullary chloride (salt) concentration.

Mechanistically, as depicted schematically in Fig. 5C, these observations suggest that some of the urea delivered to the tip of the papilla by UT-A1/UT-A3/UT-A4 and carried up by the blood through venous ascending vasa recta is not recycled in UT-B-deficient arterial descending vasa recta and thus returned to the general circulation. In contrast to the remarkably reduced urea concentrating ability in the UT-B null mice, the
defect in the concentration of all solutes as assessed by the urine-to-plasma osmolality ratio (lower by only one-third) was more modest, suggesting that UT-B plays a greater role in enabling the kidney to concentrate urea than other solutes in the urine. The selective reduction in inner medullary urea concentration supports this conclusion.

A natural model of UT-B gene deletion occurs in rare human subjects lacking the Kidd blood group Jk antigens, Jk(a−b−) (30). Erythrocytes in these subjects have low urea permeability, and it is assumed that they also lack UT-B protein in vasa recta. In two such subjects, Sands et al. (31) found a normal blood urea nitrogen and a modest −20% decline in maximum urinary concentrating ability after dehydration and vasopressin infusion (790 mosmol/kg H2O versus −980 mosmol/kg H2O in healthy subjects). The concentrating defect observed in mice in this study is substantially larger than that in humans. Blood urea concentration was elevated, and maximum urinary concentration after water deprivation decreased by 35%. This difference may be accounted for by the difference in overall concentrating ability of the two species partly because of the differences in kidney architecture and development of the renal medulla. The urinary concentrating capacity in different mammalian species is influenced by several factors, including the relative length of the papilla (much longer in mice than in humans) and the anatomy of the vascular bundles in the outer medulla (32, 33). The human kidney has a relatively simple architecture and modest concentrating ability, whereas mice have a more complex vascular architecture and greater concentrating ability.

In conclusion, the UT-B null mice manifest a unique defect in urinary concentrating capacity of the kidney affecting the ability to concentrate urea preferentially over other urinary solutes. The impaired urea recycling in the medullary vasculature results in increased blood urea concentration and decreased urine urea concentration, producing an ∼50% reduction in urea concentrating capacity. As reported in rat (18–20), mouse UT-B was also found to be expressed in several other organs in which facilitated urea transport was not suspected to occur previously, including brain, testis, spleen, ureter, and urinary bladder. The UT-B null mice will allow studies to elucidate the role of facilitated urea transport in these extrarenal organs.

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FIG. 5. Effect of UT-B deletion on urea recycling. A, plasma (top) and urine (middle) urea concentration, and urea/plasma urea concentration ratio (bottom) (S.E., n = 6) ∗, p < 0.01; ∗∗, p < 0.001. B, Osmolality (top), urea concentration (middle), and chloride concentration (bottom) in homogenized inner medullas (S.E., n = 6). C, diagram of urea recycling in the kidney of UT-B null mice. Urea is delivered normally to the tip of the papilla, but its recycling via the vascular route is compromised in UT-B null mice, leading to a greater return of urea to the general circulation.