UT-B1 proteins in rat: tissue distribution and regulation by antidiuretic hormone in kidney


UT-B1 proteins in rat: tissue distribution and regulation by antidiuretic hormone in kidney. Am J Physiol Renal Physiol 283: F912–F922, 2002. First published June 11, 2002; 10.1152/ajprenal.00359.2001.—UT-B1 is the facilitated urea transporter of red blood cells (RBCs) and endothelial cells of descending vasa recta in the kidney. Immunoblotting with a polyclonal antibody against the C-ter sequence of rat UT-B1 revealed UT-B1 as both nonglycosylated (29 kDa) and N-glycosylated (47.5 and 33 kDa) proteins in RBC membranes, kidney medulla, brain, and bladder in rat. In testis, UT-B1 was expressed only as a nonglycosylated protein of 47.5 kDa. Immunohistochemistry confirmed that the location of UT-B1 is restricted to descending vasa recta. In brain, UT-B1 protein was found in astrocytes and ependymal cells. Cell bodies and perivascular end feet of astrocytes were labeled in brain cortex, whereas astrocyte cell processes were labeled in corpus callosum. Flow cytometry analysis of RBCs revealed a good cross-reactivity of the antibody with mouse and human UT-B1. UT-B1 protein expression in rat kidney medulla was downregulated greatly by long-term [deamino-Cys1,D-Arg8]vasopressin infusion and moderately by furosemide treatment. This study discloses an uneven distribution of UT-B1 protein within astrocytes and the regulation of renal UT-B1 protein by antidiuretic hormone.

urea transporter; N-glycosylation; erythrocytes; astrocytes; [deamino-Cys1,D-Arg8]vasopressin; furosemide

Urea is the main end product of protein catabolism in mammals. It was long believed that its transmembrane movement occurs only via passive diffusion through the plasma membrane lipid bilayer. However, very high transfer rates of urea disclosed in several mammalian cell types suggested the presence of a facilitated process. The molecular basis of the massive and rapid movement of urea across the membrane of these cells rests on recently cloned urea transporters (UT). These transporters ensure a facilitated process that dissipates any transmembrane urea gradient.

UT-A1, -A2, -A3, -A4, and -A5 are seemingly encoded by the Slc14a2 gene located on chromosome 18, comprising 24 exons in rat and mouse (8, 17) and 20 exons in human (1). They are expressed mainly in the kidney medulla, where UT-A1 is the so-called vasopressin-regulated transporter. UT-A5 has been cloned in testes (9). UT-B1 is encoded by a distinct gene that was identified as the Kidd blood group locus also assigned to chromosome 18 (14). The presence of the Kidd/UT mRNA in human and rat tissues has been commonly examined by Northern blot analysis or in situ hybridization (4, 18, 19, 24, 36). UT-B1 is more broadly expressed in mammalian tissues. UT-B1 mRNA has been detected in the descending vasa recta of renal medulla, in bladder, Sertoli cells of testis, prostate, heart, astrocytes throughout the central nervous system, and in the fetal but not the adult liver. Because cells can have mRNAs without expressing respective proteins as is the case for neurons (34), tissue and cellular distribution of UT-B1 protein deserves examination. UT-B1 protein has been revealed by immunohistochemistry in endothelial cells of human and rat descending vasa recta (39) and by immunoblotting in human red blood cell (RBC) membranes (19). Very recently, using a polyclonal antibody against human UT-B1, Timmer and coworkers (33) revealed the presence of the transporter in rat brain, heart, colon, liver, and lung in addition to RBCs and kidney medulla. Cells containing UT-B1 protein in these organs have not been identified yet. Very recently, UT-B1 protein in testes has been located in Sertoli cells (7).

In RBC, the fast urea transport mediated by UT-B1 is believed to be essential for RBC stability when passing through the hypertonic renal medulla (16). In the kidney, the urea transport in vasa recta resulting from UT-B1 plays, besides UT-A1, UT-A2, and UT-A3, an important role in the urinary concentrating process (2, 3, 31, 37). Because of a countercurrent exchange process, part of the urea escaping the medulla in ascending vasa recta is trapped in the descending vasa recta endowed of UT-B1 and returns to the medulla. In this way, UT-B1 helps maintain a high medullary urea concentration and thus the hypertonic medulla re-
required for water reabsorption. The role of UT-B1 in extrarenal tissues is yet unclear. It has been speculated that, in testis, prostate, and brain, UT-B1 would favor the exit of urea, which is a by-product of L-arginine conversion into ornithine, a precursor of polyamines, by arginase activity (4). The presence of UT-B1 mRNA in the human but not rat heart (Bailly P and Carton J-P, unpublished observations) and in bladder is very intriguing. Because the liver is the main site of body urea production, rapid urea extrusion from hepatocytes should require UT(s). Whether UT-A and UT-B1 are present in the liver is still unclear. UT-A has been disclosed as protein by Klein et al. (12), and UT-B1 as both mRNA by RT-PCR and protein by immunoblotting in rat liver (33). However, UT-A1, UT-A2, and UT-B1 mRNAs were not detected by us (18, 24) in previous Northern analysis of rat and human liver.

In the present study, a polyclonal antibody directed against the carboxy terminus of rat UT-B1 was raised and characterized. It revealed an extrarenal tissue distribution and N-glycosylation state of rat UT-B1 somewhat different from that recently reported by Timmer et al. (33). This antibody was shown to recognize human and mouse UT-B1. It also revealed that UT-B1 proteins in kidney are regulated by antidiuretic hormone and furosemide.

METHODS

Antiserum production. Polyclonal antibodies were raised in rabbit against a synthetic peptide (sequence EENR-FYQNKSSAVDRPL) corresponding to the carboxy terminal 19 amino acids of rat UT-B1 (Neosystem, Strasbourg, France; 36). A cysteine residue was added to the amino terminus of the peptide to enable the conjugation of keyhole limpet hemocyanin, the carrier protein. The immunized rabbit developed enzyme-linked immunosorbent assay titers of >1:64,000. Affinity-purified antibody was obtained by extraction from antiserum using the peptide immobilized on agarose gel (SulfoLink kit; Pierce, Rockford, IL).

Collection of organs. RBCs were obtained from male Sprague-Dawley rats and male C57BL6/J mice (Iffa Credo, Arbresle, France). Human RBCs of voluntary donors with Jk-positive or the Jknull phenotype were provided by the Blood Group Reference National Center (CNRGS, Paris, France). Organs were taken from male Sprague-Dawley rats and male C57BL6/J mice (Iffa Credo, Arbresle, France). Organs were taken from male Sprague-Dawley rats.

RBC flow cytometry analysis. RBCs from fresh anticoagulated blood were washed and suspended in PBS at a concentration of 0.2% (vol/vol) and fixed with a mixture of paraformaldehyde (PFA) and glutaraldehyde (1 and 0.025%, respectively) for 15 min at room temperature. Because the antibody recognizes the intracellular carboxy end of the transporter, RBCs were permeabilized with 1% (wt/vol) N-octyl glucoside for 15 min. RBCs (~20,000) were resuspended in PBS containing 10% donkey serum and either the immune or the preimmune sera (dilution 1:200). After a 60-min incubation period, cells were washed in PBS and stained with 100 μl phycoerythrin-conjugated F(ab’) fragments of donkey anti-rabbit IgG (1:40; Beckman Coulter, Villepinte, France) for 30 min in the dark. After washing, the presence of UT-B1 in cell suspensions was analyzed by flow cytometry (FACS-Calibur; Becton-Dickinson, San Diego, CA).

RBC membrane and tissue homogenate preparation. RBC membranes were prepared by hypotonic lysis, as previously described (29). The final membrane pellet was resuspended in ice-cold isolation buffer containing 250 mmol/l sucrose, 10 mmol/l triethanolamine, and antiprotease cocktail (Roche Diagnostics, Meylan, France).

The kidney, bladder, testis, liver, and brain cerebrum were collected and placed in ice-cold isotonic saline. Kidney cortex and inner medulla were separated. About 50 mg (wet weight) of each tissue was thoroughly homogenized using a tissue homogenizer in 1 ml ice-cold isolation buffer.

Protein concentrations were assayed using a protein assay reagent (Bio-Rad, München, Germany). Samples were then diluted with isolation buffer to a protein concentration of either 2 μg/μl for tissue homogenates or 0.2 μg/μl for RBC membranes.

UT-B1 protein detection by immunoblotting. Samples containing 2 or 15 μg protein were added to Laemmli buffer [final concentration 10 mM Tris-HCl (pH 6.8), 1 mM EDTA, 1.5% SDS, and 240 mM β-mercaptoethanol] and heated for 10 min at 65°C. Proteins were then separated by electrophoresis on denaturing SDS-10% polyacrylamide minigels and transferred electrochemically overnight to a polyvinylidene difluoride membrane (PVDF, NEN, Boston, MA). Blots were blocked for 45 min at room temperature with 5% (wt/vol) nonfat milk in PBS containing 0.3% (vol/vol) Tween 20 and were incubated for 90 min at room temperature with the affinity-purified antibody (0.3 μg/ml). After washing, they were incubated for 60 min with a second antibody (goat anti-rabbit IgGs) conjugated with horseradish peroxidase (0.2 μg/ml; Promega, Madison, WI). The specificity of protein recognition by the antibody was checked by 4°C overnight preincubation of the antibody with the immunizing or nonrelevant peptides coupled to agarose gel (SulfoLink kit; Pierce), and not with free peptide, because excess free peptide bound to the PVDF membrane used in the present study, as well as to nitrocellulose membrane (7), thereby masking all possible bands. Specific bands were revealed by enhanced chemiluminescence (ECL+; Amersham, les Ulis, France).

For semiquantitative determination in regulation studies, equal protein loading was verified by Coomassie blue staining of the membranes at the end of the experiment. After scanning of the autoradiograms, the density of the specific bands was quantitated with NIH image software. Differences between groups were analyzed by Student’s t-test. They were considered significant for P < 0.05.

Protein N-deglycosylation. Two micrograms of RBC membrane proteins or fifteen micrograms of tissue homogenate proteins were incubated for 1 h at 37°C in a medium containing 0.5% SDS, 1% Nonidet P-40, 1% β-mercaptoethanol, 50 mM sodium phosphate, pH 7.5, and 50 units N-glycosidase F (PNGase; New England BioLabs, Beverly, MA). Control samples were run simultaneously in enzyme-free buffer in otherwise identical conditions.

Immunohistochemistry. Male Sprague-Dawley rats were maintained under standard laboratory conditions on a 12:12-h light-dark cycle. Food and tap water were available ad libitum.

For kidney studies, rats were anesthetized with pentobarbital sodium (50 mg/kg ip), and their kidneys were fixed by retrograde perfusion via the lower aorta with 100 ml of 4% PFA in PBS, pH 7.4, warmed at 37°C at a rate of 20 ml/min. Kidneys were sliced and postfixed in 4% PFA overnight. Kidney slices were washed extensively in PBS and infiltrated overnight in 30% sucrose in PBS at 4°C. They were then frozen in liquid nitrogen, and 10-μm sections were collected on Superfrost ++ glass slides. Nonspecific bindings were blocked by incubating the sections in PBS containing 1% BSA (PBS-BSA) for 10 min. Sections were then incubated for 90 min in PBS-BSA containing either anti-UT-B1 antiserum or...
preimmune serum (dilution 1:200). Sections were washed three times in PBS and incubated in PBS-BSA containing fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (dilution 1:50; Jackson ImmunoResearch, West Grove, PA) for 45 min at room temperature followed by three washes in PBS. They were counterstained with Evan’s blue and mounted in N-propyl gallate anti-quenching medium [0.1 M Tris (pH 8.0), 50% glycerol, and 2% N-propyl gallate] before examination with an Olympus VANOX-T fluorescence microscope.

For brain studies, double immunofluorescence staining was performed using UT-B1 antiserum or preimmune serum and monoclonal mouse anti-glial fibrillary acidic protein (GFAP) that reveals astrocytes. Rats were anesthetized with ethyl carbamate (1.25 g/kg ip) and transcardially perfused with 200 ml of 4% PFA in PBS at a rate of 20 ml/min. Brains were immersed in the fixative solution for 2 h at 4°C. Coronal sections (25 μm thick) were cut on a vibratome (Leica VT 1000 S) and collected in PBS. PBS containing 0.1% Triton X-100 and 1% BSA (PBS-T-BSA) was used for antibody dilution and washing. In the first step, free-floating sections were rinsed three times in PBS for 5 min and incubated in PBS-T-BSA for 30 min for blocking nonspecific binding sites. The sections were then incubated with the primary antibody (UT-B1 antiserum or preimmune serum, dilution 1:100) overnight at 4°C. After three washes in PBS-T-BSA, they were incubated for 2 h in the dark at room temperature with CY3-coupled sheep anti-rabbit IgG (dilution 1:200) as secondary antibody. The first step was repeated using anti-GFAP antibody (dilution 1:200) as primary antibody and FITC-coupled goat anti-mouse IgG (dilution 1:200) as secondary antibody. Sections were mounted in anti-quenching medium (Vectashield H-100; Vector Laboratories, Burlingame, CA) and examined under a laser-confocal microscope (Zeiss LSM 510) using excitation at 477 and 543 nm for FITC and CY3, respectively. The epifluorescence was recorded through a 1-μm-thick plane.

[Deamino-Cys¹, D-Arg⁸]vasopressin and furosemide administration. Male adult Sprague-Dawley rats were housed individually, during the entire experiment, in metabolic cages in a room with a 12:12-h light-dark cycle, 50% humidity, and a temperature of 20°C. After a 7-day period of adaptation to the metabolic cages, their urine output and osmolality were measured to assign them equivalently to the control or the experimental group.

[Deamino-Cys¹, D-Arg⁸]vasopressin (dDAVP; a synthetic selective V₂ agonist of vasopressin; Ferring, Malmö, Sweden) was administered to 5 rats for 6–7 days at a dose of 0.25 μg/day using osmotic minipumps (Alzet, Palo Alto, CA) implanted in the peritoneal cavity under light anesthesia. Minipumps containing isotonic saline were implanted in four control rats. Furosemide (a loop diuretic; Sigma, Saint Quentin Fallavier, France) was given to four rats for 6 days at a dose of 10 mg·100 g body wt⁻¹·day⁻¹ mixed with 20 g standard rat food. Because of this slight food restriction, rats ingested the totality of the drug. Four rats that served as controls were given 20 g/day food without furosemide. Urine was collected daily, and urine flow rate was measured. The osmolality of urine collected for the two last 24-h periods was measured according to the freezing-point procedure using a Roebeling osmometer. Plasma urea, sodium, and potassium concentrations were measured with an Astra8 Beckman analyzer.

After completion of the dDAVP treatment, rats were anesthetized with pentobarbital sodium, and a blood sample was collected for the RBC membrane preparation. Kidneys were removed, and the inner medulla was excised from kidney slices. Fragments of the inner medulla were used for semi-quantitative quantitation of UT-B1 by Western blotting.

RESULTS

Antibody specificity. The antibody specificity was shown by unreactivity with RBC membranes from a Jk⁺⁻ donor devoid of UT-B1/Kidd blood proteins, whereas an intense band was revealed in a Jk⁺⁺ positive donor (Fig. 3B). The bands revealed in RBCs, kidney medulla, testis, and brain by the antibody were completely abated when probing with the antibody preadsorbed to immunizing peptide and were normally detected with the antibody previously incubated in the presence of a nonrelevant peptide (Fig. 3B). In immunocytochemistry studies, the labeling found on kidney sections with the anti-UT-B serum was absent with the preimmune serum (Fig. 5).

UT-B1 of rat, mouse, and human RBCs. The rabbit anti-rat antibody exhibited a high cross-reactivity with murine and human UT-B1. As shown in Fig. 1, the 19-amino acid peptide corresponding to the carboxy terminus of the rat UT, used for immunization, differs by four residues from the mouse corresponding sequence (Lucien N and Bailly P, unpublished observations, and Ref. 40) and by five residues from the human sequence (20).

Fluorescence-activated cell sorter (FACS) analysis on permeabilized RBCs showed that the anti-rat antibody recognized the rat transporter but also the human and the mouse UT-B1 transporter (Fig. 2). After incubation with the immune serum, the fluorescence observed for RBCs of all three species (rat, mouse, and human) was much higher than that observed after incubation with the preimmune serum. Fluorescence was undetected on nonpermeabilized RBCs, confirming the predicted intracellular location of the carboxy end of the transporter (data not shown).

The good cross-reactivity of the antibody with mouse and human UT-B1 was also evidenced by immunoblot analysis of RBC membranes (see Fig. 4A). UT-B1 protein was mainly N-glycosylated in RBC membranes of the three species with, however, some differences about the apparent molecular masses of the different forms. Molecular masses of N-glycosylated forms were the highest for human and the lowest for the mouse. The apparent molecular masses of nonglycosylated

**Fig. 1.** Comparison of the 27 amino acids of the carboxy end of rat (r), mouse (m), and human (h) urea transporter (UT)-B1. The sequence of immunizing peptide corresponds to the last 19 amino acids of rat UT-B1. Amino acids of mouse and human differing from rat are underlined. Asterisks indicate identical amino acids for the three sequences. A cysteine residue (boxed) was added for keyhole limpet hemocyanin coupling.
protein were the highest for human and the lowest for rat (see Fig. 4A).

Tissue distribution of UT-B1. The affinity-purified antibody detected specific bands in homogenate extracts from the inner medulla (but not from the cortex) of the kidney and from brain, bladder, and testis (Fig. 3A). No band was revealed in the liver (Fig. 3A). PNGase treatment revealed that UT-B1 is present as a nonglycosylated form of ~29 kDa in renal inner medulla, bladder, and brain (Fig. 4B). However, the N-glycosylation levels differ among these tissues. The apparent molecular mass of N-glycosylated transporter in brain is 32.5 kDa, whereas in kidney, bladder, and RBCs, it is present as a diffuse band of 35–56 kDa (Fig. 4, A–C). It is noteworthy that testicular UT-B1 is present only as a nonglycosylated protein with a higher molecular mass (~47.5 kDa) than its counterpart in the other tissues (Fig. 4B). This finding did not result from endogenous testicular PNGase inhibitory activity, since PNGase was able to deglycosylate UT-B1 protein of RBC membranes mixed with testes homogenates (Fig. 4C).

Immunocytochemistry. In rat kidney cross-sections through the inner stripe of the outer medulla, immunofluorescence using the UT-B1 antiserum revealed a strong labeling of thin structures located in the central area of vascular bundles (Fig. 5A). These bundles are known to comprise narrow descending vasa recta and wider ascending vasa recta. The labeling of thin structures within vascular bundle sections strongly suggests that only the arterial descending vasa recta were recognized by the antibody, in agreement with the previously described location of UT-B1 (33, 39). Preimmune serum used at the same dilution as antiserum was unable to reveal fluorescence signals (Fig. 5B).

In brain sections, the UT-B1 antiserum revealed a labeling throughout the central nervous system (Fig. 6, A–C). UT-B1 labeling was detected in astrocytes of the pial surface coating the central nervous system (Fig. 6, A and D). In the cortex, the UT-B1 labeling was found in the center of cell bodies (Fig. 6, A and B) identified as astrocytes by the presence of radiating GFAP-labeled processes (Fig. 6, D and E). Whereas few astrocyte processes were labeled by UT-B1 antiserum in the cortical parenchyma, UT-B1 and GFAP labeling colocalized around perforating vessels (Fig. 6, A and D) and rarely around intraparenchymal microvessels (Fig. 6, B and E). This double staining suggests that perivascular astrocyte end feet may contain UT-B1 protein. In white matter like corpus callosum (Fig. 6, C and F) and optic tract (data not shown), UT-B1 labeling was detected in astrocyte processes identified by GFAP labeling. In addition to astrocytes, UT-B1 labeling was also detected at the periphery of ependymal cells lining the ventricles (Fig. 6C) and the central canal in the spinal cord (data not shown). Very weak unspecific signals were observed using preimmune serum at the same dilution as immune serum (data not shown).

Effect of dDAVP on kidney and RBC UT-B1 expression. Chronic administration of dDAVP reduced urine flow rate by one-half from 8.2 ± 1.1 to 4.1 ± 0.4 ml/day. Urine osmolality rose considerably from 1,713 ± 196 to 3,946 ± 132 mosmol/kgH2O. UT-B1 abundance in the inner medulla was lowered significantly by 60% in dDAVP-treated compared with sham-treated rats (Fig. 7A). Both glycosylated and nonglycosylated proteins underwent this fall under hormone treatment. RBC UT-B1 abundance was unaffected by hormone treatment (Fig. 7B).

Effect of furosemide on renal medulla UT-B1 abundance. Rats undergoing chronic furosemide treatment had a much higher urine output (31.5 ± 5.7 vs. 8.2 ± 1.1 ml/day) and a lower urine osmolality than control rats (492 ± 82 vs. 1,713 ± 196 mosmol/kgH2O). Their plasma urea, sodium, and potassium concentrations did not differ from those of untreated rats (Table 1). Furosemide administration did not alter the abundance of the nonglycosylated form of UT-B1 protein but decreased by 30% that of glycosylated protein (Fig. 8).

DISCUSSION

With the use of a specific antibody against the carboxy terminus of rat UT-B1, the present study reveals that UT-B1 protein is expressed in several rat tissues,
with different levels of N-glycosylation. It also identifies cells containing UT-B1 protein in the kidney and brain. The antibody cross-reacts with the murine and human UT-B1 transporters and reveals species-related differential N-glycosylation for UT-B1 in RBC membranes. Finally, we demonstrate that the renal UT-B1 protein abundance is regulated by antidiuretic hormone and furosemide.

Antibody cross-reactivity. The antibody exhibits a good cross-reactivity with the murine and human transporters in their native form in RBC, as revealed by FACS, or in their denatured form, as shown by immunoblotting analysis on RBC membranes. In non-permeabilized RBCs of all three species, FACS did not record significant fluorescence when we used this antibody directed against the COOH terminus of the transporter (results not shown). This indicated that the carboxy terminus of the transporter is indeed located in the intracellular domain, as predicted by the hydropathy profile.

Tissue and cellular distribution of UT-B1 in rat. The present study shows that the kidney medulla but not the cortex, the bladder, brain, and testis, contains UT-B1 protein, thus confirming previously reported results (18, 24, 33, 36). UT-B1 protein was not detected in rat liver, a result at variance with that of Timmer et
al. (33) using an anti-human UT-B1 antibody. In the kidney medulla, the labeling of thin structures within vascular bundles is consistent with the previously reported location of UT-B1 protein in descending vasa recta of human (39) and rat (33) kidney medulla.

The current study strongly suggests that UT-B1 protein is present throughout the central nervous system in astrocytes and ependymal cells, a result congruent with that of a previous in situ hybridization study (4). It also reveals that UT-B1 protein is unevenly distributed within astrocytes and that this distribution is somewhat different between astrocytes located in the cortex and those present in the white matter. In astrocytes of cortex, the UT-B1 labeling is obvious in cell bodies and end feet surrounding perforating blood vessels or the pial surface, whereas it is rarely present in intraparenchymal processes. In astrocytes of white matter like corpus callosum, the UT-B1 labeling is found in parenchymal processes. In contrast to cortical astrocytes with a diffuse UT-B1 in the cell body, ependymal cells exhibit UT-B1 labeling at the cell periphery, suggesting a plasma membrane location.

Because astrocytes and ependymal cells are implicated in the regulation of the composition of extracellular...
lular fluid, UT-B1 may contribute to the control of extracellular urea concentration in brain, as proposed by Berger et al. (4). Because of UT-B1, astrocytes may efficiently collect excess brain interstitial urea and guide it to the perivascular end feet where urea might be taken up by endothelial cells and dumped into the vascular space or the pial surface of the cortex where it is released in the cerebrospinal fluid. UT-B1 in ependymal cells may remove urea from the brain extracellular compartment by accelerating urea transfer to cerebrospinal fluid. The stronger UT-B1 labeling in ependymal cells lining ventricles in the cerebrum and central canal in the spinal cord than in astrocyte perivascular end feet would imply that the elimination of urea from brain to blood is more powerful through the cerebrospinal fluid than by a direct passage through intraparenchymal blood vessels. The finding of UT-B1 labeling in astrocyte processes and perivascular end feet in corpus callosum is consistent with the previously reported presence of UT-B1 mRNA in corpus callosum.

Fig. 6. Double immunofluorescence labeling with UT-B1 antiserum (CY3, red; A-C) and anti-glial fibrillary acid protein (GFAP) antibody (FITC, green; D-F) on same sections in rat brain. A and D: coronal section through superficial layers of the cortex. UT-B1 is revealed on pial surface coating the central nervous system in astrocytes labeled with GFAP. UT-B1 signal is present in the center of radiating GFAP-labeled processes (arrowheads), indicating the presence in astrocyte cell bodies. Around a perforating vessel, UT-B1 labeling colocalizes with GFAP labeling (arrow). In parenchyma, few GFAP-labeled processes colocalize with UT-B1 labeling. B and E: coronal section through layers V and VI of the cortex. Intense UT-B1 labeling is present in the center of astrocyte cell bodies revealed by GFAP labeling (arrowheads). Few intraparenchymal vessels are surrounded by a thin UT-B1 labeling, which colocalizes with GFAP labeling (arrows). C and F: coronal section through the corpus callosum (CC) facing the lateral ventricle (LV). In corpus callosum, UT-B1 labeling colocalizes with GFAP labeling in astrocyte processes (arrowheads). Ependymal cells exhibit a heavy pericellular labeling (arrows). Arrowheads and arrows on the couple of views from one section were exactly placed on identical coordinates. All views were obtained following the same immunological treatment and the same acquisition parameters. Confocal microscope images through a 1-μm-thick plane. Calibration bar: 20 μm.
This suggests that high urea transport occurs in astrocyte processes of white matter. This transport in astrocytes could help remove urea derived from arginine hydrolysis in the myelin sheath.

**Tissue and species differences in UT-B1 protein expression.** Northern blot analysis had shown that, in rats, the UT-B1 gene encodes a single mRNA of ∼3.9 kb in the different organs expressing the transporter, including the kidney, brain, and testis. In humans, UT-B1 is expressed as several transcripts. For instance, there are three transcripts in the kidney, four in the brain, two in the bladder and prostate, and one in the heart and skeletal muscles, whereas no transcript was detected in testis (Bailly P and Cartron J-P, personal communication).

Glycosylation can influence the topogenesis and the function of multispanning membrane proteins. UT-A1, a UT exclusively expressed in the kidney, is present in two monomeric N-glycosylated forms whose abundance was shown to vary differently with urine concentrating activity in rats. This suggests that the extent of N-glycosylation of UTs could be of functional significance. The UT-B1 amino acid sequences in rat tissues and regulation.

**Table 1. Plasma data of rats treated with furosemide (10 mg/100 g body wt daily) for 6 days**

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<thead>
<tr>
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<th>Control</th>
<th>Furosemide</th>
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<tr>
<td>Osmolality, mosmol/kgH₂O</td>
<td>304 ± 9</td>
<td>313 ± 3</td>
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<tr>
<td>Urea, mmol/l</td>
<td>6.88 ± 1.40</td>
<td>8.90 ± 0.96</td>
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<td>Sodium, meq/l</td>
<td>145 ± 2</td>
<td>141 ± 3</td>
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<tr>
<td>Potassium, meq/l</td>
<td>4.22 ± 0.32</td>
<td>3.66 ± 0.31</td>
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Data are shown as means ± SE; n = 4 rats/group. Differences between groups were not statistically significant.

**Fig. 7. Western blot quantification of UT-B1 protein abundance in kidney inner medulla homogenates (A) and in RBC membranes (B) of vehicle-treated control (open bars) and [deamino-Cys¹⁰,D-Arg⁸]vasopressin (dDAVP)-treated (filled bars) rats. Each lane, representing a different rat, was loaded with 15 μg homogenate protein in A and with 2 μg membrane protein in B. Density of nonglycosylated (29 kDa) and glycosylated (45–56 kDa in kidney; 35–50 kDa in RBCs) forms was measured as indicated in MATERIALS AND METHODS and expressed as arbitrary units. Bars represent SE. **P < 0.01 and ***P < 0.001 by Student’s t-test.

**Fig. 8. Western blot quantification of UT-B1 protein abundance in kidney inner medulla homogenates of control (open bars) and furosemide-treated (filled bars) rats. Each lane was loaded with 15 μg protein of inner medulla homogenate from a different rat. Density was measured as indicated in MATERIALS AND METHODS and expressed as arbitrary units. Bars represent SE. *P < 0.05 by Student’s t-test.
rat, human, or mouse are devoid of O-glycosylation sites and have three potential consensus N-glycosylation sites. The N-glycosylation sites at Asn^{206} for rat UT-B1 and at Asn^{31} for human UT-B1 (15) are located in an extramembrane domain (25, 36) and are thus potentially important.

We found that UT-B1 was mainly expressed as glycosylated forms in most studied tissues. The pattern of UT-B1 protein expression in rat tissues revealed by our anti-rat polyclonal antibody differs somewhat from that revealed by anti-human polyclonal antibody and reported by Timmer et al. (33). In RBCs and kidney, both studies revealed a nonglycosylated form as a band around 30 kDa and glycosylated forms as a broad band between 40 and 54 kDa. In addition, we found that this pattern is roughly similar in RBCs of rat, mouse, and human and in the bladder as well with, however, much weaker abundance. In testes, our antibody recognized a single band of 47.5 kDa that was unaffected by PNGase treatment. Such a band was also found by Fenton et al. (7) with two other bands (32, 33) with an additional band at 98 kDa. This 47.5-kDa band exclusively found in testis may represent a UT-B isoform. On the other hand, our pattern of UT-B1 protein expression differs from the study by Timmer et al. regarding the following points: 1) Timmer et al. (33) detected a 98-kDa form in all studied organs, whereas we were unable to reveal this band in any organ; 2) we did not find any band in the liver, whereas Timmer et al., using anti-human UT-B1 antibody, found three unglycosylated UT-B1 forms in this organ; and 3) our study revealed that, in the brain, UT-B1 was present as a major glycosylated protein with apparent molecular mass ~32.5 kDa that fell to 29 kDa after incubation with PNGase F, whereas Timmer et al. found four bands that were unchanged after incubation with PNGase. It has been shown that N-glycosylation of UT-B1 is not required for its membrane targeting and urea transport activity in the oocyte expression system (Sidoux-Walter F and Cartron J-P, unpublished observations). Hence, the functional significance of tissue UT-B1 glycosylation levels needs elucidation.

The light but reproducible band around 83 kDa detected in rat kidney and RBC and mouse RBC could represent dimers of glycosylated UT-B1 protein. Such possible remaining oligomerization, in spite of reducing conditions, was commonly found for several aquaporins and recently for UT-A3 (31). It should be mentioned that such dimerization is not apparent in the human RBC membrane because no band around or above 83 kDa was detected.

Regulation of renal UT-B1 protein. In the kidney, UTs are located in the medulla. Their attributed role is to accumulate urea in this renal zone for medullary hypertonicity, a prerequisite for urine concentration. The regulation by vasopressin of UT-A1 present in collecting ducts and UT-A2 present in descending thin limbs is well documented in terms of both mRNA and proteins. UT-A1 is expressed as a 4-kb mRNA and two N-glycosylated proteins of 97 and 117 kDa in rat renal medulla (5). In vaspressin-treated rats, UT-A1 mRNA was unchanged or modestly decreased (23, 26, 28), and UT-A1 proteins shifted from a 117- to a 97-kDa glycoprotein form in inner medulla (30). We found that vasopressin caused a spatial redistribution of UT-A1 protein isoforms from terminal (decrease of the 97-kDa isoform) to initial (increase of the 117-kDa isoform) inner medulary collecting ducts (35). Regarding UT-A2, both the 3-kb mRNA and the 50-kDa protein were upregulated by vaspressin infusion (23, 37).

Regarding UT-B1, Promeneur et al. (23) reported a reduced mRNA abundance in chronically dDAVP-treated Brattleboro rats genetically devoid of endogenous vasopressin. The result of the present study in normal rats is consistent with the mRNA data on Brattleboro rats, since it reveals that chronic dDAVP infusion causes a fall in UT-B1 protein abundance. This fall involves both the glycosylated and the unglycosylated forms of the transporter.

The furosemide experiment aimed at determining whether medullary hypertonicity, rather than antidiuretic hormone per se, may regulate UT-B1 expression. By inhibiting the Na-K-2Cl transporter of thick ascending limbs, responsible for the primary event of the urinary concentrating process, furosemide considerably lowers the medullary osmolality and increases the urine flow rate, although the endogenous plasma level of vasopressin is maintained or increased (11). Our study shows that prolonged furosemide administration tends to lower UT-B1 expression without changes in blood chemistries. The present finding of underepression of medullary UT-B1 with both increased (dDAVP treatment) and decreased (furosemide treatment) medullary osmolality suggests that the expression of UT-B1 in renal medulla is unlikely regulated by ambient hypertonicity per se. A direct effect on V2 receptors of the hormone can also be excluded because endothelial cells of descending vasa recta, where UT-B1 is located, are devoid of AVP V2 receptors (21, 22, 32). Rather, because V1 receptors are present in endothelial cells (22), decreased V1 receptor-dependent AVP effects in renal medulla may be implicated in the reduction in UT-B1 expression induced by dDAVP or furosemide. Decreased V1 receptor-mediated effects during dDAVP infusion likely result from suppression of endogenous vasopressin release. During furosemide administration, the vasopressin plasma level is unaltered (11), but V1 receptor-mediated effects may be locally minimized because vasopressin concentration in the kidney inner medulla, which is normally 10- to 20-fold higher than in plasma (27), possibly falls because of reduced countercurrent shunting of water in medullary vasa recta.

Why is UT-B1 underexpressed in renal medulla of dDAVP-treated rats? Such a decrease may be an adaptive process to the reduced need for urea transport resulting from decreased production of urea within endothelial cells of descending vasa recta. In these cells, both urea and nitric oxide are formed by the L-arginine cleavage by arginase and nitric oxide synthase, respectively. It has been reported that dDAVP stimulates NO production in rat renal medulla to pre-
serve blood flow and thereby nutrient delivery to the papillae (21). It is therefore conceivable that, in dDAVP-treated rats, arginine is mobilized much more toward NO than urea formation. Such a competition is suggested by the recent finding that endothelial arginase plays a regulatory role in endothelial NO synthesis, by a counteracting effect (13, 41). Alternatively, the high urea content in inner medulla induced by dDAVP infusion may inhibit I-arginine entry in endothelial cells, as recently reported (38), thereby limiting urea production.

REFERENCES


