Abstract. Previous studies have established that the vasopressin-regulated water channel of the collecting duct, aquaporin-2, is excreted in the urine, providing a means for assessment of regulation and dysregulation of aquaporin-2 in humans. This article addresses the hypothesis that membrane transporters from upstream nephron segments are normally detectable in urine. The experiments employed rabbit polyclonal antibodies against the major Na transporters of the proximal tubule (the type 3 Na-H exchanger [NHE3]), the thick ascending limb of Henle’s loop (the bumetanide-sensitive Na-K-2Cl cotransporter [NKCC2]), and the distal convoluted tubule (the thiazide-sensitive Na-Cl cotransporter [NCC]) in immunoblotting experiments. All three of these transporters were readily detectable as high molecular weight complexes present in low-density membrane fractions from urine of normal rats. Cross linking studies of NHE3, NKCC2, and NCC revealed that high molecular weight complexes are normally present in renal tissue. The molecular weights of the complexes in urine matched those of the cross-linked complexes in native kidney tissue. The presence in urine of integral membrane proteins representative of each nephron segment raises the possibility that limited or comprehensive proteomic analysis of urine samples may be useful in clinical settings.

Previous studies have demonstrated that aquaporin-2, the vasopressin-regulated water channel of the collecting duct, is excreted in the urine (1–7). The excreted aquaporin-2 has been demonstrated to be present in small membrane vesicles (1,6). It is present chiefly in the form of a high molecular weight complex (5,6). The amount of aquaporin-2 in urine correlates with circulating vasopressin levels (1,2,4–7), and measurements of aquaporin-2 excretion have been exploited for the study of water balance abnormalities in humans (1–5,7).

The observations made with aquaporin-2 raise the possibility that regulated transporters expressed in other renal tubule segments may be detectable in urine. If so, this might permit a broader analysis of renal regulation and dysregulation than is possible through present analytical approaches. The purpose of this study was to test the hypothesis that the major sodium transporters expressed in renal proximal tubule, thick ascending limb of Henle, and distal convoluted tubule are detectable in urine from normal rats.

Materials and Methods

Antibodies

We previously developed rabbit polyclonal antibodies to the following renal sodium transporters: the type 3 Na-H exchanger (NHE3) of the proximal tubule (8), the Na-K-2Cl cotransporter (BSC1/NKCC2) of the thick ascending limb (9), and the thiazide-sensitive Na-Cl cotransporter (TSC/NCC) of the distal convoluted tubule (10). The antisera were affinity purified against the immunizing peptides as described previously (9,10). In addition, the studies used previously characterized antibodies to aquaporin-1 (11) and aquaporin-2 (12).

Animals and Experimental Protocols

Experiments were conducted in male Sprague–Dawley rats (200 to 300 g) from Taconic Farms (Germantown, NY). They received normal rat chow and water ad libitum. The rats were placed in metabolism cages for 24 h, allowing quantitative collection of urine into 15 ml of ice-cold 1 M Tris-Cl (pH 6.8) containing 1 mg/ml leupeptin (Bachem California, Torrance, CA), 1 mM sodium azide, and 0.1 mg/ml phenylmethyl sulfonyl fluoride (United States Biochemical Corporation, Toledo, OH). The urine samples were centrifuged at 1000 × g for 5 min to remove whole cells and other debris, and the pellet was discarded. Then, the supernatant was spun at 200,000 × g for 120 min with a Beckman L8-M ultracentrifuge fitted with a type 80TI rotor to obtain a membrane fraction. In some cases, an intermediate spin of 17,000 × g for 20 min was carried out using a Sorvall RC2-B refrigerated centrifuge with a SS-34 rotor (DuPont Medical Products, Newtown, CT). The resulting pellets were resuspended in isolation solution (250 mM sucrose/10 mM triethanolamine), and total protein concentration in these membrane fractions was measured using the BCA Protein Assay reagent kit (Pierce Chemical Company, Rockford, IL) before addition of 1 vol per 5 vol 5X Laemmli sample buffer (0.06 M Tris-Cl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 0.025% bromophenol blue) and heating to 60°C for 15 min.

After completion of the urine collections, the rats were killed by decapitation and the kidneys were dissected to obtain renal cortex, outer medulla, and inner medulla. These tissues were homogenized
using a tissue homogenizer (Omni 1000 fitted with a micro-sawtooth generator) in ice-cold isolation solution containing 250 mM sucrose and 10 mM triethanolamine (Calbiochem, La Jolla, CA) with 1 mg/ml leupeptin (Bachem California) and 0.1 mg/ml phenylmethyl sulfonyl fluoride (United States Biochemical Corporation). In addition to kidney samples, lung homogenates were obtained from control rats and used as negative controls. Homogenates were subjected to protein measurement and solubilized with Laemmli sample buffer as described for the urine samples above.

Electrophoresis and Immunoblotting

SDS-polyacrylamide gel electrophoresis was carried out on precast minigels of 7.5, 10, or 12% polyacrylamide (BioRad, Hercules, CA). The proteins were transferred from the gels electrophoretically to nitrocellulose membranes. After a 60-min 5% milk block, membranes were probed overnight at 4°C with the appropriate primary antibody. For this, the antibodies were diluted into a solution containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/dl sodium azide, 50 mg/dl Tween-20, and 0.1 g/dl bovine serum albumin (pH 7.5). The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase (#31463; Pierce) used at 1:5000. Sites of antibody-antigen reaction were visualized using luminol-based enhanced chemiluminescence (LumiGLO, Kirkegaard and Perry Laboratories, Gaithersburg, MD) before exposure to light-sensitive film (Kodak #165–1579 Scientific Imaging Film, Rochester, NY).

Cross-Linking

A kidney from a control rat was homogenized as above in isolation solution containing 250 mM sucrose, 20 mM Hepes, with 1 µg/ml leupeptin (Bachem California, Torrance, CA) and 0.1 mg/ml phenylmethyl sulfonyl fluoride (United States Biochemical Corporation, Toledo, OH). The starting material for the cross-linking experiments was either whole homogenate or the crude membrane fraction prepared as described above. Four different cross-linking agents were used: dimethyl pimelimidate - 2HCl (DMP; Pierce, Catalog # 21666, spacer arm length 9Å), bis(sulfosuccinimidyl)sulfaure (BS3; Pierce, Catalog # 21580, spacer arm length 11Å), bis[2-(sulfosuccinimidoxy-carbonyloxy)ethyl]sulfone (Sulfo-BSOCOES; Pierce, Catalog # 21556, spacer arm length 13Å), and sulfo-ethylene glycolbis sulfosuccinimidylsulfate (Sulfo-EGS; Pierce, Catalog # 2156, spacer arm length 16Å). The membrane samples were incubated with different concentrations of the cross-linking agents for 30 min at room temperature with bidirectional mixing following the manufacturer’s protocol. After the Laemmli buffer was added, the electrophoresis and immunoblotting procedures were performed as described above. The cross-linking serves to bridge covalently any proteins physically associated in the plasma membrane at distances comparable to the spacer arm lengths of the cross-linking agents, thereby yielding a higher molecular weight species.

Results

Figure 1 shows immunoblots loaded with membrane fractions obtained by centrifuging urine samples sequentially at 17,000 × g and 200,000 × g (after a brief centrifugation at 1000 × g to eliminate whole cells and casts). Antibodies to NHE3 of the proximal tubule, NKCC2 of the thick ascending limb, and NCC of the distal convoluted tubule labeled distinct high molecular weight bands (arrowheads) in the high-speed fraction, consistent with the presence of these proteins in small, low-density membrane vesicles, as previously seen for aquaporin-2 (1,6). The apparent molecular weights of these proteins were higher than those of the respective monomeric proteins labeled by the antibodies in kidney homogenates (NHE3, MW 86; NKCC2, MW 161; NCC, MW 160). Recognizing that most, if not all, transporter proteins are present in the native state in the form of high molecular weight complexes (13), we hypothesized that the high molecular weight bands labeled by the three antibodies represent protein complexes containing the respective transporters as previously reported for aquaporin-2 (5,6).

To test for specificity of labeling, competition experiments were carried out in which the antibodies were preincubated with synthetic peptides containing the target sequence for the antibody or with unrelated synthetic peptides. As shown in Figure 2, preincubation with the specific peptides ablated the high molecular weight bands, whereas the unrelated peptides did not affect the labeling. These results support the view that the high molecular weight bands represent complexes of NHE3, NKCC2, and NCC, respectively.

Previous studies have established that the functional form of NHE3 is an NHE3 dimer (14), and additional studies have demonstrated the presence of NHE3 regulatory proteins, viz E3KARP and NHERF (15), which bind NHE3. Thus, it is well established that the native form of NHE3 is a high molecular weight complex. To address whether NKCC2 is present in the kidney as a high molecular weight complex, we carried out cross-linking studies in a crude membrane fraction from outer medulla. As can be seen from the immunoblots shown in Figure 3, increasing concentrations of three of four of the cross linkers progressively stabilized increasingly higher molecular weight NKCC2 complexes, most likely NKCC2 homodimers, trimers, and/or tetramers. Similar cross-linking experiments for
Figure 2. Preadsorption controls for NHE3, NKCC2, and NCC. Minigels were loaded with whole kidney homogenate (10 mg), lung homogenate (10 mg), and 200,000 × g membrane fraction from urine (45 mg). The resulting immunoblots were probed with specific antibodies incubated overnight either with 1 mg unrelated peptide (“nonspecific peptide”) or with 1 mg of the peptide used originally to make each respective antibody (“specific peptide”). In the nonspecific peptide blots, the NKCC2 and NHE3 antibodies were incubated with the NCC peptide and the NCC antibody was incubated with the NHE3 peptide. The specific peptide ablated both the monomeric band in kidney and the high molecular weight band in urine in each case.

NCC are shown in Figure 4. As can be noted, increasing concentrations of two different cross linkers progressively stabilized a higher molecular weight NCC complex, most likely a dimer.

To test whether the high molecular weight complexes in urine correspond in molecular weight to complexes present in native kidney, we carried out immunoblotting with cross-linked samples from kidney and non–cross-linked samples from urine (Figure 5). For both NHE3 and NKCC2, the high molecular weight band in urine corresponded in size to the cross-linked protein in kidney. As shown, addition of 6 M urea (a denaturing agent) to the urine samples failed to disassemble the urinary protein complexes.

We also tested urinary membrane fractions for the presence of aquaporin-1 and aquaporin-2, both of which have been previously demonstrated in urine (Figure 6). As shown in Figure 6A, aquaporin-1 is present in membrane fractions isolated from rat urine. The monomeric protein (kidney and lung lanes) is present in two forms, a 28-kD nonglycosylated form and a glycosylated form centered at 40 kD. The same bands are found in a membrane fraction isolated from urine, but in addition there is a broad high molecular weight aquaporin-1 band consistent with the presence of aquaporin-1 complexes, possibly the previously described aquaporin-1 tetramers (16). Addition of 6 M urea to the urinary membrane fraction increased the amount of monomeric aquaporin-1 but did not remove the high molecular weight band. The high molecular weight band was similar to that seen in DMP cross-linked membrane samples from kidney. As shown in Figure 6B, aquaporin-2 is present in the membrane fractions from rat urine.
As previously shown (1–7). As was previously demonstrated (5,6), aquaporin-2 is also present chiefly in higher molecular weight forms.

**Discussion**

The present studies demonstrate that the kidneys of normal rats excrete readily detectable quantities of Na\(^+\) transporter proteins from the proximal tubule (NHE3), the thick ascending limb of Henle’s loop (NKCC2), and the distal convoluted tubule (NCC). They also confirm the presence of aquaporin-1 and aquaporin-2 in urine. As previously demonstrated for aquaporin-2 (1,3,6), these proteins were present in low-density membrane fractions isolated from urine by ultracentrifugation. Because whole cells were eliminated by initial low-speed centrifugation steps, it is unlikely that the appearance of these Na\(^+\) transporters in urine is due to sloughing of whole cells. It is possible that these low-density vesicles arise as part of the normal turnover of plasma membranes, perhaps via the formation of multivesicular bodies from endocytosed plasma membrane and the subsequent fusion of the outer membranes of the multivesicular bodies with the apical plasma membrane to eliminate their vesicular contents. Although we do not know the mechanisms involved in excretion of these Na\(^+\) transporter proteins, it seems likely that the presence of these proteins in urine will be exploitable in clinical studies in much the same way as have measurements of aquaporin-2 in urine (1–5,7). For example, profiling of Na\(^+\) transporters in urine could be useful in detecting and classifying acute renal failure, classifying inherited disorders of Na\(^+\) excretion, e.g., Bartter’s syndrome and Gitelman’s syndrome, or identifying renal defects associated with hypertension.
The Na\textsuperscript{+} transporters in urine are present in the form of high molecular weight complexes. Previous studies have demonstrated that many (if not all) integral membrane transporters are naturally expressed as high molecular weight complexes (13). Specific kidney proteins that have been shown to exist normally as high molecular weight complexes include NHE3 (14), aquaporin-1 (16), the type 1 Cl-HCO\textsubscript{3} exchanger AE-1 (17), Na-K-ATPase (18), the amiloride-sensitive Na channel ENaC (19), and the renal urea transporter UTA-1 (20). In addition, we have demonstrated here using chemical cross linkers that the Na-K-2Cl cotransporter NKCC2 and the thiazide-sensitive Na-Cl cotransporter NCC are normally present in native kidney membranes with a high degree of specificity for homodimers. Furthermore, the apparent molecular weight of the kidney NKCC2 complexes matched the complexes found in urine, and similar results were found with NHE3 (Figure 5) and NCC (compare Figures 1 and 4). The urinary Na\textsuperscript{+} transporter complexes seem to be extremely stable in view of their resistance to dissolution by SDS and by 6 M urea (Figures 5 and 6).

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References

The Consequences and Costs of Chronic Kidney Disease Before ESRD

Lawrence G. Hunsicker

See related article by Smith et al. (pp. 1300–1306).

ANNOUNCEMENTS

Cover picture: Strategy for therapeutic cloning and tissue engineering. For detailed information, see Koh and Atala on pages 1113–1125.

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ERRATA


In the legends to Figures 2, 3, 4, 5, and 6, all values reported in milligrams (mg) should have been reported in micrograms (µg). JASN regrets the error.


The legend to Figure 3 should read as follows: “Event-free survival by quartile of baseline total homocysteine (quartile 1 highest).” JASN regrets the error.

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