Regulation of the Proximal Tubular Sodium/Proton Exchanger NHE3 in Rats with Puromycin Aminonucleoside (PAN)–Induced Nephrotic Syndrome

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Abstract. Excessive proteinuria due to loss of glomerular permselectivity in nephrotic syndrome can cause disturbances in renal salt and water handling with edema formation. Apart from oncotic and hydrostatic mechanisms associated with hypoalbuminemia, primary derangements in renal tubular sodium transport may contribute to the pathogenesis of nephrotic edema. Whereas there is evidence for an increase of cortical collecting duct sodium reabsorption in nephrotic rats, it remains controversial whether proximal tubule sodium transport may also be activated in this condition. The regulation of the cortical NaH exchanger NHE3, the main pathway for Na reabsorption in the proximal tubule (PT), was investigated in rats with puromycin aminonucleoside (PAN)–induced nephrotic syndrome. PAN rats developed reduced GFR, severe proteinuria, and sodium retention within 3 d. After 10 d, immunoblots of brush border vesicles revealed a decreased abundance of NHE3 in nephrotic animals. However, the Na/H antiporter activity in the same vesicle preparations was not significantly altered. Antiporter activity normalized for NHE3 protein was increased by 88% in nephrotic animals (P = 0.025). Immunohistochemistry with the same polyclonal antibody as for immunoblots revealed a decrease of NHE3 abundance in PT. In contrast, immunoreactivity for the monoclonal antibody 2B9, which specifically recognizes the non–megalin-associated, transport-competent pool of NHE3, was higher in PAN-treated rats than in controls. In conclusion, increased sodium reabsorption might be associated with a shift of NHE3 from an inactive pool to an active pool, thus contributing to sodium retention in a state of proteinuria.

The nephrotic syndrome (NS) is a common manifestation of renal disease and is characterized by urinary protein loss due to glomerular damage and renal salt and water retention (1). Under regular conditions, permselectivity of intact glomerular structures ensures retention of most of the serum proteins in the vascular space (2). Even under normal conditions, up to 5 g of protein per day may be filtered by the glomeruli, which are then reabsorbed by the renal tubule (3,4). In glomerular diseases, two pathophysiologically important events occur. First, substantial amounts of protein (mainly albumin) are filtered through the damaged glomeruli and are lost into the urine. Second, increasing quantities of the filtered protein are reabsorbed by the renal tubule (5). Besides being a hallmark of glomerular disease, proteinuria has been shown to be an independent factor that induces and maintains renal damage (6). The current understanding is that protein reabsorption by the proximal tubule triggers inflammation and scarring by induction of several cytokines and growth factors (for example TGFβ) (7). The reabsorption of albumin is achieved predominantly by endocytosis. Several recent studies have suggested an interrelation of transcellular albumin transport by endocytosis and acidification of lysosomes (8,9) through endosomal Na/H exchange (10,11). The nephrotic syndrome (NS) is also accompanied by various degrees of salt and water retention and represents a major clinical problem in the management of patients with nephrosis (12,13). One proposed mechanism is systemic interstitial volume sequestration due to hypoalbuminemia and secondary Na+ retention. In addition, primary salt retention per se may contribute substantially to systemic volume expansion in NS. The nephrotic state has been associated with activation of the Na/K-ATPase in the collecting duct (14,15). The effect of nephrotic syndrome on proximal tubule Na+ transporters has not been explored. The bulk of sodium reabsorption in the proximal tubule is mediated by the apical sodium/proton exchanger NHE3 (16). Given the potential role of proximal tubular sodium/proton exchange in both tubular protein reabsorption and salt retention, we investigated the regulation of the cortical brush border membrane Na/H exchanger NHE3 in rats with nephrotic syndrome induced by puromycin amino nucleoside (PAN).

Materials and Methods

Materials and Supplies

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless stated otherwise.
Animals

Male Sprague-Dawley rats (average 200 g) were injected with either puromycin aminonucleoside (n = 9; PAN 150 mg/kg body weight intraperitoneally) or an identical volume of vehicle (H2O; n = 9). Animals were then placed separately into metabolic cages and allowed free access to distilled water. Control and PAN rats were pair-fed standard rat chow (No 9331 25 W10; Eberle Nafag AG, Gossau SG, Switzerland). Daily 24-h urine collections were performed in all animals. Urinary sodium concentration, as well as serum and urinary creatinine concentrations, were measured with an AVL984 electrode electrolyte analyzer (AVL Medical Instruments, Schaffhausen, Switzerland). Urine protein concentration was determined using the Bradford assay on a Merck Vitalab Eclipse filter photometer. Creatinine clearance was calculated from the urinary creatinine excreted over the last 24 h before sacrifice and the serum creatinine concentration from venous blood taken at sacrifice. Ten days after injection of PAN or vehicle, rats were anesthetized with 0.25 ml/kg Dormitor (Pfizer, Sandwich, Kent, UK) (1 mg/ml medetomidinid hydrochloridum) and 0.25 ml/kg Hypnorm (Janssen, New Brunswick, NJ) (0.315 mg/ml fentanyl citrate), and kidneys were excised, weighed, and placed in ice-cold phosphate-buffered saline (PBS).

Cortical Brush Border Membrane Vesicle Preparation

Renal cortical apical membrane vesicles were prepared by Mg2+ aggregation, as described previously (17). Dissected kidney cortex was homogenized in membrane buffer (300 mM mannitol, 18 mM HEPES, 5 mM EGTA, 100 mM NaCl, pH 7.50) at 4°C with a Brinkmann Polytron (Brinkmann Instruments, Westbury, NY). MgCl2 was added to the homogenate to obtain a final concentration of 15 mM, and the homogenate was pelleted at 4°C by centrifugation at 2500 × g for 30 min (Polytron; Kinematica GmbH, Kriens, Switzerland). Supernatant was transferred, an additional MgCl2 precipitation was performed, and membranes were pelleted from the final supernatant at 48,000 × g at 4°C for 30 min (RC 5c Plus; DuPont Company, Sorvall Products, Wilmington, DE). Enrichment of brush border membrane vesicles was not affected by PAN treatment, as evidenced by comparable ratios of BBM to total cortical homogeneic leucine aminopeptidase activities in control and PAN animals (×16 ± 3 versus ×13 ± 2, respectively, P = NS).

Immunoblot

Cortical brush border membrane pellets from the above preparation were resuspended in membrane buffer (see above), and protein content was assessed according to Bradford. Thirty micrograms of protein were diluted 1:5 in 5 X sodium dodecyl sulfate (SDS) loading buffer (1 mM Tris - HCl, pH 6.8, 1% SDS, 10% glycerol, 1% [vol:vol] 2-mercaptoethanol), boiled for 10 min, size fractionated by SDS-polyacrylamid gel electrophoresis on 7.5% gels, and electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, blots were probed in the same buffer for 1 h with a polyclonal anti-rat NHE3 antibody directed against amino acids 809 to 822 of the COOH-terminal cytoplasmic domain of rat NHE3 (kindly provided by Dr. Orson W. Moe, University of Texas Southwestern Medical Center, Dallas, TX) at a dilution of 1:10,000. Blots were washed in 0.05% Tween 20 in PBS one time for 15 min and two times for 5 min, incubated with a 1:10,000 dilution of peroxidase-labeled sheep anti-rabbit IgG in 5% nonfat milk and 0.05% Tween 20 in PBS for 1 h, washed as above, and then visualized by enhanced chemiluminescence (Nitro-Block II, Applied Biosystems, Foster City, CA; CDP-Star detection reagent, Amersham, Piscataway, NJ). NHE3 protein abundance was quantitated by densitometry (BioCapt software version 72.02 s for Windows, Wilbert Lourmat, France; Scion Image Beta 3 1998, Scion Corporation, Frederick, MD). Signal was measured as an integrated volume with correction for a defined background.

Na+/H+ Antiporter Assay

Na+/H+ antiporter activity was determined by the acridine orange method as described by Moe et al. (18). Cortical brush border membrane vesicles were used as prepared for western blotting, with the exception that after the final precipitation step at 48,000 × g, the pellet was resuspended in 140 mM NMDG gluconate and 5 mM MES at pH 5.5. The acid-loaded vesicles were then added to a solution containing 120 mM NMDG-gluconate, 20 mM HEPES at pH 7.50, and 6 μM acridine orange (Molecular Probes, Eugene, OR). Fluorescence signal was followed in a spectrofluorometer (λex = 493 nm, λem = 530 nm; Shimadzu 5000, Japan). The proton gradient-driven trapping of acridine orange in the vesicles provoked fluorescence quenching. Na+/H+ antiporter activity was then assayed as the rate of increase in fluorescence (acridine orange efflux) occurring in response to Na+ addition to the extravesicular space. Specific activity of the Na+/H+ antiporter was expressed as the slope of the initial Na+-dependent fluorescence increase divided by the initial quench.

Tissue Preparation for Light Microscopy and Immunohistochemistry

An additional set of animals (n = 3 for both PAN and control) undergoing identical treatment as described above (except for the following modifications) was analyzed by light microscopy and immunohistochemistry. Nine days after injection of PAN or vehicle, rats were anesthetized with an intraperitoneal injection of thiopental (0.5 mg/kg Pentothal; Abbott, Abbott Park, IL), and kidneys were fixed by vascular perfusion via the abdominal aorta as described previously (19). The fixative consisted of 3% paraformaldehyde and 0.05% picric acid in 0.06 M cacodylate buffer (pH 7.4; containing 3 mM MgCl2 and adjusted to 300 mM in sucrose) and 1% hydroxyethyl starch (HAES, Fresenius, Switzerland). After fixation, the left renal artery was clamped and the fixative in the right kidney was washed out by perfusion with 0.1 M cacodylate buffer. Both kidneys were then removed.

For light microscopy, the left unwashed kidney was cut in coronal slices and immersed for at least 24 h in the 3% paraformaldehyde solution, to which 0.1% glutaraldehyde (Fluka Chemie AG, Buchs, Switzerland) was added. Thereafter, the tissue was postfixed in 1% OsO4 and embedded in epoxy resin. Then, 1-μm-thick sections were cut from the epoxy resin–embedded tissue and stained with azure II-methylene blue. Coverslips were applied with DPX mounting medium (Agar Scientific, Stansted, Essex, UK). For immunofluorescence, coronal slices of the right kidney were mounted on cork disks, frozen in liquid propane cooled by liquid N2 and stored at −80°C until use. Four-micrometer-thick cryosections were placed on chromatum/gelatin-coated slides. Sections were pre-treated with 1% SDS in PBS for 4 min. After rinsing with PBS, they were covered with 10% normal goat serum in PBS containing 1% bovine serum albumin (PBS/BSA) for 10 min. Sections were then incubated overnight at 4°C with a mouse anti-rabbit NHE3 monoclonal antibody directed against a Maltose Binding Protein fusion protein containing C-terminal 131 amino acids of rabbit NHE3 (clone 2B9; Chemicon International, Temecula, CA) diluted 1:50, with a polyclonal anti-rat NHE3 antibody (see Immunoblot section) diluted 1:500 in PBS/BSA or with a polyclonal anti-ecto-5′-nucleotidase antibody.
Sections were then rinsed three times with PBS and covered for 1 h at room temperature with FITC-conjugated goat anti-mouse IgG and IgM (Jackson Immunoresearch Laboratories, West Grove, PA) diluted 1:50 and Cy3-conjugated goat anti-rabbit IgG (Jackson) diluted 1:1000 in PBS/BSA together with normal rat serum 1:100. Finally, the sections were rinsed with PBS, coverslips were applied with DAKO-Glycergel (Dakopatts, Glostrup, Denmark) containing 2.5% 1,4-diazabicyclo-(2.2.2)-octane (DABCO; Sigma) as a fading retardant, and the sections were examined by epifluorescence microscopy. The same protocol was followed for double-labeling, but the two primary antibodies as well as the two second antibodies were mixed.

Results
Renal Sodium and Protein Excretion; Creatinine Clearance

As shown in Figure 1, PAN-treated rats developed severe proteinuria on day 4 and beyond, whereas renal protein excretion was minimal in control animals. Similarly, PAN rats showed a significant decrease in urinary sodium excretion compared with control animals within 3 d after administration of the drug (Figure 2). A new steady state in sodium balance was achieved on day 9 in PAN animals, with a urinary sodium excretion no longer statistically different from rats not treated with puromycin. Most of the PAN rats also exhibited ascites on the day of sacrifice. Taken together, administration of puromycin resulted in the clinical equivalent of nephrotic syndrome with proteinuria as well as salt and volume retention. Creatinine clearance was significantly lower in nephrotic versus control rats (0.67 ± 0.09 versus 1.65 ± 0.15 ml/min, respectively; \( P = 0.0074 \)).

Light Microscopy

The overview of control kidneys (Figure 3a) shows intact cortex and outer stripe of medulla with open tubules. In contrast, the kidneys of rats treated with PAN exhibit an irregular pattern with many partially collapsed tubules (Figure 3b). Moreover, many dilated proximal tubules, especially in the medullary rays, can be detected. At higher magnification, the proximal tubules of control rats (Figure 4a) manifest with an intact brush border. In PAN rats (Figure 4b), a widespread shortening of the brush border takes place in proximal tubules.

Figure 1. 24-h urinary protein excretion. Protein excretion was determined from 24-h urine collections of control and puromycin aminonucleoside (PAN)-treated rats. Experimental rats developed severe proteinuria 4 d after administration of PAN, which persisted throughout the whole study period. Note the logarithmical scale of the y-axis. *\( P < 0.001 \).

Figure 2. 24-h urinary sodium excretion. Sodium excretion was determined from 24-h urine collections of control and PAN-treated rats. Whereas control rats were in sodium balance, as revealed by constant natriuresis over the whole study period, PAN rats retained sodium from day 3 through 8, as evidenced by almost undetectable sodium excretion during this period. However, natriuresis in PAN animals returned back to control levels at day 9. *\( P < 0.001 \).

Figure 3. Light microscopic overview of cortex. 1-μm-thick epon sections. (a) control kidney; (b) PAN-treated animal showing some collapsed tubules, whereas many tubules are dilated in the cortical medullary rays.
The brush border also undergoes focal destruction, especially in S2 segments. The proximal tubular cells display some variability in size, with many cells being smaller than in control animals, but some cells also with an increase in size.

**Immunofluorescence**

Two different antibodies were used for detection of NHE3 in this study. With the monoclonal antibody 2B9, immunoreactivity was homogeneously increased in PAN-treated animals (Figure 5). In contrast, with the polyclonal antibody there was an overall decrease in PAN-treated animals. The levels of immunofluorescence were similar to controls in some tubules, whereas they were very low in other tubules (Figure 6). The decrease of immunoreactivity with the polyclonal antibody may be related to the structural alteration of the brush border described above. Indeed, the immunoreactivity for two unrelated proteins of the luminal membrane, ecto-5'-nucleotidase and NaPi-IIa, decreased also (not shown).

In both control and PAN-treated animals, the intracellular distribution of NHE3 immunoreactivity was strikingly different with the two antibodies (Figure 7). NHE3 is mainly localized at the base of the brush border in the so-called intermicrovillar compartment (20–22), which the polyclonal antibody labeled almost exclusively in the present study (Figure 7). In contrast, strong labeling was detected with antibody 2B9 up to the tip of the microvilli, as described previously (20). With 2B9 the intracellular distribution pattern of immunoreactivity was somewhat altered by PAN treatment. Whereas in control animals the intermicrovillar compartment showed a distinctly higher immunofluorescence than the brush border, there was little difference between the two compartments in PAN-treated animals (Figure 7). Although the overall immunoreactivity with 2B9 was stronger in treated animals, its pattern often appeared irregular within tubular profiles at high magnification. This probably reflects the focal damage in the brush border, which has been observed also by light microscopy (see above). Indeed, the same irregular pattern was seen when adjacent sections were double-labeled with 2B9 and with an antibody against the brush border enzyme ecto-5'-nucleotidase (Figure 8).

**Na/H Exchange Activity and NHE3 Protein Abundance of Renal Cortical Brush Border Membrane Vesicles**

Na/H exchange activity in cortical brush border membrane (BBM) vesicles was not significantly different between PAN and control rats (Figure 9A). However, NHE3 immunoblot showed that NHE3 protein abundance (per unit of total BBM vesicle protein) was decreased by 38% in animals that had been treated with puromycin ($P = 0.003$; Figure 9, B and C). This
can be explained mainly by the partial disruption of the cortical brush border from PAN toxicity. To estimate specific Na/H exchange activity of intact tubules, activity measurements were normalized to cortical brush border NHE3 protein abundance, as Na/H exchange activity and NHE3 protein abundance were always determined from the same brush border vesicle preparation. Specific Na/H exchange activity was significantly increased in PAN rats by 88% versus control animals ($P = 0.032$; Figure 9D). As an internal control, Western immunoblot experiments were performed using an antibody directed against the rat sodium/phosphate cotransporter NaPi-2 (kindly provided by Dr. Jürg Biber, Institute of Physiology, University of Zurich-Irchel, Zurich, Switzerland). Unlike for NHE3, no relevant changes in NaPi-2 cortical BBM protein abundance could be detected between control and PAN treated animals (data not shown).
Discussion

It is still a matter of debate whether nephrosis is a state of underfill due to hypoalbuminemia or the result of primary renal salt and water retention from an activated renin-angiotensin-aldosterone system. Although our study was not designed to resolve this question, the findings point to a novel mechanism for primary renal sodium retention in a model of nephrosis in the rat. PAN in a dose of 150 mg/kg body wt resulted in salt retention within 3 d of administration (Figure 1), preceding overt proteinuria by 1 d (Figure 2). Besides a clear reduction in urinary sodium excretion, PAN rats also exhibited volume retention as evidenced by the development of ascites. Current evidence suggests that the collecting duct is one of the primary sites of salt retention in nephrotic syndrome (23-25). The Na/K-ATPase seems to be the major regulator of sodium reabsorption in this tubular segment in rats with proteinuria (14, 15). However, other studies have also shown increased sodium reabsorption to occur in the proximal tubule (26). Varying levels of intravascular volume status and GFR at time of study may account for the diverse findings with regard to the principal site of sodium reabsorption in nephrotic subjects. As the bulk of sodium absorption occurs in the proximal tubule, it seems reasonable to postulate that this segment contributes at least in part to salt and volume retention in the nephrotic syndrome.

The combined functional and immunohistochemical data of our study point toward an increase in activity of the Na/H exchanger in proximal tubules in PAN-induced nephrotic syndrome. Na/H exchange activity normalized to NHE3 antigen was increased by 88% in nephrotic versus control rats. As other BBM proteins, like the cortical sodium/phosphate cotransporter NaPi-2 and the ecto-5’-nucleotidase, are not affected in PAN-treated animals, we postulate a specific effect of proteinuria on proximal tubular NHE3. Furthermore, our immunohistochemical data along with recent studies by Biemesderfer et al. (20) provide the basis for a possible explanation how NHE3 activity may be regulated in the nephrotic syndrome. They demonstrated that NHE3 occurs in two different pools. The majority of NHE3 is found as a 21 S, megalin-associated, inactive form in the intermicrovillar microdomain at the base of the apical plasma membrane, whereas the remainder is present mainly in a 9.6 S active form present in brush border microvilli unrelated to megalin. There are two separate lines of evidence in the present study that suggest that the relative distribution of NHE3 is shifted toward the megalin-free, active

animals (black bar) regarding Na/H exchange activity. (B) Typical Western immunoblot from the same vesicle preparations as used for activity measurements showing substantially reduced NHE3 protein abundance in PAN rats compared with controls. (C) Quantification of NHE3 protein abundance in control and PAN-treated animals as determined by densitometry from Western immunoblot chemiluminescence signals. (D) Proximal tubular brush border Na/H exchange activity normalized for the amount of NHE3 protein abundance depicted in panels B and C. The adjusted Na/H exchange activity was increased by 88% in nephrotic rats (PAN) versus normal controls.

Figure 9. Proximal tubular Na/H antiporter activity and NHE3 protein abundance. Panel A depicts results of Na/H antiporter activity as determined from changes in intravesicular pH of cortical brush border membrane vesicles by acridine orange. No apparent difference exists in unadjusted measurements between control (white bar) and PAN-treated
pool in nephrotic animals. First, in BBM vesicles, the Na/H exchange activity per unit of NHE3 protein increased in PAN-treated rats. Second, in tissue sections, the abundance of NHE3 detected with the polyclonal antibody decreased, whereas in increased immunoreactivity could be noted with the monoclonal antibody 2B9, which is specific for the megalin-free, active form of NHE3. Megalin is a receptor for filtered proteins; increased protein filtration might therefore represent the link between PAN treatment and the postulated dissociation of NHE3 from the complexes with megalin. To substantiate this proposed link between proteinuria and activation of the Na/H exchanger, it will be necessary to examine further models of proteinuria. However, regulation of NHE3 activity in opossum kidney (OKP) cells via trafficking of NHE3 protein between the microvillar plasma membrane and a subapical compartment has been shown previously by various stimuli such as acid (22), osmolality (27), endothelin-1 (28), parathyroid hormone (29), and dopamine (30). Finally, in a preliminary report, we have demonstrated that albumin increases Na/H exchange activity and apical cell surface NHE3 antigen in OKP cells (31).

The immediate signal for Na/H exchanger stimulation could be activation of the renin-angiotensin system. Indeed, 10−11 M angiotensin II activates Na/H exchange as shown in cultured proximal tubule cells. However, the literature on changes in local and systemic angiotensin II concentrations in PAN nephrosis is controversial. Whereas some investigators found increased angiotensin II levels in nephrotic animals, others did not observe such changes. PAN-treated rats had a decreased whole-animal clearance, as revealed by the significantly lower creatinine clearance compared to control animals. Given the reduced total kidney GFR, the “normal” total cortical BBM NHE3 activity suggests a heightened level of proximal tubule reabsorption reflecting a reset level of glomerulotubular balance. The second implication of our findings relates to proteinuria. Albumin enhances Na/H exchange activity in OKP cells, as shown in a preliminary report (31). NHE3 participates in tubular albumin uptake (10,11); the increase in NHE3-specific activity may therefore be in response to the increased albumin load presented to the proximal tubule.

In conclusion, in a model of nephrotic syndrome, the abundance of NHE3, its specific activity, and its subcellular localization were altered, suggesting a novel mechanism of control of Na/H exchange in the proximal tubule in vivo.

Acknowledgments

PMA was supported by a grant from the Swiss National Science Foundation (31–54957.98) and the Hermann Klaus Foundation. JK was supported by the Novartis Science Foundation.

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