NHE3 Na\(^+/\)H\(^+\) exchanger supports proximal tubular protein reabsorption in vivo

Michael Gekle,1 Katharina Völker,1 Sigrid Mildenberger,1 Ruth Freudinger,1 Gary E. Shull,2 and Martin Wiemann3

1Physiologisches Institut, University of Würzburg, 97070 Würzburg; 2Physiologisches Institut, Universität Duisburg-Essen, 45122 Essen, Germany; and 3Department of Molecular Genetics, Biochemistry, and Microbiology, The University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Submitted 20 February 2004; accepted in final form 22 April 2004

Gekle, Michael, Katharina Völker, Sigrid Mildenberger, Ruth Freudinger, Gary E. Shull, and Martin Wiemann. NHE3 Na\(^+/\)H\(^+\) exchanger supports proximal tubular protein reabsorption in vivo. Am J Physiol Renal Physiol 287: F469–F473, 2004. —Proximal tubular receptor-mediated endocytosis (RME) of filtered proteins prevents proteinuria. Pharmacological and genetic studies in cultured opossum kidney cells have shown that the apical Na\(^+/\)H\(^+\) exchanger isoform 3 (NHE3) supports RME by interference with endosomal pH homeostasis and endocytic fusion events. However, it is not known whether NHE3 also supports proximal tubular RME in vivo. We analyzed proximal tubular protein reabsorption by microinfusion experiments in rats and investigated renal protein excretion in NHE3 knockout (Nhe3\(^{−/−}\)) mice. Inhibition of NHE3 by EIPA or S-3226 reduced the fractional reabsorption of [\(^{14}\)C]cytochrome c by \(-50\%\) during early proximal microinfusion. During early distal microinfusion, no protein reabsorption could be detected. Urinary protein excretion of Nhe3\(^{−/−}\) or heterozygous mutant mice was significantly higher compared with wild-type mice. SDS-PAGE analysis of urinary proteins revealed that Nhe3\(^{−/−}\) animals excreted proteins the size of albumin or smaller. Thus a reduction in NHE3 activity or abundance causes tubular proteinuria. These data show that NHE3 supports proximal tubular RME of filtered proteins in vivo.

urinary protein excretion; pH homeostasis

RECEPTOR-MEDIATED ENDOCYTOSIS is an essential mechanism for reabsorption of filtered proteins in the proximal tubule. In recent years, it has been shown that megalin/cubilin-mediated endocytosis is one of the most important pathways in this context (5, 6, 9, 14, 17). An important process along the endocytic pathway is the proper pH homeostasis of endosomal compartments (21, 27, 29), which influences ligand-receptor dissociation, vesicle trafficking, endosomal fusion events, and recycling to the plasma membrane (18, 19, 27, 29, 37). Endosomal pH homeostasis is maintained, at least in part, by the vacuolar-type H\(^+\)-ATPase (33). In addition, cell culture studies provided evidence for the involvement of Na\(^+/\)H\(^+\) exchanger isoform 3 (NHE3) in endosomal pH homeostasis and megalin/cubilin-mediated endocytosis (1, 11, 14–16, 23, 26). Renal proximal tubular protein reabsorption is of major importance because it prevents the loss of vitamins, hormones, and amino acids (8, 17), but at the same time enhanced tubular protein loads can induce tubulointerstitial inflammation and fibrosis (3, 13) and change proximal tubular gene expression (30). The mechanisms leading to protein-induced inflammation or fibrosis involve the expression of pathophysiologically relevant mediators such as RANTES and MCP-1, transcription factors such as NF-κB, and activation of mitogenic pathways during enhanced protein reabsorption (3, 12, 22, 38). Using proximal tubule-derived opossum kidney cells, we showed previously that prevention of apical protein uptake by inhibition of NHE3 reduces the activation of inflammatory and fibrotic events (40). Thus, if the cell culture data were to hold true for the situation in vivo, then NHE3 would be a potential therapeutic target for the treatment of proteinuria-induced tubulointerstitial nephropathies. The present study was performed to test whether NHE3 supports proximal tubular protein endocytosis also in vivo. For this purpose, we performed micropuncture experiments in rats and determined urinary protein excretion in NHE3 knockout mice (34).

METHODS

Materials. [\(^{14}\)C]Cytochrome c (850 kBq/mg) was obtained from Biotrend (Cologne, Germany). S-3226 and EIPA were kindly provided by Dr. H.-J. Lang (Aventis, Frankfurt, Germany). All other materials were obtained from Sigma (Deisenhofen, Germany), if not stated otherwise.

Preparation of rats for microinfusion experiments in vivo. Male Wistar rats (Charles River, Konstanz, Germany) weighing 210–290 g were fed an Altromin standard diet and had free access to water. The rats were anesthetized with 120 mg/kg body wt Inactin (Byk-Gulden, Constance, Germany). After a polyethylene tube was inserted into the trachea and two catheters were inserted into the jugular vein, the animals were infused intravenously with Ringer solution at a rate of 50 μl/min. The kidney was prepared for micropuncture as described elsewhere (20).

Microinfusion experiments. After identification of the nephron section by intravenous injection of lissamine green SF (Chroma-Gesellschaft, Königsmünster, Germany) at a bolus dose of 0.02 ml of a 100 g/l solution titrated with NaOH to pH 7.4, the tubule was micropunctured using glass capillaries. They had ground tips (outer tip diameter 10–12 μm) and were mounted on a microperfusion pump (36). Puncture sites were the earliest superficial loop of the proximal tubule (‘early proximal’), which represents a distance from the glomerulus of ~0.3–1.2 mm. The microinfused solution was a Ringer solution containing [\(^{14}\)C]Cytochrome c and [\(^{3}\)H]Inulin. Microinfusion at a rate of 10 nl/min lasted for 20 min. Starting shortly before microinfusion, the ipsilateral urine was collected from a urethral catheter in 15-min fractions for 60 min, and radioactivity of each fraction was counted in a liquid scintillation spectrometer (Packard Instruments, Frankfurt, Germany). Urinary [\(^{14}\)C]label recovery (frac-
tional excretion (FE) was calculated from $[\text{[H]urine}]_{\text{[14C]perfusion}}/\text{[14C]urine-}[\text{[H]perfusion}}$. Fractional reabsorption (FR) is $1 - \text{FE}$. As a control, the urine of the contralateral kidney was collected from a bladder catheter. Here, radioactivity did not exceed the background level.

Preparation of mice for clearance experiments. Wild-type, heterozygous, and homozygous NHE3-deficient mice were obtained from an established colony that was generated by targeted gene disruption as previously described (34). All animals were derived from heterozygous crosses, and genotypes were determined by polymerase chain reaction analysis of DNA from tail biopsies. Mice were maintained on normal rodent chow and tap water in a barrier facility until the time of the experiment.

Male mice of each genotype (Nhe3 $+/+$, $n = 4$; Nhe3 $+/−$, $n = 7$; and Nhe3 $−/−$, $n = 4$) weighing between 30 and 40 g were prepared for clearance studies according to the conventional techniques modified for use in the mouse as described (25). Mice were anesthetized with thiobutabarbital (100 $\mu$g/g body wt Inactin; Research Biochemicals International, Natick, MA) and placed on a thermally controlled surgical table. After a tracheostomy, the jugular vein was cannulated with polyethylene tubing hand-drawn to a fine tip over a flame (0.3 to 0.5-mm OD). The venous catheter was connected to a syringe pump for infusion. The bladder was also cannulated with flared PE-10 polyethylene tubing for the collection of urine. Body temperature was maintained at 37.5°C, and animals were provided with a steady stream of 100% O$_2$ to breath. Four to seven samples of urine were collected from each animal. At the end of the experiment, arterial blood was obtained by left cardiac puncture. All experiments were carried out according to the national legal guidelines for animal use in research.

Analytic procedures. Protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Munich, Germany). Creatinine was determined by the Jaffe reaction. SDS-PAGE was performed as described previously (24).

Calculations and statistics. Data are presented as means ± SE; $n$ represents the number of animal or micropuncture samples. Significance of difference was tested by Student’s $t$-test or ANOVA as appropriate. Differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

Micropuncture studies in rats. To investigate the importance of NHE3 for proximal tubular protein reabsorption, we determined the FR of cytochrome c during microinfusion in rats. Cytochrome c is reabsorbed via the “megalin/cubilin pathway” (32). Thus FR-cytochrome c provides information about megalin/cubilin-mediated endocytosis. During early proximal microinfusion, FR was $\sim$45% (Fig. 1). Because of the relatively low specific activity of $[\text{14C}]$cytochrome c, we had to use a relatively high chemical concentration of $\sim$350 $\mu$mol/l in the infusion solution, resulting in partial saturation of the reabsorption capacity. During early distal microinfusion, no significant reabsorption was observed. Precipitation with trichloroacetic acid confirmed that all radioactivity was bound to protein.

Next, we tested the effect of pharmacological NHE3 inhibition with EIPA or the NHE3-specific S-3226 on cytochrome c reabsorption. To achieve complete inhibition of NHE3 and taking into consideration that the infusion solution is diluted ($\sim$5- to 6-fold) by endogenously formed ultrafiltrate, we used 100 $\mu$mol/l EIPA and 30 $\mu$mol/l S-3226 (31, 35). Figure 1 shows that both inhibitors reduced proximal reabsorption of cytochrome c by $\sim$50%. The possibility that the inhibition observed resulted from a weak base effect can be excluded because the concentrations used are too low, as we showed previously (14). In vivo, in addition, 10 mmol/l NH$_4$Cl are necessary to inhibit protein reabsorption (4). These data show, for the first time, that NHE3 supports proximal tubular receptor-mediated endocytosis of proteins in vivo, as observed previously in vitro. To further confirm this finding, we used NHE3 knockout mice as a second, independent experimental model.

Clearance studies in mice. We compared urinary protein excretion of Nhe3 $+/+$, Nhe3 $+/−$, and Nhe3 $−/−$ animals

![Fig. 1. Micropuncture experiments in rats](http://ajprenal.physiology.org/)

A: fractional reabsorption (FR) of cytochrome c was $−45\%$ during early proximal (EP) microinfusion, whereas no reabsorption was observed during early distal (ED) microinfusion. Number of animals = 8 for EP and 6 for ED. B: effect of 100 $\mu$mol/l EIPA or 30 $\mu$mol/l S-3226 on FR expressed as a percentage. C: effect of 100 $\mu$mol/l EIPA or 30 $\mu$mol/l S-3226 on cytochrome c reabsorption expressed as $\mu$g/nephron. $*P < 0.05$ vs. control; $n = 8−10$ rats.
As shown in Fig. 2, total urinary protein excretion (μg/min) was significantly higher in Nhe3 +/− and Nhe3 +/− mice compared with Nhe3 +/+ mice. Serum protein concentrations did not differ significantly in the three groups (59 ± 1 g/l). The gene-dose effects observed for urinary protein excretion correspond to the gene-dose effect for proximal tubular fluid reabsorption (25). Thus increasing protein excretion in Nhe3 +/− and Nhe3 −/− animals most probably reflects the graded reduction in NHE3 expression. It is known from previous studies (2, 25) that glomerular filtration is reduced in Nhe3 −/− animals. A recent study revealed that this reduction results from renal compensatory mechanisms due to decreased proximal tubular salt reabsorption (41). This altered glomerular filtration is also reflected by the reduced creatinine clearance measured in the present investigation (129 ± 12 μl/min for Nhe3 +/+, 116 ± 8 μl/min for Nhe3 +/−, and 94 ± 4 μl/min for Nhe3 −/− mice). The values for creatinine clearance underestimate glomerular filtration by a factor of about three due to a nonspecific color formation mainly in serum (28). However, taking this factor into account, the values obtained here are in good agreement with filtration rates determined with inulin (41). To account for reduced filtration in Nhe3 −/− animals, we expressed protein excretion normalized to creatinine in Fig. 2B. Here, the differences are even more pronounced. Total urinary protein excretion (μg/min) in Nhe3 −/− animals was 188% compared with that in Nhe3 +/+ mice. Creatinine-normalized protein excretion in Nhe3 −/− mice was 221% compared with Nhe3 +/+ mice. These data obtained from Nhe3 −/− animals confirm the hypothesis of NHE3-supported protein reabsorption.

Enhanced protein excretion can result from enhanced protein filtration or reduced protein reabsorption (10). Although glomerular filtration is reduced in Nhe3 −/− animals and there is no evidence for altered glomerular protein sieving, we characterized the urinary proteins by SDS-PAGE (gel loading was normalized for creatinine, i.e., a volume equivalent to 3 μg of creatinine was loaded). With respect to the size of proteins, tubular proteinuria is characterized by enhanced excretion of proteins the size of albumin or smaller. As shown in Fig. 3, this was precisely the case for Nhe3 +/− or Nhe3 −/− animals. In Nhe3 +/− animals, excretion of proteins ≤24 kDa was enhanced, whereas for larger proteins there was no difference. In Nhe3 −/− animals, excretion of proteins ≤24 kDa was also enhanced. In this group, we observed in addition an increased excretion of larger proteins. Most likely, the 60- to 70-kDa band corresponds to albumin and the 48-kDa band could correspond to vitamin D-binding protein (5). Protein excretion of Nhe3 −/− animals, therefore, resembles the pattern ob-
served in megalin-deficient mice or in CLC-5-deficient mice, which are both typical models of tubular proteinuria (7, 39). Enhanced excretion of larger proteins, typical for glomerular proteinuria, was not observed. Thus proximal tubular NHE3 deficiency leads to reduced protein reabsorption.

The data presented in this study show for the first time that proximal tubular NHE3 supports protein endocytosis not only in cultured cells but also in vivo. Given the fact that the Na+/H+ -transporting function as well as the regulation of NHE3 are very comparable in vivo and in cultured cells, we think that the mechanisms linking NHE3 and endocytosis in cultured cells (14–16) are also suitable to explain our in vivo findings. Enhanced tubular protein reabsorption, for example, after glomerular injury, is one of the major factors determining the development of tubulointerstitial injury (3). Therefore, inhibition of excessive protein reabsorption would be a rational therapeutic strategy to prevent such damage. In line with this, cell culture data suggest that prevention of apical protein uptake by inhibition of NHE3 reduces the activation of inflammatory and fibrotic events (40). Our data presented in the present study provide a rational basis for evaluating the above-mentioned strategy in vivo.

ACKNOWLEDGMENTS

We thank Dr. S. Silbernagl for stimulating discussions and Dr. H. Koepsell for support with handling of the mice.

GRANTS

The work was supported in part by the Wilhelm-Sander-Stiftung (grant to M. Gekle) and by National Institutes of Health Grant DK-50594 (to G. E. Shull).

REFERENCES


