**Experimental nephrotic syndrome leads to proteolytic activation of the epithelial sodium channel (ENaC) in the mouse kidney**

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**Abstract**

Proteolytic activation of the renal epithelial sodium channel ENaC involves cleavage events in its α- and γ-subunits and is thought to mediate sodium retention in nephrotic syndrome (NS). However, detection of proteolytically processed ENaC in kidney tissue from nephrotic mice has been elusive so far.

We used a refined Western blot technique to reliably discriminate full-length α- and γ-ENaC and their cleavage products after proteolysis at their proximal and distal cleavage sites (designated from the N-terminus), respectively. Proteolytic ENaC activation was investigated in kidneys from mice with experimental NS induced by doxorubicin or inducible podocin deficiency with or without treatment with the serine protease inhibitor aprotinin.

Nephrotic mice developed sodium retention and increased expression of fragments of α- and γ-ENaC cleaved at both the proximal and more prominently at the distal cleavage site, respectively. Treatment with aprotinin but not with the mineralocorticoid receptor antagonist canrenoate prevented sodium retention and upregulation of the cleavage products in nephrotic mice. Increased expression of cleavage products of α- and γ-ENaC was similarly found in healthy mice treated with a low salt diet, sensitive to mineralocorticoid receptor blockade. In human nephrectomy specimens, γ-ENaC was found in the full-length form and predominantly cleaved at its distal cleavage site.

In conclusion, murine experimental NS leads to aprotinin-sensitive proteolytic activation of ENaC at both proximal and more prominently distal cleavage sites of its α- and γ-subunit, most likely by urinary serine protease activity or proteasuria.

**New and Noteworthy**

This study demonstrates that murine experimental nephrotic syndrome leads to aprotinin-sensitive proteolytic activation of ENaC at both the α- and γ-subunit, most likely by urinary serine protease activity or proteasuria.

**Introduction**

The epithelial sodium channel (ENaC) is expressed in the distal nephron and plays a decisive role in the regulation of body sodium homeostasis. A specific feature of ENaC is its complex posttranslational processing by proteolysis leading to removal of inhibitory peptide tracts and changes in channel gating (1, 2). ENaC is composed of three subunits (α,β,γ) and proteolytic cleavage takes place at proximal and distal cleavage sites (designated from the N terminus, Fig. 1A, C) within the extracellular domains of the α- and γ-subunit. Furin, an intracellular serine protease found in the Golgi apparatus, is thought to cleave α-ENaC at the proximal and distal cleavage site whereas the γ-subunit is only cleaved once at the proximal cleavage site during intracellular maturation (3, 4). A second extracellular cleavage event in distal γ-ENaC leads to the full activation of the channel and is mediated by membrane-bound or extracellular serine proteases such as prostasin (5, 6) or tissue kallikrein (7). Whereas uncleaved ENaC has a low open probability, removal of the inhibitory tracts from α-ENaC and γ-ENaC increases the open probability to a state of maximal channel activation (8). Using mutants of α-ENaC and γ-ENaC subunits, proteolytic activation of γ-ENaC was found to have a dominant effect on channel activity, stimulating channel activation in the absence of proteolysis of α-ENaC (9).

Proteolytic activation of ENaC by aberrantly filtrated serine proteases or proteasuria is thought to occur during nephrotic syndrome, thereby causing sodium retention and edema (10-14). This concept is supported by our findings that treatment with the serine protease inhibitor aprotinin prevented sodium retention in mice with experimental nephrotic syndrome as did treatment with the ENaC blocker amiloride (13, 15, 16). A limitation of these studies was the failure to demonstrate the expression of cleaved ENaC subunits, particularly that of cleaved γ-ENaC in kidney lysates from nephrotic mice by Western blot analysis. This was in sharp contrast to WB analyses of γ-ENaC expressed in oocytes which using the same antibody revealed the expression of fully cleaved γ-ENaC after addition of chymotrypsin, plasmin or plasma kallikrein (17, 18).

In rat ENaC, there are 6, 12, and 5 consensus sites (Asn-X-Ser/Thr) for N-glycosylation in the extracellular domains of the α-, β-, and γ-subunits, respectively (19) that are required subunits for maturation and surface expression (20). In MDCK cells expressing αβγ-ENaC, N-glycosylation was found to involve all ENaC subunits (21). This finding was reproduced in rats in 2006 by Ergönül et al. (22). In both studies, ENaC subunits were deglycosylated using the enzyme Peptide:N-glycosidase F (PNGaseF). Recently, Frindt et al. reported the identification of fully cleaved γ-ENaC in kidneys from healthy mice and rats after deglycosylation using PNGaseF (23). In their discussion, they encouraged efforts to translate these findings to rodents with experimental nephrotic syndrome to finally prove the occurrence of proteolytic activation of γ-ENaC *in vivo*.

In this study, we tested the hypothesis that experimental nephrotic syndrome leads to proteolytic activation of the α- and particularly of the γ-subunit of ENaC in kidneys from nephrotic mice. We can demonstrate that nephrotic syndrome leads to increased expression of cleavage products of both α- and γ-ENaC which was prevented by the serine protease inhibitor aprotinin. Moreover, salt intake, treatment with ENaC-blocker triamterene or aldosterone also modulated expression of ENaC cleavage products.

**Methods**

*Mouse studies*

Experiments were performed on 3-month-old wild-type 129S1/SvImJ mice of both sex at a ratio of 1:1 which are susceptible to induction of experimental nephrotic syndrome by doxorubicin (24, 25). In addition, mice with inducible podocin deficiency were used as an alternative nephrotic mouse model (B6-Nphs2tm3.1Antc\*Tg(Nphs1-rtTA\*3G)8Jhm\*Tg(tetO-cre)1Jaw or *nphs2Δipod*) (15). Genotyping was done using PCR as described (15). Mice were kept on a 12:12-h light-dark cycle and fed a standard diet (ssniff, sodium content 0.24% corresponding to 104 µmol g-1, Soest, Germany) with tap water *ad libitum*. Experimental nephrotic syndrome was induced in 129S1/SvImJ after a single intravenous injection of doxorubicin (14.5 µg g bw-1, Medac, Germany) as developed by our group (24-26). *Nphs2Δipod* were induced by a 14-day treatment with doxycycline in the drinking water (2 g L-1 with 5% sucrose). Treatment with the serine protease inhibitor aprotinin (6000 KIU mg-1, Loxo, Heidelberg, Germany) was performed using custom-made subcutaneous pellets with a matrix-driven sustained release (Innovative Research of America, Florida, USA). Aprotinin-containing or placebo pellets consisting of the matrix only were surgically implanted subcutaneously on the back of the mice on day 3 after end of induction as shown (26). The optimal dose chosen after dose-finding studies was 1 mg in doxorubicin-induced nephropathy and 2 mg per day in *nphs2Δipod* mice. To block the effects of aldosterone, the MR antagonist canrenoate (aldactone) was offered in the drinking bottle (400 mg L-1) starting on day 3 after doxorubicin injection. Due to restrictions by the regulating authority and the IACUC which would not allow to study mice in metabolic cages for sodium balance over 5 or more days, we kept single mice in normal cages and collected spontaneously voided urine in the morning between 8 and 9 am. Daily food and fluid intake were monitored by weighing the food pellets and the water bottle. Mice were euthanized on day 4, 8 and 14 as designated and blood and kidneys were collected.

To study the impact of salt intake and certain drugs on proteolytic processing of ENaC *in vivo*, we treated healthy 129S1/SvImJ mice with a low salt diet (C1036, Altromin, Lage, Germany, sodium content 7 µmol g-1 food), high salt (0.9% NaCl in tap water), triamterene (200 mg L-1 in drinking water), aldosterone (10 µg·g-1 bw in 1 µl·g-1 DMSO i.p.), dexamethasone (10   
µg·g-1 bw in 10 µl saline i.p.), or canrenoate (400 mg L-1 in the drinking bottle, corresponding to ~80 µg·g-1 bw). Urine was collected in metabolic cages before and during treatment over two days, respectively. Mice were euthanized after that and blood and kidneys were collected. All animal experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the German law for the welfare of animals, and they were approved by local authorities (Regierungspraesidium Tuebingen, approval number M5/16, M5/17, M02/19M).

*Human samples*

The urology department provided small snap-frozen samples of human kidney from nephrectomy specimens of patients with renal cell carcinoma. Histologic analysis confirmed that samples contained healthy kidney tissue. Prior to nephrectomy, patients had given informed consent to make the tissue available for research. The asservation of kidney samples was approved by the local ethics committee (379/2010BO2).

*Laboratory measurements*

Urinary protease activity was measured using the chromogenic substrate S-2251 (Haemochrom, Essen, Germany). 3 µL urine and 50 µL 2 mM substrate was incubated for 1 h at 37°C with or without aprotinin (final concentration 20 µg mL-1). Absorption was analyzed at 405 nm on a 96-well plate reader (Biotek EL800, VT, USA). The difference between the optical density with or without aprotinin reflected the specific activity of serine proteases. Values were expressed as relative units (1000\*Delta absorption @405 nm) after 1 h incubation.

Urinary creatinine was measured with a colorimetric Jaffé assay (Labor+Technik, Berlin, Germany), urinary protein concentration using the Bradford method (Bio-Rad Laboratories, Munich, Germany) and urinary sodium concentration with flame photometry (Eppendorf EFUX 5057, Hamburg, Germany). Both urinary protein and sodium concentration from voided urine samples were normalized to the urinary creatinine concentration. Plasma aldosterone was measured using an ELISA kit (IBL, Hamburg, Germany).

*Western blot from kidney tissue of mice and humans*

Half the kidney per mouse was sliced, and the cortex was dissected using a scalpel. Nephrectomy specimens were used as provided from the urology department. Homogenization was performed using a Dounce homogenisator in 1 ml lysis buffer containing 250 mM sucrose, 10 mM triethanolamine HCl, 1.6 mM ethanolamine and 0.5 EDTA at pH 7.4 (all Sigma)(27). During all preparation steps, aprotinin (40 µg mL-1) and a protease inhibitor cocktail (final concentration 0.1 x stock; mini-complete, Roche) was present to avoid ENaC cleavage *in vitro*. Homogenates were centrifuged at 1,000 g for removal of the nuclei. Subsequently, the supernatant was centrifuged at 20,000 g for 30 min at 4°C, and the resulting pellet containing plasma membranes was resuspended and diluted to a concentration of 5 mg· L-1. This yielded higher ENaC signals compared to centrifugation at 300,000 g. Samples were deglycosylated using PNGaseF according to the manufacturer´s instructions (NEB, Ipswich, USA). First, samples were denaturated with a glycoprotein denaturing buffer. Samples were then incubated with glycobuffer, NP-40 and PNGaseF for 1h at 37°C. Native samples without deglycosylation were boiled in Laemmli buffer at 70°C for 10 min. Subsequently, 20 µg of sample was loaded on an 8% (γ-ENaC) or 4-15% (α- and β-ENaC) polyacrylamide gel for electrophoresis. After transfer to nitrocellulose membranes, the blocked blots were incubated with the primary antibodies against the ENaC subunits overnight at 4°C after 1:1000 dilution in blocking buffer (Licor, Lincoln, USA). After detection of α-ENaC, the membranes were stripped for detection of β-ENaC. ENaC subunits were detected with a fluorescent secondary antibody labelled with IRDye 800CW or IRDye680RD and a fluorescence scanner (Licor Odyssey, Lincoln, USA). For loading control, total protein was measured using Revert Total Protein Stain (Licor, Lincoln, USA).

*Primary Antibodies*

Antibodies against murine α- and β-ENaC were raised in rabbits against the amino acids 45-68 for α-ENaC and 617-638 for β-ENaC using a commercial service (Pineda, Berlin, Germany). Anti-γ-ENaC was purchased from Stressmarq (SPC-405, Viktoria, Canada). This antibody had been raised in rabbits against the C-terminal amino acids 634–655 of γ-ENaC (Fig. 1A, C, E). All antibodies were based on the peptide sequences first introduced and validated by Masilamani et al. (28) and used by many other researchers in the field such as in the work of Frindt et al. (23). The antibodies against α- and β-ENaC had been affinity-purified while anti-γ-ENaC had been purified with protein A according to the manufacturer. To confirm that the observed bands are specific for α- and -ENaC, the primary antibody was blocked after preincubation with custom-made immunogenic peptides (20x excess by molarity) overnight at 4 °C. In addition, the antibodies were validated using lysates from *Xenopus oocytes* expressing murine αβγ-ENaC against lysates from sham-injected oocytes. Oocyte lysates were kindly provided by Dr. Alexandr Ilyaskin and Prof. Christoph Korbmacher (University of Erlangen, Germany).

*Statistical analysis*

Data are provided as means with SEM. Data were tested for normality with the Kolmogorov-Smirnov-Test, D'Agostino and Pearson omnibus normality test and Shapiro-Wilk-Test. Variances were tested using the Bartlett´s test for equal variances. Accordingly, data were tested for significance with parametric or nonparametric ANOVA followed by Dunnett´s, Dunn´s, or Tukey's Multiple Comparison post-test, paired or unpaired Student’s t-test, or Mann-Whitney U-test where applicable using GraphPad Prism 9, GraphPad Software (San Diego, CA, www.graphpad.com). To analyze more than 18 samples with Western blot, two or more gels were loaded each with 2-3 samples from all groups to be compared (Fig. 2/4). The signal of the samples of the healthy control group in each gel was averaged and set to 100%. The effect of the treatment on the band density was expressed relative to this value. The reported mass values of the bands are the arithmetic mean of the values obtained from all blots. A p value <0.05 at two-tailed testing was considered statistically significant. Densitometric analysis of the Western blots was done using Image Studio Version 3.1.4 and Empiria Studio Version 1.3.0.83 (Licor).

**Results**

*Deglycosylation of kidney lysates leads to a shift in the migration of all ENaC subunits and reveals the expression of γ-ENaC cleavage products*

Western blot analysis from murine kidney cortex samples for α-ENaC revealed three bands at 86, 25 and 21 kDa all of which disappeared after application of the immunogenic peptide (Fig. 1A, D). For β-ENaC, there was only a single band at 88 kDa corresponding to the full-length subunit which is not proteolytically processed (Fig. 1B, E). For γ-ENaC, there was a band at 82 kDa and below this a blurry signal extended until 69 kDa (Fig. 1C, F). Application of the immunogenic peptide blocked these signals.

Deglycosylation of the same samples using Peptide:N-glycosidase F (PNGaseF) led to characteristic shifts of the migration pattern of all ENaC subunits. α-ENaC migrated at 80, 23 and 19 kDa which could also be blocked by application of the immunogenic peptide (Fig. 1D). Migration of the band for full-length β-ENaC was shifted by 15 kDa to 73 kDa and unexpectedly, there appeared a second band at 102 kDa (Fig. 1E). The most significant change was obtained for γ-ENaC. As shown in Fig 1F, the migration pattern changed with three separate bands arising at 68, 57 and 50 kDa all of which were blocked by application of the immunogenic peptide (Fig 1F). The molecular mass of the obtained bands approximated the expected mass of full length (74kDa), proximally cleaved (58 kDa) and distally cleaved γ-ENaC (53 kDa, Fig. 1F). The obtained difference between full-length and proximally cleaved γ-ENaC was 11 kDa and that between proximally cleaved and fully cleaved γ-ENaC 7 kDa which was also in good agreement to the expected values (15 and 5 kDa, respectively). In conclusion, these results using a commercially available rabbit antibody against C-terminal γ-ENaC and deglycosylated kidney lysates reproduce the results from the work of Frindt et al. (23).

*Experimental NS in mice leads to increased expression of proximally and distally cleaved α- and γ-ENaC*

The above-mentioned results set the basis to investigate the presence of proteolytic activation of ENaC by cleavage of its α- and γ-subunit in experimental nephrotic syndrome in mice which has been a missing proof in our previous efforts (13, 15, 16, 18). After induction of nephrotic syndrome by doxorubicin, mice rapidly developed proteinuria along with proteasuria, urinary sodium retention and body weight gain (Fig. 2A-D). In a previous study, renal sodium retention as evidenced from urine collections for 24 h had been demonstrated in doxorubicin-injected nephrotic mice (16). Plasma aldosterone concentration was significantly increased in nephrotic mice (Fig. 2E). Similar changes were observed in mice with inducible podocin deficiency as published previously (15). In both models, for unknown reasons sodium retention and body weight gain was spontaneously reversed thereafter which has been earlier described for nephrotic rats (29) and seems to be characteristic for experimental nephrotic syndrome in rodents.

In doxorubicin-injected nephrotic mice, expression of ENaC subunits was analyzed at different time points using Western blot. In doxorubicin-injected nephrotic mice, expression of full length α-, β- and γ-ENaC was not appreciably altered on day 4 before the nephrotic phase with sodium retention and body weight gain, and during the nephrotic phase on day 8 whereas there was an increased expression of full length β- and γ-ENaC on day 14 after the nephrotic phase (Fig. 2F, H-N). The cleavage product of α-ENaC at 25 kDa was upregulated at day 8 and then normalized at day 14 (Fig. 2I). The cleavage product of α-ENaC at 21 kDa was of very low abundance and became slightly visible in nephrotic mice at day 8 (Fig 2F, J). The expression of the cleavage product of γ-ENaC at 57 kDa and most prominently that at 50 kDa was markedly increased on day 8 and 14, reaching statistical significance (Fig. 2M, N). On day 14 after reversal of sodium retention, expression of the γ-ENaC cleavage product at 50 kDa tended to decrease while the cleavage product at 57 kDa was still increased (Fig. 2N).

*Effect of the serine protease inhibitor aprotinin and the mineralocorticoid receptor antagonist canrenoate on proteolytic ENaC activation in experimental nephrotic syndrome*

To study the impact of urinary serine protease activity and aldosterone on sodium retention and the expression of the ENaC subunits in experimental nephrotic syndrome, we treated mice with the serine protease inhibitor aprotinin and the MR antagonist canrenoate, beginning on day 3 after induction. As shown in Fig. 2A-E, aprotinin did not reduce proteinuria, but inhibited urinary serine protease activity and prevented sodium retention while reducing hyperaldosteronism. Canrenoate did not have an effect on all of these parameters.

In WB analyses of doxorubicin-treated mice, aprotinin had no effect on the expression of the distal cleavage products of α- and γ-ENaC at 25 and 50 kDa, respectively, on day 4 (Fig. 2F-N). However, on day 8, aprotinin completely inhibited the upregulation of these cleavage products seen in untreated nephrotic mice. In contrast, treatment of nephrotic mice with canrenoate had no effect on the expression of these cleavage products.

To replicate these results in another nephrotic mouse model, we analyzed kidneys from mice with inducible podocin deficiency. As shown in Fig. 3, we could reproduce the increased expression of the distal cleavage products of α- and γ-ENaC at 25 and 50 kDa whereas the expression of the other bands was not altered. In aprotinin-treated nephrotic mice the expression of these cleavage products was normalized or even suppressed (Fig. 3).

*Proteolytic activation of ENaC can be stimulated in healthy mice in vivo*

To further validate the physiological significance of the obtained cleavage products of α- and γ-ENaC, we subjected healthy wild-type mice to maneuvers that we thought might also involve proteolytic activation of ENaC. For stimulation, we used a low salt intake, ENaC blockade using triamterene and administration of aldosterone and dexamethasone. For suppression we used a high salt intake and the MR antagonist canrenoate. Kidneys were harvested after 48 h of treatments to capture the changes in proteolytic processing that are expected to take place rapidly (30).

The 24-hour urinary sodium excretion under the various treatments is shown in Fig. 4A. The changes of 24-hour urinary sodium excretion were, if not compensated, paralleled by changes in the body weight (Fig. 4B) and mirrored by opposite changes of the plasma aldosterone concentrations (Fig. 4C).

Fig. 4D-L show the corresponding Western blot analyses and the densitometry from kidney cortex lysates of these mice. Compared to control conditions, treatments had no effect on the expression of full-length α- and β-ENaC (Fig. 4F, I) whereas the expression of full-length γ-ENaC tended to be higher in high salt-treated mice and lower in low-salt or aldosterone treated mice (Fig. 4J). Treatments also modulated proteolytic processing of α-ENaC and γ-ENaC. Stimulation of ENaC activity such as low salt intake or administration of aldosterone or triamterene increased the expression of proximally and distally cleaved α- and γ-ENaC (Fig. 4 G, H, K, L). In contrast, high salt intake led to reduced expression of cleaved α- and γ-ENaC. Treatment with dexamethasone or canrenoate had no appreciable effect on the expression of ENaC subunits.

To investigate whether stimulation of proteolytic ENaC processing by low salt diet involves the mineralocorticoid receptor, additional experiments were carried out with simultaneous canrenoate treatment of the mice. As shown in Fig. 5, canreonate treatment inhibited all effects induced by low salt diet such as upregulation of the distal cleavage products of α- and γ-ENaC at 25 and 50 kDa and supression of full-length γ-ENaC.

*Expression of γ-ENaC in kidney cortex from human nephrectomy specimens*

Finally, we aimed to reproduce the results with human kidney samples obtained from nephrectomy specimens (n=16). These patients (9 males / 7 females) had a median age of 69 years (interquartile range 65; 75) and a normal kidney function (median estimated GFR 83 mL min-1 1.73m-2 [68; 94]). According to urine dipstick testing, slight proteinuria (+) was present in 6 patients, while 8 patients had evidence of microhematuria. Western blot analysis from these samples for α-ENaC and β-ENaC using the same custom-made antibodies as used for mouse samples as well as antibodies from a commercial vendor did not result in plausible signals. However, for γ-ENaC there were three bands at 81, 63 and 48 kDa and deglycosylation using PNGaseF shifted the molecular mass to 68, 56 and 45 kDa. Application of the immunogenic peptide blocked all of the observed bands obtained from native and deglycosylated samples (Fig. 6A-C). As in mouse samples, the obtained bands were suggestive of full length, proximally and distally cleaved γ-ENaC, respectively, however, their molecular mass was lower than the expected mass calculated from the amino acid sequence. The difference between full-length and proximally cleaved γ-ENaC was 12 kDa and that between proximally and distally cleaved γ-ENaC 11 kDa which was in fair agreement to the expected values (15 and 5 kDa, respectively). The expression was highly variable in this small sample, spanning a factor of 10 (Fig. 6D).

**Discussion**

This study demonstrates the stimulation of proteolytic activation of α- and γ-ENaC in the mouse kidney under physiological conditions and most importantly in experimental nephrotic syndrome which was a missing proof in our previous efforts (13, 15, 16, 18). The detection and discrimination of cleavage products of γ-ENaC in Western blot was accomplished using another antibody as used previously and, most importantly, after deglycosylation of the kidney lysates which was recently reported by Frindt et al (23), simultaneously to our efforts (6, 31). One possible reason for the improved separation of the γ-ENaC cleavage products after deglycosylation might be related to the improved binding of SDS to the protein chain lacking the negatively charged N-glycosylated residues. This will lead to a more homogenous charge of both cleavage products so that the mass difference of 5 kDa between the products is better resolved during electrophoresis.

In the study of Frindt et al., γ-ENaC expression was investigated in both mice and rats as well as in ENaC-expressing FRT cells using a custom-made antibody directed against the same C-terminal sequence of γ-ENaC as that of the commercial antibody used in this study (23). In both rats and FRT cells, the authors found three bands migrating at 65, 55 and 50 kDa, which were interpreted to represent full-length, furin-cleaved and doubly cleaved γ-ENaC. The observed migration pattern of γ-ENaC in that study was identical to that observed in mouse kidneys of our study. Currently, proteolytic activation of ENaC is thought to occur sequentially by the intracellular serine protease furin cleaving α-ENaC twice and γ-ENaC once, leaving a final proteolytic event at the distal γ-ENaC (4, 8). This concept is mainly derived from heterologous expression systems such as the above mentioned FRT cells, mouse collecting duct cells (32) or oocytes from *Xenopus laevis* (6) which, however, might be confounded by endogenous serine proteases. Moreover, this concept suggests that proteolytic activation of α-ENaC is almost complete and cannot be increased further. Our data demonstrate that ENaC is proteolytically processed under physiological and nephrotic conditions at both the α- and γ-subunit *in vivo*. This suggests a complex action of different serine proteases or cascades thereof that must not necessarily involve furin. Therefore, instead of referring to furin- and doubly cleaved α- and γ-ENaC, we preferred to refer to these cleavage products as proximally and distally cleaved α- and γ-ENaC designated from the N-terminus of the subunit, respectively. This allows a neutral view on proteolytic events in both subunits. For α-ENaC using a N-terminal antibody, we noticed two cleavage products at 25 and 21 kDa, the latter of which was almost absent in the healthy state. For γ-ENaC using a C-terminal antibody, there were two cleavage products at 57 and 50 kDa. The data are compatible with proteolytic activation by removal of inhibitory tracts from both subunits (Fig. 7). However, the data could also be interpreted that proteolytic activation could also occur by a single distal cleavage at both subunits without complete removal of the inhibitory tract. This notion is particularly suggested by the results obtained with the N-terminal antibody against α-ENaC which might distinguish proximal and distal cleavage. In low-salt treated or nephrotic mice, we found a dominant expression of the larger cleavage product at 25 kDa which might indicate absence of proximal cleavage that would be indicated by the formation of the 21 kDa fragment. However, without identification of the sequence of these cleavage products, it is impossible to tell whether they truly represent proximal or distal cleavage or if one of them might be a functionally irrelevant cleavage product. For γ-ENaC, the distinction between proximal and distal cleavage is even impossible to delineate using the C-terminal antibody and, unfortunately, N-terminal antibodies or cleavage site-specific antibodies were not available. It is conceivable that a single cleavage event in both distal cleavage sites may be sufficient to activate ENaC, particularly at the γ-subunit which seems to have a very high impact on channel activation (9). In *Xenopus* oocytes expressing αβγ-ENaC, addition of serine proteases such as trypsin or chymotrypsin results in a strong increase of channel activity (6, 17, 18, 33). In single-channel recordings, it was found that addition of trypsin increased the number of open channels without an effect on channel expression (33, 34). This finding was explained by recruitment of near-silent channels that are expressed at the cell surface without previous proteolytic activation and results from a single proteolytic event by an aprotinin-sensitive extracellular protease (34).

The most robust finding of the Western blot analyses underlying figure 2-4 was the upregulation of the cleavage products of α- and γ-ENaC at 25 and 50 kDa, respectively, representing cleavage at the distal cleavage site of both subunits. Remarkably, there was no difference between proteolytic activation in nephrotic mice regardless of the model (doxorubicin or podocin deficiency) and also healthy mice. Up to now, the identity of the membrane-bound or soluble serine proteases that mediate proteolytic activation of ENaC remain unclear, which is particularly true for the nephrotic syndrome. A recent study by our group has characterized the abundance of all serine protease in healthy and nephrotic urine using proteomics (35). Whereas in health renally expressed serine proteases such as urokinase-type plasminogen activator (uPA) or prostasin were identified, the most dominant serine protease in nephrotic urine was plasminogen in addition to other proteases from the coagulation and complement system. However, we could demonstrate that mice deficient for uPA or plg still developed sodium retention in experimental nephrotic syndrome, indicating that the uPA-plasminogen axis seems not to be essential for proteolytic ENaC activation in the nephrotic mouse models (15, 16, 36, 37). Noteworthy, aprotinin was similarly effective in nephrotic plg-deficient mice (15). Another study identified the cysteine protease cathepsin B in the urine of podocin-deficient nephrotic mice that stimulated amiloride-sensitive currents in collecting duct cells (38). Further efforts are required to identify the relevant serine proteases both in health and in nephrotic syndrome.

We confirmed the stimulation of cleavage of γ-ENaC by a low salt diet as reported by Frindt et al. (23). In addition, we found increased cleavage at the α-subunit. Furthermore, our data with canrenoate demonstrate that ENaC cleavage under a low salt diet is mediated by aldosterone (Fig. 5). Currently, it is unclear how aldosterone exerts this MR-dependent effect and which proteases are involved. Candidates include membrane-bound serine proteases such as matriptase or prostasin. We previously reported that the scaffold function of prostasin is necessary for full proteolytic ENaC activation of the γ-subunit in healthy mice under ENaC blockade with triamterene (6). However, enzymatic activity of prostasin was not essential and prostasin expression was not increased despite excessive hyperaldosteronism in these mice, pointing to another serine protease responsible for proteolytic ENaC processing. In this and the previous study by Essigke et al. (6), ENaC blockade by triamterene has proven to be a strong stimulus of proteolytic ENaC activation. This could be related to the increased aldosterone plasma concentration detected soon after ENaC blockade (16), but also to the effects of reduced intracellular sodium concentration [Na+]i which is a strong stimulus of maturation and proteolytic ENaC processing in cultured cells (39). Along the lines, a low sodium diet could also induce proteolytic ENaC activation by reducing [Na+]i.

In contrast to the findings in healthy mice, MR blockade did not prevent proteolytic ENaC activation in nephrotic mice despite hyperaldosteronism, which is consistent with the notion that aberrantly filtered serine proteases are responsible for proteolytic ENaC activation in experimental nephrotic syndrome (Fig. 2). Accordingly, treatment of nephrotic mice with aprotinin prevented the formation of ENaC cleavage products and sodium retention. It is also remarkable that proteolytic ENaC activation is prevented by aprotinin in nephrotic but not in healthy mice (31), suggesting a different mechanism of proteolytic ENaC activation in healthy vs. nephrotic mice, possibly related to membrane-bound proteases vs. soluble proteases. Despite the strong evidence in favor of proteolytic ENaC activation by proteasuria, a possible role of hyperaldosteronism in sodium retention cannot be denied completely as hyperaldosteronism is expected to exert independent effects on ENaC expression and proteolytic activation. According to an integrative concept proposed by our group, overfill caused by proteasuria and underfill indicated by hyperaldosteronism are not mutually exclusive and might coexist, e.g. in murine doxorubicin-induced nephrotic syndrome or minimal change disease in children (11). As a consequence, both factors will act synergistically to stimulate ENaC activation and sodium retention.

In human nephrectomy samples, a very similar migration pattern of γ-ENaC and its cleavage products could be obtained as in mice. The shift of full-length γ-ENaC upon deglycosylation was 14 kDa and in agreement with the value reported by Zachar et al. who demonstrated a shift of 13-15 kDa (40). Interestingly, distally cleaved γ-ENaC at 48 kDa was detectable in native samples without deglycosylation and the expression was much higher than that of proximally cleaved γ-ENaC at 63 kDa which was barely detectable. These findings are consistent with the existence of proteolytic activation of γ-ENaC in humans which was also suggested by an immunohistochemical study using an antibody against distally cleaved γ-ENaC (41). In that study this cleavage product migrated between 42 and 45 kDa which is in good agreement to the values obtained in this study. Unfortunately, we were not able to demonstrate proteolytic activation of ENaC in human nephrotic syndrome due to the lack of tissue from such patients.

A limitation of the present study might be the analysis of whole-cell kidney lysates which cannot distinguish between intra- and extracellularly expressed ENaC. To isolate cell-surface expressed ENaC Frindt and Palmer developed an *in vivo* biotinylation method which has been successfully implemented in rats and mice (27, 42, 43). However, we think that our results regarding the expression of the distally cleaved γ-ENaC reflects channel activation *in vivo* as the final proteolysis of γ-ENaC is thought to take place in the extracellular space on the cell surface. Even after internalization, distally cleaved γ-ENaC can be recycled to the cell-surface (44). Therefore, the increased expression of distally cleaved γ-ENaC clearly points to stimulated sodium transport which could be induced by dietary and pharmacological maneuvers as well as by induction of proteasuria during nephrotic syndrome. It is remarkable that the cleavage product of γ-ENaC at 50 kDa indicating distal cleavage by an extracellular serine protease behaved as a biomarker paralleling renal sodium avidity. The modulation of the expression of this cleavage product also indicates that in the mouse kidney not all γ-ENaC subunits are cleaved and that there is a channel subpopulation that can be proteolytically activated. Noteworthy, concomitant proteolytic activation of α-ENaC indicated by the expression of the 25 kDa band was also prominent in these conditions.

**Perspectives and Significance**

This study presents the missing link between experimental nephrotic syndrome and proteolytic activation of ENaC *in vivo*. Inhibition of proteolytic ENaC activation could emerge as a new therapeutic approach in nephrotic syndrome.

**Figure 1. Expression of ENaC subunits in kidney cortex of healthy wild-type mice and the effect of deglycosylation**

**A-C** Localisation of the immunogenic sequences of the used antibodies against murine α-, β- and γ-ENaC. In α- and γ-ENaC, the proximal and distal cleavage sites (designated from the N-terminus, respectively) are depicted. The antibody against N-terminal α-ENaC is supposed to detect full-length α-ENaC at 79 kDa (699 aa) and two N-terminal fragments with a mass of 27 kDa (231 aa) and 24 kDa (205 aa). The antibody against C-terminal β-ENaC is supposed to detect full-length β-ENaC at 72 kDa (638 aa). The antibody against C-terminal γ-ENaC is supposed to detect full-length γ-ENaC at 74 kDa (655 aa) and C-terminal fragments with a mass of 58 kDa (512 aa) after proximal cleavage and at 53 kDa (469 aa) after distal cleavage, respectively. Mass values are calculated from the amino acid sequences (omitting any N-glycosylations).

**D-F** Western blot of kidney lysates for the expression of ENaC subunits. Deglycosylation of the samples with PNGase F induces a shift (white arrows) of the migration of the bands by 6 kDa for full-length and 2 kDa for proximally and distally cleaved α-ENaC, by 15 kDa for full-length β-ENaC and by 14 kDa for full-length γ-ENaC. Moreover, deglycosylation reveals two additional bands for γ-ENaC, representing proximally and distally cleaved γ-ENaC. Administration of the blocking peptide for α- and γ-ENaC attenuated all bands. Note that deglycosylation negatively impacted on the appearance of the bands representing the cleavage products of α-ENaC which was due to PNGase F (37 kDa).



**Figure 2. Expression of ENaC subunits in kidney cortex from doxorubicin-induced nephrotic mice with or without treatment with the serine protease inhibitor aprotinin (APR) or the mineralocorticoid receptor antagonist canrenoate (CAN)**

**A-D** Course of proteinuria, proteasuria, urinary sodium excretion and body weight.

**E** Plasma aldosterone concentration under different time points and treatments

**F** Western blot of the expression of α-, β- and γ-ENaC under different time points and treatments. Signal intensity is depicted using a color spectrum to achieve highest resolution between weak and strong bands. Western blot of the expression of α-, β-ENaC was performed using native samples. The analysis of the expression of γ-ENaC was done using PNGaseF treated samples. Blots were additionally stained for the total protein content (TPS).

**G** Color bars representing the signal intensity.

**H-N** Densitometric analysis of the WB results from two gels and n=5-6 per group normalized for the total protein signal of each lane. Values are expressed relative to the average of the healthy control group.

\* indicates significant difference to healthy mice, # indicates significant difference to untreated mice

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**Figure 3. Expression of ENaC subunits in kidney cortex from podocin-deficient nephrotic mice with or without treatment with the serine protease inhibitor aprotinin (APR)**

**A** Expression of the α-, β- and γ-ENaC as analyzed by Western blot from plasma membrane proteins. Western blot of the expression of α-, β-ENaC was performed using native samples. The analysis of the expression of γ-ENaC was done using PNGaseF treated samples. Blots were additionally stained for the total protein content (TPS).

**B** Color bars representing the signal intensity

**C-I** Densitometric analysis of the WB results (n=4 per group) normalized for the total protein signal of each lane.

\* indicates significant difference to healthy mice



**Figure 4. Effect of salt intake or drug treatment on expression of ENaC subunits in kidney cortex of mice**

**A-C** 24-hour urinary sodium excretion, body weight change and plasma aldosterone concentration under control (C), high (HS) or low salt (LS) diet as well as after treatment with aldosterone (ALDO), triamterene (TRIAM), dexamethasone (DEX) and canrenoate (CAN).

**D** Expression of the α-, β- and γ-ENaC as analyzed by Western blot from plasma membrane proteins. Western blot of the expression of α-, β-ENaC was performed using native samples. Expression of γ-ENaC was done using PNGaseF treated samples. Blots were additionally stained for the total protein content (TPS).

**E** Color bars representing the signal intensity

**F-L** Densitometric analysis of the WB results from two gels and n=5-6 per group normalized for the total protein signal of each lane. Values are expressed relative to the average of the healthy control group.

\* indicates significant difference to control mice

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**Figure 5. Effect of blockade of the mineralocorticoid receptor on expression of ENaC subunits in kidney cortex of mice under a low salt diet**

Mice were treated for 2 days with a low salt diet (LS) with or without canrenoate (+ CAN) in the drinking bottle (400 mg/L, corresponding to an intake of ~80 µg/g).

**A** Expression of the α-, β- and γ-ENaC analyzed by Western blot from plasma membrane proteins. Western blot of the expression of α-, β-ENaC was performed using native samples. Expression of γ-ENaC was done using PNGaseF treated samples. Blots were additionally stained for the total protein content (TPS).

**B** Color bars representing the signal intensity

**C-H** Densitometric analysis of the WB results normalized for the total protein signal of each lane. Values are expressed relative to the average of the healthy control group (C).

\* indicates significant difference to control mice



**Figure 6. Expression of γ-ENaC in human nephrectomy specimens and the effect of deglycosylation**

**A** Expression of the γ-subunit of ENaC from kidneys of 15 patients undergoing tumor nephrectomy. Western blots from plasma membrane proteins with or without treatment with PNGaseF for deglycosylation. Blots were additionally stained for the total protein content (TPS). The sample marked with the asterisk was used on both blot for normalization.

**B** Administration of the blocking peptide for γ-ENaC attenuated all bands. The alignment of the samples was rearranged as indicated by the dashed line.

**C** Color bars representing the signal intensity of each blot

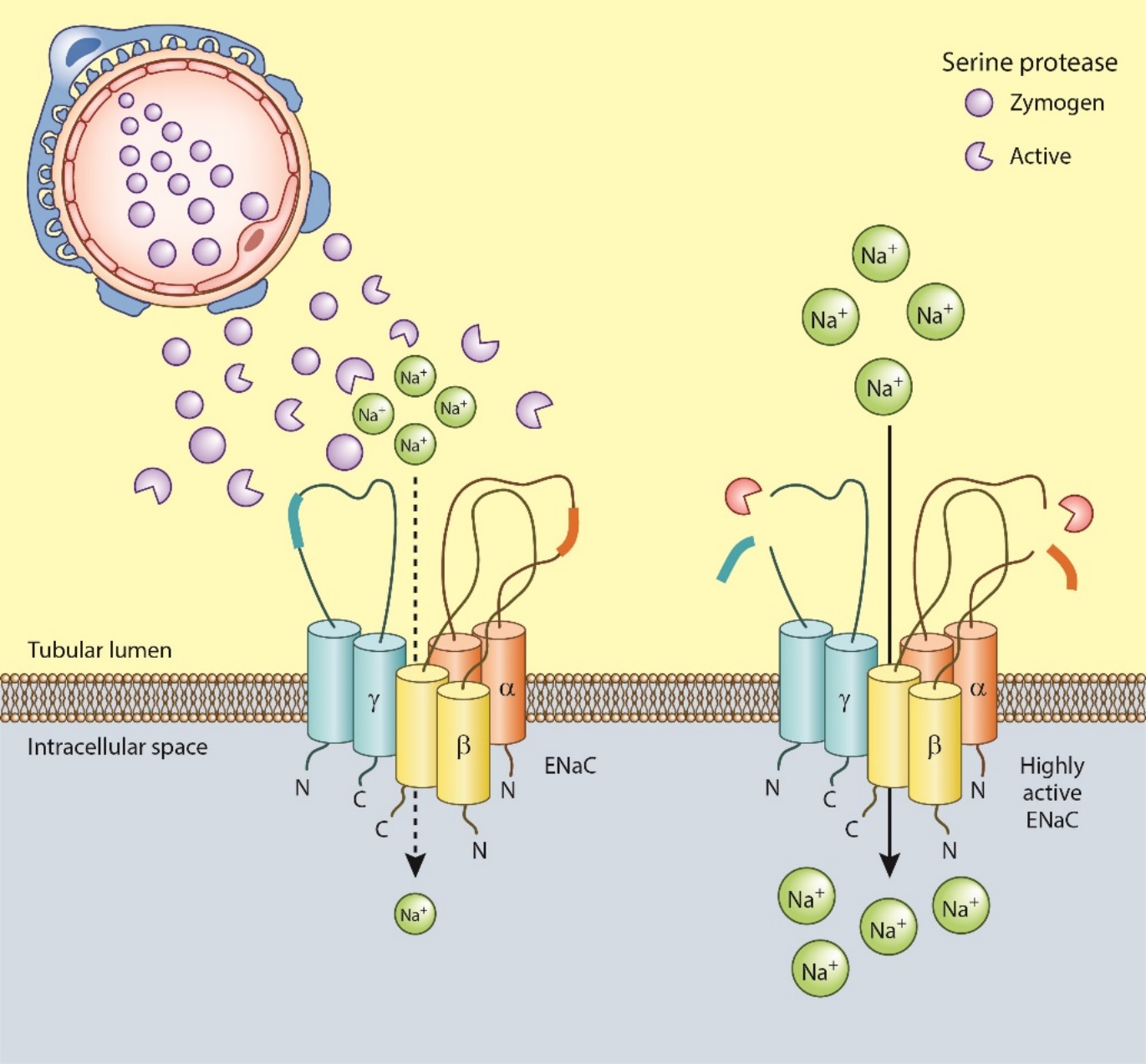
**D** Densitometric analysis of the expression of full length γ-ENaC and its cleavage product (48 kDa resp. 45 kDa) normalized for the total protein signal of each lane.

Note that the proximal cleavage product at 63 kDa resp. 45 kDa does not reach a sufficient signal to noise ratio and therefore was not analysed with densitometry.

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**Figure 7. Current model of proteolytic ENaC activation in experimental nephrotic syndrome**

Glomerular proteinuria leads to aberrant filtration of serine proteases which activate ENaC by cleavage at both the α- and γ-subunit, leading to removal of inhibitory tracts. However, the cleavage at the distal site of both α- and more importantly γ-ENaC might also be sufficient to maximally stimulate the channel. The identity of the essential serine protease(s) is not known, and there is the possibility that the α- and γ-subunit are cleaved by different serine proteases.



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