

# Proteolytic Activation of the Epithelial Sodium Channel in Nephrotic Syndrome by Proteasuria: Concept and Therapeutic Potential

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## Abstract

The epithelial sodium channel (ENaC) is expressed in the aldosterone-sensitive distal nephron, and it determines the final urinary sodium concentration. To ensure sodium preservation, ENaC-mediated sodium transport is redundantly regulated by several mechanisms. Among them, activation by proteases is a special feature that leads to the removal of inhibitory tracts from the  $\alpha$ - and  $\gamma$ -subunits, thus maximizing the channel open probability. Proteolytic ENaC activation by aberrantly filtered proteases or proteasuria has been implicated in the pathogenesis of edema formation and sodium retention in nephrotic syndrome. This concept was strongly supported by the finding that sodium retention in nephrotic mice could be prevented by treatment with either the ENaC blocker amiloride or the serine protease inhibitor aprotinin. In clinical practice, loop diuretics such as furosemide are most commonly used for the antiedematous treatment of patients with nephrotic syndrome. ENaC blockade using amiloride or triamterene could serve as an alternative with better efficacy; however, clinical data are scarce, and ENaC blockade poses the threat of hyperkalemia. This review starts with a case vignette that highlights the therapeutic potential of pharmacological ENaC blockade in treatment-resistant nephrotic edema and continues with discussing the concept of proteolytic ENaC activation in nephrotic syndrome and its therapeutic potential.

**Keywords:** ENaC, nephrotic syndrome, proteasuria, amiloride

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## Case Vignette

A 56-year-old male patient presented to our international unit at the University Hospital Tübingen, Germany, for severe treatment-refractory nephrotic syndrome due to primary membranous glomerulonephritis. The disease was diagnosed 2 years ago after manifestation of edema. Biopsy was performed, and he was treated with  $4 \times 375$  mg/m<sup>2</sup> of rituximab, which showed no effect on proteinuria. Then, he was transiently treated with tacrolimus, which had to be discontinued due to worsening of kidney function. For the treatment of edema, he was prescribed with  $1 \times 40$  mg furosemide per day. In addition, he was administered valsartan  $1 \times 160$  mg. On examination, he had massive peripheral edema, and his blood pressure was elevated to 170/98 mm Hg. Bioimpedance spectroscopy revealed an overhydration of

13 L. Laboratory analyses revealed that the plasma creatinine concentration was 2.5 mg/dL, the estimated glomerular filtration rate (GFR) was 27 mL/min/1.73 m<sup>2</sup>, the plasma Na<sup>+</sup> concentration was 139 mM, and the plasma K<sup>+</sup> concentration was 4.5 mM. Proteinuria was very high at 15.4 g/g creatinine, of which albuminuria accounted for 10.0 g/g creatinine and was accompanied by protein and albumin depletion in the plasma (total protein level, 4.6 g/dL and plasma albumin level, 1.6 g/dL). The antibody titer against PLA2-receptor was 1:640, which indicated active primary membranous glomerulonephritis.

Assuming that the overhydration was presumably mediated by proteolytic activation of the epithelial sodium channel (ENaC), the diuretic treatment regimen was switched to a fixed-dose combination of the ENaC



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blocker amiloride (5 mg) with 50 mg hydrochlorothiazide (Amiloretik® 5/50), while discontinuing furosemide. Within the subsequent 4 weeks, the patient lost 16 kg of body weight, and edema was almost completely resolved. This was accompanied by a reduction in blood pressure to values of <120/80 mm Hg. After reducing the dose of Amiloretik® by 50%, the body weight increased again; so, the dose was adjusted again to one tablet. Under Amiloretik® treatment, the kidney function was stable (plasma creatinine concentration, 1.9 mg/dL; estimated GFR, 37 mL/min/1.73 m<sup>2</sup>) and proteinuria decreased to 9 g/g creatinine, which allowed mild recovery of total plasma protein and plasma albumin levels (increased to 4.8 and 1.8 g/dL, respectively). The plasma Na<sup>+</sup> concentration was 144 mM, and the plasma K<sup>+</sup> concentration was 4.0 mM. In addition to diuretic treatment, the patient received another course of 2×1 g of rituximab. After 3 months of treatment, the patient returned to his home country for further follow-up. He was advised to continue Amiloretik® to maintain his current weight and prevent sodium retention due to persisting proteinuria.

### Pathophysiology of Sodium Retention in Nephrotic Syndrome

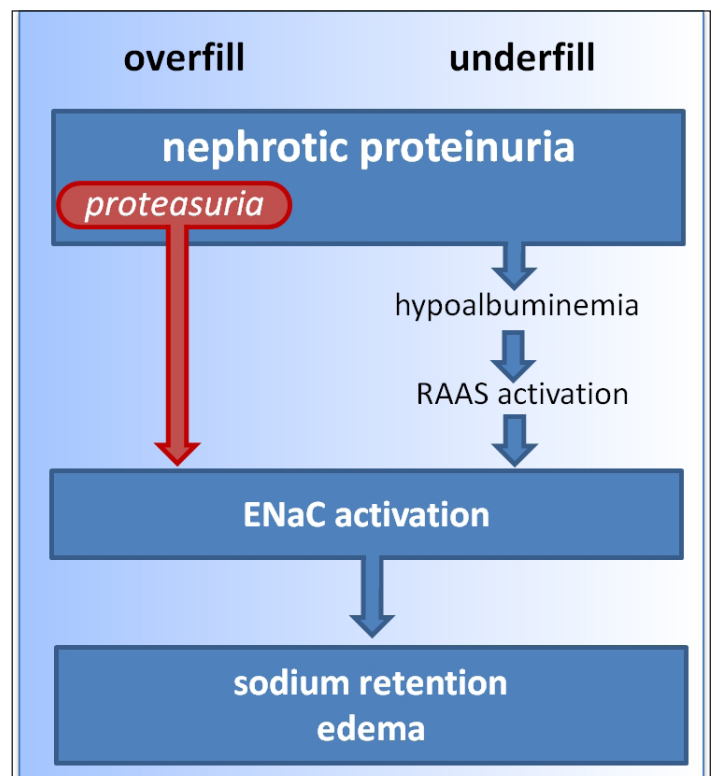
Nephrotic syndrome is the most extreme manifestation of proteinuric kidney disease and is characterized by high proteinuria and expansion of the extracellular volume due to renal sodium retention. Clinically, patients develop edema in the lower extremities and typically in the eyelids after overnight rest. In addition, fluid may accumulate in the pleura, peritoneum, and rarely, the pericardium. Two opposing mechanisms, namely, the underfill and the overfill theories have been put forward to explain sodium retention (1, 2). According to the underfill theory formulated by Epstein about 100 years ago (3), hypoalbuminemia due to urinary protein losses leads to intravascular volume depletion or underfilling that provokes secondary sodium retention by the kidneys to restore the intravascular volume. This is mainly mediated by a stimulated renin–angiotensin–aldosterone-system (RAAS) that involves the stimulation of ENaC by aldosterone (1, 4). In contrast, the overfill theory, first formulated by Meltzer et al. (5) in 1979, states that sodium retention is primarily caused by the diseased kidney due to tubular defect that leads to sodium avidity without any signs of volume depletion or a stimulated RAAS (6-8).

So far, the exact mechanisms that explain sodium retention by nephrotic kidneys remain unclear. Over the last few years, proteolytic activation of the ENaC by aberrantly filtered active serine proteases or proteasuria has been put forward as a mechanism that could explain sodium retention (9). There is strong evidence that proteasuria can be considered as a key mechanism of sodium retention in patients with nephrotic syndrome.

In an attempt to reconcile the apparent discrepancy between the underfill and overfill theories, we developed a scheme that integrates both theories (Figure 1). According to this scheme, underfill and overfill represent the two ends of a continuous spectrum of ENaC-mediated sodium retention. This implies

that these theories are not mutually exclusive and can coexist in the same patient (9). According to the reconciled scheme, proteasuria as a part of nephrotic proteinuria leads to sodium retention by direct endoluminal ENaC activation in agreement with the overfill theory. When proteinuria is sufficient to induce hypoalbuminemia and underfill, ENaC is additionally activated by RAAS. Therefore, underfill can superimpose on any patient with nephrotic syndrome and edema primarily due to proteasuria and overfill. Clinically, patients with nephrotic syndrome due to minimal change disease often have superimposed underfill, particularly pediatric patients.

It seems reasonable to assume that the quality and quantity of excreted serine proteases can make a difference with regard to proteolytic ENaC activation and sodium retention. Urinary composition can vary according to the underlying glomerular disease (diabetic nephropathy or immunological disease or others) and the extent of podocyte damage (podocytopathy). Although proteasuria follows overall proteinuria and albuminuria, variations in the excretion of serine proteases could explain the occurrence of high proteinuria without sodium retention.



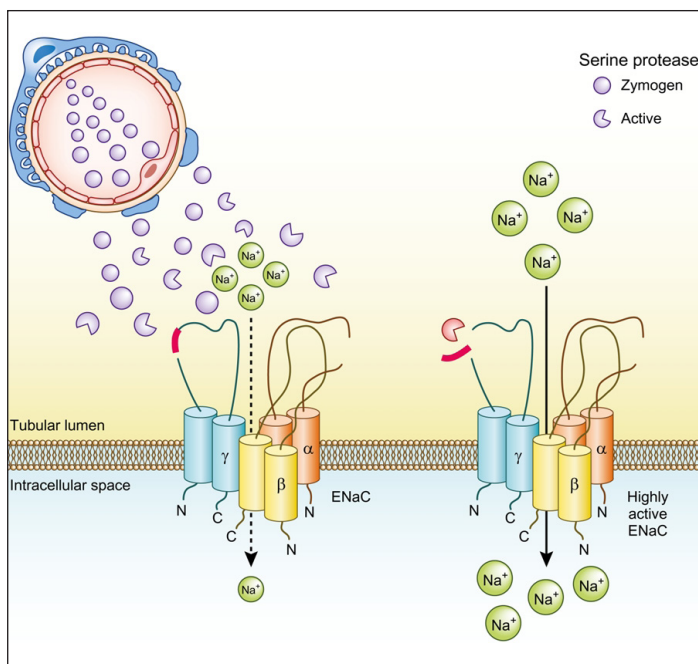
**Figure 1.** Sodium retention in nephrotic syndrome. Under- and overfill theories represent two ends of a continuous spectrum and some patients may be situated in between. Proteasuria as part of nephrotic proteinuria leads to sodium retention by direct endoluminal ENaC activation in agreement with the overfill theory. When proteinuria is sufficient to induce hypoalbuminemia and underfill, ENaC is additionally activated by the RAAS. There is a continuous relationship between both, and underfill can superimpose on any nephrotic patient with edema primarily due to proteasuria and overfill. Analogous to (9).

### Proteolytic ENaC Activation in Nephrotic Syndrome

Animal experiments have indicated that the distal tubule expressing ENaC is the site of sodium retention in nephrotic syndrome (10, 11). This is supported by the finding that treatment with the ENaC blocker amiloride prevented volume retention in both nephrotic rats and mice (10, 12, 13). Further data demonstrated that ENaC activation in experimental nephrotic syndrome is not dependent on the action of mineralocorticoid receptor (13-15). Among the complex and abundant regulatory mechanisms for ENaC including aldosterone (16, 17), a special feature of ENaC is its complex posttranslational regulation by serine proteases. This leads to endoluminal channel activation by the cleavage of specific sites in the extracellular domains of the  $\alpha$  and  $\gamma$ -subunits (18-20). Proteolytic cleavage at three sites (two in  $\alpha$ -ENaC and one in  $\gamma$ -ENaC) occurs by the intracellular serine protease furin during maturation before the channel reaches the plasma membrane (21). A second cleavage event in  $\gamma$ -ENaC is mediated by extracellular serine proteases distal to the furin site at specific cleavage sites, thereby leading to the release of a peptide (43 amino acids) in length and maximizes the channel open probability (Figure 2). Serine proteases involved in the physiological regulation of ENaC, are the membrane-anchored prostaticin (22), and soluble tissue kallikreins (23).

Under the pathophysiological conditions of nephrotic syndrome, serine proteases with large molecular weight are aberrantly filtered from the plasma and can mediate the second cleavage event in  $\gamma$ -ENaC, thereby leading to full channel activa-

tion. The relevance of this mechanism has been recently shown in wild-type mice with experimental nephrotic syndrome that were protected from proteolytic ENaC activation and sodium retention by treatment with the broad-spectrum serine protease inhibitor aprotinin (13). So far, the exact identity of the essential serine protease(s) is unknown, and it continues to be the focus of current research of the authors' group. Previously, Svenningsen et al. (24) proposed that in nephrotic syndrome, ENaC might be proteolytically activated by the serine protease plasmin after the aberrant filtration of plasminogen (Plg) from damaged glomeruli and its conversion to plasmin by the tubular urokinase-type plasminogen activator (uPA). Since then, the concept of proteolytic ENaC activation by aberrantly filtered plasminogen has been embraced as an attractive explanation for sodium retention in nephrotic syndrome (25-27); however, there was a lack of definitive proof from a knockout model (28). This gap of knowledge was recently closed by the authors' group using mice deficient in either uPA (uPA<sup>-/-</sup>) or plasminogen (plg<sup>-/-</sup>) that were studied for sodium retention after the induction of experimental nephrotic syndrome (12, 29). Contrary to the above-mentioned concept of uPA/Plg-activating ENaC in nephrotic syndrome, nephrotic uPA<sup>-/-</sup> and plg<sup>-/-</sup> mice were not protected from sodium retention compared with wild-type mice; however, both had almost no or absent urinary plasmin activity. The results of these studies strongly argued against the essential role of uPA/plg in mediating the proteolytic ENaC activation in experimental nephrotic syndrome. Currently, there is an ongoing research on this topic, and the exact identity of the essential serine protease(s) or cascade remains to be elucidated.



**Figure 2.** Model of ENaC-mediated sodium retention in nephrotic syndrome. In glomerular disease, increased permeability for proteins larger than albumin leads to the excretion of aprotinin-sensitive serine proteases that, once active, may activate ENaC by cleavage at its  $\gamma$ -subunit, thereby releasing an inhibitory peptide (red) and stimulating Na<sup>+</sup> resorption. Analogous to (9).

Proteasuria in nephrotic syndrome reflects the translocation and excretion of proteases from the plasma compartment into the urine. Since plasma proteases are very similar, if not identical, in rodents and humans, there is no reason to assume that there are fundamental differences in the role of proteasuria in promoting proteolytic ENaC activation between these species. Therefore, the results from the rodent models of nephrotic syndrome should also be valid for humans. For instance, patients with acute nephrotic syndrome exhibited an increased urinary excretion of aprotinin-sensitive proteases as those observed in the nephrotic mice (13). However, it must be underscored that there is only weak evidence from human studies to support a role for proteolytic activation of ENaC in nephrotic syndrome. The most specific evidence in favor of proteolytic ENaC activation in humans originates from a study that involved proteinuric patients who underwent nephrectomy for kidney cancer (30). Using antibodies specific for differentially cleaved  $\gamma$ -ENaC, the authors demonstrated staining for furin-mediated cleavage under physiologic conditions and positive staining for a second-hit processing of  $\gamma$ -ENaC in histological nephrectomy specimens.

### Therapeutic Potential of ENaC Blockade in Nephrotic Syndrome

In nephrotic syndrome, ENaC inhibition might be a good choice as highlighted in the case vignette above. Remarkably, the

presented patient had persistent edema despite ongoing diuretic treatment with furosemide. Earlier reports have shown a reduced efficacy of furosemide in patients with nephrotic syndrome, which is indicated by a lower urinary sodium-to-urinary furosemide ratio and a shift of the dose—response curve to the right with a lower maximum value in patients with nephrotic syndrome (31, 32). Importantly, the blunted effect of furosemide was not related to the binding to tubular proteins or altered pharmacokinetics. Proteolytic ENaC activation in nephrotic syndrome predicts that the dose—response curve would be shifted to the left. In accordance with this observation, the response to a single dose of amiloride is enhanced in nephrotic mice compared with healthy mice (Figure 3) (12, 33). Moreover, daily treatment of nephrotic mice with amiloride prevents sodium retention after the onset of proteinuria (Figure 3) (12, 13). This effect was achieved by a single dose per day by inducing natriuresis for several hours due the relatively long half-life of amiloride that ranges between 6 and 9 hours (12, 34). Triamterene is another ENaC pore blocker, which is very similar to amiloride, and can be used at an equivalent dose (mg) that is tenfold higher (35). As with amiloride, it is marketed only as a fixed-dose combination with hydrochlorothiazide (50 mg/50 mg).

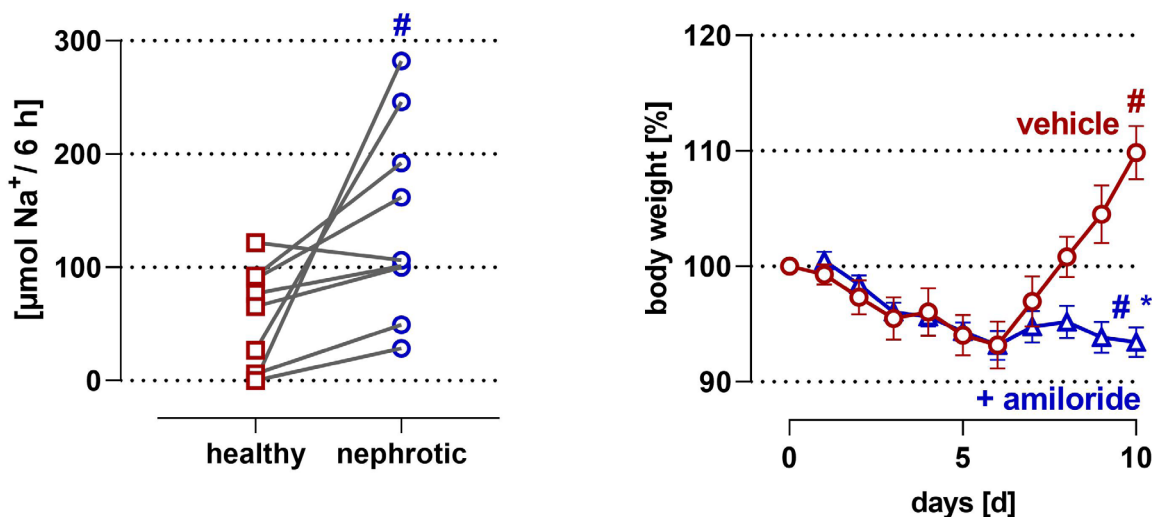
In addition to the regulation of activity, ENaC membrane expression can be suppressed using the mineralocorticoid antagonists (MRAs) spironolactone or eplerenone. In addition, newer drugs of this class are being tested in trials (36). MRAs prevent the genomic upregulation of ENaC in high aldosterone or low-salt intake conditions (37). However, ENaC that is present in the

membrane may be activated by proteasuria; therefore, MRAs are expected to be less efficient in the prevention of ENaC-mediated sodium retention compared with direct ENaC inhibition by amiloride or triamterene. In keeping with this observation, nephrotic animals with lack of aldosterone after adrenalectomy (14) or aldosterone-resistance [deficiency of the serum-and-glucocorticoid kinase 1, (15)] developed sodium retention; however, there was one-third reduction in the maximal body weight of SGK1 knockout mice.

The animal data clearly suggest that ENaC blockade has a high therapeutic potential to treat sodium retention in patients with nephrotic syndrome. However, one must bear in mind that ENaC activity is essential for potassium secretion and kaliuresis. This was most impressively illustrated in mice with an inducible deletion of  $\gamma$ ENaC in adulthood that led to the development of fatal hyperkalemia and acidosis within a few days after the induction of ENaC deletion and could only be rescued by a potassium-free diet (38). The fear of inducing life-threatening hyperkalemia certainly limits amiloride treatment in clinical practice, particularly in patients with kidney failure (39-42). Therefore, ENaC inhibition (or MRAs) cannot be recommended in patients with a reduced GFR and/or hyperkalemia.

### Clinical Evidence of ENaC Blockade

Although available since the 1960s, ENaC blockers such as amiloride or triamterene are underutilized in clinical nephrology. One reason is the fact that there is a lack of controlled clinical studies that show an improved efficacy of amiloride over other diuretic regimens such as the most commonly used loop diuretic-



**Figure 3.** Stimulation of ENaC-mediated sodium transport and prevention of sodium retention by the ENaC blocker amiloride in nephrotic mice. Left: In a sequential experimental design, amiloride-sensitive natriuresis was studied in the wild-type mice before and after the induction of experimental nephrotic syndrome by injecting the ENaC blocker amiloride. Compared to the healthy state, amiloride induced significantly higher natriuresis during six hours in the nephrotic state. Values were corrected for natriuresis after vehicle injection.

Right: After the induction of experimental nephrotic syndrome on day 0 by doxorubicin, daily doses of amiloride prevented the body weight (bw) gain in nephrotic mice as compared to the vehicle-treated mice. Data are adopted from (12, 33).



ics (furosemide). Similar to the case presented above, Hinrichs et al. (43) reported a case of a patient with severe hypertension in whom the addition of amiloride to a regimen containing RAAS blockade and a loop diuretic disrupted the refractory edematous state. Two other recent case reports describe the efficacy of the ENaC blocker triamterene in resolving nephrotic edema (44, 45). In diabetic patients with nephropathy and proteinuria, a single oral amiloride dose failed to induce an enhanced natriuresis compared with that in diabetic patients without nephropathy (46). However, proteinuria was not in the nephrotic range (1.1 g vs. 0.1 g per day). In a double-blind randomized trial with a crossover design, amiloride was compared to hydrochlorothiazide in nine patients with proteinuria and type 2 diabetes (47). Both diuretics were equally effective in lowering the blood pressure. However, two patients treated with amiloride developed hyperkalemia and acute kidney injury. It must be added that the amiloride was administered at a high dose of 20 mg per day. Like with any diuretic, potent saluresis can impose acute prerenal failure, particularly in the presence of nephrotic syndrome with underfill. Currently, more controlled studies are needed to support the use of amiloride in the treatment of nephrotic edema.

Pediatric patients with nephrotic syndrome are often treated with a combination of spironolactone with furosemide (48). In adult patients with chronic kidney disease, a meta-analysis found that low-dose spironolactone treatment (mostly 25 mg) reduced proteinuria by 30%-40% and also lowered blood pressure (49). It is noteworthy that these effects were achieved on top of a renin-angiotensin blockade. The incidence of severe hyperkalemia of >6.0 mM was not significantly increased, whereas GFR was reduced in some patients (49). It is likely that reduction of ENaC-mediated sodium retention by spironolactone might have accounted for these effects.

### Targeting Proteasuria as a New Therapeutic Strategy in Nephrotic Syndrome

The prevention of sodium retention in experimental nephrotic syndrome by the serine protease inhibitor aprotinin is a proof of the principle that the inhibition of proteasuria could be a new therapeutic approach in patients with nephrotic syndrome (13). In comparison to ENaC blockade with amiloride, the inhibition of excessive urinary serine protease activity could protect from ENaC overactivation without interfering with basal ENaC function. However, before the translation of inhibition of proteasuria to clinical medicine, more research must be conducted to reveal the exact identity of the pathophysiologically relevant proteases. This would enable the improved targeting of those and would avoid the use of broad-spectrum protease inhibitors such as aprotinin or camostat (50, 51). These drugs have the potential to exert negative effects on the other proteases of the plasma compartment that serve important physiological functions. Currently, aprotinin is not available since it has been withdrawn from the market in 2008 owing to side effects, wherein kidney events have been described (52).

### CONCLUSION

The activation of ENaC by aberrantly filtered active plasma proteases or proteasuria seems to be a key mechanism of sodium retention in nephrotic syndrome. The pharmacological inhibition of ENaC by diuretics such as amiloride or triamterene promises to be a potent approach to treat nephrotic edema, particularly in case of a treatment-refractory state. However, there is a lack of high-quality evidence from controlled trials for supporting the ENaC inhibition in nephrotic syndrome. One must be cautious to avoid hyperkalemia and acute prerenal failure during ENaC inhibition that needs a close follow-up of the patient and titration of the dose. Future research will possibly identify the essential components of proteasuria that could be inhibited specifically without interfering with basal ENaC function.

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