Aprotinin prevents proteolytic epithelial sodium channel (ENaC) activation and volume retention in nephrotic syndrome

Bernhard N. Bohnert[1,2,3,7], Martina Menacher[1,7], Andrea Janessa[1], Matthias Wörn[1], Anja Schork[1,2,3], Sophie Daiminger[1], Hubert Kalbacher[4], Hans-Ulrich Häring[1,2,3], Christoph Daniel[5], Kerstin Amann[5], Florian Sure[6], Marko Bertog[6], Silke Haerteis[6], Christoph Korbmacher[6] and Ferruh Artunc[1,2,3]

[1]*Department of Internal Medicine, Division of Endocrinology, Diabetology, Vascular Disease, Nephrology and Clinical Chemistry, University Hospital Tübingen, Tübingen, Germany;* [2]*Institute of Diabetes Research and Metabolic Diseases (IDM) of the Helmholtz Center Munich at the Eberhards Karls University of Tuebingen, Tübingen, Germany;* [3]*German Center for Diabetes Research (DZD) at the Eberhards Karls University of Tuebingen, Tübingen, Germany;* [4]*Interfaculty Institute of Biochemistry, Eberhards Karls University of Tuebingen, Tübingen, Germany;* [5]*Institute of Pathology, Friedrich-Alexander University Erlangen-Nürnberg (FAU), Erlangen, Germany; and* [6]*Institute of Cellular and Molecular Physiology, Friedrich-Alexander University Erlangen-Nürnberg (FAU), Erlangen, Germany*

Volume retention in nephrotic syndrome has been linked to activation of the epithelial sodium channel (ENaC) by proteolysis of its γ-subunit following urinary excretion of serine proteases such as plasmin. Here we tested whether pharmacological inhibition of urinary serine protease activity might protect from ENaC activation and volume retention in nephrotic syndrome. Urine from both nephrotic mice (induced by doxorubicin injection) and nephrotic patients exhibited high aprotinin-sensitive serine protease activity. Treatment of nephrotic mice with the serine protease inhibitor aprotinin by means of subcutaneous sustained-release pellets normalized urinary serine protease activity and prevented sodium retention, as did treatment with the ENaC inhibitor amiloride. In the kidney cortex from nephrotic mice, immunofluorescence revealed increased apical γ-ENaC staining, normalized by aprotinin treatment. In *Xenopus laevis* oocytes heterologously expressing murine ENaC, aprotinin had no direct inhibitory effect on channel activity but prevented proteolytic channel activation. Thus, our study shows that volume retention in experimental nephrotic syndrome is related to proteolytic ENaC activation by proteasuria and can be prevented by treatment with aprotinin. Hence, inhibition of urinary serine protease activity might become a therapeutic approach to treat patients with nephrotic-range proteinuria.

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Correspondence: *Ferruh Artunc, Department of Internal Medicine, Division of Endocrinology, Diabetology, Angiology and Nephrology, University Hospital of Tübingen, Otfried-Mueller-Strasse 10, 72076 Tübingen, Germany. E-mail: ferruh.artunc@med.uni-tuebingen.de*

[7]MM and BNB share first authorship.

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Nephrotic syndrome is characterized by proteinuria, edema, hypoalbuminemia, and hyperlipidemia; it is the most severe manifestation of proteinuric renal disease. The pathogenesis of edema formation in nephrotic syndrome remains debatable; both underfill and overfill theories have been proposed.[1] Studies in nephrotic rats have suggested that the distal tubule expressing the epithelial sodium channel (ENaC) is the site of sodium retention.[2] In addition to regulation by the mineralocorticoid hormone aldosterone,[3] a special feature of ENaC is its complex post-translational regulation by proteases, which cleave specific sites in the extracellular domains of the α- and γ-subunits.[4] Recent evidence suggests that proteolytic ENaC activation by urinary proteases may contribute to sodium retention in nephrotic syndrome.[5,6] Protein-rich urine samples from both rats[7] and patients[8] who have nephrotic syndrome have been found to activate ENaC currents *in vitro*, presumably as a result of proteolysis of the γ-subunit of ENaC by serine proteases excreted in the urine.[9] Currently, the serine protease plasmin has been implicated in promoting ENaC activation and volume retention during proteinuria.[7,10] Plasmin cleaves the γ-subunit of ENaC at a distinct site and induces a robust increase in ENaC currents *in vitro*.[10,11] In humans, a close correlation of urinary plasmin excretion with proteinuria has been found in preeclampsia[8] and diabetic nephropathy.[12,13] We have found a strong association of plasminuria with overhydration, as determined from bioimpedance spectroscopy in a large sample of patients with chronic kidney disease (CKD).[14]

Targeting urinary plasmin activity by pharmacologic inhibitors may be an interesting therapeutic approach, given the putative role of plasminuria in mediating volume retention and possibly podocyte injury.[15] Plasmin can be inhibited by the serine protease inhibitor aprotinin, which competitively

interacts with its catalytic site, and by tranexamic acid, which inhibits plasminogen conversion into plasmin after occupying lysine-binding sites at the kringle domains of plasminogen. Camostat is an orally available serine protease inhibitor that is active against plasmin; it was originally developed in Japan for treatment of pancreatitis. Anecdotal reports suggest that camostat has beneficial effects in patients who have nephrotic syndrome.[16,17] In addition, camostat has been reported to reduce blood pressure in salt-sensitive hypertensive rats, probably by preventing proteolytic ENaC activation, as suggested by the detection of partially but not fully cleaved γENaC.[18,19] The inhibition of urinary plasmin activity requires the availability of these drugs in the tubular fluid. Aprotinin, as a small polypeptide with 58 amino acids (6.5 kDa), and tranexamic acid, as a water-soluble organic acid, are eliminated exclusively via glomerular filtration. Camostat is rapidly degraded by plasmatic esterases into 2 metabolites that are excreted in the urine, one of which has preserved inhibitory activity.[20] Therefore, these drugs, which have negligible plasma protein binding, can reach therapeutically relevant concentrations in the tubular fluid, making them candidates for a pharmacologic intervention to inhibit tubular protease activity in experimental nephrotic syndrome. Rats that have experimental heart failure and developed plasminuria, aprotinin treatment resulted in a blunted response to ENaC blockade by benzamil, suggestive of reduced ENaC activity.[21]

In this study, we tested the hypothesis that pharmacologic inhibition of urinary serine protease activity *in vivo* may reduce volume retention in nephrotic mice. Therefore, we used the inhibitors aprotinin, camostat, and tranexamic acid and tested their effect on volume retention in a model of experimental nephrotic syndrome developed by our group.[22,23] We found that aprotinin treatment abolishes volume retention by preventing proteolytic ENaC activation.

RESULTS

Experimental nephrotic syndrome in mice features all the hallmarks of human nephrotic syndrome

Following a single injection of doxorubicin, mice with proteinuria exceeding 140 mg per mg creatinine developed nephrotic syndrome characterized by hypoalbuminemia (Figure 1a), body weight (BW) gain with ascites (Figure 1b; Supplementary Figure S1A), and hyperlipidemia evidenced by lipemic plasma (Supplementary Figure S1B). Although food and fluid intake remained fairly constant, except for a modest decrease during the initial days after doxorubicin treatment (Supplementary Figure S1C), urinary sodium/creatinine and urinary Na/K ratios dropped dramatically during the first 10 days, indicating that sodium retention caused the BW gain (Figure 1b; Supplementary Figure S1D). Urinary activity to cleave the amide bond of the chromogenic substrate S-2251 increased and was paralleled by a 1000-fold increase in urinary plasmin(ogen) excretion, as measured by both ELISA and a decrease in plasma plasmin(ogen) concentration (Figure 1c).

Urinary amidolytic activity was inhibited competitively *in vitro* by the serine protease inhibitors aprotinin (50% inhibitory concentration [IC_{50}] 56 [23; 137] nM) and camostat (IC_{50} 2.4 [1.1; 4.9] μM) but not by tranexamic acid (Figure 1d). Urinary amidolytic activity was also sensitive to inhibition by antiplasmin (IC_{50} 51 [40; 66] nM), indicating that plasmin activity accounted for the vast proportion of urinary amidolytic activity against S-2251. Similar inhibition curves were obtained when amidolytic activity of purified plasmin was analyzed (Supplementary Figure S2). The IC_{50} values were not significantly different (Supplementary Table S1), except for those for camostat (IC_{50} 0.4 [0.4; 0.5] μM, $P = 0.0003$).

Patients who have nephrotic syndrome display aprotinin-sensitive urinary serine protease activity

In 10 patients with acute nephrotic syndrome and nephrotic-range proteinuria (as characterized in Supplementary Table S2 and by Schork *et al.*[14]), we detected strong urinary amidolytic activity that was almost absent in 15 healthy subjects (Figure 2a and b; Supplementary Table S2). In nephrotic patients, this activity was largely sensitive to aprotinin and accounted for 73% ± 7% of total activity, whereas this proportion was only 10% ± 3% in healthy subjects ($P < 0.0001$). The increased urinary amidolytic activity in the nephrotic patients paralleled the expansion of extracellular volume, as quantified by bioimpedance spectroscopy (Figure 2c; Supplementary Table S2). These findings confirm that nephrotic syndrome in both humans and mice leads to excretion of urinary serine proteases that might be involved in volume retention.

Treatment of nephrotic mice with aprotinin prevents volume retention

To test the effect of pharmacologic inhibition of urinary serine protease activity *in vivo,* we treated 3 groups of nephrotic mice with aprotinin, camostat, and tranexamic acid, respectively, delivered by sustained-release pellets. After inducing nephrotic syndrome, we implanted the pellets subcutaneously on day 3 and followed nephrotic mice until day 10. Nephrotic mice given placebo pellets served as controls. After induction of nephrotic syndrome, the level of proteinuria (Figure 3a) as well as food and fluid intake was similar in all treatment groups (Supplementary Figure S3A and B). Urinary amidolytic activity was suppressed by aprotinin but not by camostat or tranexamic acid (Figure 3b). Although camostat and tranexamic acid–treated nephrotic mice had BW gain similar to that of placebo-treated nephrotic mice, aprotinin-treated nephrotic mice were protected from BW gain (Figure 3c). Accordingly, the urinary sodium/creatinine ratio was normalized in aprotinin-treated nephrotic mice compared with the other groups (Figure 3d; Supplementary Figure S3C). Treatment with aprotinin prevented the reduction in plasma sodium concentration seen in the other nephrotic groups (Table 1). Compared with healthy mice, nephrotic mice of all groups tended to have higher plasma