**Urokinase-type plasminogen activator (uPA) is not essential for epithelial sodium channel (ENaC)-mediated sodium retention in experimental nephrotic syndrome**

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**Abstract**

Aim. In nephrotic syndrome, aberrantly filtered plasminogen (plg) is converted to active plasmin by tubular urokinase-type plasminogen activator (uPA) and thought to lead to sodium retention by proteolytic activation of the epithelial sodium channel (ENaC). This concept predicts that uPA is an important factor for sodium retention and that inhibition of uPA might be protective in nephrotic syndrome.

Methods. Activation of amiloride-sensitive currents by uPA and plg were studied in *Xenopus laevis* oocytes expressing murine ENaC. In doxorubicin-induced nephrotic mice, uPA was inhibited pharmacologically by amiloride and genetically by the use of uPA-deficient mice (*uPA-/-*).

Results. Experiments in *Xenopus laevis* oocytes expressing murine ENaC confirmed proteolytic ENaC activation by a combination of plg and uPA which stimulated amiloride-sensitive currents with concomitant cleavage of the ENaC γ-subunit at the cell surface. Treatment of nephrotic wild-type mice with amiloride inhibited urinary uPA activity, prevented urinary plasmin formation and sodium retention. In nephrotic mice lacking uPA (*uPA-/-*), urinary plasmin formation from plg was suppressed and urinary uPA activity absent. However, in nephrotic *uPA-/-* mice sodium retention was not reduced compared to nephrotic *uPA+/+* mice. Amiloride prevented sodium retention in nephrotic *uPA-/-* mice which confirmed the critical role of ENaC in sodium retention.

Conclusion. uPA is responsible for the conversion of aberrantly filtered plasminogen to plasmin in the tubular lumen *in vivo*. However, uPA dependent plasmin generation is not required for ENaC-mediated sodium retention in experimental nephrotic syndrome.

234 words

**Introduction**

Heavy proteinuria, sodium retention and edema are hallmarks of patients with nephrotic syndrome. Strong evidence has emerged that aberrantly filtered serine proteases resulting in proteasuria cause sodium retention in nephrotic syndrome by activating the epithelial sodium channel (ENaC) through proteolysis of its γ-subunit 1-3. This is supported by our recent finding that in mice with experimental nephrotic syndrome treatment with the serine protease inhibitor aprotinin prevented sodium retention 4. Currently, plasmin is thought to be the main serine protease responsible for ENaC activation during nephrotic syndrome 1, 2, 5-7. Plasmin is formed by cleavage of aberrantly filtered plasminogen (plg) by urokinase-type plasminogen activator (uPA), which is expressed in the tubular epithelium and detectable in the urine 6, 8-10. Plasmin has been shown to increase amiloride-sensitive whole-cell currents in *Xenopus laevis* oocytes heterologously expressing ENaC 6, 11, 12 . In patients with proteinuric kidney disease, active plasmin was found in the urine and shown to correlate with extracellular volume and overhydration 11. This finding suggests a pathophysiological role of plasmin in sodium retention of humans with proteinuric kidney disease.

Sodium retention in rats with experimental nephrotic syndrome has been found to be abrogated by amiloride, a well-known blocker of ENaC 13. However, amiloride is also an inhibitor of uPA with a *Ki* value of 7 µM 14 which is below the concentrations reached in the urine of amiloride-treated rats 8. Therefore, amiloride might exert a dual effect in nephrotic syndrome by inhibiting ENaC and plasminogen cleavage in the tubule. Amiloride treatment in nephrotic rats has indeed been found to result in attenuated urinary plasmin generation by uPA that is expected to reduce proteolytic ENaC activation 6, 8. In addition to these indirect effects of uPA on sodium retention, uPA itself may also play a direct role. Firstly, uPA was reported to be aberrantly filtered into the urine in nephrotic rats 8. Secondly, active uPA activates currents of human ENaC by proteolysis in the oocyte expression system 15 and ENaC-mediated transepithelial sodium transport has been reported to be reduced in uPA deficient primary murine tracheal epithelial cells 16. Thirdly, uPA is also sensitive to aprotinin 17 and could at least partly be a target of aprotinin’s inhibitory effect on sodium retention in nephrotic syndrome 4.

In the present investigation, we confirmed proteolytic ENaC activation by a combination of uPA and plasminogen *in vitro* and aimed to define the significance of uPA in ENaC-mediated sodium retention *in vivo* by using a murine model of nephrotic syndrome. Mice constitutively lacking uPA (*uPA-/-*) were subjected to experimental nephrotic syndrome induced by doxorubicin 18-20. For comparison, we also treated nephrotic wild-type mice with amiloride to pharmacologically inhibit uPA. We demonstrate that uPA activates plasminogen in the tubule *in vivo*. Interestingly, uPA deficiency does not prevent sodium retention in nephrotic syndrome.

**Results**

***A combination of urokinase-type plasminogen activator (uPA) and plasminogen (plg) stimulates murine ENaC in Xenopus laevis oocytes and is associated with cleavage of γ-ENaC at the cell surface***

We have previously reported that a combination of plg and uPA, which leads to the formation of plasmin, increased ENaC-mediated amiloride-sensitive whole-cell currents (ΔIami) in *X. laevis* oocytes expressing rat ENaC 6. However, a similar effect on murine ENaC has not been shown so far. Here, we confirm that exposing oocytes for 30 min to a combination of plg (1 mg ·mL-1) and uPA (150 IU·mL-1) also activates heterologously expressed murine ENaC while neither plg nor uPA individually altered ΔIami. In contrast, plasmin had the expected stimulatory effect on murine ENaC(Fig. 1a-g). Although previously reported 15, we did not find a stimulation of neither murine nor human ENaC by pre-incubating oocytes for 12 hours in 2,000 IU·mL -1 uPA (Suppl. Fig. 1). Importantly, amiloride in a concentration of 500 μM prevented the stimulation of ΔIami by uPA in combination with plg (Suppl. Fig 2a,b) which is consistent with the well-known inhibitory effect of amiloride on uPA (Suppl. Fig. 2c and 14). Interestingly, 500 µM amiloride also inhibited the proteolytic activity of plasmin and its stimulatory effect on ENaC (Suppl. Fig. 3).

In biotinylation experiments performed in parallel to the whole-cell current measurements, we analyzed γ-ENaC cleavage at the cell surface after preincubation of the oocytes in control solution, uPA and/or plg, as well as in plasmin. In non-injected oocytes, γ-ENaC-specific signals were absent. The predominant γ-ENaC fragment detected at the cell surface of untreated -ENaC expressing oocytes had a molecular mass of ~76 kDa (Fig. 1h,i) which is the result of cleavage by endogenous convertases like furin. Coincubation with uPA and plg or incubation with plasmin led to a shift to ~67 kDa reflecting additional cleavage distal to the furin site which was not evident in oocytes preincubated with plg or uPA alone (Fig. 1i).

These results demonstrate that uPA is essential for plg to stimulate murine ENaC by proteolysis of its γ-subunit. In the presence of aberrantly filtered plg as observed in nephrotic syndrome, urinary uPA activity could therefore mediate ENaC activation *via* plasmin formation in nephrotic mice and thereby contribute to increased renal sodium retention.

***Dose-finding studies to determine the effect of amiloride on uPA activity in wild-type mice***

In urine samples from healthy wild-type 129S1/SvImJ mice, amiloride inhibited uPA activity with an IC50 of 6±1 µM (Fig. 2a). By comparison, the IC50 of amiloride on ENaC currents is around 0.1 µM 21, 22. After application of amiloride in two different doses (5 and 10 µg/g bw i.p.), urinary amiloride concentration peaked 2-4 h after injection and exceeded the concentration required for a 90% uPA inhibition (IC90) in mice treated with the higher dose (Fig. 2b). Both doses induced similar natriuresis after 4 h (Fig. 2c). Notably, natriuresis was significantly reduced 24 h after injection pointing to a counterregulation as evidenced by increased aldosterone secretion 10 h after injection (Fig. 2d). From these dose-finding data we concluded that amiloride given once daily in a dose of 5 and particularly 10 µg/g bw should effectively inhibit urinary uPA and ENaC activity in wild-type mice *in vivo*, at least during the first 8 h after administration.

***Effect of amiloride on uPA-mediated urinary plasmin generation and sodium retention in experimental nephrotic syndrome***

To determine the effect of pharmacological inhibition of uPA by amiloride on sodium retention *in vivo*, we studied the course of experimental nephrotic syndrome in wild-type mice treated with vehicle or amiloride. Compared to the healthy state, amiloride induced a stronger natriuresis following a single dose in the nephrotic state of the same mouse which is consistent with activation of ENaC *in vivo* (Fig. 2e,f). Following doxorubicin injection, mice of all three treatment groups developed similar proteinuria (Fig. 3a). Body weight was reduced to the same extent in all three groups in the first 5-6 days following doxorubicin injection (Fig. 3b) due to transiently lower food and fluid intake (Suppl. Fig. 4a,b). Thereafter, vehicle-treated mice gained weight and developed ascites indicating sodium retention. This was paralleled by reduced urinary sodium excretion in vehicle-treated mice (Fig. 3c) and a positive sodium balance derived from a subset of mice studied in metabolic cages (Suppl. Fig. 5). After initiation of amiloride treatment on day 5 and onset of proteinuria, body weight gain was dose-dependently inhibited and coincided with a marked natriuresis in samples taken 4 h after injection (Fig. 3b,c). Compared to healthy mice, plasma aldosterone was increased in vehicle- and to a stronger extent in amiloride-treated nephrotic mice (Fig. 3d).

During the course of nephrotic syndrome urinary uPA activity increased (Fig. 3e) which can be explained by aberrant filtration of uPA at the glomerulus 8. High dose amiloride treatment suppressed uPA activity in samples taken 4 h after injection whereas uPA activity returned to normal values in samples taken 24 h after injection (Fig. 3e). uPA-mediated cleavage of plg and formation of active plasmin were analyzed using a chromogenic substrate and Western blot. As shown in Figure 3f, urinary plasmin activity increased after onset of proteinuria in vehicle-treated mice indicating proteasuria. In amiloride-treated nephrotic mice, urinary plasmin activity was suppressed in samples taken 4h after amiloride-injection and was subsequently normalized in samples taken 24 h after injection. In Western blot analysis, vehicle-treated mice excreted both plg zymogen at 105 kDa and the heavy chain at 75 kDa indicating cleavage at the activation bond between R561/V562(Fig. 4a,b). The 22 kDa light chain harboring the active site of plasmin was not detected by the used antibody that is directed against the heavy chain of plg. In agreement with the results on uPA and plasmin activity, amiloride treatment was accompanied by the absence of plg cleavage in samples taken 4 h after injection (Fig. 4a,b) that was again detectable at the end of the dosing interval (Fig. 4c,d).

Table 1 shows the effect of experimental nephrotic syndrome on plasma parameters. Compared to healthy wild-type mice, nephrotic mice developed marked hypoalbuminemia, lower plasma Na+ and higher plasma K+ concentration. Renal function was reduced as evidenced by increased urea concentration. Except for a slightly higher plasma Na+ concentrationthere was no difference between vehicle- and amiloride-treated nephrotic mice.

In conclusion, these results show that amiloride treatment effectively prevents sodium retention in nephrotic syndrome by a possible dual action on ENaC and plasmin formation as a result of inhibition of uPA. To define the impact of uPA on ENaC activation in nephrotic syndrome we next studied mice lacking uPA (*uPA-/-*).

***ENaC activity is not different in healthy mice lacking uPA (uPA-/-)***

Urinary uPA activity was reduced in healthy heterozygous *uPA+/-* mice and was completely absent in *uPA-/-* mice (Fig. 5b). Urinary amiloride and sodium excretion after injection of 5 and 10 µg ·g -1 amiloride was not different in *uPA+/+* and *uPA-/-* mice (Suppl. Fig. 6a,b) as well as sodium preservation under low salt conditions (Suppl. Fig. 6c). Plasma aldosterone concentration was not different in in *uPA+/+* versus *uPA-/-* mice maintained under control, low salt or post-amiloride conditions (Suppl. Fig. 6d). Plg is the main substrate of uPA and might be altered in uPA deficiency. As shown in Suppl. Fig. 7, plasma plg concentration was similar in *uPA-/-* and *uPA+/+* mice and urinary plg excretion was absent before induction of nephrotic syndrome. In summary, these results indicate normal renal ENaC activity in healthy *uPA-/-* mice.

***Mice lacking uPA (uPA-/-) are not protected from sodium retention in nephrotic syndrome***

To determine whether uPA is essential for sodium retention *in vivo*, we studied the course of experimental nephrotic syndrome in mice lacking uPA (*uPA*+/- and*uPA*-/-) and their wildtype littermates (*uPA+/+*). Following doxorubicin injection all genotypes developed similar proteinuria (Fig. 5a). During the course of nephrotic syndrome, urinary uPA activity tended to increase in wild-type *uPA*+/+ mice and *uPA*+/- mice, however, this did not reach statistical significance (Fig. 5b). Urinary plasmin activity became measurable after induction of nephrotic syndrome in *uPA*+/+ mice and *uPA*+/- mice whereas plasmin activity was almost absent in *uPA*-/- mice (Fig. 5c). In nephrotic *uPA*+/+ *and uPA*-/- mice,there was no difference in urinary plasmin(ogen) excretion, and plasma plasminogen concentration fell to the same level (Suppl. Fig. 7). In Western blot analysis from urine samples, plasminogen cleavage indicated by the presence of the 75 kDa heavy chain was readily detectable in nephrotic *uPA*+/+ mice and to a lesser extent in *uPA*+/- mice (Fig 5d,e). In agreement with the activity assay, plasminogen cleavagewas almost not detectable in urine samples from nephrotic *uPA*-/- mice.

Amiloride-sensitive natriuresis was similar in nephrotic *uPA*-/- mice compared to nephrotic *uPA*+/+ mice (Suppl. Fig. 8a,b). Importantly, after induction of nephrotic syndrome daily urinary sodium excretion dropped significantly in all genotypes (Fig. 5f) and nephrotic *uPA*+/+, *uPA*+/- and *uPA*-/- mice gained body weight to the same extent (Fig. 5g). Amiloride treatment of *uPA*-/- mice was similarly effective in preventing sodium retention (Fig. 5g), however, *uPA*-/- mice had increased sensitivity to amiloride resulting in weight loss and mortality with the higher dose (10 µg ·g -1). Urinary amiloride concentration was higher in nephrotic *uPA*-/- mice compared to *uPA*+/+ mice following the 5 µg·g -1 dose (Suppl. Fig. 8c) which could be explained by a lack of amiloride binding to uPA in the tubule. As a consequence, nephrotic *uPA*-/- mice had worsened hyperkalemia and acidosis (Table 1). In nephrotic mice of all genotypes, plasma aldosterone was increased reaching highest values in amiloride-treated *uPA*-/- mice (Fig. 5h).

***Western blot for ENaC subunit expression from kidney cortex demonstrates aldosterone-stimulated furin-cleavage of α- and γ-ENaC in nephrotic mice***

Western blot analyses of kidney cortex for α-ENaC revealed three bands at 87, 26 and 22 kDa which disappeared after application of the immunogenic peptide (Suppl. Fig. 9). For γ-ENaC, there were multiple bands at 82, 71, 62, 50 and 45 kDa and application of the immunogenic peptide blocked those at 82 kDa, 71 and 45 kDa, but not those at 62 and 50 kDa (Suppl. Fig. 9). The largest bands at 87 kDa and 82 kDa most likely represent full-length α-ENaC and γ-ENaC, respectively, whereas the bands at 26 kDa and 22 kDa represent furin cleavage products of α-ENaC and that at 71 kDa furin-cleaved γ-ENaC. For β-ENaC, there was only a single band at 85 kDa (Suppl. Fig. 9) corresponding to the full length subunit which is not proteolytically processed.

As shown in Fig. 6, furin cleavage of α- and γ-ENaC was found to be increased in nephrotic mice of both genotypes with the strongest effect observed in nephrotic wild-type mice treated with amiloride. A modest increase in β-ENaC was also observed in nephrotic animals. Aldosterone is known to increase overall ENaC expression and furin cleavage of α-ENaC and γ-ENaC 23, 24. Therefore, the observed changes in ENaC expression and cleavage pattern are consistent with the increased plasma aldosterone levels in nephrotic mice (Fig. 3d and 5h) with the highest plasma aldosterone values observed in nephrotic animals treated with amiloride.

Unlike in biotinylated cell surface protein samples obtained from oocytes (Fig. 1h), we could not detect a specific band corresponding to fully-cleaved γ-ENaC between 65 to 67 kDa in renal cortex tissue from healthy wild-type or nephrotic mice. In these latter samples the portion of ENaC protein localized in the apical membrane was probably below the detection limit of our Western blot analysis. The band at 45 kDa represents a cleavage product of γ-ENaC of unknown significance. To analyze the influence of N-glycosylation of γ-ENaC (at up to 5 sites 25) on the migration pattern, we treated the samples with the deglycosylating enzyme N-glycosidase F. As shown in Suppl. Fig. 10 the migration pattern changed leaving only two bands at 71 and 50 kDa in both healthy and nephrotic wild-type mice that were blocked by the immunogenic peptide. Again, we could not discriminate a specific band that could correspond to fully-cleaved γ-ENaC in mouse tissue. Similar effects of N-glycosidase treatment on γ-ENaC in rat kidney have been reported previously 26.

**Discussion**

Our study confirms that urinary uPA activity is responsible for the conversion of aberrantly filtered plasminogen to plasmin in the tubular lumen of nephrotic mice as previously shown for rats 6, 8. This was observed both after pharmacological inhibition of uPA by amiloride and most clearly in mice lacking uPA which excreted plasminogen exclusively as a zymogen in the urine. As a result, appreciable urinary plasmin activity was absent in *uPA-/-* mice, although trace amounts of plasmin generated by other serine proteases such as plasma kallikrein cannot be excluded 27, 28 and may be relevant for proteolytic ENaC activation11. The most striking result of this study was that uPA activity was not essential for sodium retention in experimental nephrotic syndrome and that *uPA-/-* mice developed similar sodium retention as wild-type mice. This finding was surprising and unexpected given the finding that plasmin generated by uPA leads to proteolytic ENaC activation in *X. laevis* oocytes expressing murine or human ENaC (Fig. 1, Suppl. Fig. 1A and C) and should contribute to sodium retention in nephrotic syndrome 1, 2, 5-7. Using the same nephrotic model, we have found that treatment with the serine protease inhibitor aprotinin prevented sodium retention with similar efficacy as amiloride 4. This indicates that sodium retention in experimental nephrotic syndrome is caused by activated serine proteases in the urine, but it leaves open whether plasmin or another serine protease is more relevant. Besides plasmin and uPA, aprotinin also inhibits other serine proteases such as plasma kallikrein, or tissue-expressed prostasin and tissue kallikreins 29-31. Future studies will be required to identify those serine proteases essential for sodium retention in nephrotic syndrome.

The strength of this study is the application of experimental nephrotic syndrome to uPA knockout mice to elucidate the role of uPA in sodium retention *in vivo*. This approach has previously been used in nephrotic mice lacking plasma kallikrein. These mice were also not protected from sodium retention although plasma kallikrein was found to directly cause proteolytic ENaC activation *in vitro* 27. For plasmin, data from knockout mice are lacking and its role has been based on indirect evidence inferred by ENaC stimulation *in vitro* 6, 32 and association with sodium retention in patients 11. Our study suggests that, candidate proteases contributing to proteolytic ENaC activation *in vitro* have to be studied in appropriate animal models *in vivo* to determine their role in sodium retention in nephrotic syndrome.Unfortunately, detection and reliable quantification of ENaC cleavage occurring at the luminal surface of renal tubules remains an unmet experimental challenge. Thus, at present definitive proof is still missing that in nephrotic syndrome filtered and activated proteases increase the portion of fully cleaved γ-ENaC at the cell surface of renal tubular cells.

Ji et al. have previously reported that two-chain uPA, which is the active form of uPA, stimulated human ENaC-mediated currents by about 6-fold when used in a concentration of 2,000 IU/ml with an exposure time of 12 hours 15. We could not replicate these findings in our study neither with murine nor with human ENaC (Suppl. Fig. 1). This makes it unlikely that species differences are responsible for these discrepant observations which we cannot explain at present. Interestingly, the two-chain uPA used by Ji et al. was generated from single-chain uPA by incubation with immobilized plasmin. Therefore, trace amounts of contaminating plasmin in this uPA preparation may be responsible for the observed stimulatory effect on ENaC. Indeed, even low concentrations of plasmin are sufficient to stimulate ENaC currents in oocytes when incubation time is prolonged 11. Our oocyte data demonstrate that uPA is essential to mediate plg conversion and proteolytic ENaC activation *in vitro*. However, as discussed above, absence of uPA *in vivo* does not rule out the possibility that trace amounts of plasmin sufficient for proteolytic ENaC are present in nephrotic urine.

The fact that amiloride was similarly effective in preventing sodium retention as in wild-type mice shows that the antiedematous effect of amiloride in nephrotic syndrome is mainly related to its inhibitory effect on ENaC and not mediated by inhibition of uPA or plasmin. The high efficacy of amiloride in experimental nephrotic syndrome can be explained by the proteolytic activation of ENaC and is corroborated by the finding that in nephrotic mice amiloride-sensitive natriuresis was increased compared to healthy mice (Supp. Fig. 7 and 27). Results of the present study and those from studies in nephrotic rats provide a rationale for ENaC blockade using amiloride to treat edema in human nephrotic syndrome 13. However, there is a lack of clinical studies supporting this 33. It is tempting to speculate that in addition to ENaC blockade targeting proteasuria might evolve as a new and superior therapeutic approach to treat sodium retention in nephrotic patients 34.

In conclusion, we show that tubular uPA converts aberrantly filtered plasminogen to active plasmin in nephrotic syndrome. However, uPA is not essential for ENaC activation and sodium retention in nephrotic mice. Thus, in nephrotic syndrome uPA inhibition does not contribute to the antiedematous effect of amiloride which results from direct ENaC inhibition.

**Materials and methods**

*Two-Electrode Voltage-Clamp*

Oocytes were collected from *Xenopus laevis* with approval of the animal welfare officer for the University of Erlangen-Nürnberg as described 4, 12, 35, 36. Defolliculated stage V-VI oocytes were injected with cRNA encoding murine -, -, and -ENaC (0.05ng or 0.2 ng of cRNA/subunit). Oocytes were incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 5 mM Hepes, pH 7.4) and were studied two days after injection using the two-electrode voltage-clamp technique (TEVC) as described previously 12, 35, 36. Amiloride-sensitive whole-cell currents (ΔIami) were determined at a holding potential of –60 mV by washing out amiloride (2 µM or 10 µM as indicated) with amiloride-free ND96 and subtracting the whole-cell currents measured in the presence of amiloride from the corresponding whole-cell currents recorded in the absence of amiloride. To determine the stimulatory effect of plasminogen (from human serum, Roche Diagnostics, Mannheim, Germany), human urokinase-type plasminogen activator (Urokinase HS medac, Wedel, Germany) or human plasmin (from human plasma, -aminocaproic acid- and lysine free, Merck), ΔIami was detected in an individual oocyte before and after its exposure to protease-supplemented ND96 or to ND96 alone as control. To recover from the first measurement of ΔIami, the oocyte was placed for ~1 min in ND96. Subsequently, the oocyte was transferred to 100 μl of protease-supplemented ND96 or ND96 alone as control and incubated for 30 min before the second measurement of ΔIami.

*Detection of *-*ENaC cleavage products at the cell surface using a biotinylation approach*

To detect expression and cleavage of -ENaC at the cell surface of ENaC expressing oocytes, we used a biotinylation approach essentially as described 4, 12, 27, 36. Biotinylated cell surface proteins were studied by western blot analysis using a rabbit anti-murine -ENaC antibody and a secondary horseradish peroxidase-labeled goat anti-rabbit antibody (sc-2054, Santa Cruz, CA, USA). To validate separation of cell surface proteins from intracellular proteins by biotinylation, blots were stripped and reprobed using a β-actin antibody (Sigma-Aldrich, Schwalbach, Germany)37.

*Mouse studies*

Experiments were performed on 3-month-old wild-type and uPA deficient (*uPA-/-*) mice of both sex. Imported B6-*Plautm1Mlg-/-* mice 38 were backcrossed over 5 generations onto a 129 S1/SvImJ background to confer susceptibility to experimental nephrotic syndrome 18, 19. Genotyping was done using PCR as described 39. 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, Soest, Germany) with tap water ad libitum. Experimental nephrotic syndrome was induced after a single intravenous injection of doxorubicin (14.5 µg · g bw-1, Teva, Germany) as developed by our group 18-20. This model does not lead to histological changes in the heart 40. Mice were kept in their normal cages to reduce stress after doxorubicin injection. Studies of nephrotic mice in metabolic cages to collect 24 h urine samples over 10 days are not feasible due to inadequate food and sodium intake which negatively affects sodium retention. In addition, it would not be permitted by German Institutional Animal Care and Use Committees (IACUC) to study mice in metabolic cages for more than 7 days. Therefore, sodium balance could not be studied over the entire time. Alternatively, urine was collected in metabolic cages before and after induction of nephrotic syndrome over two and one day, respectively, in a subset of mice. During the course of nephrotic syndrome, samples of spontaneously voided urine were collected in the morning between 8 and 9 am 2 days before (baseline) and up to 10 days following doxorubicin injection, and daily food and fluid intake were monitored by weighing the food pellets and the water bottle. Sodium balance was inferred from urinary sodium excretion in relation to food intake and body weight change. Amiloride was administered intraperitoneally after urine collection between 8 and 9 am once daily in a dose of 5 or 10 µg/g bw dissolved in 5 µl/g bw injectable water (Ampuwa, Fresenius Kabi Deutschland, Bad Homburg, Germany) starting from day 5. In these mice, additional urine samples were collected 4 h after injection since urinary amiloride concentration showed a peak between 2 to 4 h after injection. Vehicle-treated mice received only injectable water. Blood samples were drawn before induction and at sacrifice on day 10. In healthy *uPA+/+* and *uPA-/-* mice, renal sodium handling was studied in metabolic cages with 2 days of a control diet (C1000, Altromin, Lage, Germany, sodium content 110 µmol ·g -1) followed by a 4 day low salt diet (C1036, Altromin, Lage, Germany sodium content 10 µmol · g -1 food). 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 M6/14).

*Laboratory measurements*

Urinary activity of uPA and plasmin was measured using the chromogenic substrates PNAPEP 1344 and S-2251, respectively (Haemochrom, Essen, Germany). 3 µl urine and 50 µl 2 mM substrate was incubated for 1 h at 37°C with or without a specific inhibitor for uPA (UK122, Santa Cruz Biotechnology, CA, USA, final concentration 27 µg ·mL -1) and plasmin (anti-plasmin, Loxo, Heidelberg, Germany, 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 the inhibitors reflected the specific activity of uPA or plasmin. Values were expressed as relative units (1000\*Delta absorption @405 nm) and normalized to urinary creatinine concentration.

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 as well as fecal sodium content (after dissolution in nitric acid) with flame photometry (Eppendorf EFUX 5057, Hamburg, Germany). Both urinary protein and sodium concentration were normalized to the urinary creatinine concentration. Plasma aldosterone was measured using an ELISA kit (IBL, Hamburg, Germany), plasma albumin using a fluorometric kit against mouse albumin as standard (Active motif, Carlsbad, USA). Urinary and plasma plasminogen concentration were measured using an ELISA kit (Loxo, Heidelberg, Germany) that detects both plasmin and plasminogen as indicated by plasmin(ogen). Blood gas analysis was done using an IL GEM® Premier 3000 blood gas analyzer (Instrumentation Laboratory, Munich, Germany). Urinary amiloride concentration was measured fluorometrically according to method of Baer et al. 41.

*Western blot from urine samples and kidney tissue of mice*

For Western blot (WB) analysis of plasminogen excretion in the urine of nephrotic mice, SDS-PAGE on a 7.5% gel was performed with 5 µg urinary protein per lane. A rabbit anti-plasminogen antibody was used as primary antibody (ab154560, abcam). Bands were developed by chemiluminescence using secondary HRP-conjugated antibodies (NA934V, Amersham, GE healthcare) on a ChemiDoc Touch System (Biorad, Hercules, USA).

Western blot analysis of α-, β-, and γ-ENaC expression was performed as previously described 4. ENaC cleavage products were detected with a fluorescent secondary antibody labelled with IRDye 800CW and a fluorescence scanner (Licor Odyssey, Lincoln, USA). For loading control, total protein was measured using Revert Total Protein Stain (Licor, Lincoln, USA). The effect of deglycosylation was tested after incubation of the denaturated samples with PNGase F for 1 h at 37°C according to manufacturer´s instructions (New England Biolabs, Ipswich, USA).

*Primary Antibodies*

Antibodies against murine α-, β-, and γ-ENaC were raised in rabbits against the amino acids 45-68 for α-ENaC, 617-638 for β-ENaC and 634–655 for γ-ENaC (Pineda, Berlin, Germany) 42, 43. Antiserums containing antibodies against α- and γ-ENaC were purified with affinity chromatography. To confirm that the observed bands are specific for α- and -ENaC, the primary antibody was preincubated with an immunogenic peptide (20x excess by molarity) overnight at 4 °C. Plasminogen was probed using a rabbit antibody directed against amino acid residues 84-434 of the heavy chain (ab154560, abcam). This antibody detects plasminogen zymogen at 105 kDa and plasminogen heavy chain at 75 kDa after cleavage and dissociation from the light chain under reducing WB conditions.

*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 6, GraphPad Software (San Diego, CA, www.graphpad.com). Densitometric analysis of western blots was done using Image Studio Version 3.1.4 (Licor) and ImageJ 44. A p value <0.05 at two-tailed testing was considered statistically significant.

**Author contributions**

BNB, SD, MW, FFS, TS, AI, FB, JCS, AJ, DE performed experiments and prepared the data, SK, HUH, SH, CK analyzed data, revised manuscript, FA designed study, accountable for all aspects of the manuscript. All authors approved the final version of the manuscript.

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**Conflict of interests:**

None.

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**Table 1: Plasma parameters of healthy and different groups of nephrotic mice.**

Arithmetic means ± SEM (n=3-14 each)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | healthy | healthy | nephrotic | nephrotic | nephrotic | nephrotic |
|  | *wild-type*  *129S1/SvImJ* | *uPA-/-* | *wild-type / vehicle-treated* | *wild-type / amiloride-treated1* | *uPA-/- /*  *vehicle-*  *treated* | *uPA-/- / amiloride-treated2* |
| venous pH | 7.28 ± 0.01 | 7.29 ± 0.01 | 7.30 ± 0.02 | 7.30 ± 0.02 | 7.30 ± 0.02 | 7.15 ± 0.03 #,§ |
| std HCO3-, mM | 22 ± 0.4 | 23 ± 0.5 \* | 24 ± 1.2 | 23 ± 0.5 | 25 ± 1.2 | 17 ± 1.9 #,§ |
| Na+, mM | 148 ± 1 | 148 ± 1 | 138 ± 3 # | 145 ± 1 § | 143 ± 1 # | 135 ± 2 # |
| K+, mM | 4.7 ± 0.07 | 4.5 ± 0.03 \* | 5.7 ± 0.21 # | 5.5 ± 0.31 # | 6.3 ± 0.55 # | 10.4±1.1 #,§ |
| Ca++, mM | 1.10 ± 0.01 | 1.09 ± 0.02 | 1.00 ± 0.02 # | 1.04 ± 0.03 | 1.15 ± 0.06 \* | 1.15 ±0.04 |
| Hct, % | 47 ± 0 | 46 ± 1 | 46 ± 3 | 46 ± 1 | 40 ± 3 | n.a. |
| cHb, g dL-1 | 15.6 ± 0.2 | 15.1 ± 0.3 | 15.3 ± 0.8 | 15.3 ± 0.4 | 13.2 ± 1.0 | n.a. |
| urea, mg dL-1 | 37 ± 6 | 44 ± 6 | 362 ± 31 # | 381 ± 11 # | 259 ± 82 # | 154 ± 34 # |
| albumin, g L -1 | 34 ± 4 | 30 ± 3 | 6 ± 2 # | 5 ± 1 # | 8 ± 1 # | 11 ± 0 # |

# significant difference between healthy and nephrotic mice, \* significant difference between genotypes, § significant difference between amiloride-treated and vehicle-treated nephrotic mice

1 amiloride dose 10 µg g-1, 2 amiloride dose 5 µg g-1, n.a. not available

Abbreviations: std standard, Hct hematocrit, cHb calculated hemoglobin concentration

**Figure 1. A combination of urokinase-type plasminogen activator (uPA) and plasminogen (plg) stimulates murine ENaC in Xenopus laevis oocytes and is associated with cleavage of γ-ENaC**

*Xenopus laevis* oocytes expressing murine ENaC were preincubated for 30 min in protease-free vehicle solution (ND96) or in a solution containing uPA (150 IU ·mL -1 or 360 µg·mL -1), Plg (1 mg·mL -1) or uPA + Plg. Plasmin (pl, 10 µg·mL -1) served as a positive control. Amiloride-sensitive whole-cell currents (Iami) were determined before and after incubation.

(a-e) Representative whole-cell current traces are shown before (black) and after (red) incubation in the indicated test solutions. Amiloride (ami) was present in the bath solution to specifically inhibit ENaC as indicated by black bars.

(f) Summary of similar experiments as in a-e. Individual values of Iami before (-) and after (+) incubation with uPA, Plg, uPA+Plg and Pl. Measurements performed in the same oocyte are connected by a line.

(g) Summary of the individual data shown in f normalized as relative stimulatory effect on Iami.

(h) upper panel: Using a biotinylation approach and Western blot analysis γ-ENaC expressed at the cell surface was detected with an antibody against the C-terminus of murine γ-ENaC. lower panel: The same representative blot (n=4) was re-probed with an antibody against β-actin to validate separation of cell surface proteins from intracellular proteins.

(I) As positive control, presence of β-actin was confirmed in corresponding non-biotinylated intracellular protein fractions.

N indicates the number of different batches of oocytes, n the numbers of individual oocytes measured. \*\*, p<0.01, \*\*\*, p<0.001, significance between indicated groups, paired t-test (f) or compared to control (ND96), unpaired t-test (g). Error bars, SEM

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Automatisch generierte Beschreibung**

**Figure 2. Efficacy of amiloride to inhibit uPA and induce natriuresis**

(a) Inhibition of uPA activity by amiloride in the urine of healthy wild-type mice. Pooled analysis of n=5 curves from independent urine samples.

(b, c) Time course of urinary amiloride concentration and urinary sodium excretion after i.p. injection of 5 and 10 µg g -1 bw amiloride. Between 2-4 hours, urinary amiloride concentration exceeded the threshold to inhibit almost all uPA activity (IC90) when given at 10 µg g -1 bw.

(d) Plasma aldosterone concentration 10 h after injection of vehicle or amiloride.

(e) Responses to vehicle and amiloride (5 µg g -1 bw) before and after induction of nephrotic syndrome in wild-type mice.

(f) Difference of the urinary sodium excretion between vehicle and amiloride treatment was calculated for each mouse and reflects amiloride-sensitive natriuresis.

\* significant difference between vehicle and amiloride treatment, # significant difference compared to healthy mice, § significant difference between 4h and 24 h value.

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Automatisch generierte Beschreibung**

**Figure 3. Effect of amiloride in experimental nephrotic syndrome**

Time course of proteinuria (a), normalized body weight (b) and urinary sodium excretion (c) before and after induction of nephrotic syndrome and treatment with vehicle or amiloride started on day 5 after doxorubicin injection. Urine samples were collected 4 h and 24 h after i.p. injection of vehicle or amiloride (10 µg·g -1). Initial body weight was not different across the groups (range 27.4 – 28.8 g, p=0.16).

(d) Plasma aldosterone concentrations at day 10 in vehicle- and amiloride-treated mice.

(e, f) Time course of urinary uPA and urinary plasmin activity in samples taken 4 h and 24 h after injection of amiloride (10 µg·g -1).

OD optical density @405 nm.

# significant difference vs. baseline, \* significant difference between vehicle-treated and amiloride-treated nephrotic mice.

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**Figure 4. Urinary expression of plasminogen and its cleavage product in vehicle- and amiloride treated nephrotic mice as analyzed by Western blot.**

(a, c) Compared to vehicle-treated nephrotic mice, amiloride-treated nephrotic mice excreted only plasminogen zymogen but almost no plasminogen heavy chain in samples taken 4 h after injection. This indicates efficient inhibition of urokinase-mediated cleavage after injection of amiloride. In contrast, both plasminogen zymogen and plasminogen heavy chain were again detectable at the end of dosing interval after 24 h.

(b, d) Densitometric analysis of the obtained WB results.

OD optical density

\* significant difference between vehicle-treated and amiloride-treated nephrotic mice.

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Automatisch generierte Beschreibung**

**Figure 5: Course of experimental nephrotic syndrome in wild-type (*uPA*+/+) and mice with uPA deficiency (*uPA*+/- and *uPA*-/-)**

Time course of proteinuria (a), urinary uPA (b) and urinary plasmin activity (c) before and after injection of doxorubicin in wild-type mice (*uPA*+/+) and mice with uPA deficiency (*uPA*+/- and *uPA*-/-).

(d) Western blot from urinary samples for excretion of plasminogen in nephrotic wild-type (*uPA*+/+) and mice with uPA deficiency (*uPA*+/- and *uPA*-/-). Compared to wild-type *uPA*+/+ mice, heterozygous *uPA*+/- mice had reduced excretion of plasminogen heavy chain whereas *uPA*-/- mice had nearly absent excretion of plasminogen heavy chain indicating no relevant plasminogen cleavage.

(e) Densitometric analysis of the obtained WB results.

(f, g) Time course of urinary sodium excretion and normalized body weight in nephrotic wild-type (*uPA*+/+) mice and mice with uPA deficiency (*uPA*+/- and *uPA*-/-). Note that body weight was not different across the groups (range 23.4 – 24.8 g, p=0.33).

(h) Plasma aldosterone concentrations at day 10 in nephrotic wild-type mice (*uPA*+/+) and mice with uPA deficiency (*uPA*+/- and *uPA*-/-). The horizontal line represents the plasma aldosterone concentration in healthy *uPA*+/+ mice. Amiloride treatment is again paralleled by massive hyperaldosteronism.

# significant difference vs. baseline, \* significant difference between nephrotic wildtype and uPA deficient mice. OD optical density @405 nm.

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**Figure 6. Expression of ENaC subunits in healthy, vehicle-treated nephrotic, amiloride-treated nephrotic wild-type and nephrotic *uPA*-/-mice.**

(a) Original Western blots. In the blot showing γ-ENaC, the alignment of the samples was rearranged as indicated by dashed lines.

(b-d) Densitometric analysis of ENaC subunit expression and its cleavage products in kidney cortex from healthy and nephrotic mice. Arithmetic means ± SEM (n=6 each).

# significant difference compared to healthy wild-type mice, \* significant difference between vehicle and amiloride-treated nephrotic wild-type mice.

Ein Bild, das Screenshot enthält.

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**Supplemental material**

**to the manuscript**

**Urokinase-type plasminogen activator (uPA) is not required for epithelial sodium channel (ENaC)-mediated sodium retention in experimental nephrotic syndrome**

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**Supplemental methods**

*Protease activity fluorescence assay*

Proteolytic activity was quantified using the fluorogenic substrate Boc-Gln-Ala-Arg-AMC (Boc-QAR-AMC) (Boc: t-Butyloxycarbonyl; AMC: 7-Amino-4-methylcoumarin; R&D systems, Abingdon, UK). This substrate detects the activity of a wide range of trypsin-like proteases. The experimental protocol was similar to that described by Reihill et al. 2016. The substrate was used in a concentration of 20 µM using a sample volume of 100 µl. The fluorescence signal resulting from substrate hydrolysis was continuously recorded over a period up to 150 min using a 96-well TECAN plate reader (360 nm excitation/465 nm emission wavelength).

**Reference:**

Reihill JA, Walker B, Hamilton RA, Ferguson TE, Elborn JS, Stutts MJ, Harvey BJ, Saint-Criq V, Hendrick SM, Martin SL. Inhibition of Protease-Epithelial Sodium Channel Signaling Improves Mucociliary Function in Cystic Fibrosis Airways. Am J Respir Crit Care Med. 194:701-10, 2016

**Supplemental figure 1.** **A high concentration of urokinase-type plasminogen activator (uPA) (2000 IU/ml) does not significantly stimulate murine or human ENaC.**

(a, c) *Xenopus laevis* oocytes expressing murine (a, mENaC) or human (c, hENaC) ENaC were pre-incubated for 12 h in protease-free vehicle solution (ND96) or in a solution containing uPA (150 IU mL-1), uPA (2000 IU mL-1) or uPA (150 IU mL-1) + plasminogen (Plg;1 mg mL-1). Subsequently amiloride-sensitive whole-cell currents (∆Iami) were determined. The filled circles shown in (a) and (c) represent ∆Iami values measured in individual oocytes. n indicates the number of individual oocytes measured. \*\*\*, p<0.001; \*, p<0.05; ns, not significant; one-way ANOVA with Bonferroni post-hoc test

(b, d) Using the fluorogenic substrate Boc-QAR-AMC trypsin-like proteolytic activity was assessed in the incubation solution of each oocyte at the end of the pre-incubation period. The fluorescence data (RFU=relative fluorescence unit; n=7) in (b) and (d) correspond to the current data shown in a and c, respectively. Progress curves of trypsin-like activity are shown for the different pre-incubation conditions. The data shown in (b) and (c) demonstrate that the proteolytic activity of uPA (2000 IU mL-1) was similar to that of uPA (150 IU mL-1) + Plg (1 mg mL-1) but failed to stimulate ENaC currents in the oocytes. Error bars, SEM



**Supplemental figure 2.** **The ENaC stimulating effect and proteolytic activity of a combination of urokinase-type plasminogen activator (uPA) and plasminogen (plg) are inhibited by 500 µM amiloride (ami).**

*Xenopus laevis* oocytes expressing murine ENaC were pre-incubated for 30 min in protease-free control solution (ND96) or in solutions containing uPA (150 IU mL-1) + Plg (1 mg mL-1), uPA (150 IU mL-1) + Plg (1 mg mL-1) + amiloride (Ami; 500µM) or amiloride alone (ND96 + Ami).

(a)Amiloride-sensitive currents (∆Iami) were determined before (-) and after (+) pre-incubation. Measurements performed in the same oocyte are connected by a line.

(b)Summary of the individual data shown in (a) normalized as relative stimulatory effect on ∆Iami. N indicates the number of different batches of oocytes, n the numbers of individual oocytes measured. \*\*\*, p<0.001; paired *t*-test (a) or one-way ANOVA with Bonferroni post-hoc test (b).

(c)Trypsin-like proteolytic activity in the incubation solutions was detected using the fluorogenic substrate Boc-QAR-AMC (RFU=relative fluorescence unit; n=5-6). Progress curves of trypsin-like activity are shown for the different pre-incubation conditions. Error bars, SEM

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**Supplemental figure 3. Proteolytic activity of plasmin (pl) and its stimulatory effect on ENaC are inhibited by 500 µM amiloride (ami).**

*Xenopus laevis* oocytes expressing murine ENaC were pre-incubated for 30 min in protease-free control solution (ND96) or in ND96 solution supplemented with 500 µM amiloride (+Ami), with 25µg mL-1 of plasmin (+Pl) or with 25µg mL-1 of plasmin and 500 µM amiloride (+ Pl, +Ami). (a) Amiloride-sensitive currents (∆Iami) were determined before (-) and after (+) preincubation. Measurements performed in the same oocyte are connected by a line.   
(b) Summary of individual data shown in (a) normalized as relative stimulatory effect on ∆Iami. n indicates the numbers of individual oocytes measured. \*\*\*, p<0.001; paired t-test (a) or one-way ANOVA with Bonferroni posthoc test (b). (c) Trypsin-like proteolytic activity in the incubation solutions was detected using the fluorogenic substrate Boc-QAR-AMC (RFU=relative fluorescence unit; n=7). Progress curves of trypsin-like activity are shown for the different pre-incubation conditions. Error bars, SEM. (d) BOC-QAR-AMC substrate was used to detect trypsin-like proteolytic activity in ND96 solution containing plasmin (50µg mL-1) without amiloride (control) or with different concentrations (1µM, 10µM, 100µM, 500µM, 1mM) of amiloride (RFU=relative fluorescence unit; n=2). (e) Concentration-response relationship of the inhibitory effect of amiloride on proteolytic activity of plasmin. RFU values at 95 min (RFU95; mean ± SEM) from the same experiments as shown in (d) were normalized to RFU95 of control.



**Supplemental figure 4. Food and fluid intake during the course of nephrotic syndrome**

Daily food (a) and fluid (b) intake in wild-type mice treated with vehicle or amiloride (5 µg g bw-1) as well as *uPA+/+* or *uPA-/-* mice before and after induction of nephrotic syndrome by doxorubicin. Due to inappetence mice food and fluid intake transiently decreased on day 3 after injection of doxorubicin

Arithmetic means ± SEM.

# significant difference vs. baseline, \* significant difference between treatments/genotypes

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**Supplemental figure 5. Sodium balance in wild-type mice before and after induction of doxorubicin-induced nephrotic syndrome.**

Calculated Na+ intake from food and drink, urinary and fecal Na+ excretion and calculated Na+ balance in wild-type mice before and after induction of nephrotic syndrome.

Arithmetic means ± SEM.

# significant difference between healthy and nephrotic mice

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**Supplemental figure 6. Renal ENaC activity is not different in healthy *uPA-/-* mice compared to *uPA+/+* mice.**

(a, b) Time course of urinary amiloride excretion after injection of vehicle or amiloride   
(5 / 10 µg g bw-1). Urinary amiloride concentration reaches its peak after 4 h and disappears after 24 h. Natriuresis parallels urinary amiloride concentration and is lower 24 h after injection indicating counterregulation. There is no difference between healthy *uPA+/+-* mice *uPA-/-* mice.

(c) Urinary sodium excretion and body weight change (inset) on day 5 of a low salt diet.

(d) Plasma aldosterone concentration under control conditions and after amiloride injection and a low salt diet

Arithmetic means ± SEM.

# significant difference vs. baseline, \* significant difference between genotypes

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**Supplemental figure 7. Plasma and urinary plasmin(ogen) concentration in *uPA+/+* and *uPA-/-* mice before and after induction of nephrotic syndrome**

The used ELISA recognizes both plasminogen as a zymogen and heavy chain after proteolytic activation by uPA.

Arithmetic means ± SEM.

# significant difference vs. baseline, \* significant difference between genotypes

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**Supplemental figure 8. Amiloride-sensitive natriuresis and urinary amiloride concentration in healthy and nephrotic *uPA+/+* and *uPA-/-* mice.**

(a) Natriuretic effect of mice after a single injection of vehicle and amiloride. Spot urinary samples were collected 4 h after injection of vehicle and amiloride (5 µg g-1) in healthy and nephrotic wild-type *uPA+/+* mice (day 8 after induction). The injections were done one day apart in the same mouse before and after induction of nephrotic syndrome.

(b) Amiloride-sensitive natriuresis calculated from the difference of the urinary sodium excretion between vehicle and amiloride treatment was enhanced in *uPA+/+* mice in the nephrotic state compared to the healthy state. This was not the case in *uPA-/-* mice which had already high amiloride-sensitive natriuresis in the healthy state.

(c) In the urine samples, urinary amiloride was measured. Compared to healthy mice, nephrotic *uPA+/+* show diminished urinary amiloride concentration that can be explained by binding of amiloride to uPA that is aberrantly filtered into the tubular lumen. The lack of this effect explains a higher urinary amiloride concentration in *uPA-/-* mice.

Arithmetic means ± SEM.

# significant difference vs. healthy, § significant difference between vehicle and amiloride injection

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**Supplemental figure 9. Expression of ENaC subunits and their cleavage products in kidney cortex from healthy mice**

(a) Administration of the blocking peptide for α-ENaC attenuated bands at 22, 26 and 87 kDa.

(b) Probing with an anti-β-ENaC antibody revealed a single band at 85 kDa.

(c) Administration of the blocking peptide for γ-ENaC attenuated bands at 45, 71 and 82 kDa while bands at 50 and 62 kDa were not blocked.

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**Supplemental figure 10: Expression of γENaC and its cleavage products in kidney cortex from a healthy and nephrotic mouse after treatment with the deglycosylating enzyme PNGase F.**

Western blot from renal cortex showing several bands between 45 and 82 kDa in a healthy and nephrotic wild-type mouse (lane 1 and 3). Administration of the blocking peptide attenuated bands at 45, 71 and 82 kDa while bands at 50 and 62 kDa were not blocked (lane 5 and 7). After treatment with PNGase F, only two bands at 71 and 45 kDa were visible (lane 2 and 4) both of which disappeared after application of the blocking peptide (lane 6 and 8). There was no specific band that could correspond to fully-cleaved γ-ENaC in mouse tissue with an expected size between 53-65 kDa.

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