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ORIGINAL ARTICLE

ACTA PHYSIOLOGICA

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 essential for ENaC-mediated sodium retention in experimental nephrotic syndrome.

KEYWORDS

Amiloride, epithelial sodium channel (ENaC), nephrotic syndrome, plasminogen, sodium retention, urokinase-type plasminogen activator

1 | INTRODUCTION

Heavy proteinuria, sodium retention and oedema are hallmarks of patients with nephrotic syndrome. Strong evidence has emerged that aberrantly filtered serine proteases resulting in proteinuria 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 K_i 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 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.

2 | RESULTS

2.1 | 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 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 (ΔI_{ami}) 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 minutes 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 ΔI_{ami}. In contrast, plasmin had the expected stimulatory effect on murine ENaC (Figure 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 2000 IU·mL^{-1} uPA (Figure S1). Importantly, amiloride in a concentration of 500 μM prevented the stimulation of ΔI_{ami} by uPA in combination with plg (Figure S2a,b) which is consistent with the well-known inhibitory effect of amiloride on uPA (Figures S2c).[14] Interestingly, 500 μM amiloride also inhibited the proteolytic activity of plasmin and its stimulatory effect on ENaC (Figure S3).

In biotinylation experiments performed in parallel to the whole-cell current measurements, we analysed 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 αβγ-ENaC expressing oocytes had a molecular mass of ~76 kDa (Figure 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 (Figure 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.

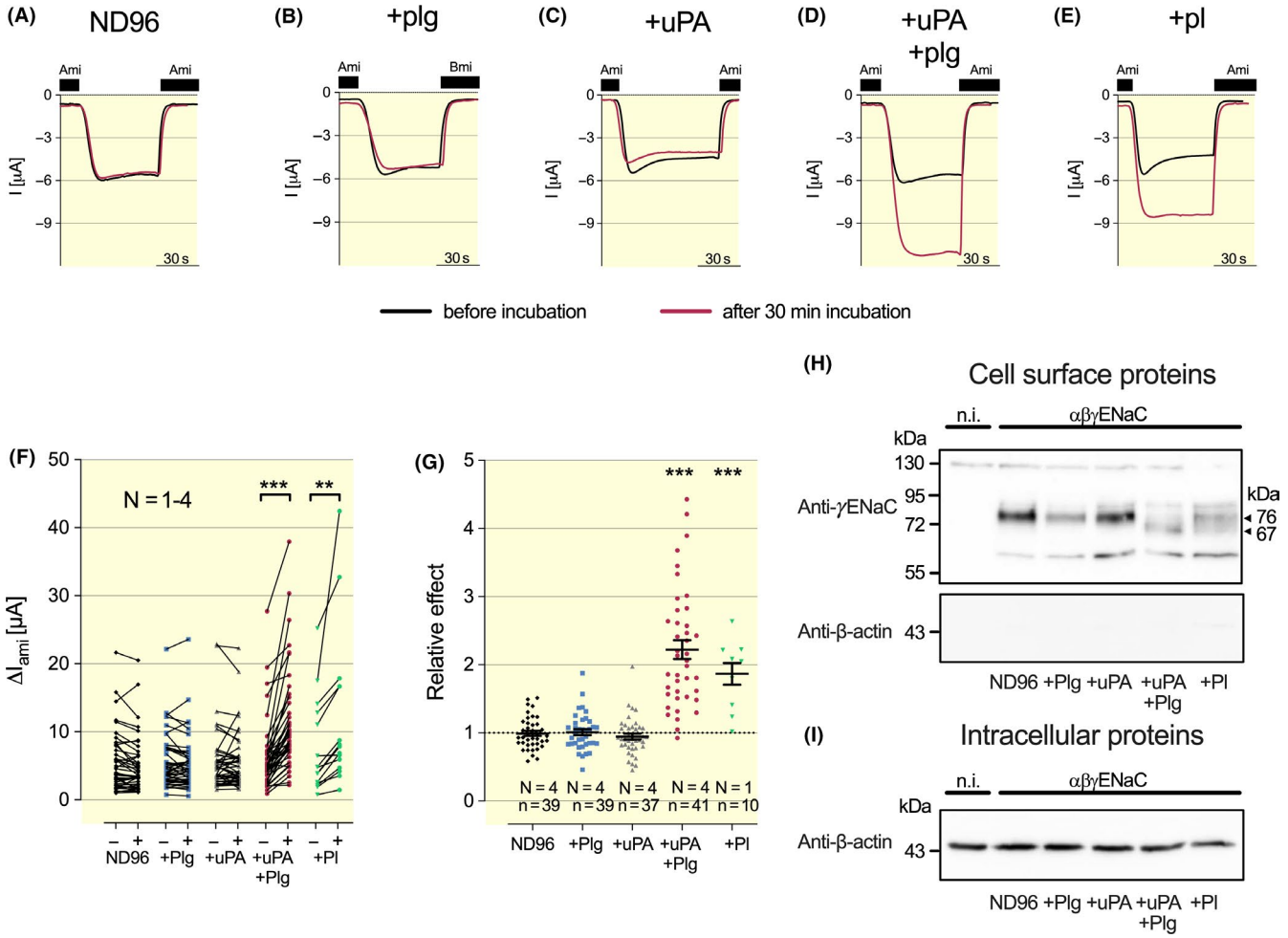

FIGURE 1 A combination of urokinase-type plasminogen activator (uPA) and plasminogen (plg) stimulates murine ENaC in Xenopus laevis oocytes and is associated with cleavage of γ-ENaC. *Xenopus laevis* oocytes expressing murine ENaC were preincubated for 30 min in protease-free vehicle solution (ND96) 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 (ΔI_{ami}) 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 ΔI_{ami} 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 ΔI_{ami}. 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. $^{**}P < .01$, $^{***}P < .001$, significance between indicated groups, paired *t* test (f) or compared to control (ND96), unpaired *t* test (g). Error bars, SEM

2.2 | 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 IC_{50} of 6 ± 1 µM (Figure 2A). By comparison, the IC_{50} of amiloride on ENaC currents is around 0.1 µM.[21,22] After application of amiloride in 2 different doses (5 and 10 µg/g bw ip), urinary amiloride concentration peaked 2-4 hours after injection and exceeded the concentration required for a 90% uPA inhibition (IC_{90}) in mice treated with the higher dose (Figure 2B). Both doses induced similar natriuresis after 4 hours (Figure 2C). Notably, natriuresis was significantly reduced 24 hours after injection pointing to a counterregulation as evidenced by increased aldosterone secretion 10 hours after injection (Figure 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 hours after administration.

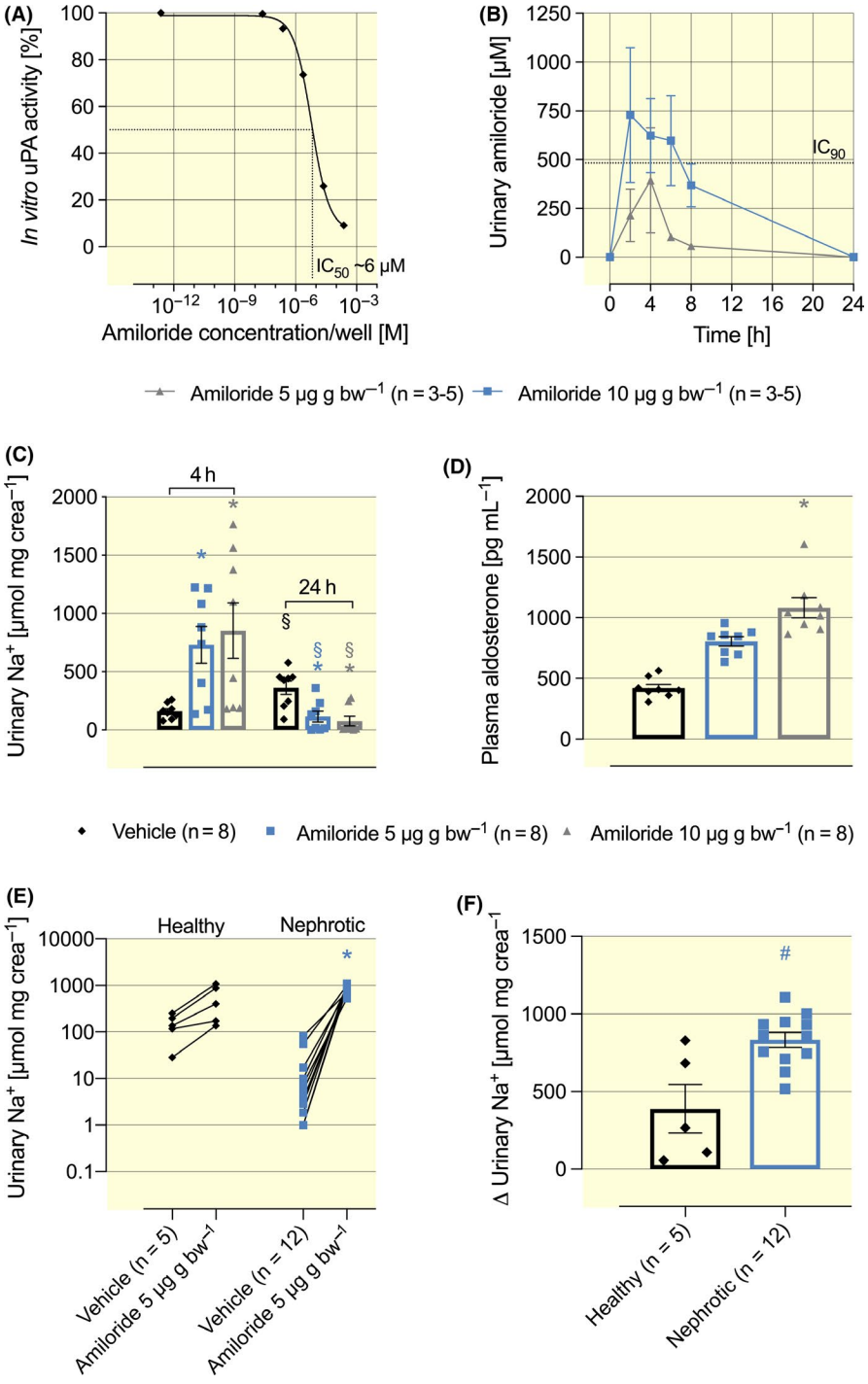

FIGURE 2 Efficacy of amiloride to inhibit uPA and induce natriuresis. A, Inhibition of uPA activity by amiloride in the urine of healthy wild-type mice. Pooled analysis of n = 5 curves from independent urine samples. B, C, Time course of urinary amiloride concentration and urinary sodium excretion after ip injection of 5 and 10 µg·g^{-1} bw amiloride. Between 2 and 4 h, urinary amiloride concentration exceeded the threshold to inhibit almost all uPA activity (IC_{90}) 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 4 and 24 h value

2.3 | 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 (Figure 2E,F). Following doxorubicin injection, mice of all 3 treatment groups developed similar proteinuria (Figure 3A). Body weight was reduced to the same extent in all 3 groups in the first 5-6 days following doxorubicin injection (Figure 3B) because of transiently lower food and fluid intake (Figure S4a,b). Thereafter, vehicle-treated mice gained weight and developed ascites indicating sodium retention.

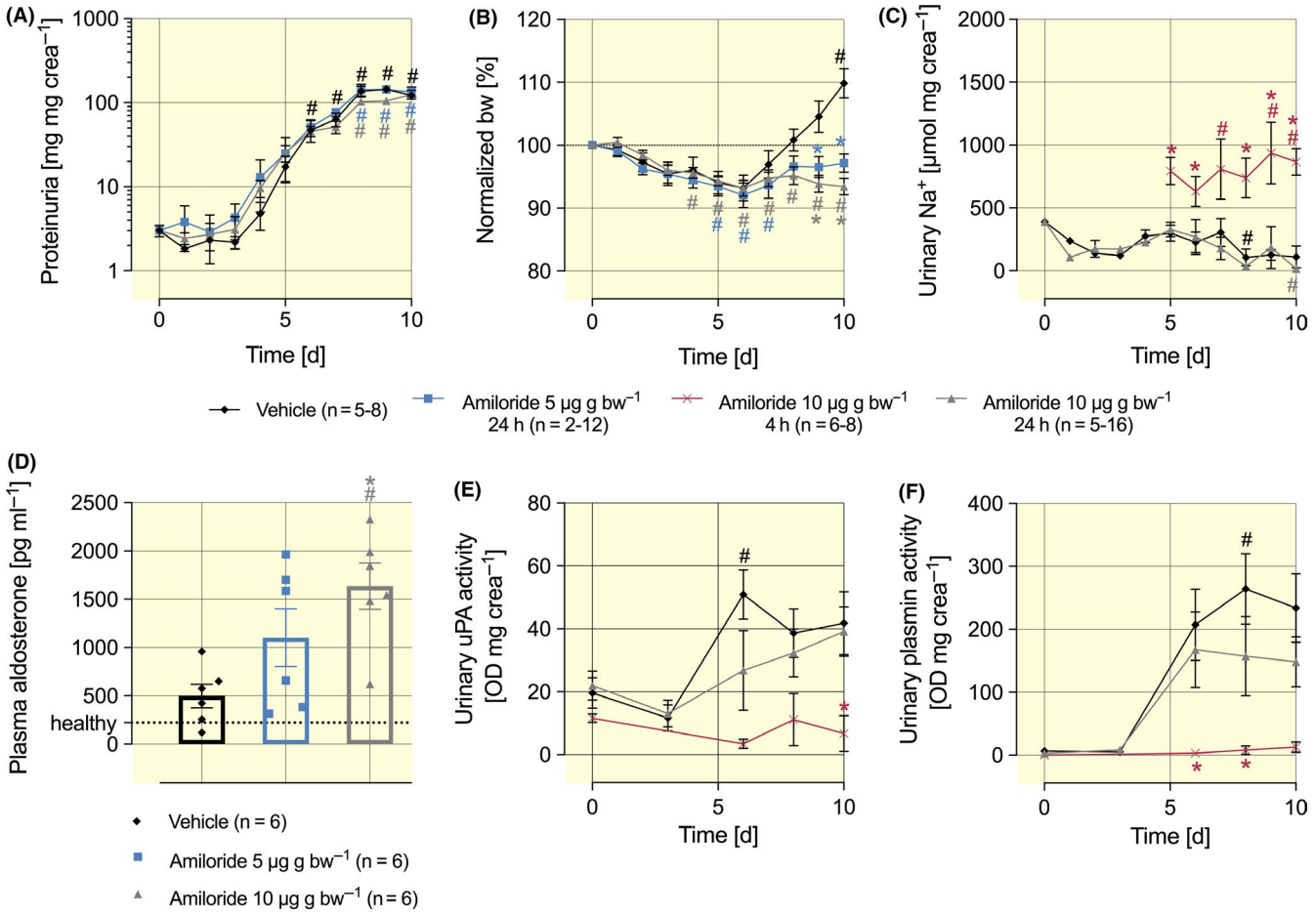

FIGURE 3 Effect of amiloride in experimental nephrotic syndrome. Time course of proteinuria A, normalized body weight B, and urinary sodium excretion C, before and after induction of nephrotic syndrome and treatment with vehicle or amiloride started on day 5 after doxorubicin injection. Urine samples were collected 4 and 24 h after ip injection of vehicle or amiloride (10 µg·g^{-1}). Initial body weight was not different across the groups (range 27.4-28.8 g, $P = .16$). 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 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

This was paralleled by reduced urinary sodium excretion in vehicle-treated mice (Figure 3C) and a positive sodium balance derived from a subset of mice studied in metabolic cages (Figure S5). 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 hours after injection (Figure 3B,C). Compared to healthy mice, plasma aldosterone was increased in vehicle- and to a stronger extent in amiloride-treated nephrotic mice (Figure 3D).

During the course of nephrotic syndrome, urinary uPA activity increased (Figure 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 hours after injection whereas uPA activity returned to normal values in samples taken 24 hours after injection (Figure 3E). uPA-mediated cleavage of plg and formation of active plasmin were analysed 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 proteinuria. In amiloride-treated nephrotic mice, urinary plasmin activity was suppressed in samples taken 4 hours after amiloride-injection and was subsequently normalized in samples taken 24 hours 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 (Figure 4A,B). The 22 kDa light chain harbouring 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 hours after injection (Figure 4A,B) that was again detectable at the end of the dosing interval (Figure 4C,D).

Table 1 shows the effect of experimental nephrotic syndrome on plasma parameters. Compared to healthy wild-type

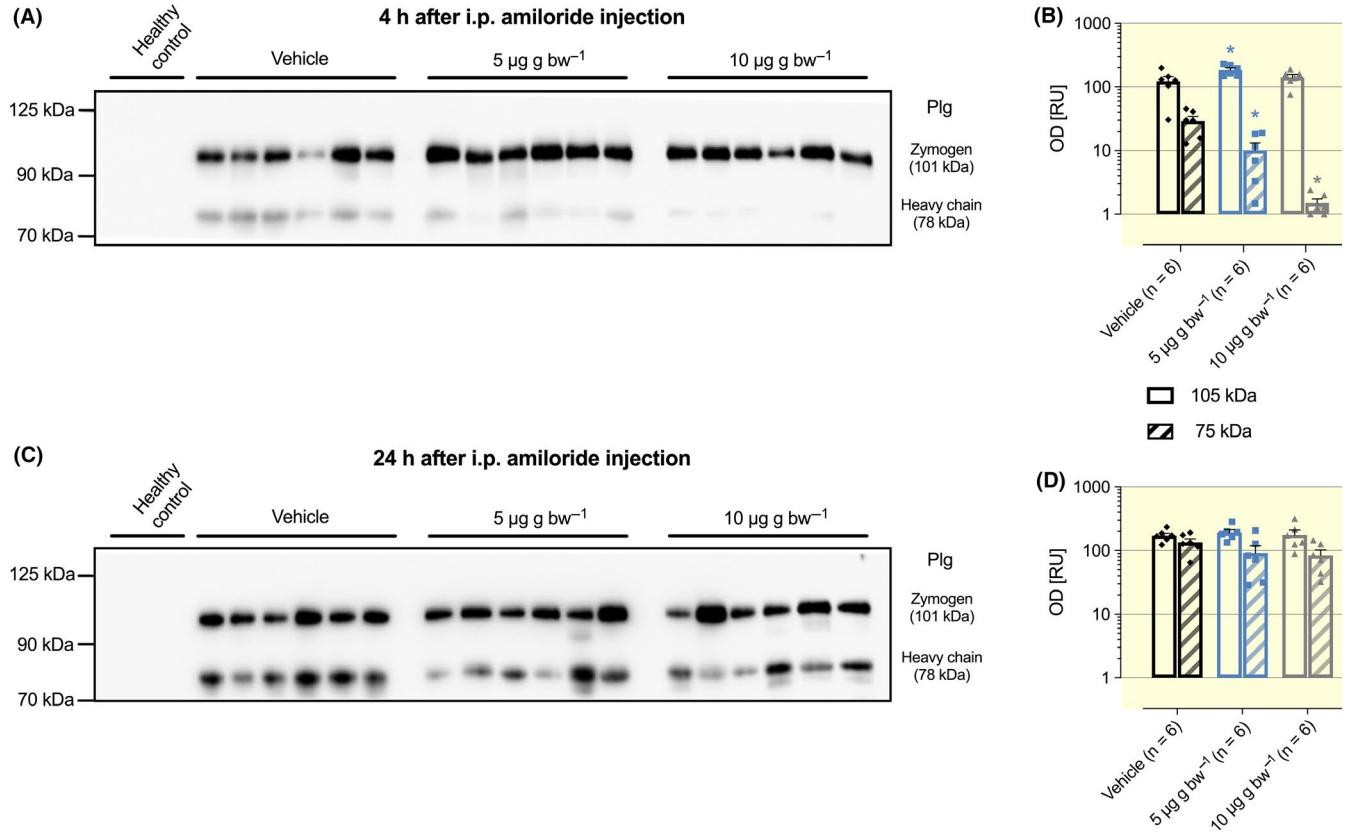

FIGURE 4 Urinary expression of plasminogen and its cleavage product in vehicle- and amiloride-treated nephrotic mice as analysed by Western blot. A,C, Compared to vehicle-treated nephrotic mice, amiloride-treated nephrotic mice excreted only plasminogen zymogen but almost no plasminogen heavy chain in 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

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^+ concentration there 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 ($uPA^{-/-}$).

2.4 | 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 (Figure 5B). Urinary amiloride and sodium excretion after injection of 5 and 10 $\mu g \cdot g^{-1}$ amiloride was not different in $uPA^{+/+}$ and $uPA^{-/-}$ mice (Figure S6a,b) as well as sodium preservation under low salt conditions (Figure S6c). Plasma aldosterone concentration was not different in in $uPA^{+/+}$ vs $uPA^{-/-}$ mice maintained under control, low salt or post-amiloride conditions (Figure S6d). Plg is the main substrate of uPA and might be altered in uPA deficiency. As shown in Figure S7, 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.

2.5 | 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 wild-type littermates ($uPA^{+/+}$). Following doxorubicin injection, all genotypes developed similar proteinuria (Figure 5A). During the course of nephrotic syndrome, urinary uPA activity tended to increase in wild-type $uPA^{+/+}$ mice and

TABLE 1 Plasma parameters of healthy and different groups of nephrotic mice

	Healthy	Healthy	Nephrotic	Nephrotic	Nephrotic	Nephrotic
	Wild-type 129S1/SvImJ	uPA$^{-/-}$	Wild-type/vehicle-treated	Wild-type/amiloride-treated[a]	uPA$^{-/-}$/vehicle-treated	uPA$^{-/-}$/amiloride-treated[b]
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$^{\#,\S}$
std HCO_3^-, mM	22 ± 0.4	23 ± 0.5*	24 ± 1.2	23 ± 0.5	25 ± 1.2	17 ± 1.9$^{\#,\S}$
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$^{\#,\S}$
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$^{\#}$

n.a., not available.

Abbreviations: std, standard; Hct, haematocrit; cHb, calculated hemoglobin concentration.

Arithmetic means ± SEM (n = 3-14 each).

[a]Amiloride dose 10 μg·g^{-1}.

[b]Amiloride dose 5 μg·g^{-1}.

[#]Significant difference between healthy and nephrotic mice.

*Significant difference between genotypes.

[§]Significant difference between amiloride-treated and vehicle-treated nephrotic mice.

uPA$^{+/-}$ mice, however, this did not reach statistical significance (Figure 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 (Figure 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 (Figure S7). 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 (Figure 5D,E). In agreement with the activity assay, plasminogen cleavage was almost not detectable in urine samples from nephrotic *uPA*$^{-/-}$ mice.

Amiloride-sensitive natriuresis was similar in nephrotic *uPA*$^{-/-}$ mice compared to nephrotic *uPA*$^{+/+}$ mice (Figure S8a,b). Importantly, after induction of nephrotic syndrome, daily urinary sodium excretion dropped significantly in all genotypes (Figure 5F) and nephrotic *uPA*$^{+/+}$, *uPA*$^{+/-}$ and *uPA*$^{-/-}$ mice gained body weight to the same extent (Figure 5G). Amiloride treatment of *uPA*$^{-/-}$ mice was similarly effective in preventing sodium retention (Figure 5G), however, *uPA*$^{-/-}$ mice had increased sensitivity to amiloride resulting in weight loss and mortality with the higher dose (10 $\mu g \cdot g^{-1}$). Urinary amiloride concentration was higher in nephrotic *uPA*$^{-/-}$ mice compared to *uPA*$^{+/+}$ mice following the 5 $\mu g \cdot g^{-1}$ dose (Figure S8c) which could be explained by the 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 (Figure 5H).

2.6 | 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 3 bands at 87, 26 and 22 kDa which disappeared after application of the immunogenic peptide (Figure S9). 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, 71 and 45 kDa, but not those at 62 and 50 kDa (Figure S9). The largest bands at 87 and 82 kDa most likely represent full-length α-ENaC and γ-ENaC, respectively, whereas the bands at 26 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 (Figure S9) corresponding to the full length subunit which is not proteolytically processed.

As shown in Figure 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 (Figures 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 (Figure 1H), we could not detect a specific band corresponding to fully cleaved γ-ENaC between 65 and 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 analyse 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 Figure S10 the migration pattern changed leaving only 2 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]

3 | 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 activation.[11] 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 (Figure 1, Figure S1A 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

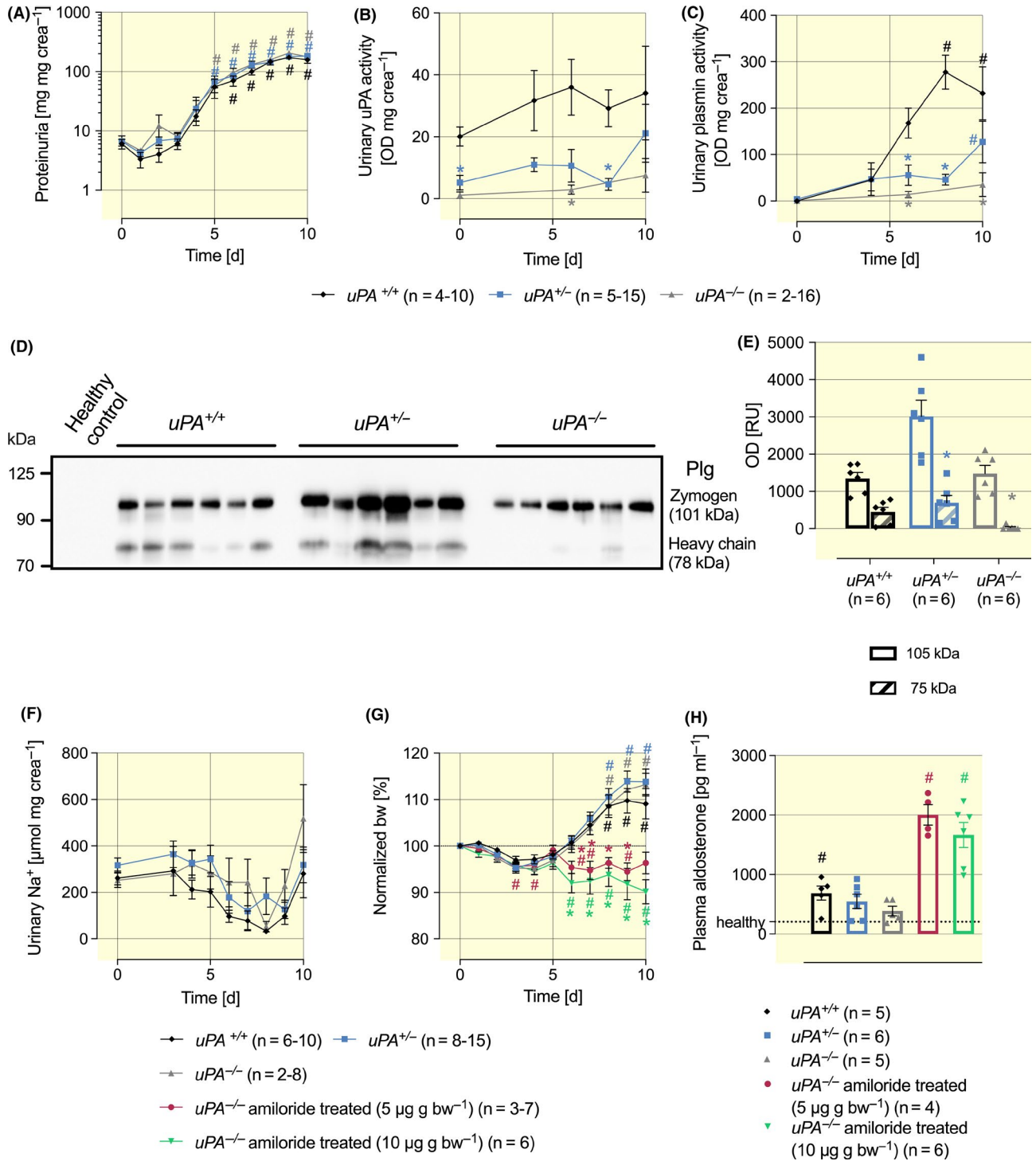

FIGURE 5 Course of experimental nephrotic syndrome in wild-type ($uPA^{+/+}$) and mice with uPA deficiency ($uPA^{+/-}$ and $uPA^{-/-}$). Time course of proteinuria A, urinary uPA B, and urinary plasmin activity C, before and after injection of doxorubicin in wild-type mice ($uPA^{+/+}$) and mice with 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, $P = .33$). *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

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

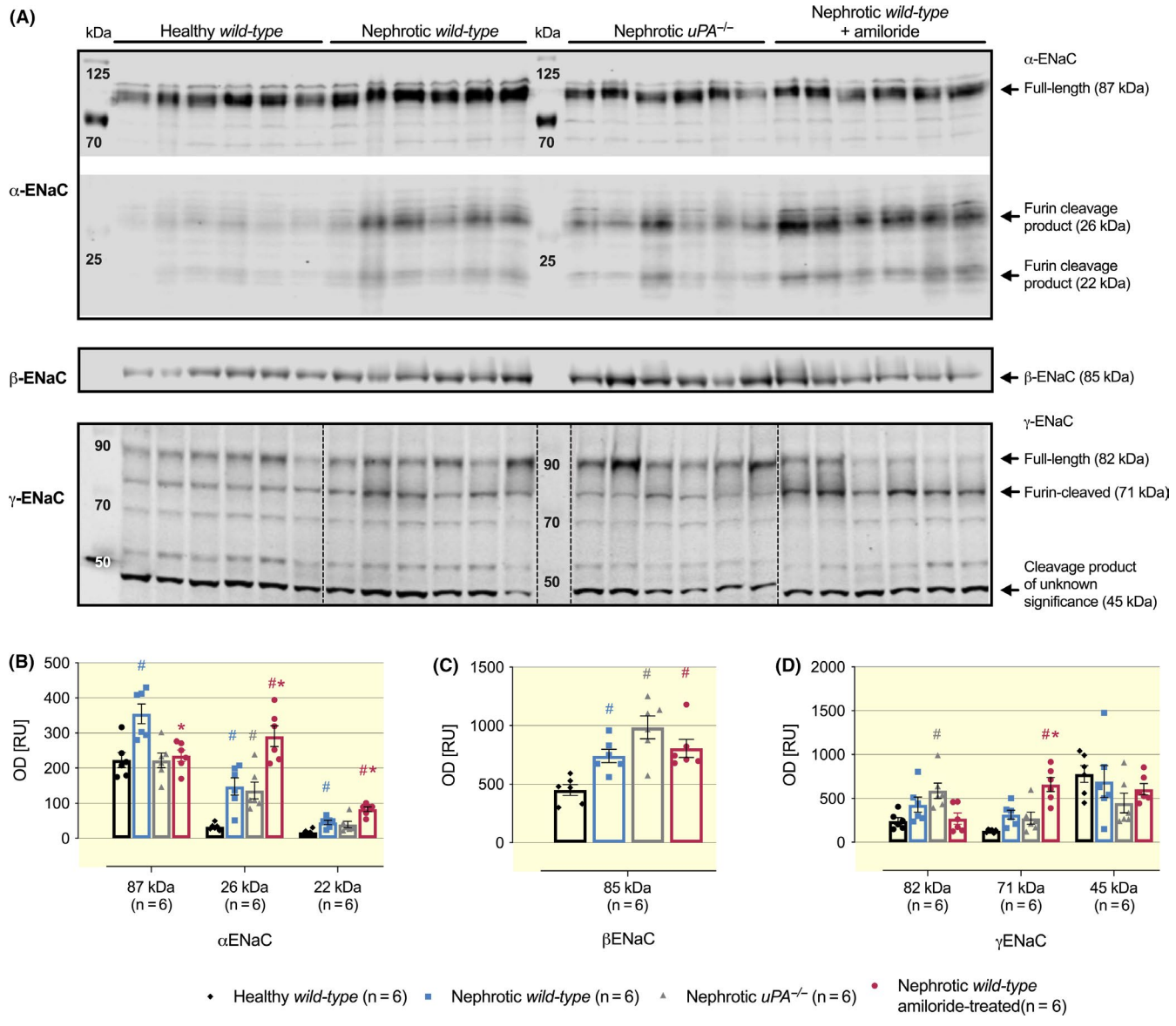

FIGURE 6 Expression of ENaC subunits in healthy, vehicle-treated nephrotic, amiloride-treated nephrotic wild-type and nephrotic *uPA*$^{-/-}$ mice. A, Original Western blots. In the blot showing γ-ENaC, the alignment of the samples was rearranged as indicated by dashed lines. B-D, Densitometric analysis 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

proteases increase the proportion of fully cleaved γ-ENaC at the cell surface of renal tubular cells.

Ji et al. have previously reported that 2-chain uPA, which is the active form of uPA, stimulated human ENaC-mediated currents by about sixfold when used in a concentration of 2000 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 (Figure S1). This makes it unlikely that species differences are responsible for these discrepant observations which we cannot explain at present. Interestingly, the 2-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 (Figures S7 and S27). Results of the present study and those from studies in nephrotic rats provide a rationale for ENaC blockade using amiloride to treat oedema 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 proteinuria 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.

4 | MATERIALS AND METHODS

4.1 | 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 α-, β- and γ-ENaC (0.05 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 2 days after injection using the 2-electrode voltage-clamp technique (TEVC) as described previously.[12,35,36] Amiloride-sensitive whole-cell currents (ΔI_{ami}) 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, ε-aminocaproic acid- and lysine free, Merck), ΔI_{ami} 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 ΔI_{ami}, the oocyte was placed for ~1 minutes in ND96. Subsequently, the oocyte was transferred to 100 μl of protease-supplemented ND96 or ND96 alone as control and incubated for 30 minutes before the second measurement of ΔI_{ami}.

4.2 | Detection of γ-ENaC cleavage products at the cell surface using a biotinylation approach

To detect expression and cleavage of γ-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 γ-ENaC antibody and a secondary horseradish peroxidase-labelled 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]

4.3 | Mouse studies

Experiments were performed on 3-month-old wild-type and uPA deficient ($uPA^{-/-}$) mice of both sex. Imported B6-$Plau^{tm1Mlg-/-}$ 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 hours urine samples over 10 days are not feasible because of 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 2 and 1 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 hours after injection since urinary amiloride concentration showed a peak between 2 and 4 hours 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, M5/16, M15/17, M1/18).

4.4 | Laboratory measurements

Urinary activity of uPA and plasmin was measured using the chromogenic substrates PNAPEP 1344 and S-2251 respectively (Haemochrom, Essen, Germany). About 3-μl urine and 50-μl 2 mM substrate was incubated for 1 hours 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 analysed 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 faecal 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]

4.5 | 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 hours at 37°C according to manufacturer´s instructions (New England Biolabs, Ipswich, USA).

4.6 | 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 γ-ENaC, the primary antibody was preincubated with an immunogenic peptide (20× 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.

4.7 | 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 $< .05$ at 2-tailed testing was considered statistically significant.

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CONFLICT OF INTERESTS

None.

AUTHOR CONTRIBUTIONS

BNB, SD, MW, FS, TS, AI, FB, JCS, AJ, DE performed experiments and prepared the data, SK, HUH, SH, CK analysed 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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SUPPORTING INFORMATION

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