ORIGINAL ARTICLE

Plasma kallikrein activates the epithelial sodium channel in vitro but is not essential for volume retention in nephrotic mice

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Abstract

Aim: Recent work has demonstrated that activation of the epithelial sodium channel (ENaC) by aberrantly filtered serine proteases causes sodium retention in nephrotic syndrome. The aim of this study was to elucidate a potential role of plasma kallikrein (PKLK) as a candidate serine protease in this context.

Methods: We analysed PKLK in the urine of patients with chronic kidney disease (CKD, n = 171) and investigated its ability to activate human ENaC expressed in *Xenopus laevis* oocytes. Moreover, we studied sodium retention in PKLK-deficient mice ($klkb1^{-/-}$) with experimental nephrotic syndrome induced by doxorubicin injection.

Results: In patients with CKD, we found that PKLK is excreted in the urine up to a concentration of 2 μg mL^{-1} which was correlated with albuminuria ($r = .71$) and overhydration as assessed by bioimpedance spectroscopy ($r = .44$). PKLK increased ENaC-mediated whole-cell currents, which was associated with the appearance of a 67 kDa γ-ENaC cleavage product at the cell surface consistent with proteolytic activation. Mutating a putative prostasin cleavage site in γ-ENaC prevented channel stimulation by PKLK. In a mouse model for nephrotic syndrome, active PKLK was present in nephrotic urine of $klkb1^{+/+}$ but not of $klkb1^{-/-}$ mice. However, $klkb1^{-/-}$ mice were not protected from ENaC activation and sodium retention compared to nephrotic $klkb1^{+/+}$ mice.

Conclusion: Plasma kallikrein is detected in the urine of proteinuric patients and mice and activates ENaC in vitro involving the putative prostasin cleavage site. However, PKLK is not essential for volume retention in nephrotic mice.

KEYWORDS
ENaC activation, epithelial sodium channel, plasma kallikrein, proteinuria, proteolytic cleavage, sodium retention

1 | INTRODUCTION

The epithelial sodium channel (ENaC) expressed in the distal nephron plays a decisive role in the maintenance of body sodium homeostasis. A specific feature of ENaC is

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Haerteis and Schork are Shared first-authorship.

its complex proteolytic processing by serine proteases leading to channel activation.[1] Proteolytic cleavage takes place at specific sites within the extracellular loops of the α- and γ-subunit but not the β-subunit. Cleavage at these sites results in the release of inhibitory tracts causing a conformational change of the channel favouring its open state.[2,3] Proteolytic cleavage at three putative furin sites (two in α-ENaC, one in γ-ENaC) probably occurs before the channel reaches the plasma membrane. The final step in proteolytic ENaC activation probably takes place at the plasma membrane where γ-ENaC is cleaved by membrane-bound proteases and/or extracellular proteases in a region distal to the furin site.[4] This correlates with stimulation of ENaC-mediated whole-cell currents and the appearance of a ~67 kDa γ-ENaC cleavage product at the cell surface.[5,6]

Sodium retention, proteinuria and oedema are hallmarks of patients with renal disease, particularly in nephrotic syndrome. Considerable evidence has emerged that aberrantly filtered serine proteases resulting in proteasuria contribute to sodium retention in nephrotic syndrome by proteolytically activating ENaC.[2,7,8] This concept is supported by our recent finding that in mice with experimental nephrotic syndrome treatment with the serine protease inhibitor aprotinin prevented sodium retention.[9] Currently, plasmin is thought to be the main serine protease responsible for ENaC activation during nephrotic syndrome.[2,7,10-12] Plasmin is formed by conversion of plasminogen by urokinase-type plasminogen activator (uPA) that is expressed in the tubular epithelium.[11,13] In patients with chronic kidney disease (CKD), plasminuria was shown to correlate with extracellular water and overhydration.[14] Both plasmin and uPA are sensitive to aprotinin[15]; however, other aprotinin-sensitive serine proteases could have a role here as well.

We hypothesized that plasma kallikrein (PKLK), an aprotinin-sensitive serine protease released into the circulation by the liver, may contribute to proteolytic ENaC activation in nephrotic syndrome. PKLK shares similarity with tissue kallikrein 1 that has been shown to be involved in proteolytic ENaC regulation.[16,17] In the healthy state, PKLK can cleave prorenin to form active renin which is expected to stimulate ENaC indirectly via hyperaldosteronism.[18] However, in proteinuric kidney disease, PKLK may be aberrantly filtrated and thus may activate ENaC by direct cleavage at the putative prostasin/tissue kallikrein site RKRK178-181.[16,19] Alternatively, PKLK could activate plasminogen and stimulate ENaC by cleavage at the putative plasmin site at K189 of γ-ENaC.[6]

In the present investigation, we aimed to define the role of PKLK in ENaC-mediated sodium retention in proteinuric renal disease and nephrotic syndrome. We analysed urine samples from patients with CKD of various aetiologies and stages for excretion of PKLK. We then correlated PKLK excretion with extracellular volume status and

overhydration as a surrogate of sodium retention. We further investigated the ability of PKLK to activate/cleave human ENaC heterologously expressed in *Xenopus laevis* oocytes. Finally, we studied ENaC activation in PKLK-deficient mice ($klkb1^{-/-}$) with experimental nephrotic syndrome induced by doxorubicin injection.

2 | RESULTS

2.1 | Active PKLK is found in urine of patients with CKD and associates with sodium retention

We studied 171 patients with stable CKD of various aetiologies and stable disease who presented consecutively to our outpatient centre and 10 inpatients with the acute nephrotic syndrome (NS). Fifteen healthy candidates for living related kidney donation were included as control group. In patients with CKD, median proteinuria was 520 mg g^{-1} creatinine (interquartile range (IQR) 146; 1485) and reached up to 6660 mg g^{-1} creatinine (IQR 5379; 8023) in patients with NS (Table 1). Compared to healthy persons, patients with CKD had increased extracellular water (ECW) and overhydration (OH) as determined from bioimpedance spectroscopy (Table 1). Patients with NS had highest values for ECW and OH.

In all samples, urinary excretion of PKLK was determined using ELISA and proteolytic activity measured with a chromogenic substrate. Median urinary PKLK concentration in 171 patients with CKD was 0.005 µg mL^{-1} (range: 0-2.1). Normalization to urinary creatinine yielded a median value of 11 µg g^{-1} crea (range: 0-3064). Urinary excretion of PKLK correlated strongly with proteinuria ($r = .75$, Figure 1A) and albuminuria ($r = .71$). In the urine of patients with nephrotic syndrome, Western blot revealed PKLK zymogen at 82 kDa and a low molecular weight band at 36 kDa (Figure 1B). The latter band corresponds to the light chain of PKLK indicating PKLK cleavage and dissociation from the heavy chain under reducing conditions. Using a chromogenic substrate, PKLK activity was detectable in 30% of the patients with CKD, but not in healthy control persons (median 0.07, range: 0-43.5 RU g^{-1} creatinine, Figure 1C) and correlated with proteinuria ($r = .44$, Figure 1D) and PKLK excretion measured with an ELISA ($r = .4116$ $P < .0001$). Patients with NS showed highest urinary PKLK concentration (median 0.12 µg mL^{-1}, range: 0.027-1.13) and activity (median 0.57 RU g^{-1} creatinine, range: 0-61.2) which was detectable in 60% (Figure 1C). The correlation of PKLK excretion with PKLK activity was $r = .4116$ ($P < .0001$).

There was a strong univariate relationship between urinary PKLK concentration and ECW as well as OH (Figure 1E,F). In multivariate regression analysis, urinary

TABLE 1 Characteristics of the study cohort

	Healthy (n = 15)	CKD patients (n = 171)	Patients with acute nephrotic syndrome (n = 10)	ANOVA P
Median age, years	50 (42; 62)	60 (48; 72)	53 (41; 69)	.1813
Gender distribution	73% ♀/27% ♂	42% ♀/58% ♂	40% ♀/60% ♂	.0630
BMI (kg m^{-2})$^{-1}$	29.2 (25.5; 30.4)	28.7 (25.6; 32.1)	23.3 (20.5; 26.3)	.0224
MDRD-GFR, mL min 1.73 m^{-2}	>60	45 (30; 68)	35 (20; 69)	<.0001
Proteinuria, mg g^{-1} crea	95 (77; 185)	520 (146; 1485)	6660 (5379; 8023)	<.0001
Albuminuria, mg g^{-1} crea	14 (7; 16)	110 (29; 850)	6050 (4841; 7568)	<.0001
Serum aldosterone, pg mL^{-1}	154 (120; 196)	119 (86; 167)	63 (7; 118)	.0015
Extracellular water (ECW), L 1.73 m^{-2}	14.8 (14.1; 15.5)	15.8 (14.7; 16.9)	18.0 (16.6; 19.4)	<.0001
Intracellular water (ICW), L 1.73 m^{-2}	17.4 (16.6; 18.8)	17.7 (16.2; 19.5)	18.3 (16.3; 19.8)	.9552
Overhydration (OH), L 1.73 m^{-2}	0 (−0.9; +0.2)	+0.2 (−0.5; +1.2)	+3.8 (+1.5; +5.7)	<.0001
ECW/ICW	0.85 (0.80; 0.88)	0.89 (0.82; 0.98)	1.03 (0.91; 1.12)	.0021
Diagnoses	n.a.	Diabetic/hypertensive nephropathy and glomerulo-sclerosis (36%), glomerulonephritis (35%), interstitial disease (2%), polycystic kidney disease (4%), unknown (23%)	Membranous glomerulonephritis (30%), IgA glomerulonephritis (30%), membranoproliferative glomerulonephritis (10%), thrombotic microangiopathy (10%), microscopic polyangiitis (10%), unknown (10%)	

n.a., not applicable.

Values are medians with interquartile range.

PKLK excretion was an independent predictor of OH similar to proteinuria; however, the influence of PKLK was not independent of proteinuria (Table 2) or albuminuria (data not shown).

2.2 | PKLK stimulates ENaC currents in Xenopus laevis oocytes expressing human ENaC

To study whether PKLK may stimulate sodium retention *via* activation of ENaC, we performed two-electrode voltage-clamp measurements using *X. laevis* oocytes heterologously expressing human ENaC. We determined amiloride-sensitive whole-cell currents (ΔI_{ami}) in each individual oocyte twice, that is before and after a 4-hours exposure to protease-free solution (Figure 2A,D), to ascending concentration of PKLK (Figure 2B,D), or to PKLK plus the selective PKLK inhibitor PKSI-527 (Figure 2C,D). Basal ΔI_{ami} values of all groups were of similar size. In control experiments, incubation in protease-free solution had a negligible effect on ENaC currents (Figure 2A,D). Exposure of the oocytes to PKLK-527 alone had no effect on ENaC currents (1.20 μA vs 1.15 μA, $P = .58$). A stimulatory effect on ΔI_{ami} was also observed

after 30-minutes exposure to 13.4 μg mL^{-1} PKLK (data not shown). Exposure to PKLK increased ΔI_{ami} in a concentration-dependent manner (Figure 2D). The effect of PKLK was prevented by PKSI-527 (Figure 2C,D).

2.3 | Activation of ENaC currents by PKLK with concomitant appearance of a γENaC cleavage product at the cell surface is prevented by mutating a putative prostasin cleavage site

The observed stimulation of ENaC currents by PKLK is likely to be the result of proteolytic cleavage occurring in the γ-subunit of the channel. To identify the site of PKLK cleavage, we used oocytes expressing wild-type α- and β-ENaC together with a γ-ENaC subunit mutated at the putative prostasin cleavage site (γRKRK178AAAA, Figure 3A). To investigate γ-ENaC fragments at the cell surface, a biotinylation approach was used.[20] ENaC-expressing oocytes (wild-type or αβγ$_{RKRK178AAAA}$) were treated for 4 hours with protease-free solution (control), or PKLK, or PKLK+PKSI-527, or plasmin (Figure 3B). Subsequently, the biotinylated γ-ENaC cleavage products were detected by Western blot (Figure 3B). In non-injected
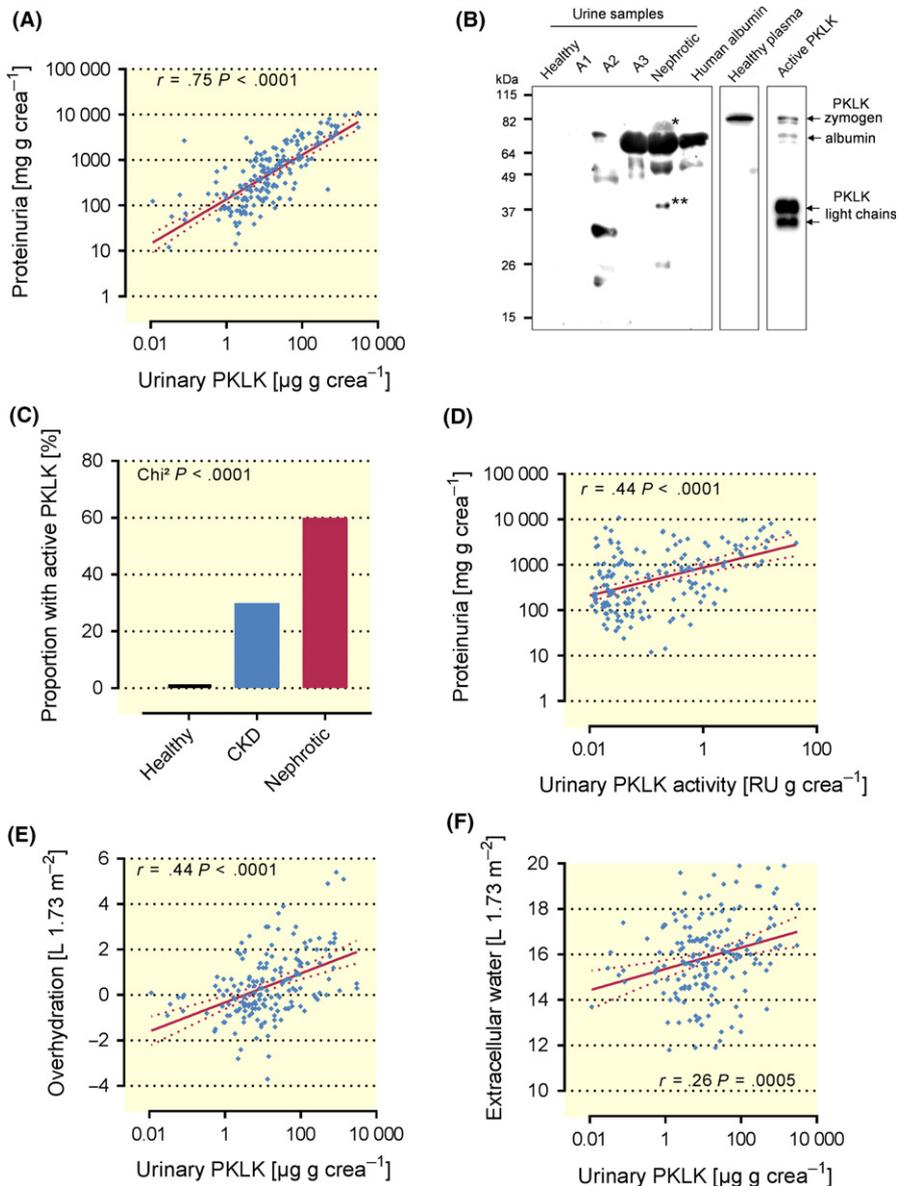
FIGURE 1 Urinary excretion of PKLK in CKD patients. (A) Correlation of the urinary excretion of PKLK with proteinuria as quantified with ELISA. (B) SDS-PAGE from a representative sample of the patient cohort including urine of a healthy control person, CKD patients with albuminuria stages A1 (<30 mg g^{-1} creatinine), A2 (30-300 mg g^{-1} creatinine), A3 (>300 mg g^{-1} creatinine) and nephrotic syndrome (NS). Only in a patient with NS PKLK is detectable as zymogen at 82 kDa (*) and at 36 kDa (**) corresponding to the light chain of PKLK. In plasma, there is only PKLK zymogen, whereas in activated PKLK only light chains at 32 and 36 kDa are visible. For optimal signal quality, WB membranes containing urine, plasma samples and positive control were developed separately. The antibody also cross-reacts with human albumin. (C) Percentage of subjects with active PKLK in healthy persons, CKD patients and patients with the acute nephrotic syndrome. (D) Correlation of urinary PKLK activity with proteinuria. (E, F) Correlation of urinary PKLK excretion with overhydration and extracellular water

oocytes, γ-ENaC-specific signals were absent (Figure 3B). The predominant γ-ENaC fragment detected at the cell surface of untreated αβγ−ENaC-expressing oocytes had a molecular mass of ~76 kDa (Figure 3B) which is the result of cleavage by endogenous proteases like furin at the so-called furin cleavage site, R138 (Figure 3A). Addition of PKLK leads to the appearance of a ~67 kDa γ-ENaC fragment reflecting additional cleavage distal to the furin site. This effect was blocked by PKSI-527 and resembled the previously described effect of plasmin[6,21] which served as control (Figure 3B). The appearance of the ~67 kDa γ-ENaC cleavage fragment was paralleled by an increase in ENaC currents (Figure 3C). Importantly, mutating the putative prostasin site prevented the appearance of the ~67 kDa γ-ENaC cleavage fragment and the stimulatory effect of PKLK on ENaC currents (Figure 3B,C). Interestingly, proteolytic ENaC activation by plasmin was preserved in the

mutant channel, which indicates that the putative plasmin cleavage site remained functional.

2.4 | Activation of ENaC by PKLK is amplified in the presence of plasminogen

Plasma kallikrein is capable of generating plasmin from plasminogen.[22,23] In this case, the plasmin cleavage site K189 in γ-ENaC may contribute to proteolytic ENaC activation.[6] Therefore, we tested the effect of adding plasminogen in the presence of PKLK on ENaC cleavage and currents (Figure 4). Exposure of wild-type ENaC-expressing oocytes to a combination of PKLK and plasminogen revealed a similar γ-ENaC cleavage fragment pattern as with PKLK alone (Figure 4A). There was a small but significant increase in ENaC current stimulation compared to PKLK alone (5.6-fold vs 7.0-fold, Figure 4B). This

TABLE 2 Determinants of overhydration in CKD patients (n = 171) as determined by multiple linear regression analysis

Covariate	Model 1 Adjusted r^2 = .48 P < .0001		Model 2 Adjusted r^2 = .46 P < .0001		Model 3 Adjusted r^2 = .48 P < .0001	
	Coefficient[a] (lower 95% to upper 95%)	P-value	Coefficient[a] (lower 95% to upper 95%)	P-value	Coefficient[a] (lower 95% to upper 95%)	P-value
y-Intercept	3.76 (0.55 to 6.97)	.0291	4.70 (1.48 to 7.91)	.0045	3.81 (0.54 to 7.07)	.0226
corrected plasma NT-pro-BNP concentration, log pg mL^{-1}	0.65 (0.36 to 0.94)	<.0001	0.66 (0.36 to 0.95)	<.0001	0.64 (0.36 to 0.94)	<.0001
Oedema (1 = present)	0.48 (0.29 to 0.67)	<.0001	0.51 (0.31 to 0.70)	<.0001	0.48 (0.28 to 0.67)	<.0001
BMI, log kg m^{-2}	−4.14 (−6.15 to 2.13)	.0001	−3.96 (−6.05 to 1.87)	.0003	−4.10 (−6.16 to 2.04)	.0001
Plasma renin activity, log ng Ang I mL^{-1} h^{-1}	−0.20 (−0.47 to 0.07)	.1483	−0.15 (−0.43 to 0.13)	.2839	−0.20 (−0.47 to 0.08)	.1576
Proteinuria, log mg g^{-1} crea	0.58 (0.30 to 0.85)	<.0001	—		0.55 (0.16 to 0.94)	.0060
Urinary PKLK excretion, log μg g^{-1} crea	—		0.29 (0.09 to 0.48)	.0033	0.03 (−0.24 to 0.29)	.8421

Selection of the variables entering the model was derived from forward stepwise multiple linear regression of parameters that were univariately correlated with the dependent variable and had a P < .05. In the models 1 and 2, proteinuria and urinary PKLK excretion were entered separately as they were closely related to each other. In model 3, both parameters were entered simultaneously. The results show that urinary PKLK excretion is a similar predictor of overhydration as is proteinuria; however, it is not independently associated with overhydration.

[a]The coefficient (or slope) is the value by which overhydration is increased when the respective covariate increases by one unit.

suggests that plasmin generated from plasminogen may enhance the stimulatory effect of PKLK. Exposure to plasminogen alone had no significant effect on ENaC currents (1.9 μA vs 2.1 μA, P = .35,[11]). Interestingly, in oocytes expressing γ-ENaC mutated at the putative prostasin cleavage site (γRKRK178AAAA), the combination of PKLK and plasminogen led to a significant increase in ENaC-mediated currents and the appearance of a faint γ-ENaC cleavage fragment of ~67 kDa (Figure 4A,B). The observed shift from the 76 to the 67 kDa band was not as complete as with plasmin (Figure 4A) consistent with the smaller ENaC current activation by PKLK/plasminogen compared to plasmin (Figure 3B). Nevertheless, these data suggest that PKLK-mediated plasmin generation can at least partially activate the mutant channel by cleaving at the preserved plasmin cleavage site.

2.5 | Mice lacking PKLK (*klkb1*$^{-/-}$) are not protected from volume retention in experimental nephrotic syndrome

To determine whether PKLK participates in oedema formation in vivo, we studied the course of experimental nephrotic syndrome in mice lacking PKLK (*klkb1*$^{-/-}$) and their wild-type littermates (*klkb1*$^{+/+}$). Following doxorubicin injection, *klkb1*$^{-/-}$ mice developed similar proteinuria on day 8 as wild-type mice (*klkb1*$^{+/+}$, Figure 5A). In Western blot analysis from urine samples of healthy *klkb1*$^{+/+}$ mice, PKLK zymogen was faintly detectable at 88 kDa

(Figure 5C). In contrast, in urine samples from nephrotic *klkb1*$^{+/+}$ mice, PKLK appeared with multiple cleavage products between 30 and 88 kDa (Figure 5C). The bands at 30 and 52 kDa most likely represent the light and heavy chain of PKLK, respectively, after dissociation of the disulphide bond under reducing conditions. In healthy plasma from *klkb1*$^{+/+}$ mice, PKLK was mainly detectable at 88 kDa which was absent in *klkb1*$^{-/-}$ mice. Using chromogenic substrate, we found that PKLK activity increased in *klkb1*$^{+/+}$ mice after induction of proteinuria (Figure 5B) paralleling the appearance of low molecular bands in the Western blot.

Under control conditions, urinary sodium excretion normalized for creatinine and ENaC-mediated sodium reabsorption as determined after amiloride injection was not different between *klkb1*$^{+/+}$ and *klkb1*$^{-/-}$ mice (Figure 6A, B). After induction of nephrotic syndrome, urinary sodium excretion dropped in both genotypes (Figure 6A) and was accompanied by a significantly increased response to amiloride (Figure 6B) indicating ENaC activation. Overall, ENaC-mediated sodium reabsorption was not different between *klkb1*$^{+/+}$ and *klkb1*$^{-/-}$ mice in both the healthy and nephrotic state (Figure 6B). Nephrotic *klkb1*$^{+/+}$ and *klkb1*$^{-/-}$ mice gained body weight to the same extent (+20.1 ± 2.4% vs +20.1 ± 2.6%, P > .05, Figure 6C) despite normal food and fluid intake (Figure 6D). Plasma parameters showed the development of marked hypoalbuminemia and hyperaldosteronism in nephrotic *klkb1*$^{+/+}$ and *klkb1*$^{-/-}$ mice compared to healthy controls; however, there was no difference between the genotypes (Table 3).
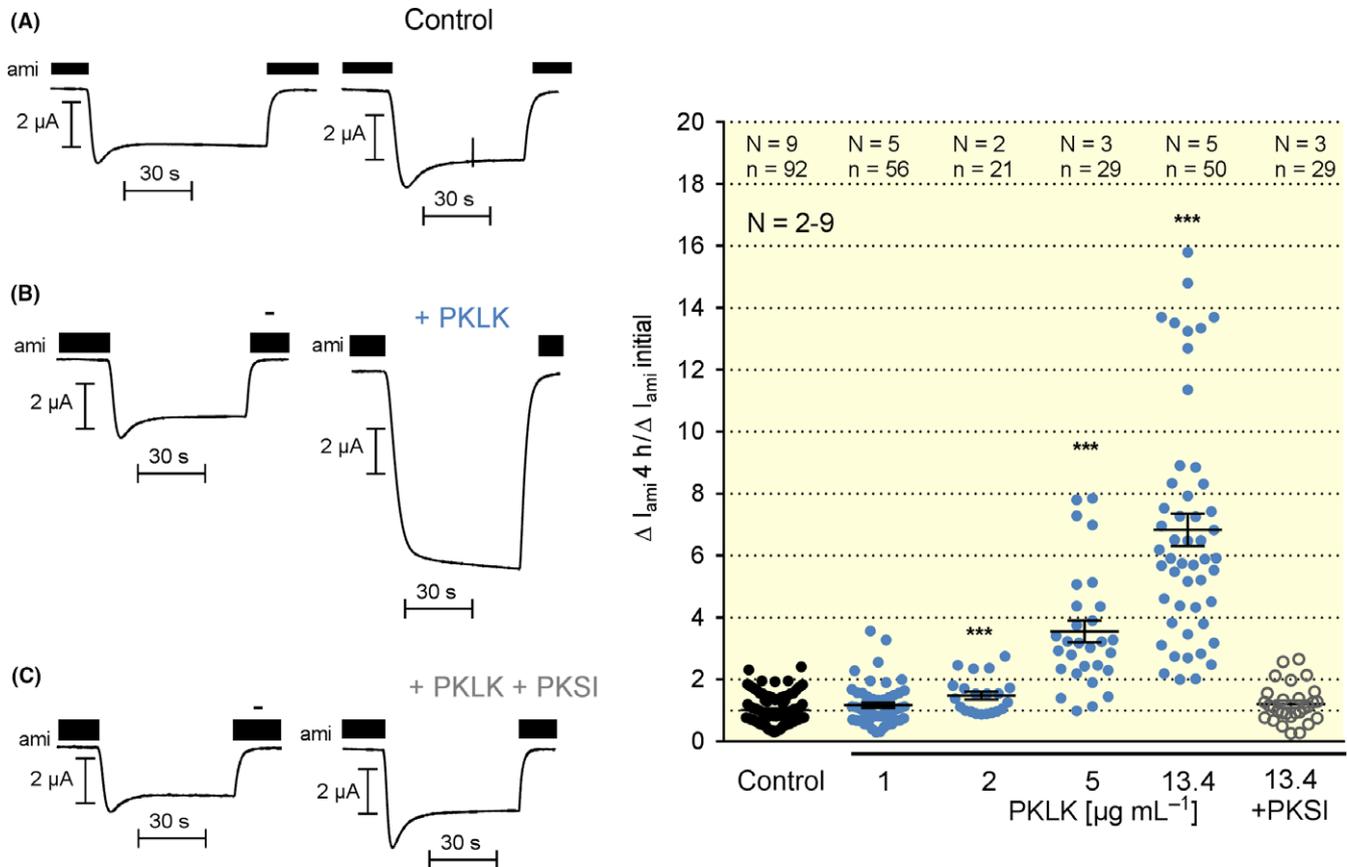
FIGURE 2 Stimulation of ENaC-mediated whole-cell currents by PKLK. *Xenopus laevis* oocytes expressing human $\alpha\beta\gamma$-ENaC were preincubated for 4 hours in protease-free solution (control) or in a solution containing PKLK or PKLK + PKSI. Amiloride-sensitive whole-cell currents (ΔI_{ami}) were determined before ($-$) and after ($+$) incubation. (A-C) Six representative whole-cell current traces from one batch of oocytes are shown. Amiloride (ami) was present in the bath solution to specifically inhibit ENaC, as indicated by black bars. (D) Individual values representing the relative stimulatory effect of PKLK on ΔI_{ami} calculated as the ratio of ΔI_{ami} measured after 4-hours incubation (ΔI_{ami} 4 hours) to the initial ΔI_{ami} (ΔI_{ami} initial) measured before incubation. N indicates the number of different batches of oocytes, n the numbers of individual oocytes measured. ***$P < .001$, unpaired t test. Error bars, SE

In addition, haematocrit, haemoglobin and plasma sodium concentration dropped in nephrotic mice reflecting dilution of plasma volume.

To analyse the contribution of PKLK to plasminogen activation in vivo, urinary plasmin activity and expression of plasminogen were analysed using chromogenic substrate and Western blot respectively. As shown in Figure 7A-C, there was no difference in either urinary plasmin activity or expression of plasminogen zymogen or plasminogen heavy chain between the genotypes arguing against a major contribution of PKLK to plasminogen cleavage/activation in vivo. The expression of γ-ENaC and its cleavage products was analysed by Western blot analyses from kidney cortex of healthy and nephrotic mice. In wild-type ($klkb1^{+/+}$) mice, multiple bands were obtained at 81, 69, 63, 50 and 45 kDa (Figure 8A,B). Application of the immunogenic peptide specifically blocked those at 81 kDa, 69 and 45 kDa, but not those at 63 and 50 kDa. The bands at 81 kDa and 69 kDa most likely represent full-length and

furin-cleaved γ-ENaC, respectively, whereas the band at 45 kDa represents a cleavage product of unknown significance. The expression pattern was not different in healthy $klkb1^{-/-}$ mice. In nephrotic $klkb1^{+/+}$ and $klkb1^{-/-}$ mice, there was a tendency towards increased expression of the bands at 81 kDa and 69 kDa, presumably as a result of hyperaldosteronism (Figure 8A,B).

3 | DISCUSSION

Our study reveals the following three novel findings: first, it demonstrates that PKLK is excreted in the urine of patients and mice with kidney disease following a close correlation to proteinuria. Secondly, PKLK was shown to stimulate ENaC currents in vitro through proteolysis of γ-ENaC at the putative prostasin/tissue kallikrein cleavage site RKRK178. The stimulatory effect of PKLK on ENaC activity and the concomitant appearance of a γ-ENaC

(A)

(B)

(C)
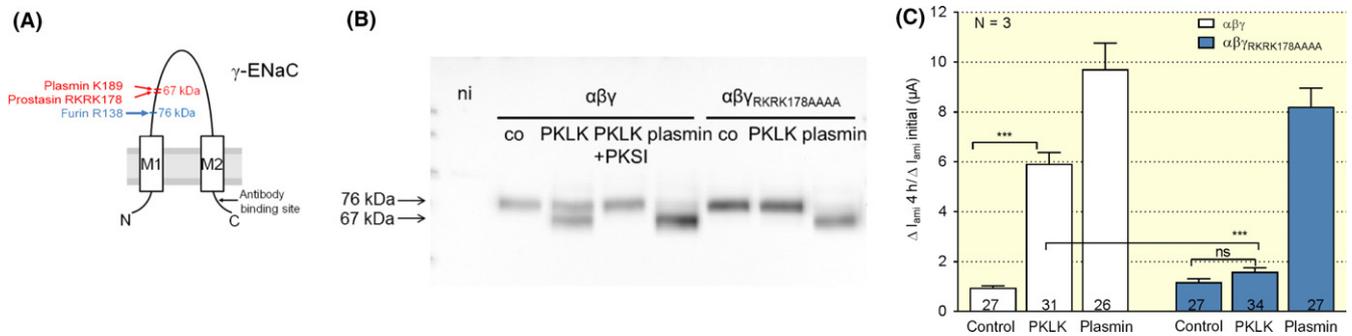
FIGURE 3 PKLK activates ENaC by cleaving γ-ENaC at the putative prostasin cleavage site. Oocytes expressing αβγ- or αβγ$_{RKRK178AAAA}$ENaC were preincubated for 4 hours in protease-free solution (control) or in solution containing either PKLK (13.4 μg mL^{-1}) −/+ PKSI or plasmin. (A) Model of the human γ-ENaC subunit showing cleavage sites for proteolytic activation and the binding site of the antibody used. (B) Expression of biotinylated γ-ENaC at the cell surface was analysed by SDS-PAGE. γ-ENaC was detected with an antibody against the C-terminus of human γ-ENaC. Representative Western blot from one batch of oocytes. (C) In parallel to the detection of the γ-ENaC cleavage fragments, ΔI_{ami} was measured. ni, non-injected oocytes. N indicates the number of different batches of oocytes. The numbers inside the columns indicate the number of individual oocytes measured. ***$P < .001$, unpaired t test. Error bars, SE

(A)

(B)
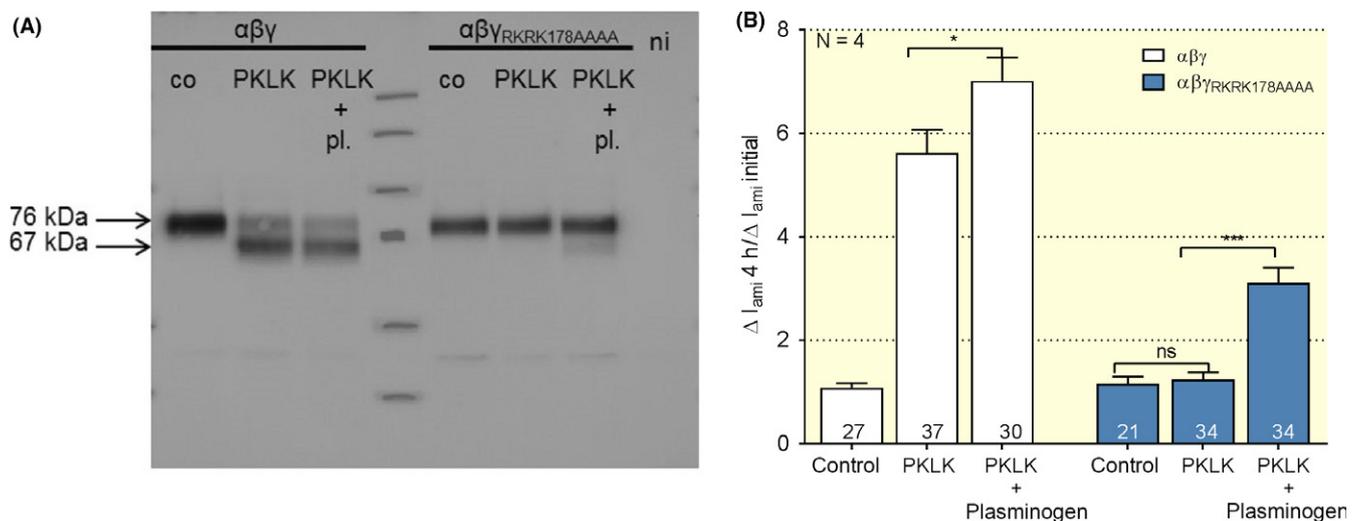
FIGURE 4 Activation of ENaC by PKLK is amplified in the presence of plasminogen. Oocytes expressing αβγ- or αβγ$_{RKRK178AAAA}$ENaC were preincubated for 4 hours in protease-free solution (control) or in solution containing either PKLK (13.4 μg mL^{-1}) or PKLK + plasminogen (pl, 0.5 U mL^{-1}). (A) Expression of biotinylated γ-ENaC at the cell surface was analysed by SDS-PAGE. γ-ENaC was detected with an antibody against the C-terminus of human γ-ENaC. Representative Western blot from one batch of oocytes. (B) In parallel to the detection of the cleavage fragments, ΔI_{ami} was measured. ni, non-injected oocytes. N indicates the number of different batches of oocytes. The numbers inside the columns indicate the number of individual oocytes measured. *$P < .1$; ***, $P < .001$, unpaired t test. Error bars, SE

cleavage product at the cell surface were prevented by mutating this cleavage site and by the PKLK inhibitor PKSI-527. Thirdly, nephrotic mice with PKLK deficiency were not protected from oedema formation. This latter finding indicates that PKLK is not essential for mediating sodium retention and that other serine proteases or mechanisms cause proteolytic ENaC activation and sodium retention in this model for nephrotic syndrome.

From a quantitative perspective, PKLK excretion in the urine was markedly lower compared to, for example, plasmin that is thought to have a pivotal role in ENaC activation in nephrotic syndrome.[11] In the present study, urinary

PKLK concentrations in patients with CKD were 5- to 10-fold lower than plasmin(ogen) concentrations reported in a previous study with the same cohort.[14] This difference is only partially explained by the lower plasma concentration of PKLK (50 μg mL^{-1})[24] compared to plasminogen (100 μg mL^{-1})[25] and therefore may be due to difference in sieving through the damaged glomeruli. An explanation could be the fact that PKLK forms a complex with high molecular weight kininogen that hinders glomerular filtration. In patients with CKD, urinary PKLK concentration seems to be lower than required for channel activation in ENaC-expressing oocytes. However, in nephrotic mice,

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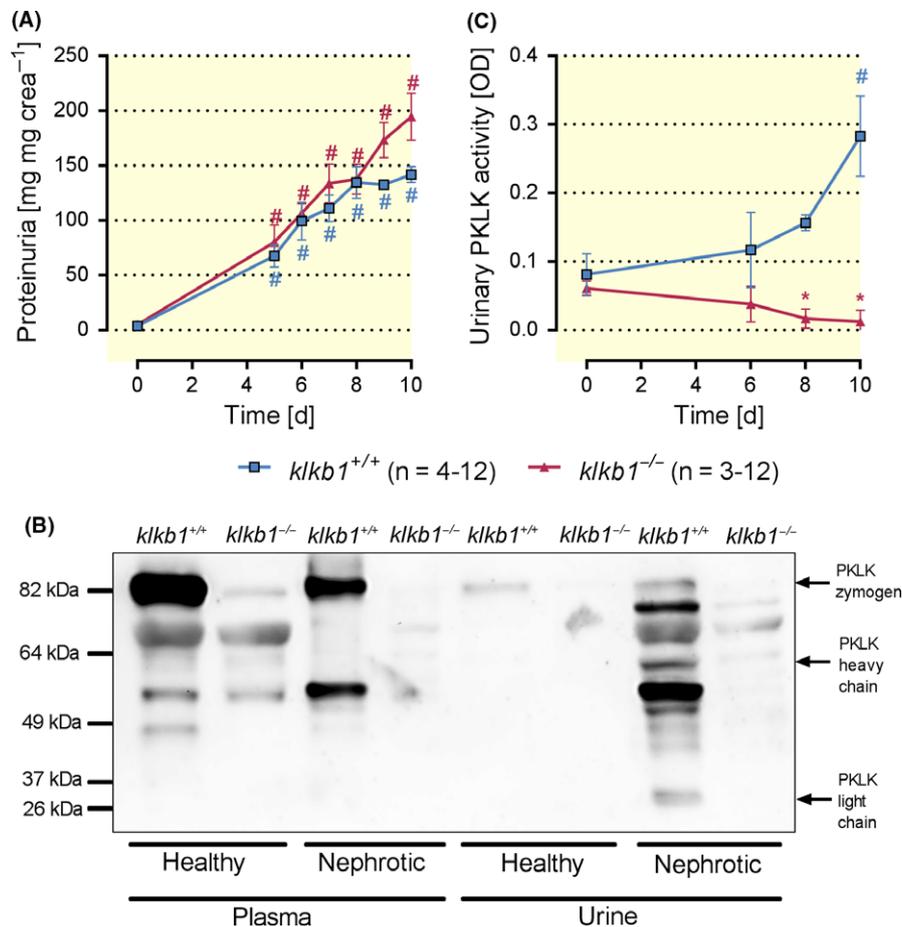
FIGURE 5 Urinary excretion of PKLK in experimental nephrotic syndrome. (A) Proteinuria in wild-type ($klkb1^{+/+}$) and PKLK-deficient mice ($klkb1^{-/-}$) after induction of experimental nephrotic syndrome by doxorubicin. (B) Western blot for PKLK expression from plasma and urine of wild-type ($klkb1^{+/+}$) mice in comparison with samples from PKLK-deficient mice ($klkb1^{-/-}$). PKLK zymogen migrates mainly at 82 kDa in plasma and urine of healthy $klkb1^{+/+}$ mice. In nephrotic $klkb1^{+/+}$ mice, the 82 kDa band for zymogen PKLK is attenuated in the plasma due to urinary loss. In nephrotic urine from $klkb1^{+/+}$ mice taken at day 8, multiple cleavage fragments of PKLK are detected. In samples of $klkb1^{-/-}$ mice, unspecific binding is visible in plasma and to a lesser extent in urine. The band at 69 kDa most likely represents albumin. (C) Urinary PKLK activity as determined with a chromogenic substrate. After induction of nephrotic syndrome, the initially close values (background) separate indicating increasing PKLK activity in the urine of wild-type ($klkb1^{+/+}$) mice. *Significant difference between genotypes, #Significant difference between treatment and baseline

urinary PKLK concentration could be higher and reach values >5 µg mL^{-1} that induced strong stimulation of ENaC currents in oocytes. Unfortunately, we could not quantify plasma and urinary PKLK concentration of nephrotic mice due to a lack of specific ELISA assays in contrast to urinary plasmin(ogen) concentration that was found to exceed >300 µg mL^{-1} in this model.[9] From this value, we estimate urinary PKLK concentration to reach at least 30 µg mL^{-1}.

It is remarkable that PKLK was found in an active form in urine samples of both proteinuric patients and mice in contrast to the plasma compartment where PKLK circulates as zymogen. This raises the question how PKLK is activated in the tubule lumen after aberrant filtration. In plasma, PKLK is mainly activated by factor XIIa and we have preliminary data showing concomitant filtration of

factor XIIa into the urinary space in nephrotic syndrome.[26] However, the activity of urinary PKLK was not sufficient to enhance urinary plasminogen conversion in vivo as observed in Western blot (Figure 7).

To our knowledge, this is the first report that the serine protease PKLK activates ENaC by proteolytic cleavage of its γ-subunit at the cell surface. Our findings reported in Figure 4 show that the combination of PKLK and plasminogen causes a significant increase in proteolytic ENaC activation. These results are remarkable, as plasminogen alone has been demonstrated to have no appreciable stimulatory effect on ENaC activity.[11] Therefore, they suggest a dual mechanism for mediating the stimulatory effect of PKLK on ENaC: first, by direct cleavage of ENaC by PKLK at the prostasin/tissue kallikrein site γRKRK178 (Figure 3), and secondly by an indirect mechanism

FIGURE 6 Role of PKLK in experimental nephrotic syndrome. (A) Urinary sodium excretion normalized for creatinine concentration before (day 0) and after induction of nephrotic syndrome showing a parallel decrease in wild-type (klkb1$^{+/+}$) and PKLK-deficient mice (klkb1$^{-/-}$). (B) ENaC-mediated sodium reabsorption inferred from the net natriuresis attributable to the injection of amiloride (10 μg/g ip). Compared to the healthy state, ENaC-mediated sodium reabsorption is stimulated in nephrotic mice of both genotypes. (C) Body weight gain from day 3 to 10 was identical in both genotypes. There was no difference in the baseline body weight between both genotypes (26.9 ± 0.9 g in klkb1$^{+/+}$ vs 26.8 ± 1.1 g in klkb1$^{-/-}$ mice, P > .05). (D) Food and fluid intake before (day 0) and after induction of nephrotic syndrome in wild-type (klkb1$^{+/+}$) and PKLK-deficient mice (klkb1$^{-/-}$). After a transient decrease owing to the acute effects of doxorubicin, food and fluid intakes stabilizes after day 3. *Significant difference between genotypes, #Significant difference between treatment and baseline
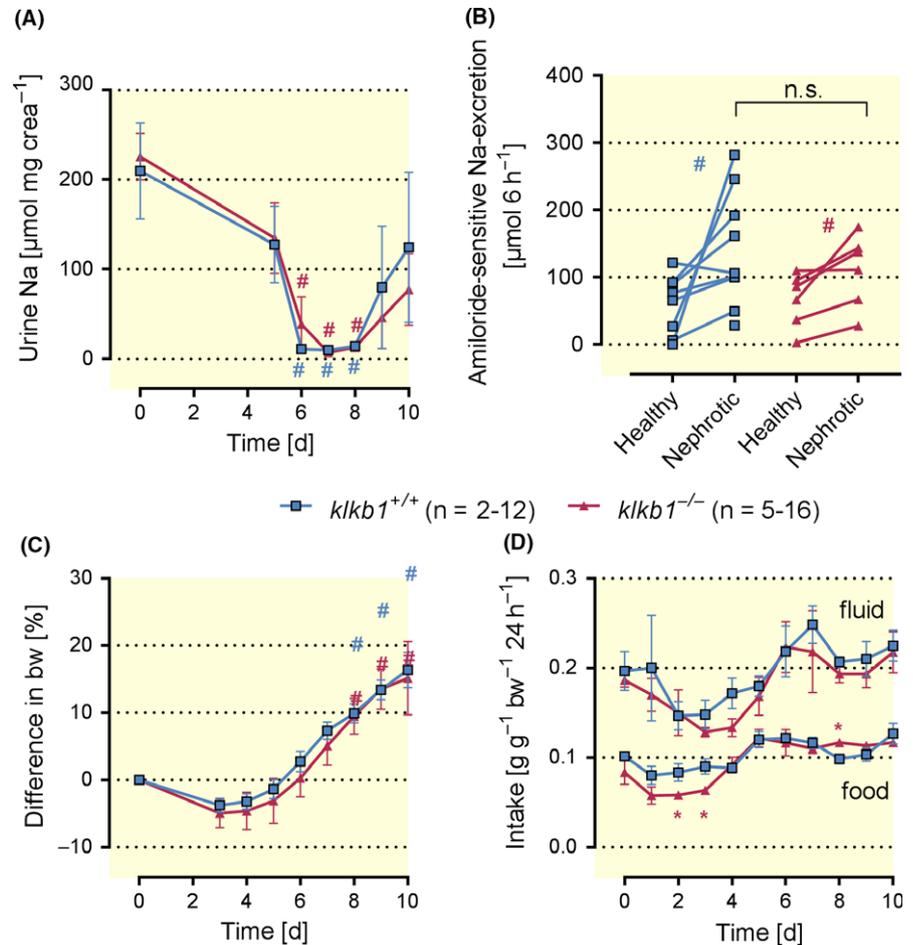
TABLE 3 Plasma parameters of healthy and nephrotic wild-type (klkb1$^{+/+}$) and klkb1$^{-/-}$ mice before and on day 10 of experimental nephrotic syndrome

	Healthy		Nephrotic	
	klkb1$^{+/+}$	klkb1$^{-/-}$	klkb1$^{+/+}$	klkb1$^{-/-}$
pH	7.24 ± 0.02	7.21 ± 0.02	7.34 ± 0.02$^{\#}$	7.31 ± 0.02$^{\#}$
std HCO_3^-, mM	21.7 ± 0.8	19.6 ± 0.7	27.9 ± 0.7$^{\#}$	25.6 ± 1.2$^{\#}$
Na^+, mM	149 ± 1	153 ± 1	140 ± 3$^{\#}$	145 ± 1$^{\#}$
K^+, mM	4.7 ± 0.2	4.7 ± 0.2	5.8 ± 0.2$^{\#}$	5.5 ± 0.2
Ca^{++}, mM	1.02 ± 0.03	0.96 ± 0.04	1.04 ± 0.03	1.05 ± 0.02
Hct, %	47 ± 1	52 ± 3	37 ± 4	39 ± 2$^{\#}$
cHbc, g dL^{-1}	15.4 ± 0.2	17.0 ± 1.0	12.3 ± 1.4	12.8 ± 0.7$^{\#}$
Urea, mg dL^{-1}	25 ± 4	20 ± 4	31 ± 4	27 ± 6
Albumin, g L^{-1}	25 ± 3	25 ± 2	7 ± 1$^{\#}$	7 ± 1$^{\#}$
Aldosterone, pg mL^{-1}	458 ± 100	356 ± 66	1806 ± 143$^{\#}$	1223 ± 336$^{\#}$

Std, standard; Hct, haematocrit; cHbc, calculated haemoglobin concentration.

Arithmetic means ± SEM (n = 6-9 each).

#Significant difference between healthy and nephrotic state.
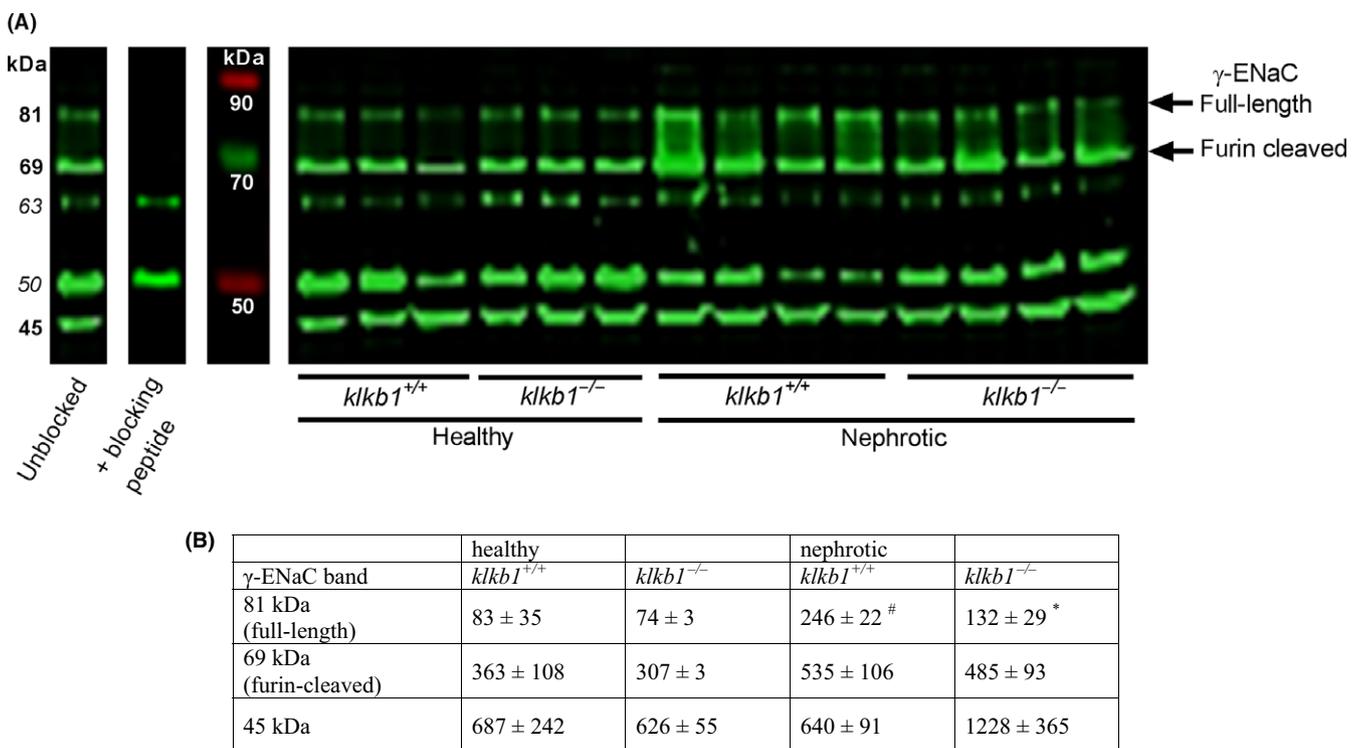
*Significant difference between genotypes.

involving PKLK-mediated generation of plasmin from plasminogen which results in cleavage at the plasmin cleavage site γK189 (Figure 4). Indeed, in the context of proteinuria, a cascade of different serine proteases may be active in the renal tubule involving a complex network of interacting proteases. However, little is known about the exact identity

(A)

(B)

(C)

klkb1$^{+/+}$ (n = 1-8)   klkb1$^{-/-}$ (n = 1-6)
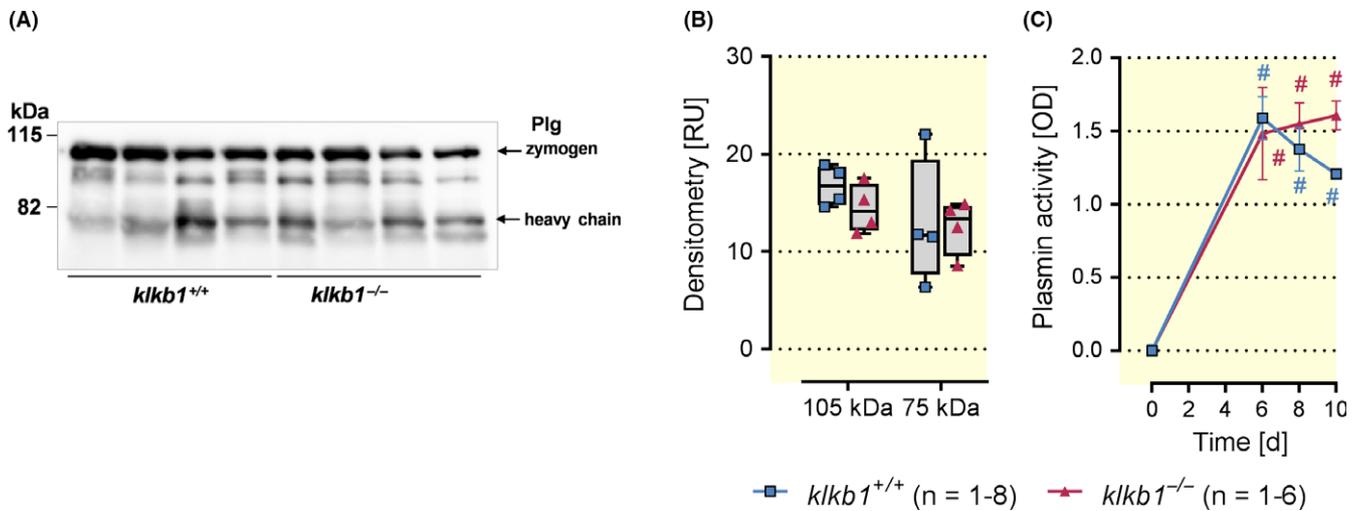
FIGURE 7 Plasminuria in nephrotic wild-type (klkb1$^{+/+}$) and klkb1$^{-/-}$ mice. (A) Western blot for urinary excretion of plasminogen detected at ~105 (zymogen) and ~75 kDa (heavy chain after cleavage and dissociation under reducing WB conditions). Each lane represents one urine sample per mouse collected at day 8. Double bands suggest cleavage products. (B) Densitometric analysis of the expression of the bands at 75 and 105 kDa. (C) Urinary plasmin activity measured with chromogenic substrate. #Significant difference between treatment and baseline

(A)

(B)

γ-ENaC band	healthy		nephrotic	
	klkb1$^{+/+}$	klkb1$^{-/-}$	klkb1$^{+/+}$	klkb1$^{-/-}$
81 kDa (full-length)	83 ± 35	74 ± 3	246 ± 22 #	132 ± 29 *
69 kDa (furin-cleaved)	363 ± 108	307 ± 3	535 ± 106	485 ± 93
45 kDa	687 ± 242	626 ± 55	640 ± 91	1228 ± 365

FIGURE 8 Expression of γ-ENaC and its cleavage products in kidney cortex from nephrotic wild-type (klkb1$^{+/+}$) and klkb1$^{-/-}$ mice. (A) Western blot from renal cortex showing multiple bands between 45 and 81 kDa. Application of the immunogenic peptide specifically blocked bands at 81 kDa, 69 and 45 kDa, but not those at 63 and 50 kDa. The bands at 81 kDa and 69 kDa most likely represent full-length and furin-cleaved γ-ENaC, respectively, whereas the other band represents a cleavage product of unknown significance. (B) Relative abundance of the obtained bands in healthy and nephrotic wild-type (klkb1$^{+/+}$) and klkb1$^{-/-}$ mice normalized to total protein per lane. Arithmetic means $\pm$ SEM (n = 3-4 each). #Significant difference between healthy and nephrotic state. *Significant difference between genotypes

of the critical serine proteases and their interactions. The phenomenon of proteases functioning in cascades is well known, for example from the serine protease cascade of the blood clotting system.[1,8] In proteinuric renal disease, these plasma serine proteases occur in the urine where they continue to interact in a complex manner.

The decisive role of urinary serine protease activity on ENaC-mediated volume retention has recently been shown by our group.[9] In that study, aprotinin treatment of nephrotic mice abolished volume retention in a similar way as treatment with amiloride. This demonstrates that the therapeutic effect of aprotinin is mediated by preventing proteolytic ENaC activation. So far, the exact identity of the serine protease(s) responsible for ENaC activation remains unknown. With this study, we can exclude that PKLK is essential, at least in the mouse model for nephrotic syndrome. Currently, plasmin is considered to be the principal serine protease responsible for ENaC activation in nephrotic syndrome. However, it is not proven in an animal model. Therefore, our combined approach to study patient samples, in vitro action and in vivo relevance of a candidate serine protease is highly valuable to elucidate the mechanism of ENaC activation in nephrotic syndrome and to identify essential serine proteases.

In conclusion, we show that PKLK is detected in the urine of proteinuric patients and mice and causes proteolytic activation of ENaC. However, this stimulatory effect of PKLK is not essential for volume retention in nephrotic mice and is most likely mediated by other serine proteases present in nephrotic urine capable of proteolytically activating ENaC.

4 | MATERIALS AND METHODS

4.1 | Patient study

This prospective study included stable ambulatory patients with CKD from the outpatient department of the University Hospital Tuebingen presenting for a routine check between September 2012 and April 2013.[14] Patients were included after they provided written informed consent. In addition, persons evaluated as potential kidney donors controls and inpatients suffering from acute nephrotic syndrome were included. The study was approved by the local ethics committee of the University of Tuebingen (259/2012MPG23).

From each patient, fluid status was assessed using the Fresenius Body Composition Monitor (BCM) that is mainly used for dry weight estimation in dialysis patients. This device utilizes bioimpedance spectroscopy with a spectrum of 50 frequencies between 5 and 1000 kHz to measure extracellular (ECW) and intracellular water (ICW) and to calculate the amount of overhydration (OH).[27,28] OH is inferred from the body composition model which divides the whole body into three compartments, that is normally hydrated lean tissue, normally hydrated adipose tissue and OH. Reference values for OH are age-independent and lie between −1 and +1 L. In addition, ultrasonography was performed to exclude pleural effusion or ascites that are not determined by the BCM monitor. Values obtained for OH and ECW were normalized to a body surface area of 1.73 m².

4.2 | Mouse studies

Experiments were performed on 3-month-old wild-type and PKLK-deficient ($klkb1^{-/-}$) mice.[29] Imported B6-$klkb1^{-/-}$ mice were backcrossed over 4 generations onto a 129 S1/SvImJ background to confer susceptibility to experimental nephrotic syndrome.[30,31] Genotyping was performed using PCR as described.[32] Mice were kept on a 12:12-hours light-dark cycle and fed a standard diet (ssniff, sodium content 0.24% corresponding to 104 μmol g^{-1}, Soest, Germany) with tap water ad libitum. Experimental nephrotic syndrome was induced after a single intravenous injection of doxorubicin (14.5 μg g^{-1} body weight, Cell Pharm, Bad Vilbel, Germany) as developed by our group.[30] Mice were kept in their normal cages to reduce distress after doxorubicin injection and proteinuria. Daily food and fluid intake were monitored by weighing the food pellets and the water bottle. Samples of spontaneously voided urine were collected in the morning 2 days before (baseline) and up to 10 days following doxorubicin injection. Blood samples were drawn before induction and at kill on day 10. Determination of ENaC-mediated sodium reabsorption was performed after intraperitoneal injection of vehicle and 10 μg g^{-1} amiloride in 5 μL g^{-1} injectable water and collection of urine for 6 hours. Mice were allowed to recover 1 day between the two injections that were carried out in the healthy and nephrotic state. ENaC-mediated sodium reabsorption was calculated from the paired difference of natriuresis following amiloride and vehicle. 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 M11/15).

4.3 | Laboratory measurements

From each patient, extra spot urine samples for the determination of PKLK concentration and activity were drawn. Urinary PKLK excretion was determined using an ELISA kit as specified by the manufacturer (Loxo, Heidelberg, Germany). The ELISA detects both the zymogen and cleaved PKLK. Urinary activity of PKLK was measured using the chromogenic substrate S-2302 (Haemochrom, Essen, Germany); 50 μL urine and 50 μL 2 mM S-2302 were incubated for 8 hours at 37°C with or without a specific inhibitor for plasmin (anti-plasmin [Loxo,

Heidelberg, Germany], final concentration 20 µg mL^{-1}) and PKLK (PKSI-527; Santa Cruz, CA, USA,[33] final concentration 15 µg mL^{-1}). UV 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 PKLK in discrimination to degradation of the substrate by plasmin and other proteases present in the urine. Values were expressed as relative units (1000*Delta absorption @405 nm) and normalized to urinary creatinine concentration. Both inhibitors had additive effects on substrate degradation indicating the specificity and validity of this approach.

In mice, urinary plasmin and PKLK activity were measured with the same approach except for a shortened incubation time of 1 hour due to high total amidolytic activity. Urinary creatinine was measured with a colorimetric assay (Labor + Technik, Berlin, Germany), urinary protein concentration using the Bradford method (Bio-Rad Laboratories, Munich, Germany) and urinary sodium concentration with flame photometry (Eppendorf EFUX 5057, Hamburg, Germany). Both urinary protein and sodium concentration 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). Blood gas analysis was carried out using an IL GEM® Premier 3000 blood gas analyser (Instrumentation Laboratory, Munich, Germany).

4.4 | Western blot from urine, plasma and kidney tissue

For Western blot analysis of PKLK in urine and plasma, SDS-PAGE on a 7.5% gel was performed with 20 µg plasma and 30 µg urinary protein per lane from either patients or mice. Human urine samples were depleted from albumin and IgG (Amgen, Germany). Mouse anti-human PKLK antibody directed against the light chain (Molecular innovations, MI, USA) and goat anti-mouse PKLK recognizing both intact and cleaved PKLK were used as primary antibodies (AF 2498, R&D systems, MN, USA). The latter antibody was raised against the PKLK fragment Gly20-Ala638 and has no crossreactivity with recombinant tissue kallikreins (KLK1, 3, 5, 7, 13 and 14). Urinary expression of plasminogen was probed using a primary antibody reacting with the heavy chain of plasminogen (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. Bands were developed by chemiluminescence using secondary HRP-conjugated antibodies (sc 2005, Santa cruz, ab 6741, abcam) on a ChemiDoc Touch System (Bio-Rad, Hercules, USA).

Western blot analysis of γ-ENaC cleavage was performed as previously described.[9] Briefly, kidneys from healthy and nephrotic wild-type ($klkb1^{+/+}$) and $klkb1^{-/-}$ mice were harvested at day 10 after induction of nephrotic syndrome. Homogenization of cortical slices was performed using a Dounce homogenizator in 1 mL lysis buffer containing 250 mM sucrose, 10 mM triethanolamine HCl, 1.6 mM ethanolamine and 0.5 EDTA at pH 7.4 (all Sigma) in the presence of protease inhibitors (aprotinin and mini-complete, Roche). After removing nuclei, total proteins were pelleted at 300 000 g for 1 hour at 4°C and after resuspension boiled in Laemmli buffer at 70°C for 10 minutes.[17] Subsequently, 40 µg was loaded on a 7.5%-polyacrylamide gel for electrophoresis. γ-ENaC was detected using an affinity-purified antibody raised in rabbit against the amino acids 634-655 (Pineda, Berlin, Germany).[3,5,9,34] After overnight incubation at 4°C in a 1:500 dilution, γ-ENaC and its cleavage products were detected with a secondary donkey anti-rabbit antibody labelled with IRDye 800CW for 1 hour at 4°C in a 1:20 000 dilution (Licor, Lincoln, USA). To test specificity of the obtained bands, the blots were probed with the primary antibody that was pretreated with the blocking peptide overnight (100× excess by weight). For loading control, total protein was measured using Revert Total Protein Stain (Licor, Lincoln, USA). Images were acquired using a fluorescence scanner (Licor Odyssey).

4.5 | Two-electrode voltage-clamp measurements using human ENaC-expressing oocytes

Oocytes were collected from *Xenopus laevis* with the approval of the animal welfare officer for the University of Erlangen-Nürnberg as described.[4-6,14] Defolliculated stage V-VI oocytes were injected with cRNA encoding human α-, β- and γ-ENaC (0.2 ng of cRNA/subunit of human wild-type or mutant $\alpha\beta\gamma_{RKRK178AAA}$ENaC). ENaC-mediated whole-cell currents were measured using the two-electrode voltage-clamp technique as previously described.[4-6] Amiloride-sensitive current (ΔI_{ami}) values were determined by washing out amiloride 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. For the determination of the stimulatory effect of activated human PKLK (Loxo, Heidelberg, Germany) or human plasmin (10 µg mL^{-1}, Merck, Darmstadt, Germany), ΔI_{ami} was determined twice in a single oocyte (ie, before and after exposure to the protease). To recover from the first

measurement of ΔI_{ami}, the oocyte was placed for 5 minutes in ND96. Subsequently, the oocyte was transferred to 150 μL of test solution (protease-supplemented ND96 or protease-free ND96 solution as control) and was incubated for 4 hours before ΔI_{ami} was determined for a second time.

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

Biotinylation experiments were performed as described previously[5,6] using 30 oocytes/group. All biotinylation steps were performed at 4°C. Oocytes were preincubated for 30 minutes either in ND96 solution or in ND96 solution containing proteases. After washing the oocytes three times with ND96 solution, they were incubated in biotinylation buffer (10 mM triethanolamine, 150 mM NaCl, 2 mM $CaCl_2$ and 1 mg mL^{-1} EZ-link sulfo-NHS-SS-Biotin, pH 9.5) for 15 minutes with gentle agitation. The biotinylation reaction was stopped by washing the oocytes twice for 5 minutes with quench buffer (192 mM glycine and 25 mM Tris-Cl, pH 7.5). Subsequently, the oocytes were lysed by passing them through a 27-gauge needle in lysis buffer (500 mM NaCl, 5 mM EDTA and 50 mM Tris-Cl, pH 7.4) supplemented with protease inhibitor mixture tablets (Complete Mini EDTA-free; Roche Applied Science) according to the manufacturer's instructions. The lysates were centrifuged for 10 minutes at 1500 g. Supernatants were incubated with 0.5% Triton X-100 and 0.5% Igepal CA-630 for 20 minutes on ice. Biotinylated proteins were precipitated with 100 μL of immunopure-immobilized NeutrAvidin-agarose (Thermo Fisher Scientific) washed with lysis buffer. After overnight incubation at 4°C with overhead rotation, the tubes were centrifuged for 3 minutes at 1500 g. Supernatants were removed, and beads were washed three times with lysis buffer; 100 μL of 2× SDS-PAGE sample buffer (Rotiload 1, Roth, Karlsruhe, Germany) was added to the beads. Samples were boiled for 5 minutes at 95°C and centrifuged for 3 minutes at 20 000 g before loading the supernatants onto a 10% SDS-polyacrylamide gel. To detect γ-ENaC cleavage fragments, we used a subunit-specific antibody against human γ-ENaC at a dilution of 1:5000. This antibody was raised in rabbit against the sequence NTLRLERAFSNQLTDTQMLDEL corresponding to the amino acids 628-649 of the C-terminus of human ENaC (Pineda, Berlin, Germany).[5,6,34] Horseradish peroxidase-labelled secondary goat anti-rabbit antibody (Santa Cruz Biotechnology) was used at a dilution of 1:50 000. Chemiluminescence signals were detected using Super-Signal West Femto chemiluminescent substrate (Thermo Fisher Scientific).

4.7 | Statistical analysis

Human data are provided as medians with interquartile or whole ranges as indicated; data from oocyte experiments and mice are provided as arithmetic means with SEM. The association of the urinary PKLK concentration with fluid status in patients with CKD was analysed by univariate parametric correlation. Multivariate linear regression analyses were performed to identify independent determinants of overhydration. Selection of the variables entering the final least squares model was derived from forward, stepwise multiple linear regression of parameters that were univariately correlated with the dependent variable and had a $P < .05$. The residuals of each model were tested for normality. Human data were analysed using JMP 10.0.1 (SAS Institute, Cary, NC, USA). Mouse and electrophysiological data were analysed using Graph-Pad Prism (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Densitometric analysis of Western blots was carried out using ImageJ[35] and Image Studio Version 3.1.4 (Licor).

CONFLICT OF INTEREST

None.

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