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

Silke Haerteis[1*], Anja Schork[2-4*], Thomas Dörffel[2], Bernhard N. Bohnert[2-4], Regina Nacken[1], Matthias Wörn[2], Mengyun Xiao[2], Daniel Essigke[2], Andrea Janessa[2], Alvin H. Schmaier[5], Edward P. Feener[6], Hans-Ulrich Häring[2-4], Marko Bertog[1], Christoph Korbmacher[1], Ferruh Artunc[2-4]

[1] Institute of Cellular and Molecular Physiology, Friedrich-Alexander University Erlangen-Nürnberg, Germany.

[2] Department of Internal Medicine, Division of Endocrinology, Diabetology, Vascular Disease, Nephrology and Clinical Chemistry, University Hospital Tübingen, Germany.

[3] Institute of Diabetes Research and Metabolic Diseases (IDM) of the Helmholtz Center Munich at the University Tübingen, Germany.

[4] German Center for Diabetes Research (DZD) at the University Tübingen, Germany.

[5] Division of Hematology and Oncology, University Hospitals Cleveland Medical Center and Case Western Reserve University, Cleveland, OH, USA.

[6] Joslin Diabetes Center, Boston, MA, USA.

* shared first-authorship

Address for correspondence:
Ferruh Artunc, MD
University Hospital Tübingen
Department of Internal Medicine
Division of Endocrinology, Diabetology, Angiology and Nephrology
Otfried-Mueller-Str.10
72076 Tübingen, Germany
E-mail: ferruh.artunc@med.uni-tuebingen.de
Tel. +49-7071-2982711
Fax +49-7071-2925215

Key words: ENaC activation – epithelial sodium channel (ENaC) – plasma kallikrein – proteinuria – sodium retention – proteolytic cleavage

Short title: plasma kallikrein activates ENaC

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 analyzed 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 CKD patients, we found that PKLK is excreted in the urine up to a concentration of 2 µg mL^{-1} which was correlated with albuminuria (r=0.71) and overhydration as assessed by bioimpedance spectroscopy (r=0.44). 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:

PKLK 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.

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 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 favoring 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 γENaC 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 edema 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 analyzed urine samples from patients with CKD of various etiologies and stages for excretion of PKLK. We then correlated PKLK excretion with extracellular volume status and overhydration as 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.

Results

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

We studied 171 patients with stable CKD of various etiologies and stable disease who presented consecutively to our outpatient center and 10 inpatients with acute nephrotic syndrome (NS). 15 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, CKD patients 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 CKD patients 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=0.75, Fig 1a) and albuminuria (r=0.71). 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 (Fig. 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 CKD patients, but not in healthy control persons (median 0.07, range 0-43.5 RU g^{-1} creatinine, Fig. 1c) and correlated with proteinuria (r=0.44, Fig. 1d) and PKLK excretion measured with an ELISA (r=0.4116 $p<0.0001$). 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% (Fig. 1c). The correlation of PKLK excretion with PKLK activity was r=0.4116 ($p<0.0001$).

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

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, i.e. before and after a 4 h exposure to protease-free solution (Fig. 2a,d), to ascending concentration of PKLK (Fig. 2b,d), or to PKLK plus the selective PKLK inhibitor PKSI-527 (Fig. 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 (Fig. 2a,d). Exposure of the oocytes to PKLK-527 alone had no effect on ENaC currents (1.20 µA vs. 1.15 µA, p=0.58). A stimulatory effect on ΔIami was also observed after 30 min exposure to 13.4 µg/ml PKLK (data not shown). Exposure to PKLK increased ΔI_{ami} in a concentration-dependent manner (Fig. 2d). The effect of PKLK was prevented by PKSI-527 (Fig. 2c,d).

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 wildtype α- and β-ENaC together with a γ-ENaC subunit mutated at the putative prostasin cleavage site (γRKRK178AAAA, Fig. 3a). To investigate γ-ENaC fragments at the cell surface, a biotinylation approach was used [20]. ENaC expressing oocytes (wildtype or $\alpha\beta\gamma_{RKRK178AAAA}$) were treated for 4 h with protease-free solution (control), or

PKLK, or PKLK+PKSI-527, or plasmin (Fig. 3b). Subsequently, the biotinylated γ-ENaC cleavage products were detected by western blot (Fig. 3b). In non-injected oocytes, γ-ENaC-specific signals were absent (Fig. 3b). The predominant γ-ENaC fragment detected at the cell surface of untreated αβγ-ENaC expressing oocytes had a molecular mass of ~76 kDa (Fig. 3b) which is the result of cleavage by endogenous proteases like furin at the so-called furin cleavage site, R138 (Fig. 3a). Addition of PKLK lead 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 (Fig. 3b). The appearance of the ~67 kDa γ-ENaC cleavage fragment was paralleled by an increase in ENaC currents (Fig. 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 (Fig. 3b,c). Interestingly, proteolytic ENaC activation by plasmin was preserved in the mutant channel, which indicates that the putative plasmin cleavage site remained functional.

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

PKLK 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 (Fig. 4). Exposure of wildtype ENaC expressing oocytes to a combination of PKLK and plasminogen revealed a similar γ-ENaC cleavage fragment pattern as with PKLK alone (Fig. 4a). There was a small but significant increase in ENaC current stimulation compared to PKLK alone (5.6-fold vs. 7.0-fold, Fig. 4b). This 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=0.35, [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 (Fig. 4a,b). The observed shift from the 76 to the 67 kDa band was not as complete as with plasmin (Fig. 4a) consistent with the smaller ENaC current activation by PKLK/plasminogen compared to plasmin (Fig. 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.

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

To determine whether PKLK participates in edema formation *in vivo*, we studied the course of experimental nephrotic syndrome in mice lacking PKLK (*klkb1*$^{-/-}$) and their wildtype littermates (*klkb1*$^{+/+}$). Following doxorubicin injection *klkb1*$^{-/-}$ mice developed similar proteinuria on day 8 as wild-type mice (*klkb1*$^{+/+}$, Fig. 5a). In Western blot analysis from urine samples of healthy *klkb1*$^{+/+}$ mice, PKLK zymogen was faintly detectable at 88 kDa (Fig. 5c). In contrast, in urine samples from nephrotic *klkb1*$^{+/+}$mice PKLK appeared with multiple cleavage products between 30 and 88 kDa (Fig. 5c). The bands at 30 and 52 kDa most likely represent the light and heavy chain of PKLK, respectively, after dissociation of the disulfide 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 (Fig. 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 (Fig. 6a,b). After induction of nephrotic syndrome, urinary sodium excretion dropped in both genotypes (Fig. 6a) and was accompanied by a significantly increased response to amiloride (Fig. 6b) indicating ENaC activation. Overall, ENaC-mediated sodium reabsorption was not different between *klkb1*$^{+/+}$ and *klkb1*$^{-/-}$ mice in both the healthy and

nephrotic state (Fig. 6b). Nephrotic *klkb1*$^{+/+}$ and *klkb1*$^{-/-}$ mice gained body weight to the same extent (+20.1±2.4% vs. +20.1±2.6%, p>0.05, Fig. 6c) despite normal food and fluid intake (Fig. 6d). Plasma parameters showed 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). In addition, hematocrit, hemoglobin and plasma sodium concentration dropped in nephrotic mice reflecting dilution of plasma volume.

To analyze the contribution of PKLK to plasminogen activation *in vivo*, urinary plasmin activity and expression of plasminogen was analyzed using chromogenic substrate and Western blot, respectively. As shown in Fig. 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 were analyzed 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 (Fig. 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 (Fig. 8a,b).

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 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 edema 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 e.g. plasmin that is thought to have a pivotal role in ENaC activation in nephrotic syndrome [11]. In the present study, urinary PKLK concentrations in CKD patients 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 CKD patients, urinary PKLK concentration seems to be lower than required for channel activation in ENaC expressing oocytes. However, in nephrotic mice, 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 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 (Fig. 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 Fig. 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 (Fig. 3), and secondly by an indirect mechanism involving PKLK mediated generation of plasmin from plasminogen which results in cleavage at the plasmin cleavage site γK189 (Fig. 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 of the critical serine proteases and their interactions. The phenomenon of proteases functioning in cascades is well known, e.g. 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.

Materials and methods

Patient study

This prospective study included stable ambulatory CKD patients 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 to 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 i.e. 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 1.73m².

Mouse studies

Experiments were performed on 3-month-old wildtype 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 done using PCR as described [32]. 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 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 was 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 sacrifice on day 10. Determination of ENaC-mediated sodium reabsorption was done after intraperitoneal injection of vehicle and 10 µg/g amiloride in 5 µl/g injectable water and collection of urine for 6 h. Mice were allowed to recover 1 day between the two injections that were done 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).

Laboratory measurements

From each patient, extra spot urine samples for 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 was incubated for 8 h 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 analyzed at 405 nm on a 96-well plate reader (Biotek EL800, VT, USA). The difference between the optical density with or without the inhibitors reflected the specific activity of 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 was measured with the same approach except for a shortened incubation time of 1 h 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 done using an IL GEM® Premier 3000 blood gas analyzer (Instrumentation Laboratory, Munich, Germany).

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 (Biorad, Hercules, USA).

Western blot analysis of γ-ENaC cleavage was performed as previously described [9]. Briefly, kidneys from healthy and nephrotic wildtype (*klkb1*$^{+/+}$) and *klkb1*$^{-/-}$ mice were harvested at day 10 after induction of nephrotic syndrome. Homogenization of cortical slices was performed using a Dounce homogenisator 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 h at 4°C and after resuspension boiled in Laemmli buffer at 70°C for 10 min [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 h 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 (100x 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).

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

Oocytes were collected from *Xenopus laevis* with 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 wildtype 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 (i.e. before and after exposure to the protease). To recover from the first measurement of ΔI_{ami}, the oocyte was placed for 5 min 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 h before ΔI_{ami} was determined for a second time.

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 min 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 min with gentle agitation. The biotinylation reaction was stopped by washing the oocytes twice for 5 min 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 min at 1,500 g. Supernatants were incubated with 0.5% Triton X-100 and 0.5% Igepal CA-630 for 20 min 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 min at 1,500 g. Supernatants were removed, and beads were

washed three times with lysis buffer. 100 µl of 2x SDS-PAGE sample buffer (Rotiload 1, Roth, Karlsruhe, Germany) was added to the beads. Samples were boiled for 5 min at 95 °C and centrifuged for 3 min 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 1:5,000. 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-labeled secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:50,000. Chemiluminescence signals were detected using Super-Signal West Femto chemiluminescent substrate (Thermo Fisher Scientific).

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 CKD patients was analyzed 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 were derived from forward, stepwise multiple linear regression of parameters that were univariately correlated with the dependent variable and had a p-value < 0.05. The residuals of each model were tested for normality. Human data were analyzed using JMP 10.0.1 (SAS Institute, Cary, NC, USA). Mouse and electrophysiological data were analyzed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). Densitometric analysis of western blots was done using ImageJ [35] and Image Studio Version 3.1.4 (Licor).

Acknowledgments

We thank Sandra Rüb, MD, Claus Geiger, Antje Raiser, Manfred Depner, Sonja Mayer, Christina Lang, and Lorenz Reeh for their valuable technical assistance during the study. We further thank Dr. Hubert Kalbacher for providing the blocking peptide and affinity-purification of the anti γ-ENaC antibody.

This study was supported by a grant from the German Research Foundation (DFG, AR 1092/2-1).

Conflict of interests:

None.

References

1. Rossier, BC, Stutts, MJ: Activation of the epithelial sodium channel (ENaC) by serine proteases. *Annual review of physiology,* 71**:** 361–379, 2009.
2. Kleyman, TR, Carattino, MD, Hughey, RP: ENaC at the cutting edge: regulation of epithelial sodium channels by proteases. *The Journal of biological chemistry,* 284**:** 20447–20451, 2009.
3. Haerteis, S, Schaal, D, Brauer, F, Brüschke, S, Schweimer, K, Rauh, R, Sticht, H, Rösch, P, Schwarzinger, S, Korbmacher, C: An inhibitory peptide derived from the α-subunit of the epithelial sodium channel (ENaC) shows a helical conformation. *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology,* 29**:** 761–774, 2012.
4. Diakov, A, Bera, K, Mokrushina, M, Krueger, B, Korbmacher, C: Cleavage in the {gamma}-subunit of the epithelial sodium channel (ENaC) plays an important role in the proteolytic activation of near-silent channels. *The Journal of physiology,* 586**:** 4587–4608, 2008.
5. Haerteis, S, Krappitz, A, Krappitz, M, Murphy, JE, Bertog, M, Krueger, B, Nacken, R, Chung, H, Hollenberg, MD, Knecht, W, Bunnett, NW, Korbmacher, C: Proteolytic activation of the human epithelial sodium channel by trypsin IV and trypsin I involves distinct cleavage sites. *The Journal of biological chemistry,* 289**:** 19067–19078, 2014.
6. Haerteis, S, Krappitz, M, Diakov, A, Krappitz, A, Rauh, R, Korbmacher, C: Plasmin and chymotrypsin have distinct preferences for channel activating cleavage sites in the gamma subunit of the human epithelial sodium channel. *The Journal of general physiology,* 140**:** 375–389, 2012.
7. Passero, CJ, Hughey, RP, Kleyman, TR: New role for plasmin in sodium homeostasis. *Curr Opin Nephrol Hypertens,* 19**:** 13-19, 2010.
8. Ray, EC, Rondon-Berrios, H, Boyd, CR, Kleyman, TR: Sodium retention and volume expansion in nephrotic syndrome: implications for hypertension. *Adv Chronic Kidney Dis,* 22**:** 179-184, 2015.
9. Bohnert, BN, Menacher, M, Janessa, A, Worn, M, Schork, A, Daiminger, S, Kalbacher, H, Haring, HU, Daniel, C, Amann, K, Sure, F, Bertog, M, Haerteis, S, Korbmacher, C, Artunc, F: Aprotinin prevents proteolytic epithelial sodium channel (ENaC) activation and volume retention in nephrotic syndrome. *Kidney international,* 93**:** 159-172, 2018.
10. Svenningsen, P, Andersen, H, Nielsen, LH, Jensen, BL: Urinary serine proteases and activation of ENaC in kidney--implications for physiological renal salt handling and hypertensive disorders with albuminuria. *Pflügers Archiv : European journal of physiology,* 467**:** 531–542, 2015.
11. Svenningsen, P, Bistrup, C, Friis, UG, Bertog, M, Haerteis, S, Krueger, B, Stubbe, J, Jensen, ON, Thiesson, HC, Uhrenholt, TR, Jespersen, B, Jensen, BL, Korbmacher, C, Skøtt, O: Plasmin in nephrotic urine activates the epithelial sodium channel. *Journal of the American Society of Nephrology : JASN,* 20**:** 299–310, 2009.
12. Svenningsen, P, Friis, UG, Versland, JB, Buhl, KB, Moller Frederiksen, B, Andersen, H, Zachar, RM, Bistrup, C, Skott, O, Jorgensen, JS, Andersen, RF, Jensen, BL: Mechanisms of renal NaCl retention in proteinuric disease. *Acta physiologica (Oxford, England),* 207**:** 536-545, 2013.
13. Staehr, M, Buhl, KB, Andersen, RF, Svenningsen, P, Nielsen, F, Hinrichs, GR, Bistrup, C, Jensen, BL: Aberrant glomerular filtration of urokinase-type plasminogen activator in nephrotic syndrome leads to amiloride-sensitive plasminogen activation in urine. *Am J Physiol Renal Physiol,* 309**:** F235-241, 2015.
14. Schork, A, Woern, M, Kalbacher, H, Voelter, W, Nacken, R, Bertog, M, Haerteis, S, Korbmacher, C, Heyne, N, Peter, A, Haring, HU, Artunc, F: Association of Plasminuria with Overhydration in Patients with CKD. *Clinical journal of the American Society of Nephrology : CJASN,* 11**:** 761-769, 2016.
15. Fritz, H, Wunderer, G: Biochemistry and applications of aprotinin, the kallikrein inhibitor from bovine organs. *Arzneimittel-Forschung,* 33**:** 479-494, 1983.

16. Patel, AB, Chao, J, Palmer, LG: Tissue kallikrein activation of the epithelial Na channel. *American journal of physiology Renal physiology,* 303**:** F540-550, 2012.
17. Picard, N, Eladari, D, El Moghrabi, S, Planes, C, Bourgeois, S, Houillier, P, Wang, Q, Burnier, M, Deschenes, G, Knepper, MA, Meneton, P, Chambrey, R: Defective ENaC processing and function in tissue kallikrein-deficient mice. *J Biol Chem,* 283**:** 4602-4611, 2008.
18. Schmaier, AH: The plasma kallikrein-kinin system counterbalances the renin-angiotensin system. *The Journal of Clinical Investigation,* 109**:** 1007-1009, 2002.
19. Bruns, JB, Carattino, MD, Sheng, S, Maarouf, AB, Weisz, OA, Pilewski, JM, Hughey, RP, Kleyman, TR: Epithelial Na+ channels are fully activated by furin- and prostasin-dependent release of an inhibitory peptide from the gamma-subunit. *J Biol Chem,* 282**:** 6153-6160, 2007.
20. Krappitz, M, Korbmacher, C, Haerteis, S: Demonstration of proteolytic activation of the epithelial sodium channel (ENaC) by combining current measurements with detection of cleavage fragments. *Journal of visualized experiments : JoVE*, 2014.
21. Passero, CJ, Mueller, GM, Rondon-Berrios, H, Tofovic, SP, Hughey, RP, Kleyman, TR: Plasmin activates epithelial Na+ channels by cleaving the gamma subunit. *The Journal of biological chemistry,* 283**:** 36586–36591, 2008.
22. Colman, RW: Activation of plasminogen by human plasma kallikrein. *Biochemical and biophysical research communications,* 35**:** 273-279, 1969.
23. Miles, LA, Greengard, JS, Griffin, JH: A comparison of the abilities of plasma kallikrein, beta-Factor XIIa, Factor XIa and urokinase to activate plasminogen. *Thrombosis research,* 29**:** 407-417, 1983.
24. Bjorkqvist, J, Jamsa, A, Renne, T: Plasma kallikrein: the bradykinin-producing enzyme. *Thrombosis and haemostasis,* 110**:** 399-407, 2013.
25. Marchal, E, Montagne, P, Cuilliere, ML, Bene, MC, Faure, G: Microparticle-enhanced nephelometric immunoassay of human plasminogen. *Journal of clinical laboratory analysis,* 10**:** 85-90, 1996.
26. Wörn, M, Alenazi, F, Kalbacher, H, Heyne, N, Artunc, F: Characterization and quantification of proteasuria in nephrotic syndrome. *9th Annual Meeting of the German Society for Nephrology.* Mannheim, 2017 pp http://www.abstractserver.com/publication/nephro2017/nephro2017Abstracts.PDF.
27. Chamney, PW, Wabel, P, Moissl, UM, Muller, MJ, Bosy-Westphal, A, Korth, O, Fuller, NJ: A whole-body model to distinguish excess fluid from the hydration of major body tissues. *The American journal of clinical nutrition,* 85**:** 80–89, 2007.
28. Moissl, UM, Wabel, P, Chamney, PW, Bosaeus, I, Levin, NW, Bosy-Westphal, A, Korth, O, Muller, MJ, Ellegard, L, Malmros, V, Kaitwatcharachai, C, Kuhlmann, MK, Zhu, F, Fuller, NJ: Body fluid volume determination via body composition spectroscopy in health and disease. *Physiological measurement,* 27**:** 921–933, 2006.
29. Simao, F, Ustunkaya, T, Clermont, AC, Feener, EP: Plasma kallikrein mediates brain hemorrhage and edema caused by tissue plasminogen activator therapy in mice after stroke. *Blood,* 129**:** 2280-2290, 2017.
30. Artunc, F, Nasir, O, Amann, K, Boini, KM, Haring, HU, Risler, T, Lang, F: Serum- and glucocorticoid-inducible kinase 1 in doxorubicin-induced nephrotic syndrome. *Am J Physiol Renal Physiol,* 295**:** F1624-1634, 2008.
31. Bohnert, BN, Daniel, C, Amann, K, Voelkl, J, Alesutan, I, Lang, F, Heyne, N, Haring, HU, Artunc, F: Impact of phosphorus restriction and vitamin D-substitution on secondary hyperparathyroidism in a proteinuric mouse model. *Kidney & blood pressure research,* 40**:** 153-165, 2015.
32. Stavrou, EX, Fang, C, Merkulova, A, Alhalabi, O, Grobe, N, Antoniak, S, Mackman, N, Schmaier, AH: Reduced thrombosis in Klkb1-/- mice is mediated by increased Mas receptor, prostacyclin, Sirt1, and KLF4 and decreased tissue factor. *Blood,* 125**:** 710-719, 2015.
33. Okada, Y, Tsuda, Y, Tada, M, Wanaka, K, Hijikata-Okunomiya, A, Okamoto, U, Okamoto, S: Development of plasma kallikrein selective inhibitors. *Biopolymers,* 51**:** 41-50, 1999.

34. Haerteis, S, Krueger, B, Korbmacher, C, Rauh, R: The delta-subunit of the epithelial sodium channel (ENaC) enhances channel activity and alters proteolytic ENaC activation. *J Biol Chem,* 284**:** 29024-29040, 2009.
35. Rasband, WS: ImageJ. *U S National Institutes of Health, Bethesda, Maryland, USA,* https://imagejnihgov/ij/, 1997-2016.

Table 1: Characteristics of the study cohort.

Values are medians with interquartile range.

	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)	0.1813
gender distribution	73% ♀ / 27% ♂	42% ♀ / 58% ♂	40% ♀ / 60% ♂	0.0630
BMI, kg $m^{2\ -1}$	29.2 (25.5; 30.4)	28.7 (25.6; 32.1)	23.3 (20.5; 26.3)	0.0224
MDRD-GFR, mL min $1.73m^{-2}$	>60	45 (30; 68)	35 (20; 69)	<0.0001
proteinuria, mg g^{-1} crea	95 (77; 185)	520 (146; 1485)	6660 (5379; 8023)	<0.0001
albuminuria, mg g^{-1} crea	14 (7; 16)	110 (29; 850)	6050 (4841; 7568)	<0.0001
serum aldosterone, pg mL^{-1}	154 (120; 196)	119 (86; 167)	63 (7; 118)	0.0015
extracellular water (ECW), L $1.73m^{-2}$	14.8 (14.1; 15.5)	15.8 (14.7; 16.9)	18.0 (16.6; 19.4)	<0.0001
intracellular water (ICW), L $1.73m^{-2}$	17.4 (16.6; 18.8)	17.7 (16.2; 19.5)	18.3 (16.3; 19.8)	0.9552
overhydration (OH), L $1.73m^{-2}$	0 (-0.9; +0.2)	+0.2 (-0.5; +1.2)	+3.8 (+1.5; +5.7)	<0.0001
ECW/ICW	0.85 (0.80; 0.88)	0.89 (0.82; 0.98)	1.03 (0.91; 1.12)	0.0021
diagnoses	n.a.	diabetic/ hypertensive nephropathy and glomerulo-sclerosis (36%), glomerulo-nephritis (35%), interstitial disease (2%), polycystic kidney disease (4%), unknown (23%)	membranous glomerulonephritis (30%), IgA glomerulonephritis (30%), membrano-proliferative glomerulonephritis (10%), thrombotic microangiopathy (10%), microscopic polyangiitis (10%), unknown (10%)	

n.a. not applicable

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

Selection of the variables entering the model were derived from forward stepwise multiple linear regression of parameters that were univariately correlated with the dependent variable and had a p-value < 0.05. 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.

	model 1		model 2		model 3	
	adjusted $r^2 = 0.48$ $p < 0.0001$		adjusted $r^2 = 0.46$ $p < 0.0001$		adjusted $r^2 = 0.48$ $p < 0.0001$	
covariate	coefficient* (lower 95%-upper 95%)	p-value	coefficient* (lower 95%-upper 95%)	p-value	coefficient* (lower 95%-upper 95%)	p-value
y-intercept	3.76 (0.55-6.97)	0.0291	4.70 (1.48-7.91)	0.0045	3.81 (0.54-7.07)	0.0226
corrected plasma NT-pro-BNP concentration, log pg mL^{-1}	0.65 (0.36-0.94)	<0.0001	0.66 (0.36-0.95)	<0.0001	0.64 (0.36-0.94)	<0.0001
edema (1=present)	0.48 (0.29-0.67)	<0.0001	0.51 (0.31-0.70)	<0.0001	0.48 (0.28-0.67)	<0.0001
BMI, log kg m^{-2}	-4.14 (-6.15-2.13)	0.0001	-3.96 (-6.05--1.87)	0.0003	-4.10 (-6.16--2.04)	0.0001
plasma renin activity, log ng Ang I mL^{-1} h^{-1}	-0.20 (-0.47-0.07)	0.1483	-0.15 (-0.43-0.13)	0.2839	-0.20 (-0.47-0.08	0.1576
proteinuria, log mg g^{-1} crea	0.58 (0.30-0.85)	<0.0001	---		0.55 (0.16-0.94)	0.0060
urinary PKLK excretion, log µg g^{-1} crea	---		0.29 (0.09-0.48)	0.0033	0.03 (-0.24-0.29)	0.8421

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

Table 3: Plasma parameters of healthy and nephrotic wildtype (*klkb1*$^{+/+}$) and *klkb1*$^{-/-}$ mice before and on day 10 of experimental nephrotic syndrome.
Arithmetic means ± SEM (n=6-9 each)

	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 #

significant difference between healthy and nephrotic state
* significant difference between genotypes

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

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 acute nephrotic syndrome.
(d) Correlation of urinary PKLK activity with proteinuria.
(e-f) Correlation of urinary PKLK excretion with overhydration and extracellular water.

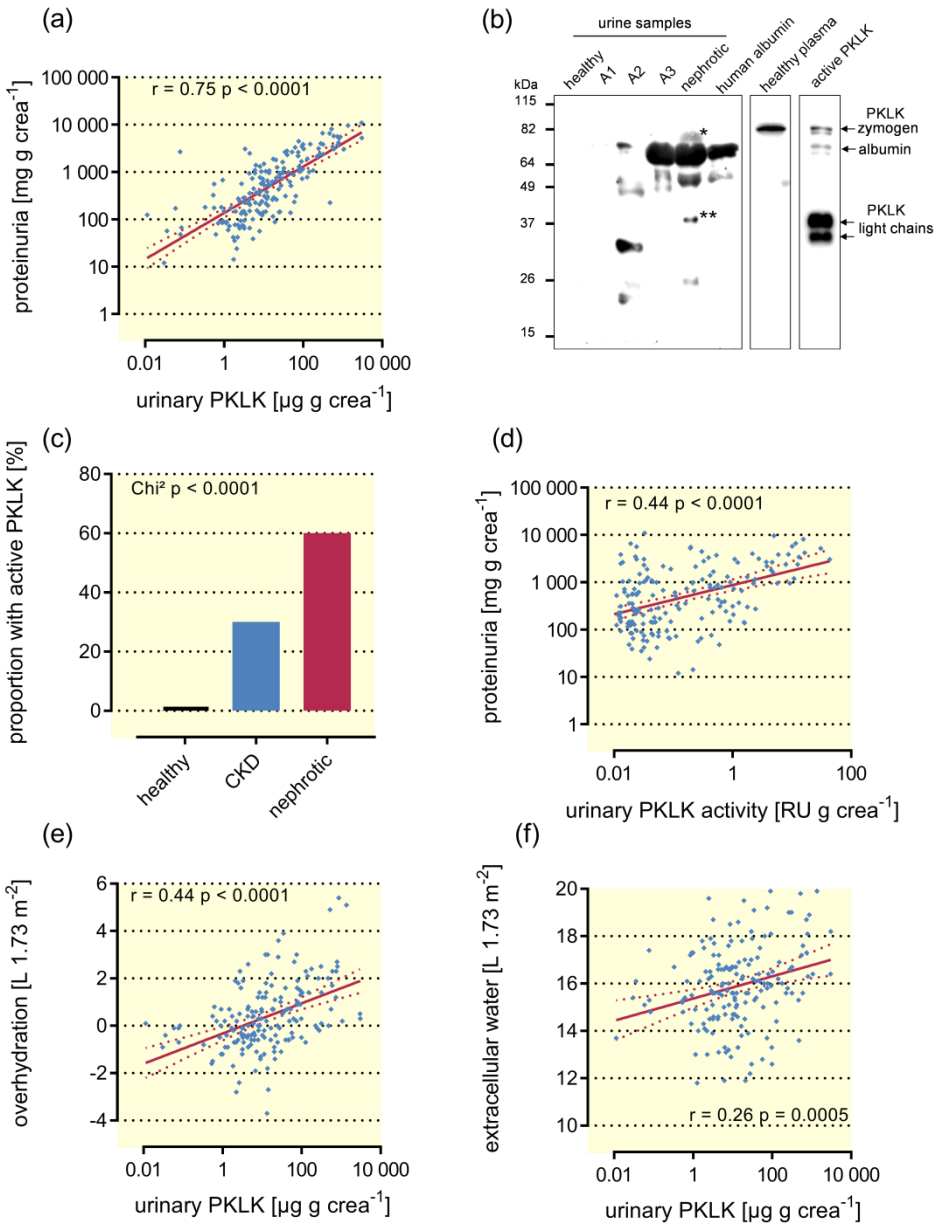


Figure 2. Stimulation of ENaC-mediated whole-cell currents by PKLK.
Xenopus laevis oocytes expressing human αβγ-ENaC were preincubated for 4 h in protease-free solution (control) or in a solution containing PKLK or PKLK + PKSI. Amiloride-sensitive whole-cell currents (ΔI_{ami}) were determined before (-) 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 4h incubation (ΔI_{ami} 4 h) 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. ***, $p<0.001$, unpaired t test. Error bars, S.E.

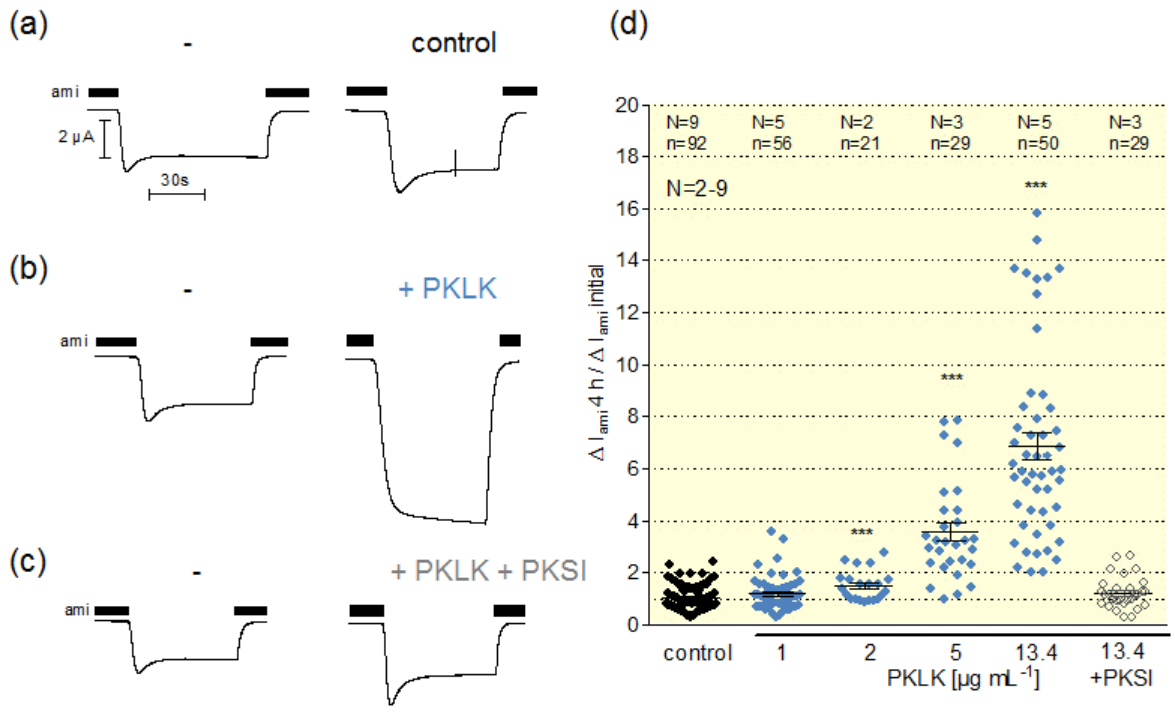


Figure 3. PKLK activates ENaC by cleaving γ-ENaC at the putative prostasin cleavage site.

Oocytes expressing αβγ- or $\alpha\beta\gamma_{RKRK178AAAA}$ENaC were preincubated for 4 h in protease-free solution (control) or in solution containing either PKLK (13.4 µg/ml) -/+ 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 analyzed 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<0.001, unpaired t test. Error bars, S.E.

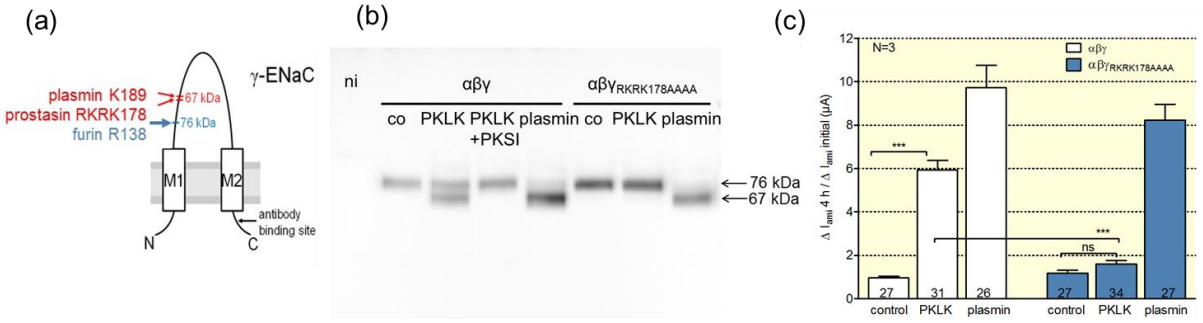


Figure 4. Activation of ENaC by PKLK is amplified in the presence of plasminogen.
Oocytes expressing αβγ- or $\alpha\beta\gamma_{RKRK178AAAA}$ENaC were preincubated for 4 h in protease-free solution (control) or in solution containing either PKLK (13.4 µg/ml) or PKLK + plasminogen (pl, 0.5 U mL^{-1}).
(a) Expression of biotinylated γ-ENaC at the cell surface was analyzed 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<0.1; ***, p<0.001, unpaired t test. Error bars, S.E.

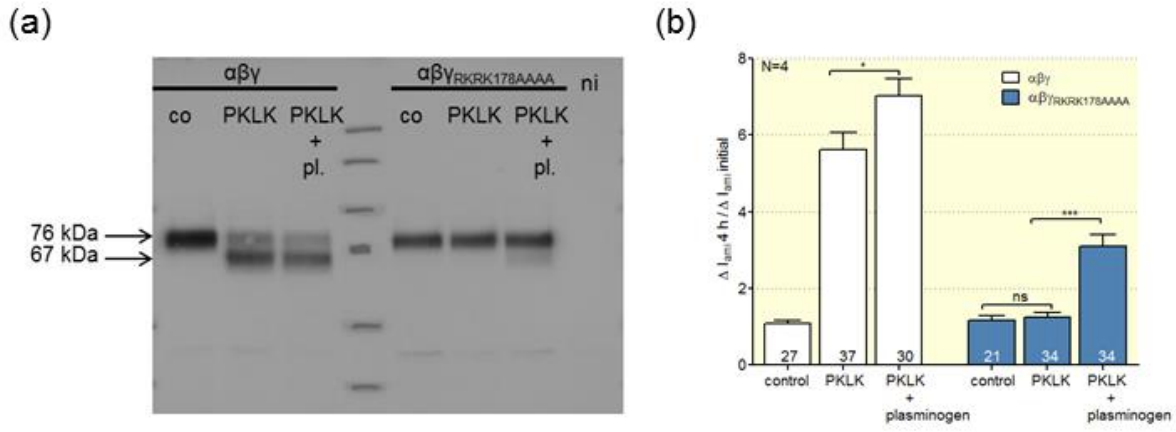


Figure 5. Urinary excretion of PKLK in experimental nephrotic syndrome.
(a) Proteinuria in wildtype (*klkb1*$^{+/+}$) and PKLK-deficient mice (*klkb1*$^{-/-}$) after induction of experimental nephrotic syndrome by doxorubicin.
(b) Western blot for PKLK expression from plasma and urine of wildtype (*klkb1*$^{+/+}$) mice in comparison to 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 wildtype (*klkb1*$^{+/+}$) mice.

* significant difference between genotypes, $^{\#}$ significant difference between treatment and baseline.

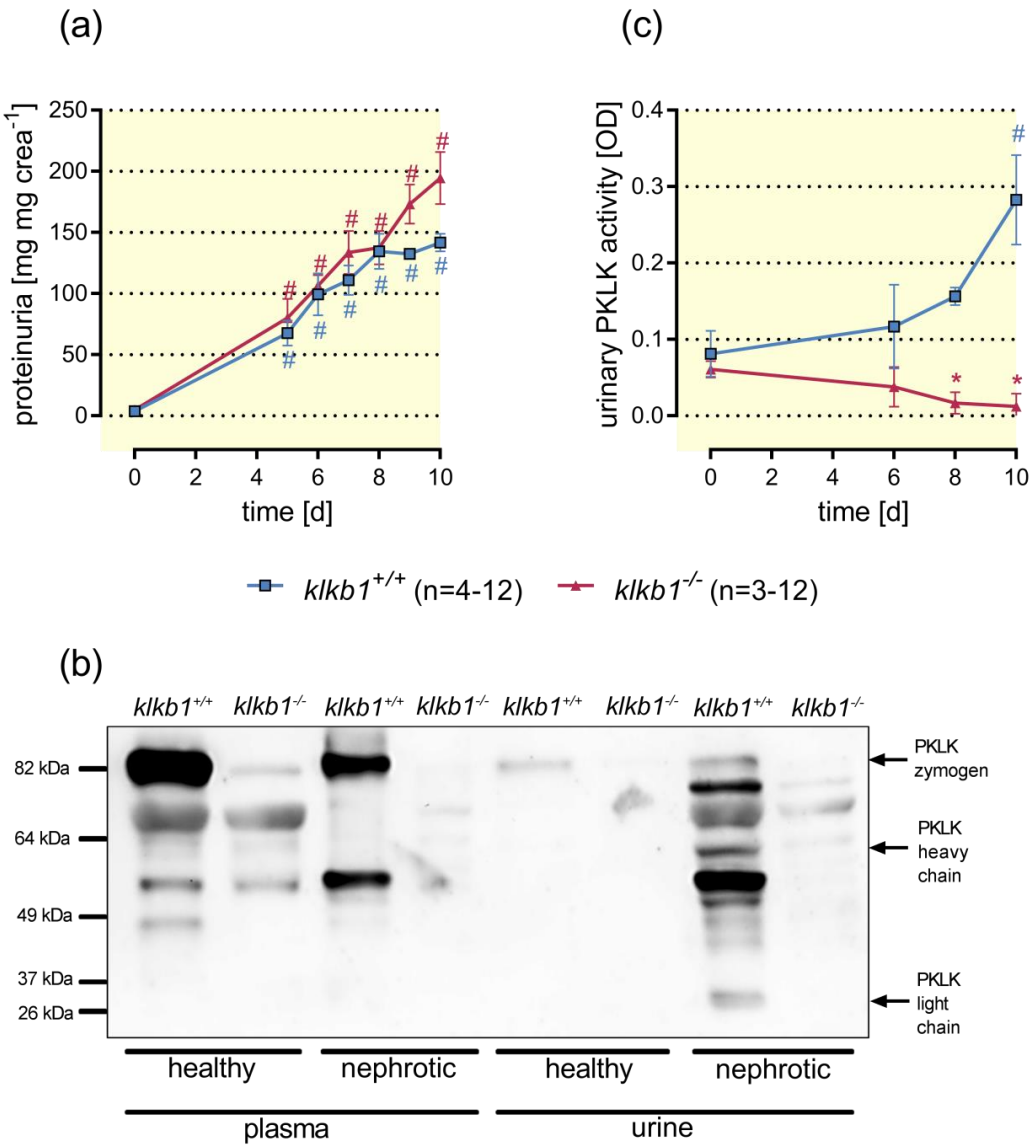


Figure 6. Role of PKLK in experimental nephrotic syndrome.
(a) Urinary sodium excretion normalized for creatinine concentration before (day 0) and after induction of nephrotic syndrome showing a parallel decrease in wildtype (*klkb1*$^{+/+}$) and PKLK-deficient mice (*klkb1*$^{-/-}$).
(b) ENaC-mediated sodium reabsorption inferred from the net natriuresis attributable to the injection of amiloride (10 µg/g i.p.). 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>0.05).
(d) Food and fluid intake before (day 0) and after induction of nephrotic syndrome in wildtype (*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.

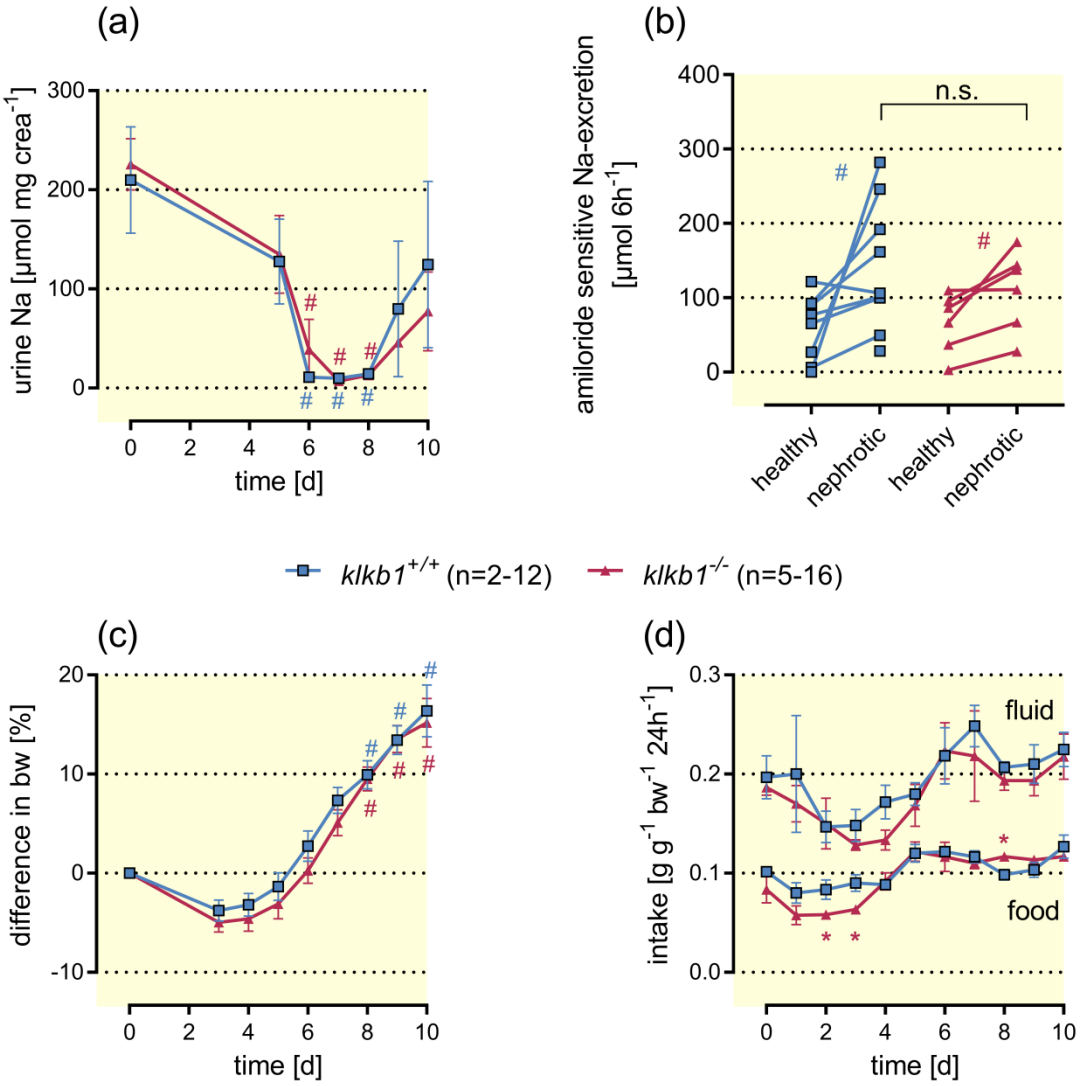


Figure 7. Plasminuria in nephrotic wildtype (*klkb1*$^{+/+}$) and *klkb1*$^{-/-}$ mice

(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.

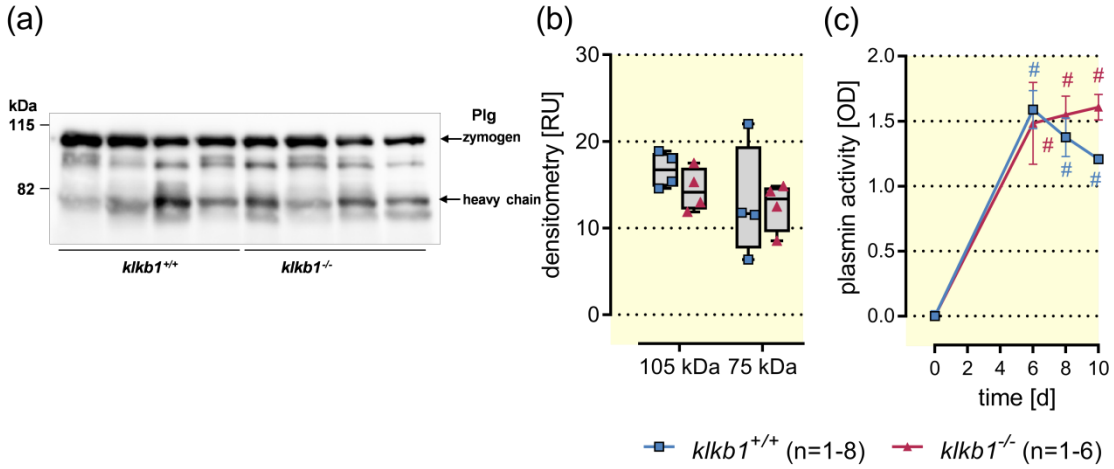
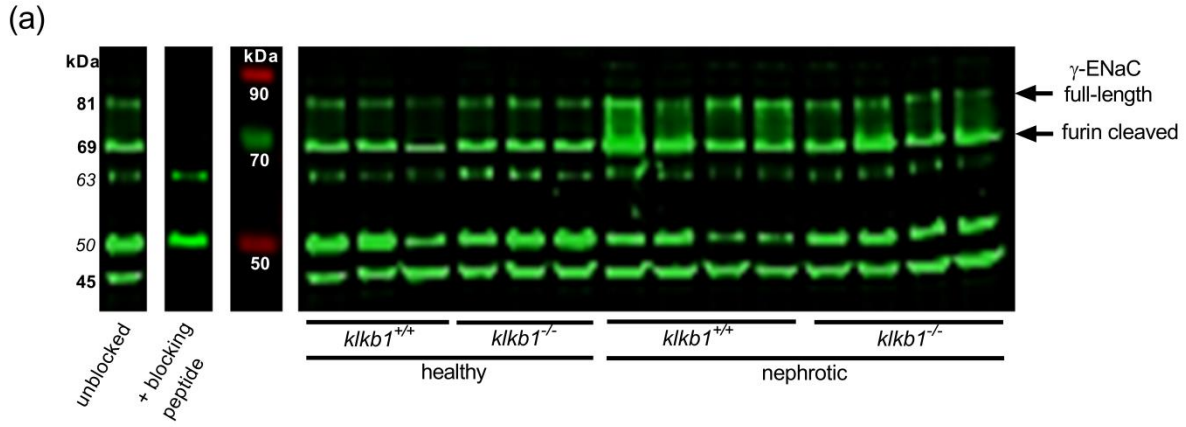


Figure 8. Expression of γ-ENaC and its cleavage products in kidney cortex from nephrotic wildtype (*klkb1*$^{+/+}$) and *klkb1*$^{-/-}$ mice

(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 wildtype (*klkb1*$^{+/+}$) and *klkb1*$^{-/-}$ mice normalized to total protein per lane.

	healthy		nephrotic	
γ-ENaC band	*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

Arithmetic means ± SEM (n=3-4 each)
$^{\#}$ significant difference between healthy and nephrotic state, * significant difference between genotypes.