Aberrant glomerular filtration of urokinase-type plasminogen activator in nephrotic syndrome leads to amiloride-sensitive plasminogen activation in urine

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Abstract

In nephrotic syndrome, aberrant glomerular filtration of plasminogen and conversion to active plasmin in pre-urine is thought to activate proteolytically ENaC and contribute to sodium retention and edema. The ENaC blocker amiloride is an off-target inhibitor of urokinase-type plasminogen activator (uPA) \textit{in vitro}. It was hypothesized that uPA is abnormally filtered to pre-urine and is inhibited in urine by amiloride in nephrotic syndrome. This was tested by determination of Na$^+$-balance, uPA protein and activity and amiloride concentration in urine from rats with puromycin aminonucleoside (PAN) induced nephrotic syndrome. Urine samples from 6 adult and 18 pediatric patients with nephrotic syndrome were analyzed for uPA activity and protein. PAN-treatment induced significant proteinuria in rats which coincided with increased urine uPA protein and activity, increased urine protease activity and total plasminogen/plasmin concentration and Na$^+$ retention. Amiloride (2mg/kg/24h) concentration in urine was in the range 10-20 µmol/L and reduced significantly urine uPA activity, plasminogen activation, protease activity and sodium retention in PAN rats, while proteinuria was not altered. In paired urine samples, uPA protein was significantly elevated in urine from children with active nephrotic syndrome compared to remission phase. In 6 adult nephrotic patients, urine uPA protein and activity correlated positively with 24h urine protein excretion. In conclusion, nephrotic syndrome is associated with aberrant filtration of uPA across the injured glomerular barrier. Amiloride inhibits urine uPA activity which attenuates plasminogen activation and urine protease activity \textit{in vivo}. Urine uPA is a relevant target for amiloride \textit{in vivo}.

Introduction

Edema associated with nephrotic syndrome (NS) is caused not only by altered Starling forces, but with a significant contribution from a primary impairment of renal NaCl excretion: In the unilateral nephrotic rat model (20), sodium retention is associated with the nephrotic kidney
only; the primary site of sodium retention is located beyond the distal convoluted tubule; (13, 44)
the renin-angiotensin-aldosterone system is suppressed in the majority of nephrotic conditions
and while edema is insensitive to adrenalectomy, albumin infusion, ACE inhibitors, AT1
blockers, dexamethasone and mineralocorticoid antagonists, it is sensitive to amiloride (4-7, 24,
25, 30). These observations were recently reviewed as the “overfill hypothesis” by Siddall and
Radhakrishnan (33). With no or minor change in the abundance and membrane association of
renal ENaC protein in nephrotic syndrome, the sensitivity to amiloride was enigmatic (14, 15,
35, 44) until a potential coupling was made to proteolytic activation of ENaC by extracellular
proteases (39). ENaC is composed of alpha, beta and gamma subunits, and the gamma subunit
requires cleavage by 2 enzymes acting in series to attain full activation (8). A range of serine
proteases may cleave the gamma subunit and enhance ENaC activity in vitro (1, 9, 41) and likely
also in physiological conditions, e.g. in response to low NaCl intake or high aldosterone, in vivo
(16, 17, 38). Our previous studies showed the aberrant presence of a soluble serine protease
identified as plasmin in urine from nephrotic rats and patients (35). Pathological filtration of the
zymogen plasminogen and activation to plasmin in the urinary space is likely to occur through
urokinase-type plasminogen activator (uPA). uPA is present in normal urine in small amounts
and thought to be secreted by the tubular epithelium (26). Since uPA circulates in plasma, it is
possible that uPA is filtered in increasing amounts during nephrotic syndrome. Amiloride, in
addition to directly blocking ENaC, is a potent inhibitor of uPA in vitro (40). We previously
observed a tendency to an increased plasminogen/plasmin ratio in nephrotic rats treated with
amiloride (35). In the present study it was hypothesized that uPA is aberrantly filtered along with
plasminogen in nephrotic syndrome and that amiloride prevents tubular activation of
plasminogen to plasmin by inhibition of urinary uPA. The hypothesis was addressed by
administration of amiloride to PAN-induced nephrotic syndrome in rats and in an observational study in urine samples from patients with nephrotic syndrome.

Materials and Methods

Human urine samples were collected from n=6 patients at the outpatient clinic at Department of Nephrology, Odense University Hospital. Only inclusion criterion was nephrotic range proteinuria, and no medical history or other data was recorded in agreement with the Regional Ethical Committee approval. Samples were anonymous. Aliquots of urine samples were kept at -80°C. Paired urine sample from children diagnosed with nephrotic syndrome was collected in a Danish multicenter study, approved by the Regional Ethics Committee, at debut of nephrotic syndrome and at time of remission. The original cohort with patient characteristics and methods of collection has previously been described in detail (2). The present substudy on uPA was approved by the Regional Ethic Committee (1-10-72-306-13). Of the 21 originally collected samples, only 18 had sufficient remaining volume for the paired analyses in the present study. No post hoc selection of patients has been performed.

Methods

Animal Experiments. Animals were housed at the Biomedical Laboratory at The University of Southern Denmark. All procedures were done in accordance with the Danish national guidelines for the care and handling of animals and to the published guidelines from the national institute of health. Male Spraque-Dawley rats, 8 weeks of age (Taconic Europe A/S, Ejby, DK) were used for the experiments. Rats were kept on a 12:12 hours light:dark cycle and had free access to tap water and standard pathogen-free rat chow containing 0.2% sodium (1324 – maintenance diet – Rats / Mice, Altromin, Lage, DE ). Three days prior to the puromycin aminonucleoside (PAN)
injection rats were shifted to milli-Q (MQ) water and a gel formula of rat chow by mixing agar (ash: 2-4.5%, Sigma-Aldrich, St. Louis, MO, USA) and milli-Q water with granulated standard rat chow to avoid contamination of urine and feces with chow. Recipe proportions for, Agar:Chow:MQ water was 1:30:50, respectively. MQ water was preheated to 60-65°C before chow and agar were added. Two days prior to the PAN inj., rats were separated and placed individually in metabolic cages. Control 24 hour urine collection (day -1) was collected after a 24 hour adjustment period in the metabolic cage. PAN (Sigma-Aldrich) 15 mg / 1 ml isotonic NaCl / 100 g BW was administered by intraperitoneal injection at day 0. Rats were kept with free access to gel-chow and MQ water throughout the experiment and daily intake was recorded. Amiloride (2 mg / kg) was dissolved in an isotonic NaCl solution and administered subcutaneously from day 4 at 9 AM. Control and PAN (vehicle/control) groups were injected with vehicle isotonic NaCl in equivalent amounts. The 24 hour urine samples were aliquoted and kept at -20°C. Twenty seven rats were included in the study. Fifteen rats had intraperitoneal PAN injection but 3 out of 15 rats did not develop adequate proteinuria (defined as: urine protein excretion > 0.1 g / 24 h /100 g BW on day 4). These rats were excluded from the study. Of the 12 rats that developed proteinuria, 6 rats were treated with amiloride (2 mg / kg BW) administered s.c. once daily from day 4 (PAN + Amiloride group) and 6 rats received vehicle-amiloride (PAN group). Of the 12 vehicle-PAN injected rats, 6 rats were subcutaneously injected with vehicle-amiloride (Control group) and 6 rats were injected with amiloride (Amiloride group).

Urine Analyses. Urine samples were centrifuged at 16.000 ×g for 30 seconds and experiments were done using the supernatant. In general, human spot urine samples were calibrated for creatinine concentration in e.g. western blotting, whereas rat samples drawn from 24h-urine collections were not normalized.
Urinary total protein was determined on the Cobas Mira Plus device using ABX Pentra reagents urinary total protein CP, ref: A11A01642 (Triolab A/S, Brøndby, Denmark).

Urinary sodium and potassium concentration was determined by flame photometry using the Instrumentation Laboratory 943 device (ILS Laboratories Scandinavia, Allerød, Denmark).

Western immunoblotting To remove excess IgG from the urine, samples were mixed with Dynabeads-Protein G (2x) (Novex, life technologies) and incubated for 10 minutes. The samples were mixed with NuPAGE® LDS Sample Buffer (4X) (Invitrogen™, Carlsbad, CA, USA) and NuPAGE® Sample Reducing Agent (2X) (Invitrogen™) and run on Tris-HCL ready gels 7.5% (plasmin/plasminogen detection) or Mini-PROTEAN TGX gels 4-15% (uPA detection) (Bio-Rad Laboratories, Hercules, CA, USA). The gel was blotted on an Immobilon®-P Transfer Membrane pore size 0.45 µm (Millipore Corporation, Billerica, MA, USA).

Plasmin/plasminogen: 3 µL of crude urine was loaded. After blotting, the membrane was subsequently blocked in TBST with 5% skimmed milk and hereafter subjected to primary anti-plasminogen antibody 1:5000 (ab6189, abcam®, Cambridge, UK) followed by secondary Polyclonal Rabbit Anti-Goat Immunoglobulins/HRP (Dako, Glostrup, DK). uPA: Human urine (5g/L creatinine) or 10 µL rat urine was loaded. After blotting, the membrane was blocked in TBST with 5% skimmed milk and hereafter subjected to primary Anti-uPA antibody 1:250 (sc-6831, Santa Cruz, Heidelberg, Germany), secondary antibody was polyclonal rabbit anti goat immunoglobulins/HRP (Dako). Blots were developed using the ECL Prime Western Blotting Detection Reagent system (Amersham™, Buckinghamshire, UK) with Amersham Hyperfilm™ MP (Amersham™). Optical densities of the bands were determined with Quantity One 4.03 software (Bio-rad).
Zymography 2.5 µL of crude urine was loaded into each well in 10% Zymogram (gelatin) Gels (Novex®, Carlsbad, CA, USA) following manufactures protocol. Incubation period with Zymogram Developing Buffer (10X) (Novex®) was 24 hours at 37°C. Urinary uPA activity Samples were diluted allowing measurements in the range 0-40 units uPA per ml according to manufactures protocol (ECM600, Chemicon® International, Millipore Corporation). To eliminate non-uPA specific background reading, the measurement at time = 10 minutes was subtracted the final reading after 5 hours. uPA enzyme-linked immunoassay kit human urine samples were diluted 1:4 and run in duplicate according to manufactures protocol (ab108917, Abcam, Cambridge, UK). Hydrophilic Interaction Liquid Chromatography (HPLC) analysis of Amiloride Chemicals: Amiloride hydrochloride (CRS, European Pharma) and Metformin hydrochloride was purchased from Sigma-Aldrich, Steinheim, Germany. Stock solutions of 1 mg/mL were prepared in Milli-Q water. Working solutions were also prepared in Milli-Q water. All solutions were prepared in amber glass vials to protect from daylight. Acetonitrile (CHROMASOLV for liquid chromatography) was purchased from Sigma-Aldrich, Steinheim, Germany. Ammonium acetate was purchased from Fluka Chemie GmbH, Buchs, Schweiz. HPLC conditions: The High-Performance Liquid Chromatography system was a LaChrom 7000 serie system and consisted of an L-7100 pump, an L-7250 autosampler, an L-7300 column oven, an L-7400 UV-detector and a D-7000 interface module (Merck-Hitachi, Japan). The analytical column was a Luna HILIC (3 µm) 150 x 4.6 mm column equipped with a HILIC SecurityGuard precolumn (Phenomenex, Torrance, CA). The mobile phase consisted of acetonitrile: 100 mM ammonium acetate, pH 5.8 (93:7) and was used at a flow rate of 1.5 mL/min. Amiloride was detected at a UV-wavelength of 362 nm and eluted at 6.2 min; whereas metformin was detected at 234 nm and eluted at 7.8 min. The total analysis time was 10 minutes. Sample preparation: A volume of
100 μL of the urine sample was transferred to a 9 mL conical glass test tube. Aliquots of 30 μL 50 μg/mL metformin (internal standard); 30 μL 5M NaOH and 2125 μL acetonitrile were also added, and the test tube was whirly mixed for 30 sec. The sample was then centrifuged at 3000 g in 2 minutes, and 150 μL was transferred to a 0.3 mL HPLC vial. The samples were protected from daylight during sample preparation. A sample volume of 50 μL was injected onto the column.

Calibration curves with 5 concentrations in a range from 2.5 – 20 μg/mL spiked in blank urine was produced each day of analysis. Quality control samples were also included in each series. The interday variability was < 2% and the mean precision was 98 %. The limit of quantification with the applied method was 0.15 ug/mL.

Statistic evaluation Data from children urine sample analysis was evaluated by paired Student’s t test. Two-way analysis of variance (2 way-ANOVA) was applied when comparing the four groups with 2 categorical variables (w/wo PAN; w/wo amiloride), followed by Bonferroni’s Multiple Comparison post hoc test to determine significant differences between groups. For all data, P values below 0.05 were considered significant. Statistical analyses were performed using Prism 5 software (GraphPad).
Results

Nephrotic syndrome is associated with changes of uPA in urine

On the first days after treatment, puromycin aminonucleoside (PAN) administration led to progressive and significant proteinuria in the rats that stabilized at day 4 post injection (Figure 1A). Amiloride treatment did not alter 24h urine protein excretion in control rats or in PAN-treated rats (Figure 1A). Western immunoblotting of crude rat urine samples from control and amiloride-treated rats collected at day -1, 4 and 6 post-PAN injection, revealed a weakly detectable protein with expected size of two-chain active uPA (33 kDa) (Figure 1B). In PAN-treated rats, a protein of 33 kDa compatible with uPA was consistently present at days 4 and 6 post-PAN (Figure 1B) at short exposure time. Comparison of individual rat 24h urine samples at day 4 post-PAN treatment showed uPA protein at low levels in control and amiloride treated rats (Figure 1C). PAN-treatment led to a significant and similar increase in uPA between PAN-vehicle and PAN-amiloride as judged from densitometry (Figure 1C, diagram).

Effect of amiloride on urine uPA activity in PAN nephrotic rats

PAN-induced nephrotic syndrome was associated with significantly increased uPA enzyme activity in urine at days 4 and 5 compared to control and amiloride treated rats (Figure 2A). Amiloride treatment (2 mg/kg/day) of PAN-nephrotic rats attenuated significantly urine uPA activity compared to vehicle-treated nephrotic rats at days 4 and 5 post PAN (Figure 2A). PAN treatment resulted in appearance of plasminogen (~100 kDa) and plasmin (~70-75 kDa) protein in rat urine at day 4 (Figure 2B). Urine samples from control rats at the same time point (day 4 post-vehicle), did not display plasmin or plasminogen (Figure 2B, right). Amiloride treatment of PAN rats altered the plasminogen/plasmin-ratio towards a lower level of plasmin (Figure 2B). The shift in plasminogen/plasmin-ratio coincided with diminished protease activity in amiloride-
treated PAN rats evaluated by gelatin zymography (Figure 2C). Urine samples from non-proteinuric control rats did not display detectable protease activity when loaded in similar zymogram gels and digested for 24 hour (data not shown). Amiloride concentration in 24h urine collected at day 4 post-PAN was on the order 10-20 µmol/L (Figure 2D-left) and was higher in PAN-amiloride rats. Amiloride urine excretion per 24h was not significantly different between PAN nephrosis and vehicle treatment. (Figure 2D-right).

uPA in human nephrotic syndrome patient urine samples

To examine human correlate, 18 paired urine samples from acute, idiopathic, childhood nephrotic syndrome patients were compared by ELISA in the acute and remission phases. Urine uPA/creatinine concentration ratio was significantly decreased at remission compared to acute phase (Figure 3A). Separation of the urine samples under denaturing conditions followed by immunoblotting for uPA showed in 15 samples a significant presence of a protein at 33 kDa compatible with uPA in the acute phase while in the remission phase the signal was decreased in 9 samples (data not shown). Urine uPA activity (Figure 3B) and uPA protein concentration by ELISA (Figure 3C) correlated directly with 24h urine protein excretion in 6 adult nephrotic syndrome patients. Immunoblotting of urine samples from the adult nephrotic syndrome patients and 3 healthy controls displayed significant uPA immunoreactive protein (Figure 3D).

Effect of amiloride on Na+ and water excretion in PAN nephrotic rats

Urine sodium excretion decreased significantly within the first 24 hours after PAN which persisted throughout the study compared to control rats (Figure 4A). The initial decline in sodium excretion is caused predominantly by a decreased intake as shown in detail before (35) and as seen by the negative balance in panel 4C. Administration of amiloride at day 4-6 post-
PAN induced a significant increase in sodium excretion compared to PAN-vehicle (Figure 4A). Control rats increased sodium excretion transiently in the first 24h after amiloride (Figure 4A). At day 6, the PAN-amiloride rats had sodium excretion similar to control rats. PAN-treatment resulted in accumulation of free ascites fluid in the abdominal cavity that was significantly diminished by amiloride treatment through days 4-6 post PAN and measured at day 7 at termination (Figure 4B). Since food intake varied just after PAN treatment as shown before (35), urine sodium balance was calculated and PAN rats accumulated sodium coincident with proteinuria at day 3-4 post-PAN compared to control rats (Figure 4C). Amiloride abolished the difference (Figure 4C). No significant difference was observed in the intake of food or water between PAN + amiloride vs. PAN or control vs. amiloride, respectively.

Discussion

The present study shows in patients and in experimental animals that nephrotic syndrome is associated with significantly increased, reversible, urinary excretion of urokinase-type plasminogen activator (uPA) and that amiloride, a K⁺-sparing diuretic, significantly reduces urine uPA activity; plasminogen-to-plasmin activation in urine; urinary protease activity and renal sodium retention and ascites formation in nephrotic rats. It is concluded that ENaC is necessary for Na⁺ retention and edema formation in nephrotic syndrome in rats and that urine uPA is a relevant target for amiloride. Human urine samples corroborated that increased urine uPA level is associated with nephrosis.

The significant natriuretic effect of amiloride in PAN-induced nephrotic syndrome in rats is well established (15, 35). A single study in pediatric patients confirms a significant natriuretic action at least equivalent to furosemide (14). The effect has mainly been attributed to the direct blockade of ENaC (32). The present data suggest a potential contribution of amiloride to
natriuresis also by attenuation of plasminogen to plasmin activation (Figure 5). The present data
do not allow discrimination between the direct ENaC inhibition and the attenuated activity of
uPA/plasmin in the natriuretic action of amiloride. The data support that amiloride inhibits urine
uPA activity by competitive antagonism since overall proteinuria and uPA protein abundance
were not diminished. Binding of pro-uPA to the uPA receptor (uPAR) leads to activation of uPA
and amiloride is able to reduce LPS-induced uPAR expression in podocytes and cancer cells (43,
45). It can therefore not be excluded that reduced podocyte uPAR expression by amiloride has
contributed to decreased urine uPA activity. In vitro, amiloride inhibits uPA with a $K_i$ of 7
µmol/L (40) and such concentrations were surpassed in the present rat urine samples with a daily
dosage of 2mg/kg amiloride. This concentration range may be reached also in human urine (34).
Because uPA has been shown in vitro also to activate ENaC directly (11, 21), inhibition of uPA
by amiloride may thus attenuate proteolytic activation of ENaC directly and indirectly through
diminished plasmin (10, 15, 19, 35). In high concentrations, plasmin directly cleaves $\gamma$-ENaC,
while at lower concentrations, it depends on a cascade with prostasin as the final mediator (29,
36). There may be other therapeutic benefits from inhibition of aberrant plasmin activation by
amiloride in urine such as prevention of the negative modulation of TRPV5 by plasmin (37).
Moreover, uPA/uPAR activity has been associated with glomerular affection in diabetic
nephropathy (22, 27). As to the origin of urine uPA, it circulates freely in plasma and is
synthesized along the tubular epithelium (31, 42) where immunoreactive uPA is associated with
the collecting duct (35). The abrupt increase in urine uPA with induction of glomerular damage
in rats and the decline in children with nephrosis in remission indicate aberrant filtration as a
significant contributor but clearly with a baseline level visible in normal control urine. In
nephrotic syndrome, plasma plasminogen decreases (2) while it increases in urine where it
correlates with albumin and since it is not produced by the epithelium, it derives predominantly
from aberrant filtration. The inactive 55 kDa single-chain uPA precursor is activated by proteolytical cleavage by various proteases including plasmin which generates a high molecular weight (HMW) active two-chain enzyme held together by a single disulfide-bridge (18, 28). The denaturing western blotting revealed a product in urine compatible with the heavy chain (Figures 1 and 3). The precursor and active HMW-uPA bind with high affinity to the uPA receptor uPAR (12). The present data suggest that no inactive single chain is present in urine. This could be due to an amplifying mutual activation between uPA and plasminogen/plasmin. The significant positive correlation between urine uPA and protein in adult nephrosis patients further supports that pathophysiological filtration of uPA from plasma across an injured glomerular barrier may account for the increase in urine uPA. A similar observation on plasma uPA has been previously observed (3). The combined urinary loss of fibrinolytic plasminogen and uPA may be causally related to the hypercoagulable, prothrombotic state associated with nephrotic syndrome (23).

Conclusion

Urokinase-type plasminogen activator is aberrantly co-filtrated with plasminogen through the injured glomerular filtration barrier in human and experimental nephrotic syndrome where it promotes activation of plasminogen to plasmin in pre-urine. Amiloride attenuates urine uPA activity which may by an additional beneficial therapeutic target to counter ENaC-mediated sodium retention and edema formation.

Disclosure

The authors have no disclosure to report.
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Legends to figures

Figure 1

The figure shows the effect of puromycin aminonucleoside (PAN) at day 0 with and without amiloride treatment from day 4 on urinary protein excretion and uPA protein abundance in urine from rats. **A** Four days after PAN injection, protein excretion was significantly elevated in PAN injected rats compared to non-PAN inj. control rats whereas no significant difference was observed between PAN and PAN+amiloride treated rats (two way ANOVA p<0.0001, n=6-8). **B** western immunoblotting for uPA protein in urine showed that uPA was present at low levels in control-vehicle rats and prior to PAN injection (day -1). There were increased urine uPA levels at day 4 and 6 after PAN treatment (n=6). **C** uPA protein level in urine was compared between groups at day 4 with significant proteinuria. Level of uPA was significantly increased in PAN rats compared to vehicle-treated rats. Amiloride treatment did not affect uPA protein abundance in vehicle or PAN rats (n=6). Data were evaluated by one way ANOVA followed by Bonferroni Post Hoc test. * Statistically significant difference between groups at P<0.05.

Figure 2

Effect of amiloride on uPA activity in PAN nephrotic rats **A** Curves show time course of uPA activity in urine from PAN nephrotic rats +/- amiloride and controls. PAN treatment significantly elevated uPA activity and amiloride reduced significantly uPA activity compared to vehicle-treated PAN rats. Two way ANOVA with Bonferroni post-test. *:PAN vs. Control, ¤: PAN vs. PAN+Amiloride. **B** Western blotting for plasminogen/plasmin in urine from n = 3 vehicle-treated PAN rats, n = 3 amiloride treated PAN rats and n = 2 non-PAN vehicle-treated control rats. Urine samples are from day 4 post-PAN injection. Plasminogen was detected at ~100 kDa and plasmin was detected at ~70-75 kDa only in PAN-treated rats amiloride caused a shift in the
plasmin/plasminogen-ratio towards the inactive plasminogen being the most prominent form in 
the amiloride treated urine.

PAN-rat nephrotic urine protease activity by zymography shows activity co-migrating with 
pure human plasmin control. Amiloride treatment reduced protease activity judged from 
inspection of the zymogram. Total amiloride concentration in rat urine day 4 post PAN 
injection and 24 hours after amiloride I.P. injection (2 mg/kg). Amiloride concentration in 24h 
urine samples was significantly higher in PAN-treated rats, whereas no difference was observed 
in 24 h urine amiloride excretion between PAN treatment and control. Unpaired students t-test, 
*: p<0.05.

Figure 3

uPA excretion in urine samples from nephrotic patients A Level of uPA in paired crude urine 
samples from pediatric patients (n=18) diagnosed with acute first onset or relapse nephrotic 
syndrome. uPA/creatinine ratio was significantly lower at remission. Paired t-test, *:p<0.05. B-C 
uPA in human adult spot urine samples from nephrotic patients and controls. B shows urinary 
uPA activity and C uPA immunoreactive protein measured by ELISA. uPA level and activity 
both correlate significantly to urinary protein D Immunoblot detecting only active 2-chain uPA 
(33 kDa) in crude urine samples from adult patients (n=6) with nephrotic range proteinuria and 3 
healthy controls. Expected molecular weight of intact pro- uPA is 54 kDa and after activation 
and denaturation, the antibody recognizes the heavy chain expected at 33 kDa.

Figure 4

A Urinary 24 hour sodium excretion from the 4 groups: PAN, PAN + amiloride, control 
(vehicle) and amiloride. Urinary sodium excretion was significantly reduced in the PAN treated
rats (+/- amiloride) compared to controls (+/- amiloride). The initial drop is caused by lower intake as seen below in panel c. Significant difference was observed between PAN and PAN + amiloride at day 6. Control and amiloride was significantly different at day 4, 24 hours after amiloride. 2-way ANOVA and subsequent Bonferroni post hoc test results are indicated on the graph. *: PAN vs. control, ◊: control vs. amiloride and ^= PAN vs. PAN+amiloride.

B Bar graph shows volume of ascites fluid collected at termination of the experiment, day 7. Rats from the group of PAN + amiloride displayed significantly less accumulation of ascites fluid in the abdomen compared to rats in the group of PAN+vehicle (2-way ANOVA, *p<0.05 and ***p<0.001, Post-hoc test was Bonferroni's Multiple Comparison Test).

C The curve shows sodium balance calculated by subtracting 24h excreted urinary sodium from daily sodium intake through chow and injection. Feces sodium excretion was not measured. In the acute phase after PAN, there is net loss of Na+ through urine. As proteinuria develops, and despite lower intake of chow, PAN rats accumulate more sodium compared to control rats from day 3 coinciding with the development of proteinuria. *: p<0.05, two way ANOVA followed by post-hoc Bonferroni multiple comparison test.

Figure 5

Effect of amiloride on uPA and ENaC in nephrotic syndrome. uPA is aberrantly co-filtered with plasminogen through the injured glomerular filtration barrier during nephrotic syndrome and is also thought to be secreted by the tubular epithelium. In the pre-urine, uPA activates plasminogen to plasmin. Plasmin may also activate pro-uPA. Plasmin activates the gamma subunit of ENaC through proteolytical cleavage leading to sodium retention. Amiloride attenuates not only urine uPA activity but also has a direct inhibitory effect on ENaC.
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Figure 1

A

![Graph showing proteinuria (μg/24h/100g BW) over days. The x-axis represents days (Day -1, Day 4, Day 5, Day 6), and the y-axis represents proteinuria. The graph shows three lines: PAN, Amiloride, and Control.](image)

B

![Western blot showing protein bands at 37 kDa and 25 kDa.](image)

C

![Western blot showing protein bands at 37 kDa and 25 kDa.](image)
Figure 2

A

B

C

D
Figure 4

A

B

C