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

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Stæhr 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–F241, 2015. First published May 13, 2015; doi:10.1152/ajprenal.00138.2015.—In nephrotic syndrome, aberrant glomerular filtration of plasminogen and conversion to active plasmin in urine are thought to activate proteolytically epithelial sodium channel (ENaC) and contribute to sodium retention and edema. The ENaC blocker amiloride is an off-target inhibitor of urokinase-type plasminogen activator (uPA) in vitro. It was hypothesized that uPA is abnormally filtered to 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 (2 mg·kg–1·24 h–1) 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 with remission phase. In six adult nephrotic patients, urine uPA protein and activity correlated positively with 24 h 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 in vivo. Urine uPA is a relevant target for amiloride in vivo.

ENaC; proteinuria; edema; proteolysis; sodium
from children diagnosed with NS were collected in a Danish multicenter study, approved by the Regional Ethics Committee, at debut of NS 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 Institutes of Health. The experimental protocol was approved by the Danish Animal Experiments Inspectorate under the Danish Ministry of Justice. Furthermore, experiments were approved by the veterinary board at the animal facility at University of Southern Denmark. Male Sprague-Dawley rats, 8 wk of age (Taconic Europe A/S, Ejby, DK), were used for the experiments. Rats were kept on a 12:12-h 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 before the PAN injection, rats were used for the experiments. Rats were kept on a 12:12-h 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 before the 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, St. Louis, MO) and MQ 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 before the PAN injection, rats were separated and placed individually in metabolic cages. Control 24-h urine collection (day −1) was collected after a 24-h adjustment period in the metabolic cage. PAN (Sigma; 15 mg·1 ml isotonic NaCl−1·100 g body wt−1) 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-h 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 of 15 rats did not develop adequate proteinuria (defined as: urine protein excretion > 0.1 g·24 h−1·100 g body wt−1 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 body wt) administered subcutaneously 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 s 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 24-h 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 (2×; Novex, Life Technologies) and incubated for 10 min. The samples were mixed with NuPAGE LDS Sample Buffer (4×; Invitrogen, Carlsbad, CA) and NuPAGE Sample Reducing Agent (2×; Invitrogen) and run on Tris-HCl ready gels 7.5% (plasmin/plasminogen detection) or MiniPROTEAN TGX gels 4–15% (uPA detection; Bio-Rad Laboratories, Hercules, CA). The gel was blotted on an Immobilon-P Transfer Membrane ( pore size 0.45 μm; Millipore, Billerica, MA). Plasmin/ plasminogen:3 μl of crude urine was loaded. After being blotted, the membrane was subsequently blocked in TBST with 5% skimmed milk and hereafter subjected to primary anti-plasminogen antibody 1:5,000 (ab6189, Abcam, Cambridge, UK) followed by secondary polyclonal rabbit anti-goat immunoglobulins/horseradish peroxidase (HRP; Dako, Glostrup, DK). uPA:human urine (5 g/l creatinine) or 10 μl rat urine was loaded. After being blotted, 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. Crude urine (2.5 μl) was loaded into each well in 10% Zymogram (gelatin) gels (Novex, Carlsbad, CA) following the manufacturer’s protocol. Incubation period with Zymogram Developing Buffer (10×; Novex) was 24 h at 37°C.

Urine uPA activity. Samples were diluted allowing measurements in the range 0−40 U uPA/ml according to the manufacturer’s protocol (ECM600, Chemicon International, Millipore). To eliminate non-uPA-specific background reading, the measurement at time = 10 min was subtracted from the final reading after 5 h.

uPA enzyme-linked immunosassay kit. Human urine samples were diluted 1:4 and run in duplicate according to the manufacturer’s protocol (ab108917, Abcam, Cambridge, UK).

HPLC Analysis of Amiloride

Chemicals. Amiloride hydrochloride (CRS, European Pharma) and metformin hydrochloride were purchased from Sigma (Steinheim, Germany). Stock solutions of 1 mg/ml were prepared in MQ water. Working solutions were also prepared in MQ water. All solutions were prepared in amber glass vials to protect from daylight. Acetonitrile (CHROMASOLV for liquid chromatography) was purchased from Sigma. Ammonium acetate was purchased from Fluka Chemie GmbH, Buchs, Schweiz.

HPLC conditions. The HPLC system was a LaChrom 7000 series 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 × 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 min.

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 5 mM NaOH, and 2,125 μl acetonitrile were also added, and the test tube was whirly mixed for 30 s. The sample was then centrifuged at 3,000 g in 2 min, and 150 μl were 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 to 20 μg/ml spiked in blank urine were produced each day of analysis. Quality control samples were also included in each series. The interday variability was <2% and the mean precision was...
The limit of quantification with the applied method was 0.15 μg/ml.

**Statistic evaluation.** Data from children’s urine sample analysis were evaluated by paired Student’s t-test. Two-way ANOVA was applied when comparing the four groups with 2 categorical vari-
ables (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, PAN administration led to progressive and significant proteinuria in the rats that stabilized at day 4 postinjection (Fig. 1A). Amiloride treatment did not alter 24-h urine protein excretion in control rats or in PAN-treated rats (Fig. 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; Fig. 1B). In PAN-treated rats, a protein of 33 kDa compatible with uPA was consistently present at days 4 and 6 post-PAN (Fig. 1B) at short exposure time. Comparison of individual rat 24-h urine samples at day 4 post-PAN treatment showed uPA protein at low levels in control and amiloride-treated rats (Fig. 1C). PAN treatment led to a significant and similar increase in uPA between PAN-vehicle and PAN-amiloride as judged from densitometry (Fig. 1C, diagram).

Effect of Amiloride on Urine uPA Activity in PAN Nephrotic Rats

PAN-induced NS was associated with significantly increased uPA enzyme activity in urine at days 4 and 5 compared with control and amiloride-treated rats (Fig. 2A). Amiloride treatment (2 mg·kg\(^{-1}\)·day\(^{-1}\)) of PAN-nephrotic rats attenuated significantly urine uPA activity compared with vehicle-treated nephrotic rats at days 4 and 5 post-PAN (Fig. 2A). PAN treatment resulted in appearance of plasminogen (~100 kDa) and plasmin (~70–75 kDa) protein in rat urine at day 4 (Fig. 2B). Urine samples from control rats at the same time point (day 4 postvehicle) did not display plasmin or plasminogen (Fig. 2B, right). Amiloride treatment of PAN rats altered the plasminogen/plasmin ratio toward a lower level of plasmin (Fig. 2B). The shift in plasminogen/plasmin ratio coincided with diminished protease activity in amiloride-treated PAN rats evaluated by gelatin zymography (Fig. 2C). Urine samples from nonproteinuric control rats did not display detectable protease activity when loaded in similar zymogram gels and digested for 24 h (data not shown). Amiloride concentration in 24-h urine collected at day 4 post-PAN was on the order 10–20 \( \mu \)mol/l (Fig. 2D, left) and was higher in PAN-amiloride rats. Amiloride urine excretion per 24 h was not significantly different between PAN nephrosis and vehicle treatment (Fig. 2D, right).

uPA in Human NS Patient Urine Samples

To examine human correlate, 18 paired urine samples from acute, idiopathic, childhood NS patients were compared by ELISA in the acute and remission phases. Urine uPA/creatinine concentration ratio was significantly decreased at remission compared with acute phase (Fig. 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 (Fig. 3B) and uPA protein concentration by ELISA (Fig. 3C) correlated directly with 24-h urine protein excretion in 6 adult NS patients. Immunoblotting of urine samples from the adult NS patients and three healthy controls displayed significant uPA-immunoreactive protein (Fig. 3D).

**Effect of Amiloride on Na\(^+\) and Water Excretion in PAN Nephrotic Rats**

Urine sodium excretion decreased significantly within the first 24 h after PAN which persisted throughout the study compared with control rats (Fig. 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 Fig. 4C. Administration of amiloride at day 4–6 post-PAN induced a significant increase in sodium excretion compared with PAN-vehicle (Fig. 4A). Control rats increased sodium excretion transiently in the first 24 h after amiloride (Fig. 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 (Fig. 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 with control rats.

**Fig. 5. Effect of amiloride on uPA and epithelial sodium channel (ENaC) in nephrotic syndrome.** uPA is aberrantly cofiltered with plasminogen through the injured glomerular filtration barrier during nephrotic syndrome and is also thought to be secreted by the tubular epithelium. In the preurine, 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.
Amiloride abolished the difference (Fig. 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 NS is associated with significantly increased, reversible, urinary excretion of uPA and that amiloride, a K⁺-sparking diuretic, significantly reduces urine uPA activity, plasminogen-to-plasmin activation in urine, urinary protease activity, renal sodium retention, and ascites formation in nephrotic rats. It is concluded that ENaC is necessary for Na⁺ retention and edema formation in NS 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 NS 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 (Fig. 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 Ki of 7 μmol/l (40) and such concentrations were surpassed in the present rat urine samples with a daily dosage of 2 mg/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 γ-ENaC, while at lower concentrations, it depends on a cascade with plasminogen-to-plasmin activation 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 with a baseline level visible in normal control urine. In NS, 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 (Figs. 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 pathophysiologically 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 NS (23).

In conclusion, uPA is aberrantly coagulated with plasminogen through the injured glomerular filtration barrier in human and experimental NS where it promotes activation of plasminogen to plasmin in preurine. Amiloride attenuates urine uPA activity which may be an additional beneficial therapeutic target to counter ENaC-mediated sodium retention and edema formation.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.S., K.B.B., R.F.A., F.N., and G.R.H. performed experiments; M.S., K.B.B., and F.N. analyzed data; M.S., K.B.B., P.S., and B.L.J. interpreted results of experiments; M.S. and K.B.B. prepared figures; M.S., K.B.B., and B.L.J. drafted manuscript; M.S., K.B.B., R.F.A., P.S., G.R.H., C.B., and B.L.J. edited and revised manuscript; M.S., K.B.B., and F.N. analyzed data; M.S., K.B.B., P.S., and B.L.J. conceived and designed the experiments; M.S. and K.B.B. prepared figures; M.S., K.B.B., and B.L.J. interpreted results of experiments; M.S. and K.B.B. performed experiments; M.S., K.B.B., and F.N. analyzed data; M.S., K.B.B., P.S., and B.L.J. performed experiments; M.S. and K.B.B. prepared figures; M.S., K.B.B., and B.L.J. interpreted results of experiments. P. Bie is thanked for editorial advice.

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