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

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Submitted 31 March 2015; accepted in final form 7 May 2015

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 “overflow hypothesis” by Siddall and Radhakrishnan (33). With no or minor change in the abundance and membrane association of renal epithelial sodium channel (ENaC) protein in NS, 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 α-, β-, and γ-subunits, and the γ-subunit requires cleavage by two enzymes acting in series to attain full activation (8). A range of serine proteases may cleave the γ-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 are 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 NS. 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 NS and that amiloride prevents tubular activation of plasminogen to plasmin by inhibition of urinary uPA. The hypothesis was addressed by administration of amiloride to puromycin aminonucleoside (PAN)-induced NS in rats and in an observational study in urine samples from patients with NS.

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 were recorded in agreement with the Regional Ethical Committee approval. Samples were anonymous. Aliquots of urine samples were kept at −80°C. Paired urine samples...
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 Ethics 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 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 (amiloride group) and 6 rats were injected with amiloride (2 mg/kg body wt) 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·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.

Urineary 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).

Urineary 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 Mini-PROTEAN 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). Western immunoblotting. 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.

**HPLC conditions.** The HPLC 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 system. 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 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 μmol/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. PAN rats accumulated more sodium compared with control rats from day 3. **P < 0.05, 2-way ANOVA followed by post hoc Bonferroni multiple comparison test.**

**Fig. 4.** A: urinary 24-h 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 with controls (±amiloride). The initial drop is caused by lower intake as seen below in C. Significant difference was observed between PAN and PAN + amiloride at day 6. Control and amiloride were significantly different at day 4, 24 h after amiloride. Two-way ANOVA and subsequent Bonferroni post hoc test results are indicated on the graph. *PAN vs. control. **PAN vs. amiloride. B: bar graph shows volume of ascites fluid collected at termination of the experiment, day 7. Rats from the PAN + amiloride group displayed significantly less accumulation of ascites fluid in the abdomen compared with rats in the PAN + vehicle group (2-way ANOVA, *P < 0.05 and ***P < 0.001, post hoc test was Bonferroni’s multiple comparison test). C: curve shows sodium balance calculated by subtracting 24-h 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 with control rats from day 3. **P < 0.05, 2-way ANOVA followed by post hoc Bonferroni multiple comparison test.**

**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 γ-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 INHIBITS uPA IN NEPHROTIC SYNDROME**

*AJR Renal Physiol • doi:10.1152/ajprenal.00138.2015 • www.ajprenal.org*
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+-sparing 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 prostatin 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 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 proteolytic 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 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 NS (23).

In conclusion, uPA is aberrantly cofiltered 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.

**ACKNOWLEDGMENTS**

V. Monrad, M. Rytt Hansen, L. Teusch, K. Andersen, I. Nissen (Dept. of Cardiovascular and Renal Research, Institute of Molecular Medicine), and B. Damby Sorensen (Clinical Pharmacology, Institute of Public Health) are thanked for skillful technical assistance. P. Bie is thanked for editorial advice.

**GRANTS**

The study was funded by Strategic Research Council (Danish Innovation Foundation), The Research Council for Health and Disease, The Lundbeck Foundation, The Danish Kidney Association, Helen and Ejnar Bjørnow’s Foundation, The Region of Southern Denmark, The AP Møller Foundation, and Odense University Hospital.

**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**


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