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a study in rats and humans

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Nephrotic syndrome is associated with increased plasma K\textsuperscript{+} concentration, intestinal K\textsuperscript{+} losses and attenuated urinary K\textsuperscript{+} excretion – studies in rats and humans

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Abstract

The present study tested the hypotheses that nephrotic syndrome (NS) leads to renal K\(^+\) loss due to augmented ENaC activity followed by down-regulation of renal K\(^+\) secretory pathways by suppressed aldosterone. The hypotheses were addressed by determining K\(^+\)-balance and kidney abundance of K\(^+\)- and Na\(^+\) transporter proteins in puromycin aminonucleoside (PAN)-induced rat nephrosis. Effect of amiloride and angiotensin II-AT1 and mineralocorticoid receptor (MR) antagonists were tested. Glucocorticoid-dependent MR activation was tested by suppression of endogenous glucocorticoid with dexamethasone. Urine and plasma samples were obtained from pediatric NS-patients in acute and remission-phase. PAN-nephrotic rats had ENaC dependent Na\(^+\) retention; displayed lower renal K\(^+\)-excretion but elevated intestinal K\(^+\) secretion that resulted in less cumulated K\(^+\) in NS. Aldosterone was suppressed at day 8. The NS-associated changes in intestinal, but not renal, K\(^+\) handling responded to suppression of corticosterone, while ATI and MR blockers and amiloride had no effect on urine K\(^+\) excretion during NS. In PAN-nephrosis, kidney protein abundances of ROMK and γENaC were unchanged while NCC was suppressed and Na\(^+\)-K\(^+\)-ATPase increased. Acute pediatric NS patients displayed suppressed urine Na\(^+\)/K\(^+\) ratio compared to remission and elevated plasma K\(^+\) concentration, while fractional K\(^+\) excretion did not differ. Acute NS is associated with less cumulated K\(^+\) in a rat model while acute NS-patients have elevated p-potassium and normal renal fractional K\(^+\) excretion. In NS rats, K\(^+\) balance is not coupled to ENaC activity but results from opposite changes in renal and fecal K\(^+\) excretion with contribution from corticosteroid-MR driven colonic secretion.
Introduction

Rats with experimentally induced nephrotic syndrome (NS) display renal Na⁺ retention and ascites. A transient elevation in plasma concentrations of renin, angiotensin and aldosterone (RAAS) is observed in this experimental model followed rapidly by significant suppression (10, 11, 24, 40). Na⁺ retention, both in nephrotic rats and patients, is not dependent on aldosterone (14, 26) and is not alleviated by albumin infusion or ACE inhibition in patients (8). Na⁺ retention is associated with the nephrotic kidney only (23), even in ex vivo isolated perfused PAN-nephrotic kidney (17). In rats, Na⁺-retention is significantly attenuated by amiloride, an ENaC-blocker (14, 26, 40), and amiloride was as efficient as furosemide to mobilize Na⁺ and decrease weight in a small cohort of pediatric NS patients (13). With variable data of changes in ENaC subunit protein abundances (10, 24) and down-regulation of most renal Na⁺-transporters in experimental NS (10, 24), these findings suggest a direct stimulation at the level of ENaC activity. Soluble serine proteases in urine e.g. urokinase, kallikrein, prostasin and plasmin cause proteolytic activation of ENaC in vitro (40, 41) and both urokinase, kallikrein and plasmin are present in NS urine (21, 39, 40). Increased ENaC activity generates a lumen-negative transepithelial potential that favors K⁺-secretion along distal convoluted tubule (DCT2), connecting tubule (CNT) and cortical collecting duct (CCD). Despite the elevated plasma aldosterone early in NS and increased ENaC activity, NS has not been associated typically with renal K⁺ loss and hypokalemia. By contrast, PAN-induced nephrotic rats exhibited impaired ability to excrete a dietary K⁺-challenge and NS was associated with lower activity of the secretory renal outer medullary K⁺ channel, ROMK (16). The suppression of plasma aldosterone concentration in the later phase of NS with avid Na⁺ retention (40) would putatively counteract K⁺-losses by down-regulation of ROMK, ENaC and Na⁺-K⁺-ATPase protein abundances in kidney. The present study was performed to test the hypotheses that 1) NS is associated with transient renal
K⁺ loss secondary to proteolytic/aldosterone-driven renal ENaC activity, and 2) a subsequent decrease in secretory K⁺-channels ROMK and KCa1.1 (BK) protein abundances by suppressed plasma aldosterone. The questions were addressed by measurement of urine and feces K⁺ excretion to assess total K⁺ balance in rats in response to PAN-induced acute nephrosis with stored samples from published studies (7, 39, 40); by analysis of plasma and urine K⁺ in a published cohort of pediatric NS patients at debut and remission (1, 2) and by determination of K⁺- and Na⁺ transport proteins that directly and indirectly regulate K⁺-excretion (ROMK, BK, Na⁺-K⁺-ATPase, ENaC, NCC) by immunohistochemistry and immunoblotting in rat and human kidney tissue.
Methods and materials

K-balance was studied in urine and feces samples from 3 published interventional studies (Experiment 1, 2, 3 and 4, Figure 1A) in rats as described below and in previous publications (7, 39, 40). Clinical correlates were determined in plasma and urine samples from a cohort of acute nephrotic pediatric patients at first contact and in remission phase (1) (Figure 1A). Tissue correlates were investigated in kidneys isolated from NS rats (39, 40) and adrenalectomized rats (experiment 5) (32) and in human kidney tissue from cancer nephrectomies where patients were selected based on pre-surgery medication or proteinuria (Figure 1A). K⁺ concentration was determined when studies were performed and data were stored.

Animal experiments

Procedures conformed to the Danish national guidelines for the care and handling of animals and the published guidelines from the National Institutes of Health. The original PAN intervention studies were approved by the Danish Animal Experiments Inspectorate under the Department of Justice (#171001-096). K⁺ balances were calculated from K⁺ determinations performed previously in 4 experimental series with male Sprague-Dawley rats (Taconic M&B, Lille Skensved, Denmark) with PAN-induced NS and vehicle-control in metabolic cages (Figure 1A) as published previously (7, 39, 40). Induction of NS was achieved with a single intraperitoneal injection of PAN (Sigma, St. Louis, MO, USA), 150 mg/kg diluted in 0.9% NaCl, control rats received a vehicle injection containing 0.9% NaCl (7, 39, 40). Two days prior to the PAN injection, rats were placed individually in metabolic cages. Control urine (day -1) was collected after a 24h period in the metabolic cage. Rats had free access to gelled standard chow and water throughout the experiment and daily intakes and outputs were recorded. At day 8, rats were anaesthetized after injection of sodium pentobarbital 50 mg/kg and terminated by collection of
blood through cardiac puncture. Plasma was isolated by centrifugation at 4,000 G at 4°C. Kidneys were separated into cortex and medulla and were snap frozen along with a snip of the liver in liquid nitrogen. Blood and tissue samples were stored at -80°C. **Series 1**: PAN nephrosis (n=8) was characterized compared with control (n=6) (40). **Series 2**: the impact of RAAS on K⁺ balance was determined by use of K⁺ values obtained previously after treatment of PAN-NS rats with subcutaneous injections of both angiotensin II and mineralocorticoid receptor antagonists (candesartan 1 mg/kg, canrenoate 100 mg/kg, n=8) and compared to PAN-NS rats treated with vehicle (n=8) (40). **Series 3**: K⁺ concentration were obtained from urine and feces from a third published series of metabolic cage experiments (7) where PAN and control rats were treated with dexamethasone (100 µg/kg, to suppress endogenous corticosterone) or vehicle. In **series 4**, 2 sets of data were included from series where PAN nephrotic rats and controls were treated with amiloride 2 mg/kg or vehicle by one daily injection from day 4 after PAN treatment until day 7 (n=6 and 6); and a step-up protocol where PAN and control rats (n=6 and 6) were treated with amiloride at 0,5 mg/kg from days 4-6 and 2 mg/kg from days 7-9 (39, 40). In the amiloride-intervention series, feces were not collected. In the 4 published series, K⁺ and Na⁺ concentrations were determined as described by flame photometry when the original cohort studies were performed (7, 39, 40). Urine K⁺-concentrations were registered and stored but not reported previously. Plasma aldosterone concentration was measured by radioimmunoassay as described previously (40). Rat kidney tissue from a series of rats (adult male Sprague-Dawley rats, 275 ± 3 g, n=20) with (n=10) and without (sham surgery, n=10) adrenalectomy was available. The adrenalectomy procedure was described previously (32) and was performed after approval by The Danish Animal Experiments Inspectorate under the Department of Justice (#2012-DY-2934-0004). Rats were euthanized (50 mg/kg pentobarbital i.p.) 7 days after surgery and kidneys were removed, frozen in liquid nitrogen and stored at -80 °C until analysis.
**Human nephrotic syndrome.** Paired plasma and urine samples from nephrotic children in acute (debut) and remission phase were from a published study (1, 2). In brief, patients from 1-15 years of age with debut or relapse of NS were included in the study. NS was defined as proteinuria >40 mg/m²/day, plasma albumin <25 g/L, edema and hyperlipidemia. Remission was achieved when urinary dipstick was negative for three days. Plasma and urine from 20 patients were included in the present study. Venous blood was collected in EDTA-coated vacutainers and plasma separated by centrifugation. Urine was collected for 6h after morning void and up to 40 mL was stored. Urine and plasma samples were collected before any pharmacological treatment was initiated. One Complete Mini tablet (Roche Diagnostics, Mannheim, Germany) was added to the urine sample. Plasma and urine were stored at -80°C. The study was approved by the local scientific ethics committee and the data protection agency and patients were enrolled after written informed consent from parents as reported (2). GFR was estimated in the pediatric patients using the revised Schwartz formula as reported (2).

**Human kidney tissue:** Human kidney cortex was obtained after informed written consent from patients that underwent unilateral total nephrectomy for renal cancer and the use of tissue was approved by the ethics committee of the Region of Southern Denmark (#20010035 until December 2014, from 2015 #S-2014-0159). Patients with pre-surgery medication and/or proteinuria were identified based on medical record information. 30-45 min after extirpation, cortex, outer and inner medulla samples were snap frozen in liquid nitrogen and stored at -80°C until use for homogenization. In parallel, wedge-shaped tissue blocks were cut along the cortical-papillary axis and subsequently paraformaldehyde-immersion fixed for 48h hours and then embedded and stored in paraffin. Subsequently 5 µm sections were cut and mounted on slides for histochemical labeling.
Urinary Extracellular vesicles (uEV). Urine samples were stored with protease inhibitors from the pediatric nephrotic cohort described previously (1, 2). It was possible to analyze 8 pairs of urine samples each with 40 ml of urine for uEV isolation. Samples were thoroughly vortexed after being thawed overnight at 4°C. After 30 min centrifugation (Sorvall RC 26 Plus) of a total of 70 ml (40 ml pure urine added 30 ml ultrapure water) of the samples (3,000 g, 4 °C), the supernatants were recovered. uEVs were isolated by ultracentrifugation (Beckman Ultracentrifuge L-70) at 45,000 RPM (220.000 g, 4 °C) for 100 min. Resuspension of the pellet took place in 2x100 μL buffer (imidazole: 25 mM; sucrose: 0.3 M; pH 7.2; EDTA-disodiumsalt: 1 mM; with addition of a “mini” protease inhibitor cocktail tablet (Roche)). The uEVs were frozen at -80 °C until usage for western blot analyses. The loaded volume of uEVs were normalized to urinary creatinine concentrations; in first concentration-dose experiments, exosome fractions corresponding to 2, 5, 10 and 20 µg creatinine were loaded and separated by SDS-PAGE. Urine creatinine concentration was determined by Dept. of Clinical Biochemistry, Skejby Hospital.

Western blotting. Tissue from patients with predicted low plasma aldosterone (patients receiving RAAS blockers prior to nephrectomy), patients with predicted high aldosterone (patients receiving diuretics only prior to surgery), patients with proteinuria (dipstick) and patients receiving no medication (“controls”) were used for analyses. Tissue from control rats and PAN rats were from previously published studies (39, 40). Tissue from human or rat were cut into small pieces with a scalpel and homogenized with a glass pestle. 10 μL buffer/mg tissue (0.3 M sucrose, 25 mM Imidazol, 1 mM EDTA-disodiumsalt) with protease inhibitor (Roche Complete mini Protease Inhibitor cocktail tablet) were added to tissue samples. Samples were centrifuged at 4°C for ten minutes at 6000g and protein concentration was determined in the supernatant. Protein concentrations were determined by Lowry Protein Assay (BioRad). In initial experiments,
increasing amount of protein were loaded to roughly estimate the range where signal related to
abundance (ROMK, NCC). Proteins were subjected to SDS-PAGE (Bio-Rad) together with a size
marker (Precision Plus Protein Dual Color (Bio-Rad)) and proteins were separated at 70 mA for 60-
90 minutes in 1X MOPS SDS Running buffer (Invitrogen). Proteins were blotted to a
polyvinylidene difluoride (PVDF) membrane (immobilon-P; Millipore) and the membrane was then
blocked for one hour in 5% dry skim milk in TBST (20 mM Tris-HCL, 137 mM NaCl, 0.05%
tween 20, pH 7.6) and incubated overnight at 4°C with specific primary antibody appropriately
diluted: polyclonal rabbit anti-KCNJ1 1:500 (NPB1-82879, Novus Biologicals); anti-KCNJ1
(abcam, ab85479); monoclonal mouse anti-Slo1/BKα, clone L6/60 1:100 (NeuroMabs, cat #75-
022), rabbit anti-Na-K-ATPase α1-subunit, 1:2000 (Upstate 06-520), Rabbit anti γ-ENaC 1:20.000
(A kind gift from Prof. J. Loffing, Zürich (48)), mouse monoclonal anti-human SLC12A3 (clone 3,
developed in-house directed against the peptide sequence GEPRKVRPTLADLHSFLKQEG,
immunization and harvest is described (54) and anti-β-actin 1:10.000 (ab8227, Abcam). After
additional washes with TBST primary antibodies were detected with horseradish peroxidase (HRP)-
conjugated antibodies (DAKO) diluted 1:2000 in 5% milk-TBST and visualized with an ECL
system (PerkinElmer). The intensity of occurring bands was measured using densitometry. In
several blots, the quality of homogenates from stored rat and human kidney tissue was compared to
urine exosomes and more freshly collected tissue.

**Immunohistochemical staining for light and fluorescent microscopy.** Initial steps (dewaxing,
hydration) in immunohistochemical staining were conducted as described previously (5). After
rehydrations, antigen recovery was achieved by boiling in a microwave for 20 min in citrate buffer
(pH 6, DaKO). After this, sections were placed in TBST buffer. Rabbit anti-ROMK (renal outer
medullary K-channel, KCNJ1, Novus Biologicals NPB1-82879 was diluted 1:100 in TBST buffer
with 5% skimmed milk. Then sections were washed for 3 times for each 5 min in TBST buffer.

Rabbit anti-Na-K-ATPase α1-subunit was diluted in TBST buffer 1:100 (Upstate 06-520). For light microscopy, secondary antibodies conjugated to horseradish peroxidase were used (HRP, DakoCytomation, Denmark or ImmPRESS from Vectorlabs). For fluorescence microscopy, staining was performed similar to above and as reported in detail previously (19). Following primary ROMK and secondary anti-mouse-HRP, a Cy3-coupled TSA substrate (TSA Cyanine 3, Perkin Elmer) was added to visualize ROMK immunolocalization. Sections were subsequently boiled in Tris-EGTA buffer (TEG, 10 mM Tris, 0.5 mM EGTA, pH = 9.0) to remove bound primary and secondary antibodies and subjected to another round of immunolabeling with polyclonal goat anti-aquaporin-2 (AQP2, C-17, Santa Cruz Biotechnologies, ), polyclonal rabbit anti-Na-K-2Cl cotransporter (NKCC2; HPA014967, Sigma Aldrich), polyclonal rabbit anti-Na-Cl cotransporter (NCC; HPA028748, Sigma Aldrich) and subsequently Alexa-labeled 488 secondary antibodies. Light and fluorescent microscopy was carried out using an Olympus BX51 microscope.

**Statistical evaluation.** Statistical analysis was carried out using GraphPad Prism 7 software. Data were tested for normal distribution using D’Agostino and Pearson normality test. When testing for statistical significance in K⁺ balance in different animal experiments, two-way Analysis of Variance (ANOVA) followed by Bonferroni’s multiple Comparison Post Hoc test was applied. Correlation between urine K⁺-Na⁺ ratios and plasma aldosterone in nephrotic children were obtained by linear regression. Unpaired or paired Students t-test and were applied when appropriate. Data are shown as means ± SEM. P<0.05 was considered statistically significant.
Results

**K+ metabolism in PAN-induced nephrotic syndrome in the rat.** PAN nephrotic rats displayed significant proteinuria (shaded area in Figures 1-4), transient stimulation of plasma-renin and aldosterone with onset of proteinuria (day 3) and a subsequent suppression during Na+ and volume accumulation (day 8) (40). Eight days after induction, creatinine clearance displayed a tendency to be reduced with quite large variation between rats but there was no significant difference between control (n=15) and nephrotic syndrome rats (n=18) (Fig 1B p=0.16, Mann Whitney test). Before PAN treatment, rats in the two groups were similar with respect to food- and water intake (40) and renal and fecal K+ excretion (Figure 1C, days -1, -2 and 0). Excreted K+ was distributed with ~85% of intake disposed through urine and ~15% via feces (Figure 1C). PAN treatment (day 0) led to a significant decrease in food intake (not shown) and hence K+ intake and absorption in the first 24h (not shown), which was reflected by reduced urine K+ excretion (Figure 1C). In the phase with development of significant proteinuria (days 2-8, shaded area Figures 1C-E), dietary K+ intake was not significantly different in PAN vs. vehicle rats (not shown). Urine K+ excretion was significantly lower at day 1, 2, 4 and 5 following PAN injection in the proteinuric phase (Figure 1C). By contrast, fecal K+ excretion displayed a peak 3-4 days after PAN treatment compared to vehicle-treatment (Figure 1C). These opposite changes in renal and fecal K+ excretion maintained day-to-day positive K+-balance (Figure 1D). The day by day cumulation of K+ was positive in both groups, but attenuated significantly in NS rats at day 8 post-PAN (Figure 1D). Cumulated Na+ was also positive in both groups but significantly larger in nephrotic rats (not shown). Vehicle-treated rats had a stable urinary [Na+]/[K+] ratio, while PAN-treated rats had a transiently increased urinary [Na+]/[K+] ratio at day 9 but significantly lower urinary [Na+]/[K+] ratio at days 1, 2, 4 and 5 (Figure 1E). During the proteinuric phase (shaded area Figure 1E), fecal [Na+]/[K+] ratio was
significantly lower at days 5 and 7 in PAN treated compared with vehicle-sham rats. At day 8, plasma $K^+$ concentrations were elevated above normal physiological levels in both groups but with no difference between the two groups (5.8±0.2 vs. 5.9±0.5).

**Effect of combined MR- and ANGII-AT1 receptor antagonist treatment on $K^+$ balance in PAN nephrosis**

The involvement of MR and ANGII-AT1 receptor activation for $K^+$ balance during PAN nephrosis was tested by treatment with MR/AT1 receptor blockers canrenoate and candesartan. In response to PAN, $K^+$ intake and absorption decreased significantly and similarly with no statistical difference in feces $K^+$ excretion between groups (MR/AT1 blocker- vs vehicle treated PAN rats, Figure 2A), and resulted in a similar and negative $K^+$-balance on day 0 (Figure 2B). In the proteinuric phase, days 2-8, MR/AT1 receptor blockers had no significant effect on intestinal $K^+$ absorption, fecal $K^+$ excretion and urinary $K^+$ excretion (Figure 2A). Thus, day-to-day $K^+$ balance in the proteinuric phase was not significantly altered by MR/AT1 blockade and both experimental arms were in $K^+$ balance (Figure 2B). The daily cumulated $K^+$ showed that the initial $K^+$ loss was not gained in the experimental period and rats in both series did not achieve a positive balance (Figure 2B). At day 2 and 4 the cumulated $K^+$ was significantly lower in the MR/AT1 blocker treated group (Figure 2B). Thus AT1/MR blockers did not prevent intestinal $K^+$ secretion in the proteinuric phase and had little influence on $K^+$ metabolism in the setting of NS. The abrupt decrease in $Na^+/K^+$ concentration ratio in feces and urine after PAN treatment in series 1 was observed also in this series and not altered by AT1/MR blockers (Figure 2C). Concurrent $Na^+$ retention was not prevented (40).

**Effect of suppression of endogenous glucocorticoid by dexamethasone on $K^+$ balance in PAN nephrosis**
Dexamethasone suppressed plasma corticosterone concentration (7), while Na⁺ balance was not changed in NS rats (7). Dexamethasone lowered K⁺-intake (not shown) and attenuated intestinal K⁺ secretion at a single measuring point (Figure 3A). Urine K⁺ excretion was unchanged by dexamethasone (not shown). There was no significant difference in cumulated K⁺ between dexamethasone and vehicle (Figure 3C).

**Effect of amiloride treatment on urine K⁺ excretion in PAN nephrotic rats**

Urine K⁺ was evaluated in 2 experimental series with amiloride intervention (Figure 4). In these series, 24h food intake and urine excretion were recorded while feces were not collected. Before amiloride intervention, urine K⁺ excretion in PAN nephrosis was significantly lower in one series (Figure 4D) but when normalized for food intake, only a single day remained significant (Figure 4C, D), as observed in the previous series (Figure 1). Amiloride treatment had no significant effect on urine K⁺ in the individual series and there were no significant differences between urine K⁺ excretion whether normalized for food intake or not (Figure 4A-D).

**Potassium homeostasis in pediatric patients with nephrotic syndrome**

Pediatric patients with acute NS displayed significantly higher plasma K⁺ concentration in acute phase compared to remission (Figure 5A), while eGFR was not different in the acute phase compared to remission (ml/min/1.73 m²: 124±38 vs. 119±21 p=0.55, (1)). Urine Na⁺/K⁺ concentration ratio was significantly lower in acute phase compared with remission (Figure 5B).

Fractional urine excretion of K⁺ was not significantly different between the acute phase and remission (Figure 5C) and fractional excretion of K⁺ did not relate significantly to plasma aldosterone concentration (that was measured previously (2)) at debut (Figure 5D) and remission (not shown). At debut, but not remission (not shown), there was a direct and significant relation between plasma concentration of K⁺ and aldosterone (Figure 5E). There was a significant inverse
relation between plasma $K^+$ and eGFR at debut (Figure 5F), but not at remission (not shown). Urine
$Na^+/K^+$ concentration ratio did not relate significantly to plasma aldosterone concentration at debut
and remission ($p=0.24$ and $p=0.29$, not shown). Change in plasma aldosterone concentration
between NS and remission (delta) related directly and significantly to change in plasma $K^+$ (Fig
5G).

**Abundance of $K^+$-transport protein ROMK in human and rat kidneys**

A primary anti-ROMK antibody (anti KCNJ1; Novus Biological) reacted with a protein migrating
at the expected size for ROMK (45 kDa) in human kidney cortex homogenate and membrane-
enriched fractions (Figure 6A). A similar band migrating at 45 kDa was detected in rat kidney with
two additional products detected at just above 50 kDa and at $\sim$60 kDa (Figure 6A). A second
primary anti-ROMK antibody (anti-KCNJ1, Abcam) for which the immunizing peptide was
available yielded also a single band at $\sim$45 kDa in human kidney that was abolished by pre-
incubation with molar excess of peptide ($\sim$10, 50 and 100 times, Figure 6B). The anti ROMK
antibody from Novus Biological was used for all subsequent analyses. There were no significant
differences in the intrarenal abundance of the 45 kDa protein between cortex, outer and inner
medulla in human kidney ($n=3$ each, Figure 6C). A mouse monoclonal antibody directed against the
alpha-1 subunit of $K_{Ca1.1}$ channel (BK) at a dilution of 1:100 yielded several bands on western blots
of rat kidney homogenates but with weak to absent signal at the predicted molecular weight at $\sim$105
kDa (not shown).

**ROMK immunolocalization in human and rat kidney**

Immunohistochemical labeling of human kidney sections with an anti-ROMK antibody (anti
KCNJ1; Novus Biological) yielded positive signal associated with apical membranes of renal
tubules. Based on localization and appearance, the apical signal was significant in thick ascending
limbs (*Figure 7A-C) and the macula densa segment (arrow, Figure 7C), while staining was less consistently detected in the distal nephron and collecting duct (CD) system (arrowheads, Figure 7A-B). In human kidney sections, the ROMK signal was co-localized with NKCC2, although not all NKCC2-positive thick ascending limb (TAL) cells were ROMK-positive (Figure 7D-F). ROMK co-localized with the thiazide-sensitive NCC transporter in the apical membrane domains of distal convoluted tubule (DCT) cells (Figure 7G-I). Aquaporin-2 (AQP2) is associated with later portions of the connecting tubule (CNT) and CD principal cells in cortex in human kidney (6). ROMK labeling was found in subsets of AQP2-positive CD cells, while some AQP2-negative intercalated cells (arrowheads) did not stain (Figure 7J-L). Immunohistochemical labeling of control rat kidneys for ROMK showed signal essentially as described for human kidney, with abundant signal in apical membrane domains in TAL and macula densa, while more distal segments showed weak apical signal (Figure 7M-O). ROMK immunolabeling disappeared at the junction between inner medulla and inner stripe of outer medulla and was virtually absent in inner medulla (Figure 7O).

**Effect of PAN-nephrotic syndrome in rat and proteinuria in human on aldosterone-sensitive distal nephron K⁺ and Na⁺ transport proteins**

In whole kidney homogenate from rats at day 8 after PAN-induced NS (n=6) and vehicle control (n=6), there was no change in ROMK protein abundance (Figure 8A). In human kidney from 6 patients with proteinuria (not nephrotic syndrome), ROMK abundance was not altered compared to three controls with no proteinuria (Figure 8B). Immunohistochemical staining for ROMK was evaluated in three controls and four PAN rats obtained from experimental series 1 and 2. Overall ROMK staining was significantly associated with TAL in controls and PAN (Figure 8C-F). In PAN kidneys, immunoreactive signal was variable and less distinctly associated with apical membranes (Figure 8D, F). This was also apparent at higher magnifications in controls (Figure 8E) and PAN animals (Figure 8F). Protein abundance for BK was not different between PAN nephrotic and
vehicle-treated rats by immunoblotting and not different in human kidney from patients with
proteinuria vs. control (not shown). ROMK protein abundance in rat kidney membrane fractions
was not changed ten days after adrenalectomy (Figure 8G). ROMK protein abundance was not
altered in human kidney tissue from patients receiving AT1 inhibitors/ACE inhibitors compared to
nephrectomy tissue from patients not treated with antihypertensives (Figure 8H).
At day 8 post-PAN treatment in rats, \( \text{Na}^{+}\text{-K}^{+}\text{-ATPase} \) \( \alpha \)-subunit protein abundance was significantly
increased in PAN rat comparted with control rat kidney (Figure 9A). Full length \( \gamma \)-ENaC subunit
protein abundance that migrated at \( \sim 80 \text{ kDa} \) was not different between groups (Figure 9B). The
thiazide-sensitive transporter NCC was significantly reduced in abundance in PAN rat kidney
(Figure 9C). In human kidney tissue from proteinuric patients, there was no difference in NCC
abundance (Figure 9D). In 8 paired samples of uEVs from the pediatric NS patients, there was no
significant change in NCC abundance in acute phase compared to remission (Figure 9E).
Immunhistochemical staining of kidney section from rats with nephrotic syndrome at day 8 after
PAN treatment and vehicle-control rats showed signal associated with tubular epithelium. In NS
tissue, some tubuli were dilated, there were casts and signs of cell injury (Figure 9F). Na-K-ATPase
staining was restricted to select tubules. In control rats, some tubuli exhibited stronger signal than
others, likely cortical thick ascending limbs of Henles loop, distal convoluted tubules and
connecting tubules, while most tubules displayed a faint level of staining (Figure 9G). Because
these patterns of labelling were quite different, no firm conclusions on abundance could be drawn.
In acute phase of nephrosis, u-exosome NCC abundance did not relate significantly to plasma \( \text{K}^{+} \)
concentration in the patients (Figure 9H).

Discussion
The present study showed that experimental acute nephrotic syndrome (NS) in rat was associated
with mild but significantly attenuated accumulation of \( \text{K}^{+} \) due primarily to increased \( \text{K}^{+} \) excretion in
feces. Na\(^+\)/K\(^+\) ratios in urine of rats and patients (and in feces in rats) with NS were suppressed. As reported previously for Na\(^+\) (10, 26, 40), this change in renal K\(^+\)-handling did not depend on ANGII-AT1 receptors or aldosterone-MR pathway; it did not depend on ENaC activity and was not associated with detectable changes in renal tissue abundance of K\(^+\)-secretory protein ROMK. ROMK immunolabelling predominated in the thick ascending limb. Concurrent there was amiloride-sensitive renal Na\(^+\) retention and volume expansion with similar ENaC level (26, 40). In NS, plasma aldosterone was suppressed at day 8, as was NCC, while Na-K-ATPase was increased (24). The present data show that, in contrast to predicted by the hypothesis, ENaC-mediated Na\(^+\) retention in experimental NS was not accompanied by severe urinary K\(^+\) wasting and hypokalemia. The present study showed a significant decline from high-normal K\(^+\) plasma concentration (not overt clinical hyperkalemia) in acute phase to clinically normal concentration in remission phase in pediatric patients (Figure 5). A tendency to elevated plasma K\(^+\) concentration compared to reference values in plasma samples from acute pediatric nephrotic patients was reported previously (16). In acute phase, there was a direct relation between aldosterone and K\(^+\) in plasma, and the changes in K\(^+\) and aldosterone between debut and remission related directly. Together with the lack of relation between plasma aldosterone and urine Na\(^+\)/K\(^+\) ratio and fractional K\(^+\)-excretion in acute phase this indicated that plasma aldosterone was not driving the change in renal K\(^+\) handling. Rather, the opposite relation appeared relevant, and plasma aldosterone may thus increase in response to a primary rise in plasma K\(^+\) in acute NS. The selective down-regulation in abundance of NCC and stimulation of Na-K-ATPase with no change in \(\gamma\)-ENaC in nephrotic rat kidney has been observed before and is in line with a non-aldosterone dependent event (10, 12, 24, 50). The changes in these transport proteins were similar to those seen after a chronic dietary K\(^+\) challenge and it can be speculated that these changes rely on elevated K\(^+\) concentration where modest increase or no change in ROMK was observed (28, 42, 47, 50). An acute oral K\(^+\) load enhanced ENaC cleavage
and ENaC activity in rats and mice (18, 38, 52). A clear limitation of the present studies is the lack of consecutive plasma K⁺ concentration measurements during nephrosis and the present tissue observations from day 8 after nephrosis could be too late to detect such changes in kidney tissue ENaC but it remains a possibility that the changes could be related to a primary increase in plasma K⁺ in nephrotic syndrome.

Estimated GFR (eGFR) in acute pediatric nephrosis patients displayed a large variation with no significant change between acute and remission phases (2). There was a significant inverse relation between plasma K⁺ and eGFR in acute NS. Severe reductions in GFR is usually necessary to observe dysregulated K⁺ balance. In PAN-treated rats, creatinine clearance at day 8 varied greatly but was not changed significantly and was therefore less likely to contribute to the observed changes in K⁺. PAN injection, in addition to podocyte injury, inflicts direct tubule-interstitial injury with complement attack and inflammation (30) that could compromise K⁺-secretory pathways. In addition to renal and gastrointestinal reabsorption/secretion, plasma K⁺ concentration is regulated also by sequestration/release of potassium by the large intracellular pools in liver and skeletal muscle (reviewed in (53) (20)). Thus, changes in release or deposition of potassium from skeletal muscle and liver could override changes in renal and/or intestinal handling of potassium in nephrotic syndrome.

By whatever mechanism that plasma [K⁺] is elevated in acute NS, it appears balanced by increased fecal excretion judged from the rat data. Thus, at baseline, approximately 15% of K⁺-intake was excreted through feces. Nephrosis led to transiently and significantly enhanced intestinal K⁺ loss mirroring the transiently decreased renal excretion and the plasma aldosterone concentration peak at day 5 after NS induction (40). The distal colon is a K⁺-secretory organ sensitive to aldosterone (22, 36) and glucocorticoid (4) action, with active apical ENaC (27). The significant drop in feces
Na⁺/K⁺ concentration ratio in acute NS would be in accord with aldosterone action. Intestinal K-
handling also depends on the hydrogen potassium-ATPase type 2 (HKA2). A down-regulation of
HKA2 in acute nephrosis would contribute to increased fecal loss of potassium. HKA2 is activated
by progesterone in males and females in states of low dietary K⁺ and considered a major pathway
that (re)absorbs potassium (15). The present study did not identify the intestinal segment involved
in increased K⁺ loss, but K⁺ secretion from colon becomes increasingly important in chronic kidney
disease (3). Combined AT1/MR blocker administration tended to attenuate intestinal K⁺ excretion
at day 4-5 without reaching significance, whereas dexamethasone treatment reduced fecal K⁺
excretion in PAN treated rats. Dexamethasone suppressed the rat glucocorticoid, corticosterone (7).
Since 11βHSD-2 is down-regulated—at least in kidney—in patients and rats with NS (45, 46),
excess and unopposed glucocorticoid-MR interaction could have contributed to the rat intestinal K⁺
excretion as indicated by the effect of dexamethasone. K⁺ secretion in colon is predominantly
through BK (37) and both BK-mediated K⁺-secretion and ENaC-mediated Na⁺ absorption are more
aldosterone/MR sensitive in colon compared to kidney (44).

Regulated renal K⁺ secretion through apical small conductance channels constituted by ROMK
occurs mainly in the late distal tubule and connecting tubule (25, 28, 35, 47, 51). K⁺ currents here
are sensitive to high K⁺ feeding (35), but insensitive to aldosterone alone or to Na⁺ restriction (34,
49). The “K⁺-sensor” mechanism depends on plasma K⁺ concentration, basolateral K⁺-channels
Kir4.1/5.1 (9) and intracellular changes in Cl⁻ concentration (43) to control NCC activity and
abundance.

The present data did not show differences in BK and ROMK protein abundances in kidney tissue
homogenate from NS (or with changes in aldosterone by using tissue from adrenalectomy and ACE
inhibition), while immunohistochemistry indicated lower intensity of ROMK signal in NS. The BK
canals are associated with intercalated cells and, in agreement with our observation, renal BK
abundance is not changed in NS (29, 33). Previous data in PAN-NS rats showed that apical K⁺-currents were absent from principal cells which was corroborated by lower ROMK mRNA, protein and staining in isolated collecting ducts. The present immunostainings from PAN rat kidney confirmed near absence of apical labeling for ROMK in principal cells in line with lower urinary K⁺-excretion in NS. This could indicate a counter regulatory mechanism or injury associated with NS (16). In normal kidney, there was a marked apical, ROMK-immunopositive signal in NKCC2-positive segments, while cells co-labeling for ROMK, NCC and AQP2 were clearly less abundant, both in PAN and control rat kidney and human kidney. This distribution of ROMK was in accord with published findings in rodents (16, 28, 51) while in human kidney, ROMK has been observed in subsets of cells in TAL, including macula densa, and only in cortical part of CD (31). Because ROMK immunoreactive protein in DCT/CNT/CCD appeared far less than in TAL, the immunoblotting signal from CNT/CCD was likely outweighed by TAL signal in total homogenate. Maxi- or big-K (BK) channels are associated with intercalated cells and western signal was weak and not changed in NS (29, 33).

In summary, the present data showed that acute NS is associated with elevated plasma K⁺ concentration in pediatric patients and opposing, acute, changes of K⁺-excretion with increased intestinal/fecal excretion and impaired renal/urine excretion. The hypothesis that changes in renal K⁺-handling in NS were driven by ENaC activity and by RAAS was refuted while Na⁺/K⁺ ratios in both urine and feces were suppressed in accord with increased ENaC activity. Acute NS is a situation where intestinal compensation for impaired renal K⁺ excretion may be relevant for systemic K⁺-homeostasis.

Acknowledgements
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Legends to figures

Figure 1.

A: K⁺-balance was studied in urine and feces samples from 3 published interventional studies (Experiment 1, 2, 3 and 4 as described (7, 36, 37). Clinical correlates were determined in plasma and urine samples from a cohort of acute nephrotic pediatric patients at first contact and in remission phase (1). Tissue correlates were investigated in kidneys isolated from NS and adrenalectomized (Experiment 5) rats and in human kidney tissue from cancer nephrectomies in which some patients had coincidental proteinuria at nephrectomy.

B: Creatinine clearance determined at day 8 by plasma and urine creatinine measurements in PAN rats (n=18) and in controls (n=15). Data were normally distributed and there was no significant difference by unpaired t-test.

C: K⁺-metabolism in PAN-induced NS (n=13) and vehicle-control rats (n=11). K⁺ was measured before, during, and after PAN treatment in urine and feces sampled for 24 hours in metabolic cages. Data in panel C and D were normalized to body weight (BW) and shaded area indicates the phase with progressive proteinuria. C: Intestinal K⁺-loss and urinary K⁺-excretion; D: K⁺-balance determined as difference between intake and sum of fecal and urine excretion and cumulated K⁺-determined as the sum of daily balance; E: Na⁺/K⁺ molar concentration ratios in feces and urine. Data were tested for significance by two-way ANOVA followed by Bonferroni’s multiple Comparison Post Hoc test. ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05.

Figure 2. Effect of combined administration of the ANGII-AT1 receptor antagonist candesartan (1 mg/kg) and the MR-antagonist canrenoate (100 mg/kg) on K⁺ metabolism in PAN-induced NS.
K\(^+\) was measured before, during, and after PAN treatment in urine and feces sampled for 24 hours (40). Both groups of rats were given a single PAN injection (150 mg/kg) at day 0, while pharmacological treatment was initiated 4 days before and given throughout (40). All data were normalized to body weight (BW). Shaded area illustrates the phase with progressive proteinuria. PAN rats were n=7 and PAN rats treated with candesartan and canrenoate were n=8). A: Intestinal and urinary K\(^+\)-loss B: K\(^+\)-balance and cumulated K\(^+\); C: Na\(^+\)/K\(^+\) molar concentration ratio in feces and urine. *p<0.05 PAN vs PAN with AT1/MR blockers.

**Figure 3.** Effect of dexamethasone administration (100 µg/kg, days 4-8 after PAN) on K\(^+\) metabolism in PAN-induced NS. All data were normalized to body weight (BW). Shaded area illustrates the phase with progressive proteinuria.

A: Fecal K\(^+\)-excretion based on measurement of K\(^+\)-content in collected feces. There was an abrupt and significant decline in feces K\(^+\) in PAN-dexa group; B: K\(^+\)-balance determined as difference between intake and sum of fecal and urine excretions. C: cumulated K\(^+\) determined as the sum of daily balance. Data were evaluated by two-way ANOVA followed by Bonferroni’s multiple Comparison Post Hoc test to compare groups. **p<0.01 (PAN vs PAN DEXA), ****p<0.0001 PAN vs control. N=6 in all groups except PAN-control n=7.

**Figure 4.** Effect of amiloride administration on urine K\(^+\) excretion in PAN-induced NS. All data were normalized to body weight (BW). Data are from 2 series where amiloride was administered at 2 mg/kg from day 4-6 after PAN treatment (A-B) and at 0.5 and 2mg for 2 days consecutively from days 5-8 post PAN treatment (C-D). Shaded area illustrates the phase with progressive proteinuria. Data were evaluated by two-way ANOVA followed by Bonferroni’s multiple Comparison Post Hoc test to compare groups. *p<0.05; **p<0.01
Figure 5. Clinical parameters related to K⁺ in pediatric patients with NS in acute phase and at remission. Patient characteristics have been published previously (2). A: K⁺-concentration in plasma at debut and at remission. There was significantly higher concentration at debut (***p=0.01, n=20). B: Urine Na⁺/K⁺ ratio was significantly lower at debut compared to remission phase (**, p=0.04; n=17). C: Calculated fractional K⁺ excretion at debut and remission. No significant difference was observed (p=0.5, n=16). D: Fractional urine K⁺ excretion did not correlate significantly to plasma aldosterone at debut (r²=0.2, p=0.08, n=16). E: Plasma K⁺ concentration correlated significantly to plasma aldosterone at debut (r²=0.27, p=0.01, n=20), but not at remission (r²=0.003, p=0.8, n=20, not shown). F: eGFR correlated significantly and inversely to plasma K⁺ at debut (r²=0.7, ****p<0.0001, n=20). G: There was direct relation between changes in plasma aldosterone and K⁺ concentrations from NS to remission. Differences in n values are due to a few missing measurements in urine samples (three missing values in urine K⁺ at debut, one missing value in creatinine measurements at remission). D’Agostino and Pearson normality test were applied to test for normal distribution. Non-normally distributed data were log-transformed. Log-normal distributed data are shown in semi-logarithmic diagrams. Students paired t test and linear regression were applied. ****p<0.0001, ***p<0.001, **p<0.01 and *p<0.05.

Figure 6. Validation of antibodies used for western blotting to determine K⁺-secretory proteins ROMK in rat and human kidney tissue homogenates. Western blot with different amounts of protein from human kidney homogenate (5, 10 and 20 µg), human membrane-enriched kidney homogenate (5 and 10 µg) and rat total kidney homogenate (20 µg) incubated with primary antibody (anti-KCNJ1) from Novus Biologicals (1:500). In rat and human samples, a distinct band was detected at approximately ~45 kDa, corresponding to the molecular weight of ROMK, and more bands with higher molecular weight were present in rat kidney. B: Western blot with human kidney protein probed with primary antibody anti-KCNJ1 from Abcam. The first lane pre-incubated
with excess of the peptide antigen used for immunization. The next lane is probed with antibody
preincubated with 10X excess of antigen. The next lane 50X excess, and the last lane is incubated
with 100X excess of antigen. All lanes were loaded with 20 μg human kidney cortex protein.
Perabsorption was achieved. C: Western blot with 20 μg human homogenates from cortex, outer
medulla (OM) and inner medulla (IM) from three different patient kidneys. The membrane was
probed with the primary antibody, anti-KCNJ1, from Novus (1:500). There were no differences in
signal.

Figure 7. A-C: immunohistochemical labeling of ROMK (Novus antibody) in human kidney
cortex. Black arrowheads indicate non-immunoreactive cells (most likely intercalated cells), while
grey arrowheads indicate principal cells in CD. *Indicate TAL segments and arrows indicate
macula densa. D-F: immunofluorescent co-localization of ROMK and NKCC-2 and overlay. G-I:
Immunofluorescent co-localization of ROMK and NCC and overlay. *Indicate TAL segments. J-L:
immunofluorescent co-localization of ROMK and AQP2 and overlay. Arrowheads indicate cells a
minority of cells negative for both AQP2 and ROMK, likely intercalated cells, in CD. M-O:
Immunohistochemical labeling of ROMK in control rat kidney. Arrowhead shows apical labeling in
cTAL. O: Shows the transition from outer stripe to inner medulla with disappearance of signal.
N=3-4 for each species.

Figure 8. Effect of PAN-induced NS on ROMK protein in rat kidney. A: Immunoblotting of rat
kidney cortex homogenate showed no significant difference in ROMK protein abundance between
PAN nephrosis (n=6) and vehicle control (Ctrl) (n=6). B: Immunoblotting of human kidney cortex
homogenate from patients with (n=6) and without (n=3) dipstick-determined proteinuria (PU)
showed no significant difference in ROMK protein abundance. C-F: Immunohistochemical
labeling of rat kidney sections from control-vehicle and PAN-nephrosis rats for ROMK. ROMK
signal was associated with apical membranes in TAL and much weaker intensity in cortical portions of CNT and CCD. Following PAN, kidneys showed less distinct ROMK signal more evenly distributed in the cytoplasm of positive cells (D, F). G: Effect of adrenalectomy on ROMK protein abundance in rat kidney homogenate (ADX) (n=4) and control (n=4) rats. There was no significant difference in protein abundance between the two groups. H: ROMK protein abundance in human kidney homogenate from patients treated with ACE inhibitors/ANGII AT1 receptor antagonists prior to nephrectomy and controls with no medication before nephrectomy (n=8 and n=4). There was no significant difference in kidney tissue ROMK abundance. In all blots, β-actin was used as a loading control. Student’s unpaired t test was applied to test for differences between groups.

Figure 9.

Effect of PAN-induced NS on Na\(^+\) transport proteins in rat kidney. A: Effect of PAN-nephrosis on the protein abundance of Na\(^+\)-K\(^+\)-ATPase α1 subunit (predicted size: 110 kDa) in kidney cortex homogenate (n=6 and n=6). There was a significant increase in abundance after PAN. B: Effect of PAN-nephrosis on the protein abundance of full-length γENaC (predicted molecular weight at ca 85kDa) in rat kidney cortex homogenate (n=6 and n=6). There was no significant difference in abundance. C: Effect of PAN-nephrosis on the protein abundance of NCC (predicted size: 150kDa) in rat kidney cortex homogenate (n=6 and n=6). There was a significant suppression of protein abundance in NS kidney tissue. D: Effect of dipstick-verified proteinuria (PU) on the protein abundance of NCC abundance in human kidney cortex homogenate (n=4 and n=6). There was no significant difference in abundance when normalized to β-actin abundance. E: NCC protein abundance in urine exosomes isolated from pediatric NS patients in acute phase (NS) and remission (R) (N=8 pairs). Human kidney cortex was used as positive control (20µg protein, HCP). Diagram shows densitometric evaluation of NCC in sample pairs. By paired students t-test there was no

In A-D, β-actin (predicted size: 42 kDa) was used as a loading control. In all immunoblots, student’s unpaired t test was applied to test for differences between groups and β-actin abundance was used to normalize for loading differences. *P<0.05; ****P<0.001.
References


Figure 1

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B

C

D

E

Feces K+ (μmol/100g bw) and Urinary K+ (μmol/100g bw)

Control vs. PAN

Cumulated K+ balance (μmol/100g bw)

Control vs. PAN

Cumulated Na+ balance (μmol/100g bw)

Control vs. PAN

Urinary Na+/K+ (μmol/100g bw) and Feces Na+/K+ (μmol/100g bw)

Control vs. PAN

Pediatric patients nephrotic syndrome

Human nephrectomy tissue

Experiment 5

Human observational studies

Experiment 1

PAN vs. Control

Experiment 2

PAN control vs. PAN + AT1/MR block

Experiment 3

PAN +/- dexamethasone vs. Control +/- dexamethasone

Experiment 4

PAN + amiloride vs. Control + amiloride

Experiment 5

ADX rat

Immunohistochemistry and western blotting

Rat intervention studies

Human observational studies

Experiment 1

PAN vs. Control

Experiment 2

PAN control vs. PAN + AT1/MR block

Experiment 3

PAN +/- dexamethasone vs. Control +/- dexamethasone

Experiment 4

PAN + amiloride vs. Control + amiloride

Experiment 5

ADX rat

Immunohistochemistry and western blotting
Figure 2

A

Feces K (μmol/100g bw)

PAN + Canrenoate/candesartan

PAN control

Days

B

Cumulated K+ balance (μmol/100g bw)

PAN + Canrenoate/candesartan

PAN control

Days

C

Urinary Na+/K+ (μmol/100g bw)

PAN + Canrenoate/candesartan

PAN control

Days
Figure 3

A

Feces K+ (μmo1/100g BW)

Days

-2 -1 0 1 2 3 4 5 6 7 8

Control/Dexa  PAN/Dexa
Control/Control  PAN/Control

B

K+ balance (μmo1/100g BW)

Days

-2 -1 0 1 2 3 4 5 6 7 8

Control/Dexa  PAN/Dexa

C

Cumulative K+ balance (μmo1/100g BW)

Days

-2 -1 0 1 2 3 4 5 6 7 8

Control/Dexa  PAN/Dexa
Figure 4

A

B

C

D

Amiloride

High
Low

Days

Urinary K+/food intake (μmol/100g bw)

Control
PAN

Amiloride

****

Days

Urinary K+/food intake (μmol/100g bw)

Control
PAN

Amiloride

**

Days

Urinary K+ (μmol/100g bw)

Control
PAN

Amiloride

****

Days

Urinary K+ (μmol/100g bw)
Figure 5

A

Plasma K⁺ [mmol/L]

**

B

u-Na-K

**

C

FeK⁺

NS NS NS Remission Remission Remission

D

FeK⁺

\[ r^2 = 0.205 \]

\[ p = 0.078 \]

E

P-K⁺ [mmol/L]

\[ r^2 = 0.272 \]

\[ * p = 0.0185 \]

F

eGFR [ml/min/1.73m²]

\[ r^2 = 0.6729 \]

\[ **** p > 0.0001 \]

G

ΔAldo [pg/L]

\[ r^2 = 0.3265 \]

\[ *** p = 0.0085 \]
Figure 6

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Figure 8

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[Image of a gel showing protein bands for Ctrl and PAN with corresponding bar graph for Ctrl PAN and Ctrl PAN with INT mm on the x-axis and [kDa] on the y-axis.

B

[Image of a gel showing protein bands for Ctrl and PU with corresponding bar graph for Ctrl PU and Ctrl PU with INT mm on the x-axis and [kDa] on the y-axis.

C

[Image of a histological section labeled Ctrl PAN with a scale bar of 250 μm.

D

[Image of a histological section labeled Ctrl PU with a scale bar of 250 μm.

E

[Image of a histological section labeled Ctrl ADX with a scale bar of 50 μm.

F

[Image of a histological section labeled Ctrl ACEi AT1 with a scale bar of 50 μm.

G

[Image of a gel showing protein bands for Ctrl and ADX with corresponding bar graph for Ctrl ADX and Ctrl ADX with INT mm on the x-axis and [kDa] on the y-axis.

H

[Image of a gel showing protein bands for Ctrl, ACEi, and AT1 with corresponding bar graph for Ctrl, ACEi, and AT1 with INT mm on the x-axis and [kDa] on the y-axis.
Figure 9

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