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Hydronephrosis is associated with elevated plasmin in urine in pediatric patients and rats and changes in NCC and γ-ENaC abundance in rat kidney

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Abstract

Obstruction of urine flow at the level of the pelvo-ureteric junction (UPJO) and subsequent development of hydronephrosis is one of the most common congenital renal malformations. UPJO is associated with development of salt-sensitive hypertension, which is set by the obstructed kidney, and with a stimulated renin-angiotensin-aldosterone system (RAAS) in rodent models. This study aimed at investigating the hypothesis that i) in pediatric patients with UPJO the RAAS is activated prior to surgical relief of the obstruction; ii) in rats with UPJO the RAAS activation is reflected by increased abundance of renal aldosterone-stimulated Na transporters; and iii) the injured UPJO kidney allows aberrant filtration of plasminogen leading to proteolytic activation of the epithelial Na channel gamma subunit (γ-ENaC). Hydronephrosis due to UPJO in pediatric patients and rats was associated with increased urinary plasminogen/creatinine ratio. In pediatric patients, plasma renin, angiotensin II, urine and plasma aldosterone and urine soluble pro-renin receptor did not differ significantly before and after surgery, or compared with controls. Increased plasmin/plasminogen ratio was seen in UPJO rats. Intact γ-ENaC abundance was not changed in UPJO kidney while low-molecular cleavage product abundance increased. The Na-Cl cotransporter (NCC) displayed significantly lower abundance in the UPJO kidney compared to the non-obstructed contralateral kidney. The Na-K-ATPase alpha-subunit was unaltered. Treatment with an angiotensin-converting enzyme inhibitor (8 days, captopril) significantly lowered blood pressure in UPJO rats. It is concluded that the RAAS contributes to hypertension following partial obstruction of urine flow at the pelvo-ureteric junction with potential contribution from proteolytic activation of ENaC.
Introduction

Congenital ureteropelvic junction obstruction (UPJO) is the most common cause of hydronephrosis in the ante- and neonatal period and is detected in approximately 1% of newborns (25). The obstruction is more common on the left side, usually partial and chronic, unless surgically corrected (19). Extensive studies on hydronephrosis have been performed using an animal model of UPJO, in which the left ureter is surgically inserted into the underlying psoas muscle to create a partial and chronic obstruction of urinary flow (8). This experimental approach yielded a more discrete obstruction, distinct from the dramatic acute total occlusion model that differs a lot from the clinical scenario with UPJO. Previous studies in both rats and mice have demonstrated a significant link between the degree of UPJO-induced hydronephrosis and the development of hypertension in adult life (8, 10-12). The UPJO-induced hypertension is salt-sensitive, and associated with elevated renin (10) and aldosterone levels (24). UPJO is accompanied by kidney injuries manifested by fibrosis and inflammation on the ipsilateral side as well as proteinuria, while the morphology/histology of the contralateral kidney is basically normal apart from having enlarged glomeruli (24). Hypertension is attenuated or even cured following relief of the obstruction or, notably, by removal of the hydronephrotic kidney (12). Moreover, recent studies show that ambulatory blood pressure in pediatric patients with congenital hydronephrosis is higher compared with healthy controls, and surgical correction of the obstruction significantly lowers blood pressure (2, 3). Thus, preclinical and clinical findings indicate that the UPJO kidney contributes to the development of hypertension, which is of salt-sensitive nature. Since data from rodents suggested increased activity of the RAAS (8, 11, 24), it was hypothesized in the present study that RAAS is reversibly elevated in human pediatric patients with congenital hydronephrosis due to UPJO. The renal mechanism underlying salt-sensitivity of blood pressure is not clear. If elevated RAAS is relevant in pediatric patients, it would predict up-regulation of aldosterone-sensitive Na transport proteins in the distal nephron and
collecting ducts, in particular the apical thiazide sensitive Na-Cl co-transporter (NCC), the epithelial Na channel (ENaC) and the basolateral Na-K-ATPase. Moreover, the significant proteinuria in the obstructed kidney could be accompanied by aberrant filtration of plasma proteases into urine, to proteolytically activate ENaC and drive salt-sensitive hypertension (30-33, 35). Thus, ENaC could with unchanged protein abundance be inappropriately activated by proteolysis in the obstructed kidneys. The present study was undertaken to examine the hypotheses that i) in pediatric patients with UPJO the RAAS is activated prior to surgical relief of the obstruction; ii) in rats with UPJO the RAAS activation is reflected by increased abundance of renal aldosterone-stimulated Na transporters and sensitivity of blood pressure to a RAAS antagonist; and iii) the injured UPJO kidney allows aberrant filtration of plasminogen leading to proteolytic activation of γ-ENaC.
Methods

Ethics

Animal study: The experimental study was approved by the institutional ethics review board in Stockholm (N139/15 & N314/12). All animal procedures were performed according to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or National Institutes of Health guidelines.

Clinical study: The clinical study was approved by the regional ethical review board in Uppsala, Sweden (EPN; Protocol Number 2011/267). Every child’s guardian gave informed consent. The study adhered to the principles of the Declaration of Helsinki. For details regarding the study population (Research design and Methods) please see previously published study (2).

Collection of human plasma and urine samples

Plasma and urine samples from twelve patients with unilateral congenital hydronephrosis were analyzed. Matched plasma samples from before (UPJO+) and after surgical treatment (UPJO-) were available and analyzed from eight patients (2), while matched urine samples from ten patients (before and after surgery) were available and analyzed. Only children with hydronephrosis that was not associated with any other diseases and did not receive antihypertensive medication were included in the study. Material from eight healthy age- and sex-matched controls were included (Ctrl). The inclusion was achieved at the Pediatric Surgery Department of Uppsala University Children’s Hospital in Uppsala, Sweden. Ambulatory blood pressure was measured for 20-24 hours preoperatively and repeated six months postoperatively in the hydronephrosis group (2). Similarly, 20-24 hours ambulatory blood pressure was measured in the control group.
Animals

All urine, blood and kidney tissue samples were from male Sprague-Dawley rats (Scanbur Charles River), either sham-operated or with UPJO to induce hydronephrosis, from published series (24). UPJO was created in three week old rats as previously described (9, 11, 24). In brief, the abdomen was opened by a midline incision and the left ureter was isolated and dissected free. The underlying psoas muscle was carefully split and the ureter was positioned inside the muscle and ligated to create a partial obstruction, and the abdomen was closed again, as originally described by Carlstrom et al. (11). Sham operation was performed in the same way, without dissecting the ureter. Following surgery, all animals were left to grow with free access to standard rodent chow (normal salt diet; 0.7% NaCl) and tap water. A telemetry device (PA-C40, DSI™, St Paul, MN, USA) was implanted in 8 weeks old rats to assess cardiovascular function, as described previously (9, 11, 24). After an acclimatization period of 10 days, blood pressure was continuously recorded in unrestrained animals for 48 hours. At termination, blood was collected from the abdominal vena cava and centrifuged, and plasma was frozen for later analysis. Excised kidneys were cleaned and the hydronephrotic ratio was calculated as previously described (i.e., residual renal urine weight / renal parenchymal weight) (11). An intervention series was conducted to examine the influence of the RAAS on blood pressure after experimental UPJO. Similar to that described above; UPJO was induced and a telemetry device was implanted. After the acclimatization period, blood pressure was continuously recorded during baseline (4 days) followed by treatment with an angiotensin-converting enzyme (ACE) inhibitor (captopril, 1 mg/ml) for 8 days.

Kidney tissue

Kidney cortex tissue from six UPJO rats (six obstructed, left kidneys (UPJO+) and six non-obstructed, right kidneys (UPJO-)) was compared with nine sham-operated rat kidneys. The tissue
was cut in pieces by a scalpel and homogenized in ice cold lysis buffer (20mM Tris-HCl (Merck, Darmstadt, Germany), 150 mM NaCl (Sigma-Aldrich, Copenhagen, Denmark), 20mM NaF (Sigma-Aldrich), 10mM Na,P,O₄ (Sigma-Aldrich), 1% Triton X-100 (Sigma-Aldrich) and 1 tablet cOmplete™ tablet protease inhibitor per 50 mL (Roche Diagnostics, Sigma-Aldrich)) with an electric homogenizer, before placed on ice for 30 minutes at 4 °C. The samples were centrifuged at 12,000 rpm at 4 °C and the supernatant were collected and frozen at -80 °C before further analysis.

The specificity of the NCC antibody was tested in rat homogenate, which was separated by centrifugation into: mainly plasma-bound proteins (pellet after 17,000 g), mainly subcellular vesicles (pellet after 200,000 g) and non-membrane bound proteins (supernatant after 200,000 g).

Protein concentration was determined using DC protein assay (Bio-Rad, Copenhagen, Denmark).

**Urinary and plasma measurements**

Albumin in urine was determined using a pre-coated albumin ELISA kits for human (108788, Abcam, Cambridge, UK) and rat (KSP-198, Nordic Biosite, Täby, Sweden) respectively. In the rats total urinary protein excretion rates were quantified using DC™ protein assay (Biorad), while human urinary Na and K were measured by a routine clinical procedure by department of Clinical Biochemistry at Odense University Hospital.

Urinary creatinine measurements in human samples were available from previous experiments (2). Aldosterone concentration in plasma and urine was determined with Aldosterone ELISA (MS E-5200, LDN, Labor Diagnostika Nord, Germany). Human EDTA-plasma was used as an internal standard, 79 ± 8 pg/ml. Between-assay coefficient of variation is 10%.

EDTA-plasma (100 μl) was incubated with plasma from a nephrectomized sheep for 3 h for determination of plasma renin concentration (PRC) by radioimmunoassay of Angiotensin I through the antibody-trapping method of Poulsen and Jørgensen as previously described (26).

Concentrations were measured by the rate of AngI formation and standardized in terms of
international units per liter (IU·l$^{-1}$) by the activity of the World Health Organization (WHO)

International Standard (ref. no. 68-356; National Institute for Biological Standards and Control, Hertfordshire, UK) of which samples of 0.05 IU/I were included in every run of the renin assay. In the period of measurement, 1 IU of the WHO standard corresponded to 32 ± 5 ng AngI per hour.

Between-assay coefficient of variation was 15%. Angiotensin II (AngII) hormone concentrations in plasma were measured by radioimmunoassay (using specific antibodies and charcoal-plasma to separate bound antigen from free) after extraction performed by use of Sep-Pak C$_{18}$ columns (Waters, Millipore Corporation, Milford, MA, USA). Immunoreactivity of AngII in plasma extracts was measured using antibody Ab-5-030682 raised in rabbits. The antibody was used as described previously (6). Essentially, plasma was incubated with antibody (final dilution of 1:1 000 000) and tracer $^{125}$I-labelled AngII (Department of Clinical Physiology, Glostrup Hospital, Denmark).

Urinary plasminogen was determined using Human Plasminogen Total Antigen ELISA kit (IHPLGKT-TOT, Innovative Research, Michigan, USA). Soluble pro-renin receptor (sPRR) was determined in human urine samples with a pre-coated ELISA kit (#27782, Immuno-Biological Laboratories, Gunma, Japan). Rat plasma-renin, aldosterone and -AngII were available from previous experiments (24).

**Differential centrifugation.**

Kidneys from rat were homogenized and pelleted as described in detail previously(14) with minor modifications. Kidneys were homogenized in fractionation buffer (140 mM NaCl, 10 mM Tris-Base, 1 mM EDTA, 0.5 mM EGTA) and spun for 2,000 g for 10 min. The supernatant was spun down at 17,000 g for 30 min and saved. The resulting supernatant was then centrifuged at 200,000 g for 30 min. Both pellet and supernatant was saved for western blotting.
Western blotting

15 μg of tissue homogenate or 26 μl of urine was loaded on each gel. Prior to plasminogen detection in the urine, the samples were concentrated using Acetone (Sigma). LDS sample Buffer (NuPAGE, Invitrogen, Thermo Fischer Scientific, Life Technologies, CA, USA) and sample reducing agent (Invitrogen) was added before denaturation for 5 minutes at 95 °C. Electrophoresis were performed on a 4-12% Bis-Tris protein gel (Invitrogen) for 50-70 minutes at 200 V, with MOPS SDS running buffer (Thermo Fisher Scientific). Precision Plus Protein Dual Color Standards (Bio-Rad) were used as a size marker. The gel was transferred to a PVDF membrane (Immobilon-P, Millipore, Copenhagen, Denmark) for 1.5 hour at 35 V and subsequently blocked with 5% skimmed milk (Sigma) in TBST (20 mM Tris-HCl (Sigma), 137mM NaCl (Sigma), 0.05% Tween 20 (Sigma), pH 7.6) before being incubated with primary antibody at a shaking table at 4 °C over night. The membrane was washed 3x5 minutes and primary antibodies were detected with horseradish peroxidase (HRP)-coupled secondary antibodies (Dako, Glostrup, Denmark) and Enhanced Chemiluminescence Substrate (ECL, PerkinElmer, MA, USA) using ChemiDoc™ XRS+ System with Image Lab™ Software (Biorad). Anti-plasminogen (Abcam, 154560) 1:5000, anti-β-actin (Abcam 8227) 1:5000, anti Na-K-ATPase α1-subunit (Santa Cruz Biotechnology, 16041) 1:5000, anti-AQP2 (Santa Cruz Biotechnology, 9882) 1:2000, anti-GAPDH (Abcam, 9485) 1:4000, anti-ATP6IP2/pro-renin receptor (Abcam, 64957) 1:500, anti-ROMK/KCNJ1 (82874, Novus Biologicals, Colorado, USA) were used as primary antibodies. An antibody directed against the rat C-termini γ-ENaC (NH2-CNTLRLDSAFSSLTQTNEF-COOH) was a kind gift by Prof Johannes Loffing, University of Zurich (36). For western blotting the antibody was diluted 1:20,000. An in-house developed monoclonal mouse anti-human SLC12A3 (NCC) antibody was used for western blotting. Immunization, harvest and characterization have been described in another manuscript(16). The antibody was directed against the peptide sequence.
GEPRKVRPTLADLHSFLKQEG. Clone #3 was used for western blotting in this manuscript. In addition to characterization by western blot, this clone recognizes NCC by immunohistochemistry in wild-type but not Slc12a3-deficient mice (Data not shown). Quantification was performed using Image Lab™ software (Biorad).

Statistical analysis

Results were tested for normality using Shapiro-Wilk normality test. If not normally distributed, log-transformation was performed. Unpaired Student’s t-test, paired t-test or one-way ANOVA with Tukey’s multiple comparisons test was used to test for differences. If log-transformation did not result in normally distributed data, the statistical analyses were conducted using the nonparametric Mann Whitney test, Wilcoxon signed-rank test or Kruskal-Wallis with Dunn’s multiple comparison test. Blood pressure responses to an ACE antagonist were analyzed by two-way repeated measure ANOVA, followed by Sidak’s multiple comparisons test. When analyzing tissue on western blot, density of bands of interest was normalized to density of β-actin or GAPDH as indicated. The mean of bands revealed in sham were adjusted to 100% on each blot or examined as ratio when testing for differences between the groups. Correlations were analyzed by linear regression and Pearson’s correlation coefficient (r), after diagnostic plots of the residuals were performed. Results with p-value < 0.05 were considered statistically significant and marked with *: p<0.05, **: p<0.005. Data are expressed ± SEM. Log-transformed normally distributed data are presented in semilogarithm scale diagrams with the geometric mean and 95% confidence interval. All statistical analysis and graph plots were performed using Prism 7 (Graph Pad Software, CA, USA).
Results

Blood pressure, urinary albumin and protein levels in pediatric patients with UPJO

24-hours mean arterial pressure (MAP) data from previous experiments (2) showed significantly decreased MAP after surgical relief of the obstruction (fig.1A, p=0.02). Urine albumin/creatinine ratios were not different between patients with UPJO compared to pediatric patients without obstruction (fig.1B). Three out of ten patients in the hydrourephrotic group before surgery (UPJO+) and five out of twelve in the hydrourephrotic group after surgery (UPJO-) met the criteria for moderate albuminuria (Albumin/Creatinine Ratio > 30 mg/g), while all subjects in the control group had normal ratio. No significant difference was detected before and after surgical relief of the obstruction (fig.1C). The degree of albuminuria in the pre-operation hydrourephrosis group did not correlate with MAP or with ΔMAP before and after surgery (table 1).

Renin-angiotensin-aldosterone system in pediatric patients with UPJO

No difference in plasma renin concentration was detected between the UPJO group before (UPJO+) and after (UPJO-) or between UPJO and the control groups (fig.2A) or in delta-values before and after operation of the obstruction (fig. 2B) in the UPJO group (p=0.07, Wilcoxon signed-rank test due to lack of normal distribution also after log transformation). Plasma AngII concentration was below detection range in four cases (one from the control group, one from pre-operated group and two from the post operated group). These were not included in the analysis. No significant difference was detected between the groups (Fig. 2C) or before and after surgery (fig.2D). There was no significant difference in plasma and urine aldosterone concentrations between the groups (fig. 2E, G) and no difference before and after surgical intervention in the obstruction group (fig. 2F, H). Soluble pro-renin receptor (sPRR) was determined in urine. Nine samples, three from each group, were below detection range and were not included. No difference was detected between the
groups (fig. 2I) or before and after surgical intervention (fig. 2J). No correlation was found between 24-hours mean arterial blood pressure measurements in the hydrenphrotic group and plasma-
alosterone, urinary-aldosterone, plasma-AngII, plasma-renin or urinary-PRR respectively (table 1)  or between the Δ values of the blood pressure before and after surgery and the Δ values of the  RAAS hormones (table 1). No difference was detected in urinary Na/K ratio (not shown).

Urinary plasminogen excretion in pediatric patients with UPJO

Total plasminogen concentration in urine was below detection range of the ELISA (0.5-500 ng/mL) in the major part of the samples (i.e., three out of ten UPJO+, nine out of twelve UPJO- and two out of eight controls). These samples were excluded before analyzing. Urinary plasminogen/creatinine concentration ratio was increased in both the UPJO+ and UPJO- group compared to healthy controls (fig. 3A). No difference was detected in paired samples before and after operation (fig. 3B, only three paired samples available). Western blotting with urine samples revealed distinct signal in the hydronephrosis group only (not shown) while no signal was detected in the controls.

Effect of UPJO on mean arterial blood pressure, hydronephrosis degree and protein- and albumin excretion in the rat model

In agreement with previous studies (11, 24), UPJO was associated with increased MAP in comparison to sham-operated animals (108 ± 2 vs. 90 ± 2, p <0.0001, Fig.4A). UPJO resulted in increased 24h urinary excretion of total protein- and albumin compared to sham-group (Fig. 4B, C).

There was no association between MAP and urinary protein- and albumin excretion (table 2). The degree of hydronephrosis (HNR) correlated directly with MAP in UPJO rats (table 2) (11). Hydronephrosis was not detected in the sham-operated rats (HNR <0.05). A positive correlation was found between plasma aldosterone levels (24) and MAP (24) in the UPJO group (fig.4D, table
There was a significant (p=0.003) blood pressure reduction in response to ACE inhibition in rats with hydronephrosis (fig.4E). Although not statistically significant (p=0.05), the averaged reduction in blood pressure during the treatment period clearly trended (p=0.05) to be more pronounced in UPJO rats (delta -11 mmHg) compared with that observed in sham rats (delta -6 mmHg) (fig.4F). No relation was observed between MAP and plasma renin or AngII concentration (table 2). Likewise, in the sham-operated rats, no correlation was detected between MAP and plasma-aldosterone, renin or AngII respectively (data not shown).

Effect of UPJO on urine proteases in the rat

Plasminogen protein was detected in rat urine by western blotting (Figure 5). Rat plasma, as positive control for intact zymogen, revealed a band ≈ 90kDa, compatible with the zymogen form of plasminogen, while positive control for active plasmin (urine from nephrotic rat (NU) (31)) displayed a migration pattern with bands corresponding to zymogen and two-chain, active plasmin at 70 and 50 kDa (fig. 5A). After in vitro urine concentration, the zymogen plasminogen was detected in six out of eight sham-operated rats and in four out of eight UPJO rats, while plasmin was detected in two out of eight sham rats and six out of eight UPJO rats (fig.5B). Quantification showed increased urine plasmin/plasminogen ratio in UPJO rats compared to sham (Fig. 5C, samples with no signal were excluded, p=0.02).

Effect of UPJO on Na transport proteins in rat kidney

The abundance of aldosterone-regulated Na transport proteins in kidney tissue was determined by western blot in ipsi- and contralateral kidneys from UPJO rats and in kidneys from sham operated rats. A newly developed murine monoclonal anti-NCC antibody yielded a product with the expected molecular size at ≈150 kDa in rat kidney that was pre-absorbed by pre-incubation with
surplus of immunizing peptide (fig.6A). Immunoblotting for total NCC in UPJO and control rats revealed a band around 150 kDa corresponding to predicted molecular weight (fig. 6B). NCC protein showed lower abundance in the obstructed kidneys (UPJO+) compared to their contralateral counterpart in paired analysis (UPJO-) (fig.6E, p=0.03) and compared to sham operated controls (fig.6F, p=0.03). There was no difference between sham and UPJO- (Figure 6F, p=0.99). The obstructed kidney of Rat nb 3 appear to have very low concentration of NCC (fig. 3B). The blot was repeated with similar result (not shown). β-actin confirmed equally loading (fig. 6B, lower panel).

An antibody directed against the Na-K-ATPase α-subunit revealed two distinct bands at 110 kDa and at 150 kDa (not shown). The band around 110 kDa corresponds to the predicted size of the Na-K-ATPase (fig. 6C). No significant difference was detected between obstructed UPJO (UPJO+) and non-obstructed (UPJO-) kidney and control (fig. 6G, H). Pro-renin receptor (PRR) protein abundance was not significantly different between UPJO+ and contralateral UPJO- kidney tissue (fig.6D, I) and no significant difference was detected between means of the groups (fig. 6J). No difference in protein abundances were detected in principal cell transporters AQP2 and ROMK proteins between UPJO+ and UPJO- or between mean in the groups (fig.7A-F). Band densities were normalized to β-actin or GAPDH densities, as indicated in their diagram (β-actin shown only for NCC).

Effect of UPJO on γ-ENaC abundance in rat kidney tissue

Western blotting on kidney tissue with antibody against the C-terminus of γ-ENaC revealed three well-defined bands in rat homogenates at ≈80-90 kDa, ≈37 and 20 kDa (fig.8A). As loading- and housekeeping control, β-actin protein showed no difference in abundance between or within groups and was used to normalize all γ-ENaC densitometry values. No difference in abundance between
UPJO+, UPJO- and control was detected in the ≈80-90 kDa band (fig. 8B,C). The 37kDa band appeared with higher density in UPJO- compared to their obstructed counterpart, UPJO+ (fig.8D, p=0.047). A significant lower density in mean was detected in the ≈37kDa band in UPJO+ kidneys compared to sham (p < 0.004), while no significant difference was detected between UPJO- and sham or between the UPJO+ and UPJO- (fig. 8E). The ≈20 kDa showed significant up-regulation in the UPJO+ kidney compared to UPJO- (fig.8F, p =0.03), while no differences were detected within or between groups (Figure 8G). No correlations were detected between MAP, plasma- aldosterone, renin or AngII respectively in the UPJO group and density of neither of the bands in the obstructed kidney (table 2). Hydronephrosis correlated negatively with the 37kDa band in the obstructed, UPJO+ kidney (p=0.02), while no correlations were detected with the other bands (table 2). No correlations were detected between plasmin/plasminogen ratio and density of any revealed band (table 2, four UPJO rat samples had information on both).
Discussion

The present findings demonstrate no significant relation between blood pressure and changes in plasma renin, AngII or aldosterone concentrations in pediatric UPJO patients. In a corresponding rat model of hydronephrosis, blood pressure and aldosterone levels were elevated and correlated; aldosterone-sensitive Na-transporters were reduced (NCC) or unchanged (Na/K-ATPase and γ-ENaC), while increased γ-ENaC cleavage was observed and the elevated blood pressure in UPJO was sensitive to ACE inhibition. Data indicate that the elevated blood pressure, associated with UPJO depended significantly on RAAS in a rat model while the correlation was less clear in pediatric patients with hydronephrosis. There was enhanced urine plasmin in both rat and human associated with differential proteolytic cleavage pattern of γ-ENaC and lower NCC level in the affected ipsilateral kidney. The functional contribution of ENaC and NCC in the UPJO-associated hypertension is at present not clear but an impaired ability to excrete Na by intrarenal mechanisms in the affected kidney could be involved.

We and others have proposed a link between hydronephrosis and later development of hypertension. A study by de Waard et al suggested reduction of arterial pressure after surgical management of dilated or obstructed upper urinary tracts (13). We found in a small prospective study that systolic and diastolic pressures were reduced following surgical correction of hydronephrosis in children with pelvic-ureteric junction obstruction (3). In adult unilateral hydronephrosis, cases showed that nephrectomy or pyeloplasty turned patients normotensive (1, 13, 29, 37). Furthermore recent studies from our group showed blood pressure is elevated in pediatric patients with hydronephrosis compared with healthy, age- and sex-matched children, and their blood pressure was significantly reduced following surgical management of UPJO (2, 3).
The present study showed a significant correlation between degree of hydronephrosis and blood pressure, which is in accordance with the affected kidney as the culprit. The UPJO rat model created various degrees of hydronephrosis corresponding to the clinical setting. The variable degree of hydronephrosis likely accounts for the variation in proteinuria, albuminuria and plasminuria as well as the magnitude of blood pressure elevation. One has to take into consideration that urine collected and analyzed in the present study originates from both kidneys, likely with a lower glomerular filtration rate (GFR) at the affected side, while only the injured kidney contributes to the above mentioned urine sequelae.

ENaC importantly contributes to the regulation of Na excretion by kidneys. Plasmin(ogen) enters the pre-urine in conditions where the glomerular filtration barrier is damaged and proteolytically cleaves γ-ENaC, resulting in increased ENaC-mediated Na reabsorption (31-33, 38) and development of hypertension in the clinical setting (5, 7) (4). Pediatric patients with UPJO children showed elevated urinary plasmin(ogen) before surgery compared to controls, and UPJO rats had a higher plasmin/plasminogen ratio, indicating that plasmin may play a role in the pathogenesis of hypertension associated with hydronephrosis.

Kidney tissue from the UPJO rat provided an exceptional opportunity to study the molecular mechanisms in the ipsilateral obstructed kidney in a paired intra-individual design with comparison to the non-obstructed contralateral kidney. Cleaved γ-ENaC in UPJO rat kidney was investigated using an antibody directed against the C-terminal part of γ-ENaC (36). Based on previous studies using the same or similar antibodies (15, 21, 23) it was estimated that the band detected at ≈ 80-90 kDa was compatible with intact γ-ENaC, while unexpectedly no furin/plasmin-cleaved γ-ENaC was detected. The ≈ 37 and ≈ 20 kDa bands presumably represent additional, not specified, cleavage
products with no known physiological role. Differential changes in abundance of these moieties were observed, however, at this point there is no evidence of physiological relevance of these lower molecular weight bands. Taken together, differences in γ-ENaC cleavage were detected between the groups, although the exact characteristics and physiological relevance of the cleavage products were not clarified and the intact γ-ENaC was unchanged. An explanation could be that proteases in the proteinuric UPJO+ rat is degrading the 37kDa band further and producing the 20kDa band.

A previous study found, that most of the major Na transporters such as the Na-K-ATPase (27) NHE1 (22), NHE3 (28) and NCC (28) were down regulated in the obstructed kidney in rats with both neonatally-induced UPJO and complete unilateral ureteral obstruction (UUO). Consistent with these previous findings, decreased NCC expression was detected in the UPJO kidney in the present study, while no significant difference was observed for Na/K-ATPase. Moreover, a previous study demonstrated increased urinary water excretion 1 year after neonatally-induced UPJO (18) and a down-regulation of the water channels aquaporin AQP1, AQP2 (27) and AQP3 (17). However, in the present study no significant differences in AQP2 protein expression were detected in the collecting duct. These discrepancies could be explained by the much shorter duration of UPJO in the present study.

The RAAS has been reported to be slightly elevated in rodent models of UPJO (8, 11, 24). In agreement, the blood pressure in the UPJO rats correlated significantly with their aldosterone levels and was significantly reduced by ACE inhibition. Aldosterone (24), but not plasma-renin (24), plasma-AngII (24) or pro-renin receptor (present study) was elevated in the UPJO rats. Aldosterone exert its main effects in the distal nephron, with ENaC as its main target under physiological conditions (20, 23). However, the present observations showed no up-regulation of aldosterone-
sensitive transporters in UPJO. A study from 2006 found that ACE-inhibitor prevented the reduction in renal blood flow and prevented progression of hydronephrosis in UPJO rats, suggesting important contribution of the RAAS (34). However, in that study the authors did not make any observations regarding MAP. The same observations were made in pediatric patients with UPJO, where no stimulation of any RAAS component was observed. Taken together, circulating RAAS contributes to hypertension in the chronic phase despite no markedly elevated level and the primary change associated with the UPJO kidney could involve inappropriately impaired Na excretion by the affected kidney.

In summary, the present study demonstrated no significant changes in plasma renin, AngII, aldosterone or plasmaPRR in the clinical settings of hydronephrosis due to UPJO in pediatric patients. However, rats with UPJO showed increased aldosterone and reduction of blood pressure in response to an ACE inhibitor. In rat, UPJO was associated with unchanged or suppressed aldosterone-sensitive Na-transporter abundances in kidney tissue. Damaged glomerular filtration barrier with albuminuria and increased plasmin to plasminogen ratio in the urine as well as differences in γ-ENaC cleavage state at the affected side suggest that abnormal proteolytical activation of ENaC could play a role in the pathogenesis of hydronephrosis-induced hypertension. It is concluded that elevated blood pressure associated with UPJO in rats and likely in pediatric patients involves increased RAAS activity with no major upregulation of Na transporters. The presence of active plasmin in urine in UPJO of both rats and patients could cause abnormal proteolytic activation of ENaC and impaired ability to excrete Na.
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Conflicts of interest

None

Author contributions

RZ, BLJ and MC, conception and design of research; RZ performed experiments; AAM and MC provided human and rat samples and blood pressure measurement data; RZ analyzed data; RZ, AAM, HD, PS, BLJ, MC interpreted results of experiments; RZ prepared figures; RZ, BLJ, MC drafted manuscript; RZ, AAM, HD, PS, BLJ and MC edited and revised manuscript and approved final version of manuscript.
References


Table 1 – Correlations, human samples

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<tr>
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<th>R²</th>
<th>p-value</th>
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<td><strong>Correlation with MAP</strong></td>
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<tr>
<td>Urinary/Urinary</td>
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<td>Plasma-Aldosterone</td>
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Table 1: Test of relation between mean arterial blood pressure (MAP) and difference in MAP before and after surgical intervention (ΔMAP) with urinary albumin and RAAS components in children with ureteropelvic junction obstruction (UPJO).
Table 2 – correlations, UPJO rat

<table>
<thead>
<tr>
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<th>p-value</th>
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<td>HNR</td>
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<tr>
<td>Plasmin/plasminogen ratio</td>
<td>0.001</td>
<td>0.97</td>
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</table>

Table 2: Correlations with mean arterial blood pressure (MAP) and semi-quantitative measurement of the γ-ENaC cleavage products, 80, 37 and 20 kDa, as detected with western blot, and urinary
albumin, protein, hydronephrosis ratio (HNR), plasmin/plasminogen ratio and RAAS components in rats with ureteropelvic junction obstruction (UPJO). *=p<0.05. ′ Data obtained from previous study(24).

**Figure caption**

**Figure 1**: Ambulatory 24 hour mean arterial blood pressure (MAP) was significantly lower after surgical intervention in the pediatric patients (A, n=8, p=0.02). No difference in albumin/creatinine ratio was detected in the hydronephrotic group before (UPJO+, n=10, p=0.55) compared with after (UPJO-, n=8, p=0.57) surgical intervention and compared to the control group (n=8)(B). There was also no difference before and after surgical intervention within the hydronephrotic patient group (C, n=10, p=0.94).

**Figure 2**: No differences were detected in plasma renin concentration (A, p=0.63) plasma angiotensin II (AngII) concentration (C, p=0.24), plasma aldosterone concentration (E, p=0.56), urinary aldosterone/creatinine concentration ratio (G, p=>0.99) or soluble urinary pro-renin-receptor (PRR)/creatinine ratio (I, p=0.11) between the groups (stated p-values represent UPJO+ versus control) or before and after surgical intervention (B, p=0.08 D, p= 0.14, F, p= 0.73, H, p=0.11, I, p=0.65).

**Figure 3**: Urinary plasminogen concentration was analyzed with pre-coated ELISA assay. Significant higher total plasminogen/creatinine ratio was detected in the hydronephrotic children before undergoing operation (UPJO+, n=7, p=0.01) which was not reduced following surgery (UPJO-, n=3, p=0.03) compared to the healthy controls (n=6). In the hydronephrotic group, three out of ten in the pre-operated group (UPJO+) and nine out of twelve after surgical relief (UPJO-)
two out of eight controls were excluded, since they were below detection range (detection range 0.5-500 ng/mL).

Figure 4: Increased mean arterial blood pressure (MAP) was detected in UPJO rats compared to sham (A, p<0.001). An increased level of total protein- (B, p=0.01) and albumin urine excretion (C, p=0.04) was detected in UPJO rats compared to sham. A positive correlation was detected between mean arterial pressure (MAP) and plasma-aldosterone (D, p=0.002, R²=0.82).

Increased mean arterial blood pressure (MAP) was detected in UPJO rats compared to sham (E).

Administration of an ACE-inhibitor resulted in decreased MAP in both groups (E) and no significant difference in ΔMAP (mean MAP day 1 to 4 minus mean MAP day 5 to 12) was detected between the groups (F, p=0.055).

Figure 5: Immunoblotting experiments for plasminogen with positive control nephrotic syndrome patient urine sample displayed a band at ≈ 90 kDa (plasminogen) with a migratory pattern that corresponded to zymogen and 2-chain active plasmin migrating at 70 and 50 kDa (NU, n=1), while only the 90 kDa band (plasminogen) was detected in plasma from rat (A). Urine from UPJO and sham operated rats revealed bands ≈ 90kDa and ≈ 50 kDa in both groups (B). Increased plasmin/plasminogen ratio was detected in UPJO rats (n=6) compared to sham (n=6) (p=0.02) (C).

Figure 6: Western blot with rat kidney cortex incubated with undiluted anti-NCC antibody (HSLC12A3 clone #3) with and without pre-absorption with immunizing peptide. The kidney cortex was centrifuged at 17,000g to primarily pellet plasma membranes (A, lane 1), centrifuged at 200,000g to primarily pellet subcellular vesicles (A, lane 2) and the supernatant containing non-membrane bound proteins, including IgG (A, lane 3). It is observed that the band observed at ≈150 kDa
kDa (corresponding to NCC) in lane number one is abolished after pre-absorption with peptide, indicating specificity of the antibody. The band at ≈250 kDa is often detected in frozen samples and likely represents NCC dimers (A). Immunoblotting experiments on kidney tissue homogenate from UPJO rats (n=6), from both obstructed (indicated by +) and non-obstructed kidney (indicated by -) and sham-operated rats (n=10) were performed with antibodies specific for Na-Cl cotransporter (NCC) (B), Na/K-ATPase α-subunit (C), and Pro-renin receptor (D). NCC protein abundance was down-regulated in the obstructed kidney compared to both the non-obstructed kidney (p=0.03) and the sham-operated control (p=0.002), when corrected for β-actin abundance (E, F), while no difference in Na/K-ATPase α-subunit was detected between the obstructed and non-obstructed counterpart (G, p=0.39) or between the groups (H). No difference was detected in pro-renin receptor protein abundance (I, J). β-actin and GAPDH were used as loading controls as indicated in diagrams. Sham-operated controls were adjusted to 100 % to be able to compare different blots.

Figure 7: Immunoblotting against ROMK revealed several bands, with the band ≈ 50 kDa (indicated by arrow) corresponding to ROMK (A). Immunoblotting against AQP2 revealed one band in all tissue homogenates (B). No difference was detected in ROMK (C, D) or AQP2 (E, F). β-actin was used as loading control. Obstructed UPJO kidneys are indicated by “+” and non-obstructed kidneys by “-”. Sham adjusted to 100 %, to be able to compare different blots.

Figure 8: Western blotting experiments on kidney tissue homogenate from UPJO rats (n=6) and sham-rats (n=10) for γ-ENaC revealed 3 distinct bands in all rats at ≈ 80-90kDa, 37 and 20 kDa respectively. β-actin was used as loading control (A). No significant difference was detected in the 80kDa band (B, C). The 37kDa band was down-regulated in the obstructed kidney compared to its non-obstructed counterpart (D, p=0.049) and compared to the sham-operated control (E, p=0.004).
The 20kDa band was up regulated in the obstructed kidney compared to the non-obstructed match (F, p=0.03, Wilcoxon test, lack of normal distribution), while no difference was detected between the groups (G). Obstructed UPJO kidneys are indicated by “+” and non-obstructed kidneys by “−”. Sham was adjusted to 100 % to allow comparison between different blots.