RENAL DENERVATION ATTENUATES NADPH OXIDASE-MEDIATED
OXIDATIVE STRESS AND HYPERTENSION IN RATS WITH HYDRONEPHROSIS

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ABSTRACT

**Aim:** Hydronephrosis is associated with development of salt-sensitive hypertension. Studies suggest that increased sympathetic nerve activity (SNA) and oxidative stress play important roles in hypertension and modulation of salt-sensitivity. This study primarily aimed to examine the role of renal SNA in the development of hypertension in rats with hydronephrosis. In addition, we aimed to investigate if NADPH oxidase (NOX) function could be affected by renal denervation.

**Methods:** Partial unilateral ureteral obstruction (PUUO) was created in 3-weeks old rats to induce hydronephrosis. Sham surgery or renal denervation was performed at the same time. Blood pressure was measured during normal, high and low salt diets. Renal excretion pattern, NOX activity and expression, as well as components of renin-angiotensin-aldosterone-system (RAAS) were characterized after treatment with normal salt diet.

**Results:** On normal salt diet, PUUO rats had elevated blood pressure compared with controls (115±3 vs 87±1 mmHg, p<0.05), and displayed increased urine production and lower urine osmolality. Blood pressure change in response to salt loading (salt-sensitivity) was more pronounced in the PUUO group compared with controls (15±2 vs 5±1mmHg, p<0.05). Renal denervation in PUUO rats attenuated both hypertension (97±3mmHg) and salt-sensitivity (5±1mmHg) (p<0.05), and normalized renal excretion pattern, whereas the degree of renal fibrosis and inflammation was not changed. NOX activity and expression, as well as renin and AT1A receptor expression, were increased in renal cortex from PUUO rats, and normalized by denervation. Plasma sodium and potassium levels were elevated in PUUO rats and normalized after renal denervation. Finally, denervation in PUUO rats was also associated with reduced NOX expression, superoxide production and fibrosis in the heart.

**Conclusion:** Renal denervation attenuates hypertension and restores renal excretion pattern, which is associated with reduced renal NOX and RAAS. This study emphasizes a link between renal nerves, development of hypertension, and modulation of NOX function.
INTRODUCTION

Hypertension is very frequent in patients with renal disease and its prevalence increases as renal failure progresses. Hydronephrosis due to the pelvic-ureteric junction obstruction is a fairly common condition, with an incidence in newborns of approximately 1%. Early observations of rather well preserved kidney function in children with hydronephrosis has led to a worldwide trend towards conservative treatment, but the long-term physiological consequences of this policy are not known. In a recent prospective study we showed that surgical treatment reduces blood pressure in children with unilateral congenital hydronephrosis (2). Several studies have demonstrated a clear link between hydronephrosis, induced by partial unilateral ureteral obstruction (PUUO), and development of hypertension in both rats and mice (13-15). Blood pressure elevation in our PUUO model is associated with increased renin-angiotensin-aldosterone-system (RAAS) activity, elevated oxidative stress, reduced nitric oxide bioavailability and sensitized afferent arteriolar reactivity and renal autoregulation (8, 9, 11). Moreover, increased sympathetic nerve activity (SNA) together with oxidative stress has been suggested to accelerate the development of renovascular hypertension (21, 26, 30).

Anatomically, the sympathetic nerve terminals are found in close association with the cells of the afferent arteriole and the granular juxtaglomerular cells as well as tubular epithelial cells along the nephron (4, 5, 21, 24). On a functional basis, renal SNA modulates renin release via actions mediated by beta-adrenergic receptors located on juxtaglomerular cells (24, 48). Acute denervation blunts renal autoregulatory responses, as evident by reduced tubuloglomerular feedback, while nerve stimulation restores or sensitizes the response (38). In addition, several studies have shown that renal denervation per se reduces sodium and water reabsorption in the proximal tubules (6, 7, 28) that can contribute to the blood pressure lowering effect. It has been also shown that potassium excretion can be increased after renal denervation because of an increased sodium/potassium exchange in the late distal and collecting tubules (57). Finally,
activation of renal SNA has been associated with oxidative stress (18) that may increase renal injuries and blood pressure further (17).

Although a correlation between increased renal SNA, oxidative stress and hypertension has been described, the mechanism remains unclear and has never been investigated in hydronephrotic animals with renal and cardiovascular disease. The aim of the present study was to investigate the link between renal nerves and regulation of NADPH oxidase (NOX) in the hydronephrotic kidney and in the heart. We hypothesized that renal denervation can attenuate blood pressure elevation and salt-sensitivity in this model of renovascular hypertension, which could involve reduction of NOX-mediated oxidative stress. Although the benefits of renal denervation in patients with resistant hypertension are being debated, our findings demonstrate a link between renal sympathetic nerves and modulation of NOX function, which may influence the development or progression of renal and cardiovascular disease.
METHODS

This study was approved by the institutional ethics review board in Stockholm (ID: N314/12). All animal procedures performed conform the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes or the NIH guidelines. Male Sprague-Dawley rats (Scanbur, Charles River, Germany) were divided into four experimental groups: sham operated rats (Control), controls with unilateral renal denervation (Control+DNx), rats with partial unilateral ureteral obstruction (PUUO) to induce hydronephrosis, and PUUO with unilateral denervation (PUUO+DNx). All animals were given a standardized normal salt diet (0.7% NaCl, SD389-R36, Lactamin, Kimstad, Sweden) for 4 weeks before cardiovascular function was assessed. In one series, salt sensitivity was evaluated by measuring blood pressure and heart rate changes during normal salt diet, followed by high salt (4% NaCl, SD312-R36, Lactamin, Kimstad, Sweden) and low salt (0.02% NaCl, Lactamin, Kimstad, Sweden) diet treatments (see details below). In a second series, all animals were euthanized after cardiovascular measurements on the normal salt diet, and blood and tissue samples were processed for further analyses.

Creation of partial unilateral ureteral obstruction

A PUUO was created in 3-week-old rats to induce hydronephrosis, as describe previously (14). In short, anaesthesia with spontaneous inhalation of isoflurane (Forene®, Abbot Scandinavia AB, Kista, Sweden), 2% in air, was used and the abdomen was opened sterile through a midline incision and the left ureter was isolated and dissected free. The underlying psoas muscle was split longitudinally to from an approximately 15 mm long groove in which the ureter was placed. The muscle edges were then sutured above the ureter with two 6/0 silk sutures thus embedding the ureter in the muscle. The abdomen was closed, and the animals were allowed to wake up under a heating lamp. Sham operations were performed in the same
way, but without dissecting the ureter. All animals were then left to grow with free access to normal salt diet for 4 weeks.

Renal denervation

During the procedure to induce hydronephrosis (i.e. 3-weeks of age) the left kidney from control or PUUO rats was denervated or exposed to a sham denervation. Renal denervation was accomplished by previously validated surgical-pharmacological procedure (20). In brief, the left kidney artery and vein were exposed through the abdominal incision and isolated from the surrounding connective tissue. Mechanical denervation was performed by stripping all visible nerves along the renal arteries and veins from the aorta to the hilum of the kidney. Chemical denervation was performed by painting the renal artery with phenol (20% in ethanol) for two minutes. The artery was then carefully washed with isotonic saline. For sham denervation, the surgical procedure was the same, but the renal artery and vein were not isolated and the nerves were left intact.

Telemetric measurements

The telemetric device (PA-C40, DSI™, St Paul, MN, USA) was implanted in the adult animals, and blood pressure and heart rate were measured as previously described.(12) Telemetric measurements for 48 hours were conducted during normal, high and low salt diet conditions. Animals were kept on different sodium diets for 7 days respectively before cardiovascular data were collected.

Renal excretion measurements

Rats were housed individually in metabolism cages for 24 hours with food and water given ad libitum. Water consumption and urine production were measured gravimetrically. Sodium and potassium concentrations were determined by flame photometry (FLM3, Radiometer,
Copenhagen, Denmark), and urine osmolality by depression of the freezing point (Fiske 210 Micro-Sample Osmometer, Fiske Associates, Norwood, MA). Urinary protein content was determined by the colorimetric method of Detergent Compatible Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Plates were read using a microplate reader (model Safire II; Tecan Austria, Grödig, Austria) at 750 nm absorbance as previously described (12).

**Determination of hydrenephrotic ratio and collection of tissues and plasma**

Once the renal and cardiovascular studies had been conducted, animals were anesthetized by intraperitoneal injection of Thiobutabarbital sodium (Inactin®, 120 mg/kg body weight) whereupon the abdomen was opened using a midline incision. A macroscopic examination of both kidneys was performed. Blood was collected from v. cava and transferred to tubes containing EDTA (final concentration 2 mM), immediately centrifuged at +4° C (2000 rpm, 5 min) and stored at -80°C for later analysis. Kidneys and heart were rapidly excised, weighed, rinsed, snap frozen in liquid nitrogen and stored at -80°C for later analysis, or prepared for histology (described below). The hydrenephrotic ratios (HNR) were calculated, before freezing, in the same way as earlier described (i.e. HNR=residual urine weight/renal parenchymal weight) (60). Renal cortex and medulla was dissected on ice and frozen separately (-80 °C) for later analysis.

**NADPH oxidase activity**

Chemiluminescence technique was used to determine NOX-mediated superoxide formation. In brief, kidney cortex and medulla pieces (between 50-70 mg) were homogenized by adding a volume of phosphate-buffered saline (PBS) that equals 3 times the tissue weight, zirconium oxide beads and finally using the bullet blender. The homogenate was then centrifuged at 4°C for 20 min at 2000g. NADPH (100 μM) and lucigenin (5 μM) (Sigma-Aldrich) were added into 1/100 diluted tissue supernatant in PBS. For the hearts we followed a similar protocol. In
brief, similar heart slices from the left ventricular area (between 220-300 mg) were homogenized with the bullet blender by adding stainless steel beads. The homogenate was then centrifuged at 4°C for 20 min at 12000g. 300 μM NADPH and 5 μM lucigenin (Sigma-Aldrich) were added into 1/5 diluted tissue supernatant in PBS. In both kidneys and hearts the final NOX activity was determined by measuring the lucigenin chemiluminescence every 3 seconds for 3 minutes with the AutoLumat LB953 Multi-Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany). The chemiluminescence signal was corrected by protein quantification (Bradford protein assay, Bio-Rad Laboratories, UK) and expressed as % of respective controls. NOX activity in tissue-samples without NADPH, or in tissue-free samples with NADPH and lucigenin, the signal was similar to blank (i.e. PBS only), demonstrating specificity for NOX enzyme activity.

**Real time quantitative reverse transcription PCR**

Total RNA was isolated from kidney cortex or the heart using RNeasy Mini Kit (QIAGEN, Valencia, CA), and the cDNA was synthesized with the High Capacity cDNA Reverse transcription kit (Applied Biosystems) according to the manufacturer’s protocol with some modifications. The kidney cortex was homogenized in the bullet blender by adding RNase free zirconium oxide beads and the appropriate volume of RLT buffer. The heart was homogenized similarly but we used Trizol instead of RLT buffer and RNase free stainless steel beads to achieve a better homogenization. After homogenization we used a portion of homogenate corresponding to 25mg of tissue for RNA extraction. RNA concentration of our samples was estimated with the NanoDrop and at the end the RNA samples were diluted in order to have 2μg of RNA. This amount of RNA was converted to 2μg of cDNA in a total volume of 20μl. Quantitative PCR analysis was performed according to the Applied Biosystems 7500 standard protocol. Power SYBR Green Master mix (Applied Biosystems) was used for amplification and detection of DNA. PCR reaction was performed in 96-well
plates with 20μl mixer/well (15 μl of mastermix containing 0.25 pmol/μl of all the used
primers apart from p47phox where 0.1 pmol/μl was used instead and 5μl of cDNA
corresponding to 20ng of RNA). The annealing temperature was 53°C for p47phox, Nox2 and
p22phox, 49°C for Nox4, and renin, 50°C for p67phox and AT1 receptor. The used
housekeeping genes were GAPDH, 18S rRNA and TBP, which presented nice amplification
profile and melting curve in all different annealing temperatures. The efficiency of PCR was
calibrated according to the standard curve and the mRNA level was normalized with a
respective housekeeping gene by the ΔCt method. The final results are expressed as % of the
respective controls. Primer sequences (Table 1) and amplification profiles used for Nox2,
p22phox, p47phox, p67phox, Nox4, renin, AT1 receptor, 18S rRNA, GAPDH and TBP are
according to that previously described (1, 41).

**Plasma Analysis**

*Sodium and potassium:* Electrolyte concentrations were determined by flame photometry
(FLM3, Radiometer, Copenhagen, Denmark).

*Renin:* EDTA-plasma incubated with surplus of substrate-enriched plasma from a
nephrectomized sheep for 24 h. Plasma renin concentration (PRC) was measured by the rate
of ANGI formation and ANGI was detected by radioimmunoassay through the antibody-
trapping method of Poulsen and Jørgensen (49) with minor modification (29). Assay was
standardized in terms of international units of renin per liter (IU·L−1) by the activity of the
WHO International Renin Standard (ref. no. 68-356; National Institute for Biological
Standards and Control, Hertfordshire, UK). Samples of 0.05 IU/L were included in every run
of the renin assay. In the period of measurement, 1 IU of the WHO standard corresponded to
4.3±0.8 ng ANGI per hour. Between-assay coefficient of variation was 19%.

*ANG II:* EDTA-plasma samples (1ml) were extracted according to manufacturer (Sep-Pak
Classic C18 cartridge, WAT051910). Dried eluates were stored at -80°C until assay. A
specific anti-ANGII antibody (Ab-5-030682) was used in a final dilution of 1:1,100,000. Plasma samples incubated with antibody for 24 h and with tracer $^{125}$I-labeled ANG II (kindly provided by the Dept. of Clinical Physiology, Glostrup Hospital, Denmark) for another 24 h. Free antigen was removed by adding a charcoal-plasma suspension, and, after centrifugation, radioactivity of the supernatant was measured. The detection limit was 1-2 pg/ml. Values were corrected for the extraction recovery of unlabeled ANG II (~75% in the present analyses) added to plasma in the individual assay. Intra- and inter-assay coefficients of variation were 5 and 11%, respectively.

**Aldosterone:** ELISA kit (MS E-5200, human aldosterone, Labor Diagnostika Nord, Germany) was used to determine aldosterone. EDTA-plasma incubated with aldosterone-HRP conjugate for 1 h. as described by the manufacturer. Human EDTA-plasma pool was used as an intern standard (~87 pg/ml). Between-assay coefficient of variation was 10.5 % and intra-assay variation 6%.

**Histology**

Sagittal slices from both the kidney and heart were placed in 4% PFA solution immediately after sacrifice. All the slices were stored at 4°C and transferred to 70% ethanol solution the next day. The tissues were embedded in paraffin, sliced into 5μM sections and then stained with either Hematoxylin & Eosin or with Picroserius. For the histopathological evaluation, sections from 6 animals/group were evaluated for fibrosis and inflammation (i.e. infiltration of plasma cells and lymphocytes) in a blinded fashion. A score of 0–3 was given depending on the severity of change (0, no observable changes; 1, mild; 2, moderate; and 3, severe changes), as described previously (9, 12).

**Calculations and statistics**

Values are presented as mean±SEM. For multiple comparisons among groups, analysis of
variance (ANOVA) followed by the Fisher's post-test, were used. Scored data for the histological evaluation was analyzed by the non-parametric Kruskal-Wallis test followed by the Mann-Whitney U-test. Statistical significance was defined as $P<0.05$. 
RESULTS

Animal characteristics and renal excretion

Rats with PUUO presented elevated water intake and urine production, decreased urine sodium and potassium concentrations, increased potassium excretion, similar sodium excretion and reduced urine osmolarity compared to controls (Table 2). Renal excretion pattern in rats with PUUO+DNx was similar to that of controls. The \([\text{Na}^+]/[\text{K}^+]\) ratio was significantly decreased in the PUUO animals, and this was comparable to controls in hydronephrotic animals with denervation. Denervated controls presented similar renal excretion pattern as sham operated controls.

Telemetric measurements of blood pressure and heart rate

Mean arterial blood pressure in the PUUO group was significantly higher under normal, low and high salt diet compared to controls (Fig 1A, C, E). Rats PUUO+DNx had significantly lower blood pressure compared to the PUUO group during all salt diet periods, but still elevated compared with control rats. Salt sensitivity, determined as blood pressure changes in response to different salt diets, was more profound in the PUUO group compared to control rats (Fig 1G). In PUUO rats with denervation the salt-sensitivity was similar to controls. In subset of control animals we also looked at potential effects of renal denervation. However, Control+DNx rats had similar blood pressure levels and salt-sensitivity as the sham-operated controls (Fig 1A, C, E).

Apart from the blood pressure, we also measured the heart rate under normal, high and low salt diet (Fig 1B, D, F). Interestingly, the hydronephrotic group presented lower heart rate, under all dietary conditions, than the sham operated controls. The renal denervated PUUO rats had similar heart rates as control rats under normal and high salt diets, but presented the opposite trend on the low salt diet. Again, Control+DNx rats had similar heart rates as the sham-operated controls (Fig 1B, D, F).
Salt sensitivity in terms of heart rate changes under different diet was significantly lower in PUUO+DNx group compared to the other groups (Fig 1H).

NADPH oxidase activity

NOX activity was measured in renal cortex (Fig 2A), medulla (Fig 2B) and in the heart (Fig 2C) after the normal salt diet period. Superoxide production in cortex from the hydronephrotic kidney (4794±391 units/min/mg; n=15) was significantly higher compared to control kidneys (3139±69 units/min/mg, n=10, p<0.05. Denervation of the PUUO kidney reversed this elevation in NOX activity, with similar values as controls (Fig 2A). These differences in NOX activity among groups were observed only in the renal cortex, but not in the medulla (Control: 2448±352; PUUO: 2426±456 units/min/mg) (Fig 2B). Moreover, denervation in control rats did not influence superoxide production (Fig 2A-B). Finally, hydronephrosis was also associated with higher NOX activity in the heart (772±110 units/min/mg; n=15) compared to controls (476±55; n=10, p<0.05). Similar to that observed in kidneys, renal denervation alone did not influence NOX activity in the heart. However, in the presence of hydronephrosis, renal denervation was associated with much lower superoxide generation in the heart (Fig 2C).

NADPH oxidase mRNA expression in the kidney cortex

We analyzed mRNA expression of NOX subunits (Nox2, Nox4, p22phox, p47phox & p67phox) in the kidney cortex. The hydronephrotic kidney (PUUO, left side) displayed higher Nox2 (Fig 3A), Nox4 (Fig 3B) and p22phox (Fig 3C) levels compared with control kidneys, whereas expression of p47phox & p67phox (Fig 3D-E) were not significantly changed. Renal denervation in PUUO rats was associated with similar or even lower expression of Nox2, Nox4, p22phox and p47phox than in controls (Fig 3A-D).
right side) the expression levels of all the tested isoforms were similar to controls and significantly lower compared to the hydrenephrotic left kidney (Fig 3A-E).

**NADPH oxidase mRNA expression in the heart**

Hydrenephrosis affected the NOX expression not only in the kidney but also in the heart. The expression levels of all NOX isoforms in the left ventricular area were significantly higher in the hydrenephrotic animals (Fig 4A-E). Interestingly, renal denervation was linked with reduced or even normalized levels of Nox2, p22phox, p47phox and p67phox (Fig 4A, C, D & E). There was also a trend for reduced Nox4 expression (Fig 4B).

**Effect of PUUO on components of the Renin-Angiotensin-Aldosterone System**

As shown in Fig 5A hydrenephrotic kidneys had elevated renin mRNA expression compared with controls. Interestingly, renal denervation in PUUO rats markedly suppressed renin expression, to levels even lower than in controls. Expression of the AT1A receptor was significantly elevated in the hydrenephrotic left kidney, which was reduced to control levels with denervation (Fig 5B). Similarly to that observed with the NOX subunits, renal denervation in control rats did not affect the expression of renin or AT1A (Fig 5A-B).

Plasma Na+ and K+ levels were elevated in PUUO rats, and these were normalized by renal denervation (Fig 5C-D). In contrast to the kidney, plasma renin and ANG II levels were not different among the four groups (Fig 5E-F). Finally, plasma aldosterone was significantly higher in hydrenephrotic rats, but not significantly reduced by renal denervation (Fig 5G). Surprisingly, denervation in controls was associated with elevated potassium (Fig 5D) and aldosterone (Fig 5G) levels.

**Renal Injuries & Inflammation**
Renal injuries were estimated by proteinuria and the degree of renal inflammation and fibrosis. Interestingly, the hydronephrotic animals presented significantly higher protein excretion, which was normalized by renal denervation (Fig 6A). Renal denervation had no effect in control rats. Compared with normal renal histoarchitecture in the controls (Fig 6D), the hydronephrotic left kidneys displayed dilated pelvic areas with flattening of the papilla (Fig 6E). This was associated with mild-to-moderate inflammation that was not significantly reduced with denervation (Fig 6B, F, G, H). Hydronephrosis was also associated with higher degree of renal fibrosis, which was still present in the PUUO+DNx rats. However, quantified evaluation showed no significant difference compared to controls (Fig 6C, I, J, K). The contralateral right kidneys and the kidneys from the denervated controls had non-detectable-to-minimal levels of both inflammation and fibrosis (Fig 6B-C).

Cardiac Injuries

Hydronephrosis was associated with higher heart-to-body weight ratio (Fig 7A), suggesting cardiac hypertrophy. This was almost normalized in PUUO rats with renal denervation. Histological analysis revealed mild-to-moderate cardiac fibrosis in rats with hydronephrosis, which was reduced to minimal-to-mild levels in denervated PUUO rats (Fig 7B-E). Denervated controls were similar to the sham operated animals for all the investigated parameters (Fig 7A-B).
Emerging evidence suggest that increased SNA and oxidative stress promote or accelerate the development of hypertension (17, 18). Here we demonstrate an important link between renal nerves and regulation of NOX in the PUUO kidney, which may explain why denervation attenuates development of high blood pressure and salt-sensitivity in this model of renovascular hypertension. In contrast to that observed in animals with hydronephrosis, unilateral renal denervation had no significant impact on cardiovascular function in healthy controls. In agreement with previous studies (8, 9, 11, 13, 14, 33, 35, 43, 52), hydronephrosis in this study was associated with renal oxidative stress and development of salt-sensitive hypertension, which was coupled to renal inflammation and glomerular barrier injury with proteinuria, increased diuresis and likely impaired urine concentration mechanism. The concentrating defect, i.e. increased urine production and reduced urine osmolality in animals with PUUO, has previously been explained by atrophy of the papilla, (9, 13, 14, 52), which was also seen in this study. Although not investigated in the present study, reduced abundance of aquaporins have been demonstrated in kidneys with chronic partial obstruction, which correlated with abnormal renal function and injuries (47). Here we show that denervation of the PUUO kidney normalized urine flow, urine osmolality and Na+/K+ ratio despite similar degree of hydronephrosis, renal fibrosis and inflammation. Moreover, changes in blood pressure to different salt loadings were associated with inverse regulation of the heart rate. These normal variations in heart rate, which can be linked to activation and inhibition of the RAAS, respectively, were abolished in the denervated group.

We have previously suggested a causal link between the degree of oxidative stress in hydronephrosis and development of hypertension (8, 9). Reduced scavenging of superoxide, due to SOD1 deficiency, aggravated salt sensitivity and hypertension, whereas overexpression of SOD1 or treatment with tempol halted hypertension (9). Taken together, these previous studies suggested a crucial role of NOX in the development of hypertension. To investigate
the link between renal denervation and reduction in oxidative stress, as a mechanism for blood pressure reduction in rats with hydronephrosis, we investigated NOX activity and expression. In the cortex, but not in the medulla, NOX was significantly higher in the hydrenephrotic kidney. Enhanced NOX-derived superoxide production was associated with higher expression of the membrane bound subunits Nox2, Nox4 and p22phox, whereas intracellular p47phox and p67phox were not significantly changed. Moreover, the hydrenephrotic kidney displayed more inflammation and interstitial fibrosis, which in turn could contribute to increased oxidative stress (19, 43). Despite similar degree of renal inflammation and fibrosis, denervated PUUO kidneys displayed normal NOX activity that was associated with significant reductions in Nox2, Nox4, p22phox and p47phox expression. This is in agreement with our previous findings, which demonstrated that mice with increased antioxidant defence had lower levels of renal oxidative and were protected from PUUO-induced hypertension, but not from the development of renal inflammation and fibrosis (8, 9). These results suggest that oxidative stress, in particular superoxide, is a major contributing factor to the development of hypertension in the PUUO model. Since NOX activity and expression was inhibited by renal denervation, without significant changes in renal inflammation, we assume that this effect mainly derive from renal vascular or tubular cells rather than from inflammatory cells. Although other studies show that renal denervation prevents the inflammatory cascade following complete ureteral obstruction (34) or ANG II-induced hypertension (65), to our knowledge there are no evidence for a causative link between renal inflammation and hypertension in our model of PUUO, which is much more subtle compared to complete ureteral obstruction and is not only dependent on increased circulating levels of ANG II.

Our previous studies using the PUUO model have also suggested enhanced RAAS activity during development of hypertension and renal pathologies (14, 15). Topcu and colleagues showed that chronic inhibition of AT1 receptors in neonatal rats subjected to PUUO attenuated renal dysfunction and normalized the expression of aquaporins and Na-K-ATPase in the
obstructed kidney (59). A suggested mechanism for blood pressure lowering after renal
denervation is inhibition of RAAS in the kidney (30). NOX activity in the kidney, and blood
pressure regulation, is coupled to ANG II levels and activation of the AT$_{1A}$ receptor (3, 10,
46, 51). We found that plasma sodium and potassium levels were elevated in the PUUO rats
and normalized by renal denervation. Aldosterone was increased in PUUO rats, but none of
the RAAS components in plasma were significantly reduced by renal denervation. Although
causality was not proven in our study, one could speculate that hyperkalemia and dehydration
in the hydronephrotic animals may contribute to activation of the RAAS and then promote
sodium reabsorption (54). Interestingly, denervated controls displayed elevated plasma
potassium and aldosterone levels. We are speculating that this change in plasma aldosterone
could be a compensatory mechanism to maintain potassium homeostasis, and a likely
explanation for the normal blood pressure is the lack of concurrent change in circulating ANG
II (54).

Instead of systemic changes in RAAS components, there is accumulating evidence that
intrarenal ANG II regulation plays a major role in the pathogenesis of hypertension and renal
injury (36). In the cortex of PUUO kidneys both renin and AT$_{1A}$ receptor expression was
higher compared with controls, and denervation reduced or even normalized their expression.
Moreover, it has been shown that decreased urinary sodium-to-potassium ratio can be
associated with increased RAAS activity (25, 27). Interestingly this ratio was reduced in
hydronephrotic rats, but normalized by renal denervation. Moreover, we also observed that
renal denervation diminished PUUO-induced proteinuria. Several studies have shown
proteinuria can be promoted by the activation of intrarenal RAAS and NOX-mediated
oxidative stress, which in turn trigger a vicious cycle with further activation of RAAS and
NOX (58, 62-64).

Previous experimental studies showed that renal denervation can attenuate mRNA expression
of NOX isoforms and superoxide production in the kidney cortex (50), but also reduce
oxidative stress-induced brain and heart injuries (31, 45). Our findings demonstrate that renal
denervation affects not only the hydronphrotic kidney, but also the contralateral kidney as
well as the heart. We observed that hydronephrotic animals displayed increased heart-to-body
weight ratio and cardiac fibrosis, which was associated with increased NOX activity and
expression. All of these parameters were reduced or even normalized by renal denervation.
Accumulating evidence demonstrate a positive link between NADPH oxidase signaling in the
heart and development of cardiac hypertrophy and fibrosis (44). More interestingly, recent
publications have reported that renal denervation can attenuate both cardiac hypertrophy and
fibrosis not only in animal models (39, 61), but also in aged humans (22). Although the
underlying mechanisms are still unclear, to our knowledge this is the first study showing that
renal denervation is strongly associated with a simultaneous reduction in cardiac NADPH
oxidases, fibrosis and hypertrophy. One may speculate that hypertrophy, fibrosis and
oxidative stress in the heart reflect the degree of hypertension in our model, but this could also
be more directly linked to modulation of renal nerve activity. Sympathetic efferent and
afferent nerve fibers are located in the proximity of the renal arteries and in the pelvic region,
respectively, and are considered to be involved in the cardio-renal syndrome and in
hypertension (21, 32). Moreover, unilateral renal denervation under hypertensive pathological
conditions leads to a general sympathetic inhibition via the renorenal reflex (30). Previous
studies have proposed that denervation of efferent sympathetic nerves can reduce
inappropriate renin release and salt retention, whereas reduced firing of afferent sensory
nerves can attenuate renal-mediated activation of centrally mediated SNA (30). Further
studies are needed to specifically investigate how renal denervation influences a cardiac-
renal-neuro axis or reno-renal reflexes in the PUUO model.
In summary, chronic PUUO leads to salt-sensitive hypertension and renal inflammation with
proteinuria, which is associated with increased NOX-mediated oxidative stress in the
ipsilateral kidney. Unilateral denervation lowered blood pressure, reduced salt sensitivity,
abolished proteinuria, and reduced both cardiac hypertrophy and fibrosis. Interestingly, this was associated with normalized NOX function in the hydronephrotic kidney and the heart. It is concluded that renal nerves are important for hypertension associated with hydronephrosis, likely through modulation of cortical oxidative stress. Although under debate, renal sympathetic denervation has been shown to lower blood pressure in patients with resistant hypertension (37, 40, 42, 53, 55, 56). Also earlier clinical studies suggested that sympathectomy efficiently lowered blood pressure and prolonged life expectancy of patients with hypertension (23). The underlying mechanisms for blood pressure reduction with renal denervation are not clear. Numerous studies have shown that NOX is the major source of superoxide in the diseased kidney (3, 16), and the current study suggests a link between renal SNA, renin and AT1A receptor expression and NOX function. Although future studies are needed to prove causality, it is tempting to speculate that reduction of NOX function could be the primary mechanism whereby renal denervation prevents from development of hypertension during urinary tract obstruction.
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CONFLICT(S) OF INTEREST / DISCLOSURE(S) STATEMENT

None
REFERENCES


<table>
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<th>Gene</th>
<th>Primer 1 Sequence</th>
<th>Primer 2 Sequence</th>
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<tr>
<td>Nox2</td>
<td>r_Noxx2-fw 5'- TGTCCAAGCTGGAGTGGCAC -3'</td>
<td>r_Noxx2-rev 5'- GCACAGGCAGTGAGTAGAT -3'</td>
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<td>Nox4</td>
<td>r_Noxx4-fw 5'-GCTTGTTGAAGATCAAAACAT -3'</td>
<td>r_Noxx4-rev 5'-TCCAGAATCCAAATCCAGGT -3'</td>
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<tr>
<td>p22phox</td>
<td>r_p22phox-fw 5'-TGCGTGATCCTCATCAGACG -3'</td>
<td>r_p22phox-rev 5'-AGGACCGGACGAGCTAAGT -3'</td>
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<td>p47phox</td>
<td>r_p47phox-fw 5'-TCGCGAGAATCCATCAGACG -3'</td>
<td>r_p47phox-rev 5'-GCCGAGATCCTCATCAGACG -3'</td>
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<td>p67phox</td>
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<td>r_p67phox-rev 5'-ATAGCACCAAGATCATCTCC -3'</td>
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<td>AT1A</td>
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<td>r_AT1-rev 5'-TAGATCCCTGAGGCGGCTAATGAA -3'</td>
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<td>GAPDH</td>
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<td>r_GAPDH-rev 5'-TGAACCGGGATCGTACTGAG -3'</td>
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<td>Renin</td>
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<td>r_Renin-rev 5'-TCGCCACACCTGTTGCT -3'</td>
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<td>18S rRNA</td>
<td>r_18S-fw 5'-CTGTCGTTGCTTAGAGCTG -3'</td>
<td>r_18S-rev 5'-AGGTATCTAGAGACCAG -3'</td>
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<td>TBP</td>
<td>r_TBP-fw 5'-TTCTGGGAAATGTTGTCAGC -3'</td>
<td>r_TBP-rev 5'-CCCACCATGTTGCTGATTCT -3'</td>
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Table 2. Renal excretion data during normal salt diet

<table>
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<tr>
<th></th>
<th>Control</th>
<th>Control+DNx</th>
<th>PUUO</th>
<th>PUUO+DNx</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Water intake</strong> (ml/24h/g BW)</td>
<td>0.057 ± 0.010</td>
<td>0.049 ± 0.009</td>
<td>0.097 ± 0.011*</td>
<td>0.059 ± 0.012#</td>
</tr>
<tr>
<td><strong>Urine production</strong> (ml/24h/g BW)</td>
<td>0.044 ± 0.009</td>
<td>0.055 ± 0.010</td>
<td>0.093 ± 0.019*</td>
<td>0.055 ± 0.009#</td>
</tr>
<tr>
<td><strong>[Na⁺]</strong> (mM)</td>
<td>95 ± 14</td>
<td>90 ± 13</td>
<td>60 ± 9*</td>
<td>92 ± 12#</td>
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<tr>
<td><strong>Na⁺ excretion</strong> (µmol/24h/g BW)</td>
<td>3.53 ± 0.21</td>
<td>4.61 ± 0.61</td>
<td>3.76 ± 0.37</td>
<td>4.06 ± 0.40</td>
</tr>
<tr>
<td><strong>[K⁺]</strong> (mM)</td>
<td>148 ± 15</td>
<td>120 ± 20</td>
<td>98 ± 13*</td>
<td>131 ± 27</td>
</tr>
<tr>
<td><strong>K⁺ excretion</strong> (µmol/24h/g BW)</td>
<td>5.12 ± 0.21</td>
<td>6.04 ± 0.42</td>
<td>6.43 ± 0.57*</td>
<td>6.33 ± 0.60</td>
</tr>
<tr>
<td><strong>[Na⁺] / [K⁺]</strong></td>
<td>0.71 ± 0.03</td>
<td>0.77 ± 0.08</td>
<td>0.51 ± 0.04*</td>
<td>0.72 ± 0.09#</td>
</tr>
<tr>
<td><strong>[Osm]</strong> (mM)</td>
<td>1021 ± 83</td>
<td>789 ± 111</td>
<td>642 ± 89*</td>
<td>918 ± 106</td>
</tr>
<tr>
<td><strong>Osm excretion</strong> (mmol/24h/g BW)</td>
<td>34.0 ± 1.8</td>
<td>37.8 ± 2.1</td>
<td>41.0 ± 2.7</td>
<td>39.9 ± 3.1</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>10</td>
<td>5</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM; PUUO=partial unilateral ureteral obstruction; DNx=renal denervation of the left kidney; BW=body weight; n=number of animals

* P<0.05 compared with Controls

# P<0.05 compared with PUUO
Figure 1. Mean arterial pressure (A, C, E) and heart rate (B, D, F) in sham-operated rats (Controls, n=10), control rats with unilateral renal denervation (Control+DNx n=5), rats with partial unilateral ureteral obstruction of the left kidney (PUUO, n=15), and rats with PUUO and renal denervation of the left kidney (PUUO+DNx, n=10). Blood pressure and heart rate were measured during treatment different salt loadings in the following order: normal salt diet (A-B), high salt diet (C-D), and low salt diet (E-F). Blood pressure was significantly higher, and heart rates lower, in the PUUO group compared with controls on all diets. Animals with PUUO+DNx had significantly lower blood pressure compared with PUUO rats, yet higher blood pressure than controls, for all the diets. Salt sensitivity, i.e. difference in blood pressure level between high and low salt conditions, was more pronounced in PUUO compared with control rats (G). In the PUUO+DNx group the salt sensitivity of blood pressure was similar to controls, and the variation in heart rate was also absent (H). Values are presented as mean±SEM. * P<0.05 between indicated groups, # P<0.05 compared with normal and low salt periods within the same group.

Figure 2. NOX activity in renal cortex (A) medulla (B) and heart (C) from sham operated rats (Controls, n=10), control rats with unilateral renal denervation (Control+DNx n=6), rats with partial unilateral ureteral obstruction of the left kidney (PUUO, n=15), and rats with PUUO and unilateral renal denervation of the left kidney (PUUO+DNx, n=10). Renal cortex from rats with PUUO displayed increased NOX activity in the hydronephrotic (left) kidney compared with that of kidneys from control rats. In rats with renal denervation, NOX activity was significantly reduced and not different from that of control rats. For the renal medulla, no significant differences were found among the experimental groups. In the heart, the hydronephrotic animals displayed higher NOX activity, which was significantly reduced but not normalized in the PUUO+DNx group. In all the cases (A, B, C) the denervated controls...
displayed similar values as the sham operated animals. L, left kidney; R, right kidney. Values are presented as mean±SEM. * P<0.05 between indicated groups.

**Figure 3.** NOX mRNA expression in renal cortex from sham operated rats (Controls), denervated controls (Control+DNx), rats with partial unilateral ureteral obstruction of the left kidney (PUUO), and rats with PUUO and unilateral renal denervation of the left kidney (PUUO+DNx). Renal cortex from rats with PUUO displayed increased expression of Nox2 (A), Nox4 (B), p22phox (C), whereas p47phox (D) and p67phox (E) was not significantly increased in the hydronephrotic left kidney. In PUUO rats with renal denervation, the mRNA expression of Nox2 (A), Nox4 (B), p22phox (C) and p47phox (D) was significantly reduced in the hydronephrotic left kidney. Denervated PUUO kidneys even had reduced p22phox (C) and p47phox (D) expression compared with control kidneys. In all the cases (A-E) the denervated controls displayed similar values as sham operated animals. Values are presented as mean±SEM. n=6 per group. * P<0.05 between indicated groups, # P<0.05 compared with Controls.

**Figure 4.** NOX mRNA expression in the heart from sham operated rats (Controls), denervated controls (Control+DNx), rats with partial unilateral ureteral obstruction of the left kidney (PUUO), and rats with PUUO and unilateral renal denervation of the left kidney (PUUO+DNx). The cardiac tissue from rats with PUUO displayed increased expression of Nox2 (A), Nox4 (B), p22phox (C), p47phox (D) and p67phox (E). In rats with renal denervation the expression of Nox2 (A), p22phox (C), p47phox (D) and p67phox (E) was significantly reduced. Expression of p47phox were normalized (D), while Nox4 (B) was not significantly decreased in the PUUO+DNx group. In all the cases (A-E) the denervated controls displayed similar values as sham operated animals. Values are presented as
mean±SEM. n=6 per group. * P<0.05 between indicated groups, # P<0.05 compared with Controls

Figure 5. Renin (A), AT$_{1A}$ (B) mRNA expression in the kidney cortex, plasma levels of sodium (C), potassium (D), renin (E), ANG II (F) and aldosterone (G) from sham operated rats (Controls), denervated controls (Control+DNx), rats with partial unilateral ureteral obstruction of the left kidney (PUUO), and rats with PUUO and unilateral renal denervation of the left kidney (PUUO+DNx). Renal cortex from rats with PUUO displayed increased expression of both renin and AT$_{1A}$. In rats with renal denervation the expression of renin was markedly suppressed both the hydronephrotic left kidney and the contralateral right kidney (A), and AT$_{1A}$ expression was comparable to control values (B). Plasma sodium was significantly elevated only in PUUO rats (C) while plasma potassium was paradoxically elevated in both the DNx and PUUO groups, but normalized in the PUUO+DNx group (D). Plasma levels of renin and ANG II did not differ significantly among the groups (E-F). Aldosterone was significantly higher in PUUO rats, but not significantly changed in the PUUO+DNx rats (G). Apart from aldosterone level that was increased, the denervated controls displayed similar values as sham operated animals. Values are presented as mean±SEM. n=6 per group. * P<0.05 between indicated groups. # P<0.05 compared with Controls

Figure 6. Urinary protein excretion (A) and histological evaluation of inflammation (B) and interstitial fibrosis (C) in the renal cortex. Overview of a control kidney (D) and the left kidney from a rat with hydronephrosis (E). HE staining (D-H) and Picro Sirius staining (I-K) of kidney sections from control (D, F, I), PUUO (E, G, J), and PUUO+DNx (H, K). The hydronephrotic animals had increased proteinuria, which was normalized in PUUO rats with renal denervation. Renal denervation in control rats had no effect (A). The hydronephrotic left
kidneys displayed flattening of the papilla and increased inflammation and, which was not significantly reduced by denervation. Hydronephrosis was also associated with increased fibrosis, which was still present in the PUUO+DNx animals, but not significant compared to controls. Scale bar 30 µm. Values are presented as mean±SEM. n=6 per group. * P<0.05 between indicated groups. # P<0.05 compared with Controls

Figure 7. Heart-to-body weight ratio (A), and histological evaluation of left ventricular fibrosis (B). Picro Sirius staining of heart sections from control (C), PUUO (D), and PUUO+DNx (E). Hydronephrosis was associated with higher heart-to-body weight ratio (A) compared to controls, which was almost normalized in denervated PUUO rats. PUUO was also associated with mild-to-moderate cardiac fibrosis, which was significantly reduced in the PUUO+DNx animals (B, C D, E). Denervated controls displayed no differences compared with sham operated animals (A & B). Scale bar 60 µm. Values are presented as mean±SEM. n=6 per group. * P<0.05 between indicated groups.
A

Renal Cortex

NADPH Oxidase Activity (% vs Control)

B

Renal Medulla

NADPH Oxidase Activity (% vs Control)

C

Heart

NADPH Oxidase Activity (% vs Control)
Protein Excretion (mg/24h/g BW)

Renal Inflammation

Renal Fibrosis

Protein Excretion (mg/24h/g BW)

Renal Inflammation

Renal Fibrosis

2 mm

2 mm

2 mm

2 mm