Renal denervation attenuates NADPH oxidase-mediated oxidative stress and hypertension in rats with hydronephrosis

Maria Peleli,1* Ammar Al-Mashhadi,2,3# Ting Yang,1 Erik Larsson,4 Nils Wåhlin,5 Boye L. Jensen,6 A. Erik G. Persson,3 and Mattias Carlström1

1Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden; 2Division of Pediatric Surgery, Department of Women’s and Children’s Health, Uppsala University, Uppsala, Sweden; 3Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden; 4Department of Genetics and Pathology, Uppsala University, Uppsala, Sweden; 5Department of Pediatric Surgery, Astrid Lindgren Hospital, Karolinska Institutet, Stockholm, Sweden; and 6Department of Physiology and Pharmacology, University of Southern Denmark, Odense, Denmark

Submitted 30 July 2015; accepted in final form 28 October 2015

Peleli M, Al-Mashhadi A, Yang T, Larsson E, Wåhlin N, Jensen BL, Persson AE, Carlström M. Renal denervation attenuates NADPH oxidase-mediated oxidative stress and hypertension in rats with hydronephrosis. Am J Physiol Renal Physiol 310: F43–F56, 2016. First published November 4, 2015; doi:10.1152/ajprenal.00345.2015.—Hydronephrosis is associated with the development of salt-sensitive hypertension. Studies have suggested that increased sympathetic nerve activity and oxidative stress play important roles in hypertension and the modulation of salt sensitivity. The present study primarily aimed to examine the role of renal sympathetic nerve activity 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. Partial unilateral ureteral obstruction (PUUO) was created in 3-wk-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. The renal excretion pattern, NOX activity, and expression as well as components of the renin-angiotensin-aldosterone system were characterized after treatment with the normal salt diet. On the normal salt diet, rats in the PUUO group had elevated blood pressure compared with control rats (115 ± 3 vs. 87 ± 1 mmHg, P < 0.05) and displayed increased urinary production and lower urine osmolality. The blood pressure change in response to salt loading (salt sensitivity) was more pronounced in the PUUO group compared with the control group (15 ± 2 vs. 5 ± 1 mmHg, P < 0.05). Renal denervation in PUUO rats attenuated both hypertension (97 ± 3 mmHg) and salt sensitivity (5 ± 1 mmHg, P < 0.05) and normalized the renal excretion pattern, whereas the degree of renal fibrosis and inflammation was not changed. NOX activity and expression as well as renin and ANG II type 1A receptor expression were increased in the renal cortex from PUUO rats and normalized after renal denervation. Final denervation in PUUO rats was also associated with reduced NOX expression, superoxide production, and fibrosis in the heart. In conclusion, renal denervation attenuates hypertension and restores the renal excretion pattern, which is associated with reduced renal NOX and components of the renin-angiotensin-aldosterone system. This study emphasizes a link between renal nerves, the development of hypertension, and modulation of NOX function.

Renal denervation because of a pelvic-ureteric junction obstruction is a fairly common condition, with an incidence in newborns of ~1%. Early observations of rather well-preserved kidney function in children with hydronephrosis has led to a worldwide trend toward conservative treatment, but the long-term physiological consequences of this policy are not known. In a recent prospective study (2), we showed that surgical treatment reduces blood pressure in children with unilateral congenital hydronephrosis. Several studies have demonstrated a clear link between hydronephrosis, induced by partial unilateral ureteral obstruction (PUUO), and the development of hypertension in both rats and mice (13–15). The 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 cells of the afferent arteriole and 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 β-adrenergic receptors located on juxtaglomerular cells (24, 48). Acute denervation blunts renal autoregulatory responses, as evident by reduced tubuloglomerular feedback, whereas nerve stimulation restores or sensitizes the response (38). In addition, several studies have shown that renal denervation per se reduces Na+ and water reabsorption in proximal tubules (6, 7, 28) that can contribute to the blood pressure-lowering effect. It has been also shown that K+ excretion can be increased after renal denervation because of an increased Na+/K+ exchange in 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 the 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 a reduction of NOX-medi-
METHODS

This study was approved by the institutional ethics review board in Stockholm (N314/12). All animal procedures performed conformed with 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. Male Sprague-Dawley rats (Scanbur, Charles River) were divided into the following four experimental groups: sham-operated rats (control), control rats with unilateral renal denervation (control + DNx), rats with PUUO to induce hydronephrosis, and rats with PUUO with unilateral denervation (PUUO + DNx). All animals were given a standardized normal salt diet (0.7% NaCl, SD389-R36, Lactamin, Kistad, Sweden) for 4 wk before cardiovascular function was assessed. In one series, salt sensitivity was evaluated by measuring blood pressure and heart rate changes during the normal salt diet followed by high-salt diet treatment (4% NaCl, SD312-R36, Lactamin) and low-salt diet treatment (0.02% NaCl, Lactamin; 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 PUUO

PUUO was created in 3-wk-old rats to induce hydronephrosis, as previously described (14). In brief, anesthesia with spontaneous inhalation of isoflurane (2% in air, Forene, Abbot Scandinavia, Kista, Sweden) was used. 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 form a 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 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 the normal salt diet for 4 wk.

Renal Denervation

During the procedure to induce hydronephrosis (i.e., 3 wk of age), the left kidney from control or PUUO rats was denervated or exposed to sham denervation. Renal denervation was accomplished by previously validated surgical-pharmacological procedures (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 2 min. 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

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

Renal Excretion Measurements

Rats were housed individually in metabolism cages for 24 h with food and water given ad libitum. Water consumption and urine production were measured gravimetrically. Na⁺ and K⁺ concentrations were determined by flame photometry (FLM5, Radiometer, Copenhagen, Denmark), and urine osmolality was determined 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 the Detergent Compatible Protein Assay (Bio-Rad Laboratories, Hercules, CA). Plates were read using a microplate reader (model Safire II, Tecan Austria, Grödig, Austria) at 750-nm absorbance as previously described (12).

Determination of the Hydronephrotic Ratio and Collection of Tissues and Plasma

Once the renal and cardiovascular experiments had been conducted, all animals were anesthetized by an intraperitoneal injection of thiobutabarbitual sodium [Inactin (120 mg/kg body wt), whereupon the abdomen was opened using a midline incision. A macroscopic examination of both kidneys was performed. Blood was collected from the vena cava, transferred to tubes containing EDTA (final concentration: 2 mM), immediately centrifuged at 4°C (2,000 rpm, 5 min), and stored at −80°C for later analysis. The 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 (as described below). Hydronephrotic ratios were calculated before samples were frozen in the same way as previously described (i.e., hydronephrotic ratio = residual urine weight/renal parenchymal weight (60). The renal cortex and medulla were dissected on ice and frozen separately (−80°C) for later analysis.

NOX Activity

Chemiluminescence techniques were used to determine NOX-mediated superoxide formation. In brief, kidney cortex and medulla pieces (between 50 and 70 mg) were homogenized by adding a volume of PBS that equaled three times the tissue weight along with zirconium oxide beads, and the bullet blender was used. The homogenate was then centrifuged at 4°C for 20 min at 12,000 g. NADPH (300 μM) and lucigenin (5 μM, Sigma-Aldrich) were added into the tissue supernatant (dilution 1:100) in PBS. The heart, we followed a similar protocol. In brief, similar heart slices from the left ventricular area (between 220 and 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 12,000 g. NADPH (300 μM) and lucigenin (5 μM, Sigma-Aldrich) were added into the tissue supernatant (dilution 1:5) in PBS. In both kidneys and hearts, final NOX activity was determined by measuring lucigenin chemiluminescence every 3 s for 3 min with an AutoLumat LB953 Multi-Tube Luminescence System (Berthold Technologies, Bad Wildbad, Germany). The chemiluminescence signal was corrected by protein quantification (Bradford protein assay, Bio-Rad Laboratories) and expressed as a percentage of the respective control. In tissue samples without NADPH or in tissue-free samples with NADPH and lucigenin, the NOX activity signal was similar to the blank (i.e., PBS only), demonstrating the specificity for NOX enzyme activity.

Quantitative Real-Time RT-PCR

Total RNA was isolated from the kidney cortex or heart using the RNeasy Mini Kit (Qiagen, Valencia, CA), and 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
binder 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 the homogenate corresponding to 25 mg tissue for RNA extraction. The RNA concentration of our samples was estimated with NanoDrop, and, at the end, RNA samples were diluted for RNA extraction. The RNA concentration of our samples was estimated with NanoDrop, and, at the end, RNA samples were diluted for RNA extraction. The annealing temperature was 53°C for p47phox, Nox2, and p22phox, 49°C for Nox4 and renin, and 50°C for p67phox and the ANG II type 1A (AT1A) receptor. The housekeeping genes used were GAPDH, 18S rRNA, and TATA box-binding protein, which presented nice amplification profiles and melting curves at 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 (where Ct is the amplification threshold cycle). The final results are expressed as percentages of the respective controls. The primer sequences (Table 1) and amplification protocol. Power SYBR Green Master mix (Applied Biosystems) was used for amplification and detection of DNA. PCR was performed in 96-well plates with 20 μl Master mix containing 0.25 pmol/μl of all used primers apart from p47phox, where 0.1 pmol/μl was used instead and 5 μl cDNA corresponding to 20 ng RNA. The annealing temperature was 53°C for p47phox, Nox2, and p22phox, 49°C for Nox4 and renin, and 50°C for p67phox and the ANG II type 1A (AT1A) receptor. The housekeeping genes used were GAPDH, 18S rRNA, and TATA box-binding protein, which presented nice amplification profiles and melting curves at 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 (where Ct is threshold cycle). The final results are expressed as percentages of the respective controls. The primer sequences (Table 1) and amplification profiles used for Nox2, p22phox, p47phox, p67phox, Nox4, renin, AT1A receptor, 18S rRNA, GAPDH, and TATA box-binding protein were as previously described (1, 41).

Plasmolysis Analysis

Na⁺ and K⁺. Electrolyte concentrations were determined by flame photometry (FLM3, Radiometer, Copenhagen, Denmark).

Table 1. Sequence of primers for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nox2</td>
<td>r_Noxx2-fw 5'-TGTCCGAAGCTGAGCTGCAC-3'</td>
</tr>
<tr>
<td></td>
<td>r_Noxx2-rev 5'-GCAACAGCCGATGAGTAATG-3'</td>
</tr>
<tr>
<td>Nox4</td>
<td>r_Noxx4-fw 5'-GCTTGTGTCAGATCAAGGAAT-3'</td>
</tr>
<tr>
<td></td>
<td>r_Noxx4-rev 5'-TCCAGAAATCTCAATTCAGGT-3'</td>
</tr>
<tr>
<td>p22phox</td>
<td>r_p22phox-fw 5'-TGGGCTCGATCTCCAATCAG-3'</td>
</tr>
<tr>
<td></td>
<td>r_p22phox-rev 5'-AGGGACGCGGACAGACTAATG-3'</td>
</tr>
<tr>
<td>p47phox</td>
<td>r_p47phox-fw 5'-TCACCGCAGATCTGAGTTC-3'</td>
</tr>
<tr>
<td></td>
<td>r_p47phox-rev 5'-TCCTGATGGCTTTGGATGA-3'</td>
</tr>
<tr>
<td>p67phox</td>
<td>r_p67phox-fw 5'-GAAACGATGAAGGTATCCGTG-3'</td>
</tr>
<tr>
<td></td>
<td>r_p67phox-rev 5'-ATAGAACCAGAGACTACCTCC-3'</td>
</tr>
<tr>
<td>AT1A</td>
<td>r_AT1-fw 5'-CTCAGGCGCTTCTGCAAGAATATG-3'</td>
</tr>
<tr>
<td></td>
<td>r_AT1-rev 5'-TAGACTTCTGACGGCAGCTA-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>r_GAPDH-fw 5'-CCATTCCAGACTGTACCT-3'</td>
</tr>
<tr>
<td></td>
<td>r_GAPDH-rev 5'-TCGAGGGGAAGGACGACT-3'</td>
</tr>
<tr>
<td>Renin</td>
<td>r_Renin-fw 5'-CAGACACAGCCAGAATCTG-3'</td>
</tr>
<tr>
<td></td>
<td>r_Renin-rev 5'-TCCACACCCTGTTTGCCTTA-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>r_18S-fw 5'-CTGTGCTATTTCTCAGGC-3'</td>
</tr>
<tr>
<td></td>
<td>r_18S-rev 5'-AGGTTATCTAGCTACACC-3'</td>
</tr>
<tr>
<td>TBP</td>
<td>r_TBP-fw 5'-TTCTGGAAAAATGTTGTCG-3'</td>
</tr>
<tr>
<td></td>
<td>r_TBP-rev 5'-GCGACCAGCTTGGATGATTT-3'</td>
</tr>
</tbody>
</table>

Nox, NADPH oxidase; AT1A, ANG II type 1A receptor; TBP, TATA box-binding protein.

Renin. EDTA-plasma was incubated with a surplus of substrate-enriched plasma from a nephrectomized sheep for 24 h. The plasma renin concentration was measured by the rate of ANG I formation, and ANG I was detected by a radioimmunoassay through the antibody-trapping method of Poulsen and Jørgensen (49) with minor modifications (29). The assay was standardized in terms of international units of renin per liter by the activity of the World Health Organization International Renin Standard (Ref. 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 World Health Organization standard corresponded to 4.3 ± 0.8 ng ANG I/l. The between-assay coefficient of variation was 19%.

ANG II. EDTA-containing plasma samples (1 ml) were extracted according to the manufacturer (Sep-Pak Classic C18 cartridge, WAT051910). Dried eluates were stored at −80°C until assay. A specific anti-ANG II antibody (Ab-5-030682) was used at a final dilution of 1:1,100,000. Plasma samples were incubated with antibody for 24 h and with tracer 125I-labeled ANG II (kindly provided by the Dept. of Clinical Physiology, Glostrup Hospital) 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. An ELISA kit (MS E-5200, human aldosterone, Labor Diagnostika, Nord, Germany) was used to determine aldosterone. EDTA-plasma was incubated with aldosterone and horseradish peroxidase conjugate for 1 h as described by the manufacturer. A human EDTA-plasma pool was used as an internal standard (~87 pg/ml). The between-assay coefficient of variation was 10.5%, and the intra-assay variation was 6%.

Histology

Sagittal slices from both the kidney and heart were placed in 4% paraformaldehyde solution immediately after animal euthanization. All slices were stored at 4°C and transferred to 70% ethanol solution the next day. Tissues were embedded in paraffin, sliced into 5-μm sections, and then stained with either hematoxylin and eosin or with picrosirius. 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 (where 0 = no observable changes, 1 = mild changes, 2 = moderate changes, and 3 = severe changes), as previously described (9, 12).

Calculations and Statistics

Values are presented as means ± SE. For multiple comparisons among groups, ANOVA followed by the Fisher’s post test were used. Scored data for the histological evaluation were analyzed by a non-parametric Kruskal-Wallis test followed by a Mann-Whitney U-test. Statistical significance was defined as P < 0.05.

RESULTS

Animal Characteristics and Renal Excretion

Rats with PUOO presented elevated water intake and urine production, decreased urine Na⁺ and K⁺ concentrations, increased K⁺ excretion, similar Na⁺ excretion, and reduced urine osmolality compared with control rats (Table 2). The renal excretion pattern in rats with PUOO + DNx was similar to that of control rats. The Na⁺-to-K⁺ concentration ratio was significantly decreased in PUOO animals, and this was comparable with control animals and to hydromephic animals

AJP-Renal Physiol • doi:10.1152/ajprenal.00345.2015 • www.ajprenal.org

DENERVATION ATTENUATES OXIDATIVE STRESS AND HYPERTENSION
with denervation. Denervated control animals presented similar renal excretion pattern as sham-operated control animals.

**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 diets compared with the control group (Fig. 1, A, C, and E). Rats in the PUUO + DNx group had significantly lower blood pressure compared with the PUUO group during all salt diet periods, but blood pressure was still elevated compared with rats in the control group. Salt sensitivity, as determined by blood pressure changes in response to different salt diets, was more profound in the PUUO group compared with the control group (Fig. 1G). In PUUO rats with denervation, the salt sensitivity was similar to control rats. In a 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 sham-operated control rats (Fig. 1, A, C, and E).

In addition to blood pressure, we also measured heart rate under normal, low-, and high-salt diets (Fig. 1, B, D, and F). Interestingly, the hydrenephrotic group presented lower heart rate, under all dietary conditions, than the sham-operated control group. 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 sham-operated control rats (Fig. 1, B, D, and F).

Salt sensitivity in terms of heart rate changes under the different diets was significantly lower in the PUUO + DNx group compared with the other groups (Fig. 1H).

**NOX Activity**

NOX activity was measured in the renal cortex (Fig. 2A), medulla (Fig. 2B), and heart (Fig. 2C) after the normal salt diet period. Superoxide production in the cortex from hydrenephrotic kidneys (4.794 ± 391 U·min⁻¹·g⁻¹, n = 15) was significantly higher compared with control kidneys (3.139 ± 69 U·min⁻¹·g⁻¹, n = 10, P < 0.05). Denervation of PUUO kidneys reversed this elevation in NOX activity, with similar values as control kidneys (Fig. 2A). These differences in NOX activity among groups were observed only in the renal cortex and not in the medulla (control: 2.448 ± 352 U·min⁻¹·g⁻¹ and PUUO: 2.426 ± 456 U·min⁻¹·g⁻¹; Fig. 2B). Moreover, denervation in control rats did not influence superoxide production (Fig. 2, A and B). Finally, hydrenephrosis was also associated with higher NOX activity in the heart (772 ± 110 U·min⁻¹·g⁻¹, n = 15) compared with the control group (476 ± 55 U·min⁻¹·g⁻¹, n = 10, P < 0.05). Similar to what was observed in kidneys, renal denervation alone did not influence NOX activity in the heart. However, in the presence of hydrenephrosis, renal denervation was associated with much lower superoxide generation in the heart (Fig. 2C).

**mRNA Expression of NOX in the Kidney Cortex**

We analyzed the mRNA expression of NOX subunits (Nox2, Nox4, p22-phox, p47-phox, and p67-phox) in the kidney cortex. The hydrenephrotic kidney (PUUO, left side) displayed higher Nox2 (Fig. 3A), Nox4 (Fig. 3B), and p22-phox (Fig. 3C) levels compared with control kidneys, whereas expression levels of p47-phox and p67-phox (Fig. 3, D and E) were not significantly changed. Renal denervation in PUUO rats was associated with similar or even lower expression of Nox2, Nox4, p22-phox, and p47-phox than in control rats (Fig. 3, A–D). In the contralateral kidney (PUUO, right side), expression levels of all tested isoforms were similar to control kidneys and significantly lower compared with hydrenephrotic left kidneys (Fig. 3, A–E).

**mRNA Expression of NOX in the Heart**

Hydrenephrosis affected NOX expression not only in the kidney but also in the heart. Expression levels of all NOX isoforms in the left ventricular area were significantly higher in hydrenephrotic animals (Fig. 4, A–E). Interestingly, renal denervation was linked with reduced or even normalized levels of Nox2, p22-phox, p47-phox, and p67-phox (Fig. 4, A and C–E). There was also a trend for reduced Nox4 expression (Fig. 4B).

**Effect of PUUO on Components of the RAAS**

As shown in Fig. 5A, hydrenephrotic kidneys had elevated mRNA expression of renin compared with control kidneys. Interestingly, renal denervation in PUUO rats markedly suppressed renin expression, to levels even lower than in control rats. 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 what was observed with NOX subunits, renal denervation in control rats did not affect the expression of renin or the AT1A receptor (Fig. 5, A and B).

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

Renal Injuries and Inflammation

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

Cardiac Injuries

Hydronephrosis was associated with a 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. 7, B–E). Denervated control animals were similar to sham-operated animals for all investigated parameters (Fig. 7, A and B).

DISCUSSION

Emerging evidence suggest that increased SNA and oxidative stress promote or accelerate the development of hypertension (17, 18). In the present study, we demonstrate an important link between renal nerves and the regulation of NOX in the PUUO kidney, which may explain why denervation attenuates the development of high blood pressure and salt sensitivity in this model of renovascular hypertension. In contrast to what was observed in animals with hydronephrosis, unilateral renal denervation had no significant impact on cardiovascular function in healthy control animals. In agreement with previous studies (8, 9, 11, 13, 14, 33, 35, 43, 52), hydronephrosis in the present study was associated with renal oxidative stress and the development of salt-sensitive hypertension, which was coupled to renal inflammation and glomerular barrier injury with proteinuria, increased diuresis, and likely an 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, a reduced abundance of aquaporins has 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 the Na⁺-/K⁺ ratio despite a similar degree of hydronephrosis, renal fibrosis, and inflammation. Moreover, changes in blood pressure to different salt loadings were associated with an

![Fig. 2. NADPH oxidase (NOX) activity in the renal cortex (A), medulla (B), and heart (C) from control rats (n = 10), control + DNx rats (n = 6), PUUO rats (n = 15), and PUUO + DNx rats (n = 10). The 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, hydronephrotic animals displayed higher NOX activity, which was significantly reduced but not normalized in PUUO + DNx animals. In all cases (A–C), denervated control animals displayed similar values as sham-operated control animals. L, left kidney; R, right kidney. Values are presented as means ± SE. *P < 0.05 between the indicated groups.](http://ajprenal.physiology.org/10.1152/ajprenal.00345.2015)
Fig. 3. mRNA expression of NOX in the renal cortex from control rats, control + DNx rats, PUUO rats, and PUUO + DNx rats. The renal cortex from rats with PUUO displayed increased expression of Nox2 (A), Nox4 (B), and p22phox (C), whereas expression of p47phox (D) and p67phox (E) was not significantly increased in the hydronephrotic left kidney. In PUUO rats with renal denervation, 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 cases (A–E), denervated control animals displayed similar values as sham-operated control animals. Values are presented as means ± SE; n = 6 animals/group. *P < 0.05 between the indicated groups; #P < 0.05 compared with the control group.
inverse regulation of 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 the 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 the 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 hydronephrotic 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 hydronephrotic kidney displayed more inflammation and interstitial fibrosis, which, in turn, could contribute to increased oxidative stress (19, 43). Despite a similar degree of renal inflammation and fibrosis, denervated PUUO kidneys displayed normal NOX activity, which 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 were inhibited by renal denervation, without significant changes in renal inflammation, we assume that this effect is mainly derived from renal vascular or tubular cells rather than from inflammatory cells. Although other studies have shown that renal denervation prevents the inflammatory cascade after complete ureteral obstruction (34) or ANG II-induced hypertension (65), to our knowledge, there is no evidence for a causative link between renal inflammation and hypertension in our model of PUUO, which is much more subtle compared with complete ureteral obstruction and is not only dependent on increased circulating levels of ANG II.

Fig. 5. mRNA expression of renin (A) and the ANG II type 1A (AT1A; B) receptor in the kidney cortex as well as plasma levels of Na⁺ (C), K⁺ (D), renin (E), ANG II (F), and aldosterone (G) from control rats, control + DNx rats, PUUO rats, and PUUO + DNx rats. The renal cortex from rats with PUUO displayed increased expression of both renin and the AT1A receptor. In rats with renal denervation, expression of renin was markedly suppressed in both the hydronephrotic left kidney and contralateral right kidney (A), and AT1A expression was comparable with control values (B). Plasma Na⁺ was significantly elevated only in PUUO rats (C), whereas plasma K⁺ was paradoxically elevated in both 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 and F). Aldosterone was significantly higher in PUUO rats but not significantly changed in PUUO + DNx rats (G). Apart from the aldosterone level, which was increased, denervated control animals displayed similar values as sham-operated control animals. Values are presented as means ± SE; n = 6 animals/group. *P < 0.05 between the indicated groups; #P < 0.05 compared with the control group.
Fig. 6. Urinary protein excretion (A) and histological evaluation of inflammation (B) and interstitial fibrosis (C) in the renal cortex. D and E: overview of a control kidney (D) and a left kidney from a rat with hydronephrosis (E). D–K: hematoxylin and eosin staining (D–H) and picrosirius staining (I–K) of kidney sections from control (D, F, and I), PUUO (E, G, and J), and PUUO + DNx (H and K) rats. Hydronephrotic animals had increased proteinuria, which was normalized in PUUO rats with renal denervation. Renal denervation in control rats had no effect (A). Hydronephrotic left kidneys displayed flattening of the papilla and increased inflammation, which was not significantly reduced by denervation. Hydronephrosis was also associated with increased fibrosis, which was still present in PUUO + DNx animals but was not significant compared with control animals. Scale bars = 30 μm. Values are presented as means ± SE; n = 6 animals/group. *P < 0.05 between the indicated groups; #P < 0.05 compared with the control group.
Our previous studies using the PUUO model have also suggested enhanced RAAS activity during the development of hypertension and renal pathologies (14, 15). Topcu and colleagues (59) 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. A suggested mechanism for the blood pressure lowering after renal denervation is inhibition of the RAAS in the kidney (30). NOX activity in the kidney (and blood pressure regulation) is coupled to ANG II levels and activation of the AT1A receptor (3, 10, 46, 51). We found that plasma Na\(^+\)/K\(^+\) levels were elevated in 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 hydronephrotic animals may contribute to the activation of the RAAS and then promote Na\(^+\) reabsorption (54). Interestingly, denervated control animals displayed elevated plasma K\(^+\) and aldosterone levels. We speculate that this change in plasma aldosterone could be a compensatory mechanism to maintain K\(^+\) homeostasis, and a likely explanation for the normal blood pressure is the lack of a 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 AT1A receptor expression were higher compared with control kidneys, and denervation reduced or even normalized their expression. Moreover, it has been shown that a decreased urinary Na\(^+\)-to-K\(^+\) 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 that...
proteinuria can be promoted by activation of the intrarenal RAAS and NOX-mediated oxidative stress, which, in turn, can trigger a vicious cycle with further activation of the RAAS and NOX (58, 62–64).

Previous experimental studies have shown 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 hydronephrotic kidney but also the contralateral kidney as well as the heart. We observed that hydrenephrotic animals displayed an 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 demonstrates a positive link between NOX signaling in the heart and the 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 NOXs, 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 a 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 cardiorenal 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). A previous study (30) has 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. Further studies are needed to specifically investigate how renal denervation influences the cardiac-renal-neuron axis or renoreflexes 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 hydrenephrotic kidney and heart. We concluded that renal nerves are important for hypertension associated with hydropnephrosis, likely through the 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). In addition, a previous clinical study (23) has suggested that sympathectomy efficiently lowered blood pressure and prolonged life expectancy of patients with hypertension. The underlying mechanisms for the 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 present study suggests a link between renal SNA, renin and AT_{1A} receptor expression, and NOX function. Although future studies are needed to prove causality, it is tempting to speculate that the reduction of NOX function could be the primary mechanism whereby renal denervation prevents the development of hypertension during urinary tract obstruction.

ACKNOWLEDGEMENTS

The authors thank Mie Rytz Hansen and Susanne Hansen (Dept. of Physiology and Pharmacology, University of Southern Denmark, Odense, Denmark) and Margareta Stensdotter (Dept. of Physiology and Pharmacology, Karolinska Institutet) for the excellent technical contributions.

GRANTS

This work was supported by Swedish Research Council Grants 521-2011-2639 (to M. Carlström) and K2009-64X-03522 (to E. G. Persson). Swedish Heart and Lung Foundation Grants 20140448 and 20110589, Jeansson Foundation Grant JS2013-00064, the Bodossaki Foundation (Athens, Greece), and by KID funding from the Karolinska Institutet.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


