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ORIGINAL PAPER

Resetting of renal tissular renin–angiotensin and bradykinin–kallikrein systems after unilateral kidney denervation in rats

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Abstract The renal tissular renin–angiotensin and bradykinin–kallikrein systems control kidney function together with the renal sympathetic innervation but their interaction is still unclear. To further elucidate this relationship, we investigated these systems in rats 6 days after left kidney denervation (DNX, $n=8$) compared to sham-operated controls (CTR, $n=8$). Plasma renin concentration was unchanged in DNX vs. CTR ($p=$NS). Kidney bradykinin (BK) and angiotensin (Ang) I and II concentrations decreased bilaterally in DNX vs. CTR rats (~20 to 40%, $p<0.05$) together with Ang IV and V concentrations that were extremely low ($p=$NS). Renin, Ang III and dopamine concentrations decreased by ~25 to 50% and norepinephrine concentrations by 99% in DNX kidneys ($p<0.05$) but were unaltered in opposite kidneys. Ang II/I and KA were comparable in DNX, contralateral and CTR kidneys. Ang III/II increased in right vs. DNX or CTR kidneys (40–50%, $p<0.05$). Ang II was mainly located in tubular epithelium by immunocytological staining and its cellular distribution was unaffected by DNX. Moreover, the angiotensinergic and catecholaminergic innervation of right kidneys was unchanged vs. CTR. We found an important dependency of tissular Ang and BK levels on the renal innervation that may contribute to the resetting of kidney function after DNX. The DNX-induced peptide changes were not readily explained by kidney KA, renin or plasma Ang I generation. However, tissular peptide metabolism and compartmentalization may have played a central role. The mechanisms behind the concentration changes remain unclear and deserve further clarification.

Keywords Bradykinin · Angiotensin · Kidney · Denervation · Sympathetic

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Introduction

A host of experimental evidence has up to now demonstrated the presence of an important tissular renin–angiotensin system (RAS) in the kidneys characterized by the local intrarenal production, storage and metabolism of Ang II as well as the tubular expression of angiotensinogen and renin. The tissular RAS is supposed to control renal function independently from the systemic or plasmatic RAS that relies on renal renin secretion and plasmatic Ang I generation (Bader 2010). The kidneys contain measurable amounts of Ang I and Ang II at concentrations higher than in plasma. Ang II binds to cellular AT_1 and AT_2 receptors to control tubular, vascular and juxtaglomerular cell function. Its metabolite Ang III exhibits high affinity binding to cellular AT_2 receptors and may thus antagonize Ang II acting on its cellular AT_1 receptor (Kemp et al. 2012; Padia et al. 2009). In vivo experiments in animals using intravenous infusions of native or tagged angiotensins have consistently shown that the majority of intrarenal Ang II is cell

bound or located intracellularly within tubular epithelial cells (Li and Zhuo 2008; Schalekamp and Danser 2006; Van Kats et al. 2001). Moreover, Ang II is also secreted into, and detectable in, the urine where it is generated in the proximal tubule by urinary renin and epithelial Ang-converting enzyme (ACE) (Navar et al. 2011). However, the regulation of tissular Ang peptide production and storage in the kidneys by endogenous and exogenous factors is still largely unclear.

The excretion of salt and water by the kidneys is also controlled by the autonomic nervous system. The renal innervation interacts with the RAS to adjust renal endocrine and exocrine function according to the variable needs of the circulation. Renal sympathetic neurotransmission stimulates renin secretion by juxtaglomerular (JG) cells, mediates arterial vasoconstriction and activates tubular epithelial cells to increase Na reabsorption by cellular adrenoceptors (Johns et al. 2011). Interestingly, there is also an angiotensinergic co-phenotype of the autonomic renal innervation suggesting that Ang II is an important co-transmitter of norepinephrine (NE) in sympathetic nervous fibers (Bohlender et al. 2012). Neuronal Ang II release may be an independent source of tissular Ang II but the role of these fibers and their association with the intrarenal RAS is yet unclear. Since the renal innervation controls JG renin release and renal functional indices, it may directly control tissular Ang peptide concentrations but this is speculative thus far.

To elucidate this relationship, we used the unique model of unilateral renal denervation in experimental rats to study the impact of the autonomic nervous innervation on tissular Ang peptide concentrations and to further clarify the role of the angiotensinergic innervation. Both kidneys are neuronally linked by autonomic reno-renal reflexes operative at the spinal level where the efferent sympathetic output to one kidney is under tonic inhibitory control of the sensory afferent input from the opposite kidney. Unilateral renal denervation therefore induces ipsilateral natriuresis and diuresis and enhanced sympathetic nervous output to the contralateral kidney showing increased tubular Na retention and decreased diuresis. Under these circumstances, the renal innervation helps with preservation of a normal sodium and water balance in the body (Johns et al. 2011). We hypothesized that the denervated kidney would concomitantly show also decreased tissular Ang II concentrations to permit increased natriuresis by low tubular AT_1 receptor stimulation. Conversely, the contralateral innervated kidney should increase its tissular Ang II concentrations to additionally stimulate tubular Na reabsorption. Furthermore, its Ang III concentrations may eventually decrease to facilitate Na retention by decreased activation of tubular epithelial AT_2-receptors. Such adaptations of tissular Ang peptide concentrations could provide explanations also for the blood pressure lowering effect of renal denervation in patients with difficult-to-control hypertension (Bakris et al. 2015).

Renal dopamine (DA) is a paracrine product of proximal tubular cells and secreted quantitatively into the urine (Di Marco et al. 2007). Tissular BK and dopamine (DA) may both inhibit tubular Na reabsorption via their B_2 and D_1 receptors, respectively (Carey 2013; Gildea et al. 2012; Mamenko et al. 2012). Despite a host of studies in the past addressing the functional role of kidney BK, almost nothing is known about its regulation by the sympathetic nervous system because determinations of tissular BK are delicate and technically demanding (Rhaleb et al. 2011). Using the same rat model, our previous investigations remained inconclusive concerning the consequences of renal denervation for absolute kidney BK concentrations (Bohlender et al. 2013). We therefore reexamined this question in our present experiment together with the dynamics of tissular Ang and catecholamine concentrations. Together, we found bilateral and side-specific changes of renal Ang peptide and BK concentrations that may contribute to the pathophysiological features of the model.

Materials and methods

Animal experiments

Eight male Wistar rats aged 8 weeks were subjected to unilateral kidney denervation (DNX) and eight age-matched rats were sham operated (CTR). The animals were housed in cages under a 12 h day–night cycle, received a commercial rat diet (0.23% NaCl w/w) with free access to tap water and had standard veterinary care throughout the experiment. Surgical denervation of the left kidneys was performed following a standard protocol (Holmer et al. 1994). Briefly, rats were anesthetized by halothane and the abdominal cavity was opened by a midline incision. The left renal artery and vein were exposed and all adventitial and perivascular plexus fibers were carefully transected. The vessels were then coated with 10% alcoholic phenol solution. After local rinsing, the abdominal cavity was next surgically closed. Six days after surgery, rats were sacrificed by decapitation during short halothane anesthesia. Trunk blood was collected into pre-chilled tubes (4 °C) containing Na_2-ethylenediaminetetraacetic acid and an enzyme inhibitor cocktail as published (Nussberger et al. 1986). The kidneys were rapidly removed and tissue aliquots were either shock frozen in liquid nitrogen (−196 °C) for subsequent biochemical determinations or immediately immersion fixed in 2% formaldehyde diluted in 0.1 M phosphate buffer (pH 7.4, 4 °C) for immunocytological analysis (Imboden et al. 2009). Body weight was measured before

the experiment and at the end. All animal procedures complied with American Physiological Society guidelines and were performed in accordance with the ethical standards of the institution after permission by local authorities.

Immunocytology

Cryostat sections of 20 µm thickness were prepared from each kidney and a free floating incubation protocol was used for immunocytological stainings as described in detail elsewhere (Imboden et al. 2009; Patil et al. 2010). A protein G-purified mouse monoclonal anti-Ang II antibody (4B3) was employed to detect tissular Ang II. In dot-blot assays, the antibody showed cross-reactivity with Ang (2–8) [Ang III], Ang (3–8) [Ang IV] and Ang (4–8) [Ang V] but not with Ang I, Ang (1–7) or angiotensinogen. Its binding to Ang II covalently linked to Sepharose was specific. Further validation in kidney sections from angiotensinogen k.o. mice was not possible but its staining results consistently matched those of less sensitive polyclonal anti-Ang II antibodies (Frei et al. 2001; Patil et al. 2010). Catecholaminergic autonomic fibers were stained using a rabbit monoclonal anti-tyrosine 3-hydroxylase (TH) antibody (clone EP1533Y, Epitomics No. 2129-1). Visualization of the primary antibodies was by Cy3- or Cy5-labeled anti-mouse or anti-rabbit secondary antibodies. Nuclear desoxyribonucleic acid (DNA) was stained with 4′,6′-diamidino-2-phenylindole (DAPI). Final digital visualization and analysis was with a Leica DM6000B confocal fluorescent light microscope. Sections from DNX and sham-operated rat kidneys were assayed simultaneously and control stainings without primary or secondary antibodies were additionally performed (not shown).

Biochemical determinations

Kidney cortical Ang peptide and BK concentrations were determined after peptide extraction and reversible adsorption to bonded phase silica. A reversed phase isocratic high-pressure liquid chromatography (HPLC) separation of Ang peptides was performed before quantitative determination of Ang I, Ang II and Ang III to V by radioimmunoassay as described (Nussberger et al. 1986). The results were expressed as fmol/g wet weight. The Ang peptide recovery rates were >70% with inter-assay and intra-assay coefficients of variation <15%. The detection limits of the various Ang fragments were between 0.7 and 1.5 fmol/g. Plasma renin (PRC) and kidney renin concentrations (KRC) were determined by enzyme kinetic assay and radioimmunoassay of Ang I as published (Bohlender et al. 1998). The protein concentrations in kidney homogenates were measured according to Lowry and the results were expressed as ng Ang I/ml/h (PRC) or ng Ang I/h/g (KRC), respectively. Renal tissular BK concentrations were determined after tissue homogenization followed by extraction into buffered solution, liquid chromatography (HPLC) and specific radioimmunoassay. Additionally, kidney kallikrein-like activity (KA) was measured by enzyme kinetic assay using an artificial peptide substrate as described (Bohlender et al. 2013). To determine tissue catecholamine concentrations, renal cortical tissue aliquots were sonicated in 0.1 mol/l perchloric acid (4 °C) using a Branson sonificator for 10 s. The resulting homogenate was centrifuged at 14,000 g (4 °C) and catecholamines were extracted from the supernatant using aluminum oxide adsorption. Epinephrine (E), NE and DA were then separated and quantified by HPLC using an electrochemical detection technique (Grouzmann et al. 2012). Catecholamine concentrations were expressed as pmol/g wet weight. All laboratory determinations and staining procedures were performed under blinded conditions.

Statistics

Mean values and standard deviations (SD) were calculated. Differences between mean values were analyzed by Student's t or analysis of variance as appropriate. A $p < 0.05$ was regarded significant.

Results

Table 1 shows the body weight of CTR and DNX rats at baseline and at the end of the experiment (day 6) together with PRC at the end. There were not significant differences between the two experimental groups, respectively. The results for renal tissular catecholamines, Ang and bradykinin peptides, and renin and kallikrein concentrations are shown in Tables 2, 3 and 4. The results for control rats are given as mean values for right and left kidneys, respectively.

Table 2 shows the results for tissular catecholamine concentrations in kidneys of DNX and CTR rats. In CTR kidneys, NE concentrations were >10 times higher than E or DA concentrations. In DNX rats, NE, E and Da

Table 1 Body weight and plasma renin concentration at baseline and 6 days after surgery (end of experiment); mean ± SD

	Control $n=8$	DNX $n=8$	p
Body weight (g), baseline	219 ± 12	217 ± 15	NS
Body weight (g), day 6	253 ± 10	245 ± 16	NS
PRC (ng Ang I/ml/h), day 6	13.4 ± 4.8	12.5 ± 5.9	NS

PRC plasma renin concentration, *Ang* angiotensin, *DNX* unilateral renal denervation, *NS* not significant

Table 2 Kidney catecholamine concentrations on day 6 after surgery (end of experiment); mean ± SD

	Control	DNX	
		Right intact	Left DNX
Epinephrine (pmol/g)	3.3 ± 1.7	4.1 ± 1.2	3.8 ± 0.7
Norepinephrine (pmol/g)	333 ± 92	337 ± 51	3 ± 2**
Dopamine (pmol/g)	21.0 ± 4.1	20.4 ± 4.2	14.6 ± 4.6*

Control results represent mean values for left and right kidneys, respectively

DNX unilateral renal denervation

*$p < 0.05$ vs. right kidney; **$p < 0.01$ vs. right kidney

Table 3 Kidney renin and Ang peptide concentrations with Ang II/I and Ang III/II ratios 6 days after surgery (end of experiment); mean ± SD

	Control	DNX	
		Right intact	Left denervated
Renin (μg Ang I/h/g)	310 ± 44	296 ± 78	222 ± 88*†
Ang I (fmol/g)	445 ± 165	258 ± 35*	274 ± 76*
Ang II (fmol/g)	107 ± 25	85 ± 14*	68 ± 18*
Ang III (fmol/g)	17 ± 7	21 ± 7	11 ± 4*†
Ang IV (fmol/g)	2.1 ± 1.3	1.4 ± 0.9	1.5 ± 0.8
Ang V (fmol/g)	2.1 ± 1.7	1.0 ± 0.2	1.5 ± 1.0
Ang II/I	0.32 ± 0.17	0.34 ± 0.09	0.24 ± 0.17
Ang III/II	0.16 ± 0.07	0.24 ± 0.03*§	0.17 ± 0.05

Control results represent mean values for left and right kidneys, respectively

DNX unilateral renal denervation

*$p < 0.05$ vs. CTR; †$p < 0.05$ vs. right kidney; §$p < 0.01$ vs. left kidney

Table 4 Kidney kallikrein and bradykinin concentrations 6 days after surgery (end of experiment); mean ± SD

	Control	DNX	
	Average	Right intact	Left DNX
Kallikrein (μcat/g)	16.7 ± 1.3	17.2 ± 1.8	17.3 ± 1.8
Bradykinin (fmol/g)	114 ± 35	85 ± 17*	78 ± 17*

Control results represent mean values for left and right kidneys, respectively

DNX unilateral renal denervation

*$p < 0.05$ vs. CTR

concentrations in the right intact kidneys were not significantly different from CTR levels. In left denervated kidneys, however, NE concentrations were 99% ($p < 0.01$, mean of individual ratios) and DA concentrations were 31% ($p < 0.05$) lower compared to contralateral kidneys. E concentrations were not significantly different from those in right or CTR kidneys.

Table 3 gives the kidney renin and Ang peptide concentrations in CTR and DNX rats together with Ang II/I and Ang III/II concentration ratios. In DNX rats, KRC of the right intact kidneys was comparable to CTR levels. However, it was 27% lower in left denervated compared to right kidneys ($p < 0.05$). Next, left and right kidneys showed significantly lower tissular Ang I (39 vs. 42%) and Ang II concentrations (37 vs. 21%) compared to CTR kidneys ($p < 0.05$). The Ang II concentrations were somewhat lower in left denervated compared to right intact kidneys but side differences were statistically not significant (p = NS). Ang III concentrations in the right kidneys were not significantly different from CTR kidneys (p = NS). In left denervated kidneys, they were 54% lower compared to the opposite kidneys. Finally, tissular Ang IV and V concentrations were very low and some determinations fell below the detection limit. The individual detection limit was then used to calculate hypothetical mean values. Under these circumstances, mean Ang IV and Ang V concentrations of denervated left and contralateral right kidneys were found to be at least 30% lower than in CTR kidneys without notable side differences (p = NS).

The Ang II/I ratios (Table 3) were not significantly different in left denervated, right intact or CTR kidneys (p = NS). In contrast, innervated kidneys of DNX rats showed significantly higher Ang III/II ratios compared to denervated (41%, $p < 0.01$) or CTR kidneys (50%, $p < 0.05$). There were otherwise no notable differences between left denervated compared to CTR kidneys (p = NS).

Table 4 shows the results of our renal BK and KA determinations. In DNX rats, BK concentrations were not significantly different between left denervated and contralateral right kidneys but they were in total significantly lower (32 vs. 25%) compared to CTR kidneys ($p < 0.05$). Moreover, kidney KA was not significantly different in left vs. right kidneys or when compared to CTR levels (p = NS).

Figure 1 shows a representative immunofluorescence micrograph of a denervated kidney (Fig. 1a) and its contralateral innervated counterpart (Fig. 1b) stained for Ang II. Intense and homogenous Ang II-positive staining was observed in all parts of the tubular epithelium. In contrast, the arterioles and glomeruli showed somewhat less intense Ang II avidity. Denervated and contralateral kidneys showed no appreciable differences of their staining patterns or of regional Ang II distribution. The same results were obtained in all DNX rats. There were also no significant differences compared to CTR kidneys (data not shown).

Figure 2 shows details of the angiotensinergic and catecholaminergic innervation in left denervated and right intact kidneys from DNX rats. Contralateral kidneys with preserved innervation showed Ang II-positive

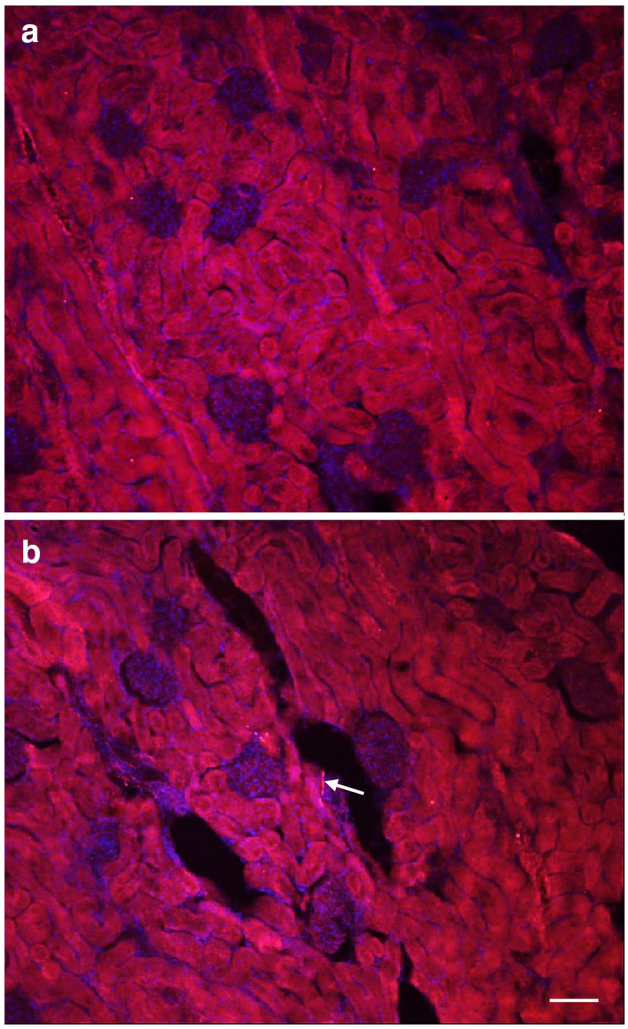

Fig. 1 Kidney sections from a denervated left (**a**) and right intact kidney (**b**) of a DNX rat stained for Ang II (*red*). There was no appreciable difference for the intensity and distribution of the Ang II staining between denervated and contralateral kidneys. The *arrow* indicates a small Ang II-positive nerve fiber bundle. DNA staining was with DAPI (*blue*); *scale bar* 100 µm

nerve bundles and fibers ubiquitously present as a dense network around arterioles (Fig. 2b) and in the pelvic area next to the urothelium (Fig. 2c). Of note, angiotensinergic fibers were not suppressed in the afferent arterioles or vicinity of juxtaglomerular cells (Fig. 2a). There was also a dense innervation of these structures by catecholaminergic fibers as exemplified by Fig. 2d. In contrast, all autonomic nerve fibers had disappeared in the denervated kidneys (Fig. 2e–h).

Discussion

As a main result, we found significantly lower tissular Ang I, Ang II and BK concentrations bilaterally in denervated and contralateral kidneys of DNX rats compared to control kidneys. There were no notable side differences despite the fact that only one kidney had lost its innervation. Moreover, also tissular Ang IV and Ang V concentrations were bilaterally decreased, albeit their concentrations were near the detection limit and the differences statistically not significant. Together, these findings suggest a concentration-dependent downstream effect on the other Ang metabolites. Tissular Ang III concentrations, however, were decreased only in the denervated kidneys but remained unchanged in the opposite kidneys compared to controls. Since the concentration changes of Ang I and Ang II were comparable in DNX and contralateral kidneys, the same bilateral mechanism should be assumed. Indeed, plasma renin activity has been associated with significant parallel changes of renal tissular Ang II concentrations but our results finally do not allow a simple answer (Nussberger 2000).

Reduced systemic Ang I generation and availability by low plasma renin or angiotensinogen was unlikely the primary cause in our model because PRC was not different in DNX and control rats. Consistently, also Jacob et al. (2005) reported unchanged plasma renin activity 6 days after unilateral renal denervation despite a decrease of blood pressure. Alternatively, diminished production of Ang I by low tissular renin could have caused the decrease of Ang I and its downstream metabolites. The decreased renal renin concentrations in the denervated kidneys would comply with this assumption. Previous studies have also reported decreased renal renin mRNA expression and reduced renin staining of juxtaglomerular cells after renal denervation (Holmer et al. 1994). However, renal renin concentrations were unchanged in the contralateral kidneys and juxtaglomerular renin was therefore not obviously responsible for the low bilateral Ang levels. Moreover, intravenous Ang infusion experiments in experimental animals have shown that arterial Ang I delivery to the kidneys would not be a significant source for intrarenal Ang II generation under unstimulated conditions or when plasma renin is low (Danser et al. 1998). Therefore, other local mechanisms have to be taken into account.

Renal extraction of Ang I and Ang II from the circulation has been described but not all compartments do this equally. The kidneys may also capture renin from the circulation and are a major site of renin degradation (Kim et al. 1988; Schalekamp and Danser 2006; Van Kats et al. 2001). Furthermore, plasma angiotensinogen is used for intrarenal Ang I generation and tubular cells were shown to express renin and angiotensinogen independently to generate intrarenal Ang I (Rhaleb et al. 2011; Yayama et al. 1995). These

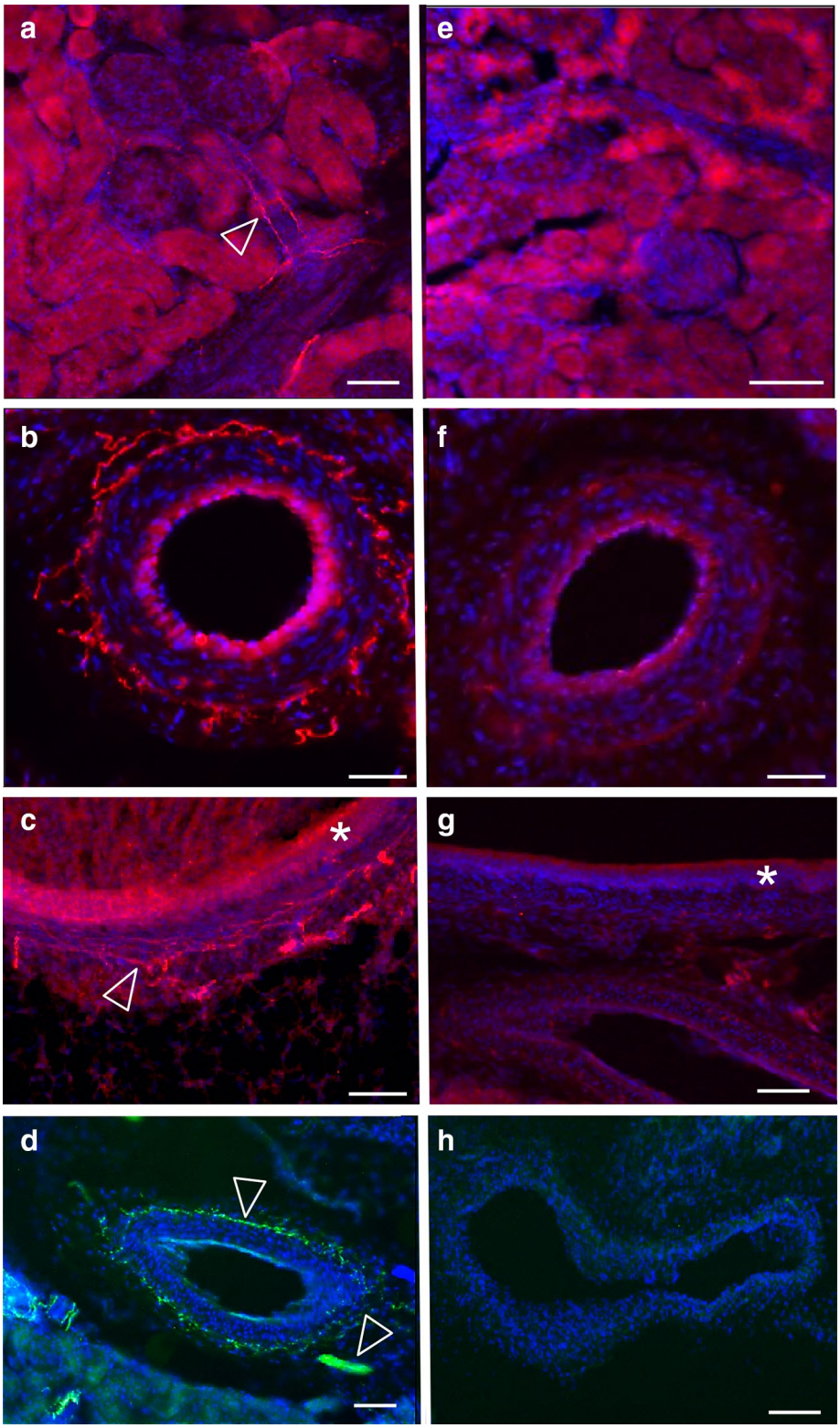

Fig. 2 Kidney sections from right intact (**a**–**d**) and left denervated kidneys (**e**–**h**) of DNX rats stained for Ang II (*red***a**–**c** and **e**–**g**) and tyrosine hydroxylase (*green***d, h**). The various panels show afferent arterioles (**a, e**), pelvic arterioles (**b, d, f, h**) and urothelium (**c, g**). The angiotensinergic and catecholaminergic innervation can be seen in different regions of right intact kidneys (**a**–**d**). Six days after denervation, all intrarenal nerve fibers had disappeared (**e**–**h**). Tubular epithelium avidly stained for Ang II as can be seen in **a, e**. DNA staining was with DAPI (*blue*). *Arrows* point to nerve fibers; *Urothelium; *scale bars* represent 50 µm (**b, d, f**) or 100 µm (**a, c, e, g, h**)

functions may have decreased bilaterally by yet unknown mechanisms. Moreover, there is accumulating evidence for an important autonomic production of Ang I and Ang II in the kidneys with quantitative Ang delivery into the venous outflow and urine. The renal interstitium contains measurable amounts of extracellular Ang II at concentrations higher than in plasma (Nishiyama et al. 2002). According to a kinetic model, this peritubular Ang II would be almost

completely bound to local AT receptors, i.e. there is only small amounts of free Ang II present in the interstitial fluid that is otherwise mostly absorbed by cell-based AT receptors (Schalekamp and Danser 2006). The cellular uptake of Ang II is mediated by AT_1 receptor internalization and can be blocked pharmacologically. Indeed, the vast majority of intrarenal Ang II has been reported to be cell associated or located intracellularly (Li and Zhuo 2008; Van Kats et al. 2001).

Low interstitial AT_1 receptor density could therefore explain the low Ang II concentrations but not the low Ang I concentrations. The Ang II/I ratio is a sensitive indicator of tissular angiotensin-converting enzyme (ACE) function responsible for the conversion of Ang I to Ang II (Campbell et al. 1991). Since this ratio was not significantly changed in DNX compared to contralateral or control kidneys, altered ACE activity did not provide an explanation. Next, peritubular Ang I and II represents only <10% of the total intrarenal amount and its alterations would not explain the much greater concentration differences after DNX. Therefore, intracellular Ang concentrations may have changed additionally.

As a notable exception, low Ang III concentrations were found only in denervated kidneys suggesting that Ang III production and metabolism were differently regulated by the renal innervation. Aminopeptidase A [APA, EC 3.4.11.7], a membrane-bound ectoenzyme, cleaves Ang II to produce Ang III that is further processed to Ang IV by Aminopeptidase N [EC 3.4.11.2]. Ang III binds avidly to cellular AT_2 receptors while Ang 1–7, another Ang II metabolite, binds to AT_2 and Mas receptors (Carey 2013; Iusuf et al. 2008; Kemp et al. 2012). Since AT_2 receptors are not known to internalize their ligands quantitatively, the low Ang III concentrations in the denervated kidneys were likely caused by extracellular mechanisms (Hein et al. 1997; Van Kats et al. 2001). We found an unchanged Ang III/II ratio in denervated kidneys and low Ang II availability may thus have caused the low Ang III concentrations rather than abnormal APA activity. In contrast, the ratio was significantly increased by almost 50% in contralateral kidneys. This would be compatible with increased APA activity but also functional compartmentalization, for example by quantitative binding of Ang III to AT_2 receptors and cellular surfaces, could have played a role.

Interestingly, the innervated kidney in our model may significantly increase its Na excretion during a high dietary salt load but not the denervated kidney (Lohmeier et al. 1999). This was primarily attributed to the effects of long-term functional sympathoinhibition. Our results now provide another hypothesis to explain this phenomenon. Indeed, the high Ang III/II ratio in innervated compared to denervated kidneys could facilitate urinary Na excretion by promoting the activation of pro-natriuretic AT_2 receptors in the presence of low tissular Ang II and AT_1 receptor stimulation. Collectively, our findings document an important dependency of tissular Ang peptide concentrations on the renal innervation that may significantly contribute to the pathophysiological features of the model.

We also investigated the possibility that the intrarenal distribution of Ang II or its presence in parts of the nephron could have changed after DNX. However, our immunocytological studies failed to detect any such differences in denervated or contralateral kidneys compared to control kidneys. During earlier immunocytological studies, Ang II has been consistently described only in afferent arterioles and JG cells but not elsewhere in the kidneys (Cantin et al. 1984; Celio and Inagami 1981; Hunt et al. 1992). At the time, polyclonal antibodies and mostly conventional light microscopic techniques were used to visualize Ang II that may have prevented its broad detection in kidney sections for methodological reasons. These negative results were in conflict with recent experimental data suggesting that renal Ang II is mostly cell bound or located within tubular cells (Li and Zhuo 2008; Schalekamp and Danser 2006; Van Kats et al. 2001). Our monoclonal anti-Ang II antibody displayed a high sensitivity and has been tested for its specificity by dot blot assays and in various tissues from different species. (Bohlender et al. 2012; Frei et al. 2001; Imboden et al. 2009; Patil et al. 2010). This antibody allowed us to detect tissular Ang II at very low concentrations and with high specificity in renal tissue using fluorescence light microscopy.

With this methodology, we were able to document the presence of Ang II in renal tissue and particularly in the tubular compartment by direct visualization of Ang II. Most Ang II was located intracellularly in the tubular epithelium representing by far the largest compartment to host tissular Ang II thereby confirming the up to now indirect evidence provided by Ang infusion studies (Schalekamp and Danser 2006). We were also interested in possible phenotypic adaptations of the angiotensinergic or catecholaminergic innervation after asymmetric renal denervation but this was obviously not the case. Such changes could have occurred as a consequence of the altered sympathetic nervous output or the functional adaptations in the kidneys. In our present experiment, we were interested only in obvious qualitative differences. At his level, however, we were unable to report any relevant alterations of the autonomic innervation in the remaining innervated kidneys that could be viewed as a characteristic of the model.

Finally, renal tissular BK concentrations were also decreased bilaterally in DNX rats together with the Ang II concentrations. Our tissular BK concentrations were consistent with literature reports supporting the validity of our laboratory determinations (Campbell et al. 1993). Renal BK is almost exclusively produced within the kidneys

where kallikrein is mainly expressed in tubular connecting ducts (Rhaleb et al. 2011). Tissular KA concentrations were unchanged in our DNX rats and accordingly did not explain the low BK levels. Other investigators have consistently reported that urinary kallikrein excretion remained unchanged in rats after renal denervation (Fejes-Tóth et al. 1978). The concentrations of the kininogen precursor could have decreased bilaterally but experimental evidence supports another explanation. Indeed, animal studies in uninephrectomized dogs have shown a significant decrease of renal interstitial fluid BK concentrations following the intrarenal arterial infusion of a renin inhibitor but not after administration of losartan, an inhibitor of AT_1 receptors. It was concluded that Ang II and AT_2 receptors could have mediated the decrease of intrarenal BK levels after renin inhibition (Siragy et al. 1996). This dependency on Ang II was later confirmed in knockout mice for AT_2 receptors showing low kidney BK concentrations compared to wild-type mice. Their renal BK levels increased after experimental intravenous infusion of Ang II (Siragy et al. 1999). Our own findings readily comply with these data and provide additional evidence for a direct link between tissular Ang II and BK concentrations in the kidneys.

Finally, the efficacy of our surgical denervation procedure and the validity of our model were confirmed by the significant drop of tissular NE concentrations (>95%) observed in all denervated kidneys. The concomitant decrease of DA concentrations was another anticipated effect in this model (Wagner et al. 1997). Interestingly, NE concentrations in the intact right kidneys of DNX rats were unchanged despite an expected increase of sympathetic nervous activity mediated by increased firing of sympathetic nerve fibers. However, this gain of sympathetic activity would cause only small changes of NE concentrations in the synaptic vicinity in view of a highly efficient catecholamine recycling into presynaptic storage vesicles. Moreover, any local shifts of NE into the interstitium would not change total tissue concentrations. Quantitative spillover of catecholamines into the circulation may have additionally prevented a detectable change of tissular NE concentrations (DiBona 2000; Eisenhofer 2001).

Dopamine is produced and released by proximal tubular cells and may stimulate tubular Na excretion via its D_1 receptors. Similarly, BK stimulates natriuresis via its tubular B_2 receptors and thereby acts synergistically with DA and Ang III binding to AT_2 receptors (Carey 2013; Rhaleb et al. 2011). The reported natriuretic effects of Ang III still require independent experimental confirmation. With this assumption, however, the low tissular dopamine, BK and Ang III concentrations may hypothetically help attenuate tubular Na losses in the denervated kidney. Conversely, the altered Ang and BK levels in the contralateral kidney may cause a resetting of its functional parameters and modify the effects of sympathetic neurotransmission. These peptide dynamics may also be important for the clinical efficacy of renal denervation in the treatment of patients with resistant hypertension (Bakris et al. 2015).

In summary, we document an important interaction between the renal innervation and the tissular RAS and kallikrein-bradykinin systems. Furthermore, most tissular Ang II in the kidneys could be located intracellularly by our immunocytological studies. Our findings have important implications for the sympathetic control of renal function and the pathophysiological features of the renal denervation model. Concomitant changes of tissular Ang and BK concentrations have to be taken into account when investigating the renal innervation and its role for kidney function and blood pressure. The mechanisms behind these concentration changes, however, still remain unclear and await further experimental clarification.

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Compliance with ethical standards

Conflict of interest None.

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