Local modulation of the natriuretic peptide system in the rat remnant kidney

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Abstract

Background. The natriuretic peptide (NP) system plays a central role in the renal adaptations to acute volume expansion. However, the modulation of the NP system in chronic renal insufficiency (CRI) remains to be elucidated. In the present study, we evaluated cardiac haemodynamics, plasma type-B natriuretic peptide (BNP) levels and the expression of natriuretic peptide receptor A (NPR-A) and NPR-C in the renal cortex (RC) and medulla (RM) of Sham and ¾ nephrectomized (¾nx) rats, up to 26 weeks after surgery.

Methods. Male Wistar–Han rats (190–220 g; $n = 49$) were randomly assigned to ¾nx or Sham surgery. Two, 10 and 26 weeks after surgery, non-invasive blood pressure (BP) and left ventricular (LV) haemodynamics were performed, and urine and blood were collected for metabolic studies and plasma BNP determination. In addition, tissue samples from RC and RM were obtained for NPR-A and NPR-C quantification (RT-PCR and western blotting) as well as NPR-A immunodetection.

Results. In ¾nx rats, the progressive interstitial fibrosis and tubular atrophy were accompanied by a time-dependent increase of BP and impaired natriuretic response to volume expansion (VE). This was accompanied in ¾nx rats by an early and time-dependent elevation of BNP circulating levels that was not associated with cardiac dysfunction or increased myocardial BNP gene expression. In ¾nx rats, NPR-A expression in the remnant RM was consistently reduced at 2, 10 and 26 weeks, and this was accompanied by an increase in NPR-C expression in the remnant RC from ¾nx rats.

Conclusions. BP elevation and compromised natriuretic response to VE in ¾nx rats is associated with increased circulating BNP levels in the absence of cardiac dysfunction. This is accompanied in ¾nx rats by both impaired NPR-A expression in the RM and upregulation of NPR-C in the RC suggesting a novel mechanism for NP resistance in CRI.

Keywords: BNP; chronic renal insufficiency; heart failure; hypertension; natriuretic peptide receptor

Introduction

The natriuretic peptides (NPs) are a family of signalling molecules mainly released by the heart to stimulate natriuresis, diuresis and vasodilation, contributing to sodium homeostasis and BP control [1–3]. The natriuretic and diuretic actions of NPs are due to both haemodynamic and direct tubular actions [4–6]. These include dilation of afferent renal arterioles and constriction of efferent arterioles, leading to increased pressure within the glomerular capillaries and enhanced single nephron glomerular filtration rate (GFR), shifts in inner medullary blood flow and inhibition of sodium transport in both proximal and distal nephron segments [7]. In the latter instance, the NPs have been shown to inhibit sodium reabsorption in the inner medullary collecting duct (IMCD), a nephron segment that handles <5% of the filtered sodium load, yet plays a pivotal role in establishing the final sodium concentration of the urine [8]. Besides the effects on sodium handling and BP control, the NP system was implicated in limiting the proliferative and fibrotic response during disease progression [9,10,11] that, by itself, was suggested to influence directly the NP system activity [12].

Although the activation of the NP system has been, so far, evaluated mainly through the NP circulating levels, evidence is accumulating suggesting a prominent role for target organ receptor expression in the control of system activity [13]. The biological effects of NP have been shown to be mainly mediated through the cell surface family of guanylyl cyclase (cGMP) linked type-A natriuretic peptide receptors (NPR-A) [14,18,19] whereas the NP clearance receptor (NPR-C) is mainly involved in the clearance of NP through receptor-mediated internalization and degradation [15–17]. The renal actions of NP can therefore be limited both by increased NPR-C renal expression and/or by NPR-A downregulation. Accordingly, several lines of evidence have suggested that resistance to NP related to changes in the renal expression of NPR-A may explain in part the maintenance of volume expansion (VE) in oedema formation conditions, namely in congestive heart failure [23,24]. In this disease, NP circulating levels are elevated and positively correlate with VE and poor prognosis [25,26].

In chronic renal insufficiency (CRI), an increase in NP circulating levels was observed and this was implicated in the compensatory increase in GFR and in the decrease in sodium reabsorption, both under normal and salt-replete conditions [20]. Although CRI is frequently associated with disturbances in cardiovascular haemodynamics, the mechanisms responsible for the increase of NP circulating levels in this condition still remain to be elucidated. Moreover, it is not known whether this is accompanied by changes in the expression of NPRs in the remnant renal cortex (RC) and renal medulla (RM) during disease progression. This is a matter of considerable importance given that (1) substantial increments of plasma ANP in patients with CRI lead to a modest natriuretic response when compared to normal controls or to glomerulonephritic patients with well-preserved renal function [21] and (2) the effects of NP on glomerular haemodynamics were suggested to occur independently from those related to the decrease of sodium reabsorption in renal tubules [22].

On the basis of the previous considerations, the aim of the present study was to evaluate the activation of the NP system in CRI and to examine whether this is accompanied by changes in the expression of renal NPR. For this purpose, we evaluated cardiac haemodynamics, BP, renal function, circulating type-B natriuretic peptide (BNP) levels, the natriuretic response to VE and the expression of both NPR-A and NPR-C in the RC and RM, from ¾ nephrectomized and Sham rats, up to 26 weeks after renal mass ablation.

Materials and methods

Animal experiments were performed according to the Portuguese law on animal welfare and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH. Pub. No. 85-23, Revised 1996).

Normotensive male Wistar–Han rats (Harlan, Barcelona, Spain; 190–220 g) were kept under controlled environmental conditions (12:12 h light/dark cycle and room temperature $22 \pm 2°C$) and were fed *ad libitum* throughout the study with ordinary rat chow (Panlab, Barcelona, Spain) containing 1.9 g kg^{-1} of NaCl. After 1 week, the animals were randomly submitted to right kidney nephrectomy and surgical ablation of both poles of the left kidney (¾nx), as previously described [27]. The mean percentage of remnant renal mass in ¾nx rats was $27 \pm 2\%$. Control animals were rats submitted to sham surgery under similar conditions (Sham). Metabolic, haemodynamic, morphometric, histological and molecular studies were performed 2 ($n = 12$), 10 ($n = 14$) and 26 weeks ($n = 17$) after surgery.

Metabolic studies

Two, 10 and 26 weeks after surgery, the rats were placed in metabolic cages (Tecniplast, Buguggiate-VA, Italy). Twenty-four-hour urine was collected to calculate creatinine clearance (C_{Creat}, ml min^{-1} kg body wt^{-1}) and fractional excretion of sodium (FE_{Na^+}, %).

Haemodynamic evaluation

BP (systolic and diastolic) and heart rate were measured weekly in conscious restrained animals (7.00–10.00 AM), using a photoelectric tail-cuff pulse detector (LE 5000, Letica, Barcelona, Spain). Four determinations were made each time, and the means were used for further analysis.

At 2, 10 and 26 weeks, the animals were anaesthetized with pentobarbital (6 mg/100 g, i.p.), placed over a heating pad and tracheostomized for mechanical ventilation with oxygen (Harvard Small Animal Ventilator, Model 683, Massachusetts, USA), with rate and volume adjustment for body weight. Anaesthesia was maintained with additional bolus of pentobarbital (2 mg/100 g) as needed. The heart was exposed through a median sternotomy and the pericardium widely opened. Left ventricular

(LV) pressures were measured with a 2F high-fidelity micromanometer (SPR-324, Millar Instruments, TX, USA) inserted into the LV. After complete instrumentation, the animal preparation was allowed to stabilize for a few minutes. Haemodynamic recordings were made with respiration suspended at end expiration. The parameters were converted online to digital data with a sampling frequency of 1000 Hz. LV pressures were measured at end-diastole and peak-systole. The peak rates of LV pressure rise (dP/dt_{max}) and pressure fall (dP/dt_{min}) were also measured. The relaxation rate was estimated with the time constant τ by fitting isovolumetric pressure fall to a monoexponential function.

At the end of the experimental protocol, the animals were euthanized and blood was collected in lithium/heparin tubes for sodium, creatinine and BNP quantification. Also, samples from the LV, RC and RM were snap frozen in liquid nitrogen and stored ($-70°C$) for mRNA quantification. In addition, renal tissue samples were paraffin-embedded and formalin-fixed for histology and immunohistochemistry.

Volume expansion

In another set of experiments, 2, 10 and 26 weeks after the surgery, the animals were anaesthetized with pentobarbital sodium (60 mg kg body wt^{-1} followed by 20 mg kg^{-1} h^{-1}; i.p.), placed on a thermostatically controlled heating table to maintain rectal temperature at 37°C and tracheostomized. The left jugular vein was catheterized by a PE50 tube (Becton Dickson, Lisboa, Portugal) for VE. After an abdominal incision, the urinary bladder was catheterized through a suprapubic incision for urine sampling. After the completion of surgical procedures, the infusion of isotonic saline (0.9%) started at a rate of 5 ml kg^{-1} h^{-1} for 120 min; during this period a urine sample was collected ($t = 0$–120 min, Basal). After this stabilization period, the VE was started by infusion of isotonic saline (0.9%) at a rate of 100 ml kg^{-1} h^{-1} during 30 min (5% body weight); during this phase, one urine sample was collected ($t = 150$ min, VE). Thereafter, the infusion was again reduced to 5 ml kg^{-1} h^{-1} for 90 min; during this recovery period, three urine samples were collected ($t = 160$ min, VER-1; $t = 170$ min, VER-2 and $t = 240$ min, VER-3).

Plasma BNP quantification

BNP levels were quantified by a competitive radioimmunoassay after extraction of peptides from plasma, according to the manufacturer's instructions (RK-011–14; Phoenix Pharmaceuticals; Belmont, CA, USA). Briefly, in each reaction, rabbit anti-peptide antibody was incubated (16–24 h at 4°C) with sample or standard and mixed with a fixed amount of ^{125}I-peptide. After a second incubation period (16–24 h at 4°C), goat anti-rabbit serum and normal rabbit serum are added and incubated for 90 min. The reaction tubes were subsequently centrifuged and the pellet counted in a Gamma counter. By measuring the amount of ^{125}I-peptide bound as a function of the standard peptide concentration, a 'standard curve' was constructed from which the BNP concentration of each sample was calculated. Results are expressed as pg/tube.

Renal histology and immunohistochemistry

Kidney sections, 4 μm thick, of paraffin-embedded formalin-fixed specimens were deparaffinized in xylene and rehydrated through graded ethanols.

Renal fibrosis. Collagen detection with Masson's trichrome staining (Goldner with light green; Bio-Optica, 011802, Milan, Italy) was performed to evaluate interstitial renal fibrosis. Sections were stained sequentially with Weigert's iron haematoxylin (10 min), picric acid alcoholic stable solution (4 min), Ponceau acid fuchsin (4 min), phosphomolybdic acid (10 min) and light green (5 min). The number of green-stained collagen intersecting points on a grid was used for interstitial fibrosis quantification in 12 randomly selected fields (×200 magnification). Results are expressed as the mean number of grid points falling on collagen.

Tubular atrophy. Tubular basement membrane (TBM) thickening was used to detect tubular atrophy, as previously described [28]. TBM was identified by periodic acid Schiff (PAS) staining (Hotchkiss-MC Manus; Bio-Optica, 04-130802, Milan, Italy). Sections were stained sequentially with periodic acid (10 min), Schiff reagent (20 min), potassium metabissulphite (2 min), fixative solution (2 min) and Mayer's Hemalum (3 min). In each experimental group, TBM thickening was measured in 100 cross-sectioned

tubules (50 tubules in cortex, 50 tubules in medulla). Results were expressed as the percentage of TBM thickened cross-sectioned tubules.

Immunolocalization of NPR-A. Kidney paraffin sections, 4 μm thick, were placed in 0.1% poly-L-lysine-covered microscopy slides (Menzel GmbH, Braunschweig, Germany) for immunohistochemical detection of NPR-A using the appropriate polyclonal primary antibody diluted to 1/250 (Abcam, Cambridge, UK). Immunoperoxidase staining was performed by an anti-rabbit secondary biotinylated antibody diluted to 1/300 (Vector Laboratories) combined with a streptavidin–peroxidase complex diluted to 1/50 (Vector Laboratories). After counterstaining with haematoxylin, the slides were observed in a light microscope (Leica Application Suite Software, Leica Microsystems, Wetzlar, Germany).

mRNA relative quantification by real-time PCR (RT-PCR)

Total mRNA was extracted through the guanidium-thiocyanate selective silica-gel membrane-binding method (Qiagen 74124, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity were assayed by spectrophotometry (Eppendorf 6131000.012).

RT-PCR. Two-step RT-PCR was used to perform relative quantification of mRNA. For each studied mRNA molecule, standard curves were generated from the correlation between the amount of starting total mRNA and PCR threshold cycle (second derivative maximum method) of graded dilutions from a randomly selected tissue sample ($r > 0.97$). For relative quantification of specific mRNA levels, 50 ng of total mRNA from each sample underwent two-step RT-PCR. A melt curve analysis of each RT-PCR and 2% agarose gels (0.5 μg/ml ethidium bromide) were performed to exclude primer–dimer formation and assess the purity of the amplification product. The GAPDH mRNA level was used as an internal control gene. The results of mRNA quantification are expressed in an arbitrary unit (AU) set as the average value of the sham group (sham = 1 AU), after normalization for GAPDH.

RT (20 μl; 10 min at 22°C, 50 min at 50°C and 10 min at 95°C) was performed in a standard thermocycler (Whatman Biometra 050-901, Göttingen, Germany): 40 U/reaction of reverse transcriptase (Invitrogen 18064-014, California, USA), 20 U/reaction of RNase inhibitor (Promega N2515, Wisconsin, USA), 30 ng/ml random primers (Invitrogen 48190-011, California, USA), 0.5 mM nucleotide mix (MBI Fermentas R0192, Ontario, Canada), 1.9 mM $MgCl_2$ and 10 mM DTT. Ten percent of the cDNA yield was used as a template for RT-PCR (LightCycler, Roche, Indianapolis, USA) using SYBR green (Qiagen 204143, Hilden, Germany) according to the manufacturer's instructions.

Specific PCR primer pairs for the studied genes were NPR-A—fw 5′-ACA CAT GCC CAG TCC CAC CCT TAC-3′ and rev 5′-AAC CGG CAG CTT CTC TTC TCC TCA-3′; NPR-C—fw 5′-GGA CCG CGA AGC CTG AGT TTG AGA-3′ and rev 5′-ATG GAC ACC TGC CCG GCG ATA CCT-3′; BNP—fw 5′-CAG AGC TGG GGA AAG AAG AG-3′ and rev 5′-GGA CCA AGG CCC TAC AAA AGA-3′ and GAPDH—fw 5′-CCG CCT GCT TCA CCA CCT TCT-3′ and rev 5′-TGG CCT TCC GTG TTC CTA CCC-3′.

Semi-quantification of NPR-A and NPR-C protein levels by the western-blotting technique

Samples from RC and RM were mixed with a sample buffer (0.35 M tris-HCl, 4% SDS, 30% glycerol, 9.3% DTT, ph 6.8, 0.01% bromphenol blue), boiled at 95°C for 5 min and separated by SDS-PAGE in 7.5% poly-acrylamide gel (80 μl of sample/well). For immunoblotting, total proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories, California, USA) and incubated overnight at 4°C with constant shaking with the specific anti-NPR-A (dilution 1/250) and anti-NPR-C (dilution 1/100) polyclonal primary antibodies (Abcam, Cambridge, UK). Protein loading was normalized using the mouse polyclonal anti-GAPDH diluted to 1/15 000 (Santa Cruz Biotechnology, USA). The immunoblots were subsequently washed and incubated at room temperature and protected from light with the fluorescently labelled donkey anti-rabbit to IgG and goat anti-mouse to IgG, both diluted to 1/20 000 (IRDye800, and IRDye700, Rockland, PA, USA). The membrane was finally washed and the signal detected by scanning using an Odyssey Infrared Imaging System at 800 nm and 700 nm (LI-COR Biosciences, Lincoln, NE, USA). The intensity values of the detected bands were evaluated using Sham as a reference group (Sham = 100%).

Statistical analysis

Group data are presented as means ± SE. Differences between experimental groups were analysed by *two-way* ANOVA followed by the Student–Newman–Keuls test for multiple comparisons. Statistical significance was set at $P < 0.05$.

Results

Morphometry and renal function

Renal mass ablation had no effects on body growth, as ¾nx rats attained the same weight at 26 weeks as Sham rats (Table 1). This was accompanied in ¾nx rats by a progressive increase in the remnant renal mass throughout the study; 26 weeks after the surgery the remnant kidney from ¾nx rats weighted 257 ± 15% more than on the day of the surgery (Table 1). Ablation of renal mass in ¾nx rats resulted in a significant and progressive increase in the heart weight from 2 to 26 weeks after the surgery (Table 1). This contrasted with that observed in Sham rats in which the heart weight increased from 2 to 10 weeks after surgery but not from 10 to 26 weeks after surgery (Table 1). As a result, 26 weeks after surgery, the heart weight of the ¾nx group was significantly higher than that of the Sham group (Table 1).

The ¾nx rats presented significant increases in plasma creatinine values throughout the study, this being accompanied by 50–60% reductions in the creatinine clearance values in comparison with Sham rats (Table 1). No progressive changes were observed between Sham and ¾nx rats in daily urinary excretion of sodium throughout the study. This resulted in that the fractional excretion of sodium in ¾nx rats was consistently increased by ∼2.5-fold throughout the study.

Ablation of renal mass in ¾nx rats resulted in a time-dependent increase of both renal fibrosis and tubular atrophy from 2 to 26 weeks after surgery (Figure 2). This contrasted with the changes observed in Sham rats in which renal fibrosis and tubular atrophy only slightly increased from 10 to 26 weeks after surgery (Figure 2). Therefore, both renal fibrosis and tubular atrophy were increased much more in ¾nx rats than in Sham animals at 10 and 26 weeks after surgery (Figure 2).

Natriuretic response to VE

Two weeks after the surgery the natriuretic response to VE was similar between ¾nx and Sham rats. In contrast, 10 weeks after the surgery the natriuretic response to VE was decreased in ¾nx rats when compared with Sham rats at VER-1 ($t = 160$ min). Moreover, 26 weeks after the surgery the natriuretic response to VE was decreased in ¾nx rats, both at VER-1 ($t = 160$ min) and VER-2 ($t = 170$ min) (Figure 1).

BP and cardiac haemodynamics

Ablation of renal mass in ¾nx rats resulted in a significant and time-dependent increase in both systolic and diastolic BPs from 2 to 26 weeks after surgery. In addition, at 26 weeks after surgery both systolic and diastolic BPs were

Table 1. Morphometry, renal function, blood pressure and heart rate in sham-operated (Sham) and $\frac{3}{4}$ nephrectomized ($\frac{3}{4}$nx) rats 2, 10 and 26 weeks after surgery

	2 weeks		10 weeks		26 weeks	
	Sham	$\frac{3}{4}$nx	Sham	$\frac{3}{4}$nx	Sham	$\frac{3}{4}$nx
Body wt (g)	255 ± 5	247 ± 5	$417 \pm 6^{\ddagger}$	$409 \pm 15^{\ddagger}$	$486 \pm 14^{\ddagger,\delta}$	$462 \pm 7^{\ddagger,\delta}$
Heart wt (g)	6.74 ± 0.02	0.77 ± 0.02	$1.05 \pm 0.03^{\ddagger}$	$1.11 \pm 0.04^{\ddagger}$	$1.08 \pm 0.06^{\ddagger}$	$1.32 \pm 0.04^{*,\ddagger,\delta}$
Renal mass increase (%)		$99 \pm 10^{\dagger}$		$191 \pm 17^{\ddagger}$		$257 \pm 15^{\ddagger,\delta}$
P_{Creat} (mg dl^{-1})	0.34 ± 0.02	$0.76 \pm 0.04^{*}$	0.31 ± 0.03	$0.69 \pm 0.07^{*}$	$0.55 \pm 0.02^{\ddagger,\delta}$	$1.27 \pm 0.17^{*,\ddagger,\delta}$
C_{Creat} (ml min^{-1} kg body wt^{-1})	8.8 ± 1.0	$3.5 \pm 0.3^{*}$	10.8 ± 1.0	$4.6 \pm 0.7^{*}$	$4.1 \pm 0.3^{\ddagger,\delta}$	$2.0 \pm 0.2^{*,\ddagger,\delta}$
FE_{Na^+} (%)	0.36 ± 0.04	$0.89 \pm 0.06^{*}$	0.23 ± 0.05	$0.67 \pm 0.10^{*}$	0.33 ± 0.04	$0.8 \pm 0.10^{*}$
Systolic BP (mmHg)	123 ± 3	$139 \pm 4^{*}$	128 ± 3	$187 \pm 6^{*,\ddagger}$	$136 \pm 2^{\ddagger,\delta}$	$209 \pm 4^{*\ddagger,\delta}$
Diastolic BP (mmHg)	80 ± 3	$106 \pm 4^{*}$	$92 \pm 2^{\ddagger}$	$142 \pm 5^{*,\ddagger}$	$101 \pm 3^{\ddagger,\delta}$	$156 \pm 4^{*,\ddagger}$
Heart rate (beats min^{-1})	415 ± 12	473 ± 32	340 ± 10	395 ± 11	322 ± 7	387 ± 9

Data are means $\pm$ SE.
Body wt, body weight; Heart wt, heart weight; P_{Creat}, plasma creatinine; C_{Creat}, creatinine clearance; FE_{Na^+}, Na$^+$ fractional excretion; BP, blood pressure.
$^{*}P < 0.05$ versus Sham; $^{\ddagger}P < 0.05$ versus 2 weeks.
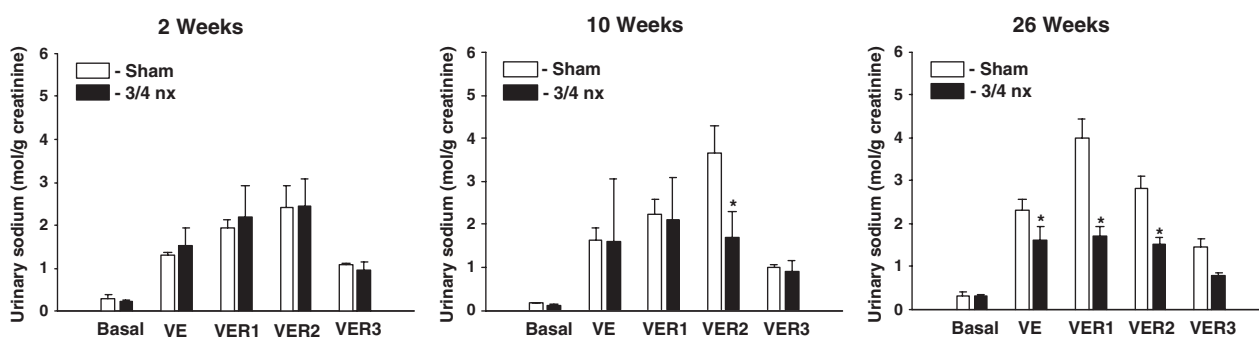
Fig. 1. Urinary sodium excretion of $\frac{3}{4}$ nephrectomized rats ($\frac{3}{4}$nx) before ($t = 0$–120 min, Basal), during ($t = 150$ min, VE) and after ($t = 160$ min, VER-1; $t = 170$ min, VER-2 and $t = 240$ min, VER-3) 5% volume expansion (VE) with isotonic saline at 2, 10 and 26 weeks after surgery. Two weeks after the surgery, no differences were observed in the natriuretic response to VE between $\frac{3}{4}$nx and Sham rats. However, the natriuretic response to VE was markedly decreased in $\frac{3}{4}$nx when compared with Sham rats at VER-1 ($t = 160$ min) 10 weeks after the surgery and both at VER-1 ($t = 160$ min) and VER-2 ($t = 170$ min) 26 weeks after the surgery. Values represent the means of 5–8 experiments per group and are expressed in mol g creatinine^{-1}. $^{*}P < 0.05$ versus Sham; $^{\dagger}P < 0.05$ versus 2 weeks; $^{\ddagger}P < 0.05$ versus 10 weeks.

increased in Sham rats in comparison with those observed at 2 and 10 weeks. Moreover, both systolic and diastolic BPs were significantly higher in $\frac{3}{4}$nx rats than in Sham rats throughout the study (Table 1).

The haemodynamic features of the two experimental groups at 2, 10 and 26 weeks after surgery are summarized in Table 2. Regarding the systolic parameters, no significant differences were detected in the maximum developed pressures (LVP$_{max}$) as well as in the index of contractility (dP/dt_{max}) between the two groups throughout the study. Also, the LV filling pressures estimated by LVEDP and the relaxation time constant τ were similar between Sham and $\frac{3}{4}$nx rats at 2, 10 and 26 weeks after surgery.

BNP circulating levels and BNP myocardial expression

Despite the absence of cardiac dysfunction, a significant elevation of BNP circulating levels was observed at some point during the study in both experimental groups (Table 3). Ablation of renal mass in $\frac{3}{4}$nx rats resulted in a significant and sustained increase in BNP circulating levels at 2 and 10 weeks after surgery with further increase at 26 weeks after renal mass ablation, whereas in Sham rats a

significant increase in BNP circulating levels was observed from 10 to 26 weeks after surgery (Table 3). As a consequence, BNP circulating levels at 26 weeks were similar between the two groups. No significant differences were observed in BNP mRNA expression of LV cells between the two groups.

Expression of NPR-A and -C in the RC and RM

In control rats, basal NPR-A and NPR-C expression differed between the RC and RM: NPR-A mRNA levels were higher in the RM (cortex: 1.0 ± 0.40 AU; medulla: 14.0 ± 3.10 AU), whereas NPR-C mRNA levels were higher in the RC (cortex: 1.0 ± 0.17 AU; medulla: 0.37 ± 0.02 AU) (Figure 3).

A time-dependent increase in NPR-A mRNA levels in the RM was observed in both experimental groups throughout the study (Figure 3). However, the mRNA expression levels of NPR-A in the RM from $\frac{3}{4}$nx rats were significantly lower than those observed in Sham rats, at 2, 10 and 26 weeks after surgery. In agreement with these findings, the protein levels of NPR-A in the remnant RM of $\frac{3}{4}$nx rats were

Table 2. Left ventricular haemodynamics in sham-operated (Sham) and ¾ nephrectomized (¾nx) rats 2, 10 and 26 weeks after surgery.

	2 weeks		10 weeks		26 weeks	
	Sham	¾nx	Sham	¾nx	Sham	¾nx
LVP_{max} (mmHg)	77.4 ± 8.0	71.5 ± 6.4	85.2 ± 15.6	102.3 ± 7.4	87.8 ± 6.8	76.3 ± 9.2
dP/dt_{max} (mmHg/s)	4212 ± 642	4114 ± 335	4716 ± 1062	5823 ± 405	4608 ± 458	3635 ± 568
dP/dt_{min} (mmHg/s)	-2259 ± 309	-2205 ± 172	-2669 ± 673	-3282 ± 391	-2582 ± 297	-2117 ± 340
LVEDP (mmHg)	2.9 ± 0.9	3.2 ± 1.3	2.3 ± 0.5	3.9 ± 0.4	3.7 ± 0.7	3.1 ± 0.5
τ (ms)	20 ± 2	17 ± 2	21 ± 2	21 ± 2	21 ± 1	22 ± 1

Data are means $\pm$ SE.
LVP_{max}, LV peak systolic pressure; dP/dt_{max} and dP/dt_{min}, peak rates of ventricular pressure rise and fall, respectively; LVEDP, LV end-diastolic pressure; τ, time constant of isovolumetric relaxation.
No significant differences were detected in the studied haemodynamic parameters between groups at 2, 10 and 26 weeks.
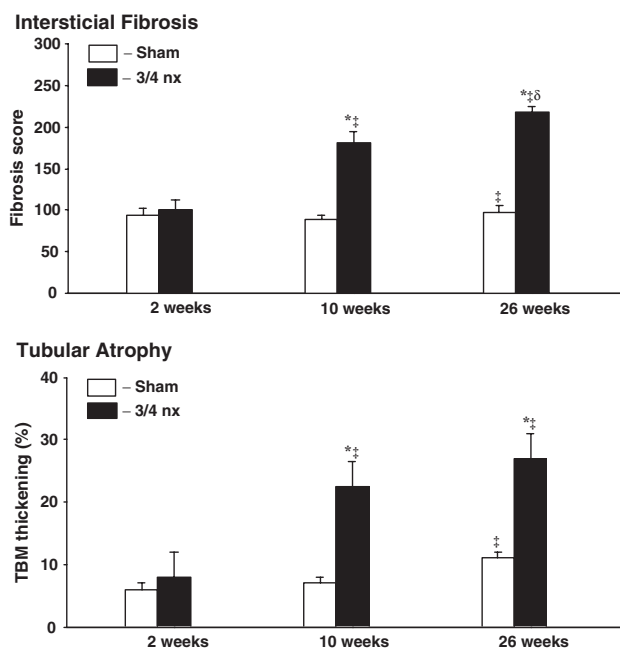
Fig. 2. Interstitial fibrosis and tubular atrophy in sham-operated (Sham) and ¾ nephrectomized (¾nx) rats 2, 10 and 26 weeks after surgery. Renal mass ablation was associated with progressive renal fibrosis at 10 and 26 weeks, as detected by increased collagen staining (Masson's trichrome) and tubular basement membrane thickening (periodic acid Schiff). Increased tubular basement membrane thickness was also observed in the Sham group at 26 weeks. $^*P < 0.05$ versus Sham; $^\ddagger P < 0.05$ versus 2 weeks; $^\delta P < 0.05$ versus 10 weeks.

Table 3. Plasma levels of BNP in sham-operated (Sham) and ¾ nephrectomized (¾nx) rats 2, 10 and 26 weeks after surgery

	2 weeks	10 weeks	26 weeks
Sham	1.35 ± 0.39	2.85 ± 0.67	$4.43 \pm 0.32^{*\ddagger}$
¾nx	$2.95 \pm 0.81^*$	$3.86 \pm 0.43^*$	$4.58 \pm 0.20^{*\ddagger}$

Data are mean $\pm$ SE in pg/reaction.
$^*P < 0.05$ versus Sham 2 weeks; $^\ddagger P < 0.05$ versus 2 weeks.

significantly lower than those observed in Sham animals at 26 weeks (Figure 4).

The expression levels of NPR-A in the RC from ¾nx rats increased throughout the study, whereas in Sham animals the expression levels of NPR-A in the RC only slightly increased at 26 weeks after surgery (Figure 3). NPR-A mRNA levels in the RC from ¾nx rats were, therefore, higher than those observed in Sham, both at 10 and 26 weeks after surgery. In contrast, at 26 weeks, the protein levels of NPR-A in the remnant RC of ¾nx rats were significantly lower than those observed in Sham animals (Figure 4).

The expression levels of NPR-C in the RC from Sham rats did not change throughout the study. In contrast, NPR-C mRNA levels in the remnant RC from ¾nx rats were higher at 10 and 26 weeks after surgery than at 2 weeks. This was accompanied at 10 and 26 weeks after surgery by higher NPR-C mRNA levels in the RC of ¾nx rats than in Sham rats (Figure 3). Accordingly, at 26 weeks the NPR-C protein levels were significantly higher in the RC of ¾nx rats than in Sham animals (Figure 5).

The expression levels of NPR-C in the RM did not change overtime in either ¾nx or Sham animals. NPR-C protein levels in the RM were only faintly detected by western blotting in both experimental groups.

NPR-A was detected by immunohistochemical staining in the RC and RM from both Sham and ¾nx rats. In agreement with the findings of decreased NPR-A protein levels in the RC and RM of ¾nx rats by western blotting, NPR-A immunodetection at 26 weeks was lower in ¾nx rats than in Sham, in both RC and RM (Figure 6).

Discussion

In the present study, BP elevation and compromised natriuretic response to VE in ¾nx rats was associated with a precocious and time-dependent increase in circulating BNP levels, in the absence of cardiac dysfunction. This was accompanied by an early, selective and sustained impaired expression of NPR-A in the RM along with an upregulation of NPR-C in the RC. Taken together, our results demonstrate a distinct modulation of NPRs in the remnant kidney that could represent a novel mechanism for NP resistance in CRI.

A distinct expression of NPR-A and NPR-C in the RC and RM was observed in Sham rats. Two weeks after the surgery, NPR-A mRNA levels in the RM were 14-fold higher than in the RC whereas the NPR-C expression in the RC was 3-fold higher than in the RM. Our
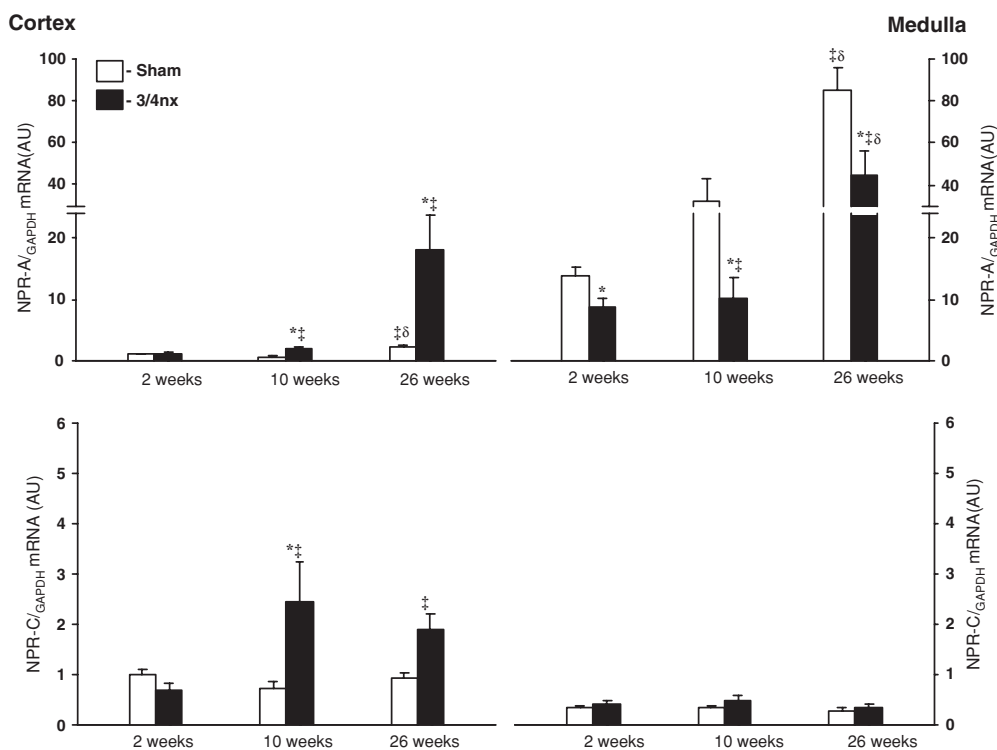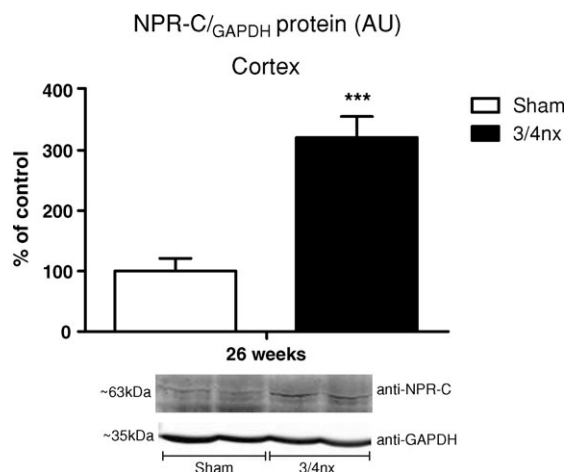
Cortex

Medulla

Fig. 3. mRNA quantification by RT-PCR of RPN-A and RPN-C in the renal cortex (RC) and medulla (RM) of sham-operated (Sham) and ¾ nephrectomized (¾nx) rats 2, 10 and 26 weeks after surgery. Basal NPR-A and NPR-C expression differed in the RC and RM, with higher mRNA levels of NPR-A detected in the RM and higher NPR-C basal expression in the RC of Sham animals. Renal mass ablation was associated with reduced expression of NPR-A in the medulla at 2, 10 and 26 weeks after surgery, with no significant differences in NPR-C expression. Differently, both NPR-A and NPR-C were upregulated in the RC in the ¾nx group. At 26 weeks, an upregulation of NPR-A expression was also evident in the RM of the Sham animals. $^{*}P < 0.05$ versus Sham; $^{\ddagger}P < 0.05$ versus 2 weeks; $^{\delta}P < 0.05$ versus 10 weeks.

NPR-A/GAPDH protein (AU)
Medulla

NPR-A/GAPDH protein (AU)
Cortex
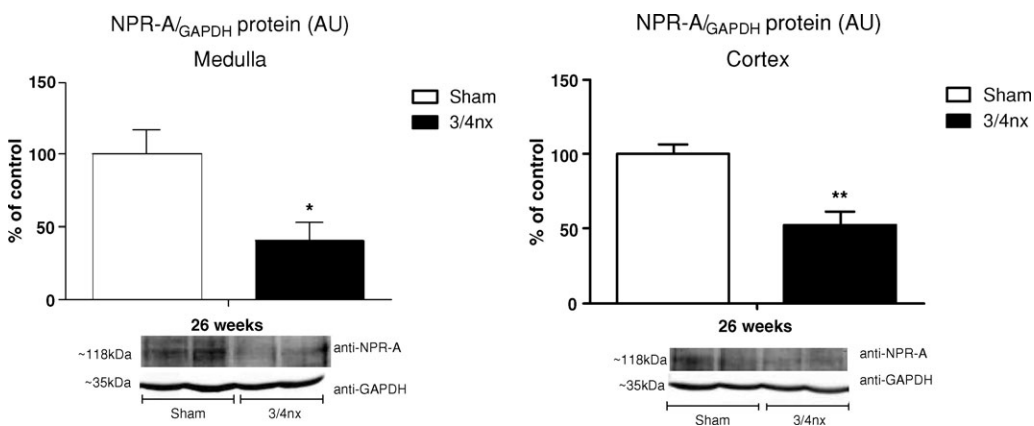
Fig. 4. NPR-A protein levels evaluated by western blotting in renal medulla (RM) and renal cortex (RC) of sham-operated (Sham) and ¾ nephrectomized (¾nx) rats 26 weeks after surgery. At 26 weeks, significantly lower protein levels of NPR-A were observed in the RM and RC of ¾nx when compared to Sham animals. Results shown as the percentage of control (Sham = 100%). $^{*}P < 0.05$ versus Sham. Bottom: representative immunoblots of NPR-A and GAPDH in the RM and RC of Sham and ¾nx rats 26 weeks after surgery (118 and 35 kDa bands, respectively).

findings are in agreement with those from previous studies performed in normal kidneys showing that the expression of NPR-A predominates in the medullary collecting duct, whereas the expression of NPR-C predominates in the cortical structures [29]. The increased expression of NPR-A in the RM fits well with the notion that the natriuresis induced by NP is mainly related to the tubular effects of these peptides [30]. On the other hand, the in-

creased expression of NPR-C in the RC suggest that this clearance receptor may play an important role in the modulation of the concentrations of NP available to interact with NPR-A in both cortical and medullar structures. This would be also in agreement with the view that the concentration of NP downstream of the glomeruli may be significantly influenced by the expression of NPR-C in the RC [31].

Fig. 5. NPR-C protein levels evaluated by western blotting in renal cortex (RC) of sham-operated (Sham) and ¾ nephrectomized (¾nx) rats 26 weeks after surgery. At 26 weeks, significantly higher protein levels of NPR-C were observed in the RC of ¾nx when compared to Sham animals. Results shown as the percentage of control (Sham = 100%). *$P < 0.05$ versus Sham. Bottom: representative immunoblots of NPR-C and GAPDH in RC of Sham and ¾nx rats 26 weeks after surgery (63 and 35 kDa bands, respectively).

Twenty-six weeks after the surgery, Sham rats presented an increase in BP going along with a decrease in GFR. This was accompanied by an increase in BNP circulating levels, in the absence of cardiac dysfunction. An elevation of BNP circulating levels was previously described in normal aged subjects in the absence of cardiac dysfunction and this was attributed to a decrease in the renal clearance of NP [32,33]. Thus, our findings agree well with the suggestion that both age and renal function should be taken in consideration when BNP circulating levels are evaluated [34,35]. However, we did not compare BNP and NT-proBNP circulating levels, which could have contributed to clarify the mechanisms involved in decreased BNP degradation/excretion observed in our study and to evaluate the role of impaired neutral endopeptidase activity as a possible mechanism for increased BNP circulating levels in decreased GFR.

A time-dependent increase in NPR-A mRNA levels was observed in the RM of Sham rats. This was accompanied by a slight increase in NPR-A mRNA levels in the RC at 26 weeks in the absence of changes in both cortical and medullar NPR-C expression overtime. Taken together our findings in Sham rats may suggest an important role of the NP system in the control of sodium balance that accompanies the increase of BP associated with ageing. Interestingly, previous human studies reported an increase in the urinary cGMP levels in essential hypertensives suggesting that they may be a marker of higher than normal tubular actions of NP [36].

Renal mass ablation in ¾nx rats was accompanied by an early and sustained increase in BNP circulating levels that was not related to an augmented myocardial production of BNP, as evidenced by the normal expression of BNP mRNA levels in ventricular cells as well as by the absence of signs of cardiac dysfunction. These results are in agreement with recent observations suggesting that an impaired renal clearance of NP could be an important determinant of BNP
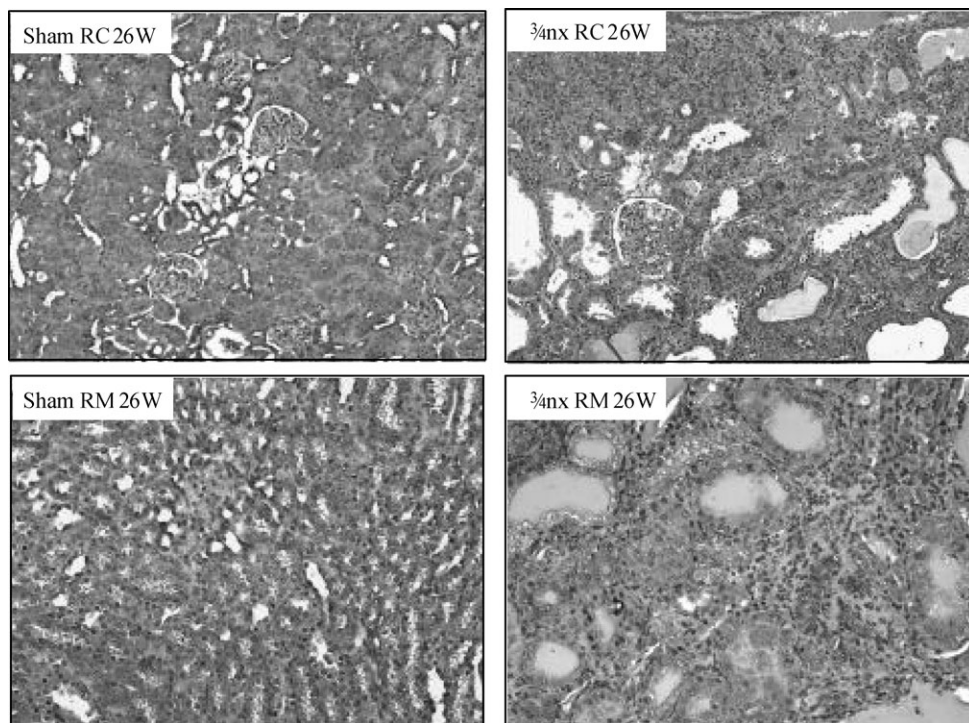
Fig. 6. Immunolocalization of NPR-A in renal medulla of sham-operated (Sham) and ¾ nephrectomized (¾nx) rats 26 weeks after surgery. NPR-A was detected by immunohistochemical staining in the tubular structures of the renal cortex (RC) and medulla (RM) from both Sham and ¾nx rats. At 26 weeks after surgery, NPR-A immunodetection in both RC and RM was lower in ¾nx rats than in Sham animals. Amplification used: 100× in the RC and 200× in the RM.

circulating levels in moderate-to-severe CRI [37,38]. In fact, ¾nx animals developed a time-dependent dramatic increase in BP and also hypervolaemia. Both hypertension and hypervolaemia result in increased plasma BNP levels independent of cardiac dysfunction. In addition, any moderate or particularly severe reduction in glomerular filtration rate results in an increase in BNP even in the absence of cardiac dysfunction. However, this was a short-term study, and cardiac dysfunction may develop in the long term also in these animals as a result of severe hypertension and/or hypervolaemia.

Despite the increase in BNP circulating levels, the ¾nx rats presented a time-dependent increase in BP and responded to VE with a blunted natriuretic response. Taken together, these findings agree well with the suggestion that the failing kidney may be hyporesponsive to NP actions [20,21]. In our study, renal mass ablation had a profound impact on NPR expression, in both the RC and RM. An early and sustained decrease in NPR-A expression in the RM of ¾nx rats was observed, and this was accompanied by a time-dependent increase in the expression of RPN-C in the RC. This later finding does not seem to be dependent on increased circulating BNP levels, given that at 26 weeks circulating BNP levels were similar between Sham and ¾nx groups and NPR-C was not upregulated in Sham. Since NPR-C in the kidney was suggested to decrease the local concentration of delivered NP [31] in a dose-dependent manner, one can speculate that the increase in NPR-C expression in the RC of ¾nx rats along with the decrease in NPR-A expression in the remnant RM of ¾nx rats may both contribute to an attenuated renal response to NP in CRI. Despite the increase in NPR-A mRNA levels observed in the RC of ¾nx rats at 26 weeks after the surgery, the protein levels of NPR-A were decreased in the RC of ¾nx rats at this time period. Although one cannot go ahead with an explanation for this apparent discrepancy, the findings of reduced NPR-A protein levels in the RC of ¾nx rats further reinforce the view of markedly attenuated effects of NP in the remnant kidney. Interestingly, patients with pulmonary hypertension were found to present increased circulating BNP levels and renal hyporesponsiveness to NP that was suggested to be located upstream to cGMP generation in renal tubules [39].

In ¾nx rats, the disturbances in sodium balance observed were accompanied by a progressive increase in tubular atrophy and interstitial fibrosis. Previous studies in animal models of immune glomerular injury have reported several antifibrotic and antiproliferative effects of the NP system [10]. Thus, one can hypothesize that the renal hyporesponsiveness to NP actions suggested by the local changes in NPR-A and NPR-C expression in the RC and RM of ¾nx rats may operate as a contributing factor to the tubular and interstitial changes observed in these animals as well as to progressive loss of renal function. However, the exact role of the NP system in the regulation of the fibrotic response in CRI progression remains to be elucidated.

In conclusion, BP elevation and compromised natriuretic response to VE in ¾nx rats was associated with increased circulating BNP levels in the absence of cardiac dysfunction. This was accompanied in ¾nx rats by both impaired NPR-A expression in the RM and upregulation of NPR-C in the RC suggesting a novel mechanism for NP resistance in CRI.

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Protein kinase C-β inhibition attenuates the progression of nephropathy in non-diabetic kidney disease

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Abstract

Background. Activation of protein kinase C (PKC) has been implicated in the pathogenesis of diabetic nephropathy where therapy targeting the β isoform of this enzyme is in advanced clinical development. However, PKC-β is also increased in various forms of human glomerulonephritis with several potentially nephrotoxic factors, other than high glucose, resulting in PKC-β activation. Accordingly, we sought to examine the effects of PKC-β inhibition in a non-diabetic model of progressive kidney disease.

Methods. Subtotally nephrectomized (STNx) rats were randomly assigned to receive either the selective PKC-β inhibitor, ruboxistaurin or vehicle. In addition to functional and structural parameters, gene expression of the podocyte slit-pore diaphragm protein, nephrin, was also assessed.

Results. STNx animals developed hypertension, proteinuria and reduced glomerular filtration rate (GFR) in association with marked glomerulosclerosis and tubulointerstitial fibrosis. Glomerular nephrin expression was also reduced. Without affecting blood pressure, ruboxistaurin treatment attenuated the impairment in GFR and reduced the extent of both glomerulosclerosis and tubulointerstitial fibrosis in STNx rats. In contrast, neither proteinuria nor the reduction in nephrin expression was improved by ruboxistaurin.