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Natriuretic peptide system modulation in uninephrectomized rats

Original article

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ABSTRACT

BACKGROUND: Cardiac activation of the natriuretic peptide (NP) system plays a major role in the renal adaptations to volume expansion (VE). However, its role after uninephrectomy (Unx) remains to be elucidated. In the present study, we evaluated cardiac hemodynamics, plasma NP-Type B (BNP) levels and NP receptor A (NPR-A) and C (NPR-C) expression in the renal cortex (RC) and medulla (RM) of Sham and Unx rats. METHODS: Male Wistar-Han rats (n=42) were randomly assigned to Unx or Sham surgery. Non-invasive blood pressure (BP), invasive left ventricular (LV) hemodynamics and natriuretic response to VE were performed and plasma was collected for BNP determination, 2, 10 and 26 weeks after surgery. In addition, samples from RC and RM were obtained for NPR-A and -C quantification (RT-PCR and western-blot). RESULTS: In Unx, blood pressure elevation and reduced natriuretic response to VE were observed. Although plasma BNP levels increased overtime, no LV dysfunction or myocardial BNP gene activation was observed in Unx. NPR-A mRNA in RC and RM were significantly increased in Unx at 26 weeks. This was accompanied by decreased NPR-C mRNA in the RM in Unx at 10 and 26 weeks, with no differences detected in the RC. CONCLUSIONS: In our experimental model of Unx, progressive BP elevation and reduced natriuretic response to VE were accompanied by modulation of NPR-A and -C expression, in both the RC and RM, in the absence of cardiac activation of the NP system. Our results suggest a local renal modulation of the NP system after Unx.

Key words: BNP; uninephrectomy; hypertension; natriuretic peptide receptor.

Introduction

Natriuretic peptides (NP) are a family of signaling molecules that play a major role in the maintenance of sodium and body volume homeostasis and in the modulation of the proliferative and fibrotic response [1,2]. B-type natriuretic peptide (BNP) synthesis occurs mainly in the ventricles in response to pressure and volume overload and exerts most of its cellular effects through the activation of the transmembrane guanylyl cyclase, natriuretic peptide receptor-A (NPR-A) [3]. Another natriuretic peptide receptor, natriuretic peptide receptor-C (NPR-C), is devoid of guanyl cyclase activity and is responsible for NP internalization and degradation [4].

Increased circulating levels of NP can be observed in congestive heart failure and is generally interpreted as an expression of the activation of the NP system. Previous evidence from a number of studies have suggested that plasmatic levels of NP are regulated both by the rate of synthesis/cardiac release of NP and by the rate of removal of the peptides from the circulation [5,6]. Additionally, as we have previously demonstrated in a rat model of chronic renal failure, modulation of target organ receptor expression may be determinant for the local bioavailability of NP and, by that mechanism, play an important role in the regional control of the NP system activity [7].

The removal of a single kidney immediately stimulates the growth and function of the remnant renal mass. This acute compensatory response is recognized during the first days after unilateral nephrectomy (Unx) and is characterized by an increase in electrolyte excretion, a mild decrease in cardiac output and a transient rise in blood pressure [8]. Some weeks later, a time-dependent increase in both systolic and diastolic blood pressure was observed, together with a sustained reduction in the natriuretic response to volume expansion, suggesting that the relative role of the natriuretic systems in the control of sodium balance may differ overtime [9]. Atrial natriuretic peptide has been previously implicated as a possible mediator of the acute renal response to contralateral renal ablation [10]. However, the role of BNP, the selective renal modulation of both the effector and clearance NPR and, more importantly, the time course of these changes after Unx were not evaluated so far.

The aim of the present study was to evaluate the activation of the NP system after Unx and to examine whether this is accompanied by changes in the expression of renal NPR. For this purpose, we evaluated cardiac hemodynamics, blood pressure, renal function, circulating BNP levels, the natriuretic response to volume expansion and the expression of both NPR-A and NPR-C in the renal cortex and medulla, from Unx and Sham rats, up to 26 weeks after surgery.

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 (European directive number 63/2010/EU, transposed to the Portuguese law by directive law 113/2013).

Normotensive male Wistar-Han rats (Harlan, Barcelona, Spain; 190-220g) were kept under controlled environmental conditions (12:12h light/dark cycle and room temperature 22±2ºC) and were fed *ad libitum* throughout the study with ordinary rat chow (Panlab, Barcelona, Spain) containing 1.9 $g.Kg^{-1}$ of NaCl. After a 7-day period of stabilization and adaptation, the animals were randomly submitted to right kidney nephrectomy (uninephrectomized - Unx), as described previously [11]. Control animals were rats submitted to sham surgery under similar conditions, however their kidneys remained intact (sham operated - Sham). After total recovery from surgery (4-6 hrs), the rats were placed in an animal facility, where they had free access to food and water. Survival rate was 100%. Metabolic, hemodynamic, morphometric, histological and molecular studies were performed at 2 (n=12), 10 (n=13) 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 hours urine was collected to calculate creatinine clearance (C_{Creat}, $ml.min^{-1}.kg\ body\ wt^{-1}$) and fractional excretion of sodium (FE_{Na+}, %).

Hemodynamics in awake animals

Blood pressure (systolic and diastolic) was 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.

Hemodynamic studies in anesthetized animals

At 2, 10 and 26 weeks the animals were anesthetized with pentobarbital (6 mg/100g, ip), 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. Anesthesia was maintained with additional bolus of pentobarbital (2mg/100g) 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, Texas, USA) inserted into the left ventricle (LV). After complete instrumentation, the animal preparation was allowed to stabilize for few minutes. Hemodynamic recordings were made with respiration suspended at end-expiration. Parameters were converted online to digital data with a sampling frequency of 1000 Hz. LV pressures were measured at end-diastole and peak-systole. Peak rates of LV pressure rise (dP/dt_{max}) and pressure fall (dP/dt_{min}) were also measured. Relaxation rate was estimated with the time constant tau (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, renal cortex (RC) and renal medulla (RM) were snap frozen in liquid nitrogen and stored (-70ºC) for mRNA quantification and semi-quantification of protein levels by Western-blotting. In addition, renal tissue samples were paraffin-embedded and formalin-fixed for histology.

Volume expansion

In another set of experiments, 2, 10 and 26 weeks after the surgery, the animals were anesthetized with pentobarbital sodium (60 $mg.kg^{-1}$ followed by $20mg.kg^{-1}.h^{-1}$; 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 volume expansion (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 the stabilization period, the VE was started by infusion of isotonic saline (0.9%) at a rate of 100 $ml.kg^{-1}.h^{-1}$ for 30 min (5% body weight); during this phase, a 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, RVE-1; t=170 min, RVE-2 and = 240 min, RVE-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, California). Briefly, in each reaction, rabbit anti-peptide antibody was incubated (16-24h at 4ºC) with sample or standard and mixed with a fixed amount of ^{125}I-peptide. After a second incubation period (16-24h at 4ºC), goat anti-rabbit serum and normal rabbit serum were added and incubated for 90 minutes. The reaction tubes were subsequently centrifuged and the pellet was 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 BNP concentration of each sample was calculated. Results are expressed as pg/tube.

Renal Histology

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 hematoxylin (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 twelve randomly selected fields (x200 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 described previously [23]. 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 methabissulphite (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.

mRNA Relative Quantification by Real-Time 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. Concentration and purity were assayed by spectrophotometry (Eppendorf 6131000.012, Hamburg, Germany).

Real-time RT-PCR: Two-step real-time 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 real-time RT-PCR. A melt curve analysis of each real-time 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 internal control gene. Results of mRNA quantification were expressed in an arbitrary unit (AU) set as the average value of the sham group (sham=1 AU), after normalization for GAPDH.

RT (20μl; 10 min at 22ºC, 50 min at 50ºC and 10 min at 95ºC) 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 real-time 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 Western-blotting

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 95ºC for 5 min and separated by SDS-PAGE in 7.5% poly-acrylamide gel (80µL of sample per well). For immunoblotting, total proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) and incubated overnight at 4ºC 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 1/15000 (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 1/20000 (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 700nm (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 were presented as means ± SE. Differences between experimental groups were analyzed using *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 Unx rats attained the same weight at 26 weeks as Sham rats (table 1). This was accompanied in Unx rats by a significant increase in the remnant renal mass: 10 and 26 weeks after the surgery the remnant kidney from Unx rats weighted 83±12% and 84±4% more respectively, than on the day of the surgery (table 1). No significant differences were observed in the heart weight between Unx and Sham groups at 2, 10 and 26 weeks after surgery (table 1).

Plasma creatinine values were significantly increased in Unx in comparison with Sham at 2, 10 and 26 weeks after surgery (table 1). Creatinine clearance values were significantly decreased in Unx in comparison with Sham at 2, 10 weeks and 26 weeks (table 1). No significant differences were observed between Sham and Unx in daily urinary excretion of sodium throughout the study. This result shows that fractional excretion of sodium was greater in Unx animals than in Sham at 2, 10 and 26 weeks (table 1).

Table 1

No significant differences were observed in both interstitial fibrosis and tubular atrophy between Unx and Sham at 2 weeks. By contrast, interstitial fibrosis and tubular atrophy were greater in Unx than in Sham group both at 10 and at 26 weeks after surgery (figure 1).

(Figure 1)

Natriuretic response to volume expansion

Two weeks after surgery the natriuretic response to VE was similar between Unx and Sham groups. By contrast, at 10 and 26 weeks after surgery the natriuretic response to VE decreased in Unx in comparison with Sham (figure 2).

(Figure 2)

Blood pressure and cardiac hemodynamics

No significant differences were observed between Sham and Unx at 2 weeks in both systolic and diastolic blood pressure (BP). By contrast, systolic and diastolic BP was higher in Unx rats than in Sham at 10 and 26 weeks (table 1).
The hemodynamic features of the two experimental groups at 2, 10 and 26 weeks after

surgery were summarized in table 2. As can be observed, both the systolic and diastolic parameters evaluated were similar between Sham and Unx throughout the study.

BNP circulating levels and BNP myocardial expression

No significant differences were observed in BNP mRNA expression of LV cells between the Unx and Sham groups at 2, 10 and 26 weeks after surgery. In addition, BNP mRNA expression of LV cells did not change significantly overtime in both experimental groups (table 3). By contrast, BNP circulating levels at 2 and 10 weeks were higher in Unx than in Sham, whereas BNP

circulating levels at 26 weeks were similar in both groups (table 4). An increase in BNP circulating levels was documented throughout the study in both Sham and Unx, with higher BNP circulating levels observed in both groups at 26 weeks when compared to 2 weeks after surgery.

Table 2

Table 3

Table 4

Expression of NPR-A and –C in the renal cortex and medulla

At 2 weeks, the expression of both NPR-A and NPR-C differed between the RC and RM in both experimental groups. The NPR-A mRNA levels were higher in the RM whereas the NPR-C mRNA levels were higher in the RC (figure 3). In addition, no significant differences were observed at 2 weeks between Unx and Sham groups in the expression of either NPR-A or NPR-C in both the RC and RM.
The expression levels of NPR-A in the RM at 10 weeks were non-significantly increased in Sham and were significantly reduced in Unx (figure 3).

Figure 3

On the other hand, a marked increase in NPR-A mRNA levels were observed at 26 weeks in both experimental groups and no significant differences were observed between the Sham and Unx at 26 weeks in the expression of NPR-A in the RM (figure 3). Accordingly, at 26 weeks the protein levels of NPR-A in the remnant RM from Unx were similar to those observed in Sham (figure 4).

Figure 4

The NPR-A mRNA levels in the RC did not differ between the two experimental groups at 10 and 26 weeks (figure 3). In addition, the expression levels of NPR-A in the RC from Unx did not change overtime (figure 3). However, a slight increase in NPR-A mRNA levels in the RC was observed in Sham at 26 weeks (figure 3).
The expression of NPR-C in the RC did not change throughout the study in both groups. In addition, no significant differences were observed between Sham and Unx overtime (figure 3). Accordingly, at 26 weeks the protein levels of NPR-C in the RC of Unx were similar to those observed in Sham (figure 5).

Figure 5

The expression of NPR-C in the RM did not change throughout the study in Sham whereas a significant decrease in NPR-C mRNA levels was observed in the RM from Unx at 10 and 26 weeks (figure 3). Thus, this result showed that NPR-C mRNA levels in the RM from Unx were lower than those observed in Sham at 10 and 26 weeks (figure 3). NPR-C protein levels in the RM were only faintly detected by Western-blotting in both experimental groups.

Discussion

In the present study, blood pressure elevation and a compromised natriuretic response to VE in Unx rats were associated with a precocious and time-dependent increase in circulating BNP levels, in the absence of cardiac dysfunction. This was accompanied in Unx rats by a late increased expression of NPR-A along with a down-regulation of NPR-C in the RM. Taken together, our results suggest a distinct modulation of NP receptors in the remnant kidney after unilateral renal ablation that seems to occur independently from the cardiac activation of the NP system.

In our Unx model, a precocious and progressive compensatory growth of the remnant renal mass was observed, complemented by an initial transient increase in urinary sodium excretion in response to isotonic saline VE. Unlike the findings observed 2 weeks after Unx, 10 and 26 weeks after renal mass ablation a time-dependent increase in both systolic and diastolic BP was accompanied by a decreased natriuretic

response to isotonic saline VE. In accordance to what was observed in our study, Unx for living kidney donation is associated with a rapid functional adaptation response of the remnant renal mass, characterized by a profound compensatory growth of the remaining kidney and by a significant increase in the fractional excretion of sodium [12,13]. Moreover, an increase incidence of hypertension and a compromised natriuretic response to VE have also been reported following kidney donation on several studies [14,15]. Interestingly, a blunted renal dopaminergic response was previously documented several weeks after unilateral renal mass ablation, implicating this neuro-humoral system in the derangement of sodium homeostasis and in the increase in blood pressure observed after Unx [16]. Despite this, the factors contributing to the renal sodium handling and to blood pressure regulation following nephrectomy have not been systematically evaluated and the long-term importance of the different natriuretic systems in this condition is still undefined.

In congestive heart failure, NP secretion from ventricular myocytes were increased in direct proportion to the degree of cardiac dysfunction, in an attempt to overcome the abnormal sodium and water retention frequently observed in more advances states of this disease [17]. In this setting, the elevation of the NP plasmatic levels was generally interpreted as an expression of the activation of the NP system and was used during the time course of the disease to monitor the response of the patient to treatment [18]. In our study, however, renal mass ablation in Unx was accompanied by an early and sustained increase in plasma BNP which was not related to an augmented myocardial production of BNP, as evidenced by the similar ventricular expression of BNP mRNA between Sham and Unx rats as well as by the absence of signs of cardiac dysfunction in both groups. Previous studies have identified several stimulating factors of NP system not directly related to cardiac dysfunction. In fact, both hypertension and hypervolemia were formerly implicated in the elevation of the plasmatic levels of BNP independently from myocardial function [19,20]. In accordance to this, in our study Unx animals developed a time-dependent increase in blood pressure and a compromised natriuretic response to sodium challenge that was paralleled by the increase in NP plasmatic levels. Also, supporting this concept is the fact that plasmatic levels of BNP similar to those documented in Unx animals at 26 weeks were also observed in the Sham group at the end of the study, alongside with the development of elevated BP.

A distinct basal expression of NPR-A and NPR-C in the RC and RM were 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. In accordance to this, previous studies in normal kidneys have documented a significant expression of NPR-A in the RM, reflecting the predominant role of NP in the modulation of the distal tubular transport of sodium [21]. The differential expression of NPR-C in the RC and RM is barely documented. NPR-C is a NP receptor subtype that does not have guanylyl cyclase activity and is responsible, among other functions, for the intracellular degradation of NP [22]. Thus, the predominant expression of NPR-C in the RC may contribute to modulate the NP levels that are made available to interact with NPR-A in both the RC and RM, independently from the plasmatic levels of BNP.

In our study, Unx had a significant impact on NPR expression, in both the RC and RM. In the RM of Unx rats, a transient decrease in the expression of NPR-A was observed 10 weeks after the surgery, which was followed by a significant increase in NPR-A mRNA levels 26 weeks after renal mass ablation. Additionally, increased levels of NPR-A were also detected in the RC of Unx at 26 weeks. By contrast, a significant decrease in the expression of NPR-C in the RM was observed in Unx rats both at 10 and 26 weeks without changes in NPR-C levels in the RC. Because the renal actions of NP can be locally determined by the balance between the expression of NPR-A and NPR-C in both the RC and RM, the up-regulation of NPR-A in the RM from Unx rats combined with the down-regulation of NPR-C in the RM from Unx rats provides evidence favoring the view that the renal NP system may behave in a compensatory way in the long-term regulation of extracellular fluid volume and BP after Unx. In support of this view are the previous studies documenting a suppressed natriuretic response to unilateral renal mass ablation in a rat model of diminished NP release obtained by right atrial appendectomy [10] as well as those showing that the blockade of circulating NP by monoclonal antibodies markedly reduced natriuretic response after Unx [23].

We have previously evaluated the expression of NPR's in the remnant RC and RM of a chronic

renal failure rat model induced by ¾ nephrectomy (¾nx) [7]. The ¾nx rat model was characterized by a severe reduction of the glomerular filtration rate along with the development of a time dependent increase of BP and blunted natriuretic response to VE. Similarly to that found after Unx in the present study, in the ¾nx rat model, an elevation of BNP plasmatic levels was observed in the absence of cardiac dysfunction. However, differently from Unx rats, the ¾nx animals presented a delayed up-regulation of NPR-C in the RC going along with a marked down-regulation of NPR-A in the remnant RM, that may have contributed both to a local resistance to the actions of the NP in this rat model of chronic kidney disease. Taken together, our results suggest that the role of the NP system in sodium homeostasis after renal mass ablation may be related with the degree of functional renal mass loss.

In Unx, 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 [2]. In fact, other experimental works have previously shown that NPR-C may not behave exclusively as a clearance receptor but could also elicit physiological functions through the inhibition of adenylyl cyclase signal transduction system, interfering with the cellular mechanisms involved in the regulation of cell growth [24]. Thus, one can hypothesize that the local changes in NPR-C expression in the RM of Unx rats, resulting in a decreased expression of this receptor, may operate as a contributing factor to the compensatory growth observed after nephrectomy as well as to the tubular and interstitial changes documented in these animals during the study. However, the exact role of the NP system in the regulation of the fibrotic response in unilateral renal mass ablation remains to be elucidated.

At the end of the study, Sham rats presented an increase in BP along with a decrease in GFR and some degree of tubular basement membrane thickening, closely mimicking the long-term adaptive response observed in Unx after renal mass ablation. A time-dependent increase in NPR-A mRNA levels was also 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. Despite the fact that the NPR expression was not formerly evaluated in ageing models, previous works have documented an activation of the NP system in normal aged subjects in the absence of cardiac dysfunction, suggesting a potential role for NP system in the adaptive response to maturation and senescence [25].

In view of these findings, we believe that uninephrectomy may impose to the kidney an accelerated form of the functional burden experienced during the process of ageing and stimulate the integrated homeostatic response oriented to the control of renal sodium handling, blood pressure regulation and fibrotic response modulation observed in the progression to senescence. Differently from what was observed in chronic kidney disease rat model, the changes in renal NPR-A and NPR-C expression levels observed after Unx provide evidence for a compensatory role of the NP system after unilateral renal mass ablation.

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Figure Legends

Figure 1. *Interstitial fibrosis and tubular atrophy in sham-operated (Sham) and uninephrectomized (Unx) rats 2, 10 and 26 weeks after surgery*. Renal mass ablation was associated with increased interstitial 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 vs. Sham; ‡ P<0.05 vs. 2 weeks; $^{\delta}$P<0.05 vs. 10 weeks.

Figure 2. *Urinary sodium excretion of uninephrectomized rats (Unx) before (t=0-120 min, Basal), during (t=150 min, volume expansion, VE) and after (t=160 min, RVE1; t=170 min, RVE2 and t=240 min, RVE3) 5% 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 Unx and Sham rats. However, the natriuretic response to VE was markedly decreased in Unx when compared with Sham rats at RVE-2 (*t*=170 min) 10 weeks after the surgery and both at REV-1 (*t*=160 min) and REV-2 (*t*=170 min) 26 weeks after the surgery. Values represent the means of 5 to 8 experiments per group and are expressed in mol.g creatinine^{-1}. *P<0.05 *vs.* Sham; †P<0.05 *vs.* 2 weeks; ‡P<0.05 *vs*. 10 weeks.

Figure 3. *mRNA quantification by Real-Time RT-PCR of RPN-A and RPN-C in the renal cortex (RC) and renal medulla (RM) of sham-operated (Sham) and uninephrectomized (Unx) rats two, ten and twenty-six 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. Uninephrectomy was associated with a transitory reduction in NPR-A expression in the RM at 10 weeks, followed by a significant increase in NPR-A mRNA levels in both the RC and RM of Unx 26 weeks after the surgery. At 26 weeks, an upregulation of NPR-A expression was also evident in the RC and RM of the Sham animals. Differently, NPR-C was downregulated in the RM of Unx group at 10 and 26 weeks after the surgery with no significant differences observed in NPR-C expression in the RC of Unx during the time course of the study. *P<0.05 vs. Sham; ‡P<0.05 vs. 2 weeks; $^{\delta}$P<0.05 vs. 10 weeks.

Figure 4. *NPR-A protein levels evaluated by Western-blotting in renal medulla (RM) of sham-operated (Sham) and uninephrectomized (Unx) rats 26 weeks after surgery.* At 26 weeks, no significant differences were observed in the protein levels of NPR-A in the RM of Unx when compared to Sham animals. Results shown as percentage of control (Sham=100%). Bottom: *Representative immunoblots of NPR-A and GAPDH in the renal medulla of Sham and Unx rats 26 weeks after surgery* (118 and 35 kDa bands, respectively).

Figure 5. *NPR-C protein levels evaluated by Western-blotting in renal cortex (RC) of sham-operated (Sham) and uninephrectomized (Unx) rats 26 weeks after surgery.* At 26 weeks, no significant differences were observed in the protein levels of NPR-C in the RC of Unx when compared to Sham animals. Results shown as percentage of control (Sham=100%). *P<0.05 *vs*. Sham. Bottom: *Representative immunoblots of NPR-C and GAPDH in renal cortex of Sham and Unx rats 26 weeks after surgery* (63 and 35 kDa bands, respectively).

TABLES

Table 1. *Morphometry, renal function, blood pressure and heart rate in sham-operated (Sham) and uninephrectomized (Unx) rats two, ten and twenty-six weeks after surgery.*

	2 weeks		*10 weeks*		*26 weeks*	
	Sham	***Unx***	***Sham***	***Unx***	***Sham***	***Unx***
Body wt, g	256±6	258±4	419±6 ‡	417±6 ‡	472±22 ‡δ	473±13 ‡δ
Heart wt, g	0.65±0.01	0.67±0.02	0.90±0.04 ‡	0.85±0.03 ‡	1.12±0.04 ‡δ	1.06±0.06 ‡δ
Renal mass increase, %		55±4		83±12 ‡		84±4 ‡
P_{Creat} ***, mg.dl*** $^{-1}$	0.33±0.02	0.45±0.01*	0.47±0.07	0.82±0.11*‡	0.54±0.03‡	0.64±0.02*‡
C_{Creat} ***, ml.min*** $^{-1}$	2.20±0.29	1.50±0.10*	2.77±0.56	1.68±0.22*	2.28±0.10	1.63±0.13*
FE_{Na+} ***, %***	0.43±0.03	0.58±0.05*	0.37±0.10	0.78±0.11*	0.28±0.03	0.45±0.06*
Systolic BP, mmHg	123±3	122±2	130±2	148±3*‡	139±3 ‡δ	160±4*‡δ
Diastolic BP, mmHg	80±3	81±3	94±2‡	109±3*‡	103±2 ‡δ	119±4*‡
Heart rate, beats.min $^{-1}$	415±12	404±8	350±8 ‡	380±9*	327±5 ‡δ	364±5*‡

Data are means ± SE; Body wt, body weight; Heart wt, heart weight; P_{Creat}, plasma creatinine; C_{Creat}, creatinine clearance; FE_{Na+}, Na^+ fractional excretion; Systolic BP, systolic blood pressure; Diastolic BP, diastolic blood pressure; *P<0.05 vs. Sham; ‡P<0.05 vs. 2 weeks; $^{\delta}$P<0.05 vs. 10 weeks.

Table 2. *Left ventricular hemodynamics in sham-operated (Sham) and uninephrectomized (Unx) rats two, ten and twenty-six weeks after surgery.*

	2 weeks		***10 weeks***		***26 weeks***	
	Sham	***Unx***	***Sham***	***Unx***	***Sham***	***Unx***
LVP_{max}, mmHg	77.7±9.8	80.6±11.1	83.9±15.4	96.3±11.1	85.6±6.7	96.7±9.8
dP/dt_{max}, mmHg/s	4258±785	4478±561	4645±1046	5738±823	4539±451	4894±629
dP/dt_{min}, mmHg/s	-2262±379	-2432±268	-2629±663	-3251±562	-25843±293	-3343±435
LVEDP, mmHg	2.8±0.7	3.0±1.2	2.2±0.5	3.4±0.7	3.6±0.6	3.9±1.0
τ, ms	21±2	18±1	21±2	19±2	20±1	18±1

Data are means ± 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 hemodynamic parameters between groups at two, ten and twenty-six weeks.

Table 3. *BNP mRNA expression in the left ventricle cells in sham-operated (Sham) and uninephrectomized (Unx) rats two, ten and twenty-six weeks after surgery.*

	2 weeks	***10 weeks***	***26 weeks***
Sham	1.00±0.11	1.21±0.51	3.98±1.37
Unx	1.06±0.16	1.34±0.41	1.36±0.65

Data are mean ± SE in AU. No significant differences were detected in the BNP mRNA levels between groups at two, ten and twenty-six weeks.

Table 4. *Plasma levels of BNP in sham-operated (Sham) and uninephrectomized (Unx) rats two, ten and twenty-six weeks after surgery.*

	2 weeks	***10 weeks***	***26 weeks***
Sham	1.44±0.43	2.85±0.75	4.51±0.37‡
Unx	2.51±0.20*	3.44±0.79*	4.30±0.20‡

Data are mean ± SE in pg/reaction. *P<.05 vs Sham; ‡P<0.05 vs. 2 weeks.

FIGURE 1

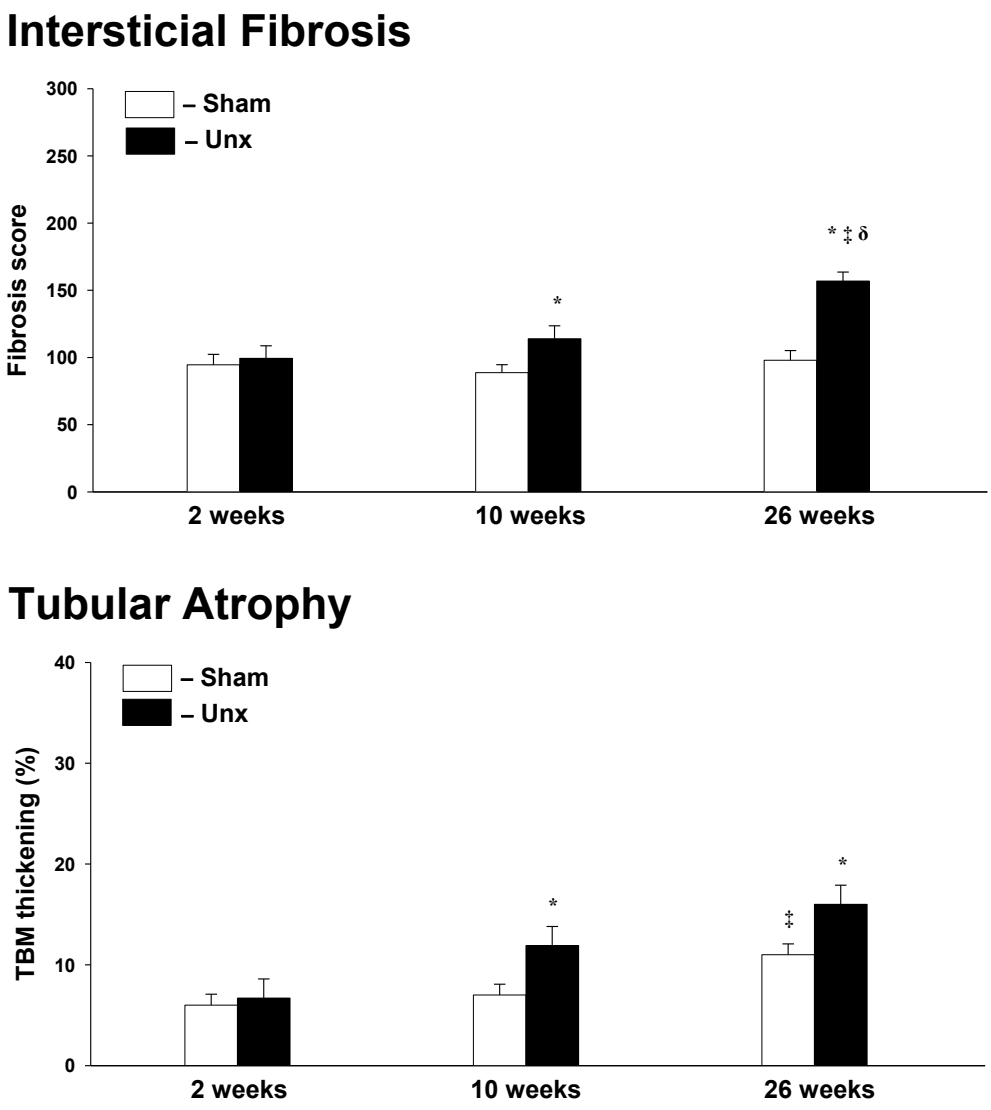

Intersticial Fibrosis
– Sham
– Unx
Fibrosis score
300
250
200
150
100
50
0
*
* ‡ δ
2 weeks
10 weeks
26 weeks
Tubular Atrophy
– Sham
– Unx
TBM thickening (%)
40
30
20
10
0
*
‡
*
2 weeks
10 weeks
26 weeks

FIGURE 2

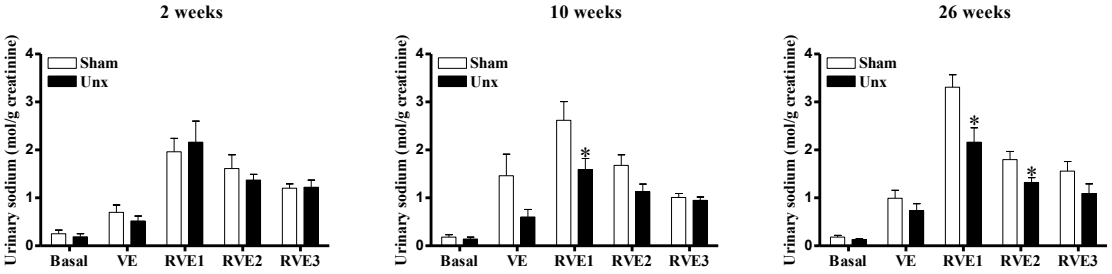

2 weeks
10 weeks
26 weeks
Sham
Unx
Urinary sodium (mol/g creatinine)
0
1
2
3
4
Basal
VE
RVE1
RVE2
RVE3
*

FIGURE 3

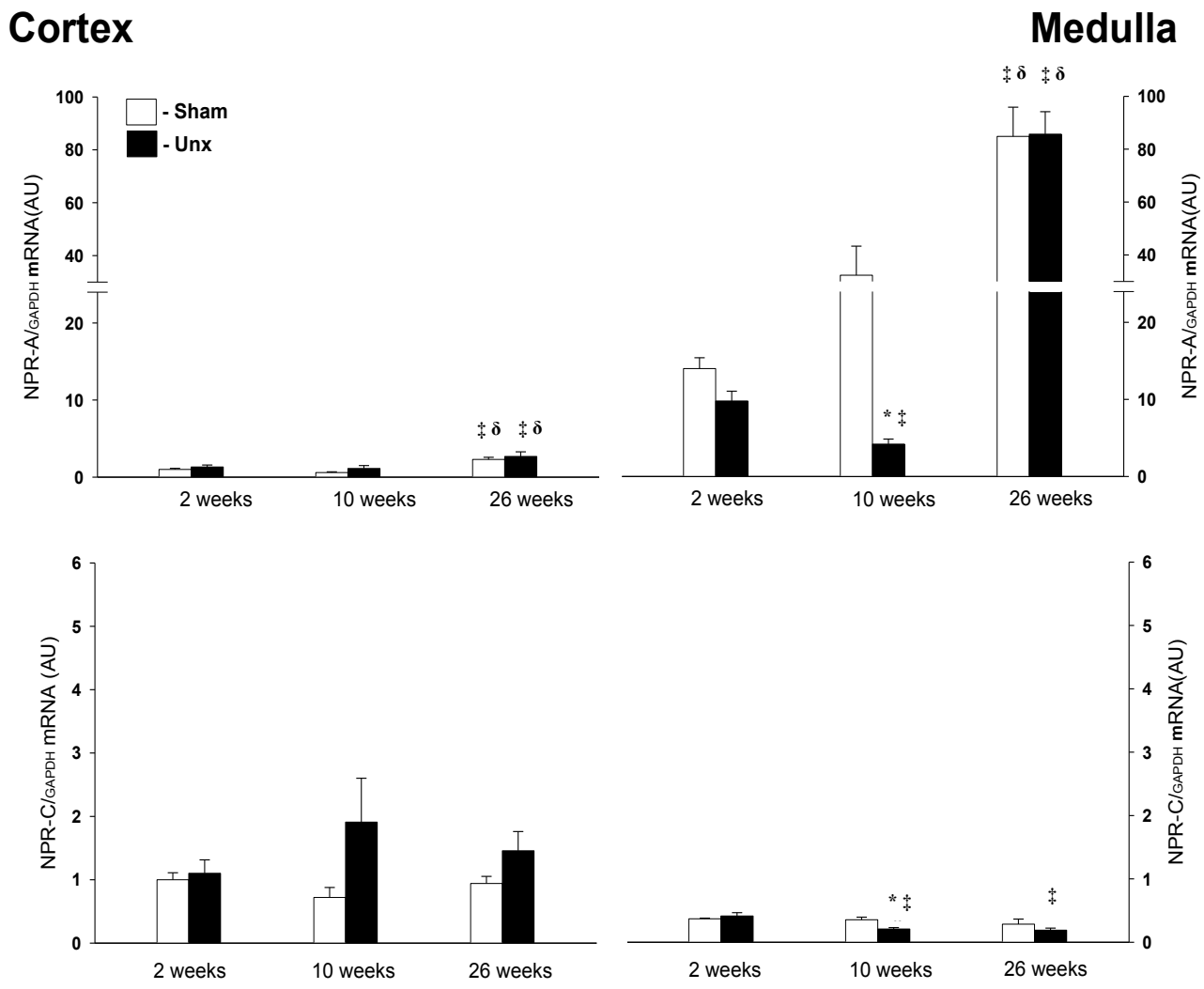

Cortex
Medulla
- Sham
- Unx
NPR-A/GAPDH mRNA(AU)
NPR-C/GAPDH mRNA (AU)
NPR-C/GAPDH mRNA(AU)
2 weeks
10 weeks
26 weeks
‡ δ
* ‡

FIGURE 4

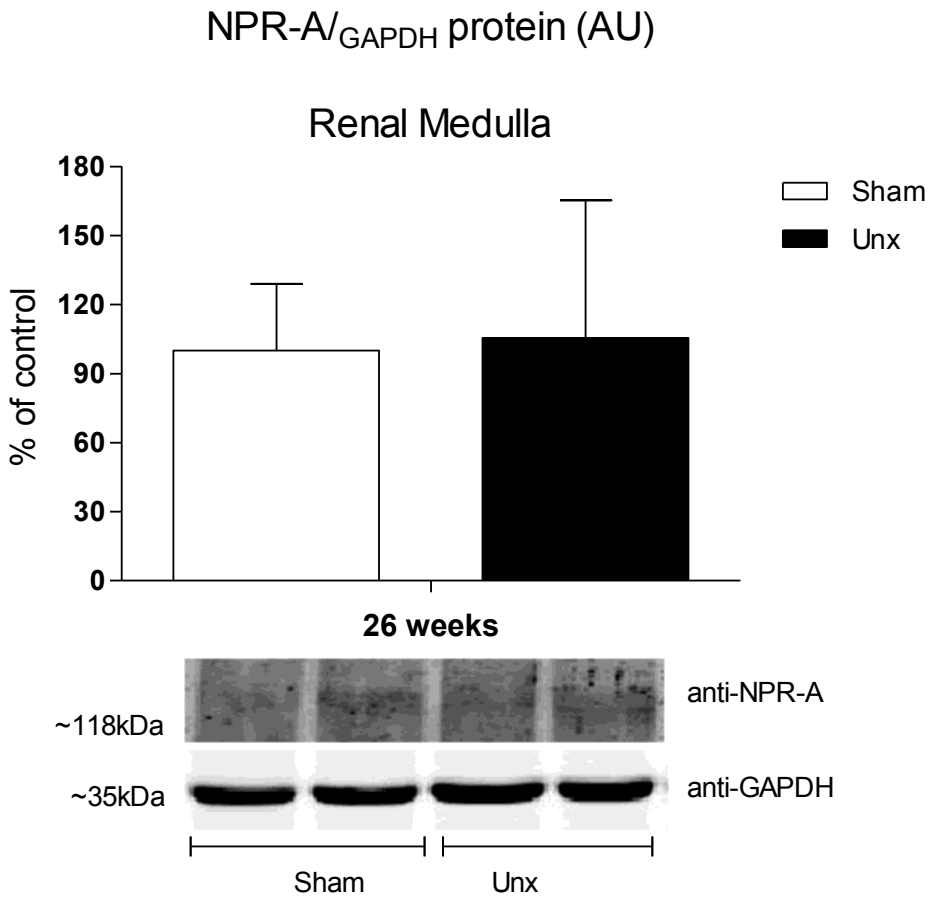
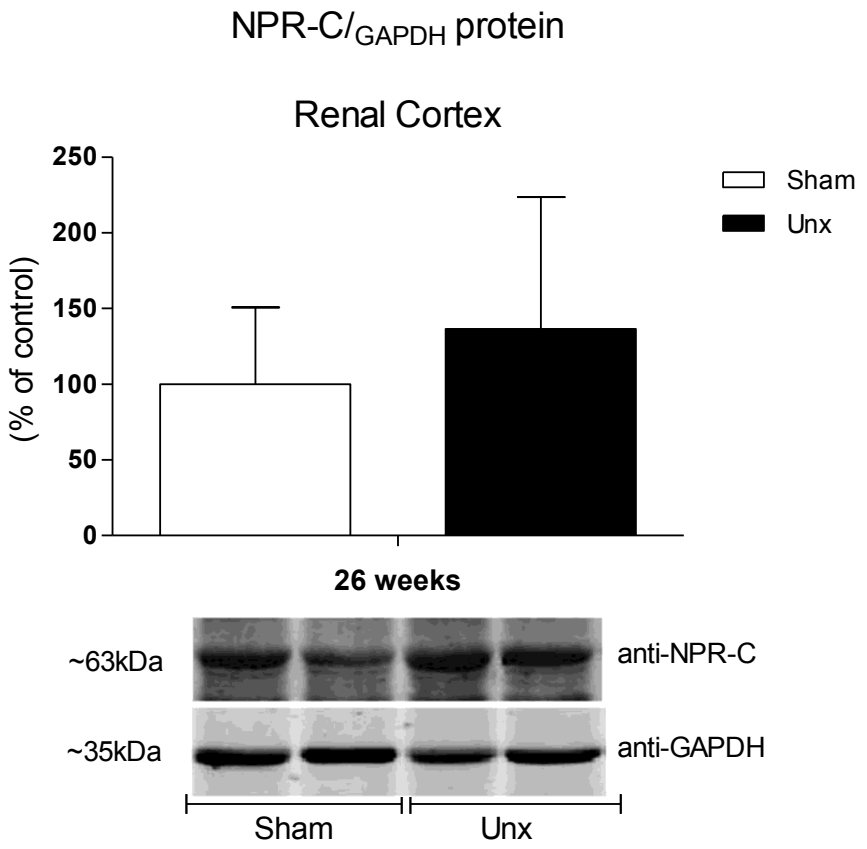

NPR-A/GAPDH protein (AU)
Renal Medulla
% of control
180
150
120
90
60
30
0
Sham
Unx
26 weeks
~118kDa
~35kDa
anti-NPR-A
anti-GAPDH
Sham
Unx

FIGURE 5


NPR-C/GAPDH protein
Renal Cortex
250
200
150
100
50
0
(% of control)
Sham
Unx
26 weeks
~63kDa
anti-NPR-C
~35kDa
anti-GAPDH
Sham
Unx