Int J Physiol Pathophysiol Pharmacol 2012;4(3):128-139
www.ijppp.org /ISSN:1944-8171/IJPPP1208003

Original Article

Disruption of *Npr1* gene differentially regulates the juxtaglomerular and distal tubular renin levels in null mutant mice

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Received August 12, 2012; accepted September 7, 2012; Epub September 20, 2012; Published September 30, 2012

Abstract: Atrial natriuretic peptide (ANP) exerts an inhibitory effect on juxtaglomerular (JG) renin synthesis and release by activating guanylyl cyclase/ natriuretic peptide receptor-A (GC-A/NPRA). Renin has also been localized in connecting tubule cells; however, the effect of ANP/NPRA signaling on tubular renin has not been determined. In the present study, we determined the role of NPRA in regulating both JG and tubular renin using *Npr1* (coding for NPRA) gene-disrupted mice, which exhibit a hypertensive phenotype. Renin-positive immunoreactivity in *Npr1*$^{-/-}$ homozygous null mutant mice was significantly reduced compared with *Npr1*$^{+/+}$ wild-type mice (23% vs 69% renin-positive glomeruli). However, after chronic diuretic treatment, *Npr1*$^{-/-}$ mice showed an increment of JG renin immunoreactivity compared with *Npr1*$^{+/+}$ mice (70% vs 81% renin-positive glomeruli). There were no significant differences in the distal tubule renin between *Npr1*$^{+/+}$ and *Npr1*$^{-/-}$ mice. However, after diuretic treatment, *Npr1*$^{-/-}$ mice showed a significant decrease in renin immunoreactivity in principal cells of cortical collecting ducts ($p<0.05$). The increased JG renin immunoreactivity after reduction in blood pressure in diuretic-treated *Npr1*$^{-/-}$ mice, demonstrates an inhibitory action of ANP/NPRA system on JG renin; however, a decreased expression of distal tubular renin suggests a differential effect of ANP/NPRA signaling on JG and distal tubular renin.

Keywords: Distal tubular and juxtaglomerular renin, immunohistochemistry, atrial natriuretic peptide receptor-A, gene -disrupted mice, hypertension

Introduction

Atrial natriuretic peptide (ANP), an endogenous cardiac hormone primarily synthesized by atrial myocytes, exerts multiple physiological effects on the cardiovascular and renal systems [1-5]. Extensive investigations have demonstrated that the acute administration of ANP reduces arterial pressures and exerts natriuretic and diuretic effects without substantive changes in glomerular filtration rate [2, 6, 7]. These effects are due, in part, to the ability of ANP to counteract the actions of the renin-angiotensin system (RAS) and antidiuretic hormone [6, 8-10]. Although three distinct natriuretic peptide receptor subtypes; natriuretic peptide receptor-A (NPRA), natriuretic peptide receptor-B (NPRB), and natriuretic peptide clearance receptor (NPRC), are present in different cells/tissue types [11-13], most of the actions of ANP on arterial pressure and sodium excretion are mediated via NPRA [9, 10, 14]. Several studies have shown that genetic mouse models with reduced or absence of expression of ANP/NPRA system exhibit elevations in arterial pressure consistent with the physiological role of this hormone-receptor system in the regulation of arterial pressure and cardiovascular, renal, and endocrine functions [15-21]. Although, it is recognized that ANP counteracts the RAS through an inhibitory effect on JG renin synthesis and release, it still remains unclear whether this is a direct and/or indirect effect of the ANP/NPRA system under *in vivo* conditions.

Complete absence of NPRA causes hyperten-

sion in mice and leads to reduced plasma and renal renin concentrations, cardiac hypertrophy and lethal vascular events similar to those seen in untreated human hypertensive patients [20, 22-25]. Our previous studies using adult hypertensive mice lacking NPRA showed an unexpected reduction of systemic and renal renin concentrations, as well as, reduced expression of kidney renin mRNA levels [9]. However, newborn *Npr1* homozygous null mutant (-/-) pups showed 2-fold higher intrarenal renin contents and mRNA levels as compared with the *Npr1* wild-type (+/+) counterparts. In contrast, the adrenal renin contents and mRNA expression levels were higher in *Npr1* (-/-) mice than in *Npr1* (+/+) adult mice [9]. These findings suggested that the inhibition of systemic and renal renin levels is a compensatory response to the elevated arterial pressure, which could prevent greater increases in blood pressure in NPRA null mutant mice. In the present study, we evaluated this possibility by treating *Npr1* (-/-) mice with a diuretic to reduce arterial pressure. Since the presence of renin in connecting tubule (CNT) cells of mice has been described [26], we evaluated the role of *Npr1* in regulating distal tubular renin protein expression using NPRA null mutant (-/-) and wild-type (+/+) mice.

Materials and methods

Breeding and genotyping of Npr1 mice colonies

Npr1 gene-disrupted mice were generated by homologous recombination in embryonic stem cells as previously described [16]. Mice were bred and maintained at the Animal Care Facility of Tulane University Health Sciences Center and the experimental protocol was approved by the Tulane University Institutional Animal Care and Use Committee (IACUC). The adult female *Npr1* mice used in the present studies were littermate progenies of C57BL6 genetic background and have been designated as follows: homozygous null mutant allele (-/-), heterozygous allele (+/-), and wild-type allele (+/+). All mice were generated from littermate crosses of *Npr1* heterozygote parents. The mice were genotyped by multiple polymerase chain reaction (PCR) analysis of DNA isolated from tail biopsies using primer A (5′-GCT CTC TTG TCG CCG AAT CT-3′), corresponding to a sequence 5′ to the mouse *Npr1* gene common to both alleles, primer B (5′-TGT CAC CAT GGT CTG ATC GC-3′) corresponding to an exon 1 sequence only present in the intact mouse allele, and primer C (5′- GCT TCC TCG TGC TTT ACG GT-3′) a sequence in the null allele. The PCR reaction for tail DNA contained: 50 mM Tris-HCl (pH 8.5), 20 mM ammonium sulfate, 1.5 mM MgCl2, 100 μM dNTPs, 10% DMSO, 40 mM primers, and 2 U of Taq DNA polymerase. The PCR was carried out using a 60 -s denaturation step at 94°C , a 60-s annealing step at 60°C, and a 60-s extension step at 72°C , respectively, for 35 cycles as previously reported [9]. PCR products were separated on a 2% agarose gel with the endogenous band of 500 base pairs (bp) and targeted band of 200 bp.

Animals and tissue collection

Animals had free access to a normal salt diet and tap water and were maintained in a temperature-controlled room regulated on a 12-hour light/dark cycle at 25°C . In the present studies, 2 groups of 16-weeks old *Npr1* female mice (body weight of 30 ± 2 g) were utilized. Group I included untreated homozygous null mutant (-/-, n=6) and wild-type (+/+, n=6) mice; and Group II included diuretic-treated homozygous null mutant (-/-, n=6) and wild-type (+/+, n=6) mice. A diuretic (bendrofluomethiazide, 10 mg/kg, during 5 days) was orally administered by gavage. Bendrofluomethiazide was selected due to its predominant action to block the Na^+/Cl^- cotransporter in the distal tubule without having a significant influence on hemodynamics [27]. At the end of experiment, animals were sacrificed by conscious decapitation; both kidneys were harvested for histological analysis and plasma was collected for renin assay.

Systolic blood pressure measurement

After a training period of 7 consecutive days, systolic arterial pressure was measured in conscious *Npr1* homozygous null mutant (-/-) and wild-type (+/+) animals, during 3 consecutive days before diuretic administration and during five consecutive days after diuretic treatments. Arterial pressures were measured using a non-invasive computerized plethysmography tail-cuff blood pressure system (Visitech System BP-2000). Mean systolic arterial pressure was calculated as the average of 6-10 sessions/day with consecutive days during pre- and post-treatment periods. Statistical analysis were performed for repeated measurements with diuretic treatments and genotypes as variables.

Renin immunohistochemistry

Freshly harvested kidneys were sagital sectioned and fixed in 10% zink-buffered formalin. Formalin-fixed kidneys were dehydrated in graded solutions of alcohol, embedded in paraffin blocks, and 3-5 μm tissue sections were cut and mounted onto slides with Vectabond (Vector Laboratories). Serial kidney sections were immunostained by the immunoperoxidase technique with modifications as described previously [28]. Immunolocalization of renin was performed using a polyclonal anti-mouse renin antibody raised in rabbit (generously provided by Dr. Tadashi Inagami, Vanderbilt University, School of Medicine, Nashville, TN) and an automatic robot system from DAKO Corporation (Carpinteria, CA), which allows exact slide time exposure to all reagents and antibody concentrations. To assist in cell localization, anti-aquaporin 2 polyclonal anti-mouse/anti-rat antibody raised in rabbit (Calbiochem-Novabiochem, Co, San Diego, CA) at a concentration of 1:500 was also assessed in each group of mice kidneys. After dewaxing in xylene, the kidney sections were dehydrated in 100, 95, and 70% alcohols and treated with absolute methanol and 0.3% H_2O_2 for 30 minutes to block peroxidase activity. After washing in phosphate-buffered saline (PBS) for 20 min, the sections were sequentially incubated at room temperature with *1)* normal blocking serum (rabbit serum) for 20 min, *2)* primary antibody, anti-peptide renin polyclonal antibody diluted in PBS containing 1% bovine serum albumin (BSA) at 1:2,000, 1:4,000, and 1:8,000 concentrations (50 μl) for 90 min, *3)* secondary antibody, biotin -conjugated rabbit anti-mouse IgG during 30 min, and *4)* avidin-biotinylated horseradish peroxidase H complex using the ABC Elite Vectastain kit (Vector Laboratories Inc., Burlingame, CA) for 45 min. Peroxidase activity was visualized with 0.1% 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co, St. Louis, MO), 0.1% nuclear fast red (Poly Scientific, Bay Shore, NY) and 0.2% H_2O_2. The slides were then washed in tap water, counterstained with hematoxylin, mounted using aqueous mounting media (Biomedia, Fisher Scientific), and coverslipped. Immunohistochemical stained slides were visualized with a 40x magnification objective of an Olympus BX51 and photographed with an integrated Magnafier SP Digital Firewire Camera System.

The specificity of immunostaining was determined by *1)* omission of the primary antibody and substitution with PBS, *2)* preadsorption of the primary antibody (1:4,000) for 72 h using 5X ($1.0x10^{-4}$ mole/L) with excess of pure porcine renin (Sigma), and *3)* Western blot using anti - renin antibody (1:1000) and proteins extracted from mouse and rat kidneys simultaneously run together with different concentrations (4, 40, and 400 ng) of porcine renin.

Analysis of renin-positive immunostaining

The analysis was performed in kidney sagital sections from both *Npr1* wild-type (+/+) and *Npr1* homozygous null mutant (-/-) mice from each group (control and treated with diuretic) as follows: a) JG renin: the total number of glomeruli with at least one visible longitudinal vessel were determined in 4 separate kidney cross sections per animal. Positive immunostaining was counted only in those glomeruli showing renin immunoreactivity in the JG cells or in a longitudinal view of the afferent arteriole. b) Distal tubular renin: The renin immunoreactivity was determined in five different microscopic fields, using an Olympus BX51 TRF microscope with a 40X magnification objective, integrated to a Magnafire SP Digital "Firewire" Camera System for image processing. The distal tubule renin immunoreactivity pattern in distinct mouse kidney sections was analyzed using an Image-Pro® Plus Software, version 4.5.1 for Windows 2000 XP (Media Cybernetics, Inc) which allows a computerized determination of the extension of positive areas (μm) and intensity of immunoreactivity (sum of density of positive tubules in an analyzed area). In order to validate the automatic computerized system analysis, two independent investigators using blinded slides, scored similar kidney sections from 10 different microscopic fields. The parameters included the number of total distal tubules with visible lumen per field, number of renin positive tubules per field, number of cells per tubule, number of renin positive cells per tubule, and intensity of renin immunoreactivity. Three separate experiments were performed and concordance was >90%.

Assays of plasma renin concentration

Blood was collected by cardiac puncture from the decapitated animals in prechilled tubes containing 5 μl of 0.25 M EDTA. Plasma was separated by centrifugation at 3,000 rpm for 20 min at 4ºC and stored at -80ºC until used. To de-

termine the plasma renin concentration, 10 µl of plasma were incubated with 225 µl of 100 mM sodium phosphate buffer (pH 6.0) containing; 1 mM diisopropyl fluorophosphate (DFP), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 2.5 µg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor, and 10 µl of maleate generation buffer according to our established protocols [9, 29]. The reaction was started by the addition of 25 µl of rat renin substrate at 37°C . The cocktail of protease inhibitors added in the reaction mixture protected the generated angiotensin I (Ang I) as well as renin from proteolytic destruction and permitted >90% recoveries. The reaction was stopped by heating in boiling water for 10 min and the generated Ang I was determined by radioimmunoassay (RIA) kit.

Statistical analysis

Statistical analysis was performed using GraphPad PRISM (version 4.0; GraphPad Software Inc., San Diego, CA). The results are presented as mean ± SEM. The differences between groups were determined by analysis of variance (ANOVA) combined with Dunnett's multiple comparison post hoc test. The probability value of $p < 0.05$ was considered significant.

Results

Systolic blood pressure

The systolic arterial pressure measurements averaged 30 mmHg higher in untreated *Npr1* homozygous null mutant (-/-) mice (131±3 mmHg) as compared with wild-type (+/+) mice (101±2 mmHg) (**Figure 1**). After chronic treatment with bendrofluomethiazide, systolic pressure decreased by an average of 14±1.5 mmHg ($P<0.05$) in *Npr1* (-/-) mice and by 10±2.0 mmHg ($P< 0.05$) in *Npr1* wild-type (+/+) mice. The magnitude of the decrease in systolic arterial pressure was greater in *Npr1* (-/-) mice than in wild-type (+/+) mice, thus allowing the comparisons of systolic arterial pressures at comparable levels. Plasma renin concentration (PRC) did not change in the diuretic-treated wild-type (+/+) mice as compared with untreated wild-type (+/+) mice (3.3±0.8 vs. 2.8±0.5 ng Ang 1. mL^{-1}. h^{-1}, respectively). In contrast, PRC increased in diuretic-treated *Npr1* (-/-) mice as compared with untreated *Npr1* (-/-) mice (5.0±1.2 vs. 2.0±0.4 ng Ang 1. mL^{-1}. h^{-1}, respectively) (**Figure 2**). PRC values in diuretic-treated *Npr1* homozygous null mutant (-/-) mice were not significantly different from those in diuretic-treated *Npr1* wild-type (+/+) mice.

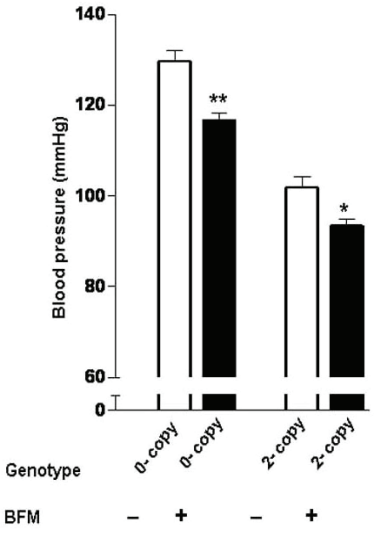

Figure 1. Analysis of blood pressure measurements in *Npr1* mice: Systolic arterial pressures were determined in conscious state by noninvasive computerized tail-cuff method (Visitech-2000) in *Npr1* homozygous null mutant (-/-) and wild-type (+/+) mice before and after treatment with diuretic bendrofluomethiazide. Untreated *Npr1* (-/-) mice, N=6; Diuretic -treated *Npr1* (-/-) mice, N=6; Untreated *Npr1* (+/+) mice, N=6; Diuretic treated *Npr1* (+/+) mice, N=6. **P*<0.05; ***P*<0.001.

Renin expression in juxtaglomerular cells

Renin immunoreactivity was observed in JG cells and on the apical side of cortical collecting tubules (CCT) and collecting duct cells (**Figure 3A**). The preadsorption of the primary antibody with porcine renin showed a substantial decrease of the JG renin immunoreactivity and its disappearance in the cortical collecting tubule cells (**Figure 3B**). Western blot analysis using proteins extracted from mouse kidney and different concentrations of pure renin peptide simultaneously confirmed the specificity of the anti-renin antibody by demonstration of a prominent band with an estimated molecular mass of 35 kDa for mouse renin and 38 kDa for pure renin protein (**Figure 3C**).

Juxtaglomerular renin immunoreactivity was evaluated determining the frequency of

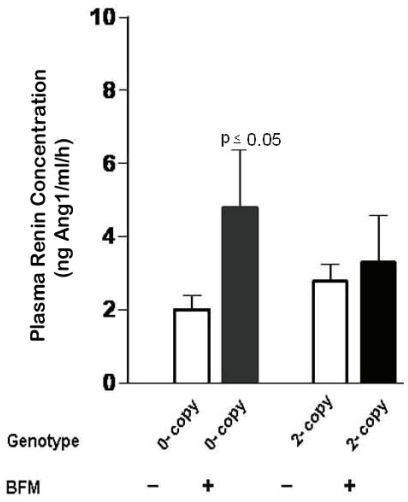

Figure 2. Determination of plasma renin concentration in *Npr1* mice: Plasma renin concentration was determined in *Npr1* homozygous null mutant (-/-) and wild-type (+/+) mice. Renin concentration was determined as described in Methods Section. Bars represent means ± SE values of *Npr1* mice genotypes as indicated. Untreated *Npr1* (-/-) mice, N=5; Diuretic -treated *Npr1* (-/-) mice, N=5; Untreated *Npr1* (+/+) mice, N=8; Diuretic treated *Npr1* (+/+) mice, N=8. *p<0.05.

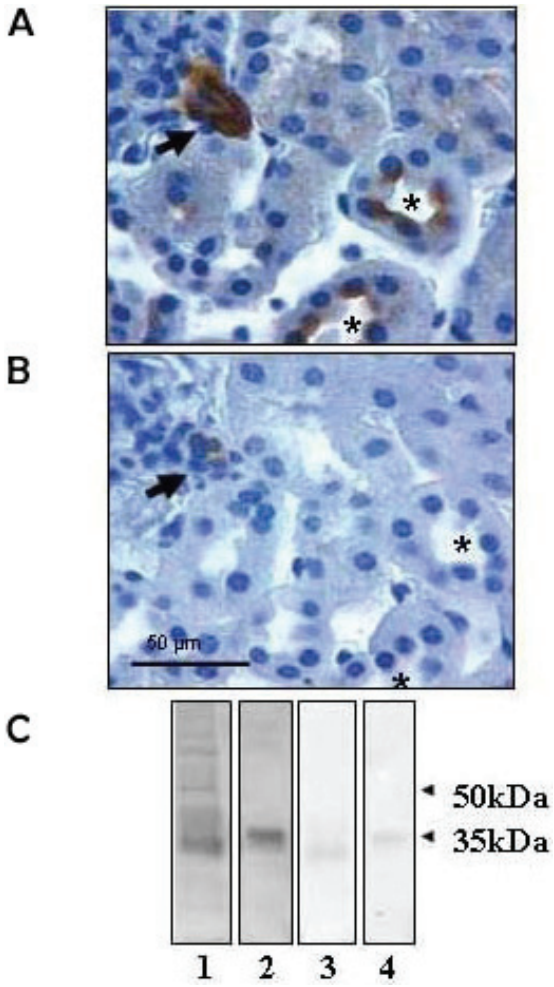

Figure 3. Immunohistochemistry and Western blot analysis of renin in juxtaglomerular and distal tubule cells: Positive renin immunostaining is shown in JGA cells (arrow) and distal tubules (asterisks) of *Npr1(-/-*) mouse kidney incubated with 1:4,000 renin antibody (Panel A). Consecutive section incubated with renin antibody preadsorbed with porcine renin demonstrates a reduction in renin immunostaining of JGA cells and complete elimination in distal tubules (Panel B). Renin protein expression was detected by Western blot analysis in mouse kidney (lanes 1 and 3; 25 µg/lane) and pure porcine renin (lanes 2 and 4; 4 ng/lane) using 1:1,000 renin antibody (lanes 1 and 2) or renin antibody preadsorbed with porcine renin (lanes 3 and 4; Panel C). Renin bands were detected at 35 kDa with minor bands at 53 and 67 kDa in mouse kidney. Band detected at 38 kDa corresponded to porcine renin. Bands detected with primary antibody were eliminated by preadsorption. Equal protein loading was demonstrated by Ponceau staining of the membrane preparations. Micrographs shown are the representative kidney sections from 5 -6 mice.

glomeruli with renin-positive immunostaining in a longitudinal view of the afferent arteriole. Although the number of counted glomeruli per kidney section was comparable between groups (**Figure 4A**), under control conditions, *Npr1* homozygous (-/-) mutant mice showed juxtaglomerular renin-positive immunoreactivity in 23% of total count glomeruli as compared with 69% in *Npr1* wild-type (+/+) mice (**Figure 4B**). However, after chronic diuretic treatment with bendrofluomethiazide, the *Npr1* homozygous null mutant (-/-) mice showed a significant increment of juxtaglomerular renin immunoreactivity up to 70% of the counted glomeruli ($P<0.001$) as compared with 81% in wild-type (+/+) mice. The increase in JG renin immunoreactivity in diuretic -treated mice was significantly greater in homozygous null mutant (-/-) mice than in wild-type (+/+) mice (**Figure 4B**).

Renin expression in cortical distal tubule cells

Distal tubules were identified based upon the absence of brush border using periodic acid Schiff (PAS) counterstaining in consecutive sections. **Figure 5A** shows the renin immunoreactiv-

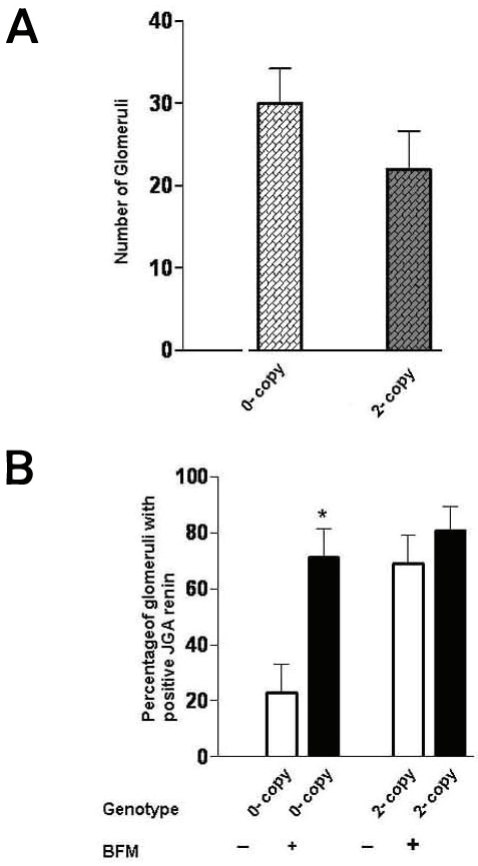

Figure 4. JG renin immunoreactivity after diuretic treatments: Comparison was made for JG renin (% renin-positive glomeruli) in control and diuretic-treated groups. Panel A shows number of glomeruli per unit area in *Npr1* (-/-) and *Npr1* (+/+) mice. Panel B shows the % positive glomeruli in control and diuretic-treated *Npr1* (-/-) and *Npr1* (+/+) mice. Untreated *Npr1* (-/-) mice, n=6; Diuretic-treated *Npr1* (-/-) mice, n=6; Untreated *Npr1* (+/+) mice, n=6; Diuretic-treated *Npr1* (+/+) mice, n=6. Values are mean ± SE. **P*<0.001 vs control group.

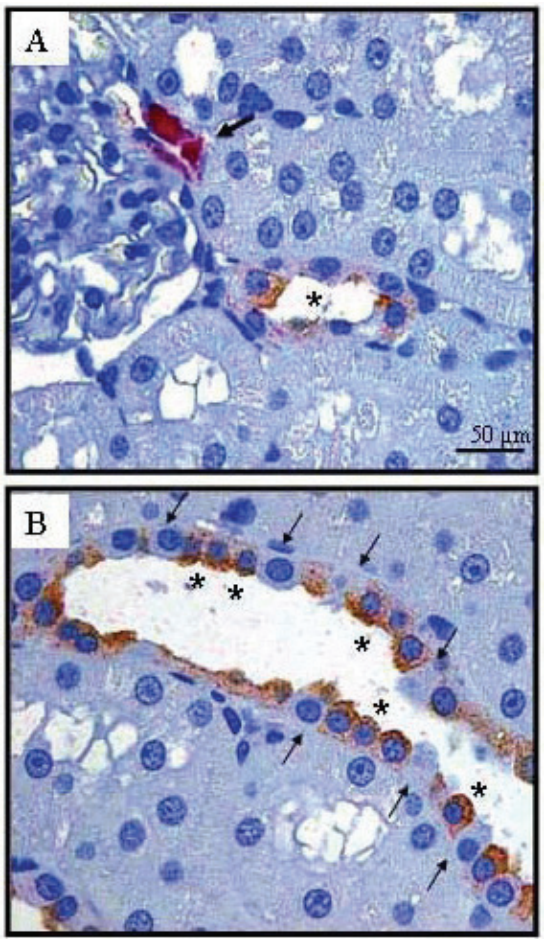

Figure 5. Immunocolocalization of renin and aquaporin 2 in kidney sections of *Npr1* mice: Panel, A shows renin immunostaining in the juxtaglomerular cells (arrows) and distal tubules (DT). Panel, B shows an immunostaining for aquaporin 2 positive cells (principal cells) associated with negative cells (intercalated cells). Renin antibody concentration was used as 1:4,000 dilution and aquaporin 2 antibody concentration was used as 1:500 dilution. G represents glomerulus and DT represents distal tubule. Micrographs shown are the representative kidney sections from 5-6 mice.

ity using renin antibody (1:4,000 dilution, DAB chromogen) characterized by the presence of positive immunoreactive staining in principal cells but negative staining in intercalated cells of the distal tubules. In a kidney consecutive section (**Figure 5B**) a comparable cellular pattern for aquaporin immunostaining is also observed using aquaporin 2 antibody (1:500 dilution, DAB chromogen). Renin immunoexpression was observed in principal cells of distal tubules and collecting ducts of both *Npr1* homozygous null mutant (-/-) and wild-type (+/+). **Figure 6A-D** shows the renin immunoexpression in JG cells and cortical collecting duct cells in *Npr1* homozygous null mutant (-/-) mice (panels A-B) and *Npr1* wild-type (+/+) mice (panels C-D) under control conditions (panels A and C) and diuretic treatments (panels B and D). Semi-quantitative analysis of renin immoreactivity in collecting duct cells showed no significant difference among *Npr1* (-/-) and *Npr1* (+/+) mice under control conditions without diuretic treatment (3,297±857 vs. 3,183±1027). However,

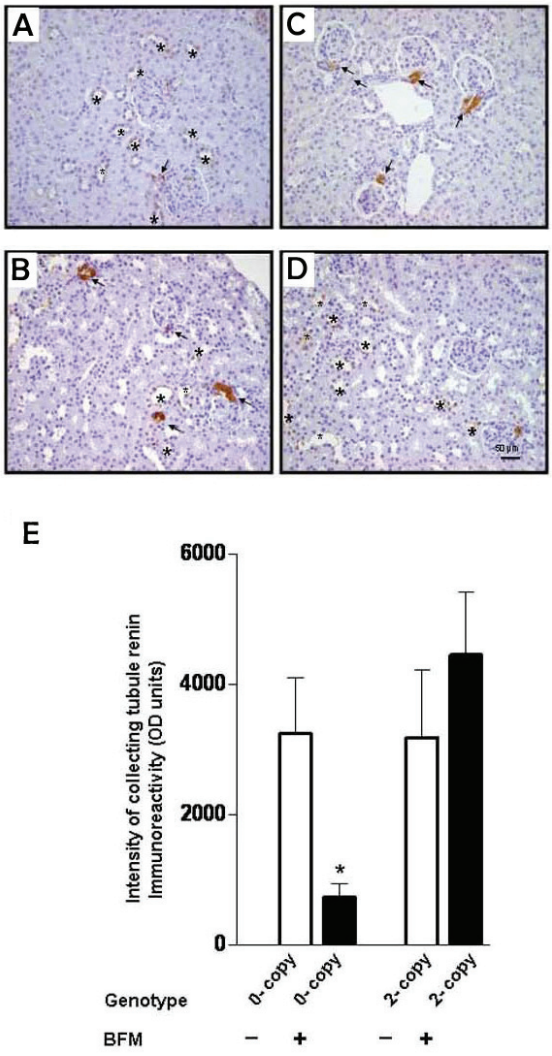

Figure 6. Effect of diuretic treatment on juxtaglomerular and distal tubular renin in *Npr1* mice: A polyclonal mouse anti-renin antibody immunostaining of paraffin-embedded kidney sections (60X, oil immersion) of *Npr1* (-/-) and *Npr1* (+/+) mice. Left panels (A and C) show immunostained sections of untreated control mice kidneys; right panels (B and D) show immunostained sections of diuretic (bendroflumethiazide) -treated mice kidneys. Upper panels represent *Npr1* (-/-) (panels A and B); lower panels represent *Npr1* (+/+) mice (panels C and D). Arrows show juxtaglomerular renin immunostaining. Dashed circles show distal tubular renin immunostaining. Renin antibody concentration was used as 1:8,000. Immunostaing shown are representative of kidney sections from 5-6 mice. Panel E shows the Comparison of distal tubular renin in control and diuretic (bendroflumethiazide) -treated *Npr1* (-/-) and *Npr1* (+/+) mice. Intensity of renin immunoreactivity was quantitated using ImagePro plus imaging analysis in control and diuretic-treated *Npr1* (-/-) mice (control, n=6; diuretic, n=6); and *Npr1* (+/+) mice (control, n=6; diuretic, n=6). OD: optical density. Values are mean ± SE. **P*<0.05 vs control group.

chronic administration of bendrofluomethiazide elicited a significant decrease in renin immunoreactivity in collecting duct cells of *Npr1 (-/-)* mice (3,297±857 vs. 736±202; p<0.001) but not in *Npr1 (+/+)* mice (3,183±1027 vs. 4,451±973) (**Figure 6E**). The intensity of tubule renin immunoreactivity was significantly greater in the diuretic-treated *Npr1 (+/+)* mice than in *Npr1 (-/-)* mice (4,451±973 vs. 736±202; *P*<0.05) (**Figure 6E**).

Discussion

We have demonstrated that JG renin-positive immunoreactivity is suppressed in adult *Npr1* homozygous null mutant (-/-) mice, which exhibited 30 mmHg higher systolic blood pressures as compared with *Npr1* wild-type (+/+) mice. In the present study, the diuretic, bendrofluomethiazide, was chronically administered to reduce the blood pressure, which increased JG renin immunoreactivity in the *Npr1 (-/-)* mice but had no significant effect on JG renin immunoreactivity in the *Npr1* (+/+) mice. In a previous study, Shi et al. [9] demonstrated that newborn *Npr1 (-/-)* pups (2 days after the birth) showed 2.5-fold higher intrarenal renin content as compared with *Npr1* wild-type (+/+) counterparts. However, both young (3-wk) and adult (16-wk) *Npr1 (-/-)* mice showed a dramatic 50-80% reduction in plasma renin concentration and renal renin mRNA expression as compared with *Npr1* wild-type (+/+) animals. Taken together, the present results indicate that in the absence of NPRA, elevated blood pressure leads to reduced expression of JG renin in the kidney, which helps to reduce the activity of the RAS and thus prevents greater increases in arterial pressure in the mutant mice. The increased JG renin immunoreactivity after diuretic treatment in *Npr1* homozygous null mutant mice supports the hypothesis that reduction in arterial pressure restores JG renin to levels similar to those seen in wild-type mice and suggests that the prevailing arterial pressure is the main determinant of JG renin. Our present findings indicate that due to the absence of ANP/NPRA signaling, the renin synthesis seems to be increased in *Npr1* (-/-) mice; however, due to an increased blood pressure, the plasma renin level is reduced in these

null mutant mice. Nevertheless, after diuretic treatment and reduction in blood pressure, the plasma renin concentration is increased. In contrast, in wild-type (+/+) mice, the functional ANP/NPRA signaling exerts an opposing effect on renin synthesis and secretion; as a result a homeostatic balance seems to be maintained.

Proximal tubule cells have been found to produce renin and contain renin mRNA [30], which may be regulated by chronic changes in salt diet, suggesting that the local RAS contributes to the renal adaptations in response to chronic alterations in NaCl [31, 32]. Renin immunoreactivity in distal nephron segments was described long ago but it was interpreted as an experimental artifact [33] or as a nonspecific uptake of filtered renin [34]. Now, more emphasis has been given to the presence of renin in distal nephron segments [26, 31, 35]. Apical renin immunostaining in connecting tubule cells as well as renin secretion in isolated connecting tubule cells, were demonstrated by Rohrwasser et al. [26]. This localization provides a basis for a potential role for action of distal tubular renin on angiotensinogen produced in proximal tubule cells, secreted directly into the tubular lumen and delivered to the distal nephron segments [31]. In the present study, we observed distal tubular renin immunoexpression in cortical collecting tubule cells, which differed according to *Npr1* gene doses and in response to diuretic treatments. Distal tubular renin immunoreactivity appeared in both *Npr1 (-/-)* and *Npr1 (+/+)* mice; however, it did not significantly change in wild-type *(+/+)* mice after diuretic treatment. In contrast to what was observed for JG renin, distal tubule renin instead substantially decreased in *Npr1* homozygous null mutant *(-/-)* mice after diuretic treatment. Lantelme et al. [31] evaluated the effects of volume depletion induced by loop diuretics and dietary sodium restriction on distal tubule renin immunoexpression and urinary excretion associated with angiotensinogen urinary excretion in mice. These authors reported an enhancement in distal tubule renin immunoexpression and in urinary renin excretion after volume depletion, secondary to diuretic treatment. According to those previous studies, this observation could be due to either one or two mechanisms: a) the marked rise in systemic renin leading to an increase in the amount of renin filtered at glomeruli, which saturates the reabsorbing capacity of proximal tubule, and/or b) activation and secretion of renin by connecting tubule cells. In the present study, after diuretic treatment, the wild-type mice showed a tendency to increase distal tubular renin-positive immunoreactivity. Nevertheless, the *Npr1* homozygous null mutant (-/-) mice instead showed a substantial decrease in the renin-positive immunostaining. In the present study, after diuretic treatment, the wild-type (+/+) mice did not exhibit a significant increase in distal tubular renin-positive immunoreactivity; in contrast, the homozygous null mutant (-/-) mice instead showed a substantial decrease in the renin-positive immunostaining. This difference suggests that the presence of ANP/NPRA signaling may be important in the regulation of distal tubular renin expression.

The present findings indicate that distal tubular renin immunoreactivity responds in concert with the JG renin in wild-type (+/+) mice but not in *Npr1* homozygous null mutant (-/-) mice. On the basis of our results, we implicate NPRA signaling is important for an enhanced distal tubular renin expression. The presence of renin in distal nephron segments can catalyze the formation of Ang I and finally the production of angiotensin II (Ang II) [36, 37]. Our previous studies indicate that it is Ang II, and not renin, which is released outside the cell [29]. On the same line of evidence, more recent studies have demonstrated that in hypertension, kidneys reflect a marked reduction in JG renin contents in response to elevated blood pressure; however, a high tubular Ang II level is maintained [38-40]. A direct action of Ang II on the luminal amiloride-sensitive sodium channel has been reported indicating a direct role of tubular Ang II in the regulation of distal tubule and collecting duct sodium reabsorption [41]. The presence of an intact distal nephron renin system may be important in the regulation of distal tubular sodium reabsorption. During volume depletion, an augmented proximal tubule angiotensinogen secretion coupled with stimulation of distal tubular renin formation could lead to increased distal formation of Ang II and enhanced Ang II-mediated sodium reabsorption [42]. However, in the *Npr1* homozygous null mutant mice, regardless of the presence of sodium and volume depletion conditions, the absence of NPRA signaling seems to prevent the distal tubular cells from increased renin protein activity in response to these stimuli. These results suggest that ANP/NPRA system may play a role in regulating the enhanced distal tubular renin expression,

an opposite effect to that of JG renin expression, which is generally suppressed by ANP/NPRA/cGMP signaling [1, 8, 9]. Our findings implicate two major interpretations: First, it seems that in the absence of ANP/NPRA signaling, the blood pressure is increased and JG renin content is decreased. As a result of this pathological compensatory mechanism, the distal tubular renin is increased in *Npr1* homozygous null mutant mice. Our present results are also supported by previous findings, which have indicated that the changes in the intrarenal tubule renin might be linked to pathological tubulointerstitial conditions [26, 35]. Secondly, it is possible that in tubular cells, ANP/NPRA signaling provides a tonic stimulus via the intracellular second messenger cGMP to enhance renin gene expression, thus increasing intracellular renin levels. However, more experimentation is needed to confirm these postulates.

In essence, the physiological effects of ANP/NPRA are catalyzed through the classic intracellular second messenger cGMP, which activates cGMP-dependent protein kinases (PKG), cGMP-dependent phosphodiesterases (PDEs), and cyclic nucleotide-gated ion channels (CNGs) [43-47]. Thus far, it is considered that ANP inhibits renin synthesis and release by activating GC-A/NPRA, which dramatically increases the cGMP levels in intact animals *in vivo* [1, 9, 48, 49]. It has also been suggested that nitric oxide-dependent cGMP production can stimulate renin secretion, without any effect on blood pressure or renal hemodynamic effects [50]. Those previous studies indicate that by selectively inhibiting phosphodiesterase-5 (PDE-5) the resultant increased levels of endogenous cGMP can inhibit phosphodiesterase-3 (PDE-3), which increases the levels of cAMP, thereby resulting in an increased renin secretion. It has also been shown that sildenafil, a potent and selective inhibitor of PDE-5 increases plasma cGMP and cAMP levels as well as renin secretion in human subjects [51]. Similarly, it has been suggested that cGMP enhances renin release by blocking the degradation of cAMP [52]. Together, the effect of cGMP on renin synthesis and release involves the protection of cAMP from PDE-3 [53]. Earlier, it has also been suggested that cGMP can exert opposing effects on renin synthesis and its secretion such as an inhibitory effect through the activation of PKG and a stimulatory effect involving the inhibition of cAMP breakdown by PDE-3 [51, 53, 54]. However, the exact mechanism by which cGMP and cAMP alter the renin synthesis and release is not well understood.

Interestingly, previous studies using Goldblat hypertensive rats, have suggested that the collecting duct renin seems to be regulated independent of the blood pressure [55]. It has also been reported that an enhanced collecting duct renin cleaves the angiotensinogen released from the proximal nephron segment to generate Ang I, which is converted to biologically active hormone Ang II [36, 56]. Evidence suggests that angiotensin converting enzyme (ACE) in the collecting duct may be responsible for the intraluminal generation of Ang II from Ang I, which can lead to an Ang II-dependent activation and increase in blood pressure [41, 57]. Systemic disruption of *Npr1* gene (coding for GC-A/NPRA) leads to volume overload, high blood pressure, and congestive heart failure. Our present results indicate that juxtaglomerular but not distal tubular renin expression is significantly suppressed in *Npr1* homozygous null mutant (-/-) mice, but no significant changes were observed in *Npr1* wild-type (+/+) mice. Intriguing was the finding that after diuretic (bendroflumethiazide) treatment of *Npr1* (-/-) mice, the juxtaglomerular renin-positive immunoexpression was increased, however, distal tubular renin expression level was decreased.

In summary, our results demonstrate that JG but not distal tubule renin protein expression is significantly suppressed in *Npr1* gene -disrupted homozygous null mutant (-/-) mice, but not in wild-type (+/+) mice. After diuretic treatment of *Npr1* null mutant (-/-) mice, JG renin-positive immunoexpression increased but distal tubular renin decreased. These data support the notion that JG and distal tubular renin are differentially regulated by the ANP/NPRA/cGMP system. It is expected that further comparative analysis of gene expression studies using *Npr1* gene-disrupted null mutant mouse model in different physiological and pathophysiological conditions, will help to clarify the importance of the interactions between the RAS and ANP/NPRA/cGMP systems in controlling RAS, sodium balance, and arterial pressure.

Acknowledgements

The authors express their gratitude to Ms. Houng Nguyen, Mr. Dale Seth, Ms. Helena

Pappas Le-Bau, and Mrs. Monique Edwards for their excellent technical assistance. We thank Mrs. Kamala Pandey for typing the manuscript. We are indebted to Dr. Oliver Smithies for providing initial breeding pairs of *Npr1* gene-targeted mouse colonies. We sincerely thank Dr. Tadashi Inagami for providing the mouse anti-renin antibody. Our special thanks go to Dr. Susan L Hamilton, Department of Molecular Physiology and Biophysics at Baylor College of Medicine, and Dr. Bharat B Aggarwal, Department of Experimental Therapeutics and Cytokine Research Laboratory at MD Anderson Cancer Center, for providing their facilities during our displacement due to Hurricane Katrina. Digital images of histological specimens were obtained at the Centralized Tulane Imaging Center and Imaging Core Facility of Hypertension and Renal Center of Excellence. This work was supported from the National Institutes of Health grant (HL62147).

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