Targeted Disruption of Guanylyl Cyclase-A/Natriuretic Peptide Receptor-A Gene Provokes Renal Fibrosis and Remodeling in Null Mutant Mice: Role of Proinflammatory Cytokines

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Binding of atrial and brain natriuretic peptides to guanylyl cyclase-A/natriuretic peptide receptor-A produces second messenger cGMP, which plays an important role in maintaining renal and cardiovascular homeostasis. Mice carrying a targeted disruption of the Npr1 gene coding for guanylyl cyclase-A/natriuretic peptide receptor-A exhibit changes that are similar to those that occur in untreated human hypertension, including elevated blood pressure, cardiac hypertrophy, and congestive heart failure. The objective of this study was to determine whether disruption of the Npr1 gene in mice provokes kidney fibrosis, remodeling, and derangement. We found that systemic disruption of the Npr1 gene causes increased renal tubular damage characterized by dilation, flattening of epithelium, and expansion of interstitial spaces in Npr1−/− (0-copy) mice. Significant increases occurred in the expression levels of TNF-α (4-fold), IL-6 (4.5-fold), and TGF-β1 (2-fold) in 0-copy null mutant mice compared with 2-copy wild-type mice. An increased epithelial-to-mesenchymal transition indicated by increased expression of α-smooth muscle actin, was observed in Npr1−/− mouse kidneys. Treatment with captopril and losartan showed a 38 and 46% attenuation in fibrosis and 30 and 42% reduction in α-smooth muscle actin immunoexpression, respectively, in 1-copy and 0-copy mice compared with 2-copy mice. Although bendroflumethiazide treatment did not show any effect. The present results demonstrate that the disruption of Npr1 gene activates proinflammatory cytokines leading to fibrosis, hypertrophic growth, and remodeling of the kidneys of mutant mice. (Endocrinology 151: 5841–5850, 2010)

Atrial natriuretic peptide (ANP) is a cardiac hormone that elicits natriuretic, diuretic, vasodilatory, and antiproliferative responses, all of which contribute to lowering blood pressure and blood volume (1–4). Natriuretic peptides belong to a family that includes at least three endogenous hormones: ANP, brain natriuretic peptide, and C-type natriuretic peptide. ANP and brain natriuretic peptide bind to guanylyl cyclase-A (GC-A)/natriuretic peptide receptor-A (NPRA), which is considered to be the major natriuretic peptide receptor that synthesizes the intracellular second messenger cGMP (5–7). Mice carrying a targeted-disruption of the Npr1 gene (encoding for GC-A/NPRA) have been shown to have hypertension, marked cardiac hypertrophy, and congestive heart failure (8–12). Our previous studies demonstrated that both glomerular filtration rate and renal plasma flow are drastically reduced in Npr1−/− gene-disrupted mice (0-copy) during and after blood volume expansion, indicating that ANP/NPRA responses to volume expansion in Npr1-null mutant mice lead to significantly diminished excretion of sodium and water (10). It has been shown that progressive loss of renal function is associated not only with the development of glomerulosclerosis but also with renal fibrosis, which is characterized by excessive deposition of ex-
tracellular matrix proteins, enhanced expression of inflammatory cytokines, and structural rearrangements that involve cellular hypertrophy, hyperplasia, and cellular proliferation (13, 14).

The ANP/NPRA signaling system has been implicated as an antihypertrophic and antiangiogenic protective mechanism during the cardiac remodeling process (8, 11, 15). ANP has been shown to inhibit fibroblast proliferation, collagen synthesis, and matrix metalloproteinase release via a cGMP-dependent mechanism (16–18). Furthermore, ANP/NPRA signaling can negatively regulate the proliferation of mesangial and vascular smooth muscle cells (19–21). Gene-targeted, Npr1 mouse models have provided strong support for physiological and pathophysiological roles of the GC-A/NPRA signaling system in regulating arterial pressure (4, 8, 10, 11, 22, 23) as well as renal functions (4, 8, 10, 11, 22, 23). To further examine the possible regulatory role of the Npr1 gene in the pathogenesis of kidney fibrosis, hypertrophy, and remodeling, we performed histological, biochemical, and molecular studies using Npr1 gene-disrupted mice.

### Materials and Methods

#### Materials

Gene-specific primers were purchased from Midland Certified Reagent (Midland, TX). Antibodies for TNF-α, IL-6, TGF-β1, and proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TRIZol reagent was obtained from Invitrogen (Carlsbad, CA). α-Smooth muscle actin (α-SMA) mouse monoclonal antibody was purchased from Sigma-Aldrich (St. Louis, MO). The creatinine kit was purchased from BioAssay System (Hayward, CA). Primary antibody for monocytes/macrophages (ED1) antibody and multiplex kit (TNF-α, IL-6, and TGF-β1) were obtained from Millipore Corp. (Bedford, MA). The ELISA kit for microalbumin was purchased from Bethyl Laboratories (Montgomery, TX).

A multiprobe template set for the ribonuclease protection assay (RPA) was purchased from Ambion (Austin, TX). [α-35S]UTP (800 Ci/mmol) at 10 mCi/ml was purchased from PerkinElmer (Waltham, MA). The cGMP assay kit was obtained from Ann Arbor, MI. All other chemicals were reagent grade.

#### Generation and genotyping of mice

Npr1 gene-disrupted mice were produced by homologous recombination in embryonic stem cells as described previously (8). These mice were bred and maintained at the animal facility of the Tulane University Health Sciences Center. Animals were handled under protocols approved by the Institutional Animal Care and Use Committee. The mouse colonies were housed under 12-h light, 12-h dark cycles at 25°C and fed regular chow (Purina Laboratories, Framingham, MA) and tap water ad libitum. All animals were littermate progenies of the C57BL6 genetic background and were designated as Npr1 gene-disrupted homozygous null mutant (Npr1−/−), heterozygous (Npr1+/−, 1-copy), and wild-type (Npr1+/*, 2-copy) mice. This study used adult (24 wk of age) male Npr1 mice. The animals were genotyped by PCR analyses of DNA isolated from tail biopsies using primer A (5′-GCTCCTTTGTCGCCAATTCT-3′), corresponding to the 5′ sequence of the mouse Npr1 gene common to both alleles (Npr1+/+); primer B (5′-TGTACCATGCTGTATCGCC-3′), corresponding to the exon 1 sequence present only in the intact allele (Npr1+/−); and primer C (5′-GCTTCCTCCTGCTTTACCGT-3′), corresponding to a sequence in the neomycin-resistance cassette present only in the null mutant allele (Npr1−/−). PCR was carried out in 25 μl of reaction mixture containing 50 mM Tris-HCl (pH 8.3), 20 mM ammonium sulfate, 1.5 mM MgCl2, 10% dimethylsulfoxide, 100 μM dNTPs, 2 U Taq DNA polymerase, and 40 μM primers with a 60-sec denaturation step at 94°C, a 60-sec annealing step at 60°C, and a 60-sec extension step at 72°C for 35 cycles using the GeneAmp 9700 (Applied Biosystems, Foster City, CA). PCR product was resolved on 2% agarose gel with the endogenous band of 500 bp and the targeted band of 200 bp.

#### Blood pressure analysis

Blood pressure was measured by a noninvasive computerized tail-cuff method using Visitech 2000 and was calculated as the average of seven to 10 sessions per day for 6 consecutive days as previously described (24).

#### Blood and tissue collection

Blood was collected by cardiac puncture under CO2 anesthesia in prechilled tubes containing 10 μl heparin (1000 U/ml). Plasma was separated by centrifugation at 3000 rpm for 20 min at 4°C and stored at −80°C until use. Animals were euthanized by administration of high concentration of CO2. Tissues were dissected, frozen in liquid nitrogen, and stored at −80°C. Simultaneously, one slice from each kidney tissue sample was kept into 10% buffered formalin overnight. Immediately thereafter, these tissues were processed for histological studies.

#### Assay of albumin and creatinine from urine and plasma

Albumin levels were measured in 24-h urine samples, collected from a metabolic cage, using the ELISA kit from Bethyl Laboratories. ELISA plates were first coated with goat anti-mouse albumin for 1 h at room temperature. The plate was then blocked with postcoat solution for 30 min. After washing, 100 μl diluted urine samples and plasma albumin standards were added to the plate. The reaction was stopped, and horseradish peroxidase antibody and its substrate were added. The amount of albumin in the urine was calculated from a standard curve based on a four-parameter fit curve in Fluostar plate reader. Plasma and urine creatinine concentrations were measured using the creatinine assay kit (BioAssay Systems) as previously described (25). The absorbance of the samples was recorded at 492 nm using a Fluostar plate reader. Creatinine levels were calculated according to the manufacturer’s protocol. Creatinine clearance rate (CCR) was calculated from the creatinine concentration in the collected urine samples, plasma concentration, and urine volume and expressed as ml/24 h.

#### Assay of renal cGMP

Frozen kidney tissue samples were homogenized in 10 vol of 0.1 M HCl containing 1% Triton X-100. The homogenate was...
heated at 95°C for 5 min and centrifuged at 600 × g at 22°C, after which the supernatant was collected. cGMP levels in kidney tissue samples were analyzed using a direct cGMP immunoassay kit as described previously (26, 27). The results are expressed as picomoles of cGMP per milligram of protein.

Morphological studies

Kidney tissues from each group were fixed in 10% buffered paraformaldehyde solution. Paraffin-embedded tissue sections (5 μm) were stained with hematoxylin and eosin (H&E) and with Masson’s trichrome for the presence of interstitial collagen fiber accumulation as a marker of renal fibrosis. The percentage of the matrix mesangial expansion relative to the total kidney area was determined by calculations made in 20 randomly selected microscopic fields in five sections per kidney for renal injury using ImagePro Plus image analysis software (Media Cybernetics, Silver Spring, MD). The ratio of fibrosis to the total kidney area was determined.

Immunohistochemistry of PCNA and α-SMA

Immunochemical staining was done on 5-μm sections of paraffin-embedded kidney tissues. Briefly, after dewaxing in xylene, the kidney sections were dehydrated by serial dilutions of alcohol (100, 90, and 70%) and then treated with absolute methanol and 0.3% H2O2 for 30 min to block peroxidase activity. After washing in PBS for 20 min, the sections were sequentially incubated at room temperature with normal blocking serum (goat serum) for 20 min, primary antibody (mouse monoclonal PCNA and α-SMA) diluted in PBS containing 1% BSA at 1:2000 dilution overnight at 4°C, secondary antibody (biotinylated goat antimouse IgG) for 30 min, and avidin-biotin horseradish peroxidase complex for 45 min, using the ABC staining kit (Santa Cruz). Peroxidase activity was visualized with 0.1% 3,3′-diaminobenzidinehydrochloride. The slides were then washed in tap water, counterstained with hematoxylin, mounted using aqueous mounting medium, and coverslipped. Immunohistochemically stained slides were visualized, and the percentage of PCNA and α-SMA-positive area to the total kidney were calculated using an Olympus BX51 camera and photographed with integrated Magnafire SP Digital Firewire camera software.

Semiquantitative RT-PCR

The primers used for TGF-β and GAPDH expression were as follows: TGF-β (5′-CTGCTGGCACAAGCATGTC-3′ (sense) and 5′-GTCCAGGCTTCAATATATAG-3′ (antisense)); those for α-SMA were 5′-AGATTTGTCGGTCACT-3′ (sense) and 5′-TTGGTGCTGACGACGCT-3′ (antisense). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control with 5′-TCCCTAGAATGTCGACGA-3′ (sense) and 5′-AGATCCACAAACGGAATA-3′ (antisense) primers. The PCR product was electropho-
resed on 2% agarose gel and quantitated by densitometry using the Alpha Innotech Imaging System.

**Ribonuclease protection assays**

The custom-made multiprobe template set for TNF-α, IL-6, collagen α-1, and the GAPDH and L32 housekeeping genes were used for the RPA. The multiprobe template set was labeled with [α-32P]UTP, using T7 RNA polymerase according to the manufacturer’s protocol (BD Biosciences). Labeled probe was allowed to hybridize with 20 μg total RNA at 56 °C for 16 h. The protected hybrid band was resolved on 5% denaturing polyacrylamide gel and exposed to radiographic film overnight at −80 °C. Densitometric analysis was done using the Alpha Innotech Imaging System.

**Antihypertensive drug treatments**

Adult Npr1 homozygous null mutant (Npr1−/−) and age-matched wild-type (Npr1+/+) mice at 4 months of age were used. All the animals were subdivided into four groups. Mice in group I were kept as positive control. Group II mice received captopril (5 mg/kg·d). Group III mice received losartan (25 mg/kg·d), and group IV mice received bendroflumethiazide (10 mg/kg·d) as oral gavage once a day for 30 d. Systolic blood pressure (SBP) was measured with Dunnett’s multiple comparisons test. We also used Student’s t test for comparison between two groups. A P value of <0.05 was considered significant.

**Statistical analysis**

All data examined are expressed as mean ± SEM. Differences between groups were determined using one-way analyses of variance with Dunnett’s multiple comparisons post hoc test. We also used Student’s t test for comparison between two groups. A P value of <0.05 was considered significant.

**Results**

Blood pressure measurements showed significant (P < 0.01) increases in SBP to 23 ± 3 mm Hg in Npr1+/− (1-copy; 124.6 ± 4.32 mm Hg) and 38 ± 4 mm Hg in Npr1−/− (0-copy; 139.4 ± 4.80 mm Hg) mice compared with the Npr1+/+ (2-copy; 101.2 ± 3.12 mm Hg) mice. We found a significant (P < 0.05) increase in the ratio of kidney weight to body weight (7.99 ± 0.22) in 0-copy mice compared with their controls (7.17 ± 0.17). Plasma creatinine levels were increased in 1-copy (0.37 ± 0.02 mg/dl; 65%; P < 0.05) and in 0-copy (0.54 ± 0.04 mg/dl; 2-fold; P < 0.01) mice compared with 2-copy (0.22 ± 0.05 mg/dl) mice. We also found a significant (P < 0.01) increase in urinary albumin levels in 1-copy (3-fold; 10.36 ± 1.60 mg/dl) and 0-copy (5-fold; 17.15 ± 2.90 mg/dl) mice compared with wild-type controls (3.36 ± 0.93 mg/dl). On the other hand, the CCR was reduced significantly by 48% in 1-copy (128.2 ± 20.2 ml/24 h; P < 0.01) and by 62% in 0-copy (92.7 ± 19.6 ml/24 h; P < 0.01) mice when compared with wild-type mice (244.7 ± 18.5 ml/24 h). Collagen content was significantly increased by 30% (P < 0.05) in 1-copy (2.03 ± 0.12 μg/g body weight) and by 2-fold (P < 0.01) in 0-copy (2.64 ± 0.16 μg/g body weight) mice compared with 2-copy (1.57 ± 0.05 μg/g body weight) mice. Moreover, the renal cGMP level was reduced progressively with the reduction in Npr1 gene copy number by 3-fold (P < 0.01) in 1-copy (6.36 ± 0.79 pmol/mg protein) and 6-fold (P < 0.001) in 0-copy (3.09 ± 0.63 pmol/mg protein) mice compared with 2-copy wild-type mice (18.60 ± 1.30 pmol/mg protein).

After staining kidney sections from each group of mice with H&E, we observed increased renal tubular damage in 1-copy and 0-copy mice compared with 2-copy mice (Fig. 1, A–C). This damage was characterized by tubular dilatation and perivascular region in 1-copy and 0-copy mice. D and H, Semiquantitative analysis of kidney sections stained with H&E and Masson’s trichrome from Npr1 gene-disrupted and wild-type mice. A and E, Corresponding photographs from wild-type (+/+) mice; B and F, corresponding photographs from 1-copy (+/−) mice; C and G, respective photographs from 0-copy (−/−) mice. B and C show a marked increase in MME (shown by arrow) in 1-copy and in 0-copy mice. F and G show an increased accumulation of collagen fibers at perivascular region in 1-copy and 0-copy mice. D and H, Semiquantitative measurements of MME changes and the percent collagen deposition, respectively. ***, P < 0.01; ***, P < 0.001. In each group, five mice were used.
tion with flattened epithelium (Fig. 1, B and C). In parallel, progressive expansion of the interstitial spaces, referred to hereafter as matrix mesangial expansion (MME), was pronounced in kidney sections from 1-copy (Fig. 1B) and 0-copy (Fig. 1C) mice. The percentage of MME was 6- and 10-fold higher in 1-copy and 0-copy mice, respectively, compared with 2-copy wild-type animals (Fig. 1D). On the other hand, kidney sections stained with Masson’s trichrome showed progressive increase in collagen deposition in the interstitial spaces in 1-copy and 0-copy mice compared with 2-copy mice (Fig. 1, E–G). The percentage of blue stained area (renal fibrosis) was evident in 1-copy (2.5-fold) and 0-copy (10-fold) mice compared with wild-type control animals (Fig. 1H). The results of immunohistochemical analysis for PCNA showed a significant in-
crease in PCNA-positive cells in the glomeruli of 1-copy ($P < 0.01$) and 0-copy ($P < 0.001$) mice as compared with 2-copy wild-type mice (Fig. 2, A–C). The percentage of glomeruli with PCNA-positive cells was increased by 4-fold ($P < 0.01$) in 1-copy mice and by 8-fold ($P < 0.001$) in 0-copy mice (Fig. 2D). A significant increase in α-SMA immunoeexpression was also found in the proximal and distal tubule cells and in arterioles of 1-copy and 0-copy mice as compared with 2-copy wild-type mice (Fig. 2, E–G). The percentage of α-SMA-positive stained cells was increased by 5-fold in 1-copy and by 10-fold in 0-copy mice compared with 2-copy mice (Fig. 2H). On the other hand, the renal PCNA protein expression was increased by 2-fold in 1-copy mice and 3-fold in 0-copy mice as compared with 2-copy counterparts (Fig. 3, A and B). The present study also showed an increased α-SMA mRNA expression in 1-copy (2-fold) and in 0-copy (3-fold) mice compared with 2-copy mice (Fig. 3, C and D). A 2-fold increase in α-SMA protein in kidney tissues was found in 0-copy mice; however, only a minor increase was observed in 1-copy mice (Fig. 3, E and F).

As shown in Fig. 4A, plasma TNF-α levels were significantly increased by 3-fold ($6.25 \pm 0.37 \text{ pg/ml, } P < 0.05$) in 1-copy mice and 6-fold ($12.8 \pm 0.43 \text{ pg/ml, } P < 0.01$) in 0-copy mice as compared with the level in 2-copy mice ($2.76 \pm 0.32 \text{ pg/ml}$). The TNF-α concentration in kidneys was also increased by 40% in 1-copy mice (15.24 \pm 0.67 pg/mg protein, $P < 0.05$) and by 2-fold in 0-copy mice.

![Fig. 2](image-url)  
**FIG. 2.** Immunohistochemical analysis of kidney sections for PCNA and α-SMA from Npr1 gene-disrupted and wild-type mice. A and E, Corresponding photographs from wild-type (+/+) mice; B and F, corresponding photographs from 1-copy (+/−) mice; C and G, respective photographs from 0-copy (−/−) mice. B and C show a marked increase in PCNA-positive cells (shown by arrow) in 1-copy and in 0-copy mice. F and G show a significant increase in α-SMA immunoeexpression in 1-copy and 0-copy mice. D and H, Semiquantitative measurements of percent positive cells for PCNA and α-SMA, respectively. ***, $P < 0.01$; ***, $P < 0.001$. In each group, five mice were used.

![Fig. 3](image-url)  
**FIG. 3.** Quantitative analysis of renal PCNA and α-SMA expression in Npr1 homozygous mutant (0-copy; n = 8), heterozygous (1-copy; n = 7), and wild-type (2-copy; n = 8) mice. A and B, PCNA protein expression and its densitometric measurements; C and D, mRNA expression of α-SMA and its densitometric values; E and F, α-SMA expression and densitometric quantitation by Western blot analysis. ***, $P < 0.01$; ***, $P < 0.001$. 

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Compared with wild-type mice (10.68 ± 0.83 pg/mg protein) (Fig. 4B). As shown in Fig. 4, C and D, there was a significant (P < 0.01) increase in plasma as well as renal IL-6 levels in 0-copy mice (48.01 ± 3.53 pg/ml and 91.61 ± 6.13 pg/mg protein, respectively) in comparison with 2-copy animals (16.83 ± 1.75 pg/ml and 36.94 ± 4.92 pg/mg protein, respectively). However, in 1-copy mice, only the renal IL-6 level was significantly increased (56.40 ± 4.13 pg/mg protein) as compared with the levels in 2-copy mice. Using Western blot analysis, we found that TNF-α protein levels in the kidney were significantly increased by 2-fold (P < 0.01) in 1-copy and 4-fold in 0-copy (P < 0.001) mice as compared with levels in wild-type mice (Fig. 4, E and F). IL-6 protein expression was also significantly increased by 3-fold (P < 0.01) in 1-copy and 4.5-fold in 0-copy (P < 0.001) mice (Fig. 4, G and H). In addition, increased accumulation of infiltrating mononuclear cells, which is a marker for inflammation were observed in kidney sections from both 1-copy and 0-copy mice compared with 2-copy mice (Fig. 5, A–C). There was a significant increase in focal accumulation of inflammatory cells at the juxtaglomerular region and at the perivascular areas in the kidney sections of 1-copy (10-fold) and 0-copy (25-fold) mice (Fig. 5D). Furthermore, ED1-positive cells (a marker for monocyte/macrophages) were found localized at the...
glomerular tuft as well as at the tubular epithelial cells in the kidney sections of 1-copy and 0-copy mice compared with 2-copy control mice (Fig. 5, E–G). The number of ED1-positive cells were significantly higher in 1-copy (5-fold) and in 0-copy (9-fold) mice compared with control animals (Fig. 5H).

TGF-β1 level in kidney tissues was increased by 2-fold in 1-copy mice (442.6 ± 29.2 pg/mg protein) and by 3-fold (769.4 ± 64.0 pg/mg protein) in 0-copy mice compared with the level in wild-type control mice (229.8 ± 17.3 pg/mg protein). TGF-β1 mRNA expression level was significantly enhanced by 1.5-fold in 1-copy (P < 0.05) and 2.5-fold in 0-copy mice compared with 2-copy animals (Fig. 6, A and B). Similarly, TGF-β1 protein expression was also up-regulated by 65% in 1-copy and by 2-fold in 0-copy mice (Fig. 6, C and D). Furthermore, we examined the kidney mRNA expression levels of TGF-α, IL-6, and collagen by RPA in all three groups of Npr1 mice (Fig. 6, E and F). The mRNA expression levels of TNF-α and IL-6 were greatly increased in 0-copy (9-fold) and 1-copy (4.5-fold) mice compared with 2-copy mice (Fig. 6F). However, the collagen α-1 mRNA expression level was significantly enhanced in 0-copy mice (6-fold; P < 0.001), whereas only a small increase was detected in 1-copy mice (2-fold; P < 0.05) compared with 2-copy wild-type mice (Fig. 6F).

Administration of captopril, losartan, and bendroflumethiazide significantly reduced the SBP in 0-copy mice (107.6 ± 2.92, 106.8 ± 3.76, and 113.8 ± 3.29 mm Hg, respectively) as compared with control mice (138.2 ± 4.09 mm Hg), and there was no significant difference between the three drug classes (Table 1). The treatment with captopril and losartan significantly reduced renal fibrosis in 0-copy mice; however, bendroflumethiazide treatment did not show any significant change in renal fibrosis of null mutant mice (Fig. 7A). The quantitative analysis showed that in captopril- and losartan-treated 0-copy mice, the renal fibrosis was reduced by 38 and 46%, respectively, as compared with untreated Npr1 null mutant mice; however, bendroflumethiazide treatment did not produce any significant change (Table 1). The immunoreactivity of α-SMA was pronounced in 0-copy mice as compared with wild-type control mice (Fig. 7B), with the quantitative analysis showing a 10-fold increase in α-SMA-positive cells in 0-copy mice compared with 2-copy mice. Captopril and losartan treatment significantly reduced α-SMA immunoreactivity in Npr1-null mutant mice; however, bendroflumethiazide did not elicit any significant effect (Fig. 7B). After treatment with captopril and losartan, α-SMA-positive cells were reduced by 30 and 42%, respectively, in 0-copy mice compared with untreated null mutant mice (Table 1).

Discussion

The present results demonstrate that Npr1 gene disruption causes renal fibrosis, hypertrophy, and extracellular matrix remodeling in mice with decreasing Npr1 gene copy numbers. We observed a significant increase in the kidney weight to body weight ratio, plasma creatinine concentrations and urinary albumin excretion, together with a
TABLE 1. Effect of antihypertensive drug treatment on blood pressure, fibrosis, and α-SMA expression in 2-copy and 0-copy mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Untreated</th>
<th>Captopril</th>
<th>Losartan</th>
<th>BFM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2-copy</td>
<td>0-copy</td>
<td>2-copy</td>
<td>0-copy</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>104.0 ± 2.60</td>
<td>138.2 ± 4.09</td>
<td>95.2 ± 3.54</td>
<td>107.6 ± 2.92</td>
</tr>
<tr>
<td>Fibrosis (%)</td>
<td>4.85 ± 0.99</td>
<td>35.98 ± 2.04</td>
<td>5.21 ± 1.36</td>
<td>22.21 ± 2.06</td>
</tr>
<tr>
<td>α-SMA (%)</td>
<td>2.65 ± 1.05</td>
<td>24.38 ± 2.80</td>
<td>2.45 ± 1.15</td>
<td>16.73 ± 1.20</td>
</tr>
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SBP, renal fibrosis, and α-SMA were determined according to the method described in Materials and Methods. The animals were either untreated or treated with captopril (5 mg/kg · d), losartan (25 mg/kg · d), and bendroflumethiazide (BFM; 10 mg/kg · d). Values are expressed as mean ± se. In each group, six mice were used for each genotype.

* P < 0.001 (untreated 2-copy vs. untreated 0-copy mice).

b P < 0.01 (untreated 0-copy vs. captopril-treated 0-copy mice).

c P < 0.01 (untreated 0-copy vs. losartan-treated 0-copy mice).

d P < 0.05 (untreated 0-copy vs. captopril-treated 0-copy mice).

Reduced CCR, which suggested an incidence of renal pathology in Npr1-null mutant mice. Increased plasma creatinine is associated with renal pathologies of tubulo-interstitial fibrosis, IgA-induced nephropathy, and polycystic kidney disease (25, 29–31). Recent findings demonstrated that monocytes and/or macrophages are overexpressed in chronic renal failure, and in turn, these cells amplify, leading to fibrosis (32). The present results showed an increased accumulation of inflammatory cells and significant increases in MME in the kidneys of 0-copy and 1-copy mice. Previous findings have suggested that the development of glomerular hypertrophy with thickening of the glomerular basement membrane seems to be proportional to glomerular enlargement and MME in renal disease states (33–36).

The present study demonstrated significant increases in both mRNA levels and plasma and tissue contents of TNF-α and IL-6 in 0-copy and 1-copy mice as compared with 2-copy mice. TNF-α has been shown to contribute to the chronic inflammation and proliferation of mesangial cells that often precedes interstitial matrix deposition and has also been implicated in obstruction-induced renal injury (37, 38). The neutralization of TNF-α ameliorates obstruction-induced renal fibrosis and dysfunction (39). Consistent with these findings, we observed concomitant rises in plasma and tissue levels of TNF-α and IL-6 in both 0-copy and 1-copy compared with wild-type mice. The increased levels of TNF-α and IL-6 appear to enhance the inflammatory state of the kidney and might lead to increased synthesis of TGF-β1, which is indicative of persistent inflammation. We found a significant (P < 0.01) increase in TGF-β1 mRNA and protein expression in both 0-copy and 1-copy mice as compared with wild-type animals. The previous reports have indicated that an increased TGF-β1 protein expression exhibited a regulatory effect on the development of MME in the kidney, and its inhibition with neutralizing antibodies prevented glomerular enlargement and attenuated excess matrix expression (40–42).
A marked reduction in cGMP levels was observed in 0-copy and 1-copy mouse kidney as compared with 2-copy wild-type mouse kidney. The activation of the ANP/NPRA system increases cGMP production in mesangial and vascular smooth muscle cells and is positively correlated with antiproliferative effects (16, 19, 20, 23). Thus, cGMP has been suggested as an important mediator of the ANP/NPRA signaling system. Here, our observation of an increased induction of PCNA in kidney tissues reflected renal hypertrophy in Npr1 gene-disrupted mice. Although there were increases in the percentages of glo- 

Meruli with PCNA-positive cells in the kidneys of both 0-copy and 1-copy mice, the severity of the increase was much pronounced in 0-copy animals.

Our data demonstrate that the development of both histological and functional changes in the kidney correlate with the progressive hypertrophy and fibrosis in Npr1-null mutant mice. To delineate the influence of increased blood pressure on renal remodeling and fibrosis, we treated 0-copy mice with three different antihypertensive drugs (captopril, losartan, and bendroflumethiazide). All three drugs significantly reduced blood pressure in 0-copy mice without significant difference between the drug classes. Interestingly, normalization of SBP seems to have a partial effect in reversing the renal fibrosis only in capto- 

Pril- and losartan-treated mice. The increased numbers of α-SMA-positive cells are considered as a major source of profibrotic cytokines and have also been implicated to cause tubulointerstitial renal fibrosis (43). Simultaneously, treatment with captopril and losartan also showed a partial attenuation in α-SMA immunoexpression (a marker for epithelial mesenchymal transition) in 0-copy mice. However, administration of bendroflumethiazide significantly lowered the SBP but did not show any salutary effect in either reversing renal fibrosis or reducing α-SMA immunooexpression in 0-copy mice.

It is conceivable that there is some residual renal damage despite treatment with three different antihypertensive agents in Npr1-null mutant (0-copy) mice compared with untreated groups. It is implicated that the ANP/NPRA/cGMP system exerts an inhibitory effect on the activation of proinflammatory and profibrotic cytokines. However, in the absence of an ANP/NPRA/cGMP signaling cascade, both proinflammatory and profibrotic cytokine genes are significantly activated to produce renal fibrosis and hypertrophy to a great extent in Npr1 0-copy mice, independent of elevated blood pressure. Thus, in the present study, the residual renal damage in 0-copy mice, despite the antihypertensive drug treatments, suggest that Npr1 gene disruption provokes a direct effect by eliciting early gene activation on renal hypertrophy and fibrosis, independent of blood pressure in null mutant mice.

In summary, the present study provides direct evidence that Npr1 gene disruption causes fibrosis in renal tissues. It also demonstrates that the event that causes fibrosis is dramatically increased with decreasing Npr1 gene copy numbers. Moreover, up-regulation of proinflammatory cytokines such as TNF-α and IL-6 as well as the profibrotic cytokine TGF-β1 was observed in the kidneys of Npr1 gene-disrupted mice. Additionally, treatment with captopril and losartan preferentially elicited improvement in renal fibrosis and remodeling in Npr1-null mutant mice.

Acknowledgments

We thank Mrs. Kamala Pandey for assistance in the preparation of this manuscript. We are indebted to Dr. Oliver Smithies for providing initial breeding pairs of Npr1 gene-targeted mice colonies. Our special thanks are due 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 M. D. Anderson Cancer Center for providing their facilities during our displacement period due to Hurricane Katrina.

Disclosure Summary: There is no disclosure to be made.

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