Genetic disruption of guanylyl cyclase/natriuretic peptide receptor-A upregulates ACE and AT1 receptor gene expression and signaling: role in cardiac hypertrophy

Elangovan Vellaichamy, Di Zhao, Naveen Somanna, and Kailash N. Pandey

Department of Physiology, Tulane University School of Medicine, New Orleans, Louisiana

Submitted 7 April 2007; accepted in final form 8 June 2007

Vellaichamy E, Zhao D, Somanna N, Pandey KN. Genetic disruption of guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA) signaling antagonizes the physiological effects mediated by the renin-angiotensin system (RAS). The objective of this study was to determine whether the targeted-disruption of Npr1 gene (coding for GC-A/NPRA) leads to the activation of cardiac RAS genes involved in the hypertrophic remodeling process. The Npr1 gene-knockout (Npr1-/-) mice showed 30–35 mmHg higher systolic blood pressure (SBP) and a 63% greater heart weight-to-body-weight ratio compared with wild-type (Npr1+/-) mice. The mRNA levels of both angiotensin-converting enzyme and angiotensin II type 1a receptor were increased by three- and fivefold, respectively, in Npr1-/- null mutant mice hearts compared with the wild-type Npr1+/- mice. In parallel, the expression levels of interleukin-6 and tumor necrosis factor-α were increased by four- to fivefold, in Npr1-/- mice hearts compared with control animals. The NF-kB binding activity in nuclear extracts of Npr1-/- mice hearts was increased by fourfold compared with wild-type Npr1+/- mice hearts. Treatments with captopril or hydralazine equally attenuated SBP; however, only captopril significantly decreased the HW/BW ratio and suppressed cytokine gene expression in Npr1-/- mice hearts. The ventricular cGMP level was reduced by almost sixfold in Npr1-/- mice compared with wild-type control mice. The results of the present study indicate that disruption of NPRA/cGMP signaling leads to the augmented expression of cardiac RAS pathways that promote the development of cardiac hypertrophy and remodeling.

angiotensin-converting enzyme; angiotensin II type 1 receptor; hypertension; cardiac hypertrophy; angiotensin II; cytokines

CARDIAC HORMONES, atrial and brain natriuretic peptides (ANP and BNP) are released into the circulation and elicit natriuretic, diuretic, vasorelaxant, and antiproliferative responses, all directed to the reduction of blood pressure and blood volume (6, 14, 24, 53). Both ANP and BNP bind to guanylyl cyclase-A/natriuretic peptide receptor A (GC-A/NPRA), which is considered the principal natriuretic peptide hormone receptor that synthesizes intracellular second messenger cGMP (10, 38, 42). Recent studies have suggested that NPRA signaling also locally antagonize cardiac growth responses to hypertrophic stimuli (4). In particular, mice carrying targeted-disruption of the Npr1 gene (encoding for NPRA) exhibit hypertension, and congestive heart failure, with sudden death occurring after 6 mo of age (34, 63). On the other hand, overexpression of NPRA reduces the blood pressure and, specifically in myocardial cells, attenuates hypertrophic agonist-induced myocyte growth (33, 66). ANP gene delivery has also been shown to attenuate cardiac hypertrophy in spontaneously hypertensive rats (21). Nevertheless, the cellular mechanisms by which the ANP/NPRA system blocks the hypertrophic growth is not well understood.

Angiotensin II (ANG II), the main active component of the renin-angiotensin system (RAS), plays an important role in the cardiovascular remodeling process associated with hypertension (2, 29, 43, 44). ANG II is implicated in the development of cardiac hypertrophy and cardiac fibrosis in humans and in experimental animal models (44, 48, 49, 65). Most effects of ANG II in the cardiovascular system are mediated through ANG II type I (AT1) receptor (58, 61, 65). Treatment with angiotensin-converting enzyme (ACE) inhibitors or AT1 blockers effectively lowers blood pressure (BP) and prevents or ameliorates myocardial hypertrophy (43, 44, 55). In addition, ANG II is considered to be a proinflammatory mediator that has a pivotal function in the inflammatory process underlying the development of vascular complications (54, 61). ANG II has also been shown to mediate hypertrophic growth in neonatal and adult myocytes by activating the interleukin-6 (IL-6) (16, 50).

ANG II has also been shown to inhibit the ANG II-mediated induction of protein kinase (PKC) and mitogen-activated protein kinases (MAPK) in vascular smooth muscle and mesangial cells (5, 23, 41). Conversely, ANG II modulates the transcriptional regulation of Npr1 gene transcription (1, 15). In addition, ANP/NPRA is reported to have an anti-inflammatory role, inhibiting TNF-α production in interferon-γ-activated macrophages and inhibiting TNF-α-induced adhesion molecule expression in endothelial cells (59, 64). In the present studies, we determined whether the disruption of the GC-A/NPRA signaling pathway activates cardiac RAS components and inflammatory mediators in the ventricular tissues of mice lacking NPRA. To this end, we have analyzed the cardiac vascular phenotypes, ventricular expression of cytokines, and RAS gene activation in Npr1-/- null mutant mice with and without antihypertensive drug treatments.

MATERIALS AND METHODS

**Materials.** Custom multiprobe set containing IL-6 and TNF-α and RNase protection assay (RPA) kits were obtained from BD Biosciences (San Diego, CA). [α-32P]UTP (3,000 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ). Proinflammatory cytokine (TNF-α and IL-6) ELISA kits were obtained from Pierce Endogen (Rockford, IL). Captopril (CAP) and hydralazine (HYZ) were obtained from Sigma (St. Louis, MO). TRIZol reagent
was purchased from Life Technologies/Invitrogen (Carlsbad, CA) and a RETROscript kit from Ambion (Austin, TX). Antibodies and oligonucleotides for NF-κB were obtained from Santa Cruz Biotechnology (San Diego, CA). All other chemicals were reagent grade.

Animals and drug treatments. Npr1 gene-disrupted mice were generated by homologous recombination in embryonic stem cells as previously described (34). Animals were bred and maintained at the vivarium facility of Tulane University Health Sciences Center and handled under protocols approved by the Institutional Animal Care and Use Committee. The mice were housed in a 12-h light-dark cycle at 25°C; they were given regular chow (Purina Laboratory) and tap water ad libitum. The Npr1 genotypes used were littermate progenies of C57BL6 genetic background and have been designated as Npr1 gene-disrupted and wild-type allelic (Npr1+/−) and wild-type allele (Npr1+×+). Experiments were performed on three groups of 4-wk-old Npr1+/− and Npr1+×+ mice (n = 16/group): group I consisted of wild-type and mutant mice and was used as a positive control; in group II, both wild-type and mutant mice received HYZ, 25 mg/kg day−1; in group III, both wild-type and mutant mice received CAP, 5 mg/kg day−1. The drugs were given orally by gavage once a day for 8 wk.

BP, cardiac hypertrophy, and fibrosis analyses. BPs were measured by a noninvasive computerized tail-cuff method as described previously (52, 53). Animals were killed by cervical dislocation. The sacrifice was carried out using a custom-made multiprobe template set for 28S and 18S bands after electrophoresis on 1.5% agarose gel. RPA for 30 min. RNA integrity was confirmed by visualization of distinct 28S and 18S bands after electrophoresis on 1.5% agarose gel. RPA probes were treated with RNase-free DNase I (1 unit/μg RNA) at 37°C for 30 min. RNA integrity was confirmed by visualization of distinct 28S and 18S bands after electrophoresis on 1.5% agarose gel. RPA was carried out using a custom-made multiprobe template set for TNF-α, IL-6, and the housekeeping genes GAPDH and L-32. The results of ACE and AT1a expression were normalized for equal loading by GAPDH amplification.

Ventricular cGMP assay. Frozen ventricular tissue samples were homogenized in 10 volumes of 0.1 M HCl containing 1% Triton X-100. Homogenate was heated at 95°C for 5 min and centrifuged at 600 g for 20 min at 22°C. The supernatants were collected and stored at −80°C until use in a cGMP assay. Ventricular tissue cGMP levels were analyzed by using a direct cGMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI).

Analysis of NIG II and thiobarbituric acid reactive substance. The concentration of ANG II was determined essentially as described previously (39). Briefly, ventricular extracts were applied to Sep-Pak cartridges (Sep-Pak C-18 columns; Waters Associates, Milford, MA), which were equilibrated with 0.1% trifluoroacetic acid (TFA). The cartridges were washed with 10 ml of 0.1% TFA-1% NaCl and were eluted with 2 ml of a mixture of methanol-water-TFA (80:19:0.1). The eluates were dried overnight with a speed-vac centrifuge. The residues were then dissolved in 100 mM Tris-acetate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.02% sodium azide, and 0.1% bovine serum albumin. Immunoreactive ANG II was measured using an RIA kit (Peninsula, Belmont, CA). Generation of reactive oxygen species (ROS) as evidenced by thiobarbituric acid reactive substance (TBARS) levels in the ventricular tissues were analyzed essentially by published methods (32). The pink-colored chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was measured colorimetrically at 532 nm. Malondialdehyde (MDA) was used as a standard, and the results are expressed as nmol MDA formed/mg protein.

Electrophoretic mobility shift assay. Nuclear and cytosolic proteins were extracted from mice hearts by the method of Dignam et al. (8). An electrophoretic mobility shift assay was performed as described previously (63). Nuclear proteins were incubated for 20 min at room temperature in 5 μl of binding buffer [50 mM Tris, pH 8.0; 750 mM KCl; 2.5 mM EDTA; 0.5% Triton-X 100; 62.5% glycerol (vol/vol); and 1 mM DTT] containing 2 μg of poly(dI-dC), 50,000 cpm radiolabeled NF-κB-specific oligonucleotides. The DNA-protein complex was resolved from the free-labeled DNA by electrophoresis using 4% (wt/vol) native polyacrylamide gel (PAGE) and autoradiography.

Inhibitory κB kinase-β activity assay. An assay of inhibitory κB kinase-β (IKK-β) activity was carried out by the method previously reported (63). Cytoplasmic proteins (200 μg) from the left ventricular tissues of Npr1 wild-type and mutant mice were immunoprecipitated with 2 μg of IKK-β antibody at 4°C for 1 h. Protein A-agarose beads were added to the supernatant and the precipitated protein complexes were eluted with 2 ml of a mixture of methanol-water-TFA (80:19:0.1). The eluates were dried overnight with a speed-vac centrifuge. The residues were then dissolved in 100 mM Tris-acetate buffer (pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.02% sodium azide. The protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Finally, the IKK-β activity was measured using an RIA kit (Peninsula, Belmont, CA).

Table 1. Systolic blood pressure, heart rate, heart weight-to-body weight ratio, cardiac collagen content, and fibrosis in Npr1+/− and Npr1−/− mice treated with and without antihypertensive drugs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Untreated</th>
<th>Hydralazine</th>
<th>Captorpl</th>
<th>cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Npr1+/−</td>
<td>Npr1−/−</td>
<td>Npr1+/−</td>
<td>Npr1−/−</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>100±4</td>
<td>132±8**</td>
<td>97±3</td>
<td>110±6***††</td>
</tr>
<tr>
<td>HW/BW ratio</td>
<td>4.6±0.3</td>
<td>7.5±0.5*</td>
<td>4.7±0.4</td>
<td>7.1±0.7**</td>
</tr>
<tr>
<td>Wt/g</td>
<td>0.1±0.02</td>
<td>0.25±0.07***</td>
<td>0.1±0.03</td>
<td>0.22±0.5**</td>
</tr>
<tr>
<td>MCA, μM²</td>
<td>415±22</td>
<td>915±73***</td>
<td>413±18</td>
<td>825±34**</td>
</tr>
<tr>
<td>Collagen, mg/g tissue</td>
<td>1.4±0.1</td>
<td>3.5±0.7***</td>
<td>1.3±0.3</td>
<td>2.4±0.2**</td>
</tr>
<tr>
<td>Fibrosis, %</td>
<td>0.8±0.3</td>
<td>25±2.7***</td>
<td>0.9±0.3</td>
<td>24±2.2***</td>
</tr>
</tbody>
</table>

Collagen concentration was quantified by measuring hydroxyproline measurements. Systolic blood pressure (SBP), heart weight-to-body weight ratio (HW/BW), wall-to-lumen ratio (Wt/g), and myocyte cross-sectional area (MCA) were measured as described in MATERIALS AND METHODS. Values are expressed as means ± SE (n = 8 animals/group). *P < 0.05, **P < 0.1, and ***P < 0.001; Npr1+/− vs. Npr1−/−; †P < 0.05, ††P < 0.01, Untreated Npr1−/− vs. antihypertensive drug treated Npr1−/− mice.
Histopathology and immunohistochemistry. Heart tissues were fixed in 4% paraformaldehyde solution. Then 5-μm thick paraffin-embedded tissue sections were stained with Masson’s trichrome for the presence of interstitial and perivascular collagen fiber accumulation as a marker of cardiac fibrosis. The ratio of interstitial fibrosis to the total left ventricular area was calculated from 20 randomly selected fields in five individual sections per heart, using image analysis software (Image-Pro Plus, MediaCybernetics, Silver Spring, MD). Hematoxylin- and eosin-stained heart sections were used to determine the myocyte cross-sectional area and to determine vascular wall thickening using Image-Pro Plus software. For immunohistochemistry, heart sections were perfused without fixation. The tissues were immediately embedded in tissue freezing medium (O.C.T compound, Marivac), frozen, and cut into 5-μm thick slices. The sections were preincubated with blocking rabbit serum for 20 min, then treated for 90 min with specific primary antibodies (AT1a receptor and NF-κB), frozen, and cut into 5-μm thick slices. The sections were preincubated with blocking rabbit serum for 20 min, then treated for 90 min with specific primary antibodies (AT1a receptor and NF-κB) and then washed and incubated with secondary biotin-conjugated rabbit anti-

RESULTS

Systolic blood pressure, cardiac hypertrophy, and fibrosis. Systolic blood pressure (SBP), ratio of heart weight to body weight (HW/BW), and fibrosis in Npr1+/+ and Npr1−/− mice hearts are shown in Table 1. The SBP of Npr1−/− mice was 32 ± mmHg higher than that in Npr1+/+ mice (132 ± 5 vs. 100 ± 4 mmHg). The HW/BW ratio was also significantly higher in Npr1−/− mice (7.5 ± 0.5, P < 0.01) than in Npr1+/+ mice (4.6 ± 0.3). In addition, significantly greater concentrations of total collagen and fibrosis (P < 0.001) were found in Npr1−/− mutant mice hearts compared with the hearts of age-matched wild-type control mice. Both CAP and HYZ treatments significantly reduced the elevated BP in Npr1+/+ mice (106 ± 4 and 110 ± 5, respectively; P < 0.01) compared with that of untreated Npr1−/− mice. CAP treatment alone significantly attenuated the HW/BW ratio (P < 0.05), coronary vessel wall thickening (P < 0.05) and myocyte cross-sectional area (P < 0.01) in the mutant mice compared with untreated

mutant mice (Table 1). However, HYZ treatment did not significantly reduce the coronary vessel wall thickening and hypertrophy in Npr1−/− mice.

ACE and AT1a receptor gene expression. The representative RT-PCR analyses of mRNA expression of ACE and AT1a

Fig. 1. Expression profiles of angiotensin-converting enzyme (ACE) and ANG II type I (AT1) genes in Npr1+/+ and Npr1−/− mice hearts. A and C: representative RT-PCR mRNA expression analysis of ACE and AT1a in Npr1+/+ and Npr1−/− mice with and without antihypertensive drug treatments. B and D: densitometry analysis of mRNA transcripts normalized to the expression of GAPDH. RT-PCR reaction was performed utilizing genespecific primers. Values are expressed as means ± SE, (n = 8/group). ***P < 0.001, Npr1+/+ vs. Npr1−/−; untreated Npr1−/− vs. antihypertensive drugs treated Npr1−/−. UT (untreated), HYZ (hydralazine), and CAP (captopril).
receptor in Npr1+/+ and Npr1−/− mouse hearts with and without CAP and HYZ treatment are shown in Fig. 1, A–D. The expression of both ACE and AT1a receptor mRNA levels were increased three- and fourfold, respectively (P < 0.001), in the hearts of untreated Npr1−/− mice compared with those of Npr1+/+ mice hearts. CAP-treated Npr1−/− mice hearts had significantly reduced expression (P < 0.001) of ACE and AT1a receptor levels compared with those of untreated Npr1−/− mice hearts. However, HYZ-treated Npr1−/− mice had no significant reduction in ACE and AT1a mRNA levels compared with untreated Npr1−/− mice. As shown in Fig. 2A, increased AT1a (panels a and b) and NF-kB (p65) immunoreactivity was evident in the vascular medium of the Npr1−/− mutant mice hearts compared with that of wild-type mice (panels c and d). Masson’s trichrome staining showed increased perivascular fibrosis in the vascular medium of mutant mice hearts compared with that of wild-type mice hearts (panels e and f). Toluidine blue staining of infiltrating mast/monocyte cells showed no significant increase in the number of these cells in the arteries of Npr1−/− compared with Npr1+/+ mice (panels g and h). Figure 2B shows the average mast/monocyte-positive cell infiltration per section in mutant and wild-type mice. Npr1−/− null mutant mice hearts showed no significant increase in the infiltration of mast/monocyte cells compared with wild-type mice hearts (Fig. 2).

Plasma and ventricular expression of IL-6 and TNF-α. Plasma concentrations of IL-6 and TNF-α in Npr1+/+ and Npr1−/− mice with and without CAP and HYZ treatments are shown in Fig. 3, A and B. The Npr1−/− mice exhibited a significant increase in the plasma concentrations of IL-6 (threelfold, P < 0.001) and TNF-α (fivefold, P < 0.001) compared with wild-type control animals. Plasma concentrations of IL-6 and TNF-α were significantly reduced (P < 0.001) in CAP-treated Npr1−/− mice compared with untreated Npr1−/− mice. In contrast, HYZ-treated Npr1−/− mice showed a significant reduction (P < 0.01) in IL-6 alone compared with untreated Npr1−/− mice. Fig. 4, A–D, shows the ventricular mRNA expression of IL-6 and TNF-α in Npr1+/+ and Npr1−/− mice with and without CAP and HYZ treatments. Significant increases occurred in the ventricular mRNA expression of IL-6 (fourfold, P < 0.001) and TNF-α (fivefold, P < 0.001) in Npr1−/− mice compared with Npr1+/+ mice. Ventricular mRNA expression of IL-6 and TNF-α were significantly reduced (P < 0.001) in CAP-treated Npr1−/− mice compared with untreated Npr1−/− mice. In contrast, HYZ-treated Npr1−/− mice exhibited a significant

Fig. 2. Immunohistochemical analysis of AT1a and NF-kB (p65 subunits) protein expression in the coronary vessels of Npr1+/+ and Npr1−/− mouse hearts. A: representative sections (a and b) showing the increased AT1a protein expression, sections (c and d) show the NF-kB protein expression in the vessels wall, sections (e and f) show the perivascular fibrosis in the coronary vessels of Npr1+/+ and Npr1−/− mice hearts and sections (g and h) shows the number of inflammatory mast/monocyte infiltrating cells in the coronary vessels of Npr1+/+ and Npr1−/− mice hearts. B: mast/monocyte-positive cells infiltration/sections. Values are expressed as means ± SE (n = 8/group). NS, nonsignificant; untreated Npr1+/+ vs. antihypertensive drugs treated Npr1−/−.
reduction ($P < 0.01$) in only IL-6 mRNA levels compared with untreated Npr1$^{-/-}$ mice hearts.

Ventricular cGMP, ANG II, and TBARS levels. The levels of cGMP and ANG II contents in ventricular tissues of Npr1$^{+/+}$ and Npr1$^{-/-}$ mice with and without CAP and HYZ treatments are presented in Table 2. Left ventricular cGMP levels were reduced by almost sixfold in Npr1$^{-/-}$ mutant mice compared with Npr1$^{+/+}$ wild-type mice. However, the ventricular ANG II content was increased by twofold in Npr1$^{-/-}$ mice compared with the wild-type mice. CAP-treated Npr1$^{-/-}$ mice had significantly reduced ($P < 0.01$) ventricular ANG II levels; however, the level of ventricular ANG II was not significantly changed in HYZ-treated Npr1$^{-/-}$ mice compared with that in untreated Npr1$^{-/-}$ mice. cGMP levels were slightly increased in CAP-treated mouse hearts, while no change was observed in HYZ-treated mutant mice. TBARS levels were increased significantly in Npr1$^{-/-}$ mutant mice hearts (38 $\pm$ 2.9, $P < 0.001$) compared with Npr1$^{+/+}$ wild-type mice hearts (25 $\pm$ 2.5). TBARS levels were significantly decreased in CAP-treated Npr1$^{-/-}$ mice hearts (23 $\pm$ 1.6, $P < 0.01$), while no change was observed in HYZ-treated mutant mice. Coronary vessel wall thickening and myocyte cross-sectional area were significantly increased in the mutant mice compared with age-matched wild-type mice (Fig. 5). Both CAP and HYZ treatments significantly reduced the elevated blood pressure in Npr1$^{-/-}$ mice (106 $\pm$ 5 and 105 $\pm$ 3, respectively; $P < 0.01$) compared with that of untreated Npr1$^{-/-}$ mice. However, CAP treatment alone significantly attenuated the coronary vessel wall thickening ($P < 0.05$) and myocyte cross-sectional area ($P < 0.01$) in the mutant mice compared with untreated mutant mice. HYZ treatment did not significantly reduce the coronary vessel wall thickening and hypertrophy in Npr1$^{-/-}$ mice.

NF-$\kappa$B binding activity. The NF-$\kappa$B binding activities in the ventricular nuclear extract isolated from Npr1$^{+/+}$ and Npr1$^{-/-}$ mice are shown in Fig. 6. The mutant Npr1$^{-/-}$ mice hearts showed a significant increase in NF-$\kappa$B binding activity of fourfold ($P < 0.001$) compared with untreated wild-type Npr1$^{+/+}$ mice. Furthermore, the IKK-$\beta$ activity was increased by threefold ($P < 0.001$) in Npr1$^{-/-}$ mice hearts compared with Npr1$^{+/+}$ mice hearts. CAP treatment showed a decrease in NF-$\kappa$B binding activity by twofold ($P < 0.001$) and IKK-$.\beta$
activity by 2.5-fold ($P < 0.001$) in Npr1$^{-/-}$ mice compared with untreated Npr1$^{-/-}$ mice. HYZ treatment did not show any change in either NF-κB binding or IKK-β activity in Npr1$^{-/-}$ mice compared with untreated Npr1$^{-/-}$ mice.

**DISCUSSION**

In the present study, Npr1$^{-/-}$ mutant mice showed a 63% increase in the HW/BW ratio compared with Npr1$^{+/+}$ control animals. Furthermore, coronary vessel wall thickening and myocyte cross-sectional area were significantly increased ($P < 0.01$) in mutant mice compared with age-matched wild-type mice. Interestingly, both antihypertensive drugs CAP and HYZ were effective in reducing the elevated SBP in mutant mice. However, CAP alone attenuated the HW/BW ratio ($P < 0.05$) while significantly reducing fibrosis and collagen content, suggesting the involvement of RAS in the hypertrophic growth of mutant mice hearts. The results also show that the decreased ventricular cGMP correlated with increased ventricular ANG II content, as well as ACE and AT1a mRNA expression in mutant mice hearts. Moreover, CAP treatment was more effective than HYZ in reducing hypertrophy and inhibiting IL-6 and TNF-α gene expression in mutant mice hearts, suggesting that the inflammatory process appears to be mediated, in part, by ANG II. Previously it has been shown that Npr1$^{-/-}$ mice have cardiac hypertrophy disproportionate to their increased BP, suggesting that the ANP/NPRA system exerts a local antihypertrophic effect on cardiac cells (20). Furthermore, mice pups lacking NPRA show, as early as 16 days after gestation, a significant increase in heart size compared with age-matched wild-type controls, supporting our present finding that blood pressure alone is not likely to be the primary cause for the development of cardiac hypertrophy in Npr1$^{-/-}$ null mutant mice (11, 22).

The ventricular expression of ACE and AT1a were found to be up-regulated by almost three- to fourfold in mutant mice hearts compared with the hearts of wild-type mice. Surprisingly, expression of both ANG II contents and AT1a mRNA were found to be increased in mutant mice hearts compared with wild-type mice hearts, further suggesting that RAS components were significantly activated in the Npr1$^{-/-}$ mutant mice hearts. Accordingly, the increased ANG II levels in the mutant mice hearts was found to be negatively correlated with ventricular cGMP levels, further supporting the finding in our previous studies that the ANP/NPRA signaling system antagonizes RAS signaling in a tissue-specific manner (32). We have previously reported that systemic and renal ANG II levels

![Fig. 5. Comparative analysis of coronary vessel wall thickening (vascular remodeling) and myocyte cross-sectional area in Npr1$^{+/+}$ and Npr1$^{-/-}$ mice hearts. A: representative sections of coronary vessels of Npr1$^{+/+}$ and Npr1$^{-/-}$ mice with and without antihypertensive drug treatments. B: representative heart sections of Npr1$^{+/+}$ and Npr1$^{-/-}$ mice with and without drug treatment.](http://physiolgenomics.physiology.org/)

Table 2. Left ventricular cGMP, ANG II, and TBARS levels in Npr1$^{+/+}$ and Npr1$^{-/-}$ mice hearts treated with and without antihypertensive drugs treatment

<table>
<thead>
<tr>
<th>Parameters</th>
<th>cGMP, pmol/mg protein</th>
<th>ANG II, pmol/mg protein</th>
<th>TBARS, nmol MDA/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Npr1$^{+/+}$</td>
<td>Npr1$^{-/-}$</td>
<td>Npr1$^{+/+}$</td>
</tr>
<tr>
<td>Untreated</td>
<td>26±5</td>
<td>5±1.2**</td>
<td>15±1.5</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>25±3</td>
<td>5±1.3**</td>
<td>14±1.2</td>
</tr>
<tr>
<td>Captopr1</td>
<td>28±6</td>
<td>10±1.6†</td>
<td>12±0.8</td>
</tr>
</tbody>
</table>

cGMP concentration was quantified using the direct competitive enzyme immunoassay kit, ANG II content was determined using RIA kit, and thiobarbituric acid reactive substance (TBARS) levels were quantified using a colorimetric assay as described in MATERIALS AND METHODS. Values are expressed as means ± SE ($n = 8$ animals/group). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$; Npr1$^{+/+}$ vs. Npr1$^{-/-}$; †$P < 0.05$, ††$P < 0.01$, Untreated Npr1$^{-/-}$ vs. antihypertensive drug-treated Npr1$^{-/-}$ mice.
are increased in newborn pups of mice lacking NPRA. However, both circulating and intrarenal ANG II and renin levels were reduced in adult Npr1 null mutant mice compared with age-matched wild-type mice (52). It has been suggested that the possible cause for the reduction in the circulating RAS might be due to the elevation of arterial pressure, which would lead to baroreceptor activation and reflex neural inhibition of renin release in the absence of functional Npr1 gene in the adult null mutant mice (52, 53). The results of this present study show that cardiac RAS is activated in the adult mutant mouse heart, even though the circulating levels of ANG II are decreased, further supporting the results of previous studies showing that cardiac RAS can be activated independently and dissociated from the circulating RAS (30, 60). The evidence also suggests that tissue levels of RAS are greatly increased in contrast to circulating RAS in ANG II-infused hypertensive rat models with elevated arterial pressure (9). The previous studies have demonstrated that all the components of the RAS can be synthesized in local tissues and that locally produced ANG II can serve as a paracrine and/or autocrine growth stimulatory factor (9, 40). Recently, it has been shown that inhibition of plasma renin activity with salt overload does not affect ventricular remodeling after myocardial infarction in rats; however, local cardiac RAS is activated, which plays a predominant role for local adaptation of the heart after myocardial infarction (7). Furthermore, previous studies have suggested that either infusion of ANG II or treatment with a high-salt diet can increase cardiac hypertrophy without significant effect on blood pressure (46, 47). Thus, it is plausible that cardiac RAS is greatly activated and plays a critical role in the ventricular remodeling process in mice lacking NPRA compared with wild-type animals.

In another model of NPRA-lacking mice, cardiac hypertrophy and fibrosis were greatly improved by blocking the AT1 receptor; the results of those studies have suggested that NPRA signaling interacts with AT1 receptor function (27). Interestingly, coactivation of ACE and AT1α expression levels has been reported in the ventricular tissue of N-nitro-1-arginine methyl ester (L-NAME)-induced hypertensive rat models (55). Treatment with ACE or AT1 receptor blockers has been shown to reduce the inflammatory phenotype in the vessel wall of L-NAME-induced hypertensive rat models (28, 55). Thus, blockade of ANG II seems to contribute to improvement in the inflammatory process in Npr1−/− mice.

The findings of this study show that disruption of ANP/NPRA signaling results in augmented expression of both circulating and ventricular cytokines (IL-6 and TNF-α), suggesting activation of the inflammatory process in vascular and ventricular tissues of Npr1−/− null mutant mice. It has been proposed that proinflammatory cytokines such as IL-6 and TNF-α are important mediators in the development of cardiac hypertrophy and heart failure (17, 25, 36, 57, 62). Cytokines have been shown to be expressed within the heart in response to either mechanical overload or ischemic injury (35, 36, 51, 62). In Npr1−/− mouse hearts compared with the hearts of age-matched wild-type mice, significant up-regulation in ventricular expression of cytokine genes was observed. Similarly, parallel increases of almost fourfold in the levels of circulating plasma IL-6 and TNF-α occurred in Npr1−/− mice compared with Npr1+/+ mice. The mechanisms underlying the stimulation of ventricular and plasma concentrations of inflammatory markers are not well understood, although the participation of early gene activation can be suggested, as can progressive mechanical stress associated with hypertension (52, 63). This affirmation is based on the present observation that a reduction in either plasma concentration or mRNA expression of inflammatory markers induced by both CAP and HYZ was accompanied by decreased blood pressure in Npr1−/− mice. The reduced expression of inflammatory markers was more pronounced in CAP-treated Npr1−/− mice than in HYZ-treated mice, suggesting that hemodynamic overload may not be the sole mechanism accounting for improvement in the inflammatory process.

The present data showed a significant increase in NF-κB (P < 0.001) levels and IKK-β activity in Npr1−/− mice hearts compared with Npr1+/+ mice, suggesting that the NF-κB signaling pathway is activated in the mutant mice hearts. The increased NF-κB binding activity was positively correlated with increased expression of cytokine genes in Npr1 null mutant mice hearts. Recent studies by several groups have implicated the activation of NF-κB as a causal event in cardiac hypertrophy (13, 26, 45). In the present study, ventricular TBARS levels were increased in the Npr1 null mutant mice hearts compared with the wild-type mice.
hearts, indicating an increase in overall oxidative stress in null mutant mice hearts. Increased levels of TBARS, an end product of lipid peroxidation, have been considered as a potential marker for oxidative stress in the pathophysiology of many forms of hypertension (18, 37). Furthermore, treatments with free radical-scavenging agents such as tempol (4-hydroxy tetramethylpiperidine-1-oxyl) significantly ameliorate oxidative stress, BP, and hypertrophic growth in different hypertensive models (31).

ANG II has been reported to be a strong activator of ROS and NF-κB after its binding to the AT1a receptor in vivo and in vitro (12, 65, 67). On the other hand, it has been shown that the ANP/NPRA system attenuates production of inflammatory mediators such as TNF-α by regulating the NF-κB pathway (19, 59). Furthermore, a recent study demonstrated that augmentation of the second messenger cGMP via chronic inhibition of cGMP-specific phosphodiesterase (PDE5A) attenuates load-induced hypertrophy, fibrosis, and myocardial dysfunction independently of changes in load, suggesting that cGMP signaling locally antagonizes hypertrophic mediators and pathways (56). Immunohistochemical analyses exhibited increased expression of AT1 and NF-κB in the vascular wall of Npr1<sup>−/−</sup> mutant mice hearts, suggesting that vascular wall remodeling and medial thickening plays a crucial role in the development of hypertrophy in null mutant mice. Figure 7 shows a diagrammatic representation of the signaling pathway involved in the cardiac remodeling process in the absence of NPRA/cGMP signaling. Our results suggest that disruption of ANP/NPRA/cGMP signaling leads to increased activation of the RAS system and proinflammatory cytokines, leading to medial thickening and perivascular fibrosis; these changes, in turn, promote cardiac remodeling, hypertrophy, and heart failure. Taken together, the results of the present study clearly demonstrate that NF-κB signaling is critically involved in the activation of inflammatory cytokine gene expression in the vascular and ventricular tissues of Npr1<sup>−/−</sup> null mutant mice.

In conclusion, the present results show that disruption of the ANP/NPRA signaling pathway resulted in cardiac hypertrophy associated with augmented expression of cytokines, ACE and AT1 receptor in Npr1<sup>−/−</sup> mice. The results indicate that a deficiency of NPRA leads to activation of RAS components in the ventricular tissues of Npr1<sup>−/−</sup> mutant mice. CAP treatment reduced the augmented expression of cytokines in Npr1<sup>−/−</sup> mice, suggesting that ANG II is involved in the activation of cardiac inflammatory mediators. Furthermore, the present study provides evidence that the increased expression of cytokines Npr1<sup>−/−</sup> gene-disrupted mice may be critical in promoting the pathways that lead to cardiac hypertrophy and congestive heart failure.

ACKNOWLEDGMENTS

We thank Gevoni Bolden and L. Charlen Binford for excellent technical assistance and Kamala Pandey for assistance in the preparation of this manuscript. We are indebted to Dr. Oliver Smithies for providing initial breeding pairs of Npr1<sup>−/−</sup> gene-targeted mice colonies. Our special thanks are due to Dr. Bharat B. Aggarwal, Department of Experimental Therapeutics and Cytokine Research Laboratory at MD Anderson Cancer Center, and to Dr. Susan L. Hamilton, Department of Molecular Physiology and Biophysics at Baylor College of Medicine, for providing their facilities during our displacement period due to Hurricane Katrina.

GRANTS

This research was supported by the National Heart, Lung, and Blood Institute Grant HL-62147 and Louisiana Board of Regents Health Excellence Fund.
REFERENCES


