Involvement of the NF-kB/Matrix Metalloproteinase Pathway in Cardiac Fibrosis of Mice Lacking Guanylyl Cyclase/Natriuretic Peptide Receptor-A

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Running Title: Matrix metalloproteinases in Npr1 null mutant mice.

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ABSTRACT:

Mice carrying targeted-disruption of Npr1 gene (coding for guanylyl cyclase/natriuretic peptide receptor-A; GC-A/NPRA) exhibit increased blood pressure, cardiac hypertrophy, and congestive heart failure similar to those seen in untreated human hypertensive patients. The objective of this study was to determine whether permanent ablation of NPRA signaling in mice alters the expression of matrix metalloproteinases (MMP-2 and MMP-9) and pro-inflammatory mediators such as tumor necrosis factor (TNF-α) leading to myocardial collagen remodeling. Here, we report that expression levels of MMP-2 and MMP-9 genes were increased by 3- to 5-fold, and the expression of TNF-α gene was enhanced by 8-fold in Npr1 homozygous null mutant (Npr1−/−) mice hearts as compared with wild-type (Npr1+/+) control mice hearts. Myocardial fibrosis, total collagen, and collagen type I/III ratio (p<0.01) were dramatically increased in adult Npr1−/− mice as compared with age-matched wild-type counterparts. Hypertrophic marker genes including β-myosin heavy chain (β-MHC) and transforming growth factor-β1 (TGF-β1), were significantly up-regulated (3- to 5-fold) in both young and adult Npr1−/− mutant mice hearts. Nuclear factor kappa-B (NF-κB) binding activity in ventricular tissues was enhanced by 4-fold with increased translocation of p65 subunit from cytoplasm to nuclear fraction in Npr1−/− mice. Our results show that reduced NPRA signaling activates MMPs, TGF-β1, and TNF-α expression in Npr1−/− mice hearts. The findings of this study demonstrate that disruption of NPRA/cGMP signaling promotes hypertrophic growth and extracellular matrix (ECM) remodeling, which leads to the development of cardiac hypertrophy, myocardial fibrosis, and congestive heart failure.
Atrial and brain natriuretic peptides (ANP and BNP) elicit natriuretic, diuretic, vasorelaxant, and anti-proliferative responses, all of which contribute to the regulation of blood pressure and blood volume homeostasis (1, 2). ANP and BNP bind to guanylyl cyclase-A/natriuretic peptide receptor-A (GC-A/NPRA), which is considered a major natriuretic peptide receptor that synthesizes intracellular second messenger, cGMP (3). Mice carrying targeted-disruption of Npr1 gene (encoding for NPRA) exhibit hypertension, marked cardiac hypertrophy, and congestive heart failure with sudden death after six months of age (4-6). On the other hand, Npr1 gene-duplicated mice have stimulated levels of guanylyl cyclase (GC) activity and increased accumulation of intracellular cGMP in a gene-dose-dependent manner and exhibit a protection against high salt-diets (7). In vitro studies have shown that ANP/NPRA system exerts growth inhibitory effects on hypertrophic agonist-induced proliferation of cardiac myocytes (8, 9), fibroblasts (10), mesangial, and human vascular smooth muscle cells (11, 12). Furthermore, transgenic mice overexpressing ANP have smaller hearts than wild-type mice, and ANP gene delivery attenuates cardiac hypertrophy in spontaneously hypertensive rats (13). Nonetheless, the molecular mechanism by which ANP/NPRA system exerts protective effects and regulates cardiac remodeling in disease state is not well understood.

Abnormal cardiac remodeling is characterized by structural rearrangements that involve myocyte hypertrophy, hyperplasia of fibroblasts, and disproportionate increases in extracellular matrix collagen deposition, which lead to myocardial fibrosis (14). Extracellular matrix (ECM) collagen is an important determinant of myocyte shape and alignment and plays regulatory roles in transduction of contractile force into overall cardiac ejection. Thus, remodeling of myocardial collagen matrix is critical in the development of ventricular diastolic and systolic dysfunctions (14). Cardiac fibroblasts are the major cell type responsible for the synthesis of fibrillar collagen
(type I and type III), and synthesis and degradation of collagen in myocardium are tightly controlled. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are the major regulators of collagen synthesis and degradation in the heart (15). Recent studies indicated that abnormal remodeling of myocardial collagen is caused by dysregulation of MMPs and their endogenous inhibitors, TIMPs (15, 16). Increased activity of MMPs or decreased levels of TIMPs have been reported in hypertrophied and failing hearts, implicating that both MMPs and TIMPs play critical roles in the process of ventricular remodeling (17). A number of factors have been linked to stimulation of fibroblast proliferation and collagen deposition in the heart, including vasoactive hormones, cytokines, and growth factors (18, 19); however, the mechanism that inhibits collagen production in heart is not well understood.

ANP/NPRA system has been implicated as an anti-hypertrophic and anti-fibrotic protective mechanism that moderates cardiac remodeling process (4, 5). ANP and BNP have been shown to inhibit fibroblast proliferation (8), collagen synthesis, and MMP release via cGMP-dependent pathway and to have a broad functional opposition to transforming growth factor-β1 (TGF-β1)-induced ECM protein synthesis in vitro (20, 21). However, in vivo studies have not been carried out to examine the role of NPRA signaling in regulation of MMPs, TIMPs, and pro-inflammatory mediators. In the present study, we have utilized Npr1 gene-disrupted mutant mouse model to determine the role of NPRA signaling in expression and activation of specific hypertrophy marker genes, MMPs, TIMPs, and extracellular matrix proteins. To our knowledge, this is the first report demonstrating that permanent ablation of NPRA signaling in mice modulates cardiac MMPs, TIMPs, and collagen remodeling, which play critical roles in cardiac hypertrophy and heart failure.
EXPERIMENTAL PROCEDURES

Materials:

Trizol reagent was obtained from Life Technologies/Invitrogen (Carlsbad, CA). Gene-specific primers were purchased from Midland Certified Reagent Company, Inc. (Midland, TX). RETROscript kit was obtained from Ambion Inc. (Austin, TX). Antibodies for MMPs (MMP-2, MMP-9), TGF-β1, TGF-β1R, β-actin, and IgG-HRP conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (San Diego, CA). Pro-inflammatory cytokine (TNF-α) ELISA kit was obtained from Pierce Endogen, Inc. (Rockford, IL) and 4′, 6-diamidino-2-phenylindole (DAP1) was from Vector Laboratories, Inc. (Burlingame, CA). RNase protection assay kit, and custom multi-probe set containing MMP-2, MMP-9, pro-collagen-I, glycerolaldehyde-3-phosphate dehydrogenase (GADPH), and L32 were from BD Biosciences (San Diego, CA). MMP-2 and MMP-9 ELISA activity assay system and [α-32P]UTP (3000 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ). Captopril, hydralazine, and bendrofluomethiazide were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade.

Generation of mice and genotyping:

Npr1 gene-disrupted mice were generated by homologous recombination in embryonic stem cells as previously described (4, 7). Animals were bred and maintained at the animal facility of Tulane University Health Sciences Center and handled under protocols approved by the Institutional Animal Care and Use Committee. The mice colonies were housed under 12 h light/dark cycles at 25° C and fed regular chow (Purina Laboratory) and tap water ad libitum. All animals were littermate progenies of C57BL6 genetic background and have been designated as
Npr1 gene-disrupted homozygous null mutant (Npr1⁻⁻), heterozygous (Npr1⁺⁻), and wild-type (Npr1⁺⁺) mice. The present study was performed using newborn pups (2-days after birth), young (4-weeks), and adult (22-weeks) Npr1 male mice. The animals were genotyped by polymerase chain reaction (PCR) analyses 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 (Npr1⁺⁺); primer B (5'-TGT CAC CAT GGT CTG ATC GC-3') corresponding to exon 1 sequence only present in the intact allele, (Npr1⁺⁺); and primer C (5'-GCT TCC TCG TGC TTT ACG GT-3'), a sequence in the neomycin resistant cassette only present in the null allele (Npr1⁻⁻). The PCR was carried out in 25 µl reaction mixture containing 50 mM Tris-HCl (pH 8.3), 20 mM ammonium sulfate, 1.5 mM MgCl₂, 10 % DMSO, 100 µM dNTPs, 2 units of Taq DNA polymerase, and 40 nM primers. The PCR was performed by use of a 60-s denaturation step at 94º C, a 60-s annealing step at 60 º C, and a 60-s extension step at 72º C, respectively, for 35 cycles using DNA-Thermal Cycler 480 as described previously (22). PCR products were resolved on a 2% agarose gel. The endogenous band is 500 base pairs (bp), and the targeted band is 200 bp.

Assessment of blood pressures, heart rate, and cardiac function:

The blood pressures and heart rate were measured by a noninvasive computerized tail-cuff method using Visitech-2000 as previously described (23). Blood pressures and heart rate were calculated as the average of 6-7 sessions/day for 6 consecutive days. Cardiac functions of young and adult Npr1⁻⁻ and Npr1⁺⁺ mice were analyzed using two-dimensional echocardiography. Animals were lightly sedated using 0.2 ml of Avertin (Aldrich) and were evaluated using M-mode transthoracic views to measure the left ventricular (LV) dimensions,
intraventricular septal wall thickness, LV posterior wall thickness, and fractional shortening. Digitized M-mode images were obtained using an ultrasound system (Toshiba Power Vision) with a 7 mHz transducer at a sweep speed of 100 mm per second. For each measurement, four consecutive cardiac cycles were traced and averaged.

**Assay of plasma and ventricular cGMP:**

Blood samples were collected in tubes containing EDTA and immediately centrifuged at 2,500 rpm for 10 min at 4° C. Plasma was separated and stored at -70° C until used. Frozen ventricular tissue samples were homogenized in 10 volumes of 0.1 M HCl containing 1% Triton X-100. The homogenate was heated at 95° C for 5 min, centrifuged at 600 x g at 22° C, and supernatant was collected. cGMP levels in plasma and ventricular samples were analyzed using a direct cGMP immunoassay kit (Assay Designs, Inc.) as previously described (24). The results are expressed as picomoles cGMP/mg protein.

**Northern blot analyses of hypertrophy marker genes:**

Total RNA was isolated from the left ventricular heart tissues from Npr1−/− and Npr1+/+ mice, using TRIzol reagent according to the manufacturer’s protocol (Invitrogen/Life Technologies). To remove genomic DNA contamination, RNA samples were treated with RNase free DNase I (1 unit/µg RNA) at 37° C for 30 min. The RNA integrity was confirmed by visualization of distinct 28 S and 18 S bands after electrophoresis on 1.5 % agarose gel. Total RNA (10 µg) was fractionated on a 1 % formaldehyde-agarose gel and transferred to Hybond nylon membrane (Amersham Biosciences) by capillary action in 10 x standard saline citrate (SSC). Blots were pre-hybridized in a hybridization solution containing 7 % SDS, 0.5 M
NaHPO₄ (pH 7.2) and 250 μg/ml salmon sperm DNA, for 5 h at 65° C, and hybridized with [γ-³²P]ATP-labeled oligonucleotide probes for 16 h at 65° C. Blots were washed three times in 2 x SSC/0.2 % SDS at room temperature for 30 min and then in 0.5 x SSC/0.2 % SDS at 65° C for 30 min before exposure to X-ray film. The sequence of oligonucleotide probes were as follows: ANP: 5’-CCG GAA GCT GTT GCA GCC TAG GTC CAC TCT GGG CTC CAA TCC TGT CAA TCC TGT CAA TCC TAC CCC CCG AAG CTG GA-3’. BNP: 5’-GTT TAA GCC TCT GGA AAA AGC TAT CTC ACA GGG CCT CTG TTT CTC CTG TAA AGT GGG TTG GGC CAT TCG GA -3’, β-MHC: 5’-GAG GGC TTC ACG GG C ACC CTT AGA GCT GGG AGC ACA AGA TCT ACT CCT CAT TCA TTC AGG CC-3’, and SERCA-2a: 5’-TCA GTC ATG CAG AGG CTG GTA GAT GTG TTG CTA ACA ACG CAC ATG CAC GCA CCC GAA CA-3’. The intensity of the bands were quantified using image density analysis software (Alpha Innotech, San Leandro, CA). The expression results of ANP, BNP, β-MHC, and SERCA-2a were normalized with GADPH.

Myosin heavy chain protein isoform shift analysis:

Myosin was extracted using the method described by Martin et al., (25). Approximately, 75 mg of left ventricular heart tissues from Npr1⁻/⁻ and Npr1⁺/⁺ mice were minced and washed with ice-cold phosphate buffered saline (PBS), pH 7.2. The minced tissues were homogenized in 3 ml of PBS with a Polytron (Brinkmann Instruments, Westbury, NY) at a setting of 4 (3-4 strokes, 30 s each) at 4º C. The homogenate was centrifuged at 1,200 x g at 4º C for 10 min. The supernatant was discarded, pellet was rewashed with 3 ml of PBS, and then recentrifuged at 1,200 x g for 10 min. The supernatant was again discarded and the pellet was dissolved in an extraction solution containing 100 mM Na₄P₂O₇, 5 mM EGTA, and 5 mM dithiothreitol (DTT),
pH 8.6. The homogenate was shaken in an ice-bath at 4°C for 60 min, after which it was centrifuged at 12,000 x g at 4°C for 2 h. The supernatant was collected and mixed with equal volume of ice-cold glycerol, and stored at -20°C. The SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as previously described (24). The gels were stained with 0.25 % Coomassie Brilliant Blue R-250 solution and destained. The α-MHC and β-MHC protein isoforms were quantified using Alpha Innotech Imaging System.

**RNase protection assay:**

RNase protection assay (RPA) was carried out using a custom made multi-probe template set for MMP-2, MMP-9, pro-collagen I (Col I), and house keeping genes GADPH and L-32. The multiple templates were labeled with [α-32P]UTP using a T7 RNA polymerase as described by the manufacturer’s protocol (BD Biosciences). Labeled probe (3 x 10^5 cpm) was allowed to hybridize with 20 µg of total RNA at 56°C for 16 h. The hybridized mRNA probes were treated with RNase A, and extracted with phenol-chloroform. Protected hybrid bands were resolved on a 5 % denaturing polyacrylamide gel and exposed to radiographic film overnight at -80°C. Densitometry was performed using Alpha Innotech Imaging System.

**MMP activity Assay:**

The left ventricular heart tissues were washed in ice-cold saline and homogenized with Polytron in an extraction buffer, pH 5.0, (1:3 w/v) containing; 10 mM cacodylic acid, 150 mM NaCl, 20 mM ZnCl₂, 1.5 mM NaN₃, and 0.01% Triton X-100 (17). The homogenate was centrifuged at 800 x g at 4°C for 10 min, and the supernatant was collected and concentrated using 30-kDa cut-off microcentrifugal filter devices. The final protein concentrations of
myocardial extracts were determined using Bio-Rad protein assay kit. The extracted samples were then aliquoted and stored at -20°C until used. MMP activity was measured by an antibody capture method essentially as described by Spinale et al., (26). The specificity and concentration dependency of MMP-2 and MMP-9 activities were initially established using commercially available MMP-2 and MMP-9 standards (1 to 12 ng/ml). Left ventricular myocardial extracts (25 µg protein) or MMP-2 and MMP-9 standards were incubated at 4°C overnight in a 96-well microtiter plate immobilized with MMP-2 or MMP-9 monoclonal antibodies (Amersham Pharmacia Biotech). After washing the plates with 10 mM Tris-HCl buffer, pH 7.6, a chromogenic peptide substrate solution was added, reaction was allowed to proceed at 37°C for 1 h, and the absorbance was recorded at 405 nm. The absorbance from the cleaved chromogenic substrate was linear with increasing MMP-2 and MMP-9 activities with an optimum substrate concentration of 0.4 mM. Assay conditions for MMP-2 and MMP-9 activities were optimized with increasing myocardial protein and substrate concentrations and incubation time periods at 37°C. In preliminary studies, MMP-2 and MMP-9 activities were linear with increasing protein concentrations up to 50 µg, substrate concentrations up to 1 mM, and incubation periods up to 90 min. The proteolytic activity was reduced by 95% in the presence of MMP-inhibitors, 1,10-phenanthroline monohydrate (1 mM) and GM 6001 (0.5 nM). Actual MMP-2/MMP-9 activities were determined by regression analysis and expressed as ng. h⁻¹.g⁻¹ heart tissues. The endogenous levels of MMP-2 and MMP-9 activities were analyzed using BIOTRACK ELISA-activity assay system (Amersham Biosciences). ELISA was performed using kit according to the manufacturer’s protocols. Total MMPs extracted from the left ventricular tissues of Npr1⁻/⁻ and Npr1⁺/⁺ mice were used for enzyme activity assays.
Zymography:

To determine the total MMPs activities in situ, fresh frozen ventricular tissue sections were incubated with gelatin-oregon green (0.5 mg/ml) in zymogram developing buffer containing; 50 mM Tris-HCl (pH 7.5), 5 mM CaCl$_2$, 1 mM PMSF, and 0.02 % NaN$_3$ at 37° C for 3 h. The slides were washed 3 times with PBS to remove unbound gelatin. Gelatinase activity resulted in the loss of quenching; therefore, an increase in activity was visualized as a linear increase in fluorescence. MMP-inhibitors such as GM 6001 and 1, 10-phenanthroline were used to show the specificities of MMPs activities. Left ventricular MMPs activities were also measured by gelatin zymography. Total MMPs (25 µg proteins) extracted from Npr1$^{-/-}$ and Npr1$^{+/+}$ mice hearts were directly loaded onto 10 % gel containing 1 mg/ml of gelatin under non-reducing conditions (17). Gels were washed twice in 2.5 % Triton X-100 (30 min each), once with substrate buffer (50 mM Tris-HCl, 5 mM CaCl$_2$, and 0.02 % NaN$_3$, pH 7.5), and then further incubated at 37° C for 24 h in fresh substrate buffer. Gels were stained with 0.25% Coomassie Brilliant Blue R-250 and destained until the lytic white bands were visible.

Treatment of Npr1 mice with MMPs inhibitor:

Young Npr1$^{-/-}$ and Npr1$^{+/+}$ mice were treated with MMP inhibitor, GM 6001 and placed randomly into four groups. Groups I and II consisted of Npr1$^{-/-}$ and Npr1$^{+/+}$ mice, which received subcutaneous injection of PBS and served as controls. Groups III and IV consisted of Npr1$^{-/-}$ and Npr1$^{+/+}$ mice, which received subcutaneous injection of MMP inhibitor GM 6001, at a dose of 100 mg/kg body weight, twice a week for 4 weeks. The ability of GM 6001 to effectively block MMPs activities was tested by incubating a zymogram gel containing ventricular protein extracts and 0.5 nM GM 6001 in incubation buffer as described above, which completely abolished
MMP-2 and MMP-9 activities. At the end of experimental period, echocardiographic analysis was performed and cardiac collagen contents, MMPs activities, fibrosis, and myocyte cross sectional areas were measured.

**Preparation of nuclear and cytosolic extracts:**

Nuclear and cytosolic proteins were extracted by the method of Dignam et al., (27) from the left ventricular tissues of Npr1\(^{-/-}\) and Npr1\(^{+/+}\) mice, immediately after the animals were sacrificed. Tissues were homogenized in an ice-cold 10 mM Tris-HCl buffer (pH 8.0) containing; 0.32 M sucrose, 3 mM CaCl\(_2\), 2 mM MgOAc, 0.1 mM EDTA, 0.5% Nonidet P-40 (NP-40), 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 4.0 µg/ml each of leupeptin, aprotinin, and pepstatin. The homogenate was centrifuged at 800 x g, and the supernatant was separated and saved as a cytosolic fraction. The resultant nuclear fraction was washed several times with homogenization buffer without NP-40. The nuclear fraction was re-suspended in a low-salt buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl\(_2\), 20 mM KCl, 0.2 mM EDTA, 25 % glycerol, 0.5 mM DTT, and 0.5 mM PMSF), incubated on ice for 5 min, and then mixed with equal volume of high-salt buffer containing; 20 mM HEPES, 1.5 mM MgCl\(_2\), 800 mM KCl, 0.2 mM EDTA, 25% glycerol, 1% NP-40, 0.5 mM DTT, 0.5 mM PMSF, and 4.0 µg/ml each of leupeptin, aprotinin and pepstatin. The mixture was incubated on ice for 30 min and centrifuged at 14,000 x g for 15 min. The supernatant was separated and stored at -70°C until used.

**Electrophoretic gel mobility shift assay:**
Electrophoretic mobility shift assay (EMSA) was performed as described by Dent et al., (28). Double stranded oligonucleotide containing the consensus-binding site for NF-kB was utilized. Oligonucleotides were end-labeled using $[^\gamma-32P]$ATP and T4 polynucleotide kinase according to the manufacturer’s protocol (New England Biolabs, Beverly, MA). Binding reaction was initiated by incubating 5 µg of nuclear proteins in 5 µl of binding buffer (50 mM Tris-HCl, pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5 % Triton-X 100, 62.5 % glycerol, and 1 mM DTT) containing 2 µg of Poly dI-dC and radiolabeled oligonucleotide (50,000 cpm) at 22º C for 20 min. Cold competitor assays were performed by adding 100-fold excess molar concentrations of unlabeled NF-kB oligonucleotide. The super-shift assay was performed with p65 antibody. The DNA-protein complex was resolved from the free labeled-DNA by electrophoresis using 5% native PAGE and visualized by autoradiography.

**IKK-kinase activity assay:**

IKK-kinase activity was assayed essentially as described by Li et al., (29). Cytoplasmic proteins (200 µg) from the left ventricular tissues of young Npr1$^{+/+}$ and Npr1$^{-/-}$ mice were immunoprecipitated with 2 µg of IKK-β antibody at 4º C for 1 h. Protein A-agarose beads (10 µl) were added and the mixture was incubated for another 1 h at 4º C. The pellet was centrifuged at 2,500 rpm for 5 min at 4º C, washed twice with lysis buffer containing; 50 mM HEPES (pH 7.4), 250 mM NaCl, 1 % Nonidet P-40, 1 mM PMSF, 5 µg/ml each of aprotinin and leupeptin, and once with kinase buffer containing 10 mM HEPES (pH 7.4), 1 mM MnCl$_2$, 5 mM MgCl$_2$, 12.5 mM β-glycerophosphate, 50 µM Na$_3$VO$_4$, 2 mM NaF, 50 µM DTT, and 10 µM ATP. The pellet was then resuspended in 15 µl of kinase buffer and the reaction was carried out in the presence of 1 µg glutathione S-transferase-IkBα substrate and 5 µCi $[^\gamma-32P]$ ATP (6,000
Ci/mmol) at 30°C for 30 min. The reaction was stopped by the addition of 3 x Laemmli loading buffer, and proteins were resolved by 15 % SDS-PAGE and autoradiography.

**Western blot analyses:**

Left ventricular tissue samples were homogenized in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1 mM EDTA, 1 % (v/v) NP-40, 0.5 % (w/v) deoxycholate, 50 mM NaF, 50 mM sodium vanadate, 0.5 mM PMSF, and 2 µg / ml each of aprotinin, and leupeptin. Tissue homogenate (20 µg proteins) was mixed with sample loading buffer and separated under reducing condition using 10 % SDS-PAGE. For MMP-2 and MMP-9, the protein samples (20 µg) were mixed with MMP-sample buffer (100 mM Tris-HCl, pH 6.8, 4 % SDS, 0.2 mM DTT, and 0.01 % Bromophenol Blue) and resolved by 8 % SDS-PAGE. The separated proteins were transferred at 100 volts to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 1 x Tris-buffered saline-Tween 20 (TBST), pH 7.5, containing 5 % non-fat milk powder for 45 min at room temperature and incubated at 4°C overnight with specific antibodies to MMPs, TIMPs, TGF-β1, TGF-β1R, p-IkB-α, and p65 at a dilution of 1:1000 in 1 x TBST containing 3 % non-fat milk powder. After three washes with 1 x TBST for 5 min each, the membrane was incubated for 1 h in HRP-conjugated anti-rabbit antibody at a dilution of 1:5000, washed three times with 1 x TBST, and developed using ChemGlow Western blot detection reagent kit (Alpha Innotech Co. San Leandro, CA). The luminescent signal was detected by Alpha Innotech Imaging System.

*Determination of hydroxyproline, TNF-α, and collagen type I/III ratio:*
The left ventricular total collagen concentration was quantified from the hydroxyproline content which was determined by the modified method of Bergman and Loxley (30). The ventricular tissues were homogenized and hydrolyzed in 6 N HCl at 110°C for 24 h in a sealed reaction vial. The hydrolyzed sample was dried using a flash evaporator and the residue was re-suspended in sterile water. The sample was treated with 0.5 ml Chloramine T, vortexed, and left for 5 min. After which, 3 ml of Ehrlich’s reagent was added to the sample, the mixture was vortexed, and left for 18 h at room temperature. The intensity of developed red coloration was measured at 558 nm. A conversion factor of 8.2 was used to convert hydroxyproline contents to total collagen concentration. Myocardial collagen was extracted using cyanogen bromide (CNBr) method and collagen type I /III ratio was determined by a procedure described by Mukherjee and Sen (31). Pro-inflammatory cytokine (TNF-α) levels in the left ventricular tissues of Npr1−/− and Npr1+/+ mice were analyzed by ELISA according to the manufacturer’s protocol (Pierce endogen, Inc.).

Measurement of cardiac hypertrophy and interstitial fibrosis:

Animals were sacrificed by cervical dislocation and hearts were isolated. Heart weight (HW), left ventricular weight (LVW), and its ratio to body weight (BW) were calculated as an index of cardiac hypertrophy. Ventricular tissues were fixed in 4 % paraformaldehyde solution. Paraffin-embedded tissue sections (5 µm) were stained with Masson’s trichrome for the presence of interstitial collagen fiber accumulation, as a marker of cardiac fibrosis. The ratio of interstitial fibrosis to the total left ventricular area was calculated from randomly selected 20 microscopic fields in five individual sections per heart, using image analysis software (Image-Pro Plus, MediaCybernetics, Inc. Silver Spring, MD).
**Antihypertensive drug treatments:**

Experiments were performed using 4-weeks young Npr1⁻/⁻ homozygous null mutant and age-matched Npr1⁺/⁺ wild-type mice. All animals were placed in four groups of 8 Npr1⁻/⁻ and 8 Npr1⁺/⁺ mice (n=16 animals/group). Group I mice were kept as a positive control; Group II mice received hydralazine (25 mg/kg/day); Group III mice received captopril (0.5 mg/kg/day); and Group IV mice received bendrofluomethiazide (10 mg/kg/day). The drugs were given orally by gavage once a day for 4-weeks. Systolic blood pressure and heart rate were measured before and during the drug treatments for 4-weeks. Animals were sacrificed at the end of drug treatments and HW/BW ratio, cardiac fibrosis, and MMP-9 activity were analyzed.

**Statistical Analysis:**

The results are presented as mean ± SEM. Differences between groups were determined by using one-way analysis of variance (ANOVA) with Dunnett’s multiple comparisons post-hoc test. The probability value of p<0.05 was considered significant.

**RESULTS**

The present study was carried out to examine whether permanent ablation of ANP/NPRA signaling activates abnormal cardiac remodeling in newborn, young, and/ or adult Npr1⁻/⁻ homozygous null mutant mice. The data presented in Table 1 show that adult Npr1⁻/⁻ mutant mice exhibited 34 ± 7 mmHg higher systolic blood pressures as compared with age-matched Npr1⁺/⁺ wild-type animals. Heart rate was significantly decreased (p<0.05) in both young and adult Npr1⁻/⁻ null mutant mice as compared with age-matched wild-type controls. The HW/BW ratio in Npr1⁻/⁻ mice was increased by 52 % (global hypertrophy) in young animals at 4-weeks of age and
it further increased to 64 % (severe hypertrophy) in adult animals at 22-weeks of age as compared with age-matched Npr1⁺/⁺ wild-type control mice. Myocyte cross-sectional area was measured in heart sections from both young and adult mutant and wild-type mice, which was found to be significantly increased (p<0.01) in young Npr1⁻/⁻ mice at 4-weeks of age and further increased in adult Npr1⁻/⁻ mice as compared with Npr1⁺/⁺ wild-type mice (Table I). M-mode echocardiographic analysis revealed that young Npr1⁻/⁻ mutant mice showed elevated septal wall thickness, posterior wall thickness, and significantly elevated left ventricular end diastolic and systolic dimensions (p<0.01) as compared with Npr1⁺/⁺ wild-type mice (Table I). The functional parameters such as fractional shortening (FS) and ejection fraction (EF) were significantly reduced in young Npr1⁻/⁻ mice. Adult null mutant mice showed further increases in septal wall thickness, posterior wall thickness, and significantly lower FS and EF (p<0.001) as compared with age-matched wild-type mice, indicating that in both young and adult Npr1⁻/⁻ mice, the cardiac function is significantly compromised. M-mode echocardiographic analysis showed a progressive cardiac hypertrophy and congestive heart failure in adult Npr1⁻/⁻ mice. Masson’s trichrome staining in the left ventricular sections of young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts are depicted in Fig.1, A-D. Blue color staining reflects the intensity of fibrosis in the heart tissues. An increased fibrosis (20 %; p<0.001) was observed in young Npr1⁻/⁻ mice and more pronounced fibrosis (35 %; p<0.001) was noted in adult Npr1⁻/⁻ mice hearts as compared with age-matched Npr1⁺/⁺ wild-type counterparts (Fig. 1 E). Heart size and LVW/BW ratio in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice at 4-weeks and 22-weeks of age are shown in Fig 1, F and G. It is evident that Npr1⁻/⁻ mutant mice have global cardiac hypertrophy at 4-weeks and severe hypertrophy with chamber dilatation at 22-weeks of age. The plasma and ventricular tissue
cGMP levels were significantly reduced by 5-fold and 6-fold in young and adult null mutant mice, respectively, as compared with age-matched wild-type mice (Fig. 2).

The representative Northern blot analyses and densitometric quantification of left ventricular hypertrophy marker genes (ANP, BNP, β-MHC, and SERCA-2a) in young and adult Npr1−/− and Npr1+/+ mice are presented in Fig. 3, A-D. Young homozygous null mutant mice exhibited an increased ventricular mRNA expression of ANP (5-fold), BNP (4-fold), and β-MHC (2-fold) as compared with age-matched Npr1+/+ wild-type animals. In contrast, SERCA-2a mRNA expression was significantly reduced (2-fold) in Npr1−/− mice as compared with Npr1+/+ wild-type controls. At 22-weeks of age, adult mutant mice displayed further increases in ANP, BNP, and β-MHC transcripts along with a decreased expression of SERCA-2a gene. Fig. 4, A and B show the representative electrophoretic pattern of α- and β-MHC protein isoforms in young and adult Npr1−/− and Npr1+/+ mice hearts. A significant (p<0.001) shift from α- to β-MHC protein isoform occurred in both young and adult mutant mice as compared with wild-type mice (Fig. 4, C and D). The left ventricular SERCA-2a protein levels were also down-regulated in both young and adult Npr1−/− mice hearts (4-fold and 5-fold, respectively, p<0.001) in comparison with age-matched Npr1+/+ wild-type control mice hearts (Fig. 4, E-G).

We determined the expression levels of MMPs, TIMPs, pro-inflammatory cytokines, and pro-collagen I in Npr1 null mutant and wild-type mice. The left ventricular total collagen concentration, collagen type I/III ratio, and ELISA quantification of active MMP-2, MMP-9, and TNF-α levels in young and adult Npr1−/− and Npr1+/+ mice are presented in Table II. Significant increases in total collagen concentrations and collagen type I/III ratio were found (p<0.01) in both young and adult Npr1−/− mutant mice hearts as compared with age-matched wild-type control mice hearts. Although, MMP-2 activity increased by 3-fold in adult Npr1−/− mice, MMP-9
activity was more pronounced in newborn, young, and adult Npr1⁻/⁻ mice hearts (4-fold and 5-fold; p<0.001; respectively). *In situ* zymography analysis of total MMPs activities in ventricular tissues showed that young and adult mutant mice exhibited 3- to 4-fold increased gelatinases activity (p<0.001), respectively, as compared with age-matched wild-type control mice (Fig. 5).

The representative RNase protection assays of MMP-2, MMP-9, and pro-collagen I mRNA transcript levels in newborn, young, and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice are presented in Fig. 6. Newborn Npr1⁻/⁻ pups displayed significant expression of mRNAs of MMP-2 (2-fold; p<0.01), MMP-9 (2-fold; p<0.01), and pro-collagen I (2-fold; p<0.01) as compared with Npr1⁺/⁺ control pups (Fig. 6, A and D). At 4-weeks, young Npr1⁻/⁻ mice continued to show an increased expression of mRNAs of MMP-2 (2-fold; p<0.01), MMP-9 (3-fold; p<0.001), and pro-collagen I (3-fold; p<0.001) as compared with Npr1⁺/⁺ wild-type mice (Fig. 6, B and E). Furthermore, at 22-weeks of age, adult Npr1⁻/⁻ mice showed more pronounced and greater increases (4-fold; p<0.001) in MMP-9 mRNA transcript (Fig. 6, C and F). The representative Western blot analyses of MMP-2 (68 kDa), MMP-9 (89 kDa), TIMP-1(28 kDa), and TIMP-2 (21 kDa) protein levels in left ventricular tissues of young Npr1⁻/⁻ and Npr1⁺/⁺ mice are shown in Fig. 7, A-D). It is evident that MMP-2 (3-fold; p<0.001) and MMP-9 (4-fold; P<0.001) protein levels were significantly increased in young Npr1⁻/⁻ mice in comparison with age-matched wild-type control mice. In contrast, TIMP-1 and TIMP-2 proteins were down-regulated in homozygous null mutant mice hearts (Fig. 7, C and D). Western blot analyses of TGF-β1 and TGF-β1R levels in young Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts are shown in Fig.7 E and F. Both TGF-β1 and TGF-β1R levels were significantly up-regulated (2- and 3-fold; p<0.001, respectively) in young Npr1⁻/⁻ mice than age-matched Npr1⁺/⁺ wild-type controls. Furthermore, newborn, young, and adult
Npr1\(^{-/-}\) mutant mice displayed significant increases in TNF-\(\alpha\) levels (8-fold; \(p<0.001\)) as compared with age-matched Npr1\(^{+/+}\) wild-type mice (Table II).

The representative gelatin zymogram depicting MMP-9 activity in newborn pups, young, and adult Npr1\(^{-/-}\) and Npr1\(^{+/+}\) mice are shown in Fig. 8 A. The lytic band of the 89-kDa corresponding to MMP-9 is more prominent in Npr1\(^{-/-}\) mice hearts than in Npr1\(^{+/+}\) wild-type mice hearts. Densitometric analysis of zymogram-lytic band showed that MMP-9 activity was increased by 2-fold in newborn pups and further increased by 4-fold in young and adult Npr1\(^{-/-}\) mutant mice in contrast with age-matched Npr1\(^{+/+}\) wild-type mice (Fig. 8 B). The observed increase in MMP-9 activity in null mutant mice was positively correlated (\(P<0.001; R=0.91\)) with an increase in left ventricular fibrosis (Fig. 8 C). To confirm the mechanistic role of MMPs in cardiac hypertrophy and fibrosis, Npr1\(^{-/-}\) mice were treated with MMP-inhibitor, GM 6001. The MMP inhibitor-treated Npr1\(^{-/-}\) mice showed a decrease in MMP-2 and MMP-9 activities by 5-fold and 3-fold (\(p<0.001\)), respectively, and a reduction in fibrosis by 75% as compared with untreated control mice (Table III). Furthermore, M-mode echocardiographic analysis showed an attenuated ventricular dilatation and improved fractional shortening in MMP inhibitor-treated Npr1\(^{-/-}\) mice as compared with untreated control mice. The changes in systolic blood pressures, HW/BW ratio, and MMP-9 activity in young Npr1\(^{-/-}\) mutant mice before and after anti-hypertensive drug treatments are shown in Fig. 9, A-C. Among the anti-hypertensive drugs selected, hydralazine (HDZ) and captopril (CAP) treatments for 4-weeks reversed the elevated blood pressures of mutant mice but only partially inhibited the elevated MMP-9 activity (HDZ: 35 % and CAP: 31 %). Bendrofluomethiazide treatment partially reduced the elevated blood pressure and MMP-9 activity in Npr1\(^{-/-}\) mutant mice. Surprisingly, the anti-hypertensive drug
treatments did not significantly inhibit cardiac hypertrophy (elevated HW/BW ratio) in Npr1⁻/⁻ mutant mice hearts (Fig. 9 B).

Electrophoretic mobility shift assay (EMSA) was carried out to analyze NF-kB activity in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts (Fig. 10, A and B). The specificity of the detected NF-kB bands were confirmed by competition analyses using a 100-fold excess molar concentrations of unlabeled NF-kB, which abrogated the p65 antibody complex formation and appearance of super-shift band. Nuclear extract isolated from young and adult Npr1⁻/⁻ mice showed a significant increases in NF-kB binding activity by almost 4-fold as compared with age-matched Npr1⁺/⁺ wild-type mice. Fig. 10, C and D show the Western blot analysis of NF-kB (p65) subunit and phosphorylated inhibitory kappa B-α (p-IkB-α) protein levels in nuclear and cytoplasmic extracts, respectively, from young Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts. Significant increases in the p65 (4-fold; p<0.001) and p-IkB-α (3-fold; p<0.001) proteins were observed in the nuclear and cytoplasmic extracts, respectively, in young Npr1⁻/⁻ null mutant mice hearts in contrast with age-matched Npr1⁺/⁺ wild-type controls. Furthermore, IKK kinase activity increased significantly (4-fold; p<0.001) in young Npr1⁻/⁻ mutant mice hearts as compared with age-matched Npr1⁺/⁺ wild-type control hearts (Fig. 10, E and F).

**DISCUSSION**

The studies presented here in demonstrate that Npr1 gene-disruption in mice provokes enhanced expression and activation of MMPs and pro-inflammatory cytokines associated with cardiac hypertrophy, fibrosis, and ECM remodeling. The gelatinases (MMP-2 and MMP-9) are considered key enzymes in matrix component degradation and have been suggested to play a critical role in matrix remodeling and left ventricular enlargement (16). Conversely, inhibition of
MMPs has been shown to limit ECM destruction and to improve myocardial structure and its function in animal models (15, 16, 32). Increased expression and activities of MMP-2 and MMP-9 in Npr1−/− null mutant mice are consistent with previously reported observations that MMPs are elevated in failing hearts of both animal models as well as humans (15, 17, 33, 34). Our present findings are consistent with the notion that an activated ANP/NPRA system can inhibit collagen synthesis (20), which advances the hypothesis that disruption of NPRA signaling pathway can lead to an activation of ECM components and exaggerated cardiac hypertrophy and remodeling. It is expected that an increase in MMP activity would result in a decreased MMP substrate; collagens. However, the contrary is usually observed; enhanced MMP activity is accompanied by increased fibrosis whereas attenuated MMP activity is associated with decreased deposition of fibrotic tissues (32, 35, 36). Indeed, this seems to be paradoxical because the total matrix collagen content is a function of both synthesis and degradation; in turn, the degraded products of matrix proteins can serve as a catalyst for collagen synthesis (32, 37). As a result, the increased deposition of inappropriately structured fibrotic tissues may occur in the myocardium. Consistent with this notion, the observed parallel increases in total collagen concentrations and MMPs levels in Npr1 null mutant mice in the present study is a function of both collagen synthesis and degradation. The differences in collagen contents between wild-type and Npr1 null mutant mice are meaningful and significant in both young and adult animals. Our results implicate that an increased MMPs levels in Npr1 null mutant mice hearts would be detrimental to normal collagenous matrix; thus, it is not surprising that the changes in the structural properties of collagen seems to be associated with the development of progressive cardiac hypertrophy and ventricular remodeling in Npr1−/− null mutant mice.
The present results also show that expression of MMP-2 and MMP-9 are significantly enhanced, and expression of TIMP-1 and TIMP-2 are attenuated in Npr1\(^{-/-}\) null mutant mice hearts. It is known that TIMPs bind to the active site of MMPs, thereby blocking their access to extracellular matrix substrates. Previously, it has been shown that TIMP-1 forms a complex with MMP-9 and effectively inhibits its activation (15). Thus, altered levels of MMPs and TIMPs observed in Npr1\(^{-/-}\) null mutant mice hearts could be implicated to promote ventricular remodeling of extracellular matrix that may contribute to abnormal cardiomyocyte architecture and organization. The treatment of Npr1\(^{-/-}\) mice with MMP inhibitor, GM-6001 attenuated the MMPs activities and cardiac fibrosis and improved ventricular dilatation, suggesting that activation of MMPs contributes to the remodeling process in mutant mice. To our surprise, MMP inhibitor-treated Npr1\(^{-/-}\) mice did not show any significant change in myocyte cross-sectional area as compared with untreated control mice. However, our echocardiographic analysis demonstrated that MMP inhibitor-treated mutant mice showed an attenuated ventricular dilation and an improved fractional shortening. Our findings suggest that MMPs seem to enhance ventricular dilation and fibrosis, however, do not play a direct role in the development of cardiac hypertrophy. Our results further support the previous findings that treatment of spontaneously hypertensive heart failure (SHHF) rats with MMP inhibitor, PD 166793, prevented cardiac dilation, preserved contractility, and showed a reduction in myocardial fibrosis compared with the untreated SHHF rats (38). Furthermore, MMPs inhibitors have been implicated to attenuate the degree of LV dilation did not have salutary effect on ventricular hypertrophy (39, 40). Nevertheless, several studies suggest a positive correlation exists between MMPs activation and ventricular remodeling process, and MMPs inhibition attenuates both fibrosis and cardiac hypertrophy (15, 16, 32). Strauss et al., (41) demonstrated that in balloon-injured arteries, the
MMP inhibitor reduced both collagen synthesis and degradation, resulting in reduced accumulation of collagen. These authors suggested that degradation of newly synthesized collagen is an important mechanism regulating collagen accumulation and that MMPs play an integral role in collagen turnover. Indeed, more experimentations are needed to delineate the exact role of MMPs in two distinct phenomena of cardiac fibrosis and hypertrophy.

Our results demonstrate that expression of SERCA-2a progressively decreased in hypertrophied left ventricular heart tissues of Npr1 null mutant mice. A decrease in SERCA-2a levels has been suggested to contribute to the slowing of relaxation in failing human hearts (42). Thus, altered levels of sarcoplasmic reticulum Ca\(^{2+}\)-handling proteins seem to underlie contractile dysfunction of the heart. The disturbances in diastolic contractile performance is correlated to an impaired uptake of Ca\(^{2+}\) into sarcoplasmic reticulum in animal models and in human dilated cardiomyopathy (43). Thus, a decreased level of SERCA-2a in Npr1 null mutant mouse hearts observed in the present study correlates well with the diastolic dysfunction of these mutant mice. Echocardiographic analysis showed that functional parameters such as fractional shortening and ejection fraction were reduced significantly in both young and adult Npr1 null mice as compared to age-matched Npr1 wild-type mice (Table I). These present data suggest that Npr1 null mice are experiencing a compromised cardiac function. Our results also provide quantitative assessment of cardiac myosin isoform expression, demonstrating a significant shift from the \(\alpha\)- to the \(\beta\)-MHC isoform in Npr1 null mice hearts. It has been shown that \(\alpha\)-MHC is detectable in non-failing myocardium and is virtually undetectable in failing and hypertrophied hearts (44). The \(\alpha\)-MHC isoform contains high ATPase activity and hearts expressing \(\alpha\)-MHC have a more rapid contractile velocity than hearts expressing \(\beta\)-MHC, which is associated with decreased myosin ATPase activity (45). However, the potential functional significance and
molecular mechanisms of changes in SERCA-2a gene expression and altered myosin composition in Npr1⁻/⁻ null mutant mice remains to be investigated in more detail.

It should be noted that defects in cardiac relaxation may trigger specific structural and molecular changes in the heart. Recently, it has been suggested that individual cardiac myocytes are significantly larger in Npr1⁻/⁻ null mutant mice at birth, and cardiac hypertrophy predominates independent of blood pressure (46, 47). Our results demonstrate that both newborn pups and young Npr1 null mutant mice display significant increases in left ventricular β-MHC mRNAs, and a decrease in SERCA-2a mRNA expression, suggesting that hypertrophic genes are probably activated at an early age before blood pressure reaches harmful high levels. To delineate, the impact of increased blood pressure on left ventricular remodeling process, we have treated the mutant mice with three different anti-hypertensive drugs (hydralazine, captopril, and bendrofluomethiazide), which have been shown to control hypertension and hypertrophy. To our surprise, normalization of systolic blood pressures did not show any salutary effect in reversing the hypertrophy and fibrosis in Npr1⁻/⁻ mice, suggesting that NPRA/cGMP signaling has a direct regulatory role on early genes involved in the cardiac hypertrophic growth and fibrosis. It has been shown that over-expression of NPRA specifically in cardiomyocytes inhibits the hypertrophic effects of isoproterenol and aortic constriction in mouse models, indicating that NPRA signaling antagonizes the cardiac growth in disease states (48). β-MHC, and SERCA-2a gene expression are considered sensitive indicators of pathological cardiac hypertrophy, but not physiological hypertrophy and have been reported as the principal biomarkers in several models of hypertrophic phenotypes (49, 50). Furthermore, increased expression of TGF-β1 and TNF-α is also associated with cardiac hypertrophy, and blocking the action of these cytokines could prevent myocardial diastolic dysfunction and heart failure (32, 51, 52). Consistent with those
previous findings, our results show that both TGF-β1 and its receptor are elevated, which may contribute to increased myocardial fibrosis and collagen synthesis in mice lacking NPRA. In agreement with our present findings, it has recently been shown that BNP, which activates, NPRA, inhibits TGF-β1-induced ECM protein synthesis in vitro (21). Plasma and ventricular cGMP levels were decreased by almost five-fold in Npr1−/− mice as compared with Npr1+/+ mice. Activation of NPRA by natriuretic peptides increases cGMP production in a number of cell types; and an increased cGMP level is positively correlated with anti-hypertrophic and anti-proliferative effects of natriuretic peptides in vitro culture conditions (8-12). Furthermore, inhibitory action of natriuretic peptides on angiotensin II-induced cardiac hypertrophy is mimicked by 8-bromo cGMP. NPRA receptor antagonist HS-142-1 has also been shown to block the anti-hypertrophic effects of ANP by inhibiting the generation of cGMP in neonatal and adult cardiac myocytes (9). Thus, cGMP, can be considered as an important mediator in anti-hypertrophic responses to ANP/NPRA signaling system.

Significant increases in NF-kB binding activity was observed in both young and adult Npr1−/− mice, suggesting that an increased activation of NF-kB occurs in absence of NPRA signaling in mutant mice hearts. NF-kB is a ubiquitous multifunctional signaling system that contributes to cell survival, apoptosis, and inflammation, and it plays a critical role in transcriptional activation of multiple genes that contribute to the development of end-organ damage (53). It has been suggested that MMPs and pro-inflammatory cytokine genes are regulated by NF-kB, however, only little is known about the cross-talk between natriuretic peptides and NF-kB pathways. The sustained induction of NF-kB has been reported in experimental animal and human heart failure conditions (54). ANP/NPRA system has been suggested to attenuate production of inflammatory mediators such as TNF-α by regulating the
NF-kB pathway (55). Furthermore, cGMP analogues have been shown to suppress the induction of vascular cell adhesion molecule-1 and expression of hypoxia-associated vascular endothelial growth factor by inhibiting the activation of NF-kB (56). Our study shows that NF-kB is activated and associated with ventricular remodeling in Npr1−/− mice, indicating a relationship between NF-kB activation and myocardial remodeling. Fig. 11 depicts the diagrammatic representation of signaling pathways involved in promoting the cardiac hypertrophy and remodeling in absence of NPRA and cGMP signaling. Our findings show that disruption of NPRA/cGMP signaling pathway abolishes the local growth moderating effects and thereby promotes the development of cardiac abnormalities independent of blood pressure. ANP/NPRA system has been suggested to regulate cardiac function, in particular relaxation; therefore, the disruption of NPRA signaling can lead to impaired cardiac relaxation resulting in specific structural and molecular changes in Npr1−/− null mutant mice hearts. Nitric oxide (NO), which also utilizes cGMP as a second messenger has been shown to prevent hypertrophy of neonatal and adult cardiac myocytes (57). In addition, NO inhibition in ex vivo aortic tissues causes a dose-dependent increase in MMP-9 expression and its activity in association with an increased NF-kB and activator protein-1 (AP-1) activity (58, 59). These findings provide strong support for the involvement of cGMP signaling axis in regulation of the MMPs and ventricular remodeling process.

In conclusion, several key findings emerge form this study. First, it provides direct evidence that MMP-2, MMP-9, and TNF-α genes are potently activated in Npr1−/− homozygous null mutant mice hearts at an early age and remain elevated in adult mutant mice. Second, it demonstrates that total collagen, collagen type I/III ratio, and myocardial fibrosis are dramatically increased in adult mutant mice hearts. Third, it identifies hypertrophic marker genes
including; β-MHC and TGF-β1, which are significantly up-regulated in ventricular tissues of both young and adult Npr1−/− mutant mice. Fourth, it shows a marked shift from the α- to the β-MHC protein isoforms and a significant decrease in SERCA-2a levels in mutant mice hearts. Finally, it shows enhanced NF-kB binding activity and accelerated translocation of p65 subunit from the cytoplasm to nuclear fraction of ventricular tissues of Npr1−/− mutant mice hearts. Together, these findings reveal several features of complex assembly that appear to be unique to cardiac hypertrophy, fibrosis, and remodeling in Npr1−/− homozygous null mutant mice.

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Table I

Blood pressure, heart rate, heart weight to body weight ratio, and cardiac structure and function analyses in young and adult Npr1<sup>+/+</sup> and Npr1<sup>-/-</sup> mice.

| Parameters                  | Young     | Adult    |
|-----------------------------|-----------|----------|
|                             | Npr1<sup>+/+</sup> | Npr1<sup>-/-</sup> | Npr1<sup>+/+</sup> | Npr1<sup>-/-</sup> |
| BW (g)                      | 26±0.8    | 25±1.2   | 31±0.7   | 32±1.4   |
| BP (mmHg)                   | 97±5      | 125±6<sup>*</sup> | 104±6    | 138±7<sup>**</sup> |
| HR (bpm)                    | 475±13    | 455±10   | 488±14   | 462±16<sup>*</sup> |
| HW/BW (mg/g)                | 4.6±0.3   | 7.0±0.5<sup>**</sup> | 4.8±0.4  | 7.9±0.7<sup>***</sup> |
| Myocyte cross-sectional area (µm<sup>2</sup>) | 370±22    | 785±43<sup>***</sup> | 420±34   | 962±82<sup>***</sup> |
| LVEDS(mm)                   | 1.82±0.02 | 2.22±0.04<sup>**</sup> | 2.05±0.03 | 2.82±0.05<sup>***</sup> |
| LVEDD(mm)                   | 3.72±0.03 | 3.95±0.05<sup>**</sup> | 3.94±0.04 | 4.33±0.07<sup>***</sup> |
| IVSTD(mm)                   | 0.63±0.03 | 0.83±0.04<sup>**</sup> | 0.65±0.03 | 0.98±0.05<sup>***</sup> |
| PWT(mm)                     | 0.90±0.02 | 1.13±0.05<sup>***</sup> | 0.93±0.02 | 1.33±0.04<sup>***</sup> |
| FS (%)                      | 51±3.5    | 44±2.2<sup>*</sup> | 48±2.3   | 35±1.5<sup>***</sup> |

Cardiac structure and function analysis, blood pressure (BP), heart rate (HR), and heart weight to body weight (HW/BW) ratio were performed as described under the Experimental...
Table I (continued)

Procedures. LVEDS, left ventricular end systolic dimension; LVEDD, left ventricular end diastolic dimension; IVSTD, interventricular sepal wall thickness (diastolic); PWT, posterior ventricular wall thickness (systolic); FS, fractional shortening (%). Values are expressed as mean ±SEM (N=8 animals in each group). * p<0.05, ** p<0.01, and *** p<0.001.
Table II

Total collagen, matrix metalloproteinases (MMP-2 and MMP-9), and TNF-α levels in the left ventricular heart tissues of newborn pups, young, and adult Npr1\(^{+/+}\) and Npr1\(^{-/-}\) mice.

| Parameters          | Newborn | Young | Adult |
|---------------------|---------|-------|-------|
|                     | Npr1\(^{+/+}\) | Npr1\(^{-/-}\) | Npr1\(^{+/+}\) | Npr1\(^{-/-}\) | Npr1\(^{+/+}\) | Npr1\(^{-/-}\) |
| Collagen (mg/g tissue) | 1.1 ±0.13 | 1.2±0.30+ | 2.1 ±0.14 | 3.24±0.33** | 2.4±0.24 | 5.62±0.52** |
| Collagen I/III ratio | 0.5±0.03 | 0.64±0.04+ | 0.7±0.05 | 1.24±0.16 ** | 0.8±0.05 | 1.52±0.21** |
| TNF-α (pg/mg protein) | 1.0± 0.4 | 8.5±0.8 *** | 1.5± 0.6 | 12.0±3 *** | 1.8±0.5 | 14.1±4 *** |
| MMP-2 (ng.h\(^{-1}\).g\(^{-1}\)) | 144± 8 | 280± 15 *** | 254± 23 | 728± 28 *** | 268± 14 | 866±26 *** |
| MMP-9 (ng.h\(^{-1}\).g\(^{-1}\)) | 26±3 | 80±5*** | 86±7 | 350±11*** | 94±8 | 540±12*** |

Collagen was quantified by measuring hydroxyproline contents. MMP-2 and MMP-9 activities in the left ventricular heart tissues of young and adult Npr1\(^{-/-}\) and Npr1\(^{+/+}\) mice were determined as described under the Experimental Procedures. The quantification of TNF-α protein levels was carried out using a ELISA assay kit. Values are expressed as mean ±SEM (N=8 animals in each group). * p<0.05, ** p<0.01, ***p<0.001, and + indicates non-significant values.
Table III

Effect of MMPs inhibitor, (GM 6001) treatment on MMPs activities and cardiac functions in wild-type (Npr1<sup>+/+</sup>) and homozygous null mutant (Npr1<sup>-/-</sup>) mice.

| Parameters                              | Without MMP inhibitor | With MMP inhibitor |
|-----------------------------------------|-----------------------|--------------------|
|                                         | Npr1<sup>+/+</sup>    | Npr1<sup>-/-</sup> |
| MMP-2 activity (ng.h<sup>-1</sup>.g<sup>-1</sup>) | 260±15                | 890±22***          |
|                                         | 230±11                | 190±7***           |
| MMP-9 activity (ng.h<sup>-1</sup>.g<sup>-1</sup>) | 96±13                 | 540±20***          |
|                                         | 71±12                 | 172±14***          |
| Collagen (mg/g tissue)                  | 2.5±0.2               | 5.6±0.3***         |
|                                         | 2.2±0.2               | 3.2±0.15***        |
| Fibrosis %                              | 0.8±0.03              | 35±2.5***          |
|                                         | 0.6±0.04              | 8.5±0.74***        |
| Myocyte cross-sectional area (µm<sup>2</sup>) | 415±22                | 915±73***          |
|                                         | 425±34                | 862±82***          |
| LVEDS (mm)                              | 2.15±0.03             | 2.92±0.06***       |
|                                         | 1.92±0.02             | 2.12±0.04***       |
| LVEDD (mm)                              | 3.98±0.04             | 4.54±0.07***       |
|                                         | 3.80±0.03             | 3.92±0.04***       |
| FS (%)                                  | 47±2.1                | 35±1.3***          |
|                                         | 49±2.2                | 45±1.5††          |
Npr1−/− and Npr1+/+ mice were treated with MMP inhibitor, GM 6001 for 4 weeks at a dose of 100 mg/kg body weight. MMP-2 and MMP-9 activities, myocyte cross-sectional area, and fibrosis in the left ventricular heart tissues of adult Npr1−/− and Npr1+/+ mice were determined as described under the **Experimental Procedures**. Collagen was quantified by measuring hydroxyproline contents. Echocardiographic measurements were carried out using an ultrasonic system as described under the **Experimental Procedures**. LVEDS, left ventricular end systolic dimension; LVEDD, left ventricular end diastolic dimension; FS, fractional shortening (%). Values are expressed as mean ±SEM (N=8 animals in each group). * p<0.05, ** p<0.01, and *** p<0.001, untreated Npr1+/+ vs untreated Npr1−/−; † p<0.05, †† p<0.01, and ††† p<0.001, untreated Npr1−/− vs GM 6001 treated Npr1−/−; ‡ p<0.05, ‡‡ p<0.01, and ‡‡‡ p<0.001, GM 6001 treated Npr1+/+ vs GM 6001 treated Npr1−/−.

|          | Npr1−/− (untreated) | Npr1−/− (GM 6001 treated) | Npr1+/+ (untreated) | Npr1+/+ (GM 6001 treated) |
|----------|---------------------|---------------------------|---------------------|---------------------------|
| MMP-2    | 100 ± 20            | 80 ± 15                   | 120 ± 25            | 90 ± 15                   |
| MMP-9    | 100 ± 20            | 80 ± 15                   | 120 ± 25            | 90 ± 15                   |
| Myocyte  | 50 ± 5              | 60 ± 6                    | 70 ± 7              | 80 ± 8                    |
| Fibrosis | 10 ± 2              | 8 ± 1                     | 12 ± 3              | 10 ± 2                    |
FIGURE LEGENDS

Figure 1: Comparative analysis of left ventricular hypertrophy and fibrosis in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts: Representative sections from young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts were subjected to Masson’s trichrome staining at 4-weeks (A and B) and 22-weeks (C and D) of age, respectively. Blue color reflects the fibrosis in the heart. E) represents the percent interstitial fibrosis in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts. F) represents hearts from young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice at 4- and 22-weeks of age, respectively, showing cardiac hypertrophy and chamber dilatation. G) represents left ventricular weight to body weight ratio in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice. Values are expressed as mean ±SEM (N=8 animals in each group). ** p<0.01 and ***p<0.001; Npr1⁻/⁻ vs Npr1⁺/⁺.

Figure 2: Plasma and ventricular cGMP levels in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice at 4- and 22-weeks of age: Panel A) shows plasma cGMP levels and panel B) shows ventricular tissue cGMP levels. cGMP concentration was determined using direct competitive enzyme immunoassay kit as described under the Experimental Procedures. Values are expressed as Mean±SEM (N=16 animals in each group. *** p<0.001 Npr1⁻/⁻ vs Npr1⁺/⁺.

Figure 3: Expression profiles of hypertrophy marker genes in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice hearts: A and B) represent Northern blots showing mRNA expression of ANP, BNP, β-MHC, and SERCA-2a in young (4-weeks) and adult (22-weeks) Npr1⁻/⁻ and Npr1⁺/⁺ mice. Total RNA (10 µg) was fractionated on 1% formaldehyde-agarose gel, transferred to nylon membrane, and hybridized with [γ-³²P]ATP-labeled oligonucleotide probe as described under the Experimental Procedures. GADPH was used as an internal control. C and D) represent
densitometric analyses of mRNA transcripts normalized with GADPH mRNA expression. Values are expressed as mean ±SEM (N=8 animals in each group). **p<0.01 and ***p<0.001; Npr1−/− vs Npr1+/+.

**Figure 4:** Analyses of α- and β-MHC protein isoform shift and SERCA-2a protein levels in young and adult Npr1−/− and Npr1+/+ mice hearts: A and B) show α- and β-MHC protein isoforms in young and adult Npr1−/− and Npr1+/+ mice hearts. C and D) represent the relative change of α- to β-MHC protein isoforms in young and adult Npr1−/− and Npr1+/+ mice hearts. E and F) represent Western blot analysis of SERCA-2a protein in the left ventricular heart tissues from young and adult Npr1−/− and Npr1+/+ mice. G) represents densitometric analysis of SERCA-2a protein bands normalized to β-actin. Values are expressed as mean ±SEM (N=8 animals in each group). ***p<0.001; Npr1−/− vs Npr1+/+.

**Figure 5:** Representative in situ zymogram showing total MMPs activities in young and adult Npr1−/− and Npr1+/+ mice: Panels A-F) represent in situ zymogram showing total gelatinase activity in heart tissues from young and adult Npr1−/− and Npr1+/+ mice at 4-weeks, and 22-weeks of age, respectively. Fresh cardiac sections (10 microns) were incubated at 37° C for 5 h with fluorogenic gelatin substrate as described under the Experimental Procedures. Green fluorescence indicates MMP activities in heart tissues of young and adult Npr1−/− mice (A and B), young and adult Npr1+/+ mice (C and D), adult Npr1−/− mice treated with MMP inhibitor, 1,10-phenanthroline monohydrate (E), and adult Npr1−/− mice treated with MMP inhibitor and stained for nuclei with DAPI (F). Each zymogram is the representative of 8-10 sections in each group of animals.
Figure 6: Expression analyses of MMP-2, MMP-9, and pro-collagen I in Npr1−/− and Npr1+/+ mice hearts: A, B, and C) represent autoradiograms of RNase protection assay (RPA) using mouse multi-probe templates (MMP-2, MMP-9, and pro-Col I) in newborn pups, young, and adult Npr1−/− and Npr1+/+ mice hearts at 2-days, 4-weeks, and 22-weeks of age, respectively. RPA was carried out as described under the Experimental Procedures. Protected hybrid bands were resolved using 5% denaturing polyacrylamide gel and autoradiography. D, E, and F) represent relative expression of MMP-2, MMP-9, and pro-collagen I mRNAs normalized to GADPH. Values are expressed as mean ± SEM (N=8 animals in each group). ** p<0.01 and ***p<0.001; Npr1−/− vs Npr1+/+.

Figure 7: Quantitative analyses of MMPs, TIMPs, TGF-β1, and TGF-β1R protein levels in left ventricular tissues from Npr1−/− and Npr1+/+ mice at 4-weeks of age: A and B) represent the Western blot and densitometric analysis of MMP-2, and MMP-9. C and D) represent Western blot and densitometric analysis of TIMP-1 and TIMP-2. E and F) represent Western blot and densitometric analysis of TGF-β1 and TGF-β1R. Values are expressed as mean ±SEM (N=8 animals in each group). * p<0.05, ** p<0.01, and ***p<0.001; Npr1−/− vs Npr1+/+.

Figure 8: Zymogram analysis of MMP-9 activity in newborn pups, young, and adult Npr1−/− and Npr1+/+ mice: A) representative zymogram showing MMP-9 activity in heart tissues from newborn pups, young, and adult Npr1−/− and Npr1+/+ mice at 2-days, 4-weeks, and 22-weeks of age, respectively. Samples (25 µg proteins) were loaded on a 10% gel containing 1 mg/ml of gelatin separated under non-reducing conditions as described under the Experimental...
Procedures. Gels were stained with 0.25% Coomassie Brilliant Blue and destained until the clear lytic bands were visible. B) represents densitometric analysis of clear lytic bands. C) represents the relationship between MMP-9 activity and percent fibrosis. M indicates MMP-9 standard. Values are expressed as mean ±SEM (N=8 animals in each group). * p<0.05 and ***p<0.001; Npr1⁻/⁻ vs Npr1⁺/⁺.

Figure 9: Effect of anti-hypertensive drug treatments on blood pressure reduction, changes in HW/BW ratio, and MMP-9 activity in young Npr1⁻/⁻ and Npr1⁺/⁺ mice: Panels A, B, and C) represent blood pressure (BP), heart weight/body weight (HW/BW) ratio, and MMP-9 activity, respectively, in young Npr1⁻/⁻ and Npr1⁺/⁺ mice. Blood pressures were measured before and after drug treatments for 4-weeks period of time. Animals were sacrificed; HW/BW ratio and MMP-9 activity were analyzed as described under the Experimental Procedures. Animals were treated with hydralazine, (HDZ, 25mg/kg/day); captopril, (CAP 0.5 mg/kg/day); and bendrofluomethiazide, (BTZ 10mg/kg/day). Values are expressed as mean ±SEM; (N=8 animals in each group). * p<0.05, ** p<0.01, and ***p<0.001. Untreated Npr1⁻/⁻ vs untreated Npr1⁺/⁺; treated Npr1⁻/⁻ vs treated Npr1⁺/⁺; untreated Npr1⁻/⁻ vs treated Npr1⁻/⁻.

Figure 10: Comparative analysis of NF-kB binding and p-IkB-α in young and adult Npr1⁻/⁻ and Npr1⁺/⁺ mice: A) represents autoradiogram of NF-kB binding activity in nuclear fraction of Npr1⁻/⁻ and Npr1⁺/⁺ mice. Cold competitor assays were performed by adding 100-fold excess molar concentrations of unlabeled NF-kB oligonucleotides. B) represents densitometric analysis of NF-kB binding activity. C) represents the Western blot analysis of p65 and p-IkB-α proteins in nuclear and cytoplasmic fractions of Npr1⁻/⁻ and Npr1⁺/⁺ mice. Nuclear and cytoplasmic
proteins (20 µg/lane) were separated using 10% SDS-PAGE, transferred onto a PVDF membrane, and incubated at 4°C overnight with p65 and p-IkB-α primary antibodies as described under the Experimental Procedures. D) represent densitometric analysis of p65 and p-IkB-α protein bands. E and F) represent the autoradiogram of IKK-kinase activity and density units in Npr1−/− and Npr1+/+ mice hearts. Values are expressed as mean ±SEM; (N=8 animals in each group). ***p<0.001; Npr1−/− vs Npr1+/+.

**Figure 11: Diagrammatic representation of NPRA signaling in cardiac hypertrophy and ECM remodeling:** Disruption of NPRA signaling leads to impaired cardiac relaxation that triggers specific structural and molecular changes in Npr1−/− mice hearts. The activated NF-kB translocates into the nucleus and activates MMPs and pro-inflammatory cytokine genes. In turn, the absence of NPRA signaling should abolish the local growth inhibitory effects of ANP and BNP, and thereby it likely promotes the development of cardiac hypertrophy and fibrosis. KHD, kinase-homology regulatory domain; CG, guanylyl cyclase catalytic domain.
Figure 2

A

Plasma cGMP
(pmols/ml)

-/-  +/+  

Young Npr1  Adult Npr1

*** P < 0.001; N=8/group

B

Ventricular cGMP
(pmols/mg protein)

-/-  +/+  

Young Npr1  Adult Npr1

*** P < 0.001; N=8/group
Figure 3

A

Young Npr1

-/-  +/+  

ANP  BNP  SERCA-2a  β-MHC  GADPH

B

Adult Npr1

-/-  +/+  

ANP  BNP  SERCA-2a  β-MHC  GADPH

C

[Graph showing mRNA expression normalized with GADPH]

***P<0.001, **P<0.01; N= 8/group

D

[Graph showing mRNA expression normalized with GADPH]

***P<0.001  *** N=8/group
Figure 8

A

MMP-9 (89 kDa)

- Newborn Npr1
- Young Npr1
- Adult Npr1

B

Density units

\[ y = 0.3829x + 1.156 \]
\[ R^2 = 0.91 \]
\[ ***P<0.001 \]

N=8/group
Figure 9

A

![Bar graph showing Systolic BP (mmHg) with -/- and +/- groups.](image)

*** P < 0.001; N=8/group

B

![Bar graph showing HW/BW ratio (mg/g) with -/- and +/- groups.](image)

*** P < 0.001; N=8/group

C

![Bar graph showing MMP-9 (ng.h^-1.g^-1) with -/- and +/- groups.](image)

†† P < 0.01, *** P < 0.001; N=8/group
Figure 10

A. 

B. 

C. 

D. 

E. 

F.
Figure 11

Growth factors TGF-beta, TNF

Cytoplasm

IKK kinase

IkBa

p50 p65

Activated NF-kB

MMP-2

MMP-9

Cytokines

Nucleus

kB-motif

Relaxation

Hypertrophy

Ca2+
P-phospholamban

PKG

cGMP

GTP

ANP

KHD

GC
Involvement of the NF-κB/matrix metalloproteinase pathway in cardiac fibrosis of mice lacking guanylyl cyclase/natriuretic peptide receptor-A
Elangovan Vellaichamy, Madan L. Khurana, Jude Fink and Kailash N. Pandey

J. Biol. Chem. published online February 13, 2005

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