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ENHANCED ACTIVATION OF PRO-INFLAMMATORY CYTOKINES IN MICE LACKING NATRIURETIC PEPTIDE RECEPTOR-A

Elangovan Vellaichamy, Kiran Kaur, and Kailash N. Pandey

Department of Physiology, Tulane University School of Medicine, New Orleans, LA 70112, USA

Abstract

Natriuretic peptide receptor-A (NPRA) is the principal receptor for the cardiac hormones ANP and BNP. Mice lacking NPRA develop progressive cardiac hypertrophy and congestive heart failure. However, the mechanisms responsible for hypertrophic growth in the absence of NPRA signaling are not yet known. In the present study, we determined whether deficiency of NPRA/cGMP signaling alters the cardiac pro-inflammatory cytokines gene expression in *Npr1* (coding for NPRA) gene-knockout (*Npr1*^{−/−}) mice exhibiting cardiac hypertrophy and fibrosis as compared with control wild-type (*Npr1*^{+/+}) mice. A significant up-regulation of cytokine genes such as TNF- α (5-fold), IL-6 (3-fold) and TGF- β 1 (4-fold) were observed in mutant mice hearts lacking NPRA as compared with the age-matched wild-type mice. In parallel, NF- κ B binding activity was almost 5-fold greater in the nuclear extract of *Npr1*^{−/−} mutant mice hearts as compared with wild-type *Npr1*^{+/+} mice hearts. Guanylyl cyclase (GC) activity and cGMP levels were drastically reduced by 10-fold and 6-fold, respectively, in ventricular tissues of mutant mice hearts relative to wild-type controls. The present findings provide direct evidence that ablation of NPRA/cGMP signaling activates inflammatory cytokines, probably via NF- κ B mediated signaling pathway, and is associated with hypertrophic growth of null mutant mice hearts.

Keywords

Natriuretic peptide receptor-A; GC activity; cGMP signaling; and cytokines

1. Introduction

Recent studies suggest that pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 β (IL- β), and interleukin-6 (IL-6) are activated within the myocardium during the experimental load-induced stress (2,23). Additionally, transforming growth factor- β 1 (TGF- β 1), TNF- α , and IL-6 have been shown to stimulate myocyte growth *in vitro* culture conditions (7,27). The aforementioned cytokines play a dual role, activating apoptosis in myocytes (13), while also functioning in a cytoprotective manner (15). Furthermore, inappropriate activation of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6) have been recognized as an important mediator in the development of endothelial dysfunction, cardiac hypertrophy, and heart failure in experimental animal models and in humans (9,17,28,30). Atrial natriuretic peptide (ANP) has been shown to inhibit the TNF- α

Address for Correspondence: Dr. Kailash N. Pandey, Department of Physiology, SL-39, Tulane University School of Medicine, 1430 Tulane Ave, New Orleans, LA 70112, USA, Tel: (504) 988-1628, Fax: (504) 988-2675, kpandey@tulane.edu.

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induced adhesion molecule expression in endothelial cells (32). ANP has also been shown to inhibit TNF- α production in interferon-gamma-activated macrophages (29).

Accumulating evidences have suggested that the guanylyl cyclase/natriuretic peptide receptor-A (GC-A/NPRA) signaling pathway is important not only in maintaining blood pressure homeostasis but is also locally involved in antagonizing cardiac growth stimulated by hypertrophic stimuli (3,4,6,24,25). In particular, 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 (12,21). Conversely, over-expression of NPRA in cardiomyocytes inhibits the hypertrophic effects of isoproterenol and aortic constriction on mouse hearts (33), and rescues the cardiac hypertrophic phenotype (12). The ANP/NPRA system has been implicated as a negative regulator of inflammation and hypertrophic growth (12,21,32). However, *in vivo* studies have not been carried out to examine the role of NPRA signaling in regulating the expression of inflammatory cytokines. To our knowledge, this is the first report demonstrating that permanent ablation of NPRA signaling activates the expression of pro-inflammatory cytokines, which plays a critical role in the development of cardiac hypertrophy and congestive heart failure.

2. Materials and Methods

2.1 Materials

Trizol reagent was obtained from Life Technologies/Invitrogen (Carlsbad, CA). cGMP assay kit was obtained from Assay Designs, Inc (Ann Arbor, MI). Custom made cytokine multi-probe set containing lymphotoxin- β (LT- β), IL-6, TNF- α , transforming growth factor- β 1 (TGF- β 1), interferon - γ (IFN- γ), macrophage inhibitory factor (MIF), ribosomal RNA (L-32) and glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) were from BD Biosciences (San Diego, CA). Antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA). T4 polynucleotide kinase, protein A-agarose, NAP-5 column, and [γ - 32 P] ATP (3000 Ci/mmol) and [α - 32 P] UTP (3000 Ci/mmol) were purchased from Amersham Biosciences (Piscataway, NJ).

2.2 Generation of *Npr1* gene-knockout mice

Npr1 gene-knockout mice were generated by homologous recombination in embryonic stem cells as previously described (21). 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 were housed under 12 h light/dark cycles at 25° C and fed regular chow (Purina Laboratory) and tap water *ad libitum*. *Npr1* genotypes used in the present studies were littermate progenies of C57/Bl6 genetic background and have been designated as *Npr1* gene-disrupted mutant allele (*Npr1*^{-/-}) and wild-type allele (*Npr1*^{+/+}). Experiments were performed using male adult (16 weeks) *Npr1*^{+/+} (wild-type) and *Npr1*^{-/-} homozygous null mutant mice.

2.2 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/ μ g 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 μ g) 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 μ g/ml salmon sperm DNA, for 5 h at 65° C, and hybridized with [γ - 32 P] ATP-

labeled oligonucleotide probes for 16 h at 65° C. Blots were washed three times in $2 \times \text{SSC}/0.2\%$ SDS at room temperature for 30 min and then in $0.5 \times \text{SSC}/0.2\%$ SDS at 65° C for 30 min before exposure to X-ray film. The sequence of oligonucleotide probes were as follows: β -myosin heavy chain (β -MHC): 5'-GAG GGC TTC ACG GGC ACC CTT AGA GCT GGG AGC ACA AGA TCT ACT CCT CAT TCA TTC AGG CC-3', Sarco/endoplasmic reticulum Ca^{2+} -ATPase-2a (SERCA-2a): 5'-TCA GTC ATG CAG AGG CTG GTA GAT GTG TTG CTA ACA ACG CAC ATG CAC GCA CCC GAA CA-3' and GADPH: 5'-GAG TGG CAG TGA TGG CAT GGA CTG TAG TCA TGT AGC CCT TCC ACG ATG CGA AAG AGT TGT -3' The intensity of the bands were quantified using image density analysis software (Alpha Innotech, San Leandro, CA). The expression results of β -MHC, and SERCA-2a were normalized with GADPH.

2.3 RNase protection assay of cytokines gene expression

RNase protection assay (RPA) was carried out using multi-probe templates consisting of LT- β , TNF- α , IL-6, TGF- β 1, IFN- γ , MIF and the control genes L32, and GADPH. The multiple template set was labeled with [α - ^{32}P]-UTP using a T7 RNA polymerase as described by the manufacturer's protocol (BD PharMingen, San Diego, CA). Labeled probes (3×10^5 cpm) were allowed to hybridize with 20 μg of total RNA for 16 h at 56° C. The hybridized mRNA probes were treated with RNase A, and extracted with phenol-chloroform. Protected hybrids were resolved on a 5% denaturing polyacrylamide gel and exposed to autoradiographic film overnight at -80° C. Densitometry was performed using Alpha Innotech Imaging System.

2.4 Preparation of cytosolic and nuclear extracts

Nuclear and cytosolic proteins were extracted by the method of Dignam et al., (5) 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 $\mu\text{g}/\text{ml}$ each of leupeptin, aprotinin, and pepstatin. The homogenate was centrifuged at $800 \times g$, and the supernatant was separated and saved as a cytosolic fraction. 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 $\mu\text{g}/\text{ml}$ each of leupeptin, aprotinin and pepstatin. The mixture was incubated on ice for 30 min and centrifuged at $14,000 \times g$ for 15 min. The supernatant was separated and stored at -80° C until used.

2.5 Western blot analysis cytokines

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 $\mu\text{g}/\text{ml}$ each of aprotinin, pepstatin, and leupeptin. Tissue homogenate (20 μg proteins) was mixed with sample loading buffer and separated under reducing condition using 10% SDS-PAGE. The separated proteins were electrotransferred onto a PVDF membrane. The membrane was blocked with 1x Tris-buffered saline-Tween 20 (TBST; 25 mM Tris, 500 mM NaCl and 0.05% Tween 20, pH 7.5) containing 5% fat-free milk, and incubated overnight in TBST containing 3% fat-free milk at 4° C with specific primary antibodies to TNF- α , IL-6, TGF- β 1, and α -tubulin. The membrane was then treated with corresponding secondary anti-rabbit or anti-mouse HRP-conjugated antibodies (1:20,000 dilution). Protein bands were visualized using the ChemGlow Western blot detection reagent kit (Alpha Innotech).

2.6 Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed as described previously (31). Double-stranded oligonucleotides containing the consensus-binding site for NF- κ B were end-labeled using [γ - 32 P]ATP and T4 polynucleotide kinase. Binding reaction was initiated by incubating 5 μ g of nuclear proteins in 5 μ l 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 μ g of Poly dI-dC and radiolabeled oligonucleotide (50,000 cpm) at 22° C for 20 min. Cold competitor assays were performed by adding 100-fold excess molar concentrations of unlabeled NF- κ B. The DNA-protein complex was resolved from the free-labeled DNA using 4% native polyacrylamide gel electrophoresis and autoradiography.

2.7 Ventricular guanylyl cyclase (GC) activity and cGMP assay

Ventricular plasma membranes were prepared as described previously (10). The GC activity was assayed as described by Leitman *et al* (16) with slight modification (10). An aliquot of plasma membrane (25 μ g) from the mutant and wild-type mice were added to a 100 μ l reaction mixture containing, Tris-HCl buffer (50 mM, pH 7.6), MnCl₂ (4 mM), GTP (0.2 mM), BSA (1 mg/ml), creatine phosphate (7.5 mM), IBMX (2 mM), and creatine phosphokinase (3 units). The membranes were incubated at 37° C for indicated time. The reaction was stopped with the addition of 900 μ l of sodium acetate buffer (50 mM, pH 6.2) and subsequently by boiling the samples for 3 min. The amount of cGMP generated was determined by direct cGMP enzyme immunoassay kit (Assay Designs, Inc., Ann Arbor, MI). To determine the total cGMP content, the 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, centrifuged at 600 $\times$ g for 20 min at 22° C, supernatants collected, and stored at -80° C until used for cGMP assay. Ventricular tissue cGMP levels were analyzed using a direct cGMP enzyme immunoassay kit.

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Software, Inc. (San Diego, CA). The results are presented as mean $\pm$ SEM. Differences between groups were determined by one-way analysis of variance (ANOVA) with student's *t* test. The probability value of $p < 0.05$ was considered significant.

3. Results

The results presented in Fig. 1 show that mutant mice *Npr1*^{-/-} displayed an increased mRNA expression of various cytokines; including: TNF- α (5-fold), IL-6 (3-fold), and TGF- β 1 (4-fold) as compared with wild-type *Npr1*^{+/+} mice (Fig. 1A, and B). LT- β and IFN- γ genes expression was found to be slightly increased in the mutant mice hearts, while MIF gene expression was not altered as compared with the wild-type mice. As depicted in Fig. 2A, there was a significant increase in the protein levels of TNF- α (4-fold), IL-6 (3-fold) and TGF- β 1 (3-fold) was noted in the null mutant mice homogenate as compared with the age-matched wild-type type control mice.

As shown in Table 1, ventricular GC activity and cGMP concentrations were drastically reduced by 10- and 5-fold, respectively, in the *Npr1*^{-/-} mutant mice, as compared with *Npr1*^{+/+} wild-type mice. Data presented in Fig. 3A, show that the expression of hypertrophic marker gene such as β -MHC was increased by more than 4-fold in *Npr1* null mutant mice hearts as compared with wild-type control mice hearts. In contrast, SERCA-2a mRNA expression was significantly reduced by almost 3-fold in mutant mice *Npr1*^{-/-} mice as compared with *Npr1*^{+/+} wild-type controls (Fig. 3B). NF- κ B binding activity was analyzed in the nuclear extract isolated from adult *Npr1*^{-/-} mutant mice hearts using electrophoretic mobility shift assay (EMSA) (Fig. 4A, and B). EMSA analysis demonstrated an increased NF-

κ B binding activity (5-fold) in the mutant mice hearts as compared with wild-type mice, confirming the increased activation of NF- κ B signaling.

4. Discussion

The results obtained from this study demonstrate that left ventricular pro-inflammatory cytokines gene expression was increased in mice null mutant lacking NPRA. Among the genes analyzed, TNF- α , IL-6, and TGF- β 1 were found to be highly up-regulated by almost 3- to 5-fold in the mutant mice hearts. Myocardial cytokine genes such as TNF- α , IL-1 β and IL-6 expression were found to be higher in patients with compensated heart failure condition, and it has been suggested that increased cytokine gene expression has adaptive role in the left ventricular remodeling process (22). Experimental induction of hemodynamic overload in the adult mammalian heart has been shown to provoke a transient increase in pro-inflammatory cytokines (2). Furthermore, cardiac-specific over expression of IL-6 and TNF- α in mice hearts has been shown to develop cardiac hypertrophy and ventricular dysfunction similar to that seen in human disease conditions, suggesting that cytokines are critically involved in the cardiac remodeling process (9,30).

In the present study, *Npr1* null mutant mice showed a drastic reduction in the ventricular GC activity (10-fold) as well as cGMP content (6-fold) as compared with wild-type mice. Anti-proliferative and anti-hypertrophic effects of the ANP/NPRA system have been reported to be mediated through the generation of cGMP (3,12,21,25,33). Furthermore, ANP/NPRA/cGMP signaling have been shown to oppose the hypertrophic agonist such as Ang II and ET-1 mediated biological effects (14,29). Hypertrophic agonist such as angiotensin II and norepinephrine, have been shown to mediate hypertrophic responses in neonatal and adult myocytes by activating IL-6 and TGF- β 1 genes (7,27). Additionally, recent evidences suggest that ANP/NPRA system has anti-inflammatory activity, and reported to inhibit the bacterial toxin (LPS) and IFN- γ -induced pro-inflammatory cytokines expression in macrophages by suppressing signal transduction pathway leading to NF- κ B activation (29,32). It has been suggested that ANP plays important role in regulating inflammatory signaling in endothelial cells, leading to reduced TNF- α -induced expression of adhesion molecules (11). The deficiency of ANP/NPRA/cGMP signaling may leads to the activation of inflammatory cytokines in hearts that may provokes the hypertrophic remodeling process in mice lacking NPRA.

SERCA-2a has been implicated as a marker for progressive hypertrophic growth and heart failure (8). It has been suggested that a decreased level of SERCA-2a is associated with impaired uptake of Ca^{2+} into sarcoplasmic reticulum and contributes to slowing of relaxation in the failing human hearts (20). Thus, an altered level of SERCA-2a seems to be associated with contractile dysfunction of the heart. In the present study, SERCA-2a gene expression was found to be significantly decreased in *Npr1* null mutant mice hearts as compared with the wild-type counterparts, suggesting a defect in Ca^{2+} handling and decreased cardiac performance in the mutant mice hearts.

In the present study, the increased NF- κ B binding activity was positively correlated with the observed increased expression of cytokine genes in the *Npr1* null mutant mice hearts. Recent studies have suggested that NF- κ B signaling seems to be involved in a number of cytokines gene expressions, and development of cardiac hypertrophy *in vivo* and *in vitro* conditions (18,26). The exact intracellular mechanisms underlying the activation of cytokines in mice lacking NPRA are not well understood. However, it can be suggested that NF- κ B-mediated signaling is involved in the synthesis of pro-inflammatory cytokines in mice lacking NPRA. ANP/NPRA/cGMP signaling attenuates the production of inflammatory mediators such as TNF- α by regulating the NF- κ B pathway (29,32). Furthermore, ANP and cGMP analogues

have been reported to suppress NF- κ B activation by inhibiting the phosphorylation of I κ B- α and its subsequent degradation in cultured cells (11). ANP has also been reported to inhibit the activation of Akt and MAPK/ERK signaling pathways (1,25). Interestingly, it has been shown that Akt and MAPK/ERK signaling directly phosphorylates p65 protein in a non-classical manner (without phosphorylation and degradation of I κ B- α) and thereby increases NF- κ B transcriptional activity (19), implicating the regulatory role of ANP/NPRA signaling in NF- κ B transactivation. Thus, it is conceivable that deficiency of ANP/NPRA signaling leads to an unbalanced activation of NF- κ B and in turn activates various cytokines and promotes hypertrophic process in mice lacking NPRA.

In summary, the present results demonstrate that disruption of NPRA/cGMP signaling results in an increased activation of cytokines in hypertrophied hearts of *Npr1* homozygous null mutant mice. The data provide strong evidence that reduced NPRA/cGMP signaling in mice lacking NPRA increases the activation of pro-inflammatory mediators, which are implicated to play a critical role in promoting cardiac hypertrophy and congestive heart failure. The findings provide novel mechanisms to demonstrate the central role of NPRA/GC/cGMP signaling in the regulation of inflammatory cytokines and development of cardiac hypertrophy.

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References

1. Abraham E. Alteration in cell signaling in sepsis. *Clin Infect Dis* 2005;41:S459–464. [PubMed: 16237648]
2. Baumgarten G, Knuefermann P, Kalra D, Gao F, Taffet GE, Michael L, Blackshear PJ, Carballo E, Sivasubramanian N, Mann DL. Load-dependent and -independent regulation of proinflammatory cytokine and cytokine receptor gene expression in the adult mammalian heart. *Circulation* 2002;2192–2197. [PubMed: 11994254]
3. Calderone A, Thaik CM, Takahashi N, Chang DL, Colucci WS. Nitric oxide, atrial natriuretic peptide, and cyclic GMP inhibit the growth-promoting effects of norepinephrine in cardiac myocytes and fibroblasts. *J Clin Invest* 1998;101:812–818. [PubMed: 9466976]
4. Cao L, Gardner DG. Natriuretic peptides inhibit DNA synthesis in cardiac fibroblasts. *Hypertension* 1995;25:227–234. [PubMed: 7843772]
5. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res* 1983;11:1475–1489. [PubMed: 6828386]
6. Drewett JG, Garbers DL. The family of guanylyl cyclase receptors and their ligands. *Endocr Rev* 1994;15:135–162. [PubMed: 7913014]
7. Gray MO, Long CS, Kalinyak JE, Li HT, Karliner JS. Angiotensin II stimulates cardiac myocyte hypertrophy via paracrine release of TGF- β 1 and endothelin-1 from fibroblasts. *Cardiovasc Res* 1998;40:352–363. [PubMed: 9893729]
8. Hasenfuss G, Reinecke H, Studer R, Meyer M, Pieske B, Holtz J, Holubarsch C, Posival H, Just H, Drexler H. Relation between myocardial function and expression of sarcoplasmic reticulum Ca(2+)-ATPase in failing and nonfailing human myocardium. *Circ Res* 1994;75:434–442. [PubMed: 8062417]
9. Hirota H, Yoshida K, Kishimoto T, Taga T. Continuous activation of gp130, a signal-transducing receptor component for interleukin 6-related cytokines, causes myocardial hypertrophy in mice. *Proc Natl Acad Sci U S A* 1995;92:4862–4866. [PubMed: 7539136]

10. Khurana ML, Pandey KN. Catalytic activation of guanylate cyclase/atrial natriuretic factor receptor by combined effects of ANF and GTP gamma S in plasma membranes of Leydig tumor cells: involvement of G-proteins. *Arch Biochem Biophys* 1995;316:392–398. [PubMed: 7840642]
11. Kierner AK, Weber NC, Vollmar AM. Induction of IkappaB: atrial natriuretic peptide as a regulator of the NF-kappaB pathway. *Biochem Biophys Res Commun* 2002;295:1068–1076. [PubMed: 12135603]
12. Kishimoto I, Rossi K, Garbers DL. A genetic model provides evidence that the receptor for atrial natriuretic peptide (guanylyl cyclase-A) inhibits cardiac ventricular myocyte hypertrophy. *Proc Natl Acad Sci U S A* 2001;98:2703–2706. [PubMed: 11226303]
13. Krown KA, Page MT, Nguyen C, Zechner D, Gutierrez V, Comstock KL, Glembotski CC, Quintana PJ, Sabbadini RA. Tumor necrosis factor alpha-induced apoptosis in cardiac myocytes. Involvement of the sphingolipid signaling cascade in cardiac cell death. *J Clin Invest* 1996;98:2854–2865. [PubMed: 8981934]
14. Kumar R, Cartledge WA, Lincoln TM, Pandey KN. Expression of guanylyl cyclase-A/atrial natriuretic peptide receptor blocks the activation of protein kinase C in vascular smooth muscle cells. Role of cGMP and cGMP-dependent protein kinase. *Hypertension* 1997;29:414–421. [PubMed: 9039136]
15. Kurrelmeyer KM, Michael LH, Baumgarten G, Taffet GE, Peschon JJ, Sivasubramanian N, Entman ML, Mann DL. Endogenous tumor necrosis factor protects the adult cardiac myocyte against ischemic-induced apoptosis in a murine model of acute myocardial infarction. *Proc Natl Acad Sci U S A* 2000;97:5456–5461. [PubMed: 10779546]
16. Leitman DC, Agnost VL, Tuan JJ, Andresen JW, Murad F. Atrial natriuretic factor and sodium nitroprusside increase cyclic GMP in cultured rat lung fibroblasts by activating different forms of guanylate cyclase. *Biochem J* 1987;244:69–74. [PubMed: 2889449]
17. Levine B, Kalman J, Mayer L, Fillit HM, Packer M. Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 1990;323:236–241. [PubMed: 2195340]
18. Li Y, Ha T, Gao X, Kelley J, Williams DL, Browder IW, Kao RL, Li C. NF-kappaB activation is required for the development of cardiac hypertrophy in vivo. *Am J Physiol Heart Circ Physiol* 2004;287:H1712–1720. [PubMed: 15142841]
19. Madrid L, Wang C, Guttridge D, Schottelius A, Baldwin AJ, Mayo M. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-kappaB. *Mol Cell Biol* 2000;20:1626–1638. [PubMed: 10669740]
20. Meyer M, Schillinger W, Pieske B, Holubarsch C, Heilmann C, Posival H, Kuwajima G, Mikoshiba K, Just H, Hasenfuss G. Alterations of sarcoplasmic reticulum proteins in failing human dilated cardiomyopathy. *Circulation* 1995;92:778–784. [PubMed: 7641356]
21. Oliver PM, Fox JE, Kim R, Rockman HA, Kim HS, Reddick RL, Pandey KN, Milgram SL, Smithies O, Maeda N. Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc Natl Acad Sci U S A* 1997;94:14730–14735. [PubMed: 9405681]
22. Oral H, Sivasubramanian N, Dyke DB, Mehta RH, Grossman PM, Briesmiester K, Fay WP, Pagani FD, Bolling SF, Mann DL, Starling MR. Myocardial proinflammatory cytokine expression and left ventricular remodeling in patients with chronic mitral regurgitation. *Circulation* 2003;107:831–837. [PubMed: 12591752]
23. Palmieri EA, Benincasa G, Di Rella F, Casaburi C, Monti MG, De Simone G, Chiariotti L, Palombini L, Bruni CB, Sacca L, Cittadini A. Differential expression of TNF-alpha, IL-6, and IGF-1 by graded mechanical stress in normal rat myocardium. *Am J Physiol Heart Circ Physiol* 2002;282:H926–934. [PubMed: 11834488]
24. Pandey KN. Biology of natriuretic peptides and their receptors. *Peptides* 2005;26:901–932. [PubMed: 15911062]
25. Pandey KN, Nguyen HT, Li M, Boyle JW. Natriuretic peptide receptor-A negatively regulates mitogen-activated protein kinase and proliferation of mesangial cells: role of cGMP-dependent protein kinase. *Biochem Biophys Res Commun* 2000;271:374–379. [PubMed: 10799305]
26. Purcell NH, Tang G, Yu C, Mercurio F, DiDonato JA, Lin A. Activation of NF-kappa B is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes. *Proc Natl Acad Sci U S A* 2001;98:6668–6673. [PubMed: 11381115]

27. Sano M, Fukuda K, Kodama H, Pan J, Saito M, Matsuzaki J, Takahashi T, Makino S, Kato T, Ogawa S. Interleukin-6 family of cytokines mediate angiotensin II-induced cardiac hypertrophy in rodent cardiomyocytes. *J Biol Chem* 2000;275:29717–29723. [PubMed: 10843995]
28. Testa M, Yeh M, Lee P, Fanelli R, Loperfido F, Berman JW, LeJemtel TH. Circulating levels of cytokines and their endogenous modulators in patients with mild to severe congestive heart failure due to coronary artery disease or hypertension. *J Am Coll Cardiol* 1996;28:964–971. [PubMed: 8837575]
29. Tsukagoshi H, Shimizu Y, Kawata T, Hisada T, Shimizu Y, Iwamae S, Ishizuka T, Iizuka K, Dobashi K, Mori M. Atrial natriuretic peptide inhibits tumor necrosis factor- α production by interferon- γ -activated macrophages via suppression of p38 mitogen-activated protein kinase and nuclear factor- κ B activation. *Regul Pept* 2001;99:21–29. [PubMed: 11257311]
30. Vanderheyden M, Paulus WJ, Voss M, Knuefermann P, Sivasubramanian N, Mann D, Baumgarten G. Myocardial cytokine gene expression is higher in aortic stenosis than in idiopathic dilated cardiomyopathy. *Heart* 2005;91:926–931. [PubMed: 15958363]
31. Vellaichamy E, Khurana ML, Fink J, Pandey KN. Involvement of the NF- κ B/matrix metalloproteinase pathway in cardiac fibrosis of mice lacking guanylyl cyclase/natriuretic peptide receptor A. *J Biol Chem* 2005;280:19230–19242. [PubMed: 15710627]
32. Vollmar AM. The role of atrial natriuretic peptide in the immune system. *Peptides* 2005;26:1086–1094. [PubMed: 15911076]
33. Zahabi A, Picard S, Fortin N, Reudelhuber TL, Deschepper CF. Expression of constitutively active guanylate cyclase in cardiomyocytes inhibits the hypertrophic effects of isoproterenol and aortic constriction on mouse hearts. *J Biol Chem* 2003;278:47694–47699. [PubMed: 14500707]

Figure 1. Expression profiles of ventricular cytokine genes expression in adult *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts

A) Representative autoradiograph showing the cytokines gene expression in adult *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts. Labeled probe (3×10^5 cpm) was allowed to hybridize with 20 μ g of RNA at 56°C for 16 h. Protected hybrids were resolved on a 5% denaturing polyacrylamide gel and exposed to radiographic film overnight at -80°C. B) Relative expression of cytokine genes normalized to the expression of L32 in adult *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts. Values are expressed as means $\pm$ SEM; $N=8$ mice/group; * $p<0.05$, ** $p<0.01$ and *** $p<0.001$; *Npr1*^{+/+} vs *Npr1*^{-/-}.

Figure 2. Analyses of ventricular TNF- α , IL-6, and TGF- β protein levels in adult *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts

A) Representative Western blot of cytokines (TNF- α , IL-6 and TGF- β) in *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts. B) Densitometric analysis of protein bands normalized with α -tubulin. Values are expressed as means $\pm$ SEM; N=8 mice /group; ***p<0.001; *Npr1*^{+/+} vs *Npr1*^{-/-}.

Figure 3. Hypertrophy marker gene expression in adult *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts
 A) Representative Northern blots showing the mRNA expression of β -MHC and SERCA-2a in *Npr1*^{-/-} and *Npr1*^{+/+} mice. B) Densitometric analysis of mRNA transcripts normalized with GAPDH mRNA expression. Values are expressed as means $\pm$ SEM; *N*=8 mice/group; ****p*<0.001; *Npr1*^{+/+} vs *Npr1*^{-/-}.

Figure 4. Nuclear NF-κB binding activity in adult *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts

A) EMSA analysis of NF-κB binding activity in the nuclear extracts of *Npr1*^{+/+} and *Npr1*^{-/-} mice hearts. The DNA-protein complex was resolved from the free-labeled DNA by electrophoresis using 4% (w/v) native polyacrylamide gel electrophoresis and autoradiography. B) Densitometric analysis of NF-κB protein bands. Values are expressed as means ±SEM; N=8 mice/group; ***p<0.001; *Npr1*^{+/+} vs *Npr1*^{-/-}.

Table 1

Guanylyl cyclase activity and total cGMP content in the left ventricular tissue of *Npr1*^{+/+} wild-type and *Npr1*^{-/-} homozygous null mutant mice

Genotype	Guanylyl cyclase activity (pmoles cGMP/mg protein/30 min)	Intracellular cGMP (pmoles/mg protein)
<i>Npr1</i> ^{+/+}	68 ± 9	28 ± 4
<i>Npr1</i> ^{-/-}	6 ± 1.1 ***	5 ± 0.8 ***

Guanylyl cyclase (GC) activity analysis was performed as described under the [Materials and methods](#) section. cGMP levels in wild-type and mutant *Npr1* mice hearts were analyzed using direct cGMP enzyme immunoassay kit. Values are expressed as means ± SEM; N=8 mice/group;

p<0.001; *Npr1*^{+/+} vs *Npr1*^{-/-}.