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Biochemical and Biophysical Research Communications 327 (2005) 106–111
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Reduced cGMP signaling activates NF-κB in hypertrophied hearts of mice lacking natriuretic peptide receptor-A

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Received 21 November 2004
Available online 9 December 2004

Abstract

Mice lacking natriuretic peptide receptor-A (NPRA) develop progressive cardiac hypertrophy and congestive heart failure. However, the mechanisms responsible for cardiac hypertrophic growth in the absence of NPRA signaling are not yet known. We sought to determine the activation of nuclear factor-κB (NF-κB) in Npr1 (coding for NPRA) gene-knockout ($Npr1^{-/-}$) mice exhibiting cardiac hypertrophy and fibrosis. NF-κB binding activity was 4-fold greater in the nuclear extract of $Npr1^{-/-}$ mutant mice hearts as compared with wild-type ($Npr1^{+/+}$) mice hearts. In parallel, inhibitory κB kinase-β activity and IκB-α protein phosphorylation were also increased 3- and 4-fold, respectively, in hypertrophied hearts of mutant mice. cGMP levels were significantly reduced 5-fold in plasma and 10-fold 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 NF-κB binding activity associated with hypertrophic growth of mutant mice hearts.

Keywords: Natriuretic peptide receptor-A; Cardiac hypertrophy; NF-κB; IκB-β-kinase; Gene-knockout; cGMP signaling

Cardiac hormones, atrial and brain natriuretic peptides (ANP and BNP), are released in the circulation and elicit natriuretic, diuretic, vasorelaxant, and antiproliferative responses, all directed to the reduction of blood pressure and blood volume [1–3]. Natriuretic peptide receptor-A/guanylyl cyclase-A (NPRA/GC-A) is considered the principal receptor for both ANP and BNP, and binding of these hormones to NPRA leads to the generation of the intracellular second messenger cGMP [4–6]. Recent studies have suggested that the ANP/NPRA signaling pathway is important not only to maintain blood pressure homeostasis but is also locally involved in antagonizing cardiac growth stimulated by hypertrophic stimuli [7–10]. 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 [11,12]. Conversely, over-expression of NPRA in cardiomyocytes inhibits the hypertrophic effects of isoproterenol and aortic constriction on mouse hearts [13], and rescues the cardiac hypertrophic phenotype [12]. However, the cellular mechanism by which NPRA/cGMP signaling regulates myocyte growth and ventricular remodeling in disease states is not known.

Nuclear factor-κB (NF-κB) is a ubiquitous inducible transcription factor that activates expression of a group of genes, including those that promote inflammatory responses, cell growth, and apoptosis [14]. NF-κB exists in an inactive cytoplasmic form, bound to the inhibitory protein, IκB. Phosphorylation and degradation of IκB are the key steps in NF-κB activation, translocation into the nucleus, and stimulation of gene expression [14]. Recent data from in vivo and in vitro studies suggested that NF-κB activation is linked to the hypertrophic

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doi:10.1016/j.bbrc.2004.11.153

responses of cardiomyocytes, which indicated that activation of NF-κB is more likely required for hypertrophic growth of cultured primary rat neonatal ventricular myocytes [15] and pressure-overloaded rat hearts [16]. Cardiac hypertrophic agonists, such as angiotensin II and endothelin-1, have also been reported to stimulate NF-κB activation [17,18]. Furthermore, NF-κB has been shown to be over-expressed during ischemic adaptation and heart failure [19]. In the present study, we have utilized the Npr1 gene-knockout mouse model to determine whether ablation of Npr1 gene activates NF-κB in association with cardiac hypertrophic growth and remodeling process. The present report demonstrates that permanent ablation of NPRA/cGMP signaling in mice activates NF-κB and its translocation from the cytoplasm to the nucleus, an important factor in the development of cardiac hypertrophy and congestive heart failure.

Materials and methods

Materials. Antibodies and oligonucleotides containing the consensus-binding site for NF-κB and IgG-HRP-conjugated secondary 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) were purchased from Amersham Biosciences (Piscataway, NJ). cGMP assay kit was obtained from Assay Designs (Ann Arbor, MI). All other chemicals were of reagent grade.

Generation of Npr1 gene-knockout mice. Npr1 gene-knockout mice were generated by homologous recombination in embryonic stem cells as previously described [11]. 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 cycle 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 16 week adult $Npr1^{-/-}$ homozygous null mutant and $Npr1^{+/+}$ wild-type male mice.

Blood pressure, cardiac hypertrophy, and echocardiographic analyses. Blood pressures of Npr1 mutant and wild-type mice were measured by a noninvasive computerized tail-cuff method using Visitech-2000 and were calculated as the average of 6–7 sessions/day for 6–8 consecutive days. Left ventricular heart weight (HW) and its ratio to body weight (BW) were calculated as an index of cardiac hypertrophy. Cardiac functional analysis was carried out using a two-dimensional echocardiography. $Npr1^{+/+}$ and $Npr1^{-/-}$ mice were lightly sedated, and left ventricular (LV) dimensions, LV posterior wall thickness, and fractional shortening were measured using an ultrasound system (Toshiba Power Vision; Model SSA380A). M-mode images were obtained using a 7 mHz transducer at a sweep speed of 100 mm/s. For each measurement, 3–4 consecutive cardiac cycles were traced and averaged. Animals were sacrificed by cervical dislocation and hearts were isolated for biochemical analyses.

Ventricular and plasma cGMP assay. Frozen ventricular tissue samples were homogenized in 10 volumes of 0.1 M HCl containing 1% Triton X-100. Homogenate was heated at 95 °C for 5 min, centrifuged at 600*g* for 20 min at 22 °C, and supernatants were collected and stored at −80 °C until used for cGMP assay. Blood samples were collected in EDTA tubes and immediately centrifuged at 2500 rpm for 10 min at 4 °C. The plasma was separated and stored at −80 °C until used. Both plasma and ventricular tissue cGMP levels were analyzed using a direct cGMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI).

Preparation of cytosolic and nuclear extracts. Nuclear and cytosolic proteins were extracted from heart tissues as previously described [20]. Ventricular 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*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 μg/ml each of leupeptin, aprotinin, and pepstatin. The mixture was incubated on ice for 30 min and centrifuged at 14,000*g* for 15 min. The supernatant was separated and stored at −80 °C until used.

Western blot analysis. For Western blots, cytoplasmic or nuclear fraction (20 μg proteins) was mixed with a sample loading buffer and separated under reducing conditions using a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were electrotransferred onto a PVDF membrane. The membrane was blocked with 1× 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 primary antibodies (1:1000 dilution). The membrane was then treated with corresponding secondary anti-rabbit or anti-mouse HRP-conjugated antibodies (1:20,000 dilution). Protein bands were visualized by enhanced chemiluminescence (ECL) plus detection system.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as previously described [21]. Double-stranded oligonucleotides containing the consensus-binding site for NF-κB were utilized. Oligonucleotides were end-labeled using [γ-^{32}p]ATP and T4 polynucleotide kinase. For binding reaction, 5 μg of nuclear proteins was incubated for 20 min at room temperature in 5 μl binding buffer (50 mM Tris–HCl, pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5% Triton X-100, 20% glycerol (v/v), and 1 mM DTT) containing 2 μg poly(dI–dC) and 50,000 cpm radiolabeled oligonucleotides. Cold competitor assays were performed by adding 100-fold excess molar concentrations of unlabeled NF-κB. The supershift assay was carried out by incubating nuclear extract with p-65 antibodies and subsequently with radiolabeled oligonucleotides. The DNA–protein complex was resolved from the free-labeled DNA using 4% native polyacrylamide gel electrophoresis and autoradiography.

Inhibitory κB kinase-β activity assay. Inhibitory κB kinase-β (IKK-β) activity assay was carried out by the method as previously reported [19]. Cytoplasmic proteins (200 μg) from the left ventricular tissues of Npr1 wild-type and mutant mice were immunoprecipitated with 2 μg IKK-β antibody at 4 °C for 1 h. Protein A–agarose beads (10 μl) were added and the mixture was incubated for another 1 h at 4 °C. The content was centrifuged at 2500 rpm for 5 min at 4 °C. The pellet was washed twice with lysis buffer (50 mM Hepes, pH 7.4, 250 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, and aprotinin and leupeptin each 5 μg/ml) and once in kinase buffer (10 mM Hepes, pH 7.4, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 12.5 mM β-glycero-2-phosphate, 50 μM Na_3VO_4, 2 mM NaF, 50 μM DTT, and 10 μM ATP). The pellet was then resuspended in 15 μl kinase buffer. The IKK-β reaction was carried out in the presence of 1 μg glutathione-*S*-transferase-IκB-α substrate and 5 μCi [γ-^{32}P]ATP (6000 Ci/mmol) at 37 °C for 30 min. The reaction was stopped by the addition of 3× Laemmli sample buffer, and the phosphorylated protein was resolved by 15% SDS–PAGE and autoradiography.

Statistical analysis. Statistical analysis was performed using GraphPad Software (San Diego, CA). The results are presented as means ± 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.

Results

The data presented in Figs. 1A–C show that NF-κB (p-65) protein levels were increased 3- to 4-fold in both cytoplasmic and nuclear fractions of hypertrophied hearts of $Npr1^{-/-}$ null mutant mice as compared with normal $Npr1^{+/+}$ wild-type mice. EMSA analysis showed an increased NF-κB binding activity in the nuclear extract isolated from $Npr1^{-/-}$ mutant mice hearts as compared with wild-type mice, confirming the translocation of NF-κB subunits from the cytoplasm into the nucleus of hypertrophied hearts (Fig. 1D, lanes 1–4). The specificity of the detected NF-κB band was confirmed by super-shift binding assay with p-65 antibody and 100× excess molar concentrations of unlabeled cold NF-κB oligonucleotides (Fig. 1D, lanes 5 and 6). The densitometric analysis of NF-κB–protein complex indicated that NF-κB binding activity increased almost 4-fold in the nuclear extracts of the $Npr1^{-/-}$ null mutant mice hearts lacking NPRA (Fig. 1E).

Figs. 2A and C show the enhanced IKK-β activity and phosphorylation of IκB-α (p-IκB-α) in the cytoplasmic extracts isolated from the Npr1 mutant and control mice hearts. Densitometric analysis revealed that Npr1 null mutant mice exhibited almost a 3-fold increase in IKK-β activity with a 4-fold parallel increase in phosphorylated IκB-α protein levels in hypertrophied hearts of mutant mice as compared with wild-type control mice hearts (Figs. 2B and D).

As shown in Table 1, blood pressures and heart weight to body weight ratio were increased significantly in $Npr1^{-/-}$ null mutant mice (7.2 ± 0.5; $p < 0.01$) as compared with $Npr1^{+/+}$ wild-type mice (4.5 ± 0.23). M-mode echocardiography analysis showed progressive cardiac hypertrophy in $Npr1^{-/-}$ mutant mice, which showed increased ventricular chamber dimensions and significantly elevated posterior wall and septal wall thickness as compared with the age-matched wild-type mice hearts (Table 1). Both plasma and ventricular cGMP levels were drastically reduced 5- and 10-fold, respectively, in the $Npr1^{-/-}$ mutant mice as compared with $Npr1^{+/+}$ wild-type mice (Fig. 3).

Discussion

The results obtained from this study demonstrate that cytoplasmic and nuclear NF-κB protein levels, IKK-β activity, and phosphorylation of IκB-α protein levels were significantly increased in hypertrophied hearts of mice lacking NPRA. In most resting cells, NF-κB is bound to its cytoplasmic inhibitory protein IκB (α, β,

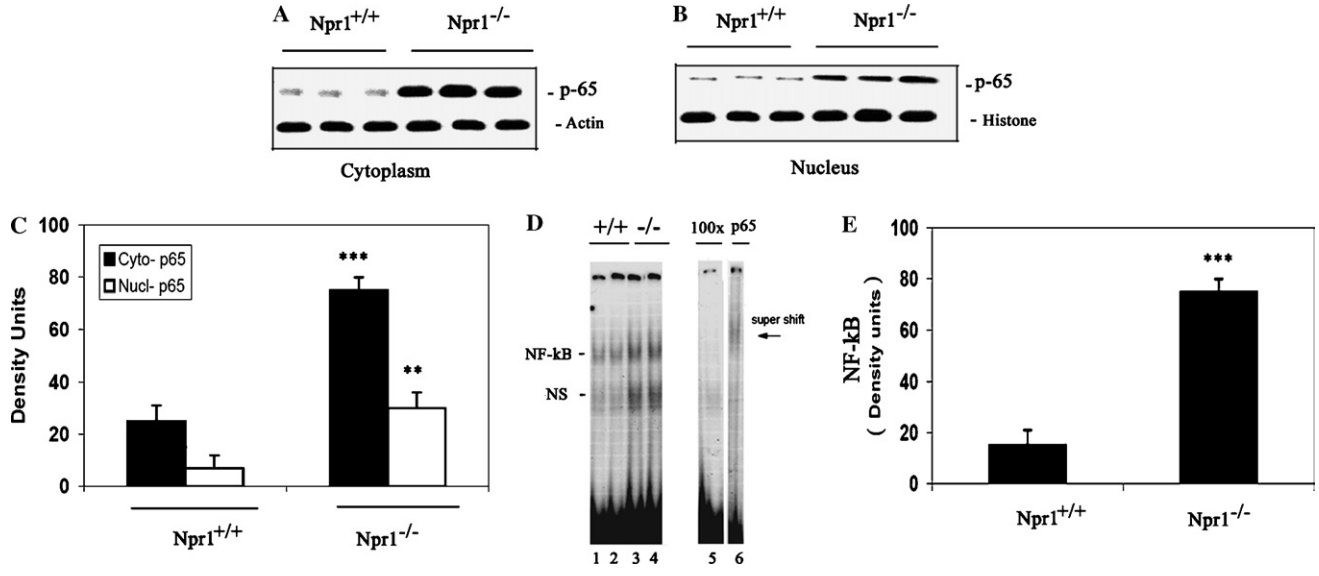

Fig. 1. Cytoplasmic and nuclear NF-κB protein levels and binding activity in hypertrophied and normal Npr1 mice hearts. Nuclear and cytoplasmic extracts were prepared from $Npr1^{+/+}$ and $Npr1^{-/-}$ mice hearts as described under Materials and methods. The NF-κB subunit p-65 protein levels in cytoplasmic and nuclear fractions were analyzed by Western blot analyses. (A,B) The Western blot analyses of p-65 protein levels in the cytoplasmic and nuclear extracts of $Npr1^{+/+}$ and $Npr1^{-/-}$ mice hearts. (C) The densitometric analysis of p-65 protein bands. (D) EMSA analysis of NF-κB binding activity in the nuclear extract 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. NF-κB-specific protein bands are labeled and NS indicates the non-specific binding. (E) Densitometric analysis of NF-κB protein bands. Values are expressed as means ± SEM; $N = 8$ mice/group; $^{**}p < 0.01$; $^{***}p < 0.001$.

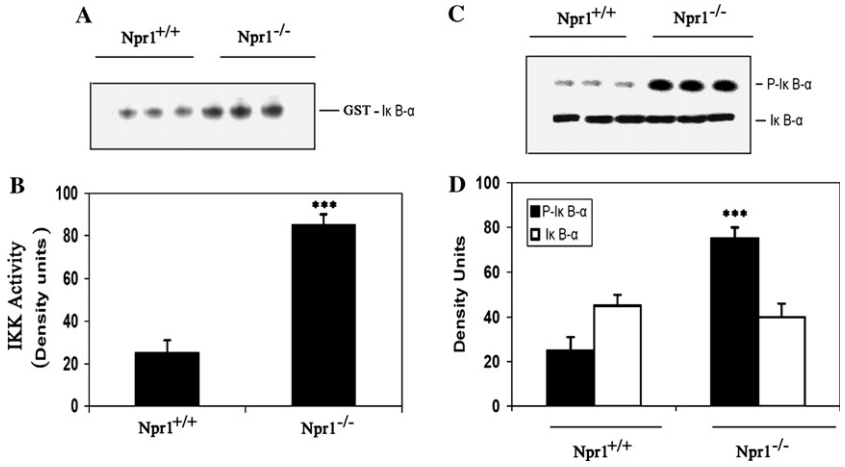

Fig. 2. IκB-β kinase (IKK-β) activity and phosphorylation of IκB-α protein levels. (A) IKK-β activity and (B) densitometric analysis of IKK-β activity in $Npr1^{-/-}$ and $Npr1^{+/+}$ mice hearts. IKK-β activity was analyzed by immunoprecipitation followed by the addition of glutathione-*S*-transferase (GST)-IκB-α substrate. Phosphorylation of IκB-α protein levels was analyzed using IκB-α antibodies as described under Materials and methods. (C) Phosphorylated IκB-α proteins levels in the cytoplasmic extract isolated from the $Npr1^{+/+}$ wild-type and $Npr1^{-/-}$ mutant mice hearts. (D) Densitometric analysis of phosphorylated IκB-α protein bands. Values are expressed as means ± SEM; $N = 8$ mice/group; $^{***}p < 0.001$.

Table 1
Blood pressure and cardiac functional analysis of $Npr1^{+/+}$ wild-type and $Npr1^{-/-}$ homozygous null mutant mice

Parameters	$Npr1^{+/+}$	$Npr1^{-/-}$
BP (mmHg)	103 ± 5	136 ± 6**
BW (g)	26 ± 4	27 ± 5
HW/BW ratio (mg/g)	4.5 ± 0.23	7.2 ± 0.5**
KW/BW ratio (mg/g)	8.5 ± 0.5	8.6 ± 0.6
LVIDS (mm)	2.12 ± 0.02	3.22 ± 0.05**
LVIDD (mm)	3.72 ± 0.04	4.76 ± 0.07***
IVST (mm)	0.64 ± 0.03	0.93 ± 0.04*
PWT (mm)	0.61 ± 0.02	0.82 ± 0.03*
FS (%)	43 ± 2.3	32 ± 1.5**

Cardiac functional parameters of wild-type and mutant Npr1 mice hearts were analyzed using M-mode echocardiographic analysis as described under Materials and methods. The parameters analyzed include; BP, blood pressure; BW, body weight; HW/BW ratio, heart weight/body weight ratio; KW/BW ratio, kidney weight/body weight ratio; LVIDS, left ventricular chamber dimension (systolic); LVIDD, left ventricular chamber dimension (diastolic); IVST, interventricular septal wall thickness (mm); PWT, left ventricular posterior wall thickness (mm); and FS, fractional shortening. Values are expressed as means ± SEM; $N = 8$ mice/group.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

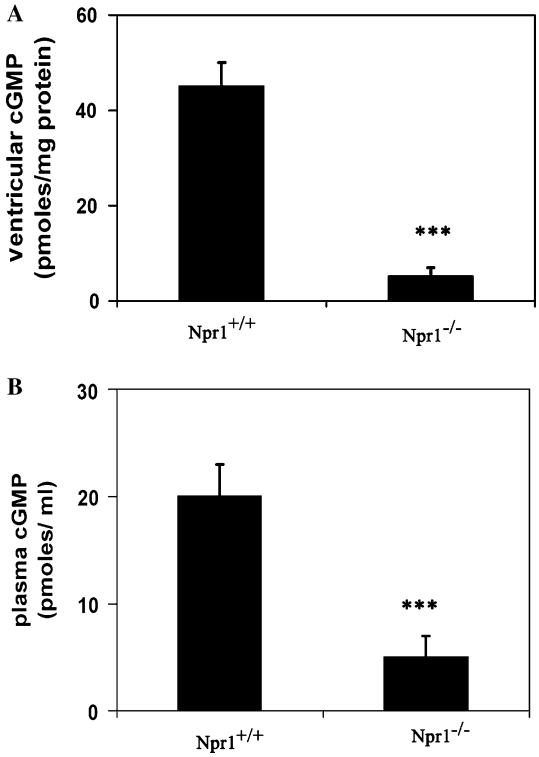

Fig. 3. Quantitation of second messenger cGMP in plasma and ventricular tissues of mutant and wild-type mice. The ventricular tissue levels of cGMP in homozygous null mutant $Npr1^{-/-}$ and wild-type $Npr1^{+/+}$ mice were determined using competitive enzyme immunoassay as described under Materials and methods. Values are expressed as means ± SEM; $N = 8$ mice/group; $^{***}p < 0.001$.

and ε) and remains in the cytoplasm in a latent form. Upon stimulation, IκB kinase is activated and in turn phosphorylates IκB protein on specific serine residues, which triggers ubiquitination-dependent degradation of IκB protein, resulting in the release of NF-κB [22,23]. Recently, it has been suggested that NF-κB activation seems to be linked with the initiation and development of cardiac hypertrophy in vivo [15,16]. Increased IKK-β activity and enhanced phosphorylation of IkBα protein levels, resulting in NF-κB translocation, have also been observed in the ischemic rat myocardium [19]. Similarly, sustained activation of NF-κB and AP-1 transcription factors has been reported in the experimental and human heart failure conditions [24].

The exact intracellular mechanisms underlying the activation of NF-κB and IKK-β activity in mice lacking NPRA are not immediately clear, although the participation of cGMP-mediated downstream signaling pathway as well as progressive mechanical stress associated with hypertension in the mutant mice can be suggested. In the present study, mutant mice showed a drastic reduction of 5- to 10-fold in the level of cGMP as compared to wild-type mice. Activation of NPRA, a GC-coupled receptor, by natriuretic peptides stimulates cGMP in a number of cell types, and the anti-proliferative and anti-hypertrophic effect of the ANP/NPRA system has been reported to be mediated through the generation of cGMP [8–10]. Furthermore, cGMP analogues have been reported to suppress vascular cell adhesion molecule-1 and hypoxia-associated vascular endothelial growth factor gene expression by inhibiting the activation of NF-κB [25]. Nitric oxide, which utilizes cGMP as a second messenger, prevents hypertrophy of neonatal and adult cardiac myocytes [26]. Recently, nitric oxide donors have been shown to inhibit the neointimal proliferation in balloon-injured arteries by suppressing NF-κB activation and by elevating vascular cGMP at the site of injury [27].

In summary, the present results demonstrate that disruption of NPRA/cGMP signaling results in an increased NF-κB binding, stimulated IKK-β enzyme activity, and enhanced IκB-α protein phosphorylation in hypertrophied hearts of Npr1 homozygous null mutant mice. The data provide strong evidence that reduced cGMP levels in mice lacking NPRA increase the activation of the NF-κB pathway, which is implicated to play a critical role in promoting cardiac hypertrophy and congestive heart failure.

Acknowledgments

Authors thank Jude Fink and L. Charlene Binford for the technical assistance during the course of this work. Our thanks are also due to Lynell Dupepe for assistance in animal surgery. We also thank Kamala Pandey for assistance in typing of the manuscript. This work was supported by grants from the National Institutes of Health (HL 62147) and Louisiana Board of Regents Health Excellent Fund.

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