Blockade of NF-κB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II

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Received 18 October 2004; received in revised form 24 April 2005; accepted 25 April 2005
Available online 24 May 2005
Time for primary review 24 days

Abstract

Objective: Nuclear factor (NF)-κB is a key transcription factor that regulates inflammatory processes. In the present study, we assessed the hypothesis that blockade of NF-κB may ameliorate ventricular hypertrophy in response to chronic infusion of angiotensin II.

Methods: Mice with targeted disruption of the p50 subunit of NF-κB (KO) were used to block the activation of NF-κB. Male KO and age-matched wild-type (WT) mice were chronically infused with angiotensin II at the rate of 0.2 (low dose) or 2 μg/kg/min (high dose) for 4 weeks.

Results: High- but not low-dose angiotensin II significantly increased systemic blood pressure and left ventricular weight in WT mice. In contrast, although the pressor response was slightly but significantly augmented, the hypertrophic effect of angiotensin II was significantly attenuated in KO mice. The attenuated hypertrophic responsiveness was confirmed histologically (cross-sectional area) and transcriptionally (atrial natriuretic peptide). Echocardiography revealed no evidence of cardiac dysfunction in angiotensin II-treated KO mice. Although phosphorylation of MAPKs, including ERK, JNK, or p38-MAPK, was not affected after 4 weeks of angiotensin II treatment in WT mice, phosphorylation of JNK was specifically abrogated in KO mice. Angiotensin II increased myocardial expression of proinflammatory cytokines and chemokines in WT mice, while expression of TNF-α and RANTES was paradoxically augmented in KO mice.

Conclusion: Blockade of NF-κB activation attenuated myocardial hypertrophy without deteriorating cardiac function. NF-κB may play an important role in cardiac hypertrophy and remodeling besides promoting inflammation.

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Keywords: Angiotensin; Cytokines; Hypertrophy; MAP kinase; Nuclear factor-κB; Cardiac remodeling

1. Introduction

Nuclear factor-kappa B (NF-κB) is a key transcription factor that regulates inflammatory processes (see Ref. [1] for review). Many stimuli activate NF-κB, including proinflammatory cytokines, lipopolysaccharide, and reactive oxygen species. Activation of NF-κB involves the phosphorylation and subsequent proteolytic degradation of the inhibitory protein IκB by specific IκB kinases. The free NF-κB (typically, a heterodimer of p50 and p65) then passes into the nucleus, where it binds to κB sites in the promoter regions of genes for inflammatory proteins such as tumor necrosis factor (TNF)-α, inducible nitric oxide synthase, and adhesion molecules. Thus the activation of NF-κB leads to a coordinated increase in the expression of many genes whose products mediate inflammatory and immune responses [1].

Recent studies have suggested that the activation of NF-κB may also play an important role in the pathogenesis of cardiac remodeling and heart failure [2]. First, NF-κB is activated in the failing human heart [3]. Second, plasma levels as well as myocardial expression of proinflammatory cytokines, including TNF-α, are increased in patients with heart failure [4,5]. Third, cardiac-specific overexpression of TNF-α causes myocardial inflammation and remodeling, resulting in the development of congestive heart failure [6].
Fourth, angiotensin II has been shown to activate NF-κB and induce TNF-α in myocardium [7]. Finally, recent in vitro studies demonstrate that activation of NF-κB is also required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes in response to angiotensin II, phenylephrine, and endothelin-1 [8,9]. Therefore, blockade of NF-κB activation may be a new therapeutic strategy for heart failure by attenuating myocardial inflammation and hypertrophy. However, little is known about in vivo effects of NF-κB inhibition in patients with heart failure or any animal models of cardiac hypertrophy and heart failure.

The purpose of the present study therefore was to assess the hypothesis that blockade of NF-κB activation might ameliorate ventricular hypertrophy and inflammation in response to chronic infusion of angiotensin II. Mice with targeted disruption of the p50 subunit of NF-κB (KO) [10] were used to block the activation of NF-κB chronically.

2. Methods

2.1. Animal models

Mice with targeted disruption of the p50 subunit of NF-κB [10], backcrossed into the FVB background more than six generations, were used to block the activation of NF-κB. These mice were born normally without any major defects. Homo-knockout mice (KO) were compared with age- and gender-matched wild type littermates (WT) in each analysis to minimize the effect of genetic background variation. Male mice at the age of 8 weeks were used unless mentioned otherwise. First, to confirm that angiotensin II induces activation of NF-κB in WT hearts but not in NF-κB KO hearts, 30 g/kg of angiotensin II was injected intraperitoneally and the heart was harvested 30 min after. Secondly, to investigate whether blockade of NF-κB activation might ameliorate ventricular hypertrophy and inflammation in response to chronic infusion of angiotensin II, an osmotic minipump (Alzet Model 2004) was implanted peritoneally and the heart was harvested 30 min after.

2.2. Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed according to the manufacturer’s instructions (Gel Shift Assay System E3300, Promega, Madison, Wisconsin, USA) as previously described [11]. Nuclear proteins were isolated using the method of Haudek et al. [12]. Protein concentrations were measured by BCA Protein Assay Reagents (PIERCE, Rockford, Illinois, USA) using bovine serum albumin (BSA) as a standard. Protein–DNA binding was carried out in a final volume of 40 μL. To each tube, 4 μL of 10× binding buffer (100 mM Tris pH 8.0, 10 mM EDTA, 40% glycerol, 1 M NaCl), 100 ng of 1,4-dithiothreitol (DTT), 4 μg of BSA, 2 μg of dIdC and 30 μg nuclear proteins was added. After the samples were incubated at room temperature for 10 min, 1 μL of 32P-labeled NF-κB probe (a double-stranded oligonucleotide corresponding to the consensus NF-κB binding site of the κ light-chain enhancer: 5′-AGTTGAGGGGACTTTCC-A3′, approximately 20000 cpm/ng) was added to each reaction and incubated for 20 min at room temperature. Samples were resolved on a 5% acrylamide gel in 0.25% TBE buffer.

2.3. Echocardiography

After 4 weeks of the treatment, echocardiography was performed using an ultrasonographic system (SSD-5500 ALOKA) as previously reported [13,14]. After anesthetization with 2.5% avertin (14 μL/g body weight, i.p., Aldrich Chemical Co.), mice were placed in a supine position. A 7.5-MHz transducer was applied to the left hemithorax. Two-dimensional targeted M-mode imaging was obtained from the short-axis view at the level of the greatest left ventricular dimension. M-mode measurements of left ventricular end-diastolic and end-systolic diameter and left ventricular anterior and posterior wall thickness were made using the leading-edge convention of the American Society of Echocardiography. End-diastole was determined at the maximal left ventricular diastolic dimension, and end-systole was taken at the peak of posterior wall motion. The percentage of left ventricular fractional shortening (FS) was calculated as FS (%) = (LVDDd – LVDs)/LVDDd × 100, where LVDd and LVDs indicate left ventricular end-diastolic and end-systolic diameter, respectively.

2.4. Tissue preparation and morphometric analysis

After echocardiography, the heart was excised, weighed, and fixed in 4% paraformaldehyde for histology or immediately frozen and stored at –80 °C for RNA or protein analysis. After hematoxylin–eosin staining, cross-sectional area of cardiomyocytes in the left ventricle was...
evaluated as previously reported [14]. The outline of 100–200 myocytes was traced in each section and NIH image system software was used to determine myocyte cross-sectional area.

2.5. Northern blot analysis

Total RNA was extracted from the left ventricle by an acid guanidium thiocyanate–phenol chloroform method (ISOGEN, Nippon Gene). RNA samples (5 μg) were electrophoresed in a formaldehyde-agarose gel, and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). The membrane was then hybridized with a 32P-labeled probes for murine atrial natriuretic peptide (ANP, nucleotides 592 to 1094, GenBank K02781) or 18S rRNA as previously reported [6]. Result of the cDNA hybridization was normalized to that of the 18S probe to correct for differences in RNA mass and efficiency of transfer. Data were in turn normalized to the mean of WT samples, arbitrarily set at 1.

2.6. Activity of mitogen-activated protein kinase (MAPK)

The left ventricle was homogenized with a lysis buffer containing 25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Na3VO4, 10 mM NaF, 1% (vol/vol) Triton X-100, 1% (vol/vol) glycerol. Equal amounts of the heart homogenate (30 μg) were separated by SDS-PAGE on 10% (wt/vol) gels and transferred onto a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Lab), and blocked with 5% skimmed milk at room temperature for 60 min. The reverse transcription (RT) product was applied to each 20 μL PCR. The PCR reactions contained 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.1% (vol/vol) Trition X-100, 1% (vol/vol) glycerol. Equal amounts of the heart homogenate (30 μg) were separated by SDS-PAGE on 10% (wt/vol) gels and transferred onto a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad Lab), and blocked with 5% skimmed milk at room temperature for 60 min. The filters were subjected to immunoblot analyses with anti-phospho-extracellular signal-regulated kinase (ERK) antibody (no. 9102; Cell Signaling Technology Inc.), anti-phospho-c-Jun NH2-terminal kinase (JNK) antibody (no. 9106; Cell Signaling Technology Inc.), anti-phospho-extracellular signal-regulated kinase (ERK) antibody (no. 9102; Cell Signaling Technology Inc.), anti-phospho-c-Jun NH2-terminal kinase (JNK) antibody (no. 9106; Cell Signaling Technology Inc.), anti-phospho-p38 antibody (no. 9211; Cell Signaling Technology Inc.). Duplicate samples were subjected to immunoblot analyses with anti-ERK antibody (no. 9102; Cell Signaling Technology Inc.), anti-JNK1 antibody (sc-474; Santa Cruz Biochemistry) for overnight at 4 °C, or anti-phospho-p38 antibody (no. 9211; Cell Signaling Technology Inc.). Immunodetection was accomplished with a horseradish anti-rabbit or anti-mouse secondary antibody (1:2000 dilution, Amersham) using an enhanced chemiluminescence kit (Amersham). The data were in turn normalized to that of GAPDH included in each template set as an internal control.

2.8. Immunohistochemistry

Sections fixed in paraformaldehyde were incubated with a goat polyclonal anti-mouse TNF-α antibody (L-19; Santa Cruz Biochemistry) for overnight at 4 °C, washed three times, and incubated with affinity-purified biotinylated rabbit anti-goat IgG for 1 h at room temperature. They were washed again and overlaid with streptavidin–biotin–peroxidase complex for 1 h at room temperature (Nichirei; Tokyo, Japan). After a final wash, the labeling was visualized with aminoethyl carbazole (Nichirei; Tokyo, Japan). Counterstaining was then performed with Mayer’s hematoxylin. The heart sections of transgenic mice with cardiac-specific overexpression of TNF-α [6] were used as positive controls.

2.9. RT-PCR

First-strand cDNA was synthesized using reverse transcriptase with oligo(dT) from 1 μg of total RNA in 20 μL reaction volume according to the manufacturer’s protocol (ReverTra Ace, TOYOBO), then 5 μL of the resulting reverse transcription (RT) product was applied to each 20 μL PCR. The PCR reactions contained 10 mM Tris–HCl, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.5 U Taq DNA polymerase (rTaq DNA polymerase; TOYOBO) and 0.1 mM PCR primers for one of angiotensin type 1 (AT1) or type 2 (AT2) receptor, or GAPDH (Table 1). PCR conditions were 30 s of denaturation at 94 °C, 30 s of annealing at 55 °C, and 1 min of extension at 72 °C. PCR cycles were limited to 26 for AT1, 32 for AT2 and 22 for GAPDH after a pilot study to avoid over-amplification. PCR products were separated by 2% agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Primer sequences for RT-PCR</th>
<th>cDNA and primer</th>
<th>Sequence of primer</th>
<th>Product size</th>
</tr>
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<tr>
<td>AT1 receptor</td>
<td>Forward</td>
<td>5′-GGAACAGCTTGGTGGTG-3′</td>
<td>555 bp</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5′-CTGAATTTCTGAAAGCTTCT-3′</td>
<td></td>
</tr>
<tr>
<td>AT2 receptor</td>
<td>Forward</td>
<td>5′-GGCCTCGATTTTAAGGAGTG-3′</td>
<td>164 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-ACGGCTGCTGGATAATGTTTC-3′</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5′-CTTGCTCGAGTCTGTCGGTG-3′</td>
<td>1064 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-AGGCGCTGCCCAGTGACAA-3′</td>
<td></td>
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AT1 and AT2 indicate angiotensin type 1 and type 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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2.10. Statistics

Results are presented as mean ± S.D. Statistical comparisons were performed by ANOVA with Student–Newman–Keuls post hoc test or two-way ANOVA where appropriate (Fig. 6). Differences were considered significant at a value of $P < 0.05$.

3. Results

3.1. Absence of NF-κB activation in NF-κB KO mice

As shown in Fig. 1, intraperitoneal injection of angiotensin II increased activation of NF-κB in the myocardium of WT mice, while it was completely abolished in NF-κB KO mice irrespective of angiotensin II treatment.

3.2. Augmented pressor response in NF-κB KO mice

Systemic blood pressure was measured twice a week after implantation of an osmotic minipump with a low or high dose of angiotensin II (0.2 or 2 mg/kg/min) or vehicle alone. There were no significant differences in systemic blood pressure between WT and NF-κB KO mice before the implantation of the osmotic minipumps (Fig. 2A,B). Although male NF-κB KO mice at the age of 8 weeks were significantly smaller (26.6 ± 1.6 g, $n = 34$) than age- and gender-matched WT littermates (28.9 ± 2.7 g, $n = 34$, $P < 0.05$), the concentration of angiotensin II was titrated carefully in accordance with the body weight in each mouse. Infusion of vehicle alone did not change systemic blood pressure either in WT or in NF-κB KO mice during 4 weeks of the treatment. In contrast, although the low dose of angiotensin II did not significantly affect systemic blood pressure in WT mice, it significantly increased it in NF-κB KO mice even after 4 weeks of the treatment (Fig. 2C). The high dose of angiotensin II significantly increased systemic blood pressure both in WT and in NF-κB KO mice (Fig. 2A,B). However, the systemic blood pressure was significantly higher in NF-κB KO mice than WT mice after 4 weeks of the treatment (Fig. 2C). These results indicated that the pressor effects of angiotensin II were significantly augmented in NF-κB KO mice.

3.3. Attenuated hypertrophic response by NF-κB KO

After 4 weeks of the treatment, the heart was harvested and weighed. Since NF-κB KO mice were slightly but significantly smaller than WT littermates, left ventricular weight of NF-κB KO mice treated with vehicle alone (76.5 ± 5.9 mg) was significantly smaller than that of WT mice (88.4 ± 7.6 mg, $P < 0.05$). However, after normalization to the body weight, there were no differences between vehicle-treated NF-κB KO and WT mice (Fig. 3A). The low dose of angiotensin II did not affect left ventricular weight of the treatment. In contrast, although the low dose of angiotensin II did not significantly affect systemic blood pressure in WT mice, it significantly increased it in NF-κB KO mice even after 4 weeks of the treatment (Fig. 2C). The high dose of angiotensin II significantly increased systemic blood pressure both in WT and in NF-κB KO mice (Fig. 2A,B). However, the systemic blood pressure was significantly higher in NF-κB KO mice than WT mice after 4 weeks of the treatment (Fig. 2C). These results indicated that the pressor effects of angiotensin II were significantly augmented in NF-κB KO mice.

Fig. 1. Electrophoretic mobility shift assay for activation of NF-κB in myocardium. Nuclear proteins were isolated from the left ventricle of wild-type (WT) or NF-κB knockout mice (KO) 30 min after injection of angiotensin II (Ang II) or saline. The activation of NF-κB was completely abolished in NF-κB KO mice.

Fig. 2. Effects of angiotensin II (Ang II) on systolic blood pressure (SBP). SBP was measured twice a week after implantation of osmotic minipumps in wild type (WT, A) and NF-κB knockout mice (KO, B). SBP after 4 weeks of the treatment with vehicle (−), low dose (+) or high dose (++) of angiotensin II was summarized in (C). Values are mean ± S.D. *$P < 0.05$ vs. vehicle, †$P < 0.05$ vs. WT.
either in WT or in NF-κB KO mice. In contrast, the high dose of angiotensin II significantly increased left ventricular weight both in WT and in NF-κB KO mice. However, left ventricular hypertrophy observed in NF-κB KO mice was significantly less than that in WT mice (Fig. 3A). These results indicated that, despite the augmented pressor response, the hypertrophic effects of angiotensin II were significantly attenuated in NF-κB KO mice.

Cross-sectional area of cardiomyocytes was evaluated to confirm the attenuated ventricular hypertrophy in NF-κB KO mice (Fig. 3B,C). Cross-sectional area of NF-κB KO mice treated with vehicle alone was similar to that of WT mice. The low dose of angiotensin II did not affect cross-sectional area of either WT or NF-κB KO mice. In contrast, the high dose of angiotensin II significantly increased cross-sectional area in WT mice but not in NF-κB KO mice. Therefore, attenuated ventricular hypertrophy in NF-κB KO mice might be related to the reduced hypertrophic response in cardiomyocytes.

To further verify the attenuated hypertrophic response in NF-κB KO mice transcriptionally, gene expression of ANP in the left ventricle was evaluated by Northern blot analysis (Fig. 3D,E). While chronic infusion of the high dose angiotensin II significantly increased myocardial expression of ANP in WT mice, this up-regulation of ANP was significantly attenuated in NF-κB KO mice. Taken together, these results suggest that the targeted disruption of the p50 subunit of NF-κB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II.

3.4. Preserved cardiac function in NF-κB KO mice

Echocardiography was performed to evaluate the effects of NF-κB KO on left ventricular function (Fig. 4). The high dose of angiotensin II significantly increased wall thickness, decreased end-diastolic dimension, and enhanced fractional shortening in WT mice. In contrast, in NF-κB KO mice, wall thickness, end-diastolic dimension, or fractional shortening was not affected by angiotensin II infusion. These results indicated that, despite the absence of ventricular hypertrophy, cardiac function was well preserved against increased afterload in NF-κB KO mice.
3.5. Absence of JNK phosphorylation in NF-κB KO mice

To elucidate the changes in hypertrophic signaling pathways, phosphorylation of MAP kinases in the myocardium was evaluated after 3-day or 4-week treatment with the high dose of angiotensin II. As shown in Fig. 5, protein levels of ERK, JNK, or p38 were not different between WT and NF-κB KO mice. Although neither ERK nor p38 was phosphorylated by 3-day or 4-week treatment, JNK was further phosphorylated by the 3-day, but not 4-week, treatment with angiotensin II. It is notable that the phosphorylation of JNK was selectively abrogated in NF-κB KO mice regardless of the treatment. Specificity of this finding was confirmed by a phospho-Western analysis after immunoprecipitation with the anti-JNK1 antibody (data not shown).

3.6. Effects of NF-κB KO on myocardial expression of cytokines

Transcript levels of proinflammatory cytokines and chemokines were evaluated by multiprobe ribonuclease...
protection assay after 4 weeks of the treatment with vehicle or the high dose of angiotensin II (Fig. 6A). The results were analyzed by two-way ANOVA and summarized in Fig. 6B. The high dose of angiotensin II significantly increased expression of RANTES, TNF-α, IL-1β, TGF-β, and MCP-1 in the myocardium. Although NF-κB KO did not affect myocardial expression of IL-1β, MCP-1, or TGF-β, it significantly increased that of TNF-α and RANTES. Since no interaction was found between NF-κB KO and angiotensin II treatment, it is suggested that induction of proinflammatory cytokines in response to chronic infusion of angiotensin II may not be mediated by NF-κB dependent pathways. To confirm the increased expression of TNF-α in NF-κB KO mice, immunohistochemical staining was performed (Fig. 7). Consistent with the transcript levels, while TNF-α staining was increased in response to angiotensin II treatment in WT mice, it was evident without angiotensin II treatment in NF-κB KO mice. Since staining was diffuse and not localized to interstitial cells, one of major sources of TNF-α was suggested to be cardiomyocytes.

3.7. Expression of angiotensin II receptors

Since targeted disruption of the p50 subunit of NF-κB may have affected expression of angiotensin II receptors in the heart, transcript levels of AT1 and AT2 receptors were evaluated by RT-PCR. As shown in Fig. 8, there was no difference in the expression of either AT1 or AT2 receptors in the heart between WT and NF-κB KO mice. Therefore, the attenuated hypertrophic response in NF-κB KO mice may not be attributable to the difference in the expression of angiotensin II receptors.

4. Discussion

We have evaluated in vivo effects of NF-κB inhibition on the development of angiotensin II-induced cardiac hypertrophy. Chronic infusion of angiotensin II, significantly increased systemic blood pressure, provoked ventricular hypertrophy, and enhanced expression of proinflammatory cytokines in the myocardium. Since the targeted disruption of the p50 subunit of NF-κB significantly attenuated ventricular hypertrophy in response to angiotensin II, it is suggested that the hypertrophic effect of angiotensin II is mediated by the activation of NF-κB. In contrast, elevation of systemic blood pressure and induction of proinflammatory cytokines were rather enhanced in NF-κB KO mice, suggesting that pressor and proinflammatory effects of angiotensin II may be mediated by pathways independent of NF-κB. Despite the absence of ventricular hypertrophy with higher blood pressure and enhanced expression of proinflammatory cytokines, echocardiography revealed no

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Fig. 6. Myocardial expression of proinflammatory cytokines and chemokines. (A) Representative images of multiprobe RPA. (B) Summarized data of cytokine expression: TNF-α, RANTES, IL-1β, MCP-1, and TGF-β. Normalized values in vehicle-treated wild type mice (WT) are arbitrarily expressed as 1. KO indicates NF-κB knockout mice. Based on two-way ANOVA, * indicates a significant effect of angiotensin II (p < 0.05) and † demonstrates that of NF-κB KO (p < 0.05). No interaction was found between angiotensin II and NF-κB.
evidence of cardiac dysfunction in NF-κB KO mice. Therefore, we conclude that NF-κB may be a new therapeutic target to attenuate ventricular hypertrophy in a setting in which angiotensin II is activated.

Recent in vitro studies [8,9] have demonstrated that the activation of NF-κB is required for hypertrophic growth of primary rat neonatal ventricular cardiomyocytes in response to G-protein-coupled receptor agonists, including phenylephrine, endothelin-1, and angiotensin II. However, it has not been clear whether the activation of NF-κB may also play an important role in the development of ventricular hypertrophy in vivo. Therefore, we took advantage of knockout mice in which the p50 subunit of NF-κB was disrupted [10]. Chronic infusion of angiotensin II was adopted as a model of ventricular hypertrophy. Two different doses of angiotensin II were infused for 4 weeks intraperitoneally. The low dose of angiotensin II (0.2 μg/kg/min) was chosen based on previous reports demonstrating the hypertrophic effect of angiotensin II without affecting systemic blood pressure [15,16]. However, in the present study, the low dose of angiotensin II did not show any hypertrophic effects either in WT or in NF-κB KO mice. It might be of interest that the low dose of angiotensin II slightly but significantly increased systemic blood pressure not in WT but in NF-κB KO mice, suggesting enhancedpressor responsiveness in NF-κB KO mice. In contrast, the high dose of angiotensin II (2 μg/kg/min) significantly increased systemic blood pressure as well as left ventricular weight in WT mice. Although the systemic blood pressure was even higher in NF-κB KO mice, the hypertrophic response was significantly attenuated by targeted disruption of the p50 subunit of NF-κB. The attenuated hypertrophic responsiveness was confirmed histologically (cross-sectional area) and transcriptionally (ANP expression). These results indicate that targeted disruption of the p50 subunit of NF-κB ameliorates myocardial hypertrophy in response to chronic infusion of angiotensin II. Taking into account the previous study that indicates that pharmacological inhibition of NF-κB with pyrrolidine dithiocarbamate was able to prevent cardiac hypertrophy in rats harboring both human renin and angiotensinogen genes [17], it is suggested that NF-κB plays an important role in the development of hypertrophy by angiotensin II.

Although the precise mechanisms by which NF-κB mediates cardiac hypertrophy remain undetermined, it is of interest that phosphorylation of JNK was abrogated in p50 knockout mice. MAP kinase signaling pathways, including ERK, JNK and p38, are supposed to play an important role in cardiac hypertrophy and remodeling, since they are phosphorylated and activated by G-protein-coupled receptor agonists such as phenylephrine, endothelin-1, and angiotensin II [18,19]. JNK is especially called stress-activated protein kinase, since it is additionally activated by cellular stresses such as reactive oxygen species and proinflammatory cytokines, including IL-1β and TNF-α [18]. Although these cellular stresses activate NF-κB as well as JNK, as far as we know, this is the first report demonstrating that the blockade of NF-κB activation abrogates JNK phosphorylation. Since the protein level of JNK is not affected in p50 knockout mice, expression or activation of upstream kinases may be modulated by NF-κB pathways.

Substrates of JNK are transcription factors, including c-Jun, ATF2, and Elk1 [18]. Inhibition of JNK has been shown to abrogate ventricular hypertrophy in vivo in response to pressure overload [20] or Goq overexpression [21]. Therefore, the attenuated hypertrophic responsiveness to angiotensin II in p50 knockout mice may be mediated by the abrogation of JNK pathways. However, a recent study suggests that the MEKK1-JNK pathway does not mediate...
cardiac hypertrophy but rather plays a protective role in pressure overload [22]. Furthermore, JNK may antagonize cardiac growth through cross-talk with calcineurin-NFAT signaling [23]. Taken together, these results indicate that further studies will be required to draw the final conclusion.

Angiotensin II has been shown to induce TNF-α biosynthesis in the adult mammalian heart with activation of NF-κB [7]. Since activation of NF-κB has been shown to induce various members of proinflammatory cytokines and chemokines, including TNF-α [1], we hypothesized that the blockade of NF-κB activation might attenuate myocardial expression of proinflammatory cytokines in response to chronic infusion of angiotensin II. However, the results did not support our hypothesis: although chronic infusion of angiotensin II increased myocardial expression of TNF-α, RANTES, IL-1β, MCP-1, and TGF-β, the targeted disruption of the p50 subunit of NF-κB rather enhanced myocardial expression of TNF-α and RANTES without affecting upregulation of IL-1β, MCP-1, and TGF-β. These results indicate that induction of proinflammatory cytokines in response to chronic infusion of angiotensin II was not mediated by NF-κB dependent pathways. Furthermore, myocardial expression of proinflammatory cytokines may not aggravate hypertrophy in this mouse model with chronic infusion of angiotensin II.

Paradoxical increase of TNF-α and RANTES expression by the blockade of NF-κB may be explained by the increased wall stress due to higher systemic blood pressure and attenuated hypertrophic responsiveness in angiotensin II-treated NF-κB KO mice. However, these cytokines were also up-regulated even without angiotensin II treatment. Therefore, it is suggested that factors other than increased wall stress should exist in NF-κB KO mice. The difference in transcriptional activity of p50–p65 heterodimers and p50–p50 homodimers [24] may be one of them. The NF-κB/Rel family consists of five subunit members, including p50, p52, c-Rel, RelA (p65), and RelB. In most cells, NF-κB is a heterodimer of p50 and p65 that is retained in the cytoplasm bound to the inhibitory protein IκB. Activation of NF-κB will occur when the specific IκB kinases phosphorylate the IκB. After chronic exposure to proinflammatory cytokines, including TNF-α, NF-κB has been shown to be converted from transcriptionally active p50–p65 heterodimers to transcriptionally inactive p50–p50 homodimers [12,24], which may act as a native negative feedback mechanism to prevent excessive inflammatory responses. The absence of p50–p50 homodimers in mice with targeted disruption of the p50 subunit therefore may account for enhanced expression of TNF-α and RANTES in response to chronic infusion of angiotensin II.

In the present study, to assure the complete and chronic inhibition of NF-κB in vivo, we used gene-manipulated mice lacking the p50 subunit of NF-κB [10], which show no developmental abnormalities, but exhibit multifocal defects in immune responses involving B lymphocytes and non-specific responses to infection; B cells do not proliferate in response to bacterial lipopolysaccharide and are defective in basal and specific antibody production. Although we did not detect any adverse effects or premature death as long as we observed, systemic inhibition of NF-κB may be deleterious in the long run. Therefore, it may be desirable to introduce cardiac-specific inhibition of NF-κB to minimize immunological detrimental effects. Furthermore, the pressor responsiveness to angiotensin II seems to be augmented in NF-κB KO mice. Although pressor responses to bolus injections of angiotensin II were similar between WT and NF-κB KO mice (data not shown), the differences in systemic blood pressure were evident after 7 days of the treatment, as shown in Fig. 2, suggesting that NF-κB KO might have augmented vascular remodeling in response to angiotensin II. Since inhibition of NF-κB activation in macrophages has been shown to increase atherosclerosis in LDL receptor-deficient mice [25], systemic inhibition of NF-κB may promote vascular injury and atherosclerosis. Finally, it remains to be determined whether inhibition of NF-κB may also attenuate myocardial hypertrophy caused by other stimuli besides angiotensin II. Although Li et al. [26] have recently reported that NF-κB is necessary for aortic banding-induced cardiac hypertrophy, further studies are required in other models of cardiac hypertrophy and heart failure, including volume overload and myocardial infarction.

In conclusion, targeted disruption of the p50 subunit of NF-κB ameliorated myocardial hypertrophy in response to chronic infusion of angiotensin II. The activation of NF-κB may play an important role in the pathogenesis of myocardial hypertrophy and remodeling besides promoting myocardial inflammation. Further studies will be warranted to verify that inhibition of NF-κB may be a promising therapeutic strategy for cardiac remodeling and heart failure.

Acknowledgements

A part of this study was conducted in Kyushu University Station for Collaborative Research. This study was supported by a grant from Kimura Memorial Heart Foundation, by the Grant for Research on Cardiovascular Disease from Japan Heart Foundation/Pfizer Pharmaceuticals Inc., and by the Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (C15590755).

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