IκBβ Attenuates Angiotensin II–Induced Cardiovascular Inflammation and Fibrosis in Mice

Shanqin Xu, Hui Zhi, Xiuyun Hou, Richard A. Cohen, Bingbing Jiang

Abstract—The development of cardiovascular fibrosis is associated with chronic inflammation, where activation of nuclear factor κB (NF-κB) signaling may play a critical role. NF-κB activation is tightly regulated by the cellular inhibitor of κB (IκB) family of proteins, such as IκBα and IκBβ. IκBα and IκBβ display different regulation kinetics in response to inflammatory stimulation. The present study tested the hypothesis that IκBα and IκBβ may have different roles in modulating cardiovascular inflammation and fibrosis, using a model of angiotensin II infusion-induced hypertension in wild-type mice and IκBβ knock-in mice, in which the IκBα gene is replaced by IκBβ cDNA (AKBI). In WT mice, subcutaneous angiotensin II infusion for 7 days induced increased perivascular and interstitial collagen deposition and fibrotic lesions, associated with myocardial interstitial hemosiderin accumulation and extensive macrophage infiltration. These effects of angiotensin II were dramatically limited in AKBI mice. Replacement of IκBα with IκBβ significantly attenuated angiotensin II infusion–induced expression of interleukin 1β, interleukin 6, monocyte chemotactic protein 1, collagen I and III, fibronectin, and tissue inhibitor of metalloproteinase 1 in the hearts. Furthermore, using cultured vascular smooth muscle cells, we demonstrated that interleukin 1β–induced NF-κB activation and monocyte chemotactic protein 1, vascular cell adhesion molecule 1, and tissue inhibitor of metalloproteinase 1 expressions were suppressed in the AKBI cells because of the replacement of IκBα with IκBβ. These results indicate that NF-κB has an essential role in mediating the cardiovascular inflammatory response to angiotensin II and suggest that targeting the balance of IκBα and IκBβ expression might be a novel therapeutic modality in preventing fibrosis in hypertensive cardiovascular disease. *(Hypertension. 2011;58:00-00.)* • Online Data Supplement

Key Words: fibrosis ■ inflammation ■ iron ■ NF-κB ■ tissue inhibitor of metalloproteinase

Cardiovascular fibrosis is the most common consequence of hypertensive disease and contributes to the development of cardiovascular dysfunction. Cardiovascular fibrosis is thought to develop as a result of a tissue repair process associated with excessive chronic inflammation. Activation of nuclear factor-κB (NF-κB) is essential for expression of numerous inflammatory mediators, including adhesion molecules, cytokines/chemokines, growth factors, and extracellular matrix (ECM) metabolic regulators that participate in the tissue repair and remodeling process. Modulation of NF-κB signaling may, therefore, influence the inflammatory and fibrotic responses in hypertensive disease.

NF-κB activation is tightly regulated by the cellular inhibitor of κB (IκB), such as IκBα and IκBβ. Various stimuli, such as interleukin (IL)-1β, tumor necrosis factor-α, and lipopolysaccharide, can induce activation of IκB kinases that phosphorylate IκB, leading to subsequent IκB ubiquitination and proteasomal degradation. NF-κB released from the IκB/NF-κB complex then translocates to the nucleus, where it initiates transcription of NF-κB–regulated genes. Although IκBα and IκBβ have overlapping functions, there are unique and nonredundant functional roles of IκBα and IκBβ in regulating immune and inflammatory response. IκBα deficiency results in mouse neonatal lethality with extensive dermatitis and granulopoiesis, whereas IκBβ deficiency does not. An IκBβ knock-in mouse model, in which the IκBα gene is replaced by IκBβ cDNA (named AKBI), develops normally but shows attenuated neutrophil recruitment and liver dysfunction after liver ischemia/reperfusion injury, suggesting that replacement of IκBα by IκBβ may not only prevent IκBα deficiency–induced mouse neonatal lethality but also protect tissue from damage in certain conditions. However, the different functional roles of IκBα and IκBβ remain largely unknown, and whether IκBα and IκBβ could have different roles in modulating cardiovascular inflammation and fibrosis has not been explored previously.

Angiotensin (Ang) II is one of the best-characterized vasoconstrictors that, at abnormally high levels, can cause hypertension, cardiovascular inflammation, and fibrosis. In vivo experiments using rodent models suggest that Ang II may contribute to fibrosis by inducing iron deposition, macrophage infiltration, and upregulation of transforming growth factor-β. Stimulation of IκBα and IκBβ by Ang II may contribute to the development of cardiovascular fibrosis.
growth factor-β1. In the present study, we used Ang II infusion-induced hypertensive models in wild-type (WT) and AKBI mice to test the hypothesis that IκBa and IκBβ may play different roles in modulating cardiovascular inflammation, iron deposition, and fibrosis. We also used cultured vascular smooth muscle cells (VSMCs) isolated from WT and AKBI mouse aortas to further uncover the different roles of IκBa and IκBβ in regulating NF-κB activation and gene expression.

**Methods**

**Animal Models**
AKBI transgenic mice were kindly made available by Cordula Stamme (Leibniz-Center for Medicine and Biosciences, Borstel, Germany). The mice were maintained on CD-1 genetic background. AKBI mice and WT CD-1 controls (both females, at 20 weeks of age) were randomly divided into 2 groups and infused with either saline or Ang II (3.2 mg/kg per day) via subcutaneously implanted osmotic pumps (ALZET, model 1007D) following a procedure as described previously. Systolic blood pressure was measured by the tail-cuff plethysmography before starting treatment and again on day 6 after Ang II infusion. Mice were euthanized on day 7 of the treatment. Blood was drawn from abdominal vein, and serum was isolated. Hearts and aortas were harvested. For histology and immunohistochemistry, the tissues were fixed with 10% buffered formalin, processed, and embedded in paraffin. For isolation of RNA, cardiac ventricles were directly added to 1 mL of TRIzol reagent (Invitrogen), and total RNA was extracted following the manufacturer’s protocol. All of the animal experiments were carried out with the approval of the institutional animal care and use committee of Boston University Medical Center and Harvard Medical School.

**Histology and Immunohistochemistry**
To assess morphological changes, Masson trichrome staining was performed in 5-μm-thick cross-sections of heart and descending aorta. Collagen was stained using Picrosirius Red. Collagen deposition (red color) and whole section area were analyzed using National Institutes of Health ImageJ. Iron (hemosiderin) accumulation was detected with Prussian blue staining. Tissue macrophage infiltration was detected by immunohistochemistry with an antibody against Mac3 antigen (online Data Supplement, please see http://hyper.ahajournals.org). To measure aortic medial thickness and adventitial fibrotic area, microscopic images of descending aorta cross-sections stained with Masson trichrome were analyzed using National Institutes of Health ImageJ.

**Cell Culture**
VSMCs were isolated from the medial layers of thoracic aorta of AKBI and WT littersmates using the explant method described previously. Cells were cultured in DMEM/F12 (Invitrogen) with 10% FBS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cells were used between passages 4 and 7. The cells at confluence were washed and maintained in DMEM/F12 with 0.1% FBS for 24 hours. The cells were then treated with or without IL-1β for designated time periods. For determination of NF-κB activation, confluent cells cultured in 24-well plates were infected with NF-κB-luciferase reporter adenovirus (Ad-NFκB-Luc) for 24 hours and then treated with IL-1β for either 4 or 16 hours. Cells were lysed and 5 μg of proteins of each cell lysate were used for luciferase activity assay using the assay kit from Promega. Relative light units were measured using the SpectraMax M5 Microplate Reader (Molecular Devices).

**Determination of Serum Cytokines**
Serum cytokines were determined by ELISA using the MILLIPLEX Mouse Cytokine/Chemokine Assay kit (MPXMCYT-70K, Millipore) following the manufacturer’s protocol.

Figure 1. AKBI mice resist developing cardiovascular fibrosis in response to angiotensin (Ang) II infusion. A, Ang II infusion increases systolic blood pressure (SBP) in wild-type (WT) and AKBI mice. Mean±SD, n=5 in each group. B, Western blot analysis of inhibitor of nuclear factor κB (IκB) and NF-κB p65 expression in heart tissues. C and D, Masson trichrome staining of heart sections. Ang II infusion causes perivascular and interstitial fibrosis in WT mice. These effects of Ang II were much less obvious in AKBI mice. LV indicates left ventricle; RV, right ventricle.
AKBI Mice Resist Developing Cardiovascular Fibrosis in Response to Ang II Infusion

Ang II infusion increased systolic blood pressure similarly in WT and AKBI mice (Figure 1A). As shown in Figure 1B, AKBI mice did not express IκBα, but showed an increased IκBβ expression in the heart tissues. There was a basal level of phosphorylated IκBα in WT mice, which was enhanced by Ang II infusion. Endogenous phosphorylated IκBβ was not detectable (data not shown). There was no difference in NF-κB p65 expression between WT and AKBI mice. Ang II infusion caused perivascular and interstitial fibrotic lesions in both left ventricles and right ventricles (Figures 1C and 1D), suggesting that Ang II–induced cardiac fibrosis is at least partially independent of increased systolic blood pressure. Ang II–induced fibrotic lesions were much less obvious in AKBI mice than in WT mice. Collagen staining showed that Ang II infusion enhanced ventricular tissue collagen deposition, which was attenuated in AKBI mice (Figure 2A and 2B). Furthermore, Ang II infusion increased aortic medial thickness in both WT and AKBI mice (Figure 2C and 2D). Interestingly, Ang II infusion increased aortic adventitial area and ECM deposition in WT mice, which was attenuated in AKBI mice (Figure 2C and 2E). These results demonstrate that replacement of IκBα with IκBβ attenuates Ang II infusion–induced fibrosis, and this effect is independent of systolic blood pressure.

AKBI Prevents Cardiac Hemosiderin Deposition and Reduces Macrophage Accumulation

Abnormal hemosiderin deposition was observed in the ventricular interstitium of all Ang II–infused WT mice but was nearly absent in those of Ang II–infused AKBI mice and saline-infused control mice (Figure 3A). Staining of serial sections revealed that hemosiderin deposition was mainly located in either the border regions of fibrotic lesions (Figure 3B) or in the regions lacking obvious fibrotic changes (Figure 3C), suggesting that Ang II–induced hemosiderin deposition may constitute an early sign of tissue damage that precedes fibrogenesis. Macrophage infiltration, as shown by positive Mac3 staining, was extensively seen in both fibrotic regions and regions with hemosiderin deposition in Ang II–infused WT mice (Figure 3B and 3C). In contrast, Ang II infusion–induced macrophage infiltration was significantly reduced in AKBI mice (Figure 3C and 3D).

AKBI Attenuates Proinflammatory and Profibrotic Gene Expression in Response to Ang II Infusion

To examine how AKBI mice attenuate Ang II infusion–induced inflammation and fibrosis, we analyzed serum cytokine levels and ventricular tissue expression of selected genes related to inflammation and ECM metabolism. Serum IL-1β and IL-6 levels significantly increased in Ang II–infused WT mice but showed no significant change in AKBI mice (Figure 4A). Serum tumor necrosis factor-α, monocyte chemotactic
protein (MCP) 1, and transforming growth factor-β1 levels did not change significantly in response to Ang II infusion (data not shown). Ang II infusion enhanced the cardiac mRNA levels of IL-1β, IL-6, tumor necrosis factor-α, MCP-1, transforming growth factor-β1, hepatocyte growth factor, and insulin-like growth factor 1 in WT mice (Figure 4B). In contrast, Ang II infusion had little or no effect on the expression of these genes in AKBI mice. Interestingly, atrial natriuretic peptide mRNA was enhanced to a similar level by Ang II infusion in WT and AKBI mice, suggesting that atrial natriuretic peptide expression is not affected by the different IkB-mediated NF-κB regulatory mechanism.

Ang II infusion significantly enhanced cardiac mRNA levels of collagen I (Col1a1), collagen III (Col3a1), fibronectin (FN-1), P4ha3 (a component of prolyl 4-hydroxylase, the key enzyme in collagen synthesis), matrix metalloprotease 12, and the tissue inhibitor of matrix metalloprotease (TIMP) 1 in WT mice, as compared with the levels in saline-infused control mice (Figure 4C). In contrast, the expression of these genes in AKBI mice did not increase significantly in response to Ang II infusion (Figure 4C), suggesting that abnormal degradation and deposition of ECM induced by Ang II infusion is inhibited by the replacement of IkBa with IkBβ.

**Differential NF-κB Activation and Gene Expression in WT and AKBI VSMCs**

We further determined the effects of replacement of IkBa with IkBβ on VSMC response to IL-1β, a major cytokine known to activate NF-κB signaling and shown to be upregulated in Ang II–infused WT mice. As shown in Figure 5A, treatment of WT cells with IL-1β resulted in IkBa phosphorylation and degradation, followed by rapid resynthesis, as evidenced by increased IkBa levels at 1 hour and 3 hours after the treatment. IkBβ was decreased during the 3-hour IL-1β treatment in WT cells. In AKBI cells, IL-1β induced a transient decrease in IkBβ levels, after which IkBβ gradually increased and restored to basal levels at 3 hours. IL-1β treatment did not change NF-κB p65 expression in WT and AKBI cells. Furthermore, IL-1β induced NF-κB activation, as shown by NF-κB–dependent luciferase activity assay, and

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**Figure 3.** Effect of angiotensin (Ang) II infusion on myocardial hemosiderin deposition and macrophage infiltration. A, Prussian blue staining for hemosiderin on heart sections. B and C, Staining of serial sections showing the relation of fibrotic lesions, hemosiderin deposition, and macrophage infiltration. D, Quantitative analysis of the number of macrophage infiltration. Mean±SD, n=5 in each group. WT indicates wild type.
the activation was significantly lower in AKBI cells than WT cells (Figure 5B). Treatment of the cells with Ang II alone did not activate NF-κB (Figure 5B). IL-1β treatment upregulated the mRNA levels of MCP-1, vascular cell adhesion molecule 1, and TIMP-1; however, the increase was significantly lower in AKBI cells than in WT cells (Figure 5C). The lower expression of TIMP-1 in AKBI than in WT cells was further demonstrated at protein levels (Figure 5A). Inhibition of IkB kinase β activity by SC-514 attenuated IL-1β-induced IL-1βα phosphorylation, IkBα and IkBβ degradation, and TIMP-1 expression in the WT cells (Figure 6B).

Discussion

In the present study, we demonstrated that the replacement of IκBα gene by IκBβ cDNA in mice results in resistance to development of cardiovascular fibrosis in response to Ang II infusion, which is evidenced by significantly reduced formation of fibrotic lesions and decreased collagen deposition in cardiac ventricular tissues, and reduced aortic adventitial fibrotic area, when compared with WT mice. Because NF-κB

is specifically targeted in the AKBI mice, these data also demonstrate the principle role of NF-κB signaling in Ang II–mediated cardiovascular fibrosis. Ang II may not directly induce NF-κB activation, at least in cultured VSMCs, which is consistent with our previous observation. The in vivo tissue inflammatory response to Ang II infusion is probably a consequence of Ang II–induced hypertrophic remodeling, which may cause ECM destruction and cell death and generate “danger signals,” triggering IL-1β production and activation of NF-κB signaling cascade.

Studies by Saito and colleagues suggested that iron and iron-mediated generation of free radicals contribute to Ang II–induced upregulation of profibrotic and inflammatory genes. Hemosiderin deposition may constitute a sign of the
Ang II–induced tissue damage and trigger excessive tissue inflammatory response and fibrogenesis, because hemosiderin deposition is associated with increased macrophage infiltration, as observed in our mouse model and reported by others in rat models.15,16,19 The reduction of hemosiderin deposition and macrophage infiltration in the interstitial regions of AKBI mouse hearts suggest that replacement of IκBα with IκBβ may protect tissue from Ang II–induced damage. Like hemosiderin, macrophage infiltration, as a marker of inflammation, has been implicated in stimulating fibrogenesis in various hypertensive animal models, including spontaneously hypertensive rats, hypertensive mice, or rats induced by infusion of Ang II, as well as rats with aortic constriction.4,16,24 In addition to less macrophage infiltration, the attenuated inflammatory response in AKBI mice is further supported by the decreased expression of numerous inflammatory genes, including IL-1B, IL-6, tumor necrosis factor-α, MCP-1, and transforming growth factor-β1. The lower expression of MCP-1, a key mediator of macrophage recruitment,4 may contribute to the reduction of macrophage infiltration observed in AKBI mouse heart tissues.

Excessive and chronic inflammation may result in maladaptive ECM remodeling and play a central role in fibrogenesis. Ang II infusion enhanced both ECM degradation and formation, as indicated by enhanced mRNA levels of matrix metalloprotease 12, TIMP-1, collagen I, collagen III, fibronectin, and P4ha3, which was markedly attenuated in AKBI mice. These gene expression data are consistent with the histological findings showing increased collagen deposition in WT mice but less in AKBI mice. A dramatically increased TIMP-1 expression may greatly contribute to the increased collagen deposition and fibrosis in Ang II–infused WT mice. Increased cardiac expression of TIMP-1 has been found to correlate with cardiac fibrosis in chronically pressure-overloaded human hearts.25 Overexpression of TIMP-1 promotes the development of liver fibrosis and may also inhibit the spontaneous resolution of liver fibrosis.26,27 In addition to its regulation of matrix metalloprotease–mediated ECM degradation, TIMP-1 has additional biological functions, including regulation of cell growth, apoptosis, and angiogenesis,28 that could also be related to its role in tissue remodeling. Importantly, IL-1B can upregulate TIMP-1 expression through activation of NF-κB, as shown in our study and reported by others.29 Our data from Ang II–infused WT and AKBI mice and from IL-1B–treated WT and AKBI cells suggest that the differential regulation of NF-κB signaling by IκBα and IκBβ may differentially regulate the TIMP-1 expression and subsequently influence the ECM deposition and fibrogenesis.

Our data support the hypothesis that IκBα and IκBβ have different roles in modulating cardiovascular inflammation and fibrosis induced by Ang II infusion. It is known that IκBα and IκBβ display different kinetics of degradation and resynthesis in response to inflammatory stimuli. On IL-1B stimulation, in WT mouse VSMCs, IκBα is rapidly degraded and then resynthesized because of the presence of NF-κB binding motifs in the IκBα gene promoter, whereas IκBβ is degraded and sustained at a low level, because its synthesis is not upregulated by NF-κB. This is consistent with previous observations in rat VSMCs and other cell types.5,30,31 Although IκBα returned to baseline levels within 1 hour after stimulation, NF-κB remained active and was not suppressed by the increased IκBα, as revealed by the increased NF-κB–driven luciferase activity at a later time point (16 hours). This may result from continuous phosphorylation and degradation of IκBα and a constantly low level of IκBβ. In AKBI mouse VSMCs that do not express IκBα, IκBβ was degraded on IL-1B stimulation and then returned to basal levels within 3 hours, probably because the knock-in IκBβ cDNA that replaces the IκBα gene is controlled under the IκBα promoter and is NF-κB inducible.11,14 However, an important difference between IκBα and IκBβ is that the increased IκBβ expression significantly attenuates NF-κB activation and reduces the expression of inflammatory genes, which could be ascribed to the lower sensitivity of IκBβ than IκBα to IκB kinase-induced phosphorylation and degradation.32 Our results, together with previous findings, indicate that IκBβ does not act the same as IκBα in regulating NF-κB activation and inflammatory gene expression principally because of its lower sensitivity to IκB kinases and support the notion that a sustained reduction of IκBβ contributes to prolonged NF-κB activation.5,31,33–35 Taken together, the deletion of IκBα and
overexpression of IκBβ in mice attenuate NF-κB-responsive inflammatory and profibrotic gene expressions, hemosiderin deposition, and macrophage infiltration in response to Ang II infusion, which slows the development of cardiovascular fibrosis in the Ang II infusion–induced hypertension.

**Perspectives**

Our data suggest that upregulating IκBβ expression or blocking IκBβ degradation may prevent excessive inflammatory response and could serve as a therapeutic target in preventing fibrosis in hypertensive disease.

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**Disclosures**

None.

**References**

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Short Title: Xu et al. IκBβ Attenuates Cardiovascular Fibrosis

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Expanded Materials and Methods

Immunohistochemistry

Tissue macrophage infiltration was detected by immunohistochemistry with an antibody against Mac3 antigen. After deparaffin and rehydration, 5-µm-thick heart tissue sections were treated with 10 mmol/L citric acid buffer (pH 6.0) at 125°C for 3 minutes in a Decloaking Chamber (Biocare Medical) to recover antigenicity. Nonspecific binding was blocked with 2.5% normal goat serum for 30 minutes. To detect macrophage, the sections were incubated overnight at 4°C with rat anti-mouse Mac3 monoclonal antibody (BD Pharmingen) at a 2 μg/mL concentration. Non-immune rat IgG (Vector Laboratories) was used in parallel as a negative control. The immunoreactivity was then detected by a polymerized reporter enzyme staining system (ImmPress Reagent, Vector Laboratories) using DAB as the substrate. Hematoxyline (Vector Laboratories) was used for nuclear counterstain.

Materials

Recombinant murine IL-1β was from PeproTech. Ang II was from Sigma. For in vivo infusion experiments, Ang II was dissolved in 0.15 mol/L NaCl solution containing 1 mmol/L acetic acid. Polyclonal antibodies against IκBβ, NF-κB p65, and monoclonal antibodies against TIMP-1 were obtained from Santa Cruz Biotechnology. Polyclonal antibodies against IκBα and p-IκBα were from Cell Signaling Technology.
## Table S1. Primers for Real time PCR

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