Overexpression of cardiac I-κBα prevents endotoxin-induced myocardial dysfunction

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Overexpression of cardiac I-κBα prevents endotoxin-induced myocardial dysfunction. Am J Physiol Heart Circ Physiol 280: H962–H968, 2001.— Nuclear factor-κ B (NF-κB) is an inducible transcription factor that regulates expression of many genes, such as tumor necrosis factor-α (TNF-α), which may contribute to myocardial dysfunction. We investigated whether cardiac NF-κB activation is involved in the development of myocardial dysfunction after lipopolysaccharide (LPS) challenge. Mice were intraperitoneally injected with LPS, and the hearts were harvested and assayed for NF-κB translocation. After LPS challenge, NF-κB activation was detected within 30 min and remained for 8 h. In transgenic mice constitutively overexpressing a nondegradable form of I-κBα (I-κBαΔN) in cardiomyocytes, myocardial NF-κB translocation was prevented after LPS challenge. Myocytes isolated from these transgenics secreted significantly less TNF-α than did wild-type cardiomyocytes after LPS stimulation. When whole hearts were excised, perfused in a Langendorff preparation, and challenged with endotoxin, I-κBαΔN transgenic hearts displayed normal cardiac function, whereas profound contractile dysfunction was observed in wild-type hearts. These data indicate that myocardial NF-κB translocates within minutes after LPS administration. Inhibition of myocyte NF-κB activation by overexpression of myocyte I-κBα is sufficient to block cardiac TNF-α production and prevent cardiac dysfunction after LPS challenge.

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addition, the receptor complex may activate the Jun NH2-terminal kinase (JNK) and p38 kinase pathways, although the precise mechanisms of these pathways in TLR4-signaling are still under investigation (31, 39).

The importance of NF-κB activation in the myocardium has been emphasized in several studies. Morishita and colleagues (30) demonstrated in rats that inhibition of NF-κB activation using a decoy oligonucleotide prevented myocardial infarction after coronary artery ligation. Shames et al. (36) determined in rats that high levels of I-κBα, induced by LPS or dexamethasone, inhibited cardiac NF-κB activation, attenuated myocardial TNF-α production, and improved cardiac contractility after a second challenge. In addition to these models, NF-κB nuclear transmigration may be involved in cardiac dysfunction after hemorrhage and ischemia and during aging (15, 23, 28).

In this study, we investigated the specific role of NF-κB translocation in mouse cardiac myocytes after endotoxin challenge. Specifically, we sought to determine the kinetics of cardiac NF-κB activation in mouse hearts after LPS challenge, and whether inhibition of NF-κB activation by overexpression of nondegradable I-κBα in cardiac myocytes would inhibit cardiac TNF-α production (an NF-κB-dependent gene) and prevent myocardial dysfunction after LPS challenge.

MATERIALS AND METHODS

Animal care. All animals were used in accordance with the guidelines of the University of Texas Southwestern Medical Center Animal Care and Research Advisory Committee and in compliance with the rules governing animal use, as published by the National Institutes of Health. Female C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME).

Transgenic design. To create a nondegradable form of I-κBα (I-κBαΔN), the NH2-terminal 36 amino acids containing the phosphorylation (serine-32 and serine-36) and ubiquitination (lysine-21 and lysine-22) sites were removed, thus rendering the mutant protein resistant to degradation after stimulation (8, 34). The cDNA encoding I-κBαΔN was amplified by polymerase chain reaction (PCR), allowing for incorporation of a Flag epitope at the NH2 terminus for immunodetection of the transgenic product. The PCR product was then cloned into the HindIII site of an expression vector containing the cardiac-myocyte-specific α-myosin heavy chain (MHC) promoter (14, 18). The transgenic construct was confirmed by automatic DNA sequencing. The expression cassette containing the α-MHC promoter and I-κBαΔN were excised with EcoRI and then injected into the pronuclei of fertilized oocytes derived from C57BL/6J mice. The oocytes were reimplanted into pseudopregnant ICR mice to generate transgenic founders backcrossed to establish I-κBαΔN transgenic lines. The genotypes of the transgenic mice were screened by PCR using transgene-specific primers.

Endotoxin challenge. Mice weighing 15–20 g were either untreated or were intraperitoneally injected with 1 mg/kg of LPS (Escherichia coli, 0111:B4, Sigma; St. Louis, MO) diluted in 100 μl of saline. At the indicated time, whole hearts were harvested, frozen immediately in liquid nitrogen, and stored at −80°C.

Protein extraction. A modified procedure based on the method of Schreiber et al. (35) was used. Hearts were thawed on ice in the presence of 400 μl of hypotonic Tris buffer (10 mM Tris·HCl pH 7.8, 5 mM MgCl2, 10 mM KCl, 0.3 mM EGTA, 0.5 mM 1,4-dithiothreitol (DTT), 0.3 M sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 μg/ml of each: aprotinin, leupeptin, and pepstatin A). The tissue was then homogenized by using a Tissuemizer (Tekmar, Cincinnati, OH) under standardized conditions (2 × 10 s, 10-s pause in between). Cells were allowed to swell on ice for 15 min. NP-40 was added to a final concentration of 0.5%, and the mixture was vortexed at full speed and centrifuged at 8,000 g for 1 min. The supernatant was saved for cytosolic protein analysis. Nuclear proteins were extracted from the pellet with 100 μl of high-salt TBS (20 mM Tris·HCl pH 7.8, 5 mM MgCl2, 320 mM KCl, 0.2 mM EGTA, 0.5 mM DTT, 1 mM PMSF, and protease inhibitors) for 15 min on ice, and centrifuged at 13,500 g for 15 min. Protein concentrations were determined using a protein assay (Bio-Rad; Hercules, CA). Extracts were stored at −80°C.

Electrophoretic mobility shift assay. Double-stranded oligonucleotide corresponding to the consensus NF-κB binding site of the murine α-light-chain enhancer (5′-AGTTGAGGG-GACTTTCGAGGC-3′) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Oligonucleotide (3.5 pmol), T4 polynucleotide kinase (5 units) in 1× kinase buffer (Promega; Madison, WI), and [γ-32P]ATP (30 μCi) (DuPont-New England Nuclear; Boston, MA) were incubated at 37°C for 60 min. Labeled probe was separated from unbound ATP using ProbeQuant G-50 micro columns from Amersham Pharmacia Biotech and stored at −20°C. Nuclear proteins (10 μg) were incubated with 500,000 counts/min of probe in the presence of salmon sperm DNA (2 μg) in 1× gel shift buffer (in mM: 20 HEPES, 50 KCl, 1 DTT, 1 EDTA, pH 7.6, and 5% glycerol) for 30 min at room temperature. The mixtures were then separated on a nondenaturing 8% polyacrylamide gel in 0.5× TBE (25 mM Tris·HCl, 25 mM borate acid, 0.5 mM EDTA). The gel was dried and exposed to X-ray film (Kodak, BioMax). Competition analyses were performed by including a 30-M excess of unlabelled double-stranded oligonucleotide in the binding reaction. Nonspecific competitor DNA contained a substance P (SP-1) binding element. Super shift experiments were performed by adding 4 μg of mouse mononclonal antibody (Santa Cruz Biotechnology; Santa Cruz, CA) to the binding reaction.

Western blotting. Cytosolic protein (50 μg) was separated on a 12% sodium dodecyl sulfate-polyacrylamide gel, then transferred onto a polyvinylidene fluoride membrane (Immobilon-P, Millipore; Bedford, MA). Membranes were washed in 1× Tris buffered saline (TBS) (20 mM Tris·HCl, 140 mM NaCl, pH 7.5), then blocked for 1 h at room temperature (1× TBS, 0.1% Tween 20, 5% nonfat dry milk), then incubated with primary antibody (rabbit polyclonal I-κBα antibody, Santa Cruz Biotechnology) or mouse mononclonal Flag antibody (Sigma) 1:1,000 in dilution buffer (1× TBS, 0.1% Tween 20, 5% nonfat dry milk) overnight at 4°C. Membranes were washed four times in 1× TBS, 0.1% Tween 20, followed by incubation with peroxidase-labeled anti-rabbit or anti-mouse IgG (1:8,000 in dilution buffer, Santa Cruz) for 1 h at room temperature. Membranes were washed four times at room temperature before the antigen-antibody complexes were detected by Super Signal (Pierce; Rockford, IL).

Primary cardiomyocyte isolation. Cardiomyocytes were harvested from untreated mouse hearts (n = 3 per group) using standard enzymatic and cell culture techniques as described earlier (42). Freshly isolated cells were plated at a density of 15,000 cells/ml and stimulated with 0, 10, 25, or 50 μg/ml of LPS for 18 h. TNF-α was measured in the supernatant by Quantikine M ELISA (R&D Systems; Minneapolis, MN).
**Evaluation of cardiac function.** Mouse hearts were hung on a Langendorff perfusion apparatus as previously described (12). Hearts (n = 5 per group) were harvested and perfused with Krebs-Henseleit buffer (in mM) composed of 118 NaCl, 4.7 KCl, 25 NaHCO₃, 1.2 MgSO₄, 1.2 KH₂PO₄, and 11 glucose with a constant flow rate of 1.5 ml/min at 38°C. Intraventricular pressure was measured with a saline-filled polyethylene tube threaded into the left ventricular chamber. Left ventricular pressure was measured with a Statham P23 ID pressure transducer attached to the cannula through the back of the heart. Left ventricular change in pressure over time (dP/dt) values were obtained using an electronic differentiator (model 7P20C, Grass Instruments; Quincy, MA). Because the heart rate varied, hearts were paced as required through an electrode attached to the right atrium (4.8–5.0 amps for 1 ms duration, Grass Stimulator). After 25 min of stabilization and recording baseline parameters, 250 μg/ml of LPS was added to the perfusate buffer for 20 min and cardiac function was measured. Hearts were then frozen until protein extraction.

**Statistical analysis.** Electrophoretic mobility shift assays and Western blots are representative results of two to three independent experiments. Values shown in graphs are given as means ± SE. The means of groups were compared using Student’s t-test; P < 0.05 was statistically significant.

**RESULTS**

**Transgenic animals.** Transgenic I-κBαΔN mice displayed no abnormalities by clinical, anatomic, or histological examination. Furthermore, these transgenics have normal life expectancies under routine laboratory conditions. The exclusive expression of I-κBαΔN in the heart was verified by Western blotting using an I-κBα specific (Fig. 1A), and a Flag specific (Fig. 1B) antibody. Endogenous I-κBα was expressed in all tissues examined, wild-type and transgenic. I-κBαΔN expression, on the other hand, was detected only in transgenic hearts, but not in other transgenic or wild-type tissues, including lung, liver, kidney, and spleen. To further confirm cardiac-specific I-κBαΔN expression, or NF-κB inhibition respectively, we challenged wild-type and transgenic mice with LPS and tested various tissues for NF-κB activation. Figure 2 demonstrates that in LPS-challenged wild-type mice, NF-κB was acti-
NF-κB activation in all tissues tested. In LPS-challenged IκBαDN transgenic mice, NF-κB did not translocate in the heart but did so in all other tissues tested.

**Kinetics of myocardial NF-κB activation.** To investigate the kinetics of cardiac NF-κB activation after LPS challenge, C57BL/6J mice were injected intraperitoneally with 1 mg/kg of LPS. At the indicated time, hearts were excised and proteins were isolated. NF-κB activation was measured in nuclear protein extracts by gel shift assays. As shown in Fig. 3A, 1 mg/kg of LPS-induced cardiac NF-κB nuclear migration within 30 min, continuing for 8 h. Super shift experiments identified p65 and p50 as the major subunits of the NF-κB complex induced by LPS, because antibodies against both proteins super shifted the NF-κB band, whereas antibodies against p52, c-Rel, and RelB did not (Fig. 3B). The addition of unlabeled NF-κB oligonucleotide competed with labeled NF-κB probe because it diminished the NF-κB-specific band, whereas addition of nonspecific, unlabeled SP-1 oligonucleotide did not influence band intensity (Fig. 3B).

**Overexpression of cardiac IκBαDN blocks myocardial NF-κB translocation.** To determine whether overexpression of a nonphosphorylatable IκBα in cardiac myocytes would inhibit NF-κB translocation after LPS stimulation, hearts from untreated wild-type and transgenic mice were hung on a Langendorff apparatus and were perfused with either saline or 250 μg/ml of LPS. Nuclear proteins were then isolated and tested for NF-κB activation. As shown in Fig. 4A, NF-κB nuclear translocation occurred in LPS-perfused wild-type hearts but not in LPS-perfused IκBαDN transgenic or saline-treated hearts. In IκBαDN transgenic animals, cardiac NF-κB activation was also blocked after intraperitoneal LPS challenge (Fig. 4B).

Cardiac TNF-α synthesis is prevented in IκBαDN-overexpressing mice. Primary isolated cardiomyocytes were stimulated with various amounts of LPS for 18 h,
and TNF-α was measured in the supernatant by ELISA. Only cardiomyocytes showing viability >75–80% were used for experiments; and the maximal decrease in cell viability during LPS incubation did not exceed 5–10%. Figure 5 demonstrates that cardiomyocytes from I-κBαΔN overexpressing mice synthesized significantly less TNF-α in response to LPS compared with WT control littermates. Relatively high LPS concentrations were required to stimulate cytokine production, consistent with previous studies from our laboratory (16).

Cardiac dysfunction is blocked in I-κBαΔN overexpressing mice. To investigate whether cardiac dysfunction differed between wild-type and I-κBαΔN transgenic mice after LPS challenge, hearts were isolated from untreated mice and hung on a Langendorff perfusion apparatus. After stabilization, hearts were perfused with 250 μg/ml of LPS and cardiac function was assayed. At this LPS concentration, wild-type mouse hearts showed significant cardiac dysfunction within a short period of time (20 min) as determined in a LPS dose-response curve (data not shown). As indicated in Fig. 6, wild-type hearts showed significant cardiac depression after LPS perfusion. In contrast, cardiac function of LPS-perfused transgenic hearts was nearly identical to nonchallenged transgenic hearts.

**DISCUSSION**

The production of cytokines, adhesion molecules, and nitric oxide by cardiomyocytes have been implicated in the progression of congestive heart failure, myocarditis, and septic cardiomyopathy. It is also known that NF-κB regulates the transcription of these genes and other inflammatory proteins. Therefore, we assessed cardiac NF-κB activation after LPS challenge and determined its impact on myocardial function and
the expression of TNF-α, a cytokine that mediates cardiac dysfunction in several experimental and clinical systems. We used a transgenic mouse model in which myocardial NF-κB activation was blocked through selective overexpression of nondegradable I-κBα by cardiomyocytes. Our results indicate that inhibition of cardiac NF-κB nuclear translocation by overexpression of nondegradable I-κBα prevents cardiac TNF-α production and blocks cardiac failure after LPS stimulation.

The activation of NF-κB by various stimuli has been investigated in a number of different cell systems and tissues (1, 2). However, only a few studies have examined NF-κB activation in primary cardiomyocytes or in whole hearts using in vivo animal models (15, 23, 28, 30, 36). Taken together, the presence of NF-κB in the nucleus suggests enhanced transcription of many NF-κB-sensitive genes. It remains to be determined which genes NF-κB activates in the heart, but evidence suggests that TNF-α, nitric oxide synthase, IL-6, intercellular adhesion molecule-1, and other cytokines may all be regulated by NF-κB (9, 19, 20, 24, 43). All of these genes code for proteins that may be involved in the depression of cardiac function. In our mouse model, a low dose of LPS induces cardiac NF-κB translocation within minutes of challenge for up to 8 h. Of note is a high, almost lethal, dose of 6 mg/kg of LPS-induced NF-κB activation for up to 36 h (unpublished observation).

The strategy of NF-κB inhibition by overexpressing a nondegradable form of I-κBα in vitro has previously been demonstrated by several groups (5, 6). Böhrer et al. (4) injected an I-κBα expression plasmid into mice 7 days before the administration of LPS, and thereby reduced LPS-mediated NF-κB activation in PBMCs and improved survival. To investigate the role of I-κBα specifically in the heart, we developed a transgenic mouse line in which expression of a murine nonphosphorylatable I-κBα coding sequence (I-κBαΔN) was driven by the murine α-MHC promoter. This promoter has been shown to be steadily activated only in cardiomyocytes (14, 18) and therefore, nondegradable I-κBα is constitutively and exclusively expressed in cardiomyocytes. Our data demonstrate that after LPS challenge, I-κBαΔN transgenic animals do not show cardiac NF-κB activation, whereas wild-type littermates respond with substantial NF-κB nuclear migration. We confirmed that locally overexpressed levels of I-κBα were potent inhibitors of LPS-mediated myocardial NF-κB translocation in vivo. Perhaps more importantly, the complete inhibition of NF-κB translocation in the heart was accomplished by targeting only myocytes, and not other cardiac cell types. At least quantitatively, cardiac NF-κB activity resides predominantly in myocytes under these conditions.

TNF-α has been detected in several human cardiomyopathy-related conditions, including congestive heart failure and septic cardiomyopathy (22, 25), and is thought to be one of a number of NF-κB-dependent genes involved in septic cardiomyopathy. At least in part, the origin of TNF-α is thought to be cardiomyocytes themselves (13, 19). In the present study, we investigated the LPS-induced TNF-α response of cardiomyocytes in the presence of NF-κB inhibition. Using freshly isolated primary cardiomyocytes, we demonstrated that local inhibition of cardiac NF-κB activation blocked cardiac TNF-α synthesis after LPS stimulation. In addition, after LPS perfusion, wild-type mouse hearts were significantly impaired in their function, consistent with previous data in feline and rat hearts (19, 36). In our transgenic mouse model, overexpression of I-κBαΔN totally blocked LPS-induced cardiac dysfunction despite a high dose of LPS perfusion. Taken together, these observations strongly support the paradigm that LPS induces NF-κB nuclear translocation, which in turn upregulates TNF-α synthesis leading to cardiac dysfunction. However, other NF-κB-dependent pathways, distinct from TNF-α, are also inhibited in our model. In addition, TNF-α (and other cytokines) signal through NF-κB-sensitive mechanisms, and inhibition of these pathways may also contribute to the improvement in cardiac function observed in our model.

In conclusion, myocardial NF-κB translocates within minutes after LPS administration. Inhibition of cardiomyocyte NF-κB activation by overexpression of myocyte I-κBα is sufficient to block cardiac TNF-α production and to prevent cardiac dysfunction after LPS challenge. Thus LPS by itself does not directly lead to myocardial failure. Rather, by activating the NF-κB signaling pathway in cardiomyocytes, LPS induces transcription of several, yet-to-be determined proteins including TNF-α, which then may initiate cardiac dysfunction. This indicates further that inhibition of cardiomyocyte NF-κB activation may be a useful clinical strategy in cardiac specific conditions, such as septic cardiomyopathy or myocarditis.

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