Cleavage of IκBα by calpain induces myocardial NF-κB activation, TNF-α expression, and cardiac dysfunction in septic mice

Xiaoping Li,1,2 Rong Luo,3 Ruizhen Chen,4 Li Song,5 Shu Zhang,1 Wei Hua,1 and Haozhu Chen4

1Cardiac Arrhythmia Center, State Key Laboratory of Cardiovascular Disease, Fuwai Hospital, National Center for Cardiovascular Diseases, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, People’s Republic of China; 2Department of Cardiology, Sichuan Academy of Medical Sciences and Sichuan Provincial People’s Hospital, Chengdu, Sichuan, People’s Republic of China; 3Medical Scientific Research Center of Guangxi Medical University, Nanning, Guangxi, People’s Republic of China; 4Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, Shanghai, People’s Republic of China; and 5Central Laboratory, Cardiovascular Institute and Fuwai Hospital, Chinese Academy of Medical Sciences, Peking Union Medical College, Beijing, People’s Republic of China

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Li X, Luo R, Chen R, Song L, Zhang S, Hua W, Chen H. Cleavage of IκBα by calpain induces myocardial NF-κB activation, TNF-α expression, and cardiac dysfunction in septic mice. Am J Physiol Heart Circ Physiol 306: H833–H843, 2014. First published January 17, 2014; doi:10.1152/ajpheart.00893.2012.—Recent studies in septic models have shown that myocardial calpain activity and TNF-α expression increase during sepsis and that inhibition of calpain activation downregulates myocardial TNF-α expression and improves cardiac dysfunction. However, the mechanism underlying this pathological process is unclear. Thus, in the present study, we aimed to explore whether IκBα/NF-κB signaling linked myocardial calpain activity and TNF-α expression in septic mice. Adult male mice were injected with LPS (4 mg/kg ip) to induce sepsis. Myocardial calpain activity, IκBα/NF-κB signaling activity, and TNF-α expression were assessed, and myocardial function was evaluated using the Langendorff system. In septic mice, myocardial calpain activity and TNF-α expression were increased, and IκBα protein was degraded. Furthermore, NF-κB was activated, as indicated by increased NF-κB p65 phosphorylation, cleavage of p105 into p50, and its nuclear translocation. Administration of the calpain inhibitors calpain inhibitor II and PD-150606 prevented the LPS-induced degradation of myocardial IκBα, NF-κB activation, and TNF-α expression and ultimately improved myocardial function. In calpastatin transgenic mice, an endogenous calpain inhibitor and cultured neonatal mouse cardiomyocytes overexpressing calpastatin also inhibited calpain activity. IκBα protein degradation, and NF-κB activation after LPS treatment. In conclusion, myocardial calpain activity was increased in septic mice. Calpain induced myocardial NF-κB activation, TNF-α expression, and myocardial dysfunction in septic mice through IκBα protein cleavage. Sepsis; calpain; tumor necrosis factor-α; IκBα/nuclear factor-κB; myocardial dysfunction

APPROXIMATELY 700,000 cases of sepsis occur each year in the United States, and this condition has an overall mortality rate of ~30% (1). Myocardial dysfunction is an early and fatal manifestation of sepsis in humans and animals (21, 25, 26), but the physiological basis of this effect and the molecular mediators that trigger myocardial dysfunction are not yet fully understood.

Calpains are a family of Ca2+-dependent intracellular cysteine proteases. Fourteen members of the calpain family have been identified to date (2). An association between calpain and myocardial dysfunction during sepsis has been reported; calpain inhibitors attenuate the circulatory failure caused by endotoxins (32) and prevented lipopolysaccharide (LPS)-induced myocardial dysfunction in rats (36). We (15) recently observed that myocardial calpain activity and TNF-α mRNA expression increased in septic mice and that inhibition of calpain activation downregulated myocardial TNF-α mRNA expression and improved cardiac dysfunction. However, there is a paucity of data describing the underlying mechanism by which calpain induces myocardial TNF-α mRNA expression in septic mice.

The expression of TNF-α mRNA might consider to be closely related to that of NF-κB. NF-κB is usually present in the cytosol in an inactive state bound to inhibitory proteins, termed “inhibitors of κB” (IκB). One common type is IκBα, which binds NF-κB in the cytoplasm and prevents its translocation to the nucleus by masking its nuclear localization signal (4). In vitro studies (17, 30) have demonstrated that both IκBα and NF-κB p65, a subunit of NF-κB, are substrates of calpain. In addition, studies (13, 35) in nonseptic models have shown that inhibition of IκBα degradation by calpain inhibitors prevented the translocation of NF-κB from the cytosol to the nucleus and inhibited the expression of many NF-κB-dependent genes, including TNF-α.

Therefore, in the present study, we proposed that IκBα/NF-κB signaling was a potential link between myocardial calpain activation and TNF-α expression. This study focused on the molecular mechanisms underlying myocardial TNF-α expression induced by calpain activation in septic mice.

MATERIALS AND METHODS

Animal preparation. Adult, pathogen-free, wild-type C57BL/6 mice (male, 6–8 wk old; 25–30 g) were used. Animals were housed under a 12:12-h light-dark cycle with food and water available ad libitum. All experimental procedures were approved by the Institutional Animal Ethics Committee of Peking Union Medical College. Transgenic mice with overexpression of calpastatin (CAST-Tg mice) were kindly provided by Dr Laurent Baud (Institut National de la Sante’ et de la Recherche Me’dicale, Paris, France) (27) through the European Mouse Mutant Archive. A total of 180 mice were divided into 6 different groups (n = 30 mice/group). Control mice (sham group) were injected intraperitoneally with 100 μl PBS, and LPS-treated mice were injected intraperitoneally with 4 mg/kg LPS from Escherichia coli serotype 055:B5 (Sigma, St. Louis, MO) dissolved in 100 μl PBS. For calpain inhibitor
Sha (10 mg/kg ip) or PD-150606 (3 mg/kg ip) treatment, LPS-treated mice were injected intraperitoneally with calpain inhibitor III or PD-150606 dissolved in 80 μl DMSO 30 min before LPS injection. Mice treated with calpain inhibitor III or PD-150606 alone were only intraperitoneally injected with calpain inhibitor III or PD-150606. All mice were euthanized by injecting of an overdose of phenobarbital (200 mg/kg ip), and the heart was removed 4 h after treatment in biological and physiological experiments. In addition, time-course experiments were performed at 0, 1, 2, 4, and 6 h after LPS injection with five mice at each time point.

Calpain activity assay. Calpain activity was measured using the fluorescence substrate N-succinyl-Leu-Leu-Val-Tyr-7-amino-4-

Fig. 1. Myocardial calpain activity and TNF-α mRNA and protein expression in septic myocardial tissues. A: mice were treated with lipopolysaccharide (LPS; 4 mg/kg ip) for 4 h, inducing an increase in myocardial calpain activity. The addition of calpain inhibitor III (CI; 10 mg/kg ip) or PD-150606 (PD; 3 mg/kg ip) prevented the increase in calpain activity. B: time course of myocardial TNF-α protein expression after LPS stimulation. TNF-α and GAPDH protein expression were determined by Western blot at 0, 1, 2, 4, and 6 h after LPS (4 mg/kg) treatment. Top, representative Western blot showing the increase in myocardial TNF-α expression; bottom, TNF-α-to-GAPDH protein ratio. C: mice were treated with LPS (4 mg/kg ip) for 4 h. Myocardial TNF-α mRNA expression increased in septic mice. The addition of calpain inhibitors CI and PD blocked TNF-α mRNA expression in septic mice. D: effects of calpain inhibitors on LPS-induced TNF-α protein expression. Mice were injected with LPS (4 mg/kg) in the presence or absence of calpain inhibitors for 4 h. Protein levels were attenuated by calpain inhibitors. Top, representative Western blot; bottom, TNF-α-to-GAPDH protein ratio. Data are means ± SE; n = 5. *P < 0.05 vs. sham or 0 h; #P < 0.05 vs. LPS.

Fig. 2. Protein levels of phosphorylated (p-)p65 subunit of NF-κB at Ser536 and IκBα in septic myocardial tissues. A: time course of NF-κB p-p65 protein expression in septic mice. Top, representative Western blot; bottom, quantitation of these data, demonstrating that LPS increased NF-κB p-p65 protein expression and reached a maximum at ∼4 h. B: representative Western blot (top) and quantitation (bottom) demonstrating the significant increase in NF-κB p-p65 protein after LPS treatment. Both calpain inhibitors, CI and PD, prevented the increase of NF-κB p65 protein phosphorylation in septic mice. C: time course of IκBα protein expression in septic mice. Top, representative Western blot; bottom, quantification of these data, demonstrating that LPS decreased IκBα protein expression, with the greatest decrease at ∼2–4 h. D: representative Western blot (top) and quantitation (bottom) showing the significant degradation of IκBα protein after LPS treatment. Both calpain inhibitors, CI (10 mg/kg ip) and PD (3 mg/kg ip), prevented the degradation of IκBα protein in septic mice. E: representative Western blot (top) and quantitation (bottom) showing no significant changes of Iκκ kinase (IKK)-α, IKK-β, and p-IκBα/β protein levels after LPS treatment. F: representative Western blot (top) and quantitation (bottom) showing no significant changes in p-IκBα protein after LPS treatment. The experiments were representative of four repeated experiments. *P < 0.05 vs. sham or 0 h; #P < 0.05 vs. LPS.
methylcoumarin (AMC; Cedarlane Laboratories, Burlington, NC), as previously described (11). This assay measures the fluorescence intensity of AMC cleaved from a peptide substrate. The fluorescence intensity of cleaved AMC was quantified with a multilabel reader (excitation: 360 nm; emission: 460 nm), and calpain activity was determined as the difference between Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent fluorescence. All experiments were performed in duplicate.

**Western blot analysis.** Protein in each sample was subjected to SDS-PAGE with a 10% gel followed by electrotransfer onto membranes. Expression levels of 1κBα/phosphorylated (p-1κBα (Ser\(^{32}\)), NF-κB p65, NF-κB p-p65, TNF-α, 1κ kinase (IKK-α)/β, p-IKK-α/β, and GAPDH protein were determined by probing the blots with specific antibodies (1:1,000, Cell Signaling, Danvers, MA) or p105/p50 antibody (1:1,000, Epitomics-Abcam) followed by enhanced chemiluminescence detection.

**Analysis of TNF-α mRNA by real-time RT-PCR.** Total RNA was extracted from heart tissue using TRIzol Reagent (GIBCO-BRL) following the manufacturer’s instructions. Real-time RT-PCR was performed to amplify mouse TNF-α and GAPDH mRNA. The primers for TNF-α and GAPDH were as follows: TNF-α, 5′-CGGAGGTGGTTGTACCTTGTC-3′ and 5′-GGGCTGGTGAGAGAATGGAT-3′; and GAPDH, 5′-AAAGGGAATCCTGGGCTACA-3′ and 5′-CAGTGT-TGGGCGTGGAGTTG-3′.

**Assay of NF-κB activity: electrophoretic mobility shift assay.** NF-κB binding to DNA was analyzed by an electrophoretic mobility shift assay (EMSA) as previously described (28) with minor modifications. Nuclear extracts (5 μg) from mouse myocardial tissues were incubated with 3²P-labeled NF-κB consensus oligonucleotide (NF-κB: 5′-AGTTAGGGACTTTCCCAGGC-3′) in the presence or absence of a 50-fold excess of cold oligonucleotide for 30 min, and the reaction mixture was then loaded onto a native 5% polyacrylamide gel and electrophoresed at 250 V in 0.5× Tris-borate-EDTA buffer. The dried gels were exposed to X-ray film (Kodak) for 16 h in cassettes with intensifying screens.

For competition experiments, a 50-fold molar excess of double-stranded unlabeled competitor oligonucleotides containing wild-type or mutant NF-κB-binding sites were coincubated with radiolabeled kB probes and nuclear extracts. For supershift assays, specific antibodies (2 μg) against p65 and p50 (Santa Cruz Biotechnology, Santa Cruz, CA) were preincubated with nuclear extracts for 15 min at room temperature before the addition of 3²P-labeled kB probes.

**Histological preparation and immunohistochemistry.** Mouse heart tissue was fixed in 4% paraformaldehyde in PBS for 24 h and subjected to standard histological processing for paraffin-embedded sections. Paraffin sections (5 μm thick) were then cut for immunohistochemistry and subsequently incubated with rabbit anti-mouse NF-κB p65 antibody (1:100, sc-109, Santa Cruz Biotechnology) and rabbit anti-mouse NF-κB p-p65 antibody (1:200, Cell Signaling) at 4°C overnight. NF-κB p65 protein was detected in the cell cytoplasm and/or in the nucleus. The expression of NF-κB p65 and p-p65 in heart tissue was visualized using routine immunoperoxidase techniques. Sections were counterstained with hematoxylin, dehydrated, and mounted using routine methods. Goat polyclonal secondary antibody (IgG) was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

**Isolated and perfused heart preparation.** Myocardial contractile function was studied using a modified Langendorff isolated heart preparation, as previously described (22). Briefly, after heparinization and ether anesthesia, mouse hearts were rapidly excised and placed into ice-cold Krebs-Henseleit (KH) buffer solution. Hearts were then mounted onto a Langendorff heart perfusion apparatus and perfused in a retrograde fashion via the aorta at a constant flow rate of 10 ml/min with aerated (95% O\(_2\)-5% CO\(_2\)) KH buffer at 37°C. Hearts were allowed to equilibrate for 30 min. Heart rate, heart work, and the maximal positive and minimal negative first derivatives of left ventricular pressure were analyzed using PowerLab software.

**Neonatal mouse cardiomyocyte culture.** Cardiomyocytes cultures were prepared as previously described with modifications (16). Briefly, ventricular myocardial tissues from neonatal mice born within 24 h were minced in a nominally Ca\(^{2+}\)- and Mg\(^{2+}\)-free Hanks balance solution. Cardiomyocytes were dispersed by 10 min of incubation with 22.5 μg/ml liberase (Roche) in D-Hanks solution at 37°C with gentle agitation. Cells were collected by centrifugation at 200 g for 5 min and resuspended in 10% FBS-containing medium 199 (Sigma). Removal of noncardiomyocytes was achieved by 90 min of preplating, after which cardiomyocytes were collected by rinsing and seeded in 1% gelatin precoated 24-well plates. Cells were incubated in a humidified atmosphere at 37°C with 5% CO\(_2\). After 48 h of cell culture, cardiomyocytes were treated with the different drugs either alone or in combination.

**Adenoviral infection of cultured neonatal cardiomyocytes.** Cardiomyocytes were infected with adenoviral vectors containing a rat calpastatin gene (Ad-CAST, ABM) or β-galactosidase (Ad-gal, Vector Bios) as a control at a multiplicity of infection of 10 plaque-forming units/cell. Adenovirus-mediated gene transfer was implemented as previously described (16). All experiments were performed after 24 h of adenoviral infection.

**Statistical analysis.** All data are presented as means ± SE. Differences between two groups were compared by an unpaired Student’s t-test. One-way ANOVA followed by the Student-Newman-Keuls test was used for multigroup comparisons. P values of <0.05 were considered statistically significant.

**RESULTS**

**Myocardial calpain activity was increased in septic mice.** LPS associated with gram-negative bacteria is recognized as a causative agent in myocardial depression during sepsis. In the present study, mice were injected intraperitoneally with LPS (4 mg/kg) to establish a model of sepsis. Calpain inhibitor III (10 mg/kg ip) or PD-150606 (3 mg/kg ip) was injected 30 min before LPS injection. At 4 h after LPS injection, mouse heart tissues were harvested for analysis. Myocardial calpain activity was increased in septic mice compared with control mice, as in our previous study (15). Both calpain inhibitor III and PD-150606 significantly inhibited the increase of myocardial calpain activity in septic mice (Fig. 1A). Neither calpain inhibitor III nor PD-150606 had an effect on myocardial calpain activity when administered alone (data not shown).

**Inhibition of calpain blocked myocardial TNF-α expression.** Consistent with our previous study (15), the expression of myocardial TNF-α mRNA levels was also increased significantly in septic mice. In the present study, we further detected myocardial TNF-α protein expression in septic mice. Western blot analyses showed that TNF-α protein levels increased at 1 h and peaked at 4 h in septic mice (Fig. 1B). Therefore, the 4-h time point was chosen to assess the role of calpain in TNF-α protein expression in subsequent experiments. The administration of calpain inhibitor III or PD-150606 did not have any evident effect on basal levels of TNF-α expression (data not shown) but significantly decreased LPS-induced myocardial TNF-α mRNA and protein expression (Fig. 1, C and D). Thus, calpain might play a role in the regulation of myocardial TNF-α expression in septic mice.

**Calpain inhibitors prevent LPS-induced myocardial NF-κB activation.** Because the expression of TNF-α is usually associated with NF-κB activation, we explored whether the expression of myocardial TNF-α expression was induced by NF-κB activation in septic mice. Phosphorylation of NF-κB p65 at Ser\(^{32}\) served as an indirect indicator of NF-κB activation,
which markedly enhances the transactivation potential of p65 (18). Western blot analysis revealed that LPS treatment did not affect total p65 protein expression but significantly increased p65 phosphorylation at Ser536 in septic mice. Therefore, the 4-h time point was also chosen to assess NF-κB activation in the following experiments. Pretreatment with either calpain inhibitor III (10 mg/kg) or PD-150606 (3 mg/kg) significantly decreased NF-κB p65 phosphorylation (Fig. 2B), suggesting that myocardial calpain participated in NF-κB activation in septic mice.

To verify the activity of translocated NF-κB, the DNA-binding activity of nuclear proteins was tested by EMSA. In septic mice, NF-κB DNA-binding activity increased at 1 h and peaked at 4–6 h after LPS treatment (4 mg/kg; Fig. 2A). Western blot analysis revealed that LPS treatment did not affect total p65 protein expression but significantly increased p-p65 protein at 1 h and peaked at 4–6 h after LPS treatment (4 mg/kg; Fig. 2B). p-p65 protein increased at 1 h in septic myocardial tissues was performed using a p65-specific antibody to evaluate NF-κB activation in septic mice.

A: time course of NF-κB nuclear translocation demonstrated by EMSA in septic mice. Mice were treated with LPS (4 mg/kg ip) for 0, 1, 2, 4, or 6 h. Top, representative NF-κB nuclear translocation; bottom, quantification, showing that LPS increased NF-κB nuclear translocation, which reached a maximum at ~4 h. Lane 1, positive control; lane 2, negative control; lanes 3 and 4, 0 h; lanes 5 and 6, 1 h; lanes 7 and 8, 2 h; lanes 9 and 10, 4 h; lanes 11 and 12, 6 h. B: LPS treatment for 4 h induced NF-κB nuclear translocation in mice. Top, representative NF-κB nuclear translocation; bottom, quantitative data demonstrating that CI (10 mg/kg ip) and PD (3 mg/kg ip) prevented the nuclear translocation of NF-κB in septic mice. Lane 1, positive control; lane 2, negative control; lanes 3 and 4, sham; lanes 5 and 6, LPS; lanes 7 and 8, LPS + CI; lanes 9 and 10, LPS + PD; lanes 11 and 12, CI; lanes 13 and 14, PD. C: transgenic mice with overexpression of calpastatin (CAST-Tg mice) treated with LPS for 4 h showed reduced NF-κB nuclear translocation. Top, representative NF-κB nuclear translocation; bottom, quantitative data demonstrating that CAST-Tg mice had reduced nuclear translocation of NF-κB during sepsis. Lane 1 and 2, black background; lanes 3 and 4, wild-type (WT) mice; lanes 5 and 6, CAST-Tg mice; lanes 7 and 8, WT mice + LPS; lanes 9 and 10, CAST-Tg mice + LPS; lane 11, competitor lane; lane 12, p65 supershift lane; lane 13, p50 supershift lane; lane 14, positive control; lane 15, negative control. Results were representative of two repeated experiments. *P < 0.05 vs. sham; #P < 0.05 vs. LPS.
Upon LPS stimulation for 2 h, subsets of cardiomyocytes were positive for p65 in the cell cytoplasm and/or nucleus. Cotreatment with either calpain inhibitor or PD-150606 for 30 min inhibited LPS-induced p65 staining in the myocardium (Fig. 4B). These results further confirmed that cardiomyocytes were the major source of p65 production in the myocardium and that blockade of calpain activation inhibited myocardial p65 expression in response to LPS.

Immunohistochemical staining also demonstrated an increase in the number of NF-κB p-p65-positive cardiomyocytes and myocardial p-p65 expression in LPS-treated mice, which peaked at ~2–4 h after LPS administration (Fig. 4C). Inhibition of myocardial calpain activity by either calpain inhibitor III or PD-150606 attenuated LPS-increased myocardial NF-κB p-p65 expression in septic mice (Fig. 4D). These data further demonstrated that NF-κB activation is dependent on calpain activity during LPS stimulation.

Effects of calpain inhibitors on myocardial dysfunction in septic mice. As we and others have previously reported (15, 32, 36), myocardial function, as represented by the maximal positive and minimal negative first derivatives of left ventricular pressure, was decreased in septic mice. The administration of calpain inhibitor III or PD-150606 markedly improved the myocardial dysfunction in septic mice (Fig. 5). These data suggested that myocardial calpain might be involved, at least in part, in the myocardial dysfunction in septic mice.
In vivo effects of calpastatin overexpression on IkBα protein degradation and NF-κB activity in endotoxemic mice. Calpain inhibitor III and PD-150606 are relatively specific inhibitors of calpain. Similarly, calpastatin is a specific inhibitor of calpain that does not inhibit the activities of any other proteases tested (9, 29). Thus, the calpastatin overexpression in vivo model is an effective method for globally inhibition of cellular calpain activity. CAST-Tg and wild-type mice (male, aged 2–3 mo) were administered PBS or LPS (4 mg/kg ip) for 4 h, and heart tissues were collected and assayed. The p50 subunit of NF-κB is from the limited cleavage of p105 precursor protein (23, 31). Therefore, in addition to p-p65, we also measured p105 and p50 protein levels to detect NF-κB activation. In agreement with a previous report (14) that showed that calpastatin was upregulated in CAST-Tg hearts, transgenic calpastatin was overexpressed in CAST-Tg hearts (Fig. 6A), further confirming the expression of exogenous calpastatin in CAST-Tg hearts. LPS significantly increased IkBα protein degradation and NF-κB activation in both CAST-Tg and wild-type hearts. However, the levels of IkBα protein degradation and NF-κB activation (NF-κB p-p65 and cleavage of p105 into p50) were significantly reduced in CAST-Tg mice compared with wild-type mice during LPS stimulation (Fig. 6, B–D). These results suggest that the in vivo overexpression of calpastatin also inhibits calpain activation, IkBα protein degradation, and NF-κB activation in hearts during LPS stimulation.

To assay NF-κB translocated activity in CAST-Tg mice, EMSA was performed. NF-κB-binding activity was prevented in CAST-Tg mice during LPS stimulation, whereas both p65- and p50-associated NF-κB activities were activated in septic mice. However, the results of a supershift assay further showed that the levels of the p65 subunit of NF-κB were greater than that of the p50 subunit during the course (Fig. 3C).

Role of calpastatin in myocardial NF-κB activation and TNF-α expression. To investigate the role of calpastatin in myocardial NF-κB activation and TNF-α expression in an in vitro model, we overexpressed calpastatin in cardiomyocytes using Ad-CAST. Cultured neonatal mouse cardiomyocytes were infected with Ad-CAST or Ad-gal as a control for 24 h and were then exposed to LPS (1 μg/ml) for 4 h. The effect of Ad-CAST infection on calpastatin upregulation in cardiomyocytes was verified in our recent study (16). Calpain activity and TNF-α mRNA were not altered by calpastatin overexpression alone in cardiomyocytes (data not shown). In LPS-stimulated cardiomyocytes, overexpression of calpastatin significantly inhibited myocardial calpain activity (data not shown), IkBα protein degradation, NF-κB activation (NF-κB p-p65), and TNF-α mRNA expression (Fig. 7). Thus, calpastatin overexpression induces calpain inhibition, which, in turn, prevents NF-κB activation and TNF-α expression in LPS-stimulated cardiomyocytes.

DISCUSSION

In the present study, we observed that myocardial calpain activity increased during sepsis and that the inhibition of calpain by calpain inhibitor III and PD-150606 or transgenic mice with overexpression of calpastatin prevented LPS-induced myocardial NF-κB signaling activation, inhibited myocardial TNF-α expression, and improved myocardial dysfunction in septic mice. The mechanism underlying this phenomenon involves calpain activation-induced IkBα degradation, which, in turn, prevented NF-κB activation and myocardial TNF-α expression in septic mice.

Previous studies have reported that myocardial calpain activity is increased in septic mice (5, 15, 36) and that μ- and m-calpain mRNA expression are increased in skeletal muscle.
of septic rats (8, 40). In the present study, we also found that myocardial calpain activity was increased in septic heart tissues. The ability of calpains to cleave cytoskeletal and myofilament proteins in vitro suggests a regulatory role for calpains in heart functions (19, 24). Previous reports (15, 32, 36) have indicated that calpain activation is involved in the process of myocardial dysfunction in sepsis. However, the molecular and cellular mechanisms that mediate the pathogenesis of calpain-induced myocardial dysfunction are still not fully understood. In septic rats, Tissier et al. (36) found that calpain activation induced myocardial dysfunction by increasing venule leukocyte rolling and endothelium leukocyte adherence. In septic mice, we observed myocardial calpain activation and TNF-α mRNA expression. However, inhibition of calpain activation reduced TNF-α mRNA expression and improved the myocardial dysfunction (15). In the present study, we further explored this mechanism and demonstrated that the IκBα/NF-κB pathway and myocardial TNF-α expression are involved in the pathological process.

It is well known that LPS induces cardiac dysfunction by stimulating the release of TNF-α from cardiomyocytes. Studies have shown that the administration of TNF-α induced septic
shock symptoms (37) and that TNF-α antibody treatment abrogated LPS-induced myocardial depression in endotoxemia (38). Studies (12, 37, 38) of in vivo and in vitro septic models demonstrated that TNF-α impairs myocardial contractility. However, the signaling pathways through which LPS induces TNF-α expression in cardiomyocytes have not been fully defined. NF-κB activation is an important mechanism for TNF-α mRNA transcription; however, little is known about the signaling pathway upstream of NF-κB, which usually exists in the cytosol as a preformed trimeric complex. The NF-κB p50/p65 protein dimer is associated with an inhibitory protein known as IκBα. When IκB-α dissociates from the NF-κB p50/p65 heterodimer (6, 39), NF-κB p50/p65 migrates to the nucleus and binds to the promoter of TNF-α, thereby initiating its transcription (34). Two distinct pathways of IκBα degradation have been reported. One pathway is dependent on phosphorylation at sites within the NH2-terminal domain of IκBα by the serine/threonine kinase complex known as the IKK complex; IKKs phosphorylate the IκBα protein on Ser32 and Ser36, a reaction that targets IκBα for ubiquitination and rapid degradation by the 26S proteasome. This phosphorylation event triggers the ubiquitination of IκBα and its subsequent degradation by the proteasome protease complex (23). Recent studies (7, 20, 33) have demonstrated that this pathway is not universal, and phosphorylation-ubiquitin-proteasome-mediated degradation of IκBα has been shown not to occur. The second pathway is regulated by calpain, which regulates the IκBα/NF-κB pathway via limited proteolysis of IκBα containing a proline-glutamic acid-serine-threonine sequence at its COOH-terminal region, and this pathway is independent of serine/threonine phosphorylation of IκBα (10, 35). Degradation of IκBα mutated at Ser32/36 (resistant to phosphorylation by IKKs) was observed in T lymphocytes treated with H2O2 (33). Furthermore, Miyamoto and co-workers (20) found that inhibitors of calpain, but not of the proteasome, ablated basal IκBα degradation in WEHI 231 cells. Another report (3) demonstrated the accumulation of IκBα in both the cytoplasm and nucleus of limb girdle muscular dystrophy type 2A patients resulted from the failure of calpain-dependent IκBα proteolysis, supporting the importance of calpain in the regulation of the NF-κB pathway. In the present study, we did not detect significant protein level changes of myocardial p-IκBα, IKK-α/β, or p-IKK-α/β (activated forms of IKKα/β) in septic mice. IκBα degradation was first observed at 2 h after LPS administration, and the pharmacological inhibitors of calpain and overexpression of calpastatin restored IκBα protein levels and inhibited NF-κB activation. This indicated that IκBα was directly degraded by calpain in septic mice, as previously reported (10, 13, 35), rather than an alternative scenario where IκBα was degraded by phosphorylation by IKKs and the ubiquitin-proteasome pathway. The temporal expression profile of IκBα and NF-κB proteins after LPS challenge suggests that IκBα degradation preceded NF-κB activation. In addition, NF-κB p65 has also been reported as a potential substrate of calpain (17). Because the total NF-κB p65 protein level was similar in control and septic mice in our study, we propose that the degradation of IκBα by calpain induces NF-κB p65 phosphorylation in septic mice. Thus, calpain induces NF-κB p65...
nuclear translocation, increasing the NF-κB-dependent expression of TNF-α.

It should be emphasized that proteasomes function in IκBα degradation, as previously established (6). Calpain inhibitor III and PD-150606 are relatively specific inhibitors of calpain. However, in the present study, the overexpression of calpastatin, a special inhibitor protein of calpain, prevented calpain activation, leading to the inhibition of NF-κB activation and downregulation of TNF-α expression both in vitro and in vivo during LPS stimulation. Therefore, the possibility that additional proteolytic cascades are affected and maybe involved in this signaling pathway can be excluded.

In conclusion, this study provides evidence showing that myocardial calpain activation plays a role in TNF-α expression in LPS-induced myocardial dysfunction through the IκBα/NF-κB signaling pathway. Therefore, inhibition of the IκBα/NF-κB signaling pathway may represent a novel therapeutic approach for the treatment of LPS-induced TNF-α expression and myocardial dysfunction.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: X.L., R.L., and L.S. performed experiments; X.L. drafted manuscript; R.L. analyzed data; R.L. interpreted results of experiments; R.L. reviewed and edited manuscript.

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